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CALCIUM OXALATE CRYSTALS IN DEVELOPING SOYBEAN SEEDS

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Abstract

Young soybean seeds contain massive amounts of calcium oxalate (CaOx) monohydrate crystals during development. A test for Ca and CaOx indicated Ca deposits and crystals initially occurred in the funiculus, where a single vascular bundle enters the ovule. Crystals formed in the integuments until the embryo enlarged enough to crush the inner portion of the inner integument. Crystals then appeared in the developing cotyledon tissues and embryo axis. The crystals formed in cell vacuoles. Dense bodies and membrane complexes were evident in the funiculus. In the inner integument, vacuoles assumed the shape of the future crystals. This presumed predetermined crystal mold is reported here for the first time for soybean seeds. As crystals near maturity, a wall forms around each crystal. This intracellular crystal wall becomes contiguous with the cell wall. Integument crystals remain visible until the enlarging embryo further crushes the integuments, then disappears. A related study revealed that the percentage of oxalate by dry mass reached 24% in the developing + 16-day (postfertilization) seeds, and then decreased during late seed maturation. CaOx formation and disappearance are an integral part of developing soybean seeds. Our results suggest that Ca deposits and crystals serve as a Ca storage function for the rapidly enlarging embryos. The oxalate, derived from one or more possible metabolic pathways, could be involved in seed storage protein synthesis.

Key Words: Calcium, crystals, development, *Glycine max*, ovule, oxalate, seed, soybean.

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Introduction

Calcium oxalate (CaOx) crystals are common within many gymnosperms and angiosperms, and they occur in different plant tissues including roots (Arnott, 1966, 1976), leaves (Horner and Whitmoyer, 1972; Horner and Zindler-Frank, 1982a), stems (Grimson and Arnott, 1983; Sakai and Hanson, 1974), seeds (Buttrose and Lott, 1978; Lott and Buttrose, 1978; Webb and Arnott, 1982, 1983), floral organs including gynoecia (Tilton and Horner, 1980) and anthers (Horner and Wagner, 1980, 1992), and root nodules (Sutherland and Sprent, 1984). Arnott and Pautard (1970), Arnott (1982), Franceschi and Horner (1980a), and Horner and Wagner (1995) have reviewed the literature of CaOx occurrence in higher plants and have listed numerous reports that correlate CaOx crystal shape to specific anatomical tissues. Crystals are rare, however, among the ferns and other lower vascular plants, but there are many reports of crystals in lichens (Wadsten and Moberg, 1985), fungi (Arnott, 1995), and algae (Friedmann *et al*., 1972; Pentecost, 1980).

Calcuim oxalate crystals are the most common form of insoluble calcium salt that occurs in plants (Arnott, 1976). The chemical makeup of CaOx crystals (hydration state; mono- or di-hydrate; Frey-Wyssling, 1981), shape, and location in a given tissue or cell type are considered to be specific for a particular species, as determined by intrinsic factors in the cell, tissue, and organ in which the crystals occur (Franceschi and Horner, 1979; Kausch and Horner, 1982). Sometimes, crystals form in special cells called crystal idioblasts. These cells have shapes, sizes, and intracellular structures quite different from noncrystal-forming cells of the same tissue (Foster, 1956; Arnott and Pautard, 1970; Horner and Wagner, 1995).

The shape(s) of CaOx crystals is(are) typically consistent within a given taxonomic group (Arnott, 1966, 1976; Arnott and Pautard, 1970). This property has prompted some researchers to correlate phylogeny with crystal shape (Heintzelman and Howard, 1948). However, studies within some crystal-producing families, such as the Leguminosae, have shown that crystal shapes do not show a discernible pattern of phylogeny. For instance, in a survey of the

Leguminosae (167 species from 89 genera), Zindler-Frank (1987) found that different shapes of CaOx crystals occur throughout the plant organs. In another study, Buss and Lersten (1972) identified prismatic and styloid CaOx crystals in tapetal cells in all three legume subfamilies (84 species from 52 genera). No correlation of crystal shape could be established among the various taxa.

Even though, crystals commonly occur on or in the cell walls of gymnosperms, most of the crystals in the angiosperms form inside cell vacuoles (Kinzel, 1989). In some angiosperm plant organs, the amount of CaOx and oxalic acid may be exceptionally high, such as, in a species of cactus (CaOx, 85% dry mass; Cheavin, 1938) and in developing soybean ovules (oxalic acid, 24% dry mass; Ilarslan *et al*., 1997). Recently, there has been an increased interest in the presence and value of plant crystals, and the special cells in which they form. However, their functional significance still remains unclear. Functional significance (Schneider, 1901) has been eluded to in some studies, such as, protection against insects and foraging animals (Thurston, 1976), contribution to strength of tissue, storage of Ca (Franceschi and Horner, 1980b), binder of toxic oxalate (Borchert, 1984), involvement in surrounding tissue degradation (Kausch and Horner, 1981; Horner and Wagner, 1980, 1992), in-plant Ca regulation (Franceschi, 1989), light gathering and reflection (Franceschi and Horner, 1980a), and salt stress and homeostasis (Hurkman and Tanaka, 1996).

A well-defined or model system for studying plant crystals is needed. Several have been utilized, such as, in *Psychotria* (Horner and Whitmoyer, 1972; Franceschi and Horner, 1979); *Lemna* (Franceschi, 1989); *Vitis* (Webb *et al*., 1995); *Canavalia* (Zindler-Frank, 1975); and *Capsicum* (Horner and Wagner, 1980, 1992). To combine crystal formation with quantification may also aid in better understanding the functional role(s) CaOx crystals play in the life of a plant or in one of its organs. Developing soybean seeds may serve this purpose because crystals seem to be associated with an integrated system of both differentiating and degenerating tissues (Ilarslan *et al*., 1997). Oxalate crystals initially were reported in soybean embryo mesophyll by Wallis (1913), and later by Winton and Winton (1932). Therefore, our objective in this study is to describe the correlation between formation and loss of CaOx crystals, and suggest the functional role(s) these crystals, crystal cells, and oxalic acid play in soybean ovule and seed development.

Materials and Methods

Normal (N) line soybean ovules (prefertilization) and seeds (postfertilization) (*Glycine max* L. Merr. cv. Harosoy, Leguminosae) were studied at -1 day (prefertilization), 0 day (fertilization) and +1 day, +3 days, +5 days, +8 days, +16 days, +24 days, +32 days, and +60 days (postfertilization). Flower buds and young fruits at these developmental stages were collected from plants in a growth chamber with a flower induction photoperiod and temperature (29°C) regime of 18 hours light during the initial four weeks, 16 hours during the fifth week, and 14 hours until maturity. Nighttime temperature was always 26°C. Flowers also were sampled from field-grown plants for comparative purposes.

Clearing method

This technique was modified from Zindler-Frank (1974) to retain the crystals. Ovules, young seeds, anthers, and ovary walls were dissected out of floral buds and placed in 95% ethyl alcohol (EtOH) overnight. Older seeds were sectioned longitudinally to provide thinner slices for viewing. To dissolve and remove cell cytoplasm, but not the crystals and cell walls, dissected seeds and seed slices, anthers, and ovary walls were treated with 2.5% Clorox (sodium hypochlorite, NaOCl; The Clorox Company, Oakland, CA]) for 8 hours (small ovules and young seeds) and for 1-2 days (older seeds). After these treatments, cleared samples were dehydrated through a graded EtOH series, then EtOH/xylene, pure xylene, and mounted in Permount (Fisher Scientific, Fort Lawn, NJ) on slides and cover slips were added (Ilarslan *et al*., 1997). The time for each step varied depending upon the size of the ovule, seed or seed slice. Crystals in clearings of whole ovules, seeds and slices were viewed by using a Leitz Orthoplan microscope (Ernst Leitz, Wetzlar, Germany) with plan-apochromatic lenses, fitted with polarizing filters. Kodak Ektachrome 64T and Techpan films (Kodak, Rochester, NY) were used to record the images.

Microscopy

Soybean ovules and young seeds at different stages of development were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in sodium/potassium phosphate buffer (0.1 M, pH 7.2) at room temperature (RT; 22°C). The ovules and seeds were dissected out of floral buds in the fixative, placed under vacuum at 15 psi (6.89 kPa) for 2 hours, and then placed in fresh fixative at 4°C for 12 hours. Fixed samples were passed through three buffer rinses, postfixed in 1% osmium tetroxide in the same buffer for 4 hours at RT, the samples were rinsed several times in the buffer, stained with 5% uranyl acetate in distilled water, and dehydrated in a graded acetone series to pure acetone. The samples were infiltrated in a series of 3:1, 1:1, 1:3 acetone/resin (v/v) to pure resin using a rotator over a 2-week period (fresh resin daily), and then embedded in Spurr's resin (hard recipe; Spurr, 1969). The same samples were used for both light microscopy (LM) and transmission electron microscopy (TEM).

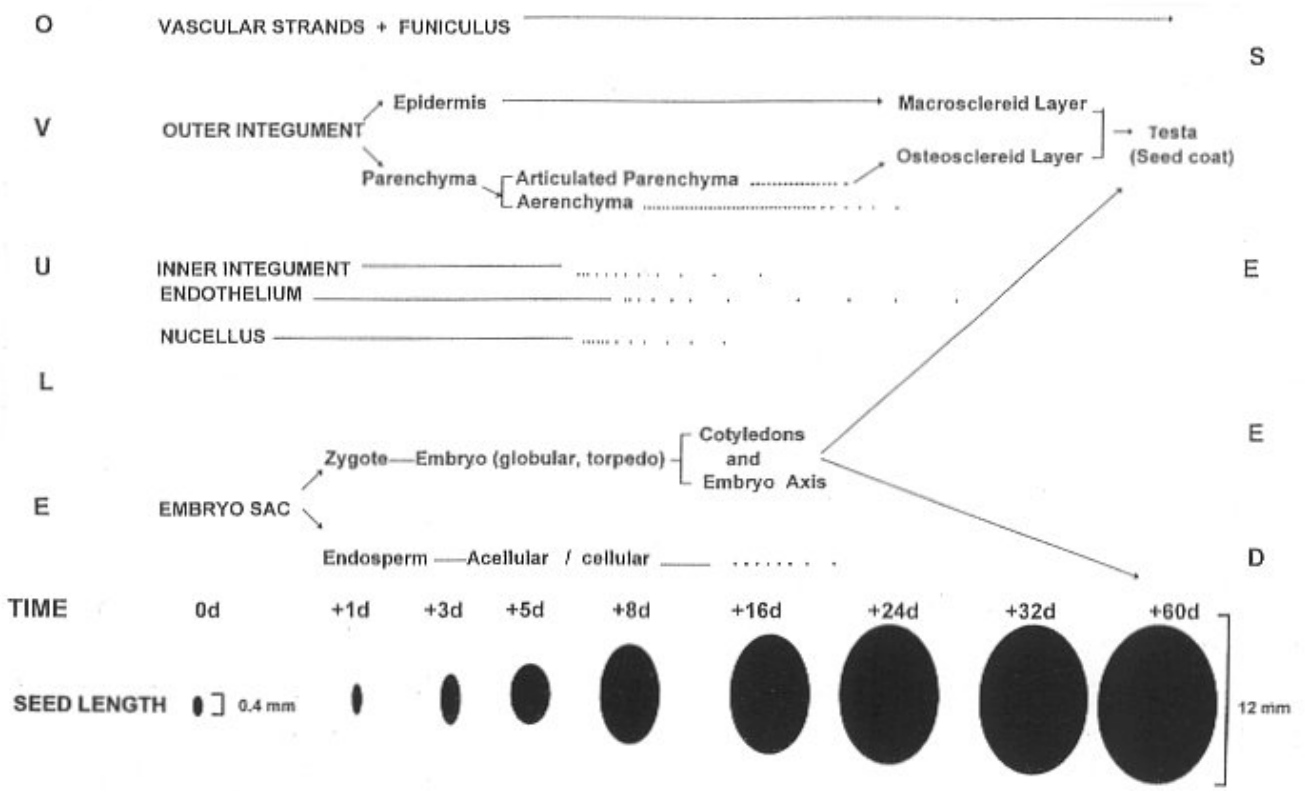


Figure 1. Diagram comparing soybean ovule and seed development from (0 day) fertilization to (+60 days) postfertilization to types of tissue formed. Lines represent time (in days) during which a tissue exists, and dotted lines represent time during which a tissue is being crushed or is degenerating.

Light Microscopy

One μm thick sections were cut with glass knives on a Reichert Ultracut E microtome (Reichert, Vienna, Austria). A triple stain of 0.13% methylene blue - 0.02% Azure II in 0.066 M NaH_2PO_4 buffer (pH 6.9) followed by 0.2% basic fuchsin in 2.5% EtOH was used with the sections for its differential contrast staining properties.

Additional sections were treated with the following histochemical staining procedures which were not adversely affected by the OsO_4 fixation: (1) periodic acid-Schiff (PAS) technique Jensen (1962), specific for water-insoluble polysaccharides; and (2) rubeanic acid (dithiooxamide), silver nitrate, and acetic acid treatment (Yasue, 1969; Horner and Wagner, 1980) for determination of CaOx. All observations and photomicrographs were recorded as described above.

Transmission electron microscopy

Thin sections (60-90 nm) were cut by using a Diatome diamond knife (Diatome-U.S., Fort Washington, PA) and stained with 5% uranyl acetate in 70% EtOH for 1 hour and in aqueous lead citrate for 30 minutes. Sections were observed using a JEOL JEM-1200 EX-II STEM (JEOL USA, Peabody, MA) operated at an accelerating voltage of 80 kV and photographed using Kodak SO 163 film.

Scanning electron microscopy (SEM)

Young seeds were prefixed and postfixed as described above under **Microscopy**; except they were dehydrated in a graded EtOH series to absolute EtOH, placed in Parafilm (American National Can, Neenah, WI) pillows filled with EtOH, sealed in the pillows, quick-frozen in liquid nitrogen, fractured with a cold razor blade, thawed in absolute EtOH, and CO_2 critical-point dried. The samples were mounted on brass discs with double-stick tape and silver cement, coated with gold and palladium (20/80) in a Polaron E5100 sputter coater (Polaron, Doylestown, PA), and viewed with a JEOL JSM35 SEM operated at an accelerating voltage of 15 to 25 kV. Polaroid Type 665 film (Polaroid Corp., Cambridge, MA) was used to record the images.

Results

Following fertilization, young seeds of soybean take about 60-70 days to mature. During this time, the seeds and their included embryos increase in size, some tissues

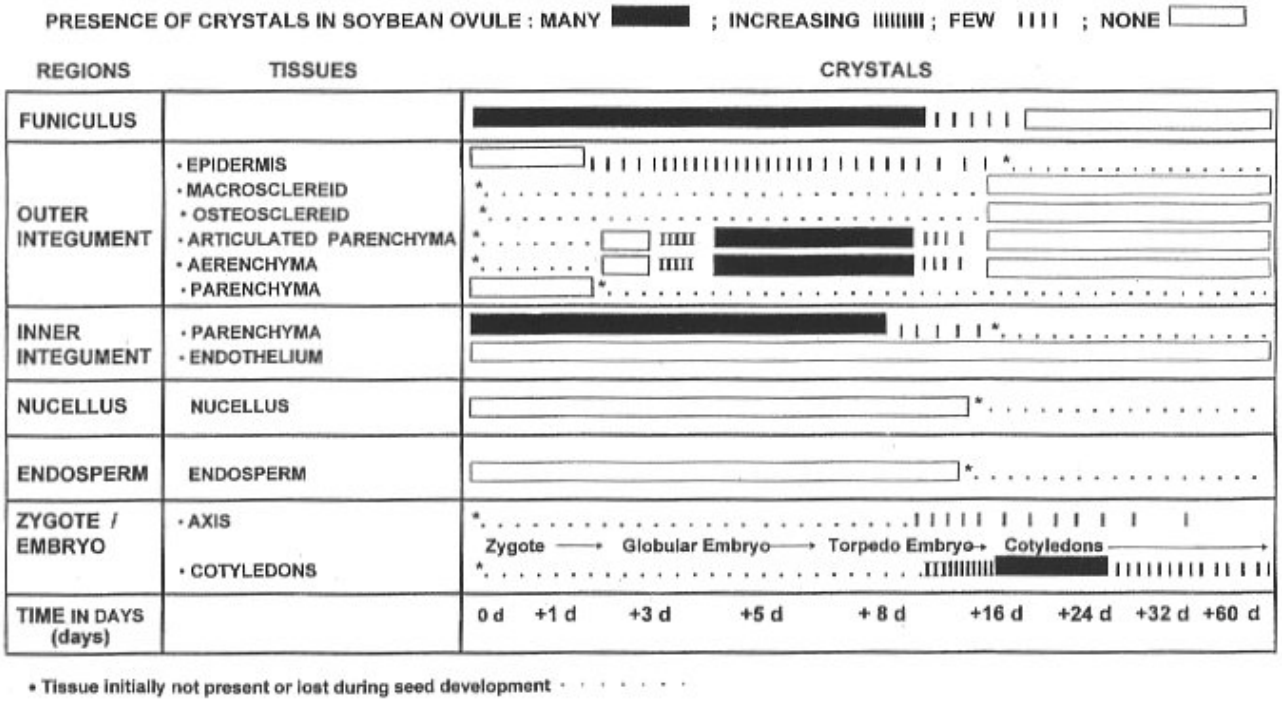
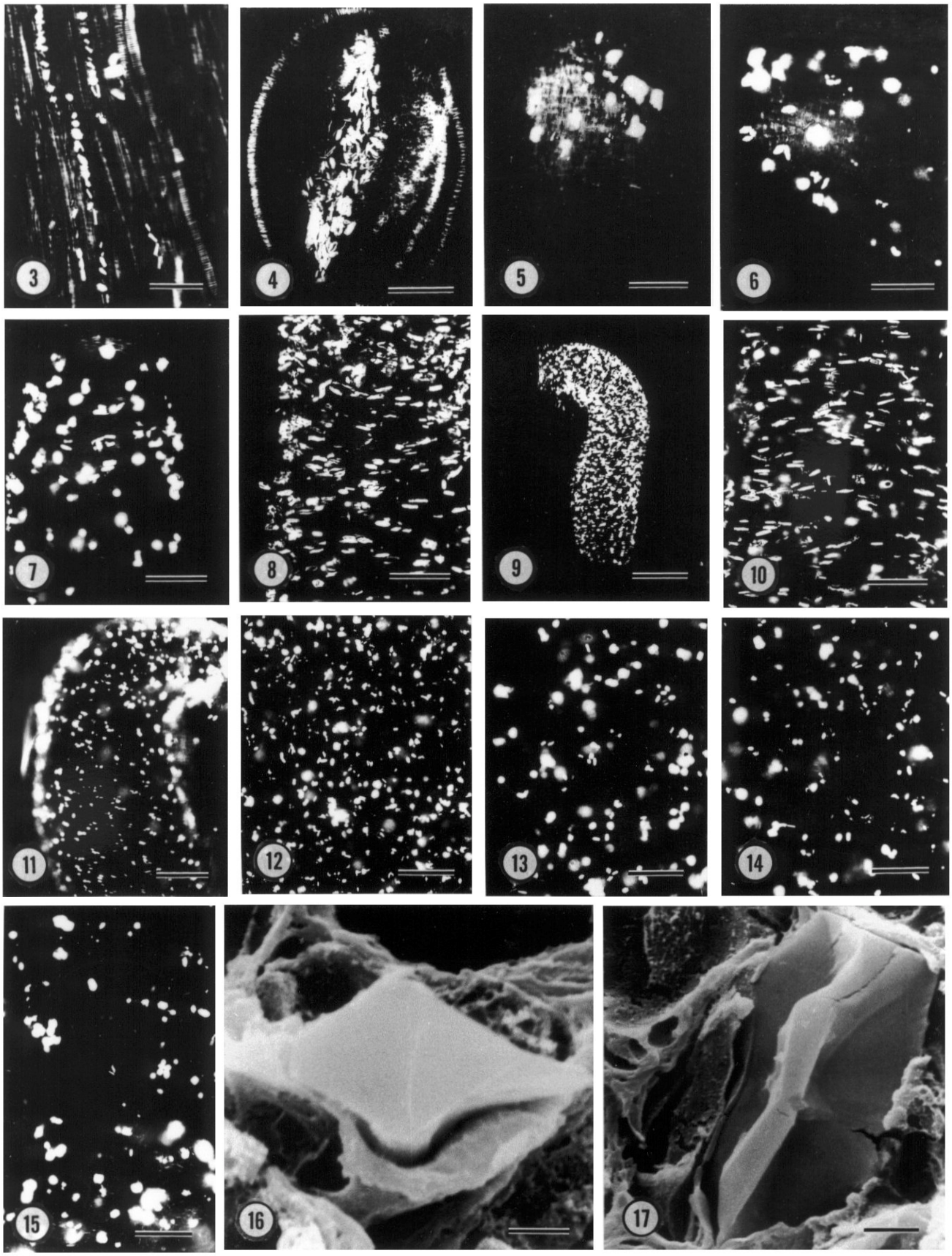


Figure 2. Diagram relating soybean ovule and seed regions to tissues to presence of crystals between (0 day) fertilization and (+60 days) postfertilization. Solid bars represent presence of many crystals; closely packed vertical lines represent increasing numbers of crystals; less closely packed lines represent few crystals; and clear bars represent absence of crystals.

Figure 3-17 (*on facing page*). Light micrographs of cleared and freeze-fractured preparations of soybean ovary wall, anther, ovules and seeds showing CaOx crystals. **Figures 3-15**. Clearings between crossed polarizers. **Figure 3**. Portion of young ovary wall with many small prismatic crystals. Bar = 50 µm. **Figure 4**. Young anther with crystals located in connective tissue between four locules. Bar = 50 µm. **Figure 5**. (-1 day; prefertilization) ovule integument with a few crystals. Bar = 50 µm. **Figure 6**. (0 day; fertilization) ovule/seed integument. Bar = 50 µm. **Figure 7**. (+1 day) seed integument. Bar = 50 µm. **Figure 8**. (+3 days) Portion of seed integument; most crystals oriented perpendicular to long axis of seed. Bar = 50 µm. **Figure 9**. (+5 days) Whole seed. Bar = 200 µm. **Figure 10**. (+5 days) Portion of seed integument showing orientation of crystals. Bar = 50 µm. **Figure 11**. (+8 days) Portion of seed integument. Bar = 200 µm. **Figure 12**. (+16 days) Portion of seed integument. Bar = 200 µm. **Figure 13**. (+24 days) Portion of embryo cotyledon. Bar = 200 µm. **Figure 14**. (+32 days) Portion of embryo cotyledon. Bar = 200 µm. **Figure 15**. (+60 days) Portion of embryo cotyledon. Bar = 200 µm. **Figure 16**. Freeze fracture through integument tissue showing single crystal in cross section. Long, tapered faces contrast with stubby faces. Bar = 1 µm. **Figure 17**. Freeze fracture through integument tissue showing a single crystal in side view with one stubby face exposed. Bar = 2 µm.

differentiate and remain through seed maturity (most of the outer integument), while others are crushed and disappear (endosperm, nucellus, inner integument, and inner portion of outer integument). The developmental fate of the ovule, seed and the embryo tissues, between 0 day (fertilization) and +60 days (postfertilization), are shown chronologically in Figure 1, as a preface to the formation and development of crystals described later. The same tissues shown in Figure 1 also are shown in Figure 2, but the emphasis in Figure 2 is on the occurrence of crystals in different seed and embryo tissues as a function of time.

The clearing technique removed all cytoplasm but retained cell walls and crystals as identified by observing the clearings between crossed polarizers. Besides the cleared ovules and seeds, both ovary walls and anthers were cleared and shown to contain crystals in some of their tissues (Figs. 3 and 4). A few crystals were present in the youngest soybean ovules observed at -1 day, and these ovules measured less than 0.4 mm in length (Fig. 5). Older cleared seeds, up to +60 days, contained varying numbers of crystals primarily in the embryo cotyledons. Crystals increased in number from -1 day through +16 days (Figs. 5-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Figures 18-30 (*on facing page*). Light micrographs of resin sections through entire young seed showing funiculus (f), outer integument (o), inner integument (i), and embryo sac (e) by using general and histochemical staining procedures. **Figure 18**. (+5 days) Near median longitudinal bright-field image of seed showing funiculus, integument tissues, and embryo sac. Bar = 500 μm. **Figure 19**. Same image as Figure 18, but viewed through crossed polarizers to show location (arrows) of crystals primarily in integument tissues. Bar = 500 μm. **Figure 20**. (+5 days) Portion of funiculus showing large cells with dense-staining bodies stained with Yasue (1969) technique. Bar = 20 μm. **Figure 21**. (+5 days) Crystals (arrows) in inner integument; endothelium is at far right and does not contain crystals. Bar = 20 μm. **Figure 22**. (+5 days) Crystals in cells of outer integument. Bar = 20 μm. **Figure 23**. Different region of Figure 22. Bar = 20 μm. **Figure 24**. (+8 days) inner integument containing crystals is partly crushed. Bar = 20 μm. **Figure 25**. (+16 days) Portion of cotyledon showing crystals in vacuoles of cells. Bar = 40 μm. **Figure 26**. (+5 days) Funiculus stained with Yasue (1969) technique; dense bodies are Ca positive. Bar = 40 μm. **Figure 27**. (+5 days) Inner integument stained with Yasue (1969) technique; crystals stain positively (black) for CaOx. Bar = 20 μm. **Figure 28**. (+5 days) Outer integument stained with Yasue (1969) technique; crystals stain positively (black). Bar = 20 μm. **Figure 29**. (+5 days) Inner integument stained with PAS technique to show non-water soluble polysaccharide walls around crystals (arrows). Bar = 10 μm. **Figure 30**. (+3 days) Inner integument stained with PAS technique to show earlier stage of crystal formation in which crystals do not have walls surrounding them. Bar = 10 μm.

12, integuments) and then seemed to decrease as shown for seeds +24 days through +60 days (Figs. 13-15, cotyledons). These qualitative observations were confirmed by a related study using a quantitative enzymatic detection assay for oxalate (Ilarslan *et al*., 1997). Those results support the observations in this study that crystals (CaOx) increased through +16 days and then decreased thereafter. This assay was used to measure total oxalate present in air-dried and cleared seeds.

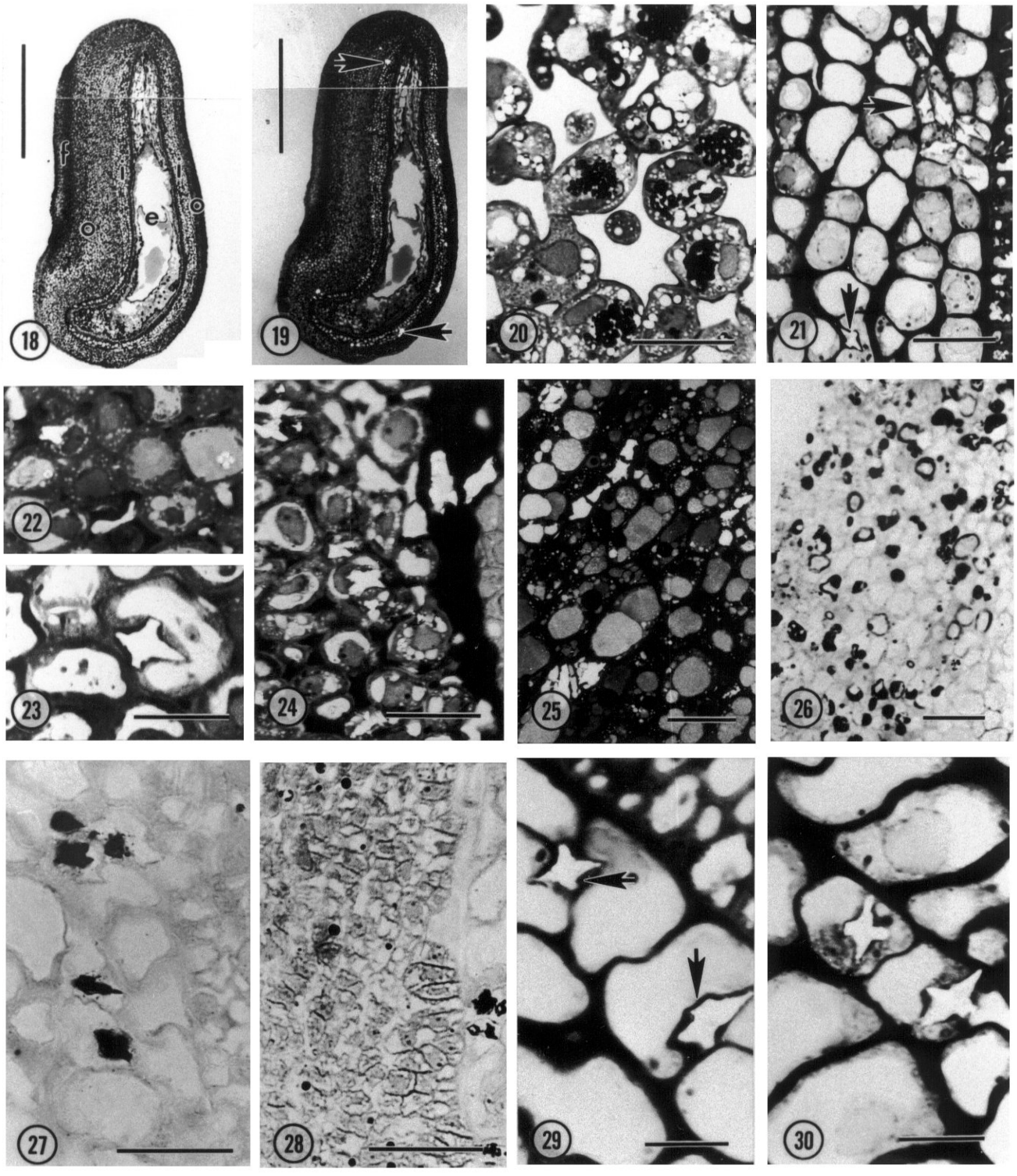
In addition, Ilarslan *et al*. (1997) isolated crystals from several different seed stages which were analyzed with X-ray diffraction and shown to be CaOx monohydrate. These crystals, isolated from cleared seeds (Ilarslan *et al*., 1997; see their Figs. 5 and 6), were prismatic as has been shown for other legumes. The crystals were either kinked (twinned) or straight (twinned). In freeze-fracture preparations of ovules observed with the SEM, the crystals were located in fractured-open cell vacuoles and seen in cross-sectional and in longitudinal views (as shown later in Figs. 16 and 17, respectively).

The clearings, in association with sections, demonstrated that early in development the crystals occurred first in the integument tissues (Figs. 5-12), and later in the cotyledons (Figs. 13-15). At earlier stages of development, the integument crystals seemed to be oriented with their long axes perpendicular to the long axis of each enlarging seed (Figs. 7-10), whereas later in development the integument (Figs. 10-12) and cotyledon (Figs. 13-15) crystals do not show any particular orientation. The crystals were highly birefringent.

Sectioned seeds were contrast enhanced by using both general and histochemical staining procedures. They showed the general young seed anatomy and association of tissues to each other in time, and location of the crystals within the various tissues (Fig. 2) by using bright-field (Fig. 18) and crossed polarizers (Fig. 19) imaging. Ca-positive dense bodies occurred first within cells of the funiculus (Fig. 20) and inner integument by 0 day. The crystals increased in number in both regions through about +8 days (inner integument; Fig. 21). Between +3 days and +5 days, crystals appeared in the outer integument tissues (Figs. 22 and 23), and slightly earlier in the epidermis. The number of crystals increased at varying times (Fig. 2) and then decreased between +8 days (Fig. 24) and +16 days as the inner integument began to be crushed by the enlarging embryo. As young embryos developed, crystals formed in the two cotyledons and the number of crystals increased between about +16 days (Fig. 25) and +24 days, and then decreased through +60 days. There also were some crystals present in the embryo axis (not shown).

Localization of Ca and CaOx were demonstrated by the Yasue (1969) technique. The greatest concentrations of Ca were in the parenchyma cells around the single vascular bundle in the funiculus, where large densely staining bodies (Fig. 26) were present. The crystals also stained positively with this technique, not only in the funiculus but also in the inner and outer integuments (Figs. 27 and 28) and in the embryo.

The PAS technique used to identify water-insoluble polysaccharides, positively stained both the cell walls and the special walls surrounding the crystals (Fig. 29). These latter walls were pressed against, and completely encased the mature crystals. Young crystals did not display these special crystal walls (Fig. 30). The combination of this crystal wall with an older crystal has been designated as a Rosanoffian crystal (Rosanoff, 1865) and it is common in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Figures 31-39 (*on facing page*). Transmission electron micrographs of cells from inner (I.I.) and outer (O.I.) integuments showing stages in crystal formation. Crystal images are holes in which crystals existed before sectioning. **Figure 31**. (+3 days) I.I. cell with membrane complex in a vacuole. Bar = 0.4 μm. **Figure 32**. (+5 days) I.I. cell showing pre-shaped crystal vacuole containing membrane complex and some crystalline material. Bar = 1 μm. **Figure 33**. (+5 days) I.I. cell with nearly mature crystal; crystal vacuole not completely filled and right region next to crystal shows electron-dense flocculate material. Bar = 1 μm. **Figure 34**. (+3 days) I.I. cell containing fully-formed crystal filling crystal vacuole; young carbohydrate wall surrounds entire crystal. Bar = 0.5 μm. **Figure 35**. (+3 d) I.I. cells each containing a single completely formed crystal. Both crystals are each surrounded by a wall and upper crystal wall contiguous with cell wall. Bar = 1 μm. **Figure 36**. (+8 days) I.I. near O.I.(larger cells to upper left). I.I. contains mature crystals and shows early stage of compression due to enlarging embryo sac. Bar = 4 μm. **Figure 37**. (+8 days) O.I. epidermal cell with vacuoles containing dense flocculate material. Bar = 1 μm. **Figure 38**. (+8 days) Portion of O.I. cell vacuole containing concentric body composed of flocculate material. Bar = 1 μm. **Figure 39**. (+8 days) Portion of O.I. cell vacuole containing concentric body similar to Figure 38; other circular bodies in vacuole are indicative of cytoplasmic inclusions. Bar = 1 μm.

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the legumes and several other taxa.

At the ultrastructure level, certain seed and embryo tissues displayed specific abilities to form crystals at different stages of development. The four tissue regions, containing crystals and localized Ca, were inner integument, outer integument, funiculus, and embryo cotyledons. In the crystal-forming tissues identified in Figure 2, the crystals typically formed within a large or central vacuole of each cell; sometimes two crystals formed per cell.

Inner Integument

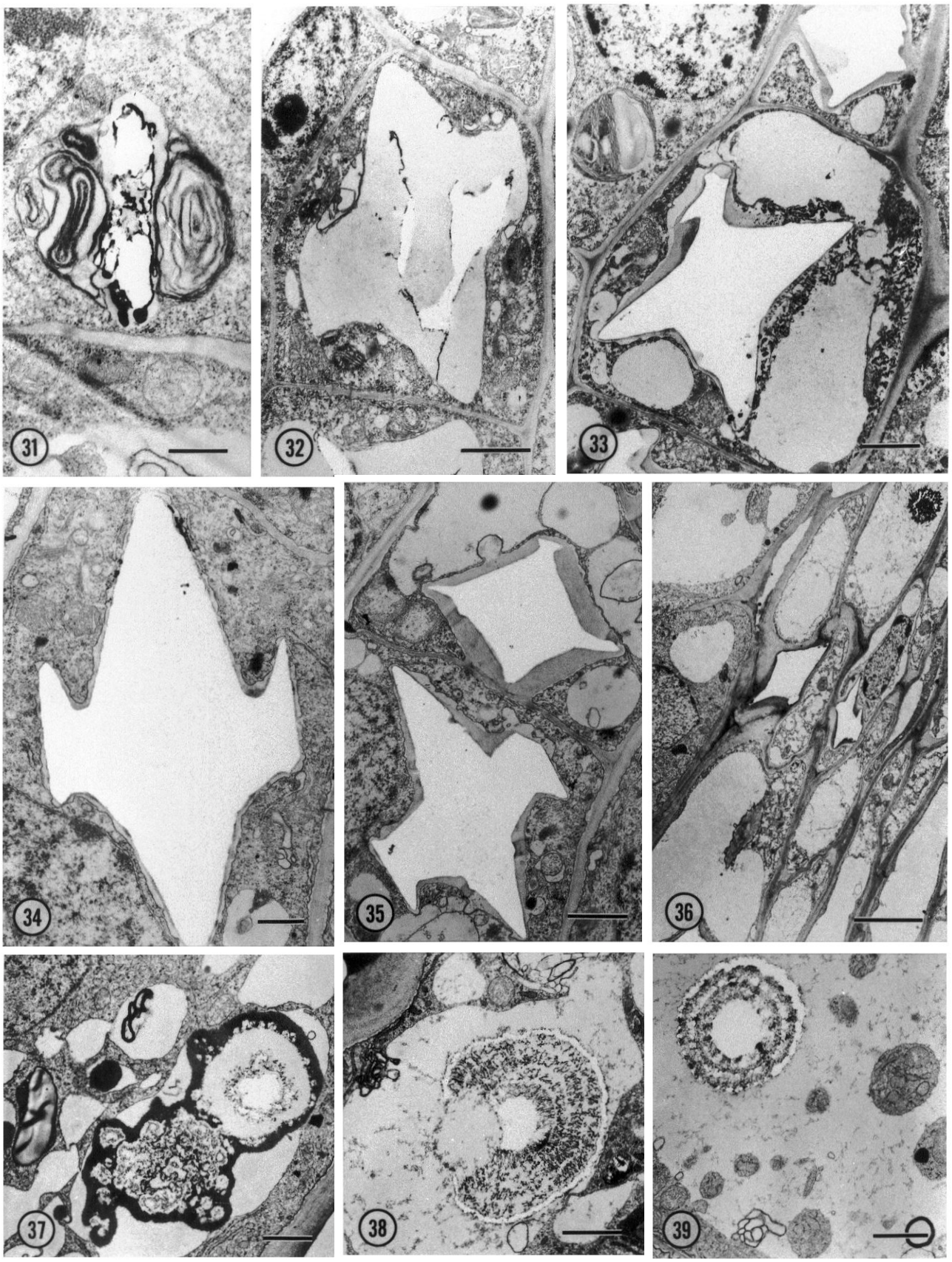
By 0 day of ovule (young seed) development, some cells in this region displayed already-formed crystals. Others had incipient crystals. In the latter cells, a vacuole either contained membrane complexes with some crystalline material (Fig. 31), or else assumed the shape of a mature crystal prior to complete crystal formation (Fig. 32). This latter observation was unique to this seed tissue in that there were no indications of any framework matrix within the vacuoles, or within the cytoplasm adjacent to the vacuole tonoplast, such as small tubules, microtubules, or microfilament bundles. In other cells with this preformed crystal shaped vacuole, crystalline material was partly or completely present (Figs. 33-35). Often membrane-like strands were associated with the incompletely formed crystals (Fig. 32); in other cell vacuoles, the crystals seemed to be completely formed without (Fig. 33) or with the crystal walls surrounding them (Figs. 34-36).

The crystal wall seemed to develop after a vacuole crystal was almost completely formed. Some vacuoles showed only part of the crystal wall present. In these instances, an electron-dense flocculate material occurred adjacent to the crystal where the wall had not yet formed (Fig. 33). This material seemed to extend the vacuole to near the cell plasmalemma adjacent to the cell wall. The crystal wall was continuous with this material. In addition, the cytoplasm adjacent to the tonoplast surrounding the crystal contained what seemed to be active dictyosomes with vesicles at their secreting faces. Although this wall was only partly visible at the time of formation, at maturity, each crystal was completely surrounded by it and it was stained uniformly positive for non-water soluble polysaccharides. Most completely-formed crystal walls seemed to be contiguous at some point with the primary wall of the cell. Thus, each mature crystal was encased in a special wall or sheath (i.e., Rosanoffian crystal) and anchored to the cell wall as shown with PAS staining at the light microscopic level (Fig. 29).

Outer Integument

The outer integument temporally differentiated into six cell layers from 0 day through +60 days (Figs. 1 and 2). The outer three layers became the seed coat and persisted through seed maturity. The outermost layer, the epidermis, and the inner two layers, the articulated parenchyma and aerenchyma, displayed crystals between +1 day and about +16 days. The epidermal crystals were very small and were not observed with the light microscope. Some cell vacuoles contained membranes, dense bodies (Fig. 37), and bodies composed of concentric layers of flocculate material with an unstained core (Figs. 38 and 39).

In the articulated parenchyma and aeren-chyma, electron-dense material also was arranged in concentric rings of membranes (Figs. 40 and 41) and flocculate material (Figs. 41-43). In some vacuoles they were elongated and gave a profile similar to that of a crystal (Figs. 44 and 45), in others they contained crystal-shaped membranes (Fig. 46) with or without portions of crystals, whereas still others contained crystals surrounded by a wall (Fig. 47). Figure 48 (+8 days) shows a series of five outer integument cells containing mature crystals with a special wall around each crystal. These cells are next to the inner integument before this tissue is crushed.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Figures 40-48 (*on facing page*). Transmission electron micrographs of outer integument (O.I.) cells showing different stages of membrane and crystal formation associated with vacuoles. Crystal images are holes in which crystals existed before sectioning. **Figure 40**. Portion of cell (+16 days) vacuole containing several concentric membrane complexes. Bar = 1 µm. **Figure 41**. Portion of epidermal cell (+8 days) with vacuole filled with membrane complexes and flocculate material. Bar = 0.6 µm. **Figure 42**. Vacuole (+8 days) with membrane complex and concentrically arranged flocculate material. Bar = 0.5 µm. **Figure 43**. Condensed concentric flocculate material in vacuole. Bar = 0.6 µm. **Figure 44**. Condensed flocculate material with crystal-like shape. Bar = 1 µm. **Figure 45**. Condensed flocculate material with both concentric inner and crystal-like outer shapes. Bar = 200 nm. **Figure 46**. Crystal within large vacuole that contains other structures. Crystal is not surrounded by a wall. Bar = 1 µm. **Figure 47**. Completely formed crystal surrounded by a wall. Bar = 1 µm. **Figure 48**. Interface between O.I. (right) and I.I. (left) showing mature crystals in five cells in inner portion of O.I. (+8 days). Each crystal is surrounded by a wall. Bar = 4 µm.

Funiculus

Many parenchyma cells around the single vascular bundle, throughout seed development, contained arrays of electron-dense material and crystals, which stained positively for Ca with the Yasue (1969) technique. Crystals and complexes of crystals usually were observed adjacent to the cell walls (Fig. 49). Vacuoles with membranes and flocculate material were common in the epidermis (Figs. 50 and 51) and interior cells (Figs. 52 and 53).

Nearer the vascular bundle were cells that contained many electron-dense bodies (Fig. 54) shown to be Ca rich as determined by the Yasue (1969) technique (Fig. 26). Some cells in this general region displayed small crystals solely in the cell walls (Fig. 55). X-ray diffraction analysis of isolated ovule crystals, however, did not show any other crystalline material besides CaOx. These wall crystals sometimes were associated with cells that contained vacuole crystals (Fig. 49).

Embryo cotyledons

As the embryo enlarged and formed its two cotyledons, crystals became evident before +16 days, when clearings were viewed between crossed polarizers. At this time the cotyledon parenchyma cells were fairly large and contained one to several large vacuoles. Some of the cell walls contained small crystals (not shown), similar to those observed in the funiculus cells early in their development. Their origin and composition are unknown but presumed to be CaOx.

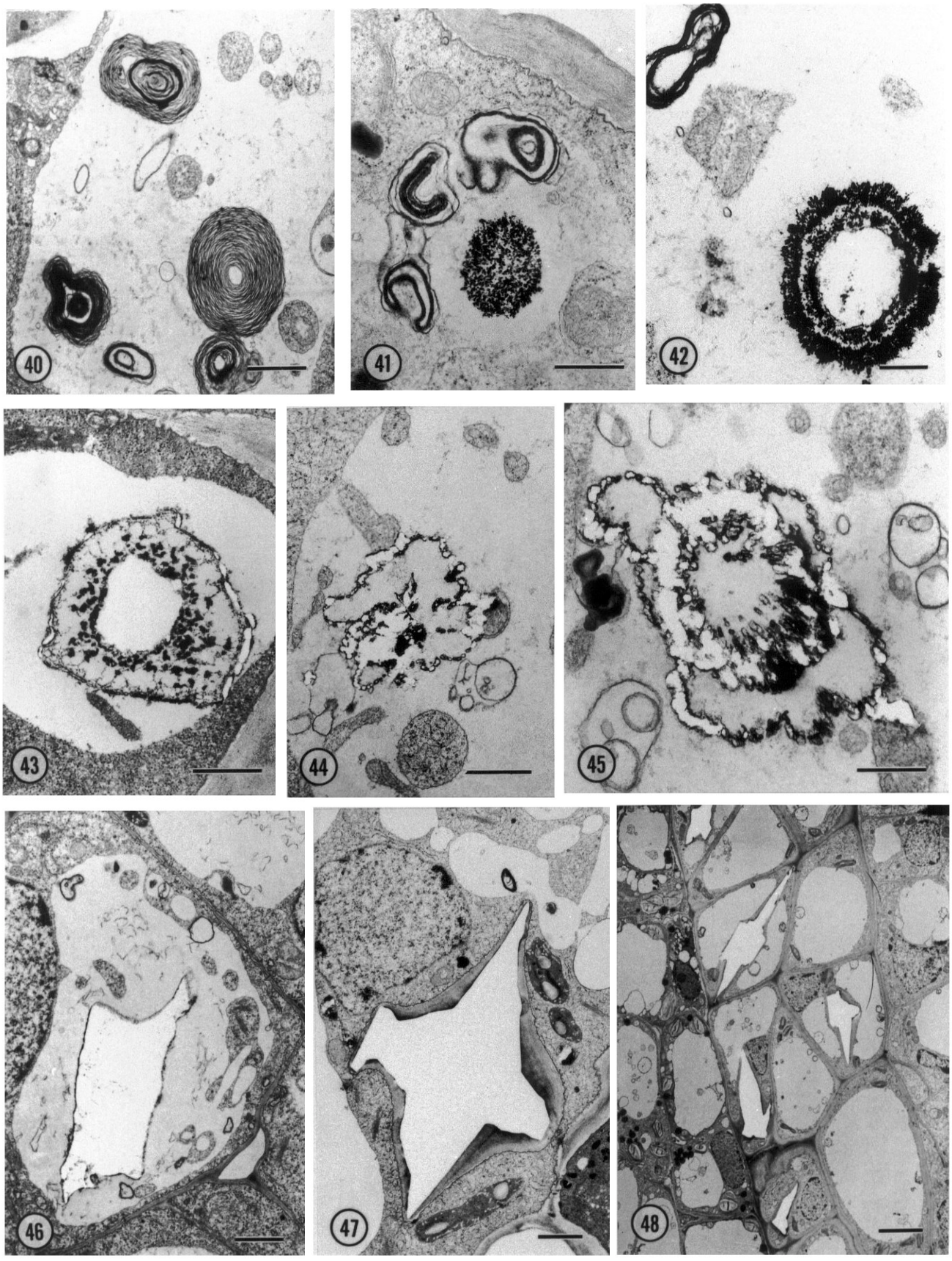
The vast majority of the cotyledon crystals (Figs. 56-61) formed between +16 days and +24 days, and they developed within the vacuoles of the cells in a manner similar to that described for the inner integument. Some of the vacuoles initially contained whorls of membranes (Fig. 56) and sometimes flocculate material (Fig. 61). Occasionally more than one crystal formed per cell. The vacuole shape, however, did not seem to mimic a crystal shape before the appearance of a crystal or as the crystal was in the process of forming (Fig. 57), as was described for the inner integument. Partly formed membrane crystal chambers showed a more discrete flocculate material at the periphery of the crystals before wall formation (Fig. 57). Some crystals that appeared well formed did not show any crystal wall (Fig. 58) while other crystals each were encased in a special wall (Figs. 59 and 60) that anchored the crystal to the cell wall (Fig. 59). Crystals were most plentiful between +16 days and +24 days, and then decreased in number thereafter. No CaOx crystals were found in storage protein bodies that formed in these cells later in development (not shown).

The results and Figure 2 can be summarized the following ways - CaOx crystals were already present in young ovules before fertilization and initially they increased in number in the funiculus and inner integument of the young seed. As the embryo enlarged and began to crush the inner integument, crystals formed and increased in number in the outer integument tissues. Later, crystals appeared in the cotyledons. At this time, the crystals in the outer integument dramatically decreased in number and disappeared. Near seed maturity, the only crystals remaining, in reduced numbers, were in the cotyledons and the funiculus.

Discussion

The majority of studies dealing with CaOx crystals in angiosperms depict their mature stage, either within the plant cells or, in a few instances, within or on their surrounding cell walls. There are few studies, however, that deal with the development of crystals and the specialized cells or crystal idioblasts in which they form (Arnott and Pautard, 1970; Franceschi and Horner, 1980a; Horner and Wagner, 1995).

Crystals have been reported in a large number of cell types and diverse tissues. Many mature seeds contain crystals (Webb and Arnott, 1982). Unfortunately, little attention has been given to developing ovules and seeds. In a related study, CaOx crystals were shown to be an integral part of developing soybean ovules and seeds, and they represented a significant portion of the dry mass of the seeds (Ilarslan *et al*., 1997). This massive amount of oxalate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Figures 49-56 (*on facing page*). Transmission electron micrographs of seed cells near funiculus epidermis and single vascular bundle, and cotyledons. Crystal images are holes where crystals existed prior to sectioning. **Figure 49**. (+8 days) Vacuole crystal with its wall attached to cell wall. Two other crystal masses are associated with cell wall. Bar = 0.5 μm. **Figure 50**. (+3 days) Epidermal cell with vacuole membrane complex associated with two crystal forming regions (arrowheads). Bar = 0.6 μm. **Figure 51**. (+8 d) Epidermal cell with vacuoles displaying membranes, flocculate material, and a crystal forming region. Bar = 2 μm. **Figure 52**. (+8 days) Portion of epidermal cell with vacuole containing dense, flocculate material and young crystal. Bar = 2 μm. **Figure 53**. (+3 days) Cells near single vascular bundle showing some vacuoles with membrane complexes; no crystals are visible. Bar = 0.6 μm. **Figure 54**. (+8 days) Cells near vascular strand containing many dense bodies that are calcium positive. No crystals occur in these cells. Bar = 4 μm. **Figure 55**. (+8 days) Cells near region shown in Figure 54. display small crystals (arrows) in cell walls but not inside cells. Bar = 2 μm. **Figure 56**. (+16 days) Cotyledon cell with extensive membranes in vacuole. No crystals are present. Bar = 1 μm.

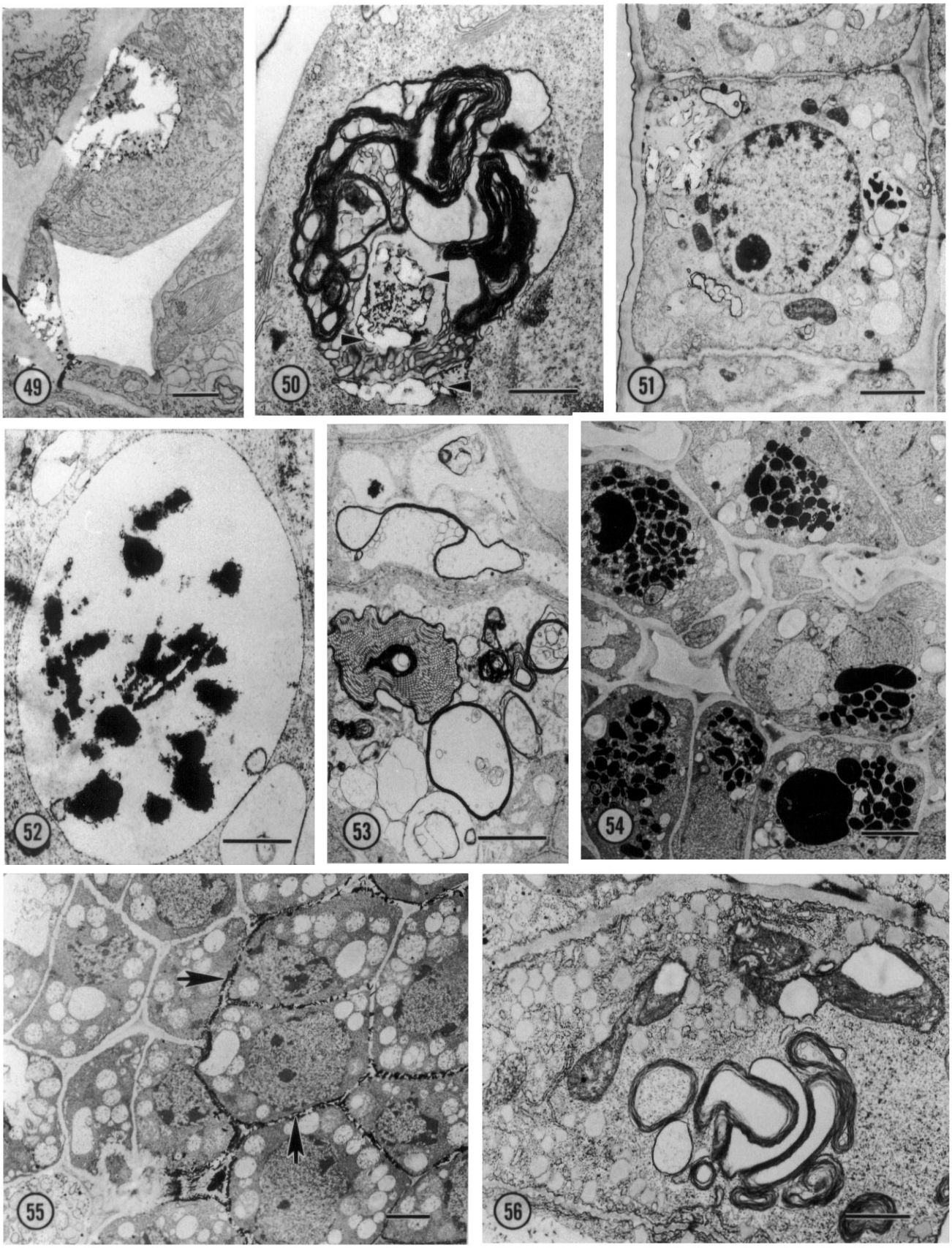
(maximum amount 24% dry mass at +16 days postfertilization), and CaOx, raises the question that has challenged researchers studying crystals for many years as to their functional significance and a tissue or organ's expenditure of energy to produce them. Ilarslan *et al*. (1997) suggested that this massive amount of oxalate serves to store Ca for later uses by the developing embryo, and the soluble or released oxalate could be involved in some way in the synthesis of seed storage protein later in embryo development.

In addition to the massive amounts of CaOx produced by some of the soybean ovule and seed tissues, the crystals disappear temporally from the integument tissues and cotyledons. This phenomenon begins during crushing of the integument tissues by the enlarging embryo. The cotyledons of the embryo also undergo loss of crystals later as they form lipids and proteins. No conclusive evidence exists for how the crystals disappear. Some possibilities are: (1) they are mechanically disrupted when some of the tissues are crushed; (2) there is a lowering of the pH in the cells that are undergoing crushing and autolysis; or (3) enzymatic (i.e., oxalate oxidase) degradation of the oxalate, initiated by the degenerating integument tissues or by the differentiating embryo tissues (Ilarslan *et al*., 1997).

The functional significance of the wall that forms around each seed crystal in a vacuole is not known. This sheathing material has been identified in various studies as cellulose (Solereder, 1908; Scott, 1941), as cellulose-lignin (Frank and Jensen, 1970), as suberin (Wattendorff, 1976b), or as a cellulosic-pectic wall material (Wattendorff, 1978). Grimson and Arnott (1983) reported that this sheath was an extension of the cell wall in *Phyllanthus*. Solereder (1908) mentioned for the Papilionoideae that the crystals very often are located in wall thickenings of the crystal-containing cells. The sheath forms between the crystal and the vacuole tonoplast; it then extends toward the cell wall. Tonoplast and plasmalemma fuse, excluding the crystal from the cytoplasmic space (Frank and Jensen, 1970; Solbrig, 1983). Even though this phenomenon is of common occurrence in some taxa, not all vacuole crystals are enclosed in this way. All other crystals studied to date, however, seem to be surrounded by either a membrane-like chamber (Arnott and Pautard, 1970) or lamellar sheath (Wattendorff, 1976a; Tilton and Horner, 1980). Some crystals have a proteinaceous or glycoprotein covering that may be external to the chambers or lamellae (Webb *et al*., 1995; Horner and Wagner, 1992).

Grimson *et al*. (1982) suggested that the crystal sheath may provide for the active elimination of a poisonous substance from the cell. The sheaths certainly may serve to isolate and maintain the crystal(s) in a state that protects the integrity of the cytoplasm of the cell or tissue. In other words, the sheaths could be an 'anti-body' response to the presence of the CaOx, however, not all crystals in other taxa develop this elaborate wall sheathing system. In the soybean ovules and seeds, these crystals do disappear as development proceeds so that the wall material is not an impediment to crystal dissolution. Even though some authors indicate that crystals may not be utilized during germination (Lott *et al*., 1982), they certainly disappear during seed development in soybean. In several other instances, presence and breakdown or absorption of CaOx crystals has been reported (Arnott and Pautard, 1970; Calmés and Piquemal, 1977; Franceschi and Horner, 1979; Tilton and Horner, 1980; Fink, 1991a, b). CaOx absorption suggests that the crystals serve as a storage source for Ca (Franceschi and Horner, 1979; Ilarslan *et al*., 1997). These observations in themselves imply a physiological role for oxalate (and the Ca) which so far has been so difficult to understand or so subtle as to evade elucidation.

Crystals in soybean embryo mesophyll and seed testa of *Phaseolus vulgaris* were described by Wallis (1913) and Haberlandt (1914), respectively. Haberlandt emphasized that they were excellent specimens for twinned crystals [Wallis (1913) had drawings of both types of crystals]. Twinned CaOx crystals were described by Solereder (1908)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and reviewed later by Arnott (1981). Kinked (twinned) and straight (twinned) crystals of CaOx monohydrate have been found in several genera of Leguminosae (Horner and Zindler-Frank, 1982b), including the fruit mesocarp of *Phaseolus vulgaris* (Winton and Winton, 1935; Grimson *et al*., 1982), and in the seed coat (Arnott and Webb, 1983). Both kinked and straight crystals were described in the developing soybean ovules and seeds in this study, and by Ilarslan *et al*. (1997). During the early and middle stages of soybean seed development both types of crystals were typically oriented with their long axes perpendicular to the elongating axis of the seed. Later there is no set orientation. It is not known what significance, if any, this orientation plays in seed and crystal development.

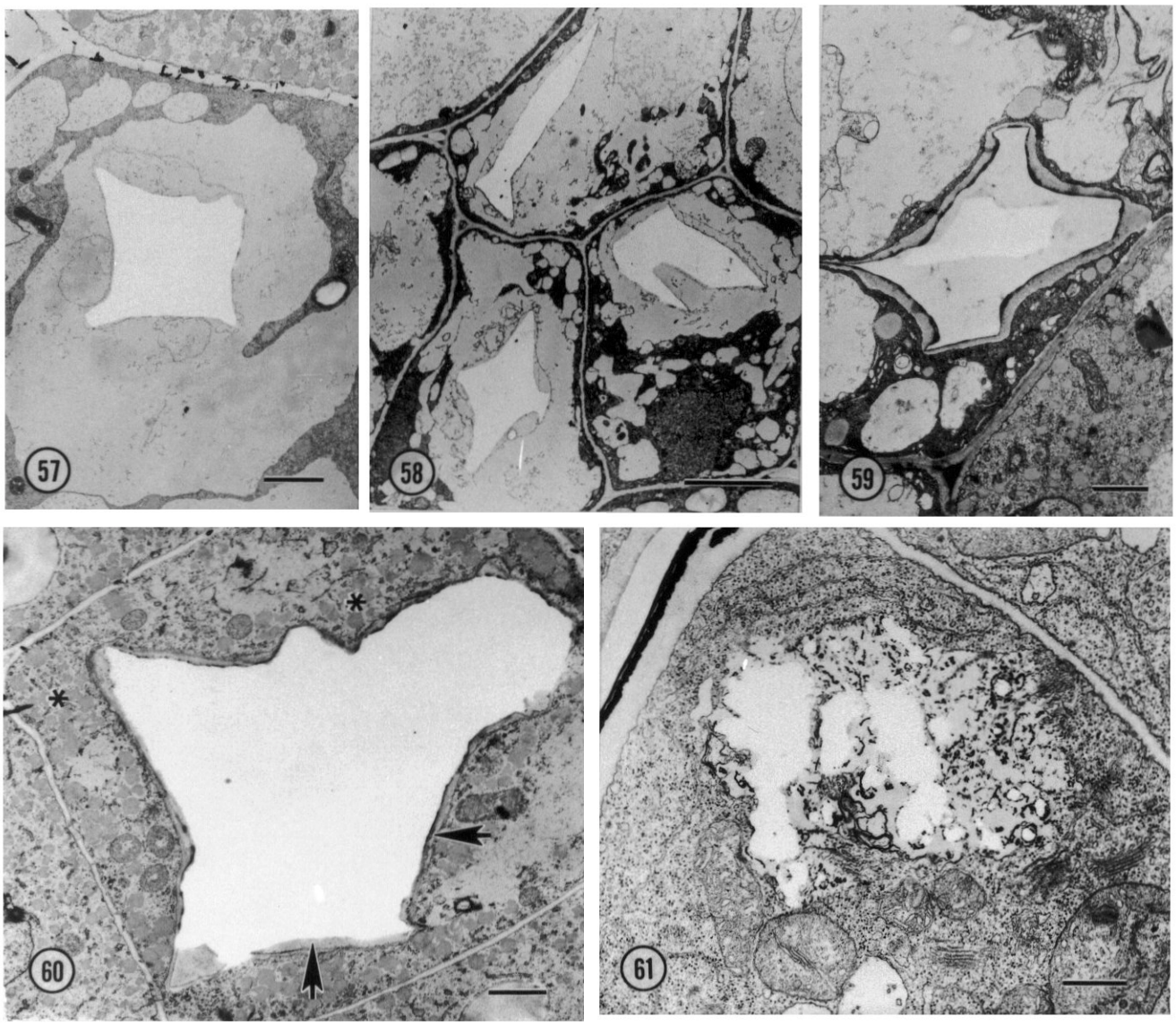
CaOx crystal shape has been considered a genetically determined characteristic (Arnott and Pautard, 1970; Franceschi and Horner, 1980a; Kausch and Horner, 1983a; b) controlled by the cell during crystal development. Arnott and Pautard (1970) suggested that a crystal chamber dictates crystal shape, as a mold, independent of crystal hydration form. Kausch and Horner (1984) noted that before crystallization, the crystal chambers in *Yucca* assumed a shape indicative of the crystals that would form within them. They mentioned that it is difficult to understand how a biological membrane could function as a mold for crystallization and crystal shape. In the inner integument cells of soybean, however, the tonoplast seems to serve that purpose. No internal vacuole matrix or cytoplasmic framework was observed outside the tonoplast in the cytoplasm. Even though the tonoplast did not seem to serve as a mold in the other seed tissues, these unique observations support those of Arnott and Pautard (1970).

Ca plays important roles within plants. It is of vital importance for cell wall formation (Ca pectate in middle lamellae) and membrane stabilization (Ca bridges phosphate and carboxylate groups of phospholipids and proteins at membrane surfaces). Within the protoplasm it plays a role as a metabolic regulator for many processes. It is involved in the maintenance of low intracellular activity of Ca ions which can be achieved by restricting the entrance of ions or by actively pumping ions out of the cytoplasm into the vacuoles (Hepler and Wayne, 1985; Kauss, 1987), and possibly into the apoplast (Kuo-Huang, 1990).

Crystal-producing cells are metabolically active and typically contain a dense cytoplasm rich in organelles, particularly mitochondria, smooth and rough endoplasmic reticulum, dictyosomes and, in some instances, plastids (Arnott and Pautard, 1970; Franceschi and Horner, 1980a; Horner and Wagner, 1995). It is accepted as fact that mitochondria in animal systems can accumulate Ca, and several studies on animal biomineralization have proposed a mechanism for calcification mediated by mitochondria. Arnott (1966) speculated that plant mitochondria may serve some role in crystal formation, possibly as energetic Ca pumps. Eilert (1974) reported that mitochondria were more numerous in *Yucca* idioblasts than other cells in the root. In addition, endoplasmic reticulum accumulates large amounts of Ca (Franceschi *et al*., 1993). In some of the crystal-forming cells of soybean, there were extensive profiles of smooth endoplasmic reticulum with extended lumens containing material.

Arnott (1995) suggested CaOx plays an important role from an evolutionary viewpoint in that oxalate can control the level of Ca to which cells are exposed. Therefore, crystal forming cells may be initiated in response to increased Ca levels, such as in developing ovule and seed tissues. The role of crystal cells might be one of localized Ca ion regulation and formation of a physiological environment such as occurs initially around integument tissues that ring the embryo. The significance of CaOx crystal formation also has been reported as a protection for cells from excess Ca (Borchert, 1984). Arnott and Webb (1983) and Tu (1989) also suggested that the crystal cell layer in the seed coat of *Phaseolus* might protect the embryo against external pests.

All of these ideas regarding the formation, presence, and loss of crystals in a wide variety of plant taxa growing in diverse environments and physical conditions lend complexity to the problem of what is the functional significance of CaOx crystals, oxalic acid, and crystal forming cells in general, and in soybean ovules and seeds, specifically. The results of this and a related study (Ilarslan *et al*., 1997) imply that considerable metabolic activity is directed toward crystal formation and dissolution. These processes are coordinated first in the integument tissues and later in the embryo cotyledons. Many studies already cited indicate the importance of Ca, both as a structural and a physiological entity. Therefore, it seems logical that the flowers, ovules and seeds that take in more Ca than they are able to use at a given time, induce specialized Ca sequestering cells to form in strategic locations within the developing issues. These cells maintain the Ca in the form of crystals until the developing embryo needs it. At that time, an appropriate physiological signal is initiated that dissolves the crystals and reclaims the Ca. Temporally, oxalic acid is available continually, but in much larger amounts later in ovule development and may contribute to seed storage protein synthesis; or it may be degraded by enzyme activity, and effectively and safely removed from the system. The metabolic formation and wide distribution of oxalate have been reviewed by Zindler-Frank (1976) and Franceschi and Loewus (1995), and complement the results presented in this study and the study by Ilarslan *et al*. (1997). We are contemplating studies to explore these former possibilities.



Figures 57-61. Transmission electron micrographs of developing embryo cotyledon cells. Crystal images are holes where crystals existed before sectioning. **Figure 57**. (+16 days) Crystal associated with membranes and flocculate material in cell vacuole. There is no crystal wall present. A few small crystals are present in cell wall. Bar = 1 μm. **Figure 58**. (+16 days) Three cells containing vacuole crystals; none with a wall. Crystals are various shapes in section. Bar = 4 μm. **Figure 59**. (+16 days) Single vacuole crystal surrounded by wall attached to cell wall. Bar = 1 μm. **Figure 60**. (+32 days) Large vacuole crystal with partial wall (arrows). Cytoplasm contains some lipid storage bodies (asterisks). Bar = 1 μm. **Figure 61**. (+16 days) Young vacuole crystal associated with membranes and flocculate material. Bar = 0.4 μm.

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Discussion with Reviewers

V.R. Franceschi: It is difficult to envision how a crystal that starts its development in the vacuole ends up surrounded by cell wall material. Is there a layer of cytoplasm or a membrane(s) between the wall and the crystal surface at maturity?

Authors: The carbohydrate special wall produced around each prismatic twinned crystal in the soybean seed also occurs around similar crystals in other parts of the soybean plant, as well as in other leguminous plants, and some non-legume taxa. As mentioned in the text, this wall does not necessarily occur around crystals in a wide variety of other taxa. In these latter taxa where a carbohydrate wall does not occur, the mature crystals may be surrounded by just a single membrane (*Psychotria punctata*; Horner and Whitmoyer, 1972), a membrane bounded by layers of lamellae (*Ornithogalum caudatum*; Tilton and Horner, 1980) or a membrane surrounded by a protein or glycoprotein matrix (*Vitis*; Webb *et al*., 1995).

Initially, the crystal forms interior to the vacuole membrane or tonoplast in association with membranes and/or flocculate material. As the crystal enlarges and begins to fill this space, wall material is deposited between the tonoplast and the crystal. It is assumed a portion of the tonoplast at some region in the cell comes in contact with the cell membrane or plasmalemma, and fuses with it, thus providing continuity of the special crystal wall with the cell wall. At this time, the crystal essentially is outside the cell protoplasm. Viewed in another way, the crystal vacuole fuses with the plasmalemma depositing its contents outside of the living cell. This is a much different situation than when the vacuole is still part of the protoplasm and considered a cell organelle.

We have no understanding as to the functional significance of either maintaining the crystal(s) within cell vacuoles versus extruding them as is the case in soybean and other selected taxa. These different methods of packaging and storing CaOx crystals is species specific, and suggests the interplay between evolutionary adaptations to physiological activities. Whatever the mechanism, it is an orderly, well orchestrated and non-disruptive process.

H.J. Arnott: On what basis did the number of crystals increase or decrease? Number per unit area?

Authors: Two methods were used: (1) a qualitative, visual method by which the crystals were seen using the clearing method; and a quantitative enzyme detection method for the presence of oxalic acid. The latter method determined the amount of unbound oxalic acid that was present in the seeds and the amount of bound oxalic acid that was present, presumably in the form of CaOx crystals. The visual method indicated an increase in the number of crystals up to +16 days, and a decrease thereafter. Even though individual crystals were not specifically counted, the impression of crystal increase or decrease could be discerned by looking at seeds at different stages of development. The results of the enzymatic method supported these observations.

E. Zindler-Frank: What is the increase of the dry mass of the seed from +16 days to maturity? Can you estimate the decrease of the crystal number per developing seed?
Authors: As Ilarslan *et al*. (1997) show, after +16 days the amount of total oxalate decreases. There is a accompanying decrease in the number of crystals. We did not try to count the number of crystals at each stage of development but used the quantitative method for change in the percent of bound oxalic acid, which we believe was in the form of CaOx.

V.R. Franceschi: I believe Prof. H.J. Arnott and others have shown that en block staining with uranyl acetate can lead to partial or total dissolution of calcium oxalate crystals. Since your samples were prepared with en block staining, is it possible that the crystal-shaped molds seen in the integument are actually due to the above effect?
Authors: In our preparations, the crystals were not dissolved as shown by observing the processed blocks as 1 µm thick sections. The crystals were always visible and polarized light, and did not seem to be diminished in their appearance or size. In addition, it was only the future crystal cells in the integument that showed this unusual shape of the tonoplast, mimicking a mold. None of the vacuoles in the other tissues mentioned showed this unusual condition. Therefore, we conclude this unique tonoplast shape is not an artifact of en block staining but a tissue specific expression of a pre-crystal condition.

H.J. Arnott: Straight and kinked crystals in *Phaseolus vulgaris* are twins - the only difference is whether or not the twin has rotated during the twinning process (Arnott and Webb, 1983).
Authors: This is also true for soybean. Each half of a kinked crystal did extinguish at different degrees of rotation between crossed polarizers, whereas both halves of the straight crystals extinguished at the same angle (Ilarslan, *et al*., 1997). There may be cases where triplets occur and they are also straight. We do not know if there is any selective advantage to either type of crystal, or what causes one to form over the other.

E. Zindler-Frank: Why does the developing seed need Ca storage? May it be that the xylem in the vascular bundle of the funiculus becomes gradually less sufficient to support the highly enlarged seed?
Authors: The embryo represents the major portion of the mature seed. It will need the Ca for an array of metabolic processes and for structural components of the cell wall and particularly the middle lamellae. We believe these needs require a ready source of Ca that cannot be met by movement of Ca in the transpiration stream via the funiculus vascular bundle. Your suggestion that the funiculus vascular bundle xylem may not be capable of transporting enough Ca later in development reinforces this idea. It is important to keep in mind that in other systems where CaOx forms, storage may not be the primary function of the Ca.