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A METHOD FOR LIGHT AND SCANNING ELECTRON MICROSCOPY OF DROUGHT-INDUCED DAMAGE OF RESTING PEANUT SEED TISSUE

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Abstract

Tissue damage creates numerous problems for the microscopic examination of resting peanut (Arachis hypogaea L. cv. Florunner) seed physical structure. This paper presents a method to deal with specific problems encountered in light microscopy (LM) and scanning electron microscopy (SEM) of drought-induced damage of peanut tissue. Major findings include: (1) improved SEM imaging through reduction of charging by increasing coating thickness from 25 nm to 30 nm; (2) improved stain affinity for LM tissue preparations by decreasing osmium tetroxide fixation time from 2 hours to 1 hour; and (3) improved tissue fixation for LM and SEM by application of a modified Karnovsky fixative (glutaraldehyde/formaldehyde combination) which proved more successful than glutaraldehyde alone for tissue preservation.

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Introduction

Light microscopy (LM) and scanning electron microscopy (SEM) of native resting peanut involve a fixation-dehydration-drying methodology that seeks to preserve the tissue so that it will retain its morphology for microscopic examination. Tissue which is damaged before fixation presents unique problems which hinder this effort. Such damaged tissue may result in poor SEM imaging, poor LM stain affinity, and poor tissue fixation for both LM and SEM.

Our laboratory was first involved with microscopic examination of drought-induced damage of resting peanut seed tissue in connection with evaluation of peanuts for a severe off-flavor problem (Young and Schadel, 1984). During that study, numerous problems with SEM imaging and LM staining preparations were encountered while adapting undamaged resting peanut seed tissue fixation-dehydration methods for use on resting peanut seed tissue with drought-induced damage. The present investigation was undertaken to discover the cause of those problems and to devise a method to improve the microscopy for evaluation of drought-induced damage of resting peanut seed tissue.

Materials and Methods

Cotyledons of resting peanut (Arachis hypogaea L. cv. Florunner) seed were examined with a dissecting microscope for physical structural characteristics. They were divided into two groups: (1) cotyledons with normal physical characteristics; and (2) cotyledons with drought-induced tissue damage. Tissue blocks (1 mm^3) of both groups were most suitably fixed in a modified Karnovsky's fixative (Karnovsky, 1965). This modified fixative was prepared by mixing 25 mL of 8% formaldehyde, 3.6 mL of 70% glutaraldehyde, and 28.6 mL of 0.2 M sodium phosphate buffer (hereafter referred to as buffer). The pH of the mixture was adjusted to 7.0. Tissue blocks were fixed under vacuum for 30 minutes at room temperature to promote initial infiltration and then fixed (not under vacuum) for 48 hours at 4°C. Following a 24 hours wash in 6 changes of 0.1 M buffer (4°C, pH 7.0), the material was post-fixed for 1 hour in 1% osmium tetroxide in 0.1 M buffer at 4°C. After post-fixation, material for LM was washed for 30 minutes in 0.1 M buffer (4°C, pH 7.0), dehydrated in a graded series of aqueous ethanol (10, 25, 50, 75, 95) to 100% ethanol, and embedded in

Spurr's low viscosity medium (Spurr 1969). Sections were cut on a Reichert ultramicrotome and stained differentially with 1% acid fuchsin and toluidine blue (Feder and O'Brien, 1968). Identically fixed tissues for SEM were dehydrated in an identical series of aqueous ethanol followed by a graded series of ethanol-amyl acetate (10, 25, 50, 75, 95, and 100% amyl acetate). Carbon dioxide was used as the transitional fluid in a Ladd Critical Point Dryer (Ladd Research Industries, Burlington, VT). After critical point drying, the specimens were mounted on aluminum stubs with aluminum tape. Two groups of tissue blocks for SEM were then coated with a gold-palladium alloy of 25 nm and 30 nm respectively in a Technics Hummer V sputter coater fitted with a Technics digital thickness monitor (Technics, Alexandria, VA). Samples were observed and photographed at 20 keV with an ETEC Autoscan Microscope (ETEC Corp., Hayward, CA).

The specific changes in our present method from our previous preparations and the reasons for those changes were:

(1) use of a modified Karnovsky's fixative rather than 4% glutaraldehyde to allow the formaldehyde component to penetrate faster than the glutaraldehyde component and temporarily stabilize structures which were subsequently more permanently stabilized by the glutaraldehyde;

(2) initial fixation under vacuum rather than at atmospheric pressure to achieve a more thorough and uniform fixation;

(3) post-fixation in 1% osmium tetroxide for 1 hour rather that 2 hours to allow less blackening of the tissue and enhance stain affinity for LM preparations;

(4) use of a Technics Hummer V Sputter Coater fitted with a Technics Digital Thickness Monitor to measure the thickness of coating that best reduced the charging of the specimen when examined with the SEM.

Results and Discussion

Scanning Electron Microscopy

Our SEM work of resting peanut seed tissue with drought-induced damage was initially hindered by problems with excessive charging of the specimen. By using the digital thickness monitor to measure the thickness of the gold-palladium coating applied to the specimen by the sputter coater, we were able to determine that the charging problem of the tissue with drought-induced damage was a result of the gold-palladium coating being too thin.

SEM of undamaged resting peanut seed epidermal tissue could be accomplished without charging artifacts when the gold-palladium coating was 25 nm thick (Fig. 1). Tissue with drought-induced damage, however, would charge when coated with 25 nm thickness (Fig. 2). By increasing the thickness of the gold-palladium coating by 20% (from 25 nm to 30 nm), we were able to minimize charging (Fig. 3). The charging problem was most likely the result of increased roughness in the surface of the samples (compare Figures 1 and 2).

Light Microscopy

LM tissue preparation using 1% osmium tetroxide for 2 hours were initially hindered by excessive blackening not only of damaged cells, but of the undamaged cells beneath the damaged cells (Fig. 4). Excessive blackening of tissue prevented us from evaluating the depth of damaged tissue because our LM staining procedures for undamaged tissue were ineffective. By reducing the length for post-fixation in 1% osmium tetroxide from 2 hours to 1 hour, we were able to use the LM stains of 1% acid fuchsin and toluidine blue more effectively since the blackening of undamaged tissue was lessened. By using the phase contrast optics on the light microscope, we observed that the fixation of the cytoplasmic network had been effectively accomplished during the decreased post-fixation period. The cytoplasmic network has a net-like appearance as a result of the oil being removed from the lipid bodies by alcohol dehydration and leaving spaces in the cytoplasm (Fig. 5).

Fixation

While initial use of a 4% glutaraldehyde fixative in 0.05 M sodium cacodylate (pH 7.0) for peanut tissue with drought-induced damage was adequate, we felt that better integrity of the cytoplasmic network in undamaged cells could be attained. This would more readily enable us to distinguish damaged cells from undamaged cells, as preservation of the cytoplasmic network is lost in severely damaged cells.

Horisberger and Vonlanthen (1980) achieved excellent fixation of soybean seed using a mixture of glutaraldehyde and formaldehyde in 0.1 M phosphate buffer (a modified Karnovsky's fixative) and a post-fixation in 1% osmium tetroxide. We have made our own modifications of a mixture of glutaraldehyde and formaldehyde followed by a post-fixation in 1% osmium tetroxide that most effectively achieves fixation of resting peanut seed tissue. Our fixative modifications proved to be excellent for observing the cytoplasmic network both with phase contrast LM (Fig. 5) and SEM (Fig. 6).

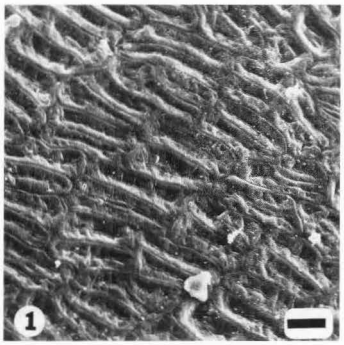
Figures 1 and 2. Scanning electron micrographs of undamaged (Fig. 1) and drought-induced damage of (Fig. 2) resting peanut seed epidermal tissue coated with 25 nm thick gold-palladium coating. Note the absence (in Fig. 1), and the presence (in Fig. 2, arrows) of charging defects (which obscure surface details). Bar = 20 micrometers.

Figure 3. Scanning electron micrograph of drought-induced damage of resting peanut seed epidermal tissue coated with 30 nm thick gold-palladium coating. Bar = 10 micrometers.

Figure 4. Phase contrast light micrograph of peanut tissue with drought-induced damage. The cellular organization of the damaged tissue is disrupted (arrows). Bar = 20 micrometers.

Figure 5. Phase contrast light micrograph of undamaged peanut tissue. Note the fixation of cytoplasmic network (c), protein bodies (p), and starch grains (st). The cytoplasmic network has a net-like appearance as a result of oil being removed from the lipid bodies by alcohol dehydration and leaving spaces in the cytoplasm. Bar = 20 micrometers.

Figure 6. Scanning electron micrograph of undamaged peanut cell. Note the integrity of cytoplasmic network (arrows) at high magnification. Bar = 1 micrometer.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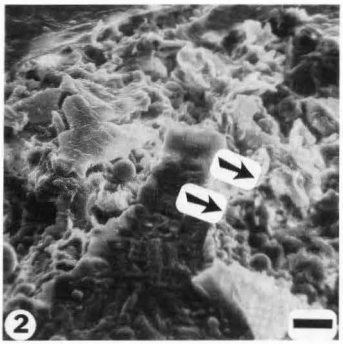
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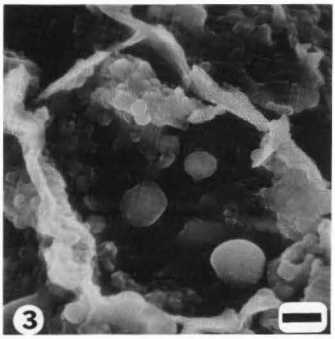
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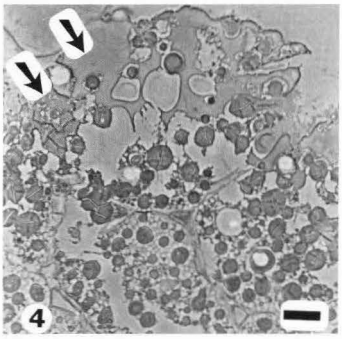
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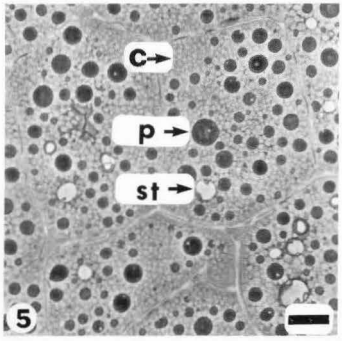
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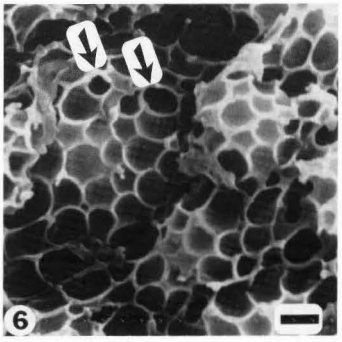
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Discussion with Reviewers

R. Taber: Would the authors distinguish between a resting peanut seed, a dormant seed, and a quiescent seed?
Authors: Resting seed, dormant seed, and quiescent seed are all synonymous terms.

R. Taber: Do the authors prefer LM examination of thick sections of peanut seed that have been embedded for transmission electron microscopy over the traditional paraffin sections? If so, what additional information is gained.
Authors: We prefer plastic-embedded sections over paraffin embedded sections because plastic embedded sections can be stained and examined without the removal of the plastic embedding medium. Paraffin-embedded sections require the removal of paraffin before staining. Our experience has been that cellular components may be lost during staining procedures which require prolonged rinsing of deparaffinized sectioned tissue.

R. Taber: Would it be difficult to use a freeze-fracture technique and cold stage to view damaged kernels? Would you expect this technique to provide the information desired?
Authors: The technique you mention should provide information about damage to lipid bodies because it would enable the lipid bodies to be examined without extracting the oil within the lipid bodies.

R. Taber: What is the advantage of using the digital thickness monitor over simply performing sequential coatings for routine examination?
Authors: The digital thickness monitor enables an investigator to reproduce an optimum coating thickness with reliability. Our experience has been that sequential coatings are time-consuming and may result in a lack of uniformity of coating thickness among different samples.