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PROTEIN BODIES IN DORMANT, IMBIBED AND GERMINATED SUNFLOWER COTYLEDONS

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Abstract

Scanning electron microscopy was used to observe the morphology and structure of protein bodies in dormant and imbibed sunflower cotyledons and to document the morphological changes in protein bodies during germination and seedling growth. In order to clearly visualize dormant seed structure, anhydrous fixation techniques were employed. Definite differences in cellular structure are seen in comparisons of dry and imbibed seed tissues. As germination proceeds, protein bodies lose their smooth spherical shape and become indented and pitted. Protein body coalescence and fusion precedes the formation of a central protein vacuole. As protein is hydrolyzed, protein vacuole density decreases, and its surface becomes granular, then fibrous, in appearance. Removal of protein from the protein vacuole appears to proceed more rapidly in cells closest to the embryonic axis. The protein vacuole becomes the main cell vacuole as remaining storage protein is hydrolyzed. The cotyledon cells undergo a gradual change in function from a quiescent storage stage, through a major exporting phase and to their final function of photosynthesis.

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KEY WORDS: Sunflower, Helianthus annuus, Seed Protein Bodies, Germination, Scanning Electron Microscopy

Introduction

Various forms of nutrient reserves are present in cells of mature seeds which are utilized by the developing seedling. Storage proteins are deposited in organelles which are usually called protein bodies (Lott, 1980). Protein bodies are membrane-bound and contain an amorphous proteinaceous matrix in which inclusions may be embedded (Weber and Neumann, 1980). The two most common types of inclusions are: 1) crystalloids, composed of crystalline protein, and 2) globoids, composed of storage phosphates, usually in the form of phytin (K, Ca, or Mg salt of myo-inositol hexaphosphoric acid). Phytin is a major form of phosphate and mineral storage in many seeds (Bewley and Black, 1978). Crystalline calcium oxalate inclusions are also present in protein bodies of some seeds (Lott, 1981). Rost (1972) used inclusion types to classify protein bodies, and the taxonomic use of protein body structure has been suggested (Buttrose and Lott, 1978).

Saio et al. (1977) investigated sunflower protein body ultrastructure and reported the presence of crystalloid-like inclusions along with globoids. However, observations by Gruber et al. (1970) as well as more recent reports (Buttrose and Lott, 1978; Allen and Arnott, 1981) indicate that crystalloids are absent from protein bodies of sunflower, likewise, crystalloids are not reported in protein bodies of several other members of Compositae, including Lactuca sativa (Paulson and Srivastava, 1968), Bidens radiata (Simola, 1969), Bidens cernua (Simola, 1973), and Helichrysam bracteatum (Buttrose and Lott, 1978).

Sunflower seeds contain 25-32% oil, depending on variety (Vaughan, 1970). Intracellular lipid-containing bodies are easily recognizable with transmission electron microscopy (TEM) of sunflower cotyledons (Buttrose and Lott, 1978) . Ultrastructural features of dormant seeds in their dry state have been difficult to observe because usual preparative techniques for electron microscopy involve aqueous solutions. Buttrose (1973) showed that water uptake by seed tissue occurs very rapidly, and, using anhydrous freeze fracture techniques, he demonstrated marked cytological changes which occurred in seed tissue upon hydration.

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Osmium tetroxide (OsO_4) vapor fixation has been used with limited success for TEM (Perner, 1965; Yatsu, 1965). More recently, Webb and Arnott (1980, 1982), used vapor fixation and scanning electron microscopy (SEM) to demonstrate hydration-related changes in zucchini cotyledons. These changes include straightening of convoluted cell walls and separation of storage lipids into distinct bodies. Several other authors have reported hydration related changes in intact seeds or in isolated tissues or organelles (Tulley and Beevers, 1976; Varriano-Marston and De Omana, 1979; Wolf and Baker, 1980; Allen and Arnott, 1981).

Protein body modification and storage protein mobilization during germination and seedling growth have been studied in several seed types with light microscopy (LM) and TEM (Ashton, 1976; Pernollet, 1978; Weber and Neuman, 1980; Lott, 1981 for reviews). SEM also has proved useful in a variety of seed investigations which include germination, and a bibliography of seed studies through 1976 in which SEM was utilized has been prepared (Brisson and Peterson, 1977).

Many variations in the details of protein body breakdown have been described from which certain generalizations can be made. Protein bodies tend to enlarge or swell very early after imbibition and many become pitted either internally or peripherally. As germination proceeds, the protein bodies within a cell fuse to form a central protein vacuole containing a relatively solid protein mass. A gradual reduction in vacuole density is observed as protein is hydrolyzed and presumably transported from the cells. As the protein vacuole empties, it becomes the main cell vacuole (Ashton, 1976).

Storage proteins are hydrolyzed to peptides or amino acids which are transported to the developing embryonic axis (Beevers, 1968; Ashton, 1976). Digestion is accomplished by proteolytic enzymes which may be contained inside the protein body or synthesized *de novo* during seedling growth (Ashton, 1976; Van Der Wilden *et al.*, 1980). The production or activation of some of these enzymes appears to be controlled by hormones produced by the embryonic axis (Penner and Ashton, 1967; Wiley and Ashton, 1967; Varner, 1964). Schnarrenberger *et al.* (1972) isolated protein bodies from sunflower cotyledons using sucrose gradient and isopycnic centrifugation. They also measured low levels of proteolytic activity in dormant cotyledons and reported activity changes and protein loss which occur after germination.

SEM, TEM, and LM were used in the present study to observe protein bodies in dormant and imbibed sunflower cotyledons and in cotyledons of growing seedlings. Digestion of storage materials during seedling growth is also described. Although the necessity for corroboration of SEM findings with other methods is understood (Clark and Glagov, 1976), it is clear that much valuable information concerning seed structure and physiology can be gained with SEM techniques.

Materials and Methods

Sunflower seeds (*Helianthus annuus* L., cv. Mammoth) were obtained through a local retail supplier from W. Atlee Burpee Company, Clinton, Iowa.

Germination

Shelled seeds were placed on several layers of filter paper in plastic petri dishes and moistened with distilled water. Seeds were allowed to germinate in the dark at 25° C for 3 days. Seedlings were exposed to light on subsequent days through day 7. Germination is considered to commence with initial imbibition, and aqueously fixed dry seeds are considered to be hydrated at 0 day germination. Seeds fixed 24 h after hydration are at day 1 germination. Three or four seedlings of medium size were chosen for each day and all specimens were fixed aqueously.

Anhydrous fixation

Dry seeds were removed from their shells and cut with a razor blade into sections less than 1 mm thick. Tissue sections were suspended on a screen in a sealed vial which contained an opened ampule of 1 g osmium tetroxide (OsO_4) crystals and allowed to fix for 4-6 weeks. Fixed sections were then placed overnight in a vacuum desiccator to draw off excess OsO_4 (Webb and Arnott, 1980).

Aqueous fixation

Cotyledons were cut with a razor blade into approximately 1 mm sections and fixed in 5% glutaraldehyde (GTA) in a 0.1 M sodium cacodylate buffer at pH 7.2 for 8-10 h. Sections were rinsed in four changes of buffer and postfixed for 3 h in 2% OsO_4 in the same buffer. Sections were again rinsed with buffer and dehydrated in a graded ethanol series.

Postfixation preparation for SEM

Anhydrously fixed sections were fractured with forceps and mounted on specimen stubs as discussed below. Aqueously fixed sections, dehydrated to 100% ethanol, were critical point dried in a Pelco unit using liquid CO_2. Dried sections were fractured with forceps and mounted on brass or aluminum specimen stubs with double-sided carpet tape. Prepared stubs were coated with a Polaron E5100 sputter coater using a gold-palladium target and viewed with a JEOL JSM-35C SEM at a working distance of 15 mm and an accelerating voltage of 15 kV.

Postfixation preparation for TEM

Aqueously fixed sections in 100% ethanol were transferred to a 50% mixture of Spurr's low viscosity embedding medium (Spurr, 1969) and ethanol. Infiltration was carried out under constant rotation in capped vials for 12 h. Vial caps were removed to allow ethanol evaporation for an additional 24 h. Sections were transferred to fresh 100% resin and placed in a vacuum desiccator for 8 h. Sections were placed in latex embedding molds with fresh plastic and polymerized overnight at 60° C.

Thin sections were cut with a Sorvall MT-2B ultramicrotome using a diamond knife. Thin sections were supported on copper grids and stained with alcoholic uranyl acetate followed by lead citrate (Reynolds, 1963) for 30 min and viewed with a Zeiss EM9 TEM.

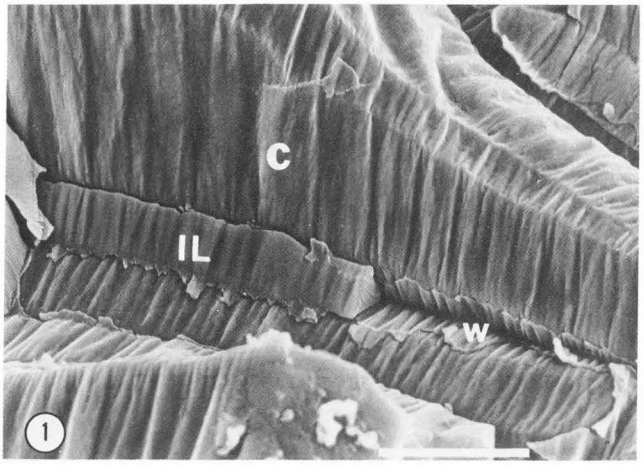
Results

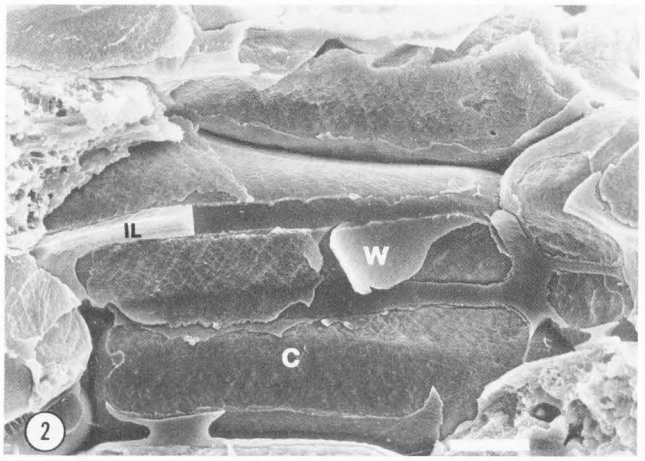
Dormant tissue

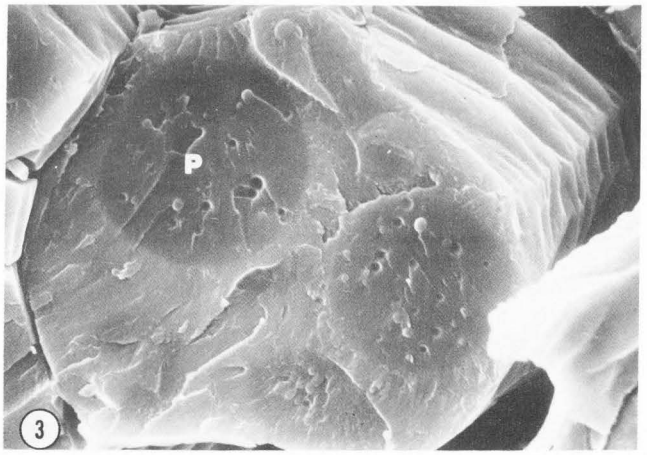
Comparisons of tissues prepared anhydrously with OsO_4 vapor fixation (OVF) and those aqueously fixed reveal marked structural changes which accompany imbibition (compare Figs. 1 and 3 with Figs. 2 and 4). External surfaces of OVF cells are transversely convoluted or compressed (Fig. 1). These convolutions are not present in aqueously fixed tissue (Fig. 2). Extracellular material which has been shown to be lipid in other seeds (see discussion) fills the intercellular spaces in OVF tissue (Fig. 1). This material is also present in most aqueously prepared tissue (Fig. 2); its occasional absence may be explained by inadequate fixation and lipid extraction during alcohol dehydration or by variation in its distribution.

Transversely fractured OVF cells appear solidly packed with storage materials (Fig. 3). Fractured protein bodies are seen as rounded areas which appear darker than surrounding regions (Fig. 3). Many small spherical protein body inclusions are seen which are most probably globoids. No separations between globoids and protein matrix which would suggest soft globoid regions are seen. External protein body surfaces in OVF cells are generally smooth but may be somewhat irregular in appearance, and no surface pattern is present. Aqueously fixed protein bodies also contain globoids, and globoid cavities or soft globoid regions, which appear as separations between globoid crystal and protein matrix, can often be discerned (Fig.4,). Globoids occasionally appear angular or granular which is an indication of partial extraction during fixation (imbibition). Protein body surfaces exhibit flattened regions surrounded by a reticulate pattern of granular cytoplasmic material. Cytoplasmic material also appears between lipid bodies (Fig. 4, arrow).

Remarkable changes in morphology of lipid reserves are seen when OVF tissues are compared with tissues imbibed during aqueous fixation. Lipid in OVF cells appears as a solid mass which surrounds protein bodies and fills the remaining cell volume (Fig. 3). No subdivisions of storage lipid into discrete bodies can be detected; rather, the entire mass fractures like glass. The border between lipid and protein bodies is also fuzzy and indistinct. Lipid in aqueously fixed cells is separated into polygonal units, and a sharp distinction between individual lipid bodies and between lipid and protein bodies is evident (Figs. 4, 5 and 6). A network of granular cytoplasm, which has been termed cytoplasmic reticulum (Webb and Arnott, 1980), is seen between lipid bodies and extends throughout the cell.







Figs. 1-6, 8-16 are SEM views of sunflower (Helianthus annuus L.) cotyledon cells.
Fig. 1. Osmium vapor fixed (OVF) dormant cells showing convoluted cell walls (W) and associated external cytoplasmic surface (C). Intercellular lipid (IL) can be seen. Bar = 10 μm.
Fig. 2. Aqueously fixed "dormant" seed showing imbibed cells. Note that cell wall (W) and cytoplasmic surfaces which are immediately interior to cell wall (C) no longer show convolutions. Intercellular lipid (IL) can be seen. Bar = 10 μm.
Fig. 3. Fractured surface of an OVF cell. Intracellular storage lipid fractures smoothly and protein bodies (P) appear embedded in a lipid matrix. Globoids and globoid holes are present. Bar = 1 μm.

Complementary faces of fractured, imbibed (aqueously fixed) tissue provide an excellent demonstration of the cellular features (Figs. 5 and 6). A fractured granular region, which possibly represents the cell nucleus, can be seen in both faces. The size and shape of the large protein body clearly demonstrate its relationship to the many lipid bodies that surround it. Clear correlation of globoid structure and number can be gained by comparing the fracture faces of four protein bodies shown in Figures 5 and 6. The smooth contour of the cell wall is a dramatic change that occurs with imbibition; one can also see the relationship between cell wall and lipid bodies. Note that the fracture process occasionally displaces globoids, and they may be seen overlaying other cell components (Fig. 5, arrow). These bodies are clearly displaced because there is no complementary hole. This also correlates with the fact that in some cases neither complementary fracture shows a globoid, but both show holes.

TEM observations of aqueously prepared material provide confirmation of the SEM as well as show new or complementary information (Fig. 7). Because of their dense nature, globoids tend to pull out during thin sectioning, or they may be extracted from sections during staining. This leaves holes in the protein bodies that are larger than actual globoids seen with SEM. Enlargement may be due to deformation of the material during sectioning or by instability and shrinkage of the plastic in the electron beam. Electron-dense cytoplasmic regions surround the protein and lipid bodies and extend throughout the cell. Occasionally, small organelles may be seen in this dense cytoplasmic matrix. As seen in SEM, the lipid bodies are more numerous than protein bodies and do not appear to contain inclusions. Intercellular spaces contain material which is similar to lipid bodies in electron density (Fig. 7, IL). This material may represent intercellular lipid.

Protein body modification and change during germination

The pattern and sequence of protein body change in sunflower cotyledons can be represented in three distinct stages: 1) intact protein bodies (imbibition through day 1 of germination) (Figs. 1-8); 2) coalescence and fusion of protein bodies (day 2 through day 3 of seedling growth) (Figs. 9-11); and 3) hydrolysis of storage proteins and vacuole formation (day 3 through day 7 of seedling growth) (Figs. 12-17). A similar sequence has been described for Cucurbita maxima cotyledons by Davis (1974), and for C. pepo (zucchini) by Webb and Arnott (1980).

1) Intact protein bodies -- Enlargement of protein bodies at day 1 following imbibition is accompanied by the appearance of large, smooth indentations in the external protein body surfaces (Fig. 8). These depressions, along with numerous smaller ones, are probably due to pressure on the expanding protein bodies from surrounding lipid bodies and cytoplasmic organelles. Fractured protein bodies reveal extensive internal degradation of the proteinaceous matrix (Fig. 9). Some internal pitting appears to extend from the protein body surface and results in small surface holes (Fig. 8, arrow). Globoids are numerous and appear to be somewhat enlarged and soft globoid zones or globoid cavities are easily observed. While it is difficult to distinguish between globoid holes and actual protein body degradation, it seems likely that at least the larger holes which contain fibrous material represent protein hydrolysis.

Mobilization of intracellular lipid is not apparent during this stage. Although lipid bodies may appear more rounded, no definite degradative changes can be seen. Intercellular lipid material is occasionally seen, but is usually eroded or pitted. Whether this erosion is artifactual or actually represents storage mobilization, has not been determined. Intercellular lipid is never seen in cotyledons of older seedlings, which does suggest that it is utilized at this time.

2) Protein body fusion -- Coalescence of protein bodies appears to occur uniformly in cells throughout the cotyledon about 2 days after germination. The process of fusion occurs over a period of 1-2 days and, in cells which exhibit very early stages of protein body fusion, separate protein body outlines can easily be discerned at the periphery of the protein mass (Fig. 10, arrows). The fractured face of the fusing protein bodies has a smooth appearance, and numerous embedded globoids are seen. More complete coalescence and condensation of fused material into a central vacuole is apparent in Figures 11 and 12. Although protein body membranes cannot be distinguished with SEM, it does appear that actual fusion does occur.

3) Protein hydrolysis and vacuole formation -- Final protein hydrolysis appears to commence after protein body fusion and during formation of the protein vacuole. As development continues, the surface of the protein vacuole first becomes granular (Fig. 11) then slightly fibrous or spongy (Fig. 12) in appearance. Globoids, which appear somewhat enlarged in previous stages, show signs of decomposition. Many globoids appear as hollow pitted shells (Fig. 11, arrows), and they are absent in more mature cells.

The change in protein vacuole appearance suggests a reduction in the amount of protein which it contains and may be described as a reduction in protein vacuole density. As protein is removed from the vacuole, its fibrous nature becomes more apparent (Fig. 13), and the strands have a somewhat beaded appearance. Protein vacuoles sometime appear subdivided by cytoplasmic strands which suggests they are composed of several smaller vacuoles which clump together near the center of most cells. Spherical cores of more solid material can be seen within many vacuoles (Fig. 13). This may indicate that protein vacuoles are hydrolized from the periphery inward.

Unlike the protein body fusion, protein hydrolysis does not occur simultaneously throughout the length of the cotyledon. Protein decomposition first begins on about day 3 in cells closest to the embryonic axis and

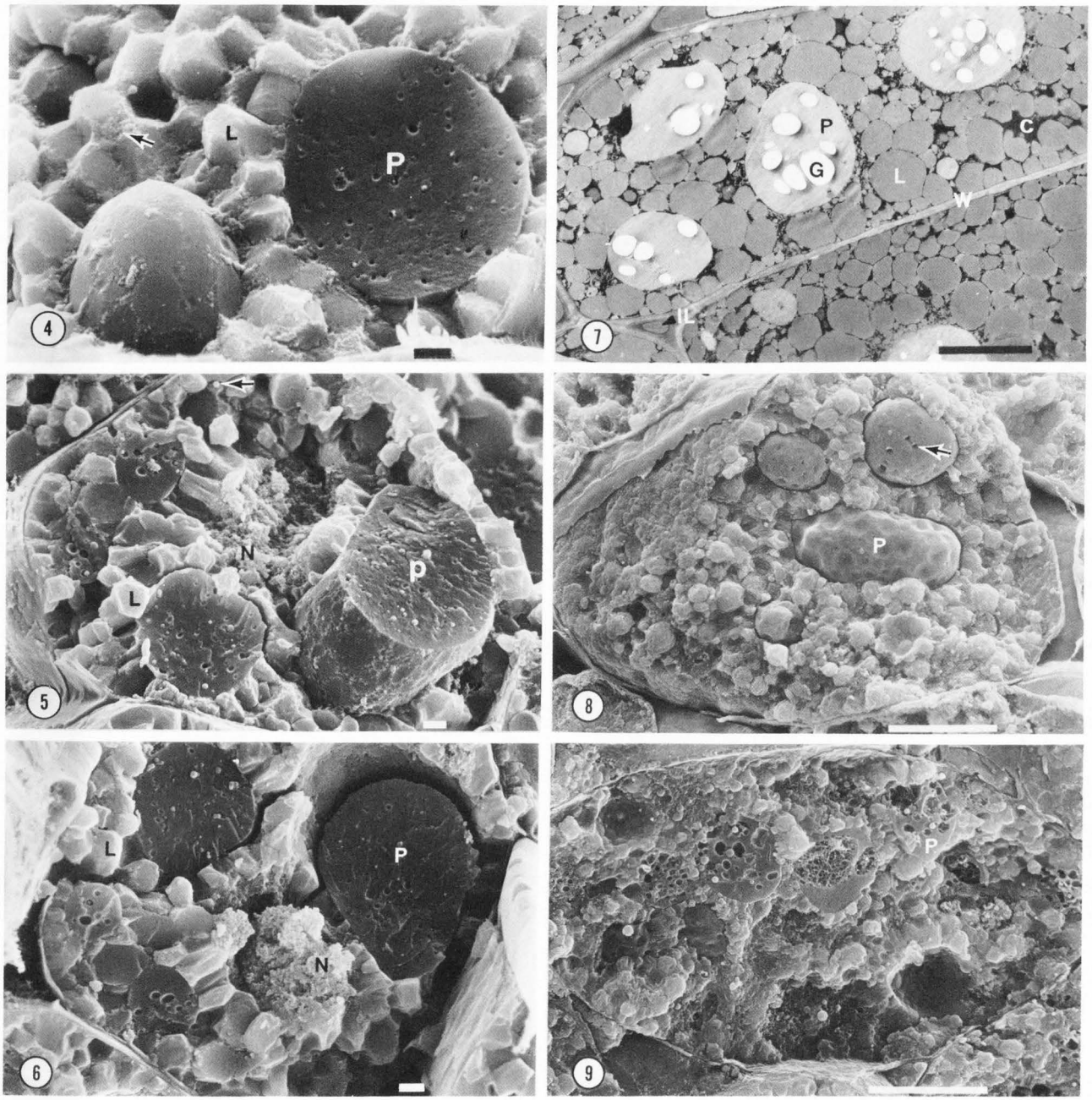


Fig. 4. Fractured surface of an aqueously fixed (imbibed) seed. Fractured protein body (P) contains many globoids and globoid holes. External protein body surface can be seen. Individual lipid bodies (L) are polygonal in shape and are separated by cytoplasmic reticulum (arrow). Bar = 1 μm.
Figs. 5 and 6. Complementary fracture faces of an aqueously fixed (imbibed) seed showing the interrelationship between protein bodies (P) and lipid bodies (L). Many globoids can be seen. Some globoids are displaced during the fracture process (arrow). Central granular area is believed to represent the nucleus (N). Bar = 1 μm.
Fig. 7: TEM view of aqueously fixed (imbibed) sunflower seed showing smooth walls (W), lipid bodies (L), protein bodies (P), and cytoplasmic reticulum (C). White areas in protein bodies represent places where the globoids (G) have dropped out. Intercellular lipid is also present (IL, arrow). Bar = 5 μm.
Fig. 8: Fractured cell at day 1 postimbibition. Protein bodies (P) show indentions and holes (arrow) in their surfaces. No intercellular lipid material is visible. Bar = 10 μm.
Fig. 9: Fractured protein bodies at day 1 postimbibition showing prominent pitting within protein bodies (P). Some larger internal holes which contain strands of fibrous material are seen. Bar = 10 μm.

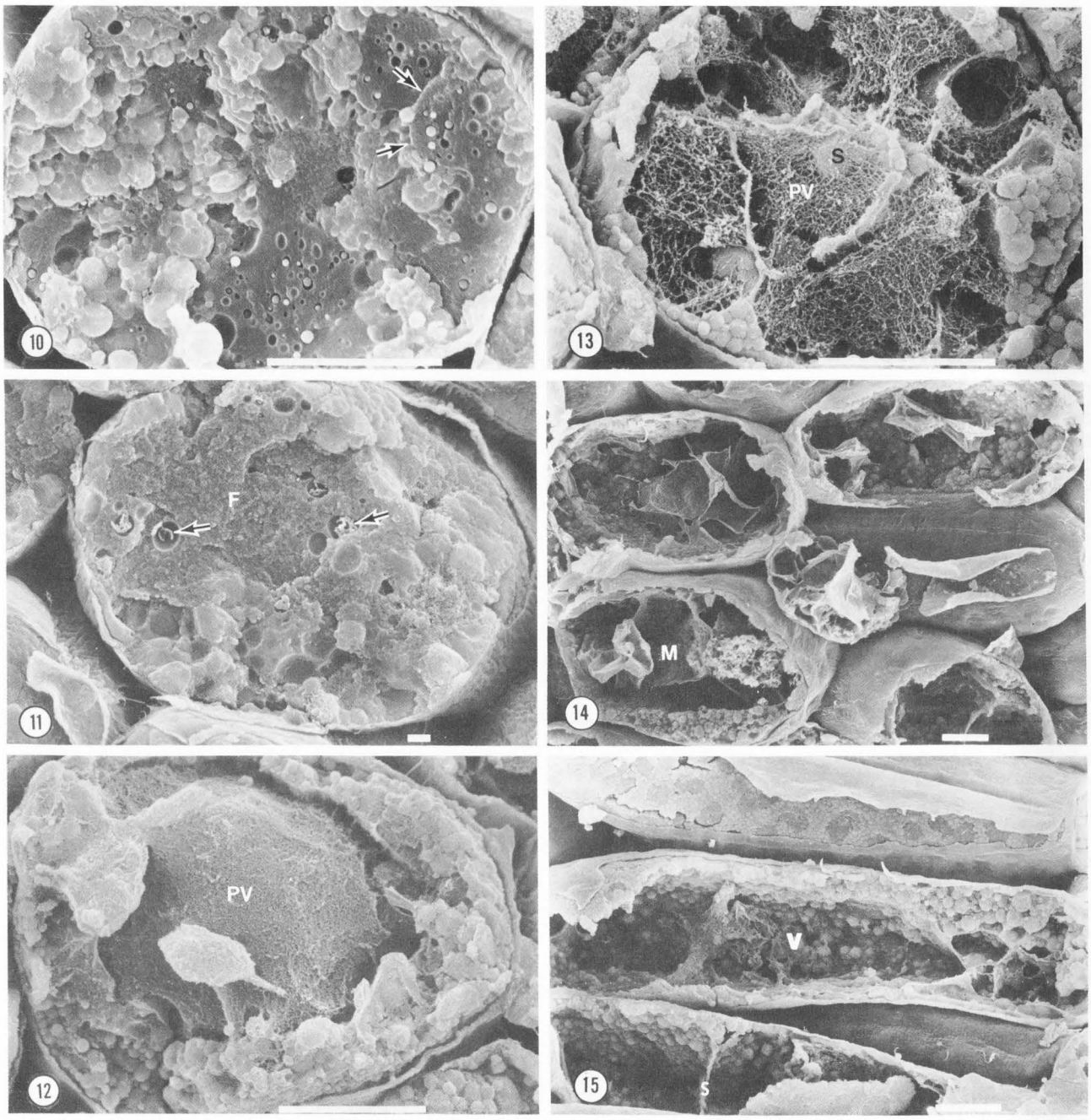


Fig. 10: Fractured cell at day 3 postimbibition showing protein bodies which appear to be coalesced. Globoids are still present. Separate protein body outlines can be seen (arrows). Bar = 10 μm.
Fig. 11: Fractured cell day 3 postimbibition showing fused protein mass (F) with enlarged globoid areas which contain remnants of globoids (arrows). Bar = 10 μm.
Fig. 12: Fractured cell day 4 postimbibition showing central protein vacuole (PV) which has a fibrous external surface. Lipid bodies and other organelles are seen at cell periphery. Bar = 10 μm.
Fig. 13: Fractured cell day 4 postimbibition showing several protein vacuoles (PV) with fibrous protein contents. A solid core of materials can be seen in the vacuoles (S). Lipid bodies and organelles are peripherally located. Bar = 10 μm.
Fig. 14: Fractured cells day 5 postimbibition showing large central vacuoles with peripheral organelles. Some vacuoles are subdivided by membraneous structures (M) and some residual protein may be seen. Bar = 10 μm.
Fig. 15: Fractured palisade parenchyma cells showing cytoplasmic strands (S) extending across central vacuole (V). Cell wall has been stripped away on upper cell to reveal external view of cytoplasm composed of spherical lipid bodies and chloroplasts. Bar = 10 μm.

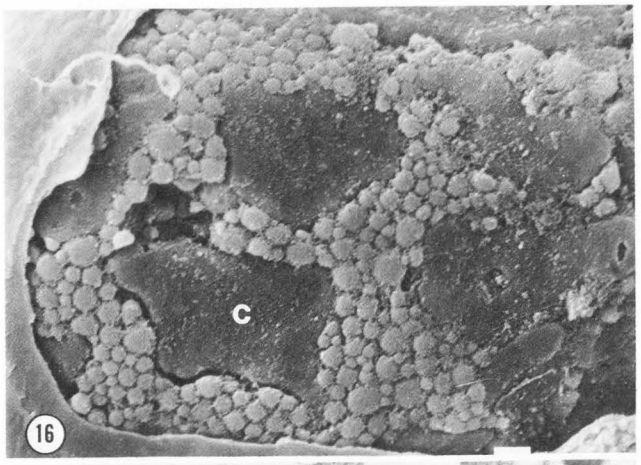
progresses to more distal regions on succeeding days. For this reason, it is possible to observe cells along the length of a single cotyledon at several different stages of protein removal. For example, the cell seen in Figure 13 was located near the axis of a day 4 cotyledon, while the cell in Figure 12 was located near the center of a similar cotyledon. Although the cell in Figure 11 was located in a day 3 cotyledon, similar cells can be seen in distal regions of day 4 cotyledons. By day 6, all cells appear to be devoid of storage proteins.

As storage protein is depleted, it is apparent that the emptied protein vacuoles become the main cell vacuole (Fig. 14). Lipid bodies and cytoplasm are restricted to the cell periphery, and the remnants of a torn membranous structure, which may represent the tonoplast, can be seen in several fractured cells (Fig. 14). These membranes appear to subdivide the vacuole space into several chambers which may represent separate vacuoles which eventually fuse. Vacuolate cells are seen throughout the cotyledon by days 6 and 7 after germination, and cell vacuoles in day 7 cotyledons are usually not subdivided.

Irregularly shaped structures can be seen immediately interior to the cell wall in appropriately fractured day 6 cells (Fig. 15). These bodies, seen at higher magnification in Figure 16, are plastids and are observed with TEM to contain grana and starch grains (Fig. 17). Lipid bodies and other organelles, which may include microbodies and mitochondria, surround plastids in these cells. Dramatic changes in lipid body structure are not observed during the period of cotyledon development studied. Lipid bodies appear essentially unaltered until protein hydrolysis is well underway. They then assume a more spherical shape and gradually become smaller on succeeding days. Small, spherical lipid bodies surround the plastids in day 6 and day 7 cells and are visible in Figures 16 and 17.

Discussion

Dormant sunflower cotyledons are compact storage structures. Cells are engorged with storage materials and appear to be longitudinally compressed, resulting in deep convolutions of the cell walls. "Wavy" cell walls have been reported in dry seed tissues by a number of authors (Lott, 1974; Perner, 1965; Buttrose, 1973; Webb and Arnott, 1980). Following hydration, cellular expansion results in straightening of convoluted cell walls. Similar phenomena have been described in both zucchini cotyledon and barley scutellum and in yucca and okra seeds (Webb and Arnott, 1980; 1982; Buttrose, 1973). Webb and Arnott (1982) have shown a close association of cytoplasm to convoluted cell walls which is maintained during cellular expansion at imbibition. They suggest cell wall compression may be important for maintaining contact between cellular components which shrink differentially during dehydration.



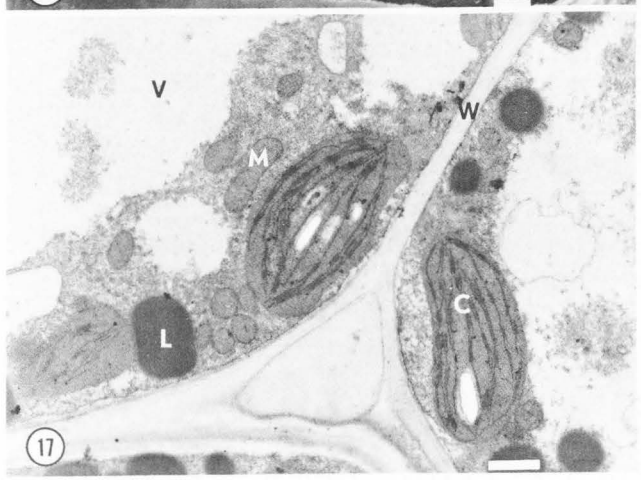


Fig. 16: External view of chloroplasts (C) and surrounding lipid bodies and other organelles in a day 5 postimbibition fractured cell. Bar = 1 μm.

Fig. 17: TEM view of day 7 postimbibition cells showing chloroplasts (C), lipid bodies (L), wall (W), central vacuole (V), and mitochondria (M). Bar = 1 μm.

Material which fills intercellular spaces has been reported in storage tissues of several oil seeds, and has been characterized histochemically as lipid (Webb and Arnott, 1980; 1981). In sunflower, this material appears to be digested within the first few days of germination and is presumed to be additional storage material; however, the details of its metabolism are unknown.

Intracellular storage lipid is present in dry cells and appears as a continuous mass which surrounds the protein bodies and fills the remaining space. Upon hydration, a membranous network of cytosol becomes hydrated and separates the lipid into distinct bodies. Webb and Arnott (1980) also observed glassy fracture surfaces of lipid in zucchini cotyledons with SEM, and by using freeze fracture replicas, they were able to detect rows of particles which outline lipid bodies in dry tissues. These particles were not visible with SEM and may represent a "dormant" or dehydrated form of membrane as

suggested by Simon (1974). Webster and Leopold (1977) also report that membranes in soybean cotyledons lose their integrity by dehydration. More recently, however, McKersie and Stinson (1980) found membranes in Lotus corniculatus seeds to be damaged by dehydration, but x-ray diffraction indicated that they maintained their laminar structure. Therefore separation of lipid bodies during imbibition may simply be due to expansion of the surrounding cytosol.

The protein bodies present in OVF tissues appear very similar to those in aqueously prepared material. Globoid inclusions are easily observed; however, crystalloid or crystalloid-like protein inclusions are never seen. These observations agree with previous reports and published micrographs of sunflower seeds and seeds of several other Compositae (Buttrose and Lott, 1978). Soft globoid inclusions, which appear as a cavity surrounding the hard, globoid crystal, are not seen in dormant tissues, but do appear and enlarge in aqueously prepared and germinated material. The development of soft globoid regions appears to be hydration dependent, or possibly due to extraction of globoid components during aqueous fixation (Buttrose and Lott, 1978; Lott et al., 1978).

Cytological changes which accompany germination represent storage reserve mobilization. Sunflower protein bodies first swell and undergo internal change, then fuse to form one or more large protein vacuoles. This pattern, with variations, is described for a large number of species and is the most commonly reported protein body fate (Ashton, 1976). Peripheral degradation of protein bodies is reported less frequently. Horner and Arnott (1966) reported internal degradation of protein bodies in the perisperm of three species of Yucca; however, in Yucca schidigera, peripheral erosion occurs. Ashton (1976) suggested that internal degradation is mediated by proteolytic enzymes (endopeptidases) present within protein bodies, and an acid proteinase has been demonstrated in sunflower protein bodies (Schnarrenberger et al., 1972). Protein body autolysis has been shown in a number of cases (Davis, 1974; Harris and Chrispeels, 1975), and protein body fusion occurs in acetone defatted, water soaked sunflower seeds within 15 minutes (Allen and Arnott, 1981). These details, plus the simultaneous occurrence of protein body fusion throughout the cotyledon, suggest that this is a programmed response automatic upon hydration, and that hydration may activate endogenous protein body enzymes. Whether these enzymes are controlled by the embryonic axis is not known at present. A dramatic increase in proteinase activity is suggested by the distinct progressive reduction in protein vacuole density which begins at about day 3 and continues through day 5 after imbibition. Biochemical data reported by Schnarrenberger et al. (1972) correspond closely with these observations. Low levels of proteinase activity were reported in dormant seeds and in seedlings 1 and 2 days after germination, followed by a rapid increase in activity and a gradual reduction of total protein on days 3-5.

Van Der Wilden, et al. (1980) have shown that proteolytic enzymes are produced in the cytosol of cotyledon cells in mung bean seedlings. These enzymes later accumulate in the protein bodies. We speculate that sunflower protein bodies are mobilized during two phases of proteolytic activity. The first which results in protein body fusion, may be facilitated by endogenous protein body enzymes. However, the second phase which results in protein vacuole hydrolysis may be mediated by enzymes produced de novo in the cytoplasm. The first phase may be necessary to release amino acids required for enzyme synthesis.

The proximity of cotyledon cells to the embryonic axis appears to influence the timing of protein digestion. Hydrolysis is initiated close to the axis and spreads progressively throughout the cotyledon. Although we have not investigated the effects of the axis on protein mobilization, these results do suggest that the second phase of enzyme activity is under hormonal control. The effect of the embryonic axis on proteinase activity has been reported in other seeds (Wiley and Ashton, 1967; Webb and Arnott, 1980), and Penner and Ashton (1967) were able to reproduce the effect of the axis with exogenous hormones in squash (Cucurbita maxima) seedlings. Hormonal control of de novo enzyme synthesis within the aleurone layer of several cereal grains is well documented (Bewley and Black, 1978); however, similar processes in dicot seeds have not been completely demonstrated. Globoid and lipid digestion in sunflower appears to coincide with the initiation of protein hydrolysis; therefore, it is possible that phytase and lipase production begin concomitantly with that of proteinase, starting at about day 3 after germination, and that the production of these enzymes is also hormonally controlled. Additional studies using excised sunflower cotyledons and exogenous hormones, along with protein synthesis inhibitors, may provide useful information in this area.

As storage protein is hydrolyzed from the cotyledon, it becomes apparent that the emptied protein vacuole is transformed into the main cell vacuole. The theory that protein bodies give rise to the vacuole is well established and supported by many authors (Ashton, 1976), and is consistent with the present study. Cytoplasmic strands, which appear to subdivide the vacuole, suggest that all protein bodies in a cell may not fuse to form a single protein vacuole, rather several protein vacuoles may form and empty (Figs. 13 and 14). These vacuoles may subsequently fuse to form the main cell vacuole. The cotyledons develop over the period of germination and seedling growth from quiescent storage structures to metabolically active photosynthetic units.

SEM, combined with various fixation techniques, is a unique and valuable tool for the investigation of seed germination and seedling development. Although TEM has been used extensively in previous seed studies and is required for comparison of SEM findings and organelle identification, many details, such as the surface texture and fibrous nature of the protein

vacuoles, could not be fully appreciated using more traditional methods. The capability of scanning large areas provides cell-by-cell comparisons throughout a specimen and facilitates correlation of cytological characteristics with respect to cellular location. Energy dispersive x-ray analysis, combined with SEM provides another valuable method for analysis. These techniques seem applicable to many comparative or developmental studies in seeds.

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Discussion with Reviewers

H. T. Horner, Jr.: In sunflower do all protein bodies fuse together to form one large protein mass or are there several masses produced per cell?
Authors: We believe that several protein masses are produced, and their masses coalesce to form a single protein vacuole such as is seen in Figure 12. Further hydrolysis in the protein vacuole reveals it to be composed of several vacuoles which may be fused at a later time.

Reviewer III: Do the authors consider that seeds imbibed in a fixation medium are representative of the physiological imbibition state? Are the differences between dormant seed and aqueous fixed "imbibed" seed ultrastructure due to biological alteration or to fixation procedures?
Authors: We believe the structural changes which accompany imbibition to be the physical result of hydration of this tissue. The differences between dormant and aqueously fixed seed structure are due to fixation in an aqueous medium which causes imbibition to occur. We are not aware of any studies which indicate that glutaraldehyde in an aqueous solution modifies the imbibition of a seed. These physical changes are presumed to occur in the "normal" germination process. We have observed cotyledons of seed soaked in water for up to 3 h before fixation, and although some protein body pitting similar to that seen in 1 day cotyledons (Figure 9) is seen in a few cells of 3 h soaked seeds, cells in 1 and 2 h soaked seeds are essentially identical to those in aqueously fixed seeds.

H. T. Horner, Jr.: How can you be sure that your anhydrous fixation procedure stabilized the seed tissue? Does the OsO_4 penetrate 1/2 to 1 mm into dry seed sections?
Authors: The major need for fixation of dry oilseed material for SEM is to stabilize the storage lipid. Protein bodies and other cytoplasmic features can be seen in acetone-extracted tissue without fixation and appear to be stable. Lipid on the other hand is released from fractured unfixed cells and obscures cellular details (Allen and Arnott, 1981). Penetration of OsO_4 depends on exposure time but generally penetration does not exceed 0.5 mm. The central regions of larger seed sections are often unfixed and can be easily distinguished visually (unfixed regions appear white), and with SEM (unfixed regions are obscured with lipid).

H. T. Horner, Jr.: Using the secondary electron mode of your SEM can you really determine density differences in your seed material?
Authors: Differences in electron density can be determined by secondary electrons (Postek, et. al., 1980). These differences allow visualization of darker, less electron dense, protein bodies embedded in lighter osmiophilic and more electron dense lipid regions in Figure 3. We have also used the phrase "reduction in density" to describe the loss of material from protein vacuoles. Protein vacuoles appear to retain their volume while the amount of storage proteins they contain appears to be reduced.

J. N. A. Lott: Does the torn membrane structure (Fig. 14) mean that you have considerable processing damage? What do complementary fracture faces reveal?
Authors: Since these cells are physically broken open during the fracture process, one

expects a certain amount of damage to intracellular components. However, membranes within the vacuole appear to divide it into subchambers which may represent separate vacuoles. Cells which fracture evenly usually exhibit similar membrane patterns in complementary faces.

H. T. Horner, Jr.: What is your explanation of the "fibrous nature" of the protein vacuole?
Authors: The protein vacuole appears to be composed of a large number of strands which become less numerous as storage protein hydrolysis progresses. This apparent fibrosity may be due to the characteristic fixation of fluid storage proteins. However, we have not observed unfixed cotyledons of seedlings, so it is difficult to speculate on the true nature of the protein in the vacuoles prior to fixation.

Reviewer IV: Would the authors care to speculate as to what may be lost where pitting is observed on the protein bodies? Is this, perhaps, due to the loss of phytin? Have the authors looked at protein bodies with energy dispersive x-ray analysis?
Authors: Protein body pitting may be due to loss of phytin; however, since globoids are present in 1 day protein bodies as well as in later stages, it does not seem likely. The strands of fibrous material seen in some of the larger internal holes (Fig. 9) resemble the fibers seen in protein vacuoles (Fig. 12). This suggests that these holes result from protein hydrolysis, although smaller holes may be globoid cavities. We have not analyzed these seeds by energy dispersive x-rays.

Reviewer IV: Did the authors compare the protein bodies from dried dormant seeds with protein bodies from fresh collections? Air drying of seeds apparently induces shrinkage (wavy cell walls), have the authors looked at fresh seed that were critical point dryed?
Authors: We have not looked at fresh collections or critical point dryed fresh seeds. It would be interesting to see when cell wall wrinkling is initiated.

Reviewer IV: Can any correlation be made from the number of protein bodies and surrounding lipid bodies as to whether the seed is from a healthy or diseased plant?
Authors: It seems reasonable that such correlations could exist. Investigations of these and other factors such as soil fertility and water availability would be interesting.

Additional Reference

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