Peanut Science (1983) 10:93-97

Specimen Preparation Techniques for Scanning Electron Microscopy of Developing Peanut Pegs[1]

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ABSTRACT

This study evaluated various specimen preparation techniques for light microscopy (LM) and scanning electron microscopy (SEM). Main conclusions from this study were:

(i) Critical point drying (CPD) was preferred over freeze drying for SEM of whole or large pieces of specimens. However, CPD did not offer any additional advantage over air drying for SEM of thin (14 μm) microtomed sections of paraffin embedded specimens.

(ii) Formaldehyde-acetic acid-alcohol (FAA) was found to be satisfactory as a general purpose fixative for LM and low magnification SEM. However, for magnifications higher than X500, where subcellular details become the subject of investigation, glutaraldehyde (GA) was found to be preferable over FAA.

(iii) Certain artifacts appeared to be related to developmental stages of the peanut fruit.

Key Words: Scanning electron microscopy, light microscopy, peanut fruit, maturation, development.

Anatomical studies of the peanut (*Arachis hypogaea* L.) fruit have generally been conducted on peanuts of a limited physiological age span. Some include only the early stages of fruit development (3,14,26,27), some describe selected developmental stages from fertilization to near maturity (6,22,24,25), and others the later stages of development (9,21,23,28). The early literature on peanut fruit anatomy has been reviewed by Jacobs (14), Reed (24), and Smith (25). Technique advances since many of these studies were conducted have made available scanning electron microscopy (SEM)[4] and transmission electron microscopy (TEM). Development of SEM has provided a research instrument which has continuously variable magnification between X28 and X100,000 with three-dimensional features and increased depth of focus and resolution. These capabilities make SEM not only a powerful tool but also a connecting link between LM and TEM where the lowest operational magnification is about X5,000. Peanut structure studies which have used SEM are confined to later stages of development (9,21,29). Halliburton et al. (9), in their anatomical study of the peanut pericarp, used both LM and SEM. SEM not only complements LM observations but, under certain circumstances where differential staining is not a primary requirement, it can even replace LM.

[1]Paper Number 8979 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27650. Use of trade names of specific materials does not constitute endorsement by the United States Department of Agriculture or the North Carolina Agricultural Research Service to the exclusion of others which also may be available.

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[4]SEM and LM denote scanning electron microscope and light microscope, respectively. Depending upon usage in the sentence, the observations may also refer to the two types of microscopy, micrographs, or microscopic techniques.

SEM's intermediate position between LM and TEM has fostered rather indiscriminate use of LM and TEM specimen preparation techniques for SEM (8, 11, 17, 19, 20). Although routinely avoided because of possible instrument contamination, direct SEM of fresh specimens has also been reported (1,13,20).

There are a variety of SEM preparation techniques, consequently several types of artifacts can be encountered (7). The situation is further confounded by the fact that SEM artifacts have been reported to be associated not only with species-specific differences, but also with different developmental stages within the same species (1). Thus, the nature and source of artifacts in a recent study on peanuts (29) lack proper identification and/or classification. In an extensive structural study of the peanut fruit and seed development, we are attempting to complement LM with SEM. In the absence of uniform techniques for SEM of plant materials in general and peanut fruits in particular, it is necessary to determine to what extent LM techniques can be used for SEM with or without further modification. Specifically, it is necessary to determine whether the widely used, but cumbersome and expensive, critical point drying (CPD) method (2,5) is indispensible for peanut SEM. Systematic examination of this and other pertinent questions, as has been done in this study, should facilitate future SEM studies of peanut fruit development with minimal artifacts.

Materials and Methods

Fixation, Dehydration and Embedding: Peanut *(Arachis hypogaea* L. cv. NC 6) plants were grown under field or greenhouse conditions, and pegs that had not penetrated the soil were used in this study. Pegs were harvested in two groups: one for SEM of whole or hand-cut specimens and the other for SEM (or LM) of thin sections of paraffin embedded specimens. The whole (or hand-cut) specimens were frozen in dry-ice (i.e. cryofixation) followed by freeze drying (FD) or were chemically fixed followed by CPD or FD; chemical fixatives used were formalin-acetic acid-alcohol (FAA) or 3% glutaraldehyde (GA) in Sorenson phosphate buffer pH 6.9 (4). Penetration of fixatives was augmented by intermittent application of partial vacuum. GA fixed specimens were washed in the buffer prior to dehydration for CPD. Specimens designated for CPD were dehydrated in a graded series of ethanol (30, 50, 75, 95, 100%) followed by a graded series of Freon 113 (30, 50, 75, 100%) in absolute ethanol using 30 min intervals. Dehydrated specimens were CPD in a Bomar SPC-1500 instrument using Freon 13 (11). Both FD and CPD samples were stored under dessication at 23 ± 2C until further processing for SEM.

Hand-cutting of specimens to be examined with SEM is done routinely following sample collection, but prior to fixation, without due consideration to the appropriate step where the cut surface to be examined should be prepared for best results. Therefore, we introduced hand-cutting at various stages of specimen preparation to obtain four major categories of specimens: a) fresh cut surface followed by FD; b) fresh cut surface, washed prior to FD; c) cut surface of chemically fixed/dehydrated specimens; and d) cut surface of dried (FD or CPD) specimens. It should be noted that these cuttings exposed the cut surface to be examined and were done in addition to other cuttings involved in specimen collection. Specimens to be paraffin (Tissue Prep, m.p. 56.5 ± 0.5 C, Fisher Scientific Co.) embedded were transferred from the chemical fixative (GA fixed specimens were washed first in buffer) to 50% ethanol and then passed through a graded series of ethanol-tertiary butyl alcohol as described by Berlyn and Miksche (4).

[5]Considerable amount of negative results pertinent to the discussion are not shown due to space considerations but can be communicated upon request.

Light Microscopy (LM): Paraffin embedded specimens were sectioned (14 μm) with a rotary microtome, and the ribbon affixed to glass slides with Haupt's adhesive, deparaffinized, stained (safranin 0 and fast green FCF), and permanently mounted (Permount, Fisher Scientific Co.) according to Berlyn and Miksche (4).

Scanning Electron Microscopy (SEM): Whole or hand-cut specimens designated for SEM were fixed and dehydrated as described earlier. The dehydrated specimens were mounted on appropriate specimen holders with a conductor-adhesive (colloidal graphite) followed by conductorization with gold-palladium coating in a Technics Hummer V Planar Magnetron Sputter coater. Coated specimens were examined at 15 kV with the aid of a JEOL T-200 scanning electron microscope. Electron micrographs were made using Polaroid P/N Type 55 film.

For SEM of thin sections, microtomed paraffin ribbons were mounted on an acid-washed metal slide, instead of a glass slide, according to the procedure of Mohapatra and Johnson (19) summarized by Hayat (11). For comparison of similar features under LM and SEM, alternating two-section lengths of the ribbon were affixed to glass and metal slides, respectively. The metal slides were deparaffinized and stained in the same manner as glass slides; however, following the final 100% ethanol step, the metal slides were divided into two groups. One group was processed for CPD by passing them through a graded series of ethanol-Freon 113 as described above using 15 min intervals, whereas the other group was passed through xylene and air dried. The air dried and CPD metal slides were cut to an appropriate size and then mounted on the specimen holder with collidal graphite adhesive, conductorized, and finally examined with the aid of the SEM as described above.

Results and Discussion

Scanning electron microscopy can be used to examine both fresh and fixed specimens; however, the former were excluded from this study to avoid instrument contamination that might result from moisture loss under the high vacuum of SEM. Therefore, results discussed herein will be limited to the specimens dehydrated in various ways prior to SEM. Following cryofixation, specimens are usually dehydrated by lyophilization or freeze-substitution (15). Inasmuch as freeze-substitution is used exclusively in conjunction with histochemical research, this approach was not used in this study. Thus, results were obtained using specimens which were freeze dried, critical point dried, or air dried following other steps in specimen preparation.

As shown in Fig. 1, structural details at the cut surface of freeze dried specimens (Fig. 1A)[5] were obscured by artifacts as compared to those in the CPD specimens (Fig. 1B). These surface artifacts are believed to be freeze dried residues of solutes present in the sap exuded at the cut surface following cutting. Thus, when the cut specimens were rinsed in water, or when specimens in the fixative or dehydrating solutions were cut, prior to FD or CPD, the surface exudates were washed out allowing the structural details to be seen as in Fig. 1B. Cut surfaces of freeze dried or critical point dried specimens were also free of surface artifacts; however, these specimens developed considerable fracture-artifacts due to the brittleness of the specimens at the time of cutting. Chemically fixed specimens were free of the surface artifacts because of the washing effects of the fixatives and dehydrating reagents. If freezing is used it is recommended that fresh specimens be washed following cutting and before freezing.

The palisade arrangement of epidermal and subepidermal cells at the cut surface was not as well preserved in FD specimens (Fig. 1C) as in CPD specimens (Fig. 1D).

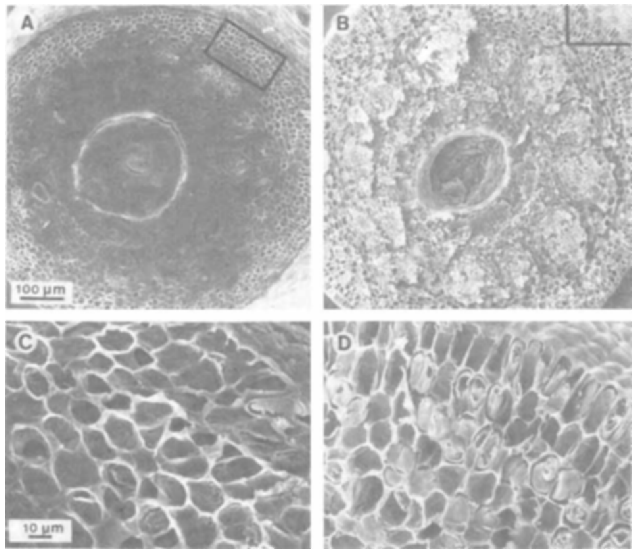

Fig. 1. Scanning electron microscope comparison of freeze dried (FD) and critical point dried (CPD) peanut pegs cut at various stages of specimen preparation.
A. Hand cut specimen followed by freeze drying without washing, X100.
B. Chemically fixed specimen hand-cut prior to critical point drying, X100.
C. Epidermal and subepidermal cells from area marked in A, X500. Note ice crystal damage.
D. Epidermal and subepidermal cells from area marked in B, X500.

These artifacts apparently resulted from ice crystal formation during freezing (15) but were not discernible at lower magnifications (compare Fig. 1A and 1C). This might explain why CPD has replaced FD for routine application in whole specimen SEM. However, FD may still be preferable over CPD in studies dealing with surface deposit of materials or organisms on various plant parts. For example, studies dealing with pollen grain or fungal spore deposit and germination may be adversely affected by CPD because repeated rinsing by fixatives and dehydrating reagents could result in the partial or complete loss of the deposited materials. No ice crystal damage could be detected when specimens fixed in FAA were freeze-dried. This may be related to different freezing properties of the FAA mixture as compared to cell sap and hardening of the cell walls and subcellular components as a result of fixation. In this connection, it may be noted that where CPD is not available, or is considered to be cumbersome, chemical fixation followed by freeze drying may be an acceptable alternative provided that it has been carefully evaluated with regard to possible species specific differences.

Microtomed thin sections prepared for LM need no drying because they are transferred to a permanent mounting medium immediately after the xylene (4). Mohapatra and Johnson (19) air dried these sections, following the xylene step, for subsequent SEM. We compared the air drying method with CPD under the assumption that surface tension associated with xylene might introduce artifacts during air drying. As shown in Fig. 2, there was no noticeable difference between air dried and CPD specimens, thus suggesting that the latter is not needed for the SEM of microtomed thin sections.

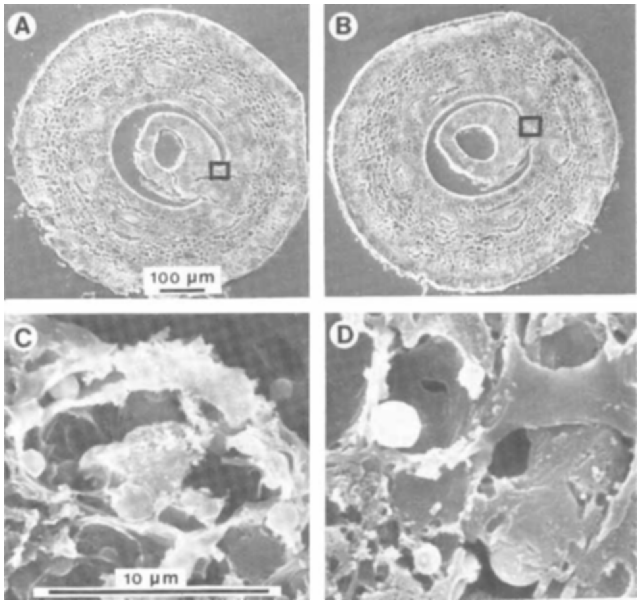

Fig. 2. Scanning electron microscope comparison of critical point dried and air-dried microtomed sections of peanut peg.
A. Critical point dried cross-section, X100.
B. Air dried cross-section, X100.
C. Higher magnification of area marked in A, X5,000.
D. Higher magnification of area marked in B, X5,000.

Judicious selection of fixatives is a critical requirement for both light and electron microscopy. The selection process must take into consideration several factors including cost, convenience, safety, specimen size and type, and intended results. Literature pertaining to the choice of various fixatives has been reviewed extensively (10,12,16,18,20). Although osmium tetroxide has been used in combination with GA as a fixative for SEM of peanuts (21,29), this fixative was omitted in our study based on the following considerations. The primary benefits of osmium tetroxide are an increase in staining contrast and conductivity of the specimen and fixation of the fine structure (10,11,12) which are not usually discernable at magnifications lower than X5,000. Thus, limited benefits would be expected to be gained from the use of osmium tetroxide where SEM studies are limited to relatively lower magnifications as compared to TEM. Furthermore, in studies such as this one where specimen size exceeds 1.0 mm, the use of osmium tetroxide is to be avoided because of the slow penetration rate of this chemical. Comparison of GA and FAA as fixatives showed that GA preserved subcellular features better, whereas FAA revealed anatomical features better (Fig. 3). These results would, however, be expected on the basis of basic consideration of both the fixatives (12). Thus, microtomed sections of GA and FAA fixed specimens had differences similar to those discussed above. Inasmuch as FAA is used usually for anatomical studies, and these sections can also be used for SEM (19), this fixative is recommended for routine SEM of hand-cut or microtomed specimens at lower magnifications. However, if SEM is to be conducted at greater than X500, GA would be the more appropriate fixative.

Fracture-artifacts were often associated with SEM of thin-sections (Figs. 4A, and 4B) but not with LM of thin-

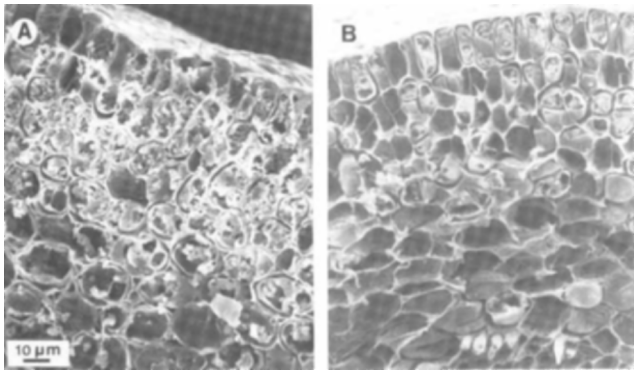

Fig. 3. Scanning electron microscope comparison of glutaraldehyde (GA) and formaldehyde (FAA) fixed peanut pegs.
A. Cross-section of GA fixed, hand-cut specimen, X500.
B. Cross-section of FAA fixed, hand-cut specimen, X500.

sections (Fig. 4C). These artifacts, however, did not result from exposure to the electron beam although the latter aggravated the artifacts already present with prolonged or repeated exposure. Since prior LM examination of specimens prepared for SEM also showed the presence of these fractures, it is presumed that the fractures resulted during handling associated with the mounting of sections on metal slides and the cutting of these slides to fit the SEM specimen holder. The fact that Mohapatra and Johnson (19) did not report these artifacts in their study on tobacco indicates that the artifacts may be related to materials being used. This is further evident from the fact that peanut fruits at more advanced stages exhibited fewer artifacts than younger fruits.

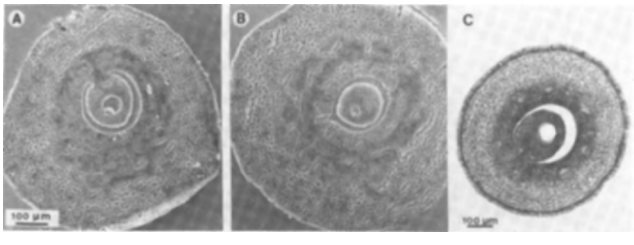

Fig. 4. Scanning and light microscope comparison of fracture-artifacts in microtomed cross-sections of peanut peg.
A. Formaldehyde (FAA) fixed section mounted on metal slide for SEM, X150.
B. Glutaraldehyde (GA) fixed section mounted on metal slide for SEM, X150.
C. GA fixed section mounted on glass slide for LM, X100.

By comparative SEM and LM of adjacent deparaffinized thin sections of tobacco leaf, Mohapatra and Johnson (19) demonstrated that SEM could be used to show the structural details of thin-sections. This was particularly true at higher magnifications, since conventional LM could not be used for magnifications beyond X1,000. Similar conclusions were drawn by McDonald et al. (17) who used the same thin section of paraffin embedded rabbit sensory cortex for SEM and LM. The use of metal slides by Mohapatra and Johnson (19) was an improvement over the glass slides of McDonald et al. (17) in terms of conductivity and materials handling. However, metal slides could not be used for LM, thus necessitating the use of adjacent sections for SEM and LM. Thus, Fig. 5 compares LM and SEM of adjacent microtomed sections. The noticeable comparable differences in the depth of focus and resolution are consistent with earlier reports by other workers (17,19). Use of metal slides has been found to present limitations in our study and might do so in other developmental studies where the specimen size is so minute that considerable morphological gradients may become obvious even between the adjacent sections. In thse situations, examination of the same section with LM and SEM, while avoiding glass slide associated problems, would be desirable. Therefore, efforts are underway in this laboratory to develop such a technique.

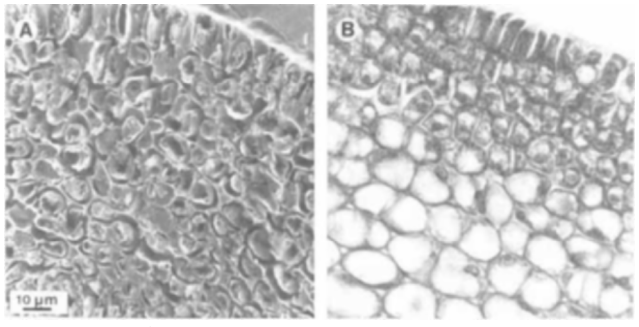

Fig. 5. Scanning electron and light microscope comparison of adjacent microtomed sections of FAA fixed peanut peg.
A. SEM of cross-sectional anatomy, X500.
B. LM of cross-sectional anatomy, X500.

Summary and Conclusions

This study was undertaken to evaluate the applicability of various specimen preparation procedures, available for biological SEM, for the SEM of peanut fruits. The major findings are:

1. Critical point drying is preferred over FD for whole specimens but is not necessary for microtomed sections of paraffin embedded specimens. However, FD of chemically fixed specimens or fresh specimens can be used with advantage under certain conditions.
2. Since studies of developmental anatomy depend on the use of thin sections obtained in close succession, this is better accomplished through the SEM of paraffin embedded specimens than through the use of single-cut surface(s) of hand-cut specimens. Multiple thin sections also permit LM and SEM of adjacent sections. In this connection, LM can be used for general anatomical investigations with supplementary/complementary application of SEM where three-dimensional information is desired.
3. FAA is recommended for LM and SEM where magnifications less than X500 are desired; however, GA should be used for higher magnification SEM, as is rountinely done for TEM.

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Accepted November 14, 1983