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Daniel M. Baker
University of Missouri-Columbia

Harry C. Minor
University of Missouri-Columbia

Merton F. Brown
University of Missouri-Columbia

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COMPARISON OF PREPARATIVE TECHNIQUES FOR SCANNING ELECTRON
MICROSCOPY EXAMINATION OF SOYBEAN SEED COATS IN SECTIONAL VIEW

Daniel M. Baker^{1*}, Harry C. Minor¹, and Merton F. Brown²

Departments of Agronomy¹ and Plant Pathology²
University of Missouri-Columbia

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Abstract

Various scanning electron microscopy preparative techniques have been used by researchers to examine sectional views of dry, mature soybean (*Glycine max* (L.) Merr.) seed coats. Such previously employed techniques were utilized in our preliminary investigations of seed coat structure, but often yielded unacceptable preservation. Consequently, eight preparative techniques were evaluated in an effort to define conditions required to obtain consistently high quality preservation of soybean seed coats in sectional view. Of the eight procedures tested with the cultivar Williams 82, razor sections and mechanical fractures of dry seed coats yielded the poorest definition of anatomical features. Samples soaked in water prior to preparation and those subjected to prolonged osmium fixation showed unacceptable alteration of the seed coat parenchyma. Best preservation was obtained with seed coats which were not subjected to chemical fixation, but which were sequentially dehydrated from 20% ethanol, cryofractured, and critical point dried. Results obtained via this protocol, with four additional soybean genotypes and cultivars, demonstrate its applicability to comparative anatomical analysis.

Introduction

The seed coat of soybean consists of three layers derived from the outer integument of the ovule (Figures 1a and 1b). The outermost layer, composed of macrosclereids, is referred to as the "palisade layer". The palisade functions as the primary protective tissue of the seed coat and is resistant to the movement of gases and water vapor (Esau, 1977). The second layer of the seed coat, which lies below the palisade, consists of osteosclereids separated by large intercellular spaces. The characteristic shape of the osteosclereids, as seen with the light microscope (Figure 1a), has resulted in this tissue being termed the "hourglass layer". These osteosclereids function in the support of the palisade layer (Carlson, 1973). The final layer of the mature seed coat is a zone of collapsed parenchyma (Figure 1b) located below the osteosclereids. In the immature seed coat (Figure 1a) assimilates are transported to the developing ovule (Thorne, 1981) through vascular tissue situated in the active parenchyma. Attached below the parenchyma is a layer of cubical cells with cytoplasm. This layer is derived from the inner integument, and therefore is not considered part of the seed coat proper. It has been termed aleurone (Carlson, 1973), endosperm (Esau, 1977), or endothelium (Thorne, 1981).

Three basic methods have been used to obtain sectional views of seed coats for scanning electron microscopy (SEM). [1] Razor sectioning techniques were used to examine pits in the soybean seed coat (Wolf et al., 1981), factors associated with water absorption by soybean seeds (Calero et al., 1981), and fungi in the osteosclereid layer of soybean seed coats (Hill and West, 1982). [2] Mechanical fracture techniques, or the free break method, were used by Yaklich et al. (1984) in their examination of seed coat permeability. [3] Cryofracture techniques were employed by Wolf and Baker (1975) in their examination of soybean proteins. With each method, the manner in which the sectional view is exposed varies, thus affecting the quality of morphological preservation obtained. The purpose of our investigation was to evaluate these basic methods used to obtain sectional views of the mature soybean seed coat,

KEY WORDS: Soybeans, *Glycine max.*, Seed Coat, Preparation, Scanning Electron Microscopy, Cryofracture Technique.

*Address for correspondence:

Daniel Baker
203 Waters Hall, UMC
Columbia, Mo. 65211 Phone No. (314) 882-2306

and to test others, to determine which yield the best results consistently.

Materials and Methods

Seed coat materials were taken from a single lot of the soybean cultivar "Williams 82". The mature harvested seeds (approximately 10% moisture content) were stored at 20 to 25 C prior to preparation. When indicated, dehydration was achieved by use of a 20, 40, 60, 80, 100% x 3, (30 mins./change) ethanol series. Cryofracture (Humphreys et al., 1974) was accomplished by placing seed coat strips in handmade Parafilm M tubes filled with absolute ethanol. Tubes were then frozen in liquid nitrogen and fractured by striking the tube gently. Fractured tubes containing the seed coat pieces were then thawed in absolute ethanol. After preparation, all specimens were mounted on stubs with copper tape, sputter coated with gold-palladium, and examined at 20 kV in a JEOL JSM-35 scanning electron microscope. The eight techniques employed in this study are listed below. [1] Razor section. Strips of seed coat materials were removed by razor. [2] Mechanical fracture. Seed coat material was removed in strips from dry seed, and fractured by bending the strip with forceps. [3] Whole seed cryofracture. Whole seeds were frozen directly in liquid nitrogen and fractured. Seed coat pieces were thawed in absolute ethanol and critical point dried (CPD) as described by Anderson (1951). [4] Water pretreatment. Whole seeds were soaked in distilled water for 10 minutes to loosen the seed coat. Seed coats were cut into strips with a razor, dehydrated in the ethanol series, and then ethanol cryofractured. The resultant pieces were critical point dried. [5] Osmium fixation. Seed coats were removed in strips from dry seeds and fixed in 1% aqueous osmium tetroxide at 4 C for periods ranging from one hour to overnight. The strips were then rinsed in distilled water, dehydrated in the ethanol series, cryofractured in absolute ethanol, and critical point dried. [6] Sequential dehydration, cryofracture, and air dried. Seed coat strips were dehydrated in the ethanol series and cryofractured in absolute ethanol. Fractured pieces were then allowed to air dry. [7] Cryofracture, and critical point dried. Seed coat strips were not dehydrated in the ethanol series but placed directly in 100% ethanol, cryofractured, and critical point dried. [8] Sequential dehydration, cryofracture, and CPD. Seed coat strips were removed from dry seeds, dehydrated in the ethanol series, cryofractured in absolute ethanol, and critical point dried.

Quality of preservation was judged on the basis of anatomical clarity of the various seed coat components. Other specific criteria used included the intactness of the macrosclereids, lack of compression and disfigurement of the osteosclereids, and the ability to observe the parenchyma and endothelium as distinct tissues. The ethanol cryofracture technique (method 8) was further tested on three Plant Introductions (P. I. 377.574, 417.479, and 416.755) as well as one additional cultivar, "Delmar".

Figure 1 [1a] Light micrograph of the seed coat of Williams 82 during seed development. Macrosclereids (ms), osteosclereids (os), parenchyma (p, arrows indicate parenchyma limits) is living, and active, and endothelium (e). Bar = 50 μ m. [1b] A diagrammatic representation of a dry, mature soybean seed coat. Macrosclereids (Ms), osteosclereids (Os), collapsed parenchyma (P), and endothelium (E).



Results and Discussion

Razor Section

Razor sectioning exposed the general tissue organization of the soybean seed coat, but smudging resulted in overall loss of cellular detail (Figure 2a). Smudging obscured the fine structure of the macrosclereids, parenchyma, and endothelium. In some sections, the osteosclereids were compressed and disfigured as a result of the mechanical forces imposed on the sample (Figure 2b). With this technique, it was not possible to distinguish the parenchyma and endothelium as separate tissues.

Mechanical Fractures

Micrographs of sectional views prepared with this procedure demonstrate the fibrous nature of the macrosclereid secondary walls, a feature not seen in razor sections. Frequently, extensive tearing of these secondary walls (Figure 3a) and compression of the osteosclereids (Figure 3b) occurred as a result of the force applied during the fracture process. As with razor sections, observation of the parenchyma and endothelium as distinct tissues was not achieved.

Whole Seed Cryofracture

Cryofracture preparations provided smooth, clean sectional views of the soybean seed coat. In this preparation, tearing of the macrosclereid secondary walls was minimized and the osteosclereids were free of compression and distortion (Figure 4). However, the parenchyma and endothelium could not be clearly distinguished as separate tissues with this simple cryofracture preparation. In addition, the random manner in which fractures occur throughout the seed coat makes the repeated examination of a specific region difficult. Comparison of the length of the osteosclereids in Figure 4 with those of the Figure 3a illustrates this problem. Osteosclereid length (vertical axis) is greatest near the hilum, and least near the side opposite the hilum. Figure 4 represents a section from the latter region, and illustrates the shorter osteosclereids present in that region.

It is relevant to note that all the preceding

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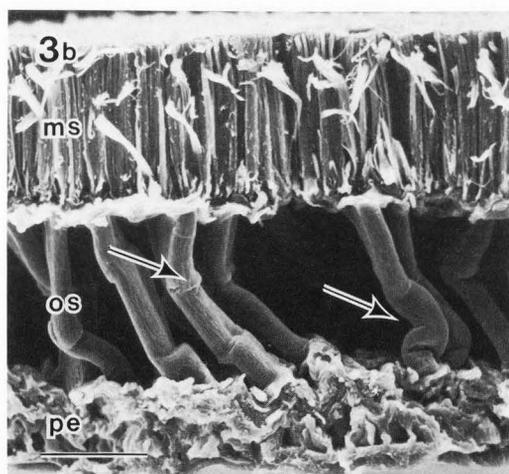
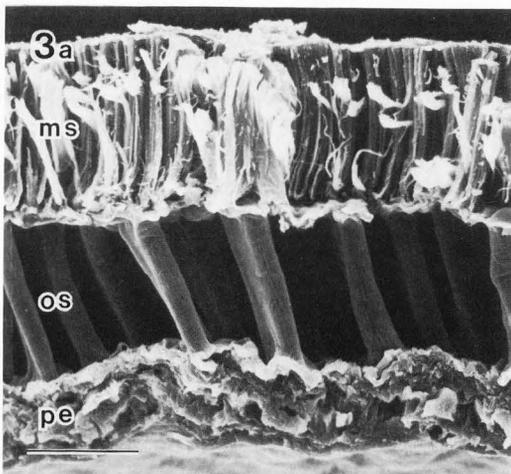
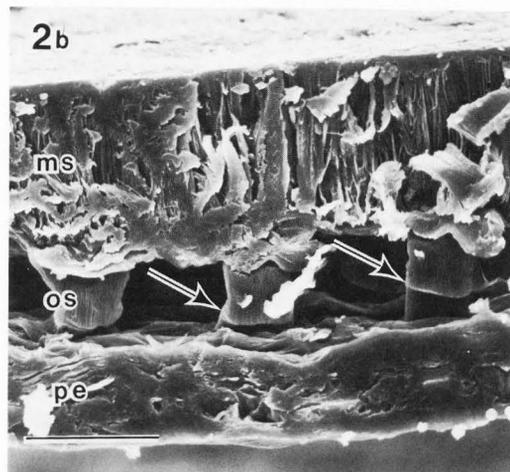
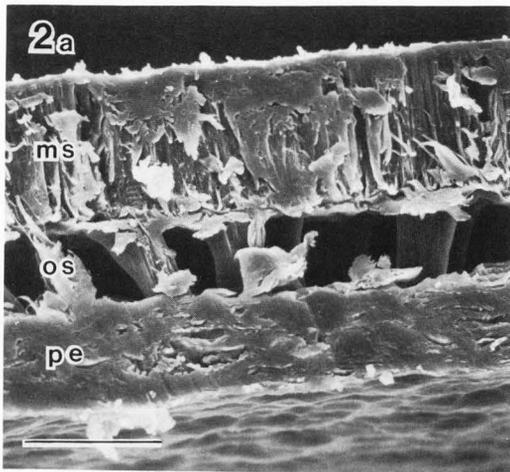
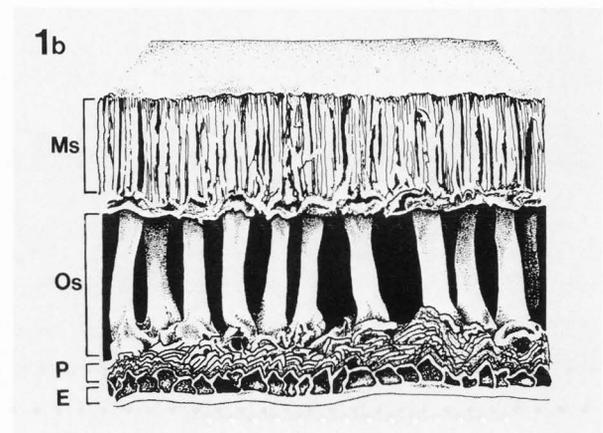
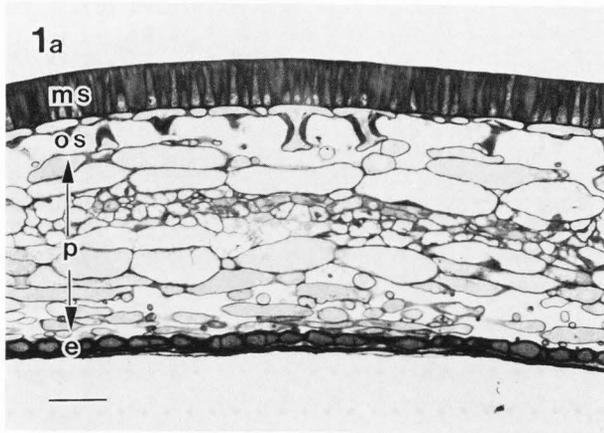


Figure 2. Razor section: [2a] Demonstrating smudging of the macroscleireids (ms), parenchyma and endothelium (pe). [2b] Compression of the osteosclereids (os, arrows). Bars = 30 μ m.

Figure 3. Mechanical fracture: [3a] Illustrating tearing of the macroscleireids (ms), [3b] Compression of the osteosclereids (os, arrows), and parenchyma and endothelium (pe). Bars = 30 μ m.

treatments involve a minimum amount of preparation prior to SEM examination. The preparations which follow are various elaborations of cryofracture technique. Different steps were employed in each preparation to define conditions required to obtain consistently high quality preservation of seed coats in sectional view.

Water Pretreatment

Wolf and Baker (1972) examined sectional views of the soybean seed coat using seeds that were presoaked in water prior to sectioning in a cryostat. In our preparation, presoaking in water was utilized prior to ethanol cryofracture. Presoaking soybean seeds in water facilitates the removal of the seed coat, and permits the observation of parenchyma and endothelium as separate tissues. However, this treatment also results in the disruption and breakdown of the seed coat structure (Figure 5). This is especially true for the cultivar Williams 82. In this cultivar, the parenchyma layer imbibes water rapidly, causing it to swell, leading to the physical separation of the parenchyma from the sclereid layers. To the unaided eye, this is observable as wrinkles in the seed coat. The expansion of the parenchyma and separation of the seed coat layers was observed by Saio et al. (1973) when studying the changes which occur in the seed coat during cooking. Since some cultivars and genotypes imbibe water rapidly, presoaking seeds prior to preparation may produce unacceptable preservation.

Osmium Fixation

Osmium fixation is commonly used in the preparation of biological specimens for scanning electron microscopy, but apparently has not been employed in the preparation of mature soybean seed coats. When osmium fixation was performed, the quality of preservation varied in relationship to the length of the fixation period. Expansion of the parenchyma layer, as demonstrated in the presoaked seed treatment, occurred as a consequence of prolonged (overnight) fixation in osmium, perhaps due to imbibition of water from the fixative solution (Figures 6a and 6b). A brief one hour fixation in osmium tetroxide resulted in little, if any, expansion of the parenchyma, and provided good anatomical preservation, including the distinction of the parenchyma and endothelium as separate tissues (Figure 6c).

Sequential Dehydration, Cryofracture, and Air Dried

Critical point drying is utilized in SEM to avoid drying artifacts in sensitive biological tissues. The need to critical point dry the rigid sclerenchyma tissues of the soybean seed coat is questionable since these tissues have thick, often lignified, secondary walls; tissues not normally considered sensitive. In this treatment, seed coat strips were dehydrated in the ethanol series, cryofractured in absolute ethanol, then allowed to air dry. Air-drying did not appear to affect the quality of preservation of the macrosclereids, parenchyma, or endothelium (Figure 7a), but resulted in some

collapse of the osteosclereids (Figure 7b).

Cryofracture, and Critical Point Dried

Sequential dehydration is routinely used in SEM preparations to provide gradual removal of cellular water. Dry, mature soybean seed coats, however, contain little water since the sclerenchyma and parenchyma are devoid of cytoplasm at this stage. These conditions could preclude the need for sequential dehydration and permit direct ethanol cryofracture. To examine this possibility, seed-coat strips were placed directly in absolute ethanol, cryofractured, then critical point dried. The micrographs show that macrosclereid and osteosclereid preservations were not affected (Figure 8a), but the parenchyma and endothelium were no longer observable as separate tissues (Figure 8b).

Sequential Dehydration, Cryofracture, and CPD

The previously discussed preparations indicate that the model technique for use in establishing consistently high quality preservation should include: an ethanol series dehydration to permit distinction of parenchyma and endothelium; cryofracture to provide smooth, clean sectional views; and critical point drying to minimize drying artifacts. Sectional views obtained utilizing this protocol showed well-preserved macrosclereids, with a minimum tearing of the secondary walls (Figures 9a and 9b); bone-shaped osteosclereids with little distortion and typical surface morphology (Figures 9a and 9c); and both parenchyma and endothelium distinguishable as separate tissues (Figures 9a and 9d). The quality of preservation obtained with this method closely resembles that of the one-hour osmium fixation preparation indicating that osmium fixation of mature seed coats is unnecessary to obtain high quality preservation.

Four additional seed sources were examined using this preparation technique (method 8) to determine its applicability to comparative anatomical analysis. P.I. 377,574 provides a unique anatomy for comparison purposes. In P.I. 377,574 a seed coat modification (Figure 10a) is

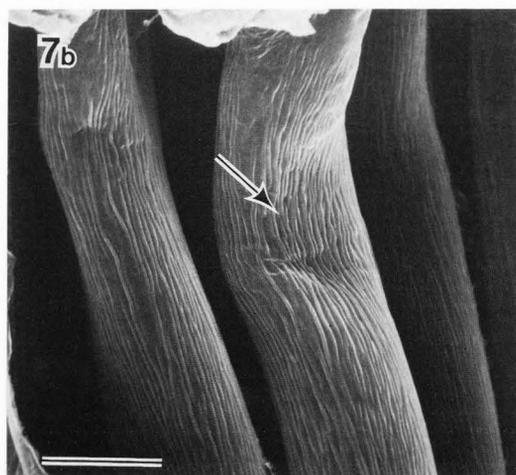
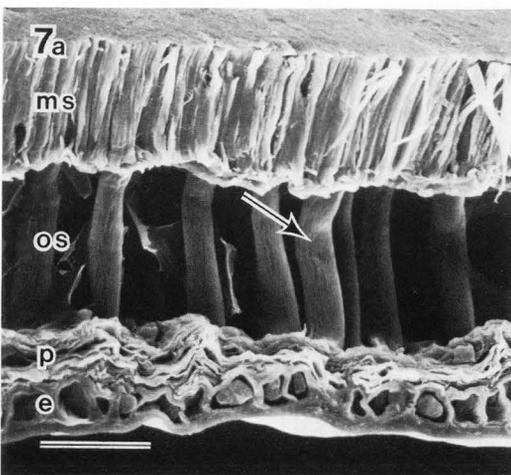
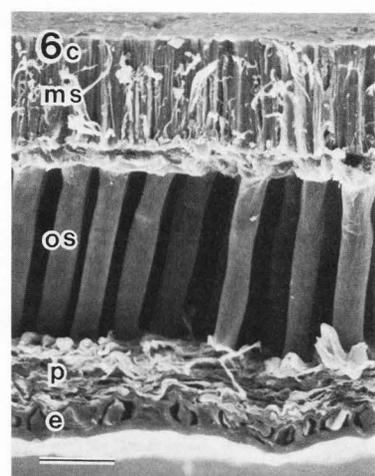
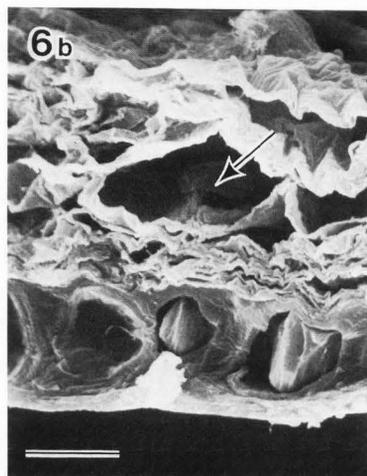
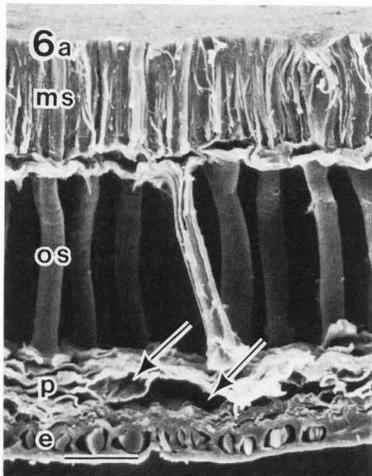
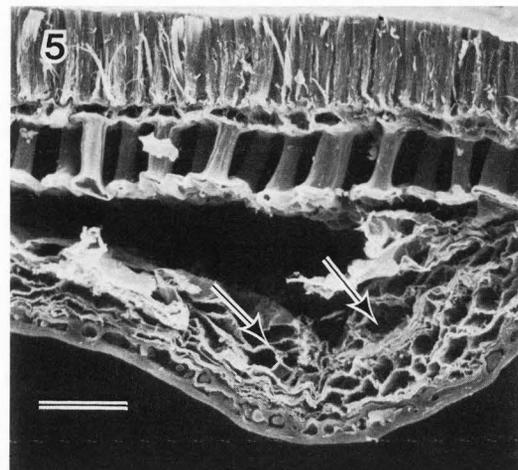
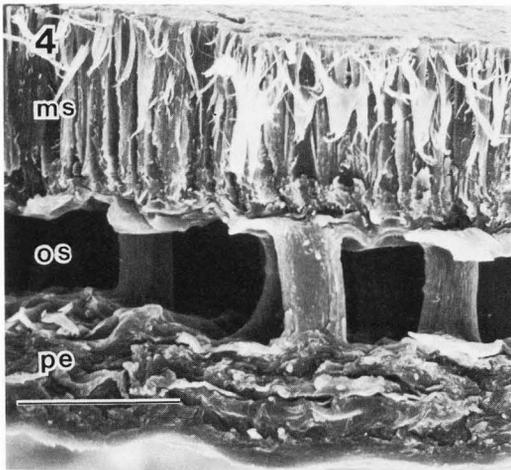
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Figure 4. Whole seed cryofracture: Macrosclereids (ms), osteosclereids (os), parenchyma and endothelium (pe). Bar = 30 μm.

Figure 5. Water pretreatment: Illustrating the expansion and separation of the parenchyma from the sclereids (arrows), Bar = 30 μm.

Figure 6. Osmium fixation: [6a] Overnight fixation period. Macrosclereids (ms), osteosclereids (os), parenchyma (p), and endothelium (e), arrows indicate expanded parenchyma, Bar = 30 μm. [6b] Higher magnification of the expanded parenchyma (arrow), Bar = 10 μm. [6c] One hour fixation period, Bar = 30 μm.

Figure 7. Sequential dehydration, cryofracture, and air dried: [7a] Macrosclereids (ms), osteosclereid collapse (os, arrow), parenchyma (p), and endothelium (e), Bar = 30 μm. [7b] Higher magnification of collapsed osteosclereid (arrow), Bar = 10 μm.

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present in the region of the pit (Dzikowski, 1936, 1937, Miksche, 1961) located on the abaxial surface of the cotyledons, approximately midway between the hilum, and the opposing side of the seed. Yaklich et al. (1984, 1985) described this region of the seed coat of the cultivar "Sooty" soybean as a dome inserted in a pit in the cotyledons, and observed their tongue and groove interconnection. Our examinations of this seed coat modification, or dome, reveal the presence of a fifth layer below the endothelium (Figure 10b). The dome adds considerably to the cross-sectional dimension in this region of the seed coat and consists of vertically elongated cells distinct from the horizontal, cubical cells of the endothelium. Other differences are also observable with this preparative technique. Anatomical comparisons show little evidence of a macrosclereid lumen in P.I. 417.479 (Figure 11a) or Delmar (Figure 11b), but a well-defined lumen is present in P.I. 416.755 (Figure 11c). These micrographs also indicate differences in the fibrous nature of the macrosclereids among these cultivars and plant introductions, whereas osteosclereids, parenchyma, and endothelium are similar in form and appearance.

Conclusions

Among the preparative techniques used to obtain sectional views of the mature soybean seed coat, razor sections and mechanical fractures yielded the poorest definition of anatomical features. Presoaked samples and those subjected to prolonged osmium fixation showed unacceptable alteration of the seed coat parenchyma. The best preservation of anatomical components was obtained with dry seed coat strips which were not subjected to fixation, but which were sequentially dehydrated, cryofractured, and critical point dried.

Acknowledgements

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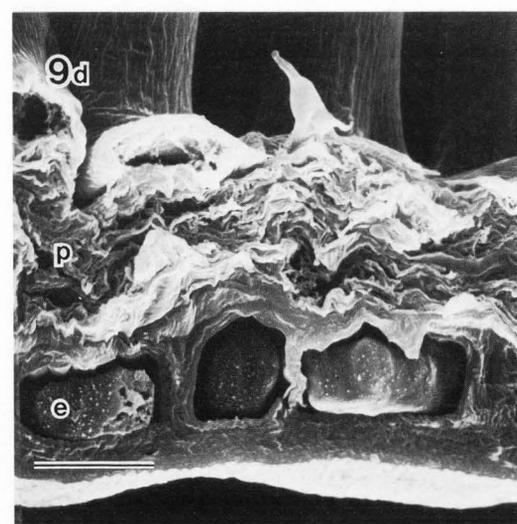
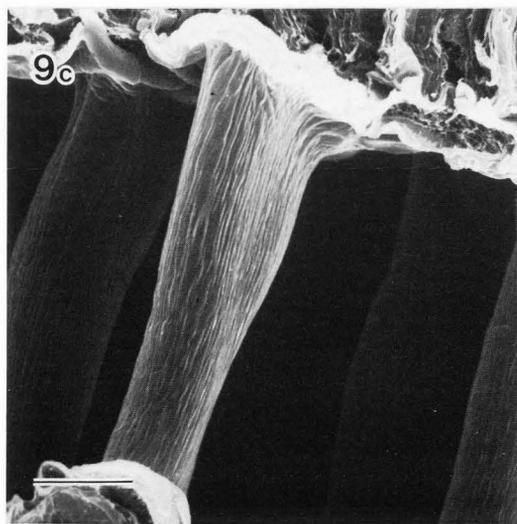
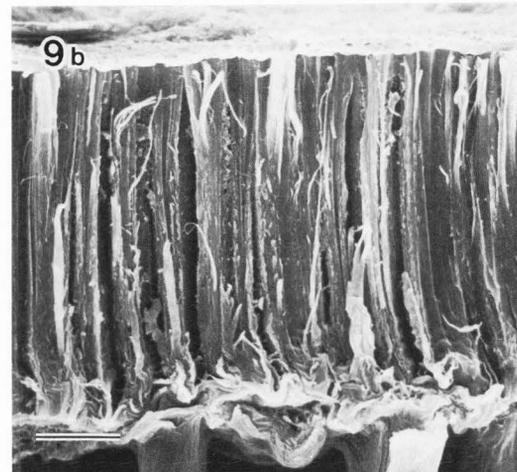
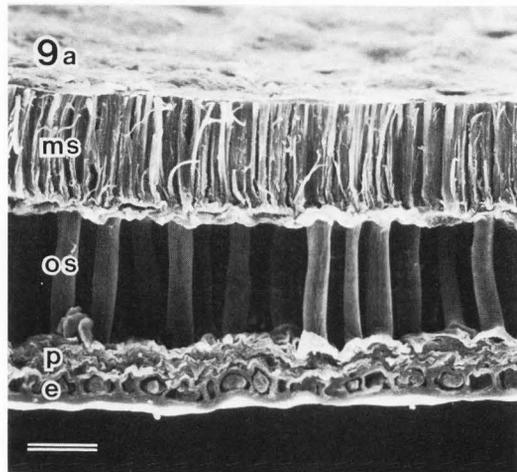
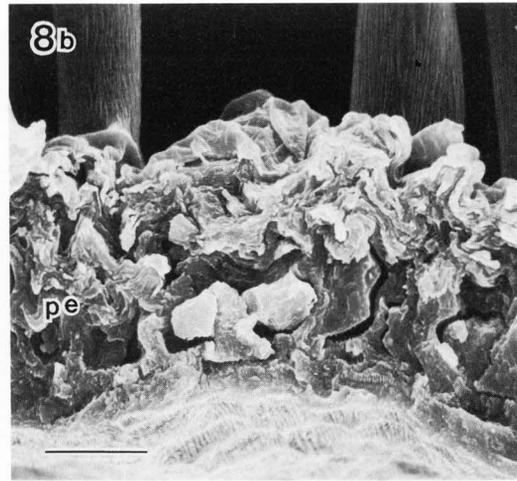
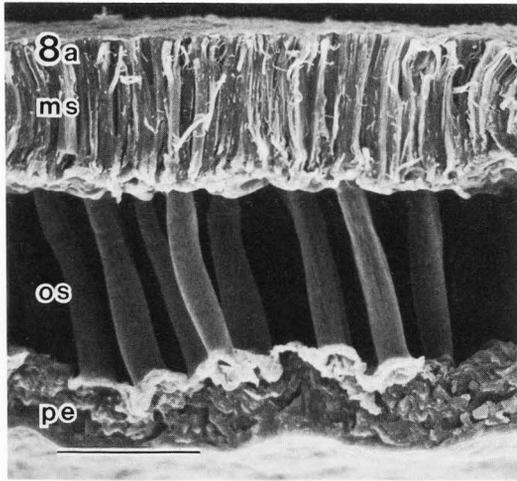
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 **Figure 8.** Cryofracture, and critical point dried: [8a] Macrosclereids (ms), osteosclereids (os), parenchyma and endothelium (pe), Bar = 30 μ m. [8b] Higher magnification of parenchyma and endothelium (pe), Bar = 10 μ m.

Figure 9. Sequential dehydration, cryofracture, and critical point dried: [9a] Macrosclereids (ms), osteosclereids (os), parenchyma (p), and endothelium (e), Bar = 30 μ m. [9b] Higher magnification of the macrosclereids, Bar = 10 μ m. [9c] Higher magnification of an osteosclereid, Bar = 10 μ m. [9d] Higher magnification of the parenchyma (p), and endothelium (e), Bar = 10 μ m.

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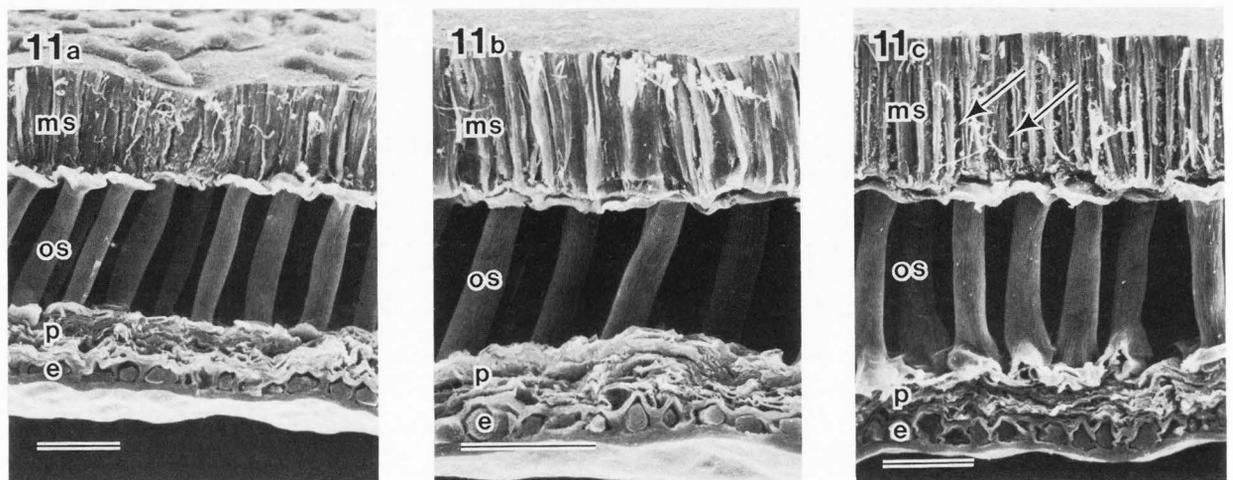
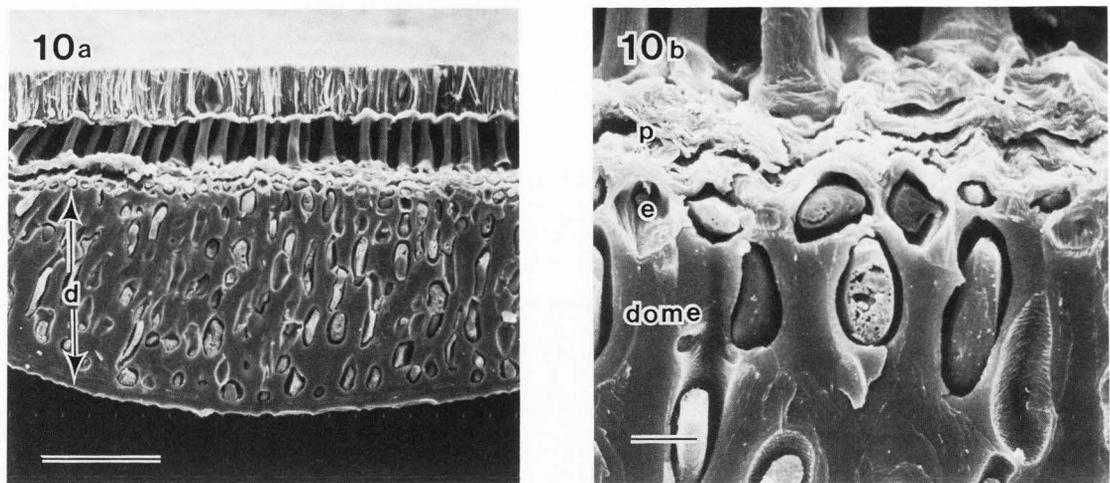


Figure 10. [10a] P. I. 377.574 seed coat modification or dome (d, arrows indicate dome limits), Bar = 100 μm . [10b] Higher magnification of the dome region showing parenchyma (p), endothelium (e), and vertical cells of the dome, Bar = 10 μm .

Figure 11. [11a] P. I. 417.479, [11b] Delmar, and [11c] P. I. 416.755, arrows indicate macrosclereid lumens. Bars = 30 μm .

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Discussion with Reviewers

R. Yaklich: In the free break method used by Yaklich et al. (1984), the seed coat was fractured across from the area they wanted to observe. This does less damage to the tissue than the mechanical fracture method outlined by the authors.

Authors: We have prepared seed coats with the technique described by Yaklich et al. and have found it to give results similar to our procedures. We also have examined fractures induced by chipping the seed coat, and fractures created by crushing whole seeds. Each of these methods (which we call mechanical fractures) results in some tissue damage. The extent of

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this damage is variable. Typically, macrosclereid damage (tearing of the secondary walls) occurs with any of the mechanical preparations, but the osteosclereids may or may not show distortion. Our purpose was to demonstrate the range of results that can be expected when mechanical fracture techniques are used.

W. Wolf: In some of our studies on naturally occurring fractures in soybean seed coats (Wolf et al., 1981) we examined the specimens directly after coating. Have you examined such fractures with and without applying method 8 (skipping the cryofracture step)? If so, have you detected any significant differences?

Authors: We have examined some naturally occurring fractures applying ethanol series dehydration and critical point drying (method 8, minus cryofracture). Since naturally occurring fractures seldom permit observation of the full sectional view (most natural fractures end at the parenchyma layer below the osteosclereids) and frequently show extensive tissue damage nothing is gained by careful application of preparative techniques. Rather it is best to desiccate, mount, coat, and examine such fractures.

K. Saio: Unique anatomy in P. I. 377.574 seems to be thick aleurone layer. Is the structure limited only in the region of the pit? Are there similar structures in the region of other soybean varieties, even if it does not develop like P. I. 377.574. Do these cultivars derived from Delmar have any different function, shape or color?

Authors: In all of the seed coats which we have examined the "thickened aleurone" layer has always been confined to the region opposite the pit in the abaxial surface of the cotyledons. This thickened aleurone occurs in a number of experimental lines which we have examined, and although we have not estimated the frequency of this characteristic, it does not appear to be a common feature of seed coats. It is present in both black and yellow soybeans and seems to occur in seeds of various sizes. Its function is not known. While Delmar is one of the cultivars we examined for comparative purposes, none of the seed coats shown are derived from Delmar.

H. Hill: I find it interesting that the authors soaked seed for 10 minutes in distilled water and caused seed coat disruption, while a 1 hour soak in 1% OsO₄ did not. Do the authors think that the 1% OsO₄ prevented seed coat disruption or were there other differences between techniques that would have caused the results?

Authors: It is likely that there is bonding occurring between osmium and the parenchyma cell wall, resulting in some degree of stabilization of the parenchyma. Also, seed coat strips in the osmium preparations were fixed at 4 C as opposed to room temperature (20 C) for the water pretreatment procedure. At lower temperatures we might expect the parenchyma to have a reduced

rate of water uptake.

H. Hill: What do the authors feel caused the compression of the parenchyma layer in Figures 3a, 4, 6a, 6b, 6c, 7a, 9a, 9d, 10c, 10d, 10e? Photomicrographs of the parenchyma layer in Thorne's paper (1981) do not show this compression?

Authors: Thorne sampled seeds at 45 to 50 days after flowering, prior to maximum dry weight accumulation (physiological maturity). At this stage of development the seed coat is active in the import of photosynthate to the cotyledons. The parenchyma is vascularized and "expanded". After physiological maturity the parenchyma of the seed coat loses its cytoplasmic contents and collapses (or compresses). It is natural for the parenchyma to be fully collapsed at harvest maturity, and therefore reasonable for all our micrographs to demonstrate this feature.

H. Hill: How many seeds and photomicrographs were used for each technique to get an overview such that comparisons can be made?

Authors: Some preparations required less examination than others to get an overview. Since both razor and mechanical preparations have been previously employed in the literature our evaluation of these preparations was based on 15 to 20 seeds and comparison with published results. The other methods presented, with exception of method 8, were based on observation of 30 to 40 seeds. Method 8 has been widely used in our studies and has given consistent results with approximately 20 genotypes and cultivars of varying sample sizes.

H. Hill: Sources I checked (Principles of Seed Science and Technology, Copeland and McDonald, p. 14; Physiology and Biochemistry of Seeds, Vol. 1, Brewley and Black, p. 41) indicate that the endothelium is considered part of the seed coat proper because the seed coat is, by definition, derived from both the inner and outer integuments.

Authors: In the case of legumes, testa is the more appropriate term to use when referring to the layers derived from both the inner and outer integuments.