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Calcium Oxalate Crystals in Developing Seeds of Soybean

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Young developing soybean seeds contain relatively large amounts of calcium oxalate (CaOx) monohydrate crystals. A test for Ca and CaOx indicated that Ca deposits and crystals initially occurred in the funiculus, where a single vascular bundle enters the seed. 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. All crystals formed in cell vacuoles. Dense bodies and membrane complexes were evident in the funiculus. In the inner integument, cell vacuoles assumed the shape of the future crystals. This presumed predetermined crystal mould is reported here for the first time for soybean seeds. As crystals in each tissue 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 crushes the integuments; the crystals then disappear. A related study revealed that the highest percent of oxalate by dry mass was reached in the developing +16 d (post-fertilization) seeds, and then decreased during late seed maturation. At +60 d, CaOx formation and disappearance are an integral part of developing soybean seeds. Our results suggest that Ca deposits and crystals functionally serve as Ca storage 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.

INTRODUCTION

Calcium oxalate (CaOx) crystals are commonly found in many gymnosperms and angiosperms, and they occur in different plant tissues including roots (Arnott, 1966, 1976, Horner *et al*., 2000), leaves (Horner and Whitmoyer, 1972; Horner and Zindler-Frank, 1982a; Lersten and Horner, 2000), 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). Reviewing the literature of CaOx occurrence in higher plants, Arnott and Pautard (1970), Arnott (1982),Franceschi and Horner (1980*a*), Horner and Wagner (1995) and Webb (1999) have listed numerous reports that correlate CaOx crystal shape to specific anatomical tissues. CaOx crystals are the most commonly occurring form of insoluble calcium salt 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 (Franceschi and Horner, 1979; Kausch and Horner, 1982). Crystals often form in special cells called crystal idioblasts. These cells have shapes, sizes, and intracellular structures quite different from non-crystal-forming cells of the same tissue (Foster, 1956; Arnott and Pautard, 1970; Horner and Wagner, 1995).

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Although crystals commonly occur on or in the cell walls of gymnosperms, most of the crystals found in angiosperms form inside cell vacuoles (Kinzel, 1989). In some angiosperm plant organs, the amount of CaOx and oxalate may be exceptionally high, such as in a species of cactus (Cheavin, 1938) and in developing soybean seeds (Ilarslan *et al*., 1997). Recently, there has been increased interest in the presence and value of plant crystals, and the special cells in which they form (Webb, 1999). However, their functional significance remains unclear, although it has been eluded to in a variety of studies (Schneider, 1901). Calcium oxalate crystals may give protection against insects and foraging animals (Thurston, 1976), contribute to the strength of the tissue and store Ca (Franceschi and Horner, 1980*b*), bind toxic oxalate (Borchert, 1984), or be involved in surrounding degrading tissue (Horner and Wagner, 1980, 1992; Kausch and Horner, 1981), in in-plant Ca regulation (Franceschi, 1989), light gathering and reflection (Franceschi and Horner, 1980*a*), and salt stress and homeostasis (Hurkman and Tanaka, 1996). Even though there is agreement that Ca is derived from the environment (Arnott and Pautard, 1970), determination of the precursor(s) of oxalate biosynthesis (Franceschi and Loewus, 1995; Loewus, 1999; Horner *et al*., 2000; Keates *et al*., 2000) may help to clarify the factors that control crystal formation, and thus its functional significance. To meet this objective, a well-defined or model system for studying plant crystals is needed. Several have been utilized, such as *Psychotria* (Horner and Whitmoyer, 1972; Franceschi and Horner, 1979), *Lemna*, (Franceschi, 1989), *Vitis* (Webb *et al*., 1995),

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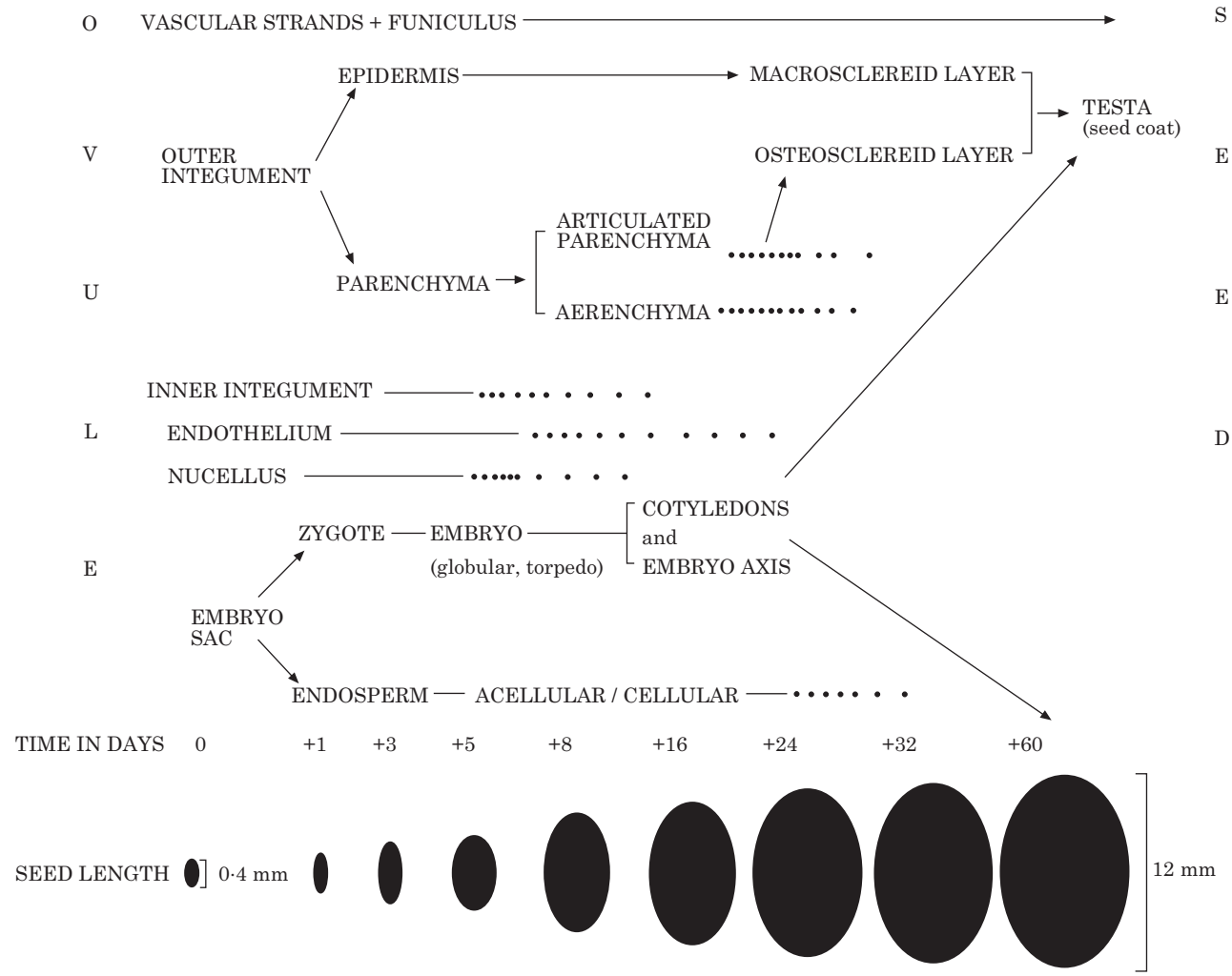


FIG. 1. Diagram comparing soybean ovule and seed development from (0 d) fertilization to (+ 60 d) post-fertilization to types of tissue formed. Lines represent time (in days) during which a tissue exists, and dotted lines represent time during which a tissue is being crushed or is degenerating.

Canavalia (Zindler-Frank, 1975), and *Capsicum* (Horner and Wagner, 1980,1992).

Combining crystal formation with quantification may also aid understanding of the functional role(s) CaOx crystals play during 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 developing and degenerating tissues (Ilarslan *et al*., 1997). Oxalate crystals were first reported in soybean embryo mesophyll by Wallis (1913), and later by Winton and Winton (1932). To our knowledge no further studies have been conducted since these early reports. Therefore, the objective of this study was to describe the relationship between formation and loss of CaOx crystals, and to suggest possible functional role(s) for the crystals, crystal cells, and oxalate during soybean seed development.

MATERIALS AND METHODS

Normal (N) line soybean (*Glycine max* L. Merr. 'Harosoy', Leguminosae) ovules (pre-fertilization) and seeds (post-fertilization) were studied at − 1d (pre-fertilization), 0 d (fertilization) and +1, +3, +5, +8, +16, +24, +32 and +60 d (post-fertilization). Flower buds and young fruits were collected at these developmental stages from plants in a growth chamber with a flower induction photoperiod and temperature (29 °C) regime of 18 h light during the initial 4 weeks, 16 h during the fifth week, and 14 h until maturity. Night-time temperatures were always 26 °C. Flowers were also 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

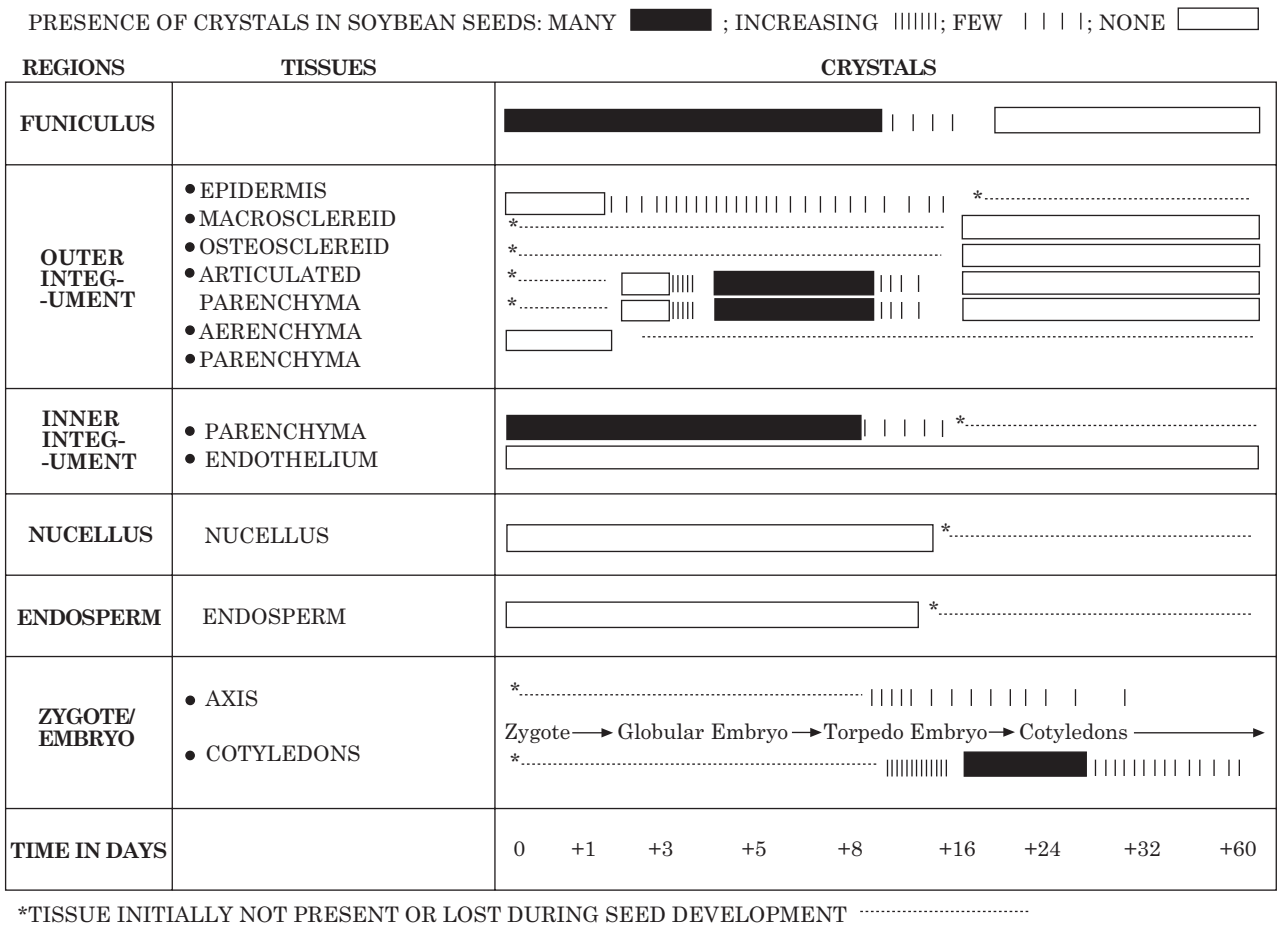


FIG. 2. Diagram relating soybean ovule and seed tissues to presence of crystals between (0 d) fertilization and (+ 60 d) post-fertilization. Solid bars represent presence of many crystals; closely packed vertical lines represent increasing numbers of crystals; less closely packed lines represent few crystals; and clear bars represent absence of crystals.

(sodium hypochlorite = NaOCl) for 8 h (small ovules and young seeds) and up to 1–2 d (older seeds). After these treatments, cleared samples were dehydrated through a graded EtOH series, then EtOH/xylene and pure xylene, and mounted in Permount (Fisher, Fort Lawn, NJ, USA) on slides; 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 using a Leitz Orthoplan microscope (Ernst Leitz Wetzlar, Wetzlar, Germany) with plan-apochromatic lenses, fitted with polarizing filters. Kodak Ektachrome 64T and Techpan films (Rochester, NY, USA) 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 2·0 % 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 h, and then placed in fresh fixative at 4 °C for 12 h. Fixed samples were passed through three buffer rinses, post-fixed in 1 % osmium tetroxide (OsO_4) in the same buffer for 4 h at RT, 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; Spurr, 1969). The same samples were used for light microscopy (LM) and transmission electron microscopy (TEM).

Light microscopy (LM)

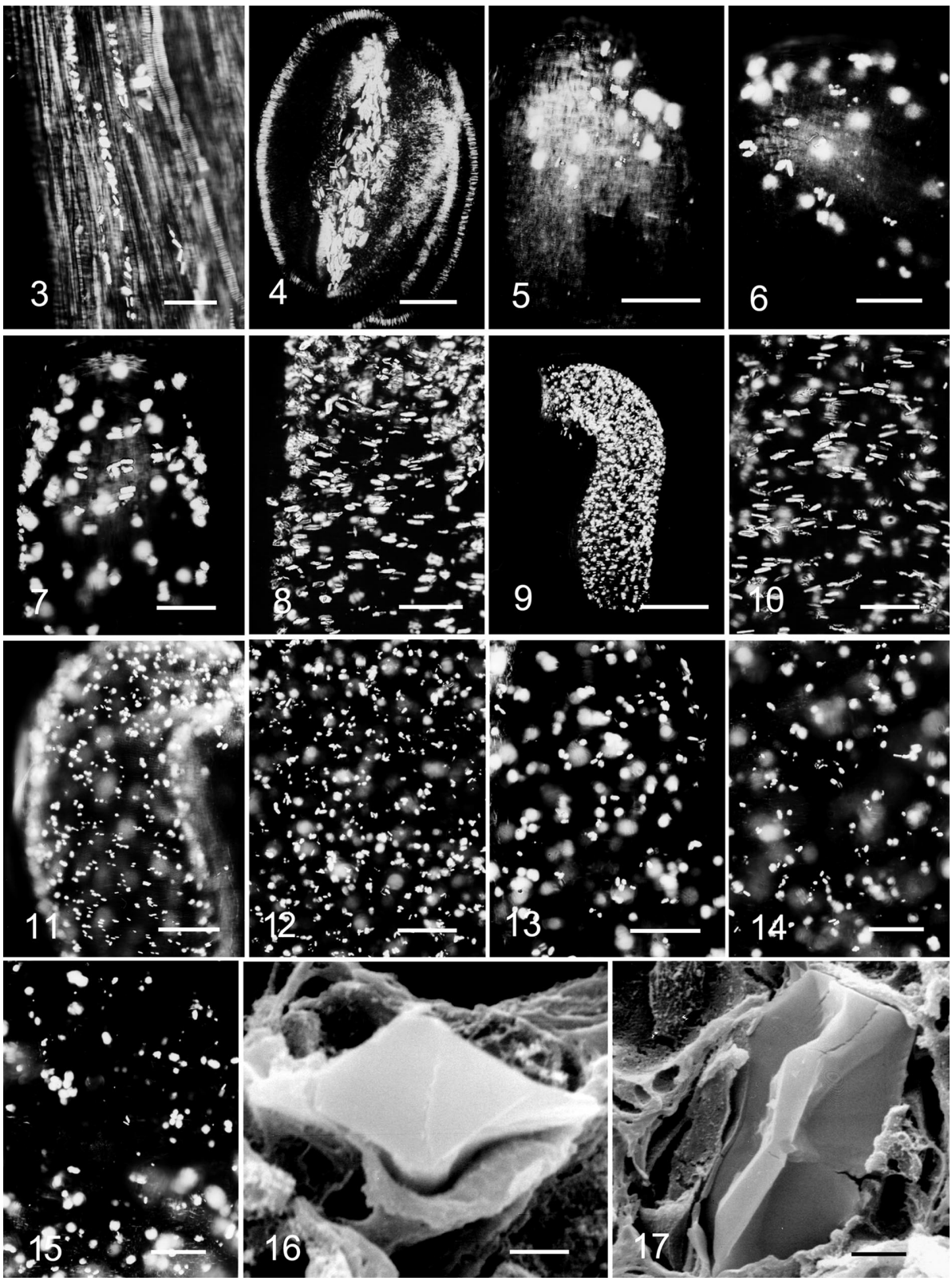
Sections were cut 1 μm thick with glass knives on a Reichert Ultracut E microtome (Vienna, Austria). A triple stain of 0·13 % methylene blue – 0·02 % Azure II in 0·066 M NaH_2PO_4 buffer (pH 6·9) followed by 0·2 % basic fuchsin in 2·5 % EtOH was used with the sections for their differential contrast 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 (Berlyn and Miksche, 1976), 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 made as described previously.

Transmission electron microscopy (TEM)

Thin sections (60–90 nm) were cut using a Diatome diamond knife (Fort Washington, PA, USA) and stained with 5 % uranyl acetate in 70 % EtOH for 1 h and in



aqueous lead citrate for 30 min. Sections were observed using a JEOL JEM-1200 EX-II STEM (JEOL USA, Peabody, MA, USA) at 80 kV and photographed using Kodak SO 163 film.

Scanning electron microscopy (SEM)

Young seeds were prefixed and postfixed in a similar manner to that described in the Microscopy section, except they were dehydrated in a graded EtOH series to absolute EtOH, placed in Parafilm (American National Can, Neenah, WI, USA) 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 (Doylestown, PA, USA), and viewed with a JEOL-JSM35 SEM at 15 to 25 kV. Polaroid Type 665 film (Cambridge, MA, USA) was used to record the images.

RESULTS

Following fertilization, soybean seeds take about 60–70 d to mature. During this time the seeds and their embryos increase in size. Some tissues differentiate and are retained 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 d (fertilization) and +60 d (post-fertilization, near maturity), are shown chronologically in Fig. 1, as a preface to the formation, development and loss of crystals described later. The same tissues shown in Fig. 1 also are shown in Fig. 2, but the emphasis in Fig. 2 is on the occurrence of crystals in different seed and embryo tissues as a function of development.

The clearing technique removed all the cytoplasm but cell walls and crystals were retained as demonstrated by observing the cleared samples between crossed polarizers. Besides the cleared ovules and seeds, both ovary walls (Fig. 3) and anthers (Fig. 4) were cleared and shown to contain crystals in some of their tissues. A few crystals were present in the youngest soybean ovules observed at −1 d (pre-fertilization), and these ovules measured less than 0·14 mm in length (Fig. 5). Older cleared seeds, up to +60 d, contained varying numbers of crystals primarily in the embryo cotyledons. Crystals increased in number from -1 d to +16 d (Figs 5–12, integuments) and then seemed to decrease as shown for seeds from +24 d to +60 d (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 until +16 d and decreased thereafter.

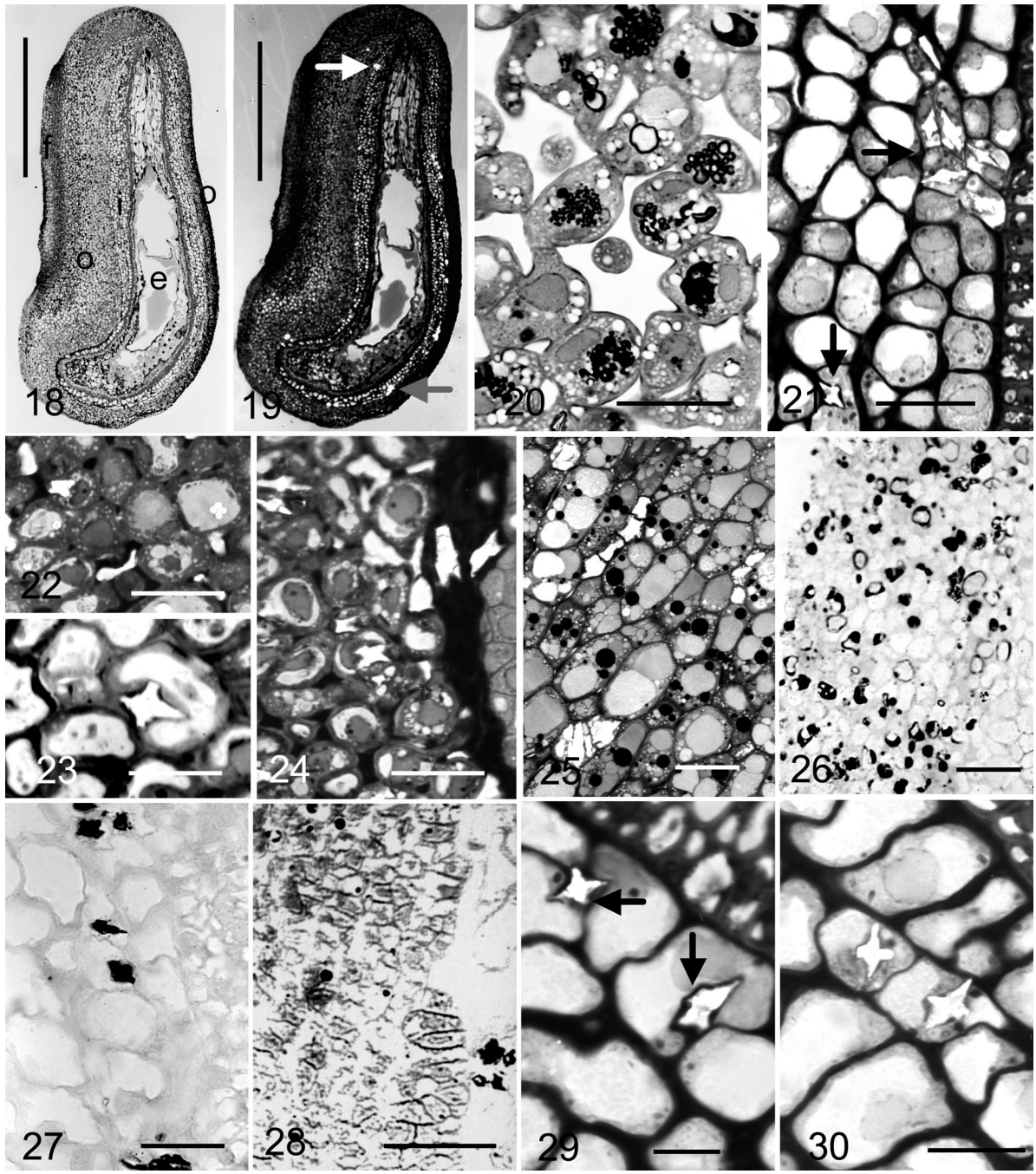
In addition, crystals isolated from seeds at different stages were analysed with X-ray diffraction and were shown to be CaOx monohydrate (Ilarslan *et al*., 1997). These crystals were all twinned prismatic crystals. In freeze-fracture preparations of seeds observed with the SEM, the crystals were located in fractured-open cell vacuoles and seen in cross-sectional view (Fig. 16) and in longitudinal view (Fig. 17).

Cleared samples, 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 seed 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 11 and 12) and cotyledon (Figs 13–15) crystals do not show any particular orientation. The crystals were highly birefringent.

Sectioned seeds were contrast enhanced using both general and histochemical staining procedures. The general anatomy of young seeds, association between tissues in time, and the location of crystals within the various tissues (Fig. 2) was shown using bright-field microscopy (Fig. 18) with partially crossed polarizers (Fig. 19). Ca-positive dense bodies first occurred within cells of the funiculus (Fig. 20) and inner integument by 0 d. The crystals increased in number in both regions until about +8 d (inner integument; (Fig. 21). Between +3 d and +5 d, crystals appeared in the outer integument tissues (Figs 22 and 23), slightly later than in the epidermis. The number of crystals increased, at varying times (Fig. 2), and then decreased between +8 d (Fig. 24) and +16 d as the inner integument was crushed by the enlarging embryo. As the young embryo developed, crystals formed in the two cotyledons and the number of crystals increased between about +16 d (Fig. 25) and +24 d, and then decreased until +60 d. Some crystals were also 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. Crystals also stained positively with this technique, not only in the funiculus but also in the

FIGS 3–17. LM of cleared and SEM of freeze-fractured preparations of soybean ovary wall, anther, ovules and seeds showing CaOx crystals. Figs 3–15. Cleared sections viewed between crossed polarizers. Fig. 3. Portion of young ovary wall with many small prismatic crystals. Bar = 50 μm. Fig. 4. Young anther with crystals located in connective tissue between four locules. Bar = 50 μm. Fig. 5. (-1 d; Pre-fertilization) ovule integument with a few crystals. Bar = 50 μm. Fig. 6. (0 d; Fertilization) ovule/seed integument. Bar = 50 μm. Fig. 7. (+ 1 d) Seed integument. Bar = 50 μm. Fig. 8. (+ 3 d) Portion of seed integument; most crystals oriented perpendicular to long axis of seed. Bar = 50 μm. Fig. 9. (+ 5 d) Whole seed. Bar = 200 μm. Fig. 10. (+ 5 d) Portion of seed integument showing orientation of crystals. Bar = 50 μm. Fig. 11. (+ 8 d) Portion of seed integument. Bar = 200 μm. Fig. 12. (+ 16 d) Portion of seed integument. Bar = 200 μm. Fig. 13. (+ 24 d) Portion of embryo cotyledon. Bar = 200 μm. Fig. 14. (+ 32 d) Portion of embryo cotyledon. Bar = 200 μm. Fig. 15. (+ 60 d) Portion of embryo cotyledon. Bar = 200 μm. Fig. 16. SEM of freeze fracture through integument tissue showing single crystal in cross section. Long, tapered faces contrast with stubby faces. Bar = 1 μm. Fig. 17. SEM of freeze fracture through integument tissue showing a single crystal in side view with one stubby face exposed. Bar = 2 μm.



FIGS 18–30. LM 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. Fig. 18. (+ 5 d) Near median longitudinal bright-field image of seed showing funiculus, integument tissues, and embryo sac. Bar = 500 μm. Fig. 19. Same image as Fig. 18. but viewed through crossed polarizers to show location (arrows) of crystals primarily in integument tissues. Bar = 500 μm. Fig. 20. (+ 5 d) Portion of funiculus showing large cells with dense-staining bodies stained with Yasue (1969) technique. Bar = 20 μm. Fig. 21. (+ 5 d) Crystals (arrows) in inner integument; endothelium is on far right and does not contain crystals. Bar = 20 μm. Fig. 22. (+ 5 d) Crystals in cells of outer integument. Bar = 20 μm. Fig. 23. Different region of Fig. 22. Bar = 20 μm. Fig. 24. (+ 8 d) Inner integument containing crystals is partly crushed. Bar = 20 μm. Fig. 25. (+ 16 d) Portion of cotyledon showing crystals in vacuoles of cells. Bar = 40 μm. Fig. 26. (+ 5 d) Funiculus stained with Yasue (1969) technique; dense bodies are Ca positive. Bar = 40 μm. Fig. 27. (+ 5 d) Inner integument stained with Yasue (1969) technique; crystals stain positively (black) for CaOx. Bar = 20 μm. Fig. 28. (+ 5 d) Outer integument stained with Yasue (1969) technique; crystals stain positively (black) as well as regions next to cell walls. Bar = 20 μm. Fig. 29. (+ 5 d) Inner integument stained with PAS technique to show non-water soluble polysaccharide walls around crystals (arrows). Bar = 10 μm. Fig. 30. (+ 3 d) 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.

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 a Rosanoffian crystal (Rosanoff, 1865) and they are common in legumes and several other taxa.

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

Inner integument

By 0 d of ovule (young seed) development, some cells in the inner integument contained fully 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 pre-formed 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 with (Figs 34–36) or without (Fig. 33) the crystal walls surrounding them.

The crystal wall seemed to develop after a vacuole crystal was almost completely formed. Only part of the crystal wall was present in some vacuoles. 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 across the vacuole almost to the cell plasmalemma adjacent to the cell wall. The crystal wall was continuous with this material (Fig. 33, arrow). In addition, the cytoplasm adjacent to the tonoplast surrounding the crystal contained what seemed to be active dictyosomes with vesicles at their secreting faces (not shown). Although this wall was only partly visible at the time of formation, at maturity, each crystal was completely surrounded by it (Fig. 35) and it was stained uniformly positive for non-water soluble polysaccharides (Fig. 29). 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 and TEM levels (Figs 29, 35 and 36).

Outer integument

The outer integument temporally differentiated into six cell layers between days 0 and +60 (Figs 1 and 2). The outer three layers became the seed coat and persisted until seed maturity. The outermost layer, the epidermis, and the inner two layers, the articulated parenchyma and aerenchyma, displayed crystals between +1 d and about +16 d. The epidermal crystals were very small and were not observed with the light microscope and were only infrequently observed with the electron microscope (not shown). 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 aerenchyma, electron-dense material was also 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 d) shows a series of four outer integument cells each containing a mature crystal with a special wall. These cells are next to the inner integument before this tissue is crushed.

Funiculus

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

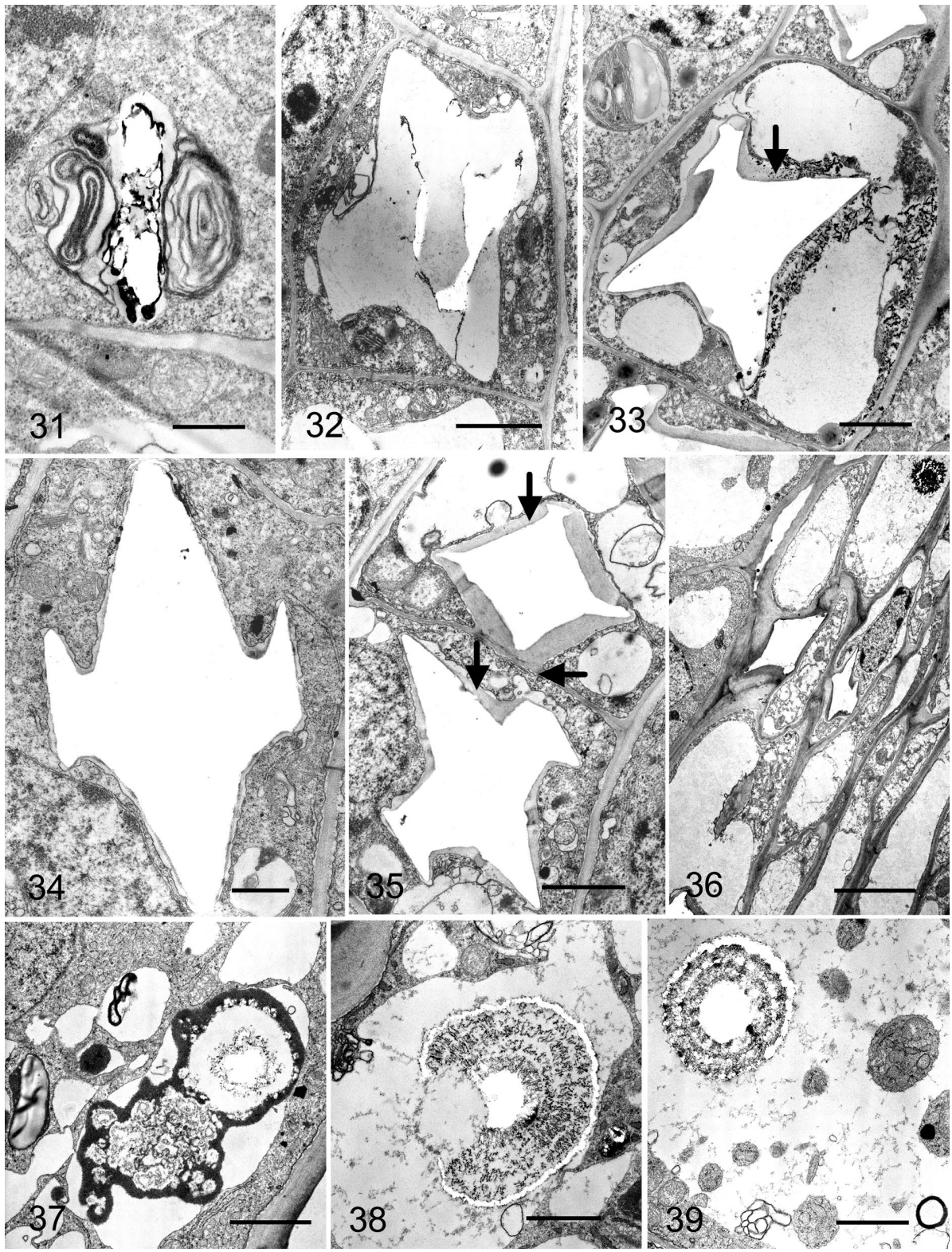
Nearer the vascular bundle were cells that contained many electron-dense bodies (Fig. 54) shown to be rich in Ca, as determined by the Yasue (1969) technique (Fig. 26). Some cells in this general region displayed small crystals solely in the cell walls (Fig. 55). X-ray diffraction analysis of isolated seed crystals, however, did not show any other crystalline material besides CaOx. These wall crystals were sometimes 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 d, when cleared sections 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 57–61) formed between +16 d and +24 d, developing 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. However, the vacuole shape 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 were encased singly in a special wall (Figs 59 and 60) that anchored the crystal to the cell wall (Fig. 59). Crystals were most abundant between +16 d and +24 d, decreasing in number thereafter. No CaOx crystals were found in storage protein bodies that formed in these cells later in development (not shown).

In summary, CaOx crystals were already present in young ovules before fertilization and they initially 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, crystals in the outer integument decreased dramatically in number and disappeared. Near seed maturity, reduced numbers of crystals were observed only in the cotyledons and the funiculus.

DISCUSSION

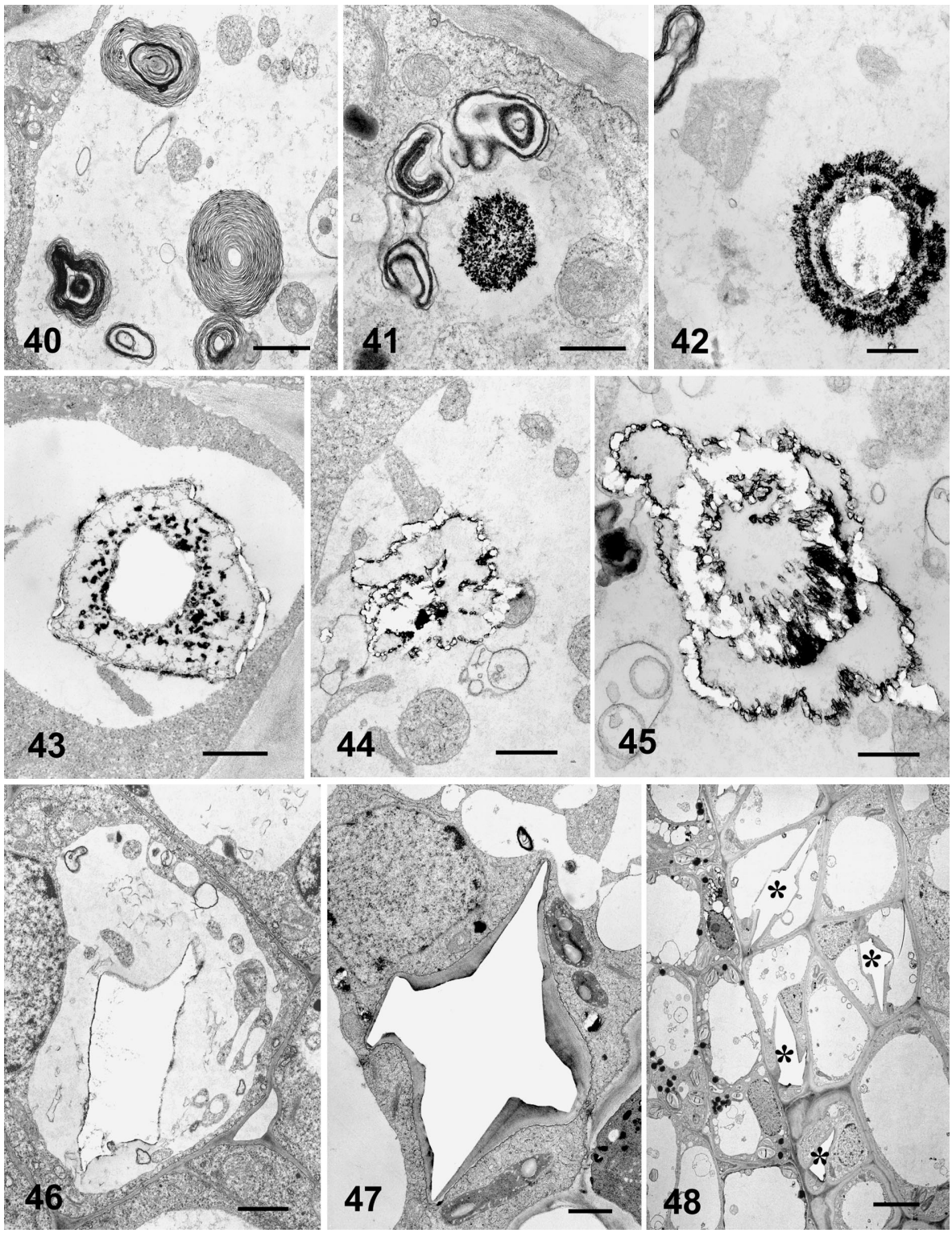
The majority of studies dealing with CaOx crystals in angiosperms depict their mature state. There are few studies, however, that deal with the development of crystals and the specialized cells or crystal idioblasts in which they form (Arnott and Pautard, 1970; Horner and Whitmoyer, 1972; Franceschi and Horner, 1980*a*). 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 their dry mass (Ilarslan *et al*., 1997). This amount of oxalate and CaOx raises questions about the functional significance of crystals and the energy expended by a tissue or organ to produce them. Ilarslan *et al*. (1997) suggested that oxalate serves to store Ca for later use by the developing embryo, and that the soluble or released oxalate could be involved in some way in the synthesis of seed storage proteins later in embryo development.

In addition to the 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 to explain how 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 within the cells that are being crushed or undergoing autolysis; or (3) degradation of the oxalate is enzymatic (i.e. oxalate oxidase), and is initiated by the degenerating integument tissues or by the differentiating embryo tissues (Ilarslan *et al*. 1997).

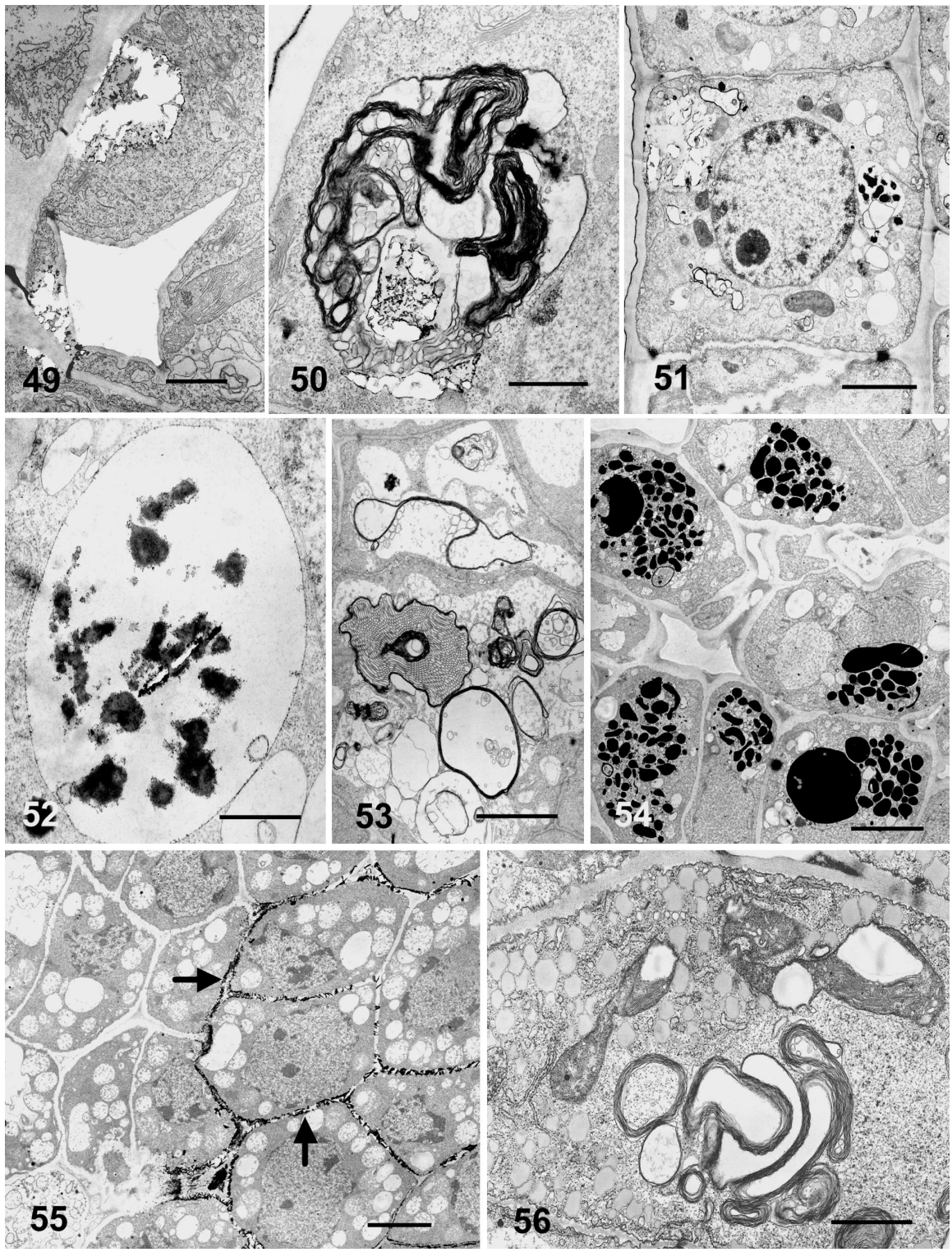
The functional significance of the wall that forms around each seed crystal in a vacuole is not known. This sheathing material has been identified in various studies as cellulose (Solereder, 1908; Scott, 1941), as cellulose-lignin (Frank and Jensen, 1970), as suberin (Wattendorff, 1976*b*), or as a cellulosic-pectic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

Grimson *et al*. (1982) suggested that the crystal sheath could provide for the active elimination of a poisonous substance from the cell. The sheaths may certainly serve to isolate and maintain the crystal(s) in a state that protects the integrity of the cytoplasm of the cell or tissue. However, not all crystals in other taxa develop this elaborate wall sheathing system. In soybean ovules and seeds, these crystals disappear as development proceeds so that the wall material is not an impediment to crystal dissolution. Although some authors have indicated that crystals may not be utilized during germination (Lott *et al*., 1982), the crystals 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, 1991*a*, *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

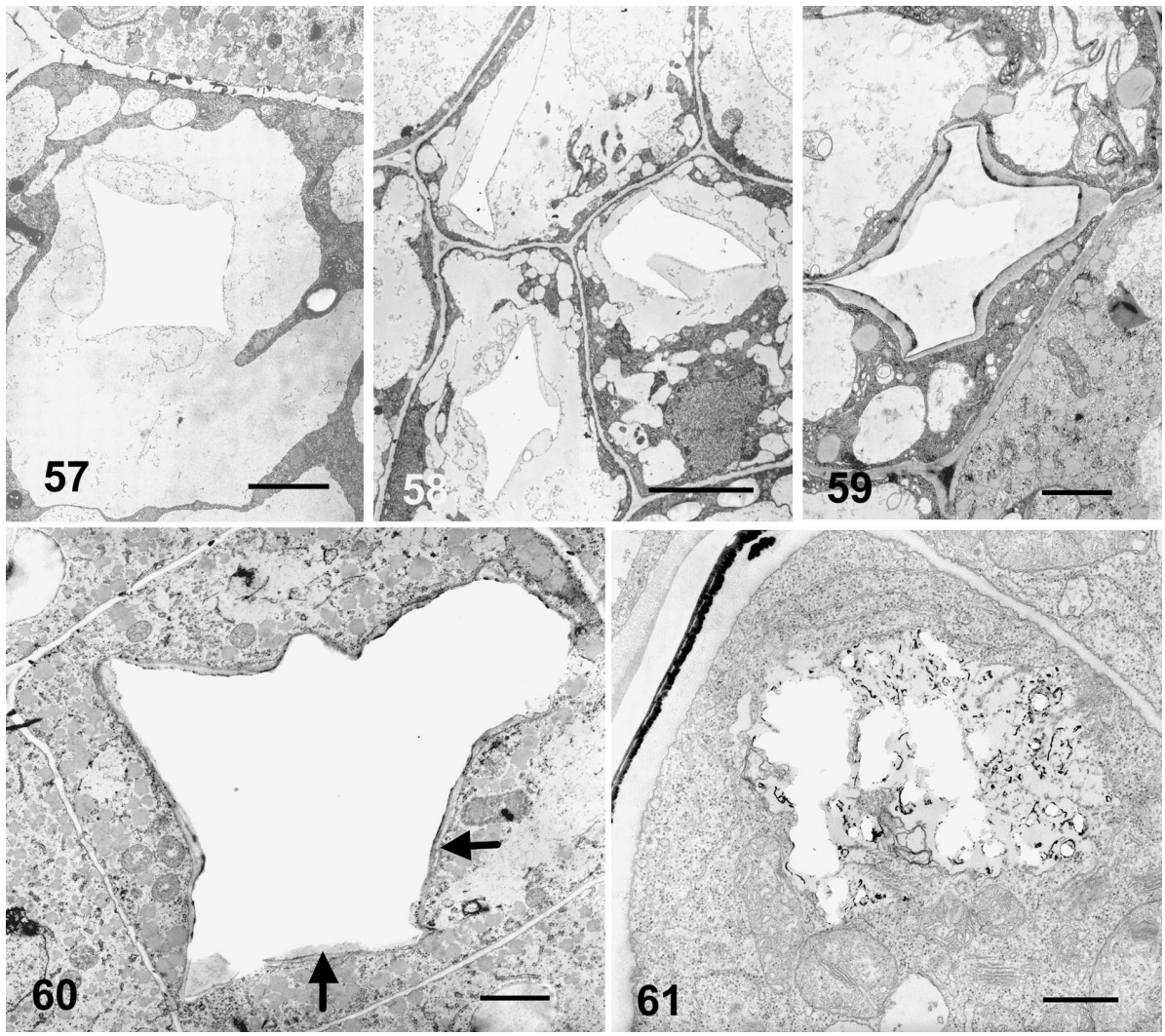
FIGS 31–39. TEM of cells from inner (I.I.) and outer (O.I.) integuments showing stages in crystal formation. Crystal images are holes in which crystals existed before sectioning. Fig. 31. (+ 3 d) I.I. cell with membrane complex in a vacuole. Bar = 0·4 μm. Fig. 32. (+ 5 d) I.I. cell showing pre-shaped crystal vacuole containing membrane complex and some crystalline material. Bar = 1 μm. Fig. 33. (+ 5 d) I.I. cell with nearly mature crystal; crystal vacuole not completely filled and region right next to crystal shows electron-dense flocculate material. Bar = 1 μm. Fig. 34. (+ 3 d) I.I. cell containing fully-formed crystal filling crystal vacuole; young carbohydrate wall surrounds entire crystal. Bar = 0·5 μm. Fig. 35. (+ 3 d) I.I. cells each containing a single completely formed crystal. Both crystals are surrounded by a wall (vertical arrow) and upper crystal wall (vertical arrow) contiguous with cell wall (horizontal arrow). Bar = 1 μm. Fig. 36. (+ 8 d) 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. Fig. 37. (+ 8 d) O.I. epidermal cell with vacuoles containing dense flocculate material. Bar = 1 μm. Fig. 38. (+ 8 d) Portion of O.I. cell vacuole containing concentric body composed of flocculate material. Bar = 1 μm. Fig. 39. (+ 8 d) Portion of O.I. cell vacuole containing concentric body similar to Fig. 38; other circular bodies in vacuole are indicative of cytoplasmic inclusions. Bar = 1 μm.



FIGS 40–48. TEM 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. Fig. 40. Portion of cell (+ 16 d) vacuole containing several concentric membrane complexes. Bar = 1 μm. Fig. 41. Portion of epidermal cell (+ 8 d) with vacuole filled with membrane complexes and flocculate material Bar = 0·6 μm. Fig. 42. Vacuole (+ 8 d) with membrane complex and concentrically arranged flocculate material. Bar = 0·5 μm. Fig. 43. Condensed concentric flocculate material in vacuole. Bar = 0·6 μm. Fig. 44. Condensed flocculate material with crystal-like shape. Bar = 1 μm. Fig. 45. Condensed flocculate material with both concentric inner and crystal-like outer shapes. Bar = 200 nm. Fig. 46. Crystal within large vacuole that contains other structures. Crystal is not surrounded by a wall. Bar = 1 μm. Fig. 47. Completely formed crystal surrounded by a wall. Bar = 1 μm. Fig. 48. Interface between O.I. (right) and I.I. (left) showing mature crystals (asterisks) in four cells in inner portion of O.I. (+ 8 d). Each crystal is surrounded by a wall. Bar = 4 μm.



FIGS 49–56. TEM of seed cells near funiculus epidermis and single vascular bundle, and cotyledons. Crystal images are holes where crystals existed prior to sectioning. Fig. 49. (+ 8 d) Vacuole crystal with its wall attached to cell wall. Two other crystal masses are associated with cell wall. Bar = 0·5 μm. Fig. 50. (+ 3 d) Epidermal cell with vacuole membrane complex associated with two crystal forming regions. Bar = 0·6 μm. Fig. 51. (+ 8 d) Epidermal cell with vacuoles displaying membranes, flocculate material, and a crystal forming region. Bar = 2 μm. Fig. 52. (+ 8 d) Portion of epidermal cell with vacuole containing dense, flocculate material and young crystal. Bar = 2 μm. Fig. 53. (+ 3 d) Cells near single vascular bundle showing some vacuoles with membrane complexes; no crystals are visible. Bar = 0·6 μm. Fig. 54. (+ 8 d) Cells near vascular strand containing many dense bodies that are calcium positive. No crystals occur in these cells. Bar = 4 μm. Fig. 55. (+ 8 d) Cells near region shown in Fig. 54 display small crystals (arrows) in cell walls but not inside cells. Bar = 2 μm. Fig. 56. (+ 16 d) Cotyledon cell with extensive membranes in vacuole. No crystals are present. Bar = 1 μm.



FIGS 57–61. TEM micrographs of developing embryo cotyledon cells. Crystal images are holes where crystals existed before sectioning. Fig. 57. (+ 16 d) 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. Fig. 58. (+ 16 d) Three cells containing vacuole crystals; none with a wall. Crystals are various shapes in section. Bar = 4 μm. Fig. 59. (+ 16 d) Single vacuole crystal surrounded by wall attached to cell wall. Bar = 1 μm. Fig. 60. (+ 32 d) Large vacuole crystal with partial wall (arrows). Cytoplasm contains some lipid storage bodies. Bar = 1 μm. Fig. 61. (+ 16 d) Young vacuole crystal associated with membranes and flocculate material. Bar = 0·4 μm.

oxalate (and the Ca) that has so far been difficult to understand or so subtle as to evade elucidation.

Wallis (1913) and Haberlandt (1914) described crystals in soybean embryo mesophyll and seed testa of *Phaseolus vulgaris*, respectively. Haberlandt emphasized that they were excellent specimens for twinned crystals [Wallis (1913) includes drawings of both types of crystals]. Twinned CaOx crystals were described by Solereder (1908) 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, 1982*b*), including the fruit mesocarp of *Phaseolus vulgaris* (Winton and Winton, 1935; Grimson *et al.*, 1982) and in the seed coat (Arnott and Webb, 1983). Kinked and straight crystals were described by Ilarslan *et al.* (1997) in developing soybean ovules and seeds, as well as in this study. 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 was no set orientation. The significance of this orientation in seed and crystal development is not known.

CaOx crystal shape has been considered a genetically determined characteristic (Arnott and Pautard, 1970; Franceschi and Horner, 1980*a*; Kausch and Horner, 1983*a*, *b*) controlled by the cell during crystal development. Arnott and Pautard (1970) suggested that a crystal chamber dictates crystal shape, as a mould, 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. These authors noted that it is difficult to understand how a biological membrane could function as a mould 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 mould in the other seed tissues, these unique observations support those of Arnott and Pautard (1970).

Calcium plays an important role within plants. It is of vital importance for cell wall formation and membrane stabilization, and within the protoplasm it acts as a metabolic regulator for many processes. It is involved in the maintenance of low intracellular activity of Ca ions that can be achieved by restricting the entrance of ions or by actively pumping ions out of the cytoplasm into 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, 1980*a*; Horner and Wagner, 1995; Horner *et al*., 2000).

Arnott (1995) suggested that CaOx has 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 (Franceschi *et al*., 1993) 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. CaOx crystal formation has also been reported to protect 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 question of the functional significance of CaOx crystals, oxalate, 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 tissues. These cells maintain the Ca in the form of crystals until the developing embryo has need for the Ca. At that time, an appropriate physiological signal is initiated that degrades the crystals and reclaims the Ca. Temporally, oxalic acid and/or oxalate are available continually, but in much larger amounts later in seed development and they may contribute to seed storage protein synthesis; or they 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, 1987) and Franceschi and Loewus (1995), and complement the results presented in this study and in an earlier study by Ilarslan *et al*. (1997). Finally, the use of the developing soybean seed as a model system is advantageous since CaOx and Ox are temporally both formed and removed as development of the embryo proceeds. Better understanding of the signals and metabolic pathway(s) that control oxalate synthesis and degradation will certainly provide useful information about the significance of this poorly understood compound.

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