

Calcium Distribution in Globoid Crystals of *Cucurbita* Cotyledon Protein Bodies¹

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JOHN N. A. LOTT, ERNEST SPITZER, AND CATHERINE M. VOLLMER
Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1 Canada

ABSTRACT

Energy-dispersive x-ray analysis was used to investigate the location of globoid crystals with relatively high Ca levels within cotyledons of *Cucurbita maxima*, *Cucurbita mixta*, and *Cucurbita andreana*. The small globoid crystals in both upper and lower epidermal cells commonly contained Ca. Ca was present in globoid crystals of all provascular regions with the exception of the very small provascular regions of *C. maxima*. In *C. maxima* and *C. mixta* cotyledons, some cases were observed where Ca was found in the globoid crystals of the first layer of mesophyll cells surrounding the provascular region, but in general Ca was absent from globoid crystals of palisade and spongy mesophyll cells. In *C. andreana*, globoid crystals of palisade and spongy mesophyll cells commonly contained at least some Ca. Cell position and cell type are factors affecting the Ca content of globoid crystals in protein bodies.

Protein bodies in seeds are important for seedling growth because they provide necessary nitrogenous compounds. It is often overlooked that protein bodies also function as the subcellular location of much of a seed's reserves of macronutrients such as P, K, Ca, and Mg. Structural investigations of storage tissue from several cucurbit seeds have revealed that protein bodies often consist of proteinaceous matrix, protein crystalloid, soft globoid, and globoid crystal regions (2, 8, 9). The protein reserves are thought to be located primarily in the proteinaceous matrix and protein crystalloid regions. The mineral reserves usually occur in the electron-dense globoid crystals and are generally considered to be largely phytin, a cation (K, Mg, Ca) salt of inositol hexaphosphoric acid (3, 4, 11-13).

Recently, Lott *et al.* (7) published results of an energy-dispersive x-ray analysis study of globoid crystal composition in 10 different regions of *Cucurbita maxima* embryos. Although P, K, and Mg were commonly found in globoid crystals in all squash embryo regions, the Ca distribution showed definite differences between embryo regions. Ca was common in globoid crystals of the radicle and stem regions. In the cotyledon, Ca levels were on average much lower than those of the root-shoot regions. Most globoid crystals from spongy or palisade mesophyll cells lacked any detectable Ca. The bulk of the Ca values for the cotyledon samples thus came from a fraction of the globoid crystals present. In this paper we report the results of EDX² analysis studies designed to discover where globoid crystals with relatively high Ca levels are located within *Cucurbita* cotyledons.

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² Abbreviation: EDX: energy-dispersive x-ray.

MATERIALS AND METHODS

Seeds. Squash seeds, *C. maxima* cv. Warded Hubbard, were obtained from Stokes Seeds Ltd., St. Catharines, Ontario. Seeds of *Cucurbita mixta* and *Cucurbita andreana* were obtained from Dr. W. P. Bemis, Department of Plant Sciences, University of Arizona, Tucson.

Fixation and Embedding. Epidermis-to-epidermis slices of dry seed tissue were cut from the base, center, and tip of *C. maxima* cotyledons and from the center of *C. mixta* and *C. andreana* cotyledons. Tissues from individual seeds were kept separate so that seed-to-seed variation could be studied. Tissue samples were glutaraldehyde fixed and embedded according to the method of Lott (3). No OsO₄ was used in these studies since it can bring about extraction of cations and even phosphorus from globoid crystals (6). Glutaraldehyde fixation alone does not seem to alter globoid crystal composition drastically in *C. maxima* (7) and thus this procedure was used in the studies reported here.

EDX Analysis. EDX analysis of 150 to 190 nm sections was carried out as described previously (7). With the exception of tiny globoid crystals in very small provascular regions and in epidermal cells, the spot size used was equal to or smaller than the size of the globoid crystal under investigation. Past work with *C. maxima* (3) showed that some proteinaceous material in the region being analyzed will not contribute greatly, if at all, to the P, K, Ca, and Mg peaks.

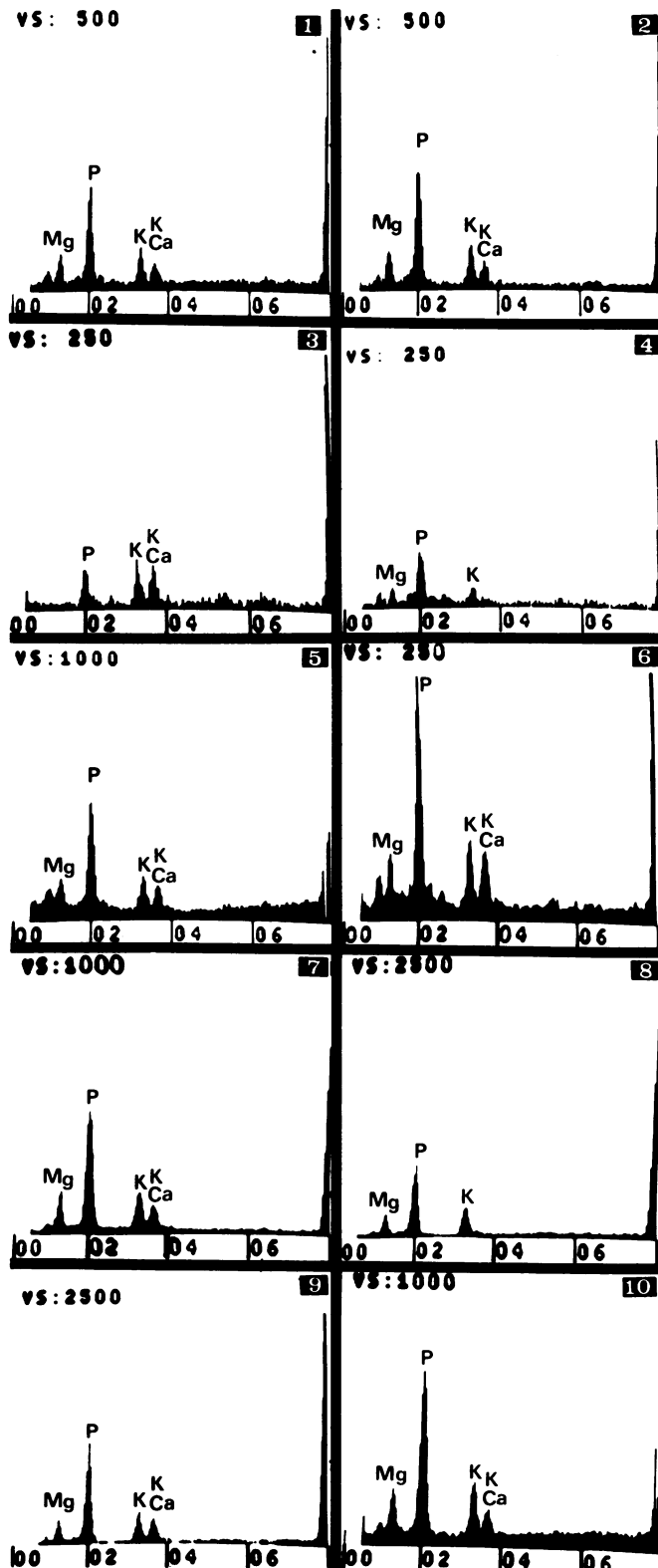
Results are presented here as EDX spectra only, but interpretation was aided by calculation of peak-to-background numbers as described by Lott *et al.* (7). Determining whether or not traces of Ca are present in a sample can be difficult because the major K_α peak for Ca centered at 3.69 keV is overlapped by the minor K_β peak of K, centered at 3.59 keV. Since the K_β peak of K is 10% of the major K_α peak of K the true Ca value can be obtained by subtracting 10% of the value for the K_α peak of K from the combined Ca K_α and potassium K_β peak.

RESULTS

The results presented here are based upon the study of several seeds of each species. Unless otherwise stated, results of studies of different seeds of the same species were comparable.

Investigation of Globoid Crystals in *C. maxima* Cotyledons. In squash cotyledons, epidermal cell protein bodies often contained no globoid crystals. When present, such globoid crystals were very small. Globoid crystals were more common in epidermal cells from the cotyledon tip than they were in epidermal cells from central and basal regions. Sometimes globoid crystals were found in a series of adjacent epidermal cells. Globoid crystals in epidermal cells usually contained at least some Ca (Figs. 1-3) although occasional exceptions were found in the cotyledon base.

No veins with mature xylem or phloem tissues were identified in the samples studied here. Transversely sectioned provascular



FIGS. 1-30. EDX spectra of globoid crystals from various tissues of cotyledons from *C. maxima*, *C. mixta*, and *C. andreana* seeds. Epidermis-to-epidermis slices from the cotyledon base, cotyledon center, and cotyledon tip regions of *C. maxima* and from the central region of the cotyledon of *C. mixta* and *C. andreana* were fixed in 5% glutaraldehyde in distilled H₂O, dehydrated, embedded in Spurr's epoxy resin, and sectioned prior to EDX analysis. Analysis time for each globoid crystal was 60 s. Elements present, emission levels in keV, and principle emission lines are as follows: magnesium 1.253 K_α; phosphorus 2.013 K_{α1,2}; 2.028 K_{α4} (10% of K_{α1,2} peak)

regions could be readily distinguished from mesophyll cells by the small cell diameters. Although provascular cells have small diameters in cross-section, they are elongated in the other plane of section. Thus, provascular cells cut longitudinally were more difficult to differentiate from mesophyll cells.

Very small provascular regions, which contained up to 10 or 12 cells in cross-section, were found in cotyledon center and cotyledon tip samples. In a given section, small provascular regions contained few globoid crystals and those that were present were much smaller than those found in mesophyll cell protein bodies. In most cases globoid crystals in very small provascular regions contained no Ca (Fig. 4).

In cross-sections of provascular areas containing numerous cells, the globoid crystals routinely contained Ca. No positional differences in globoid size or Ca content were found within a provascular region. Large and medium sized provascular areas in the cotyledon tip (Fig. 5), base (Fig. 6), and center (Figs. 7 and 9) all had Ca-containing globoid crystals. Such globoid crystals were generally larger than those of very small provascular regions but smaller than those of mesophyll cells.

In most cases examined, the globoid crystals of mesophyll cells adjacent to a provascular region contained no Ca. There is thus a great difference between the Ca content of globoid crystals within a provascular region (Fig. 7) and those in the first mesophyll cell next to a provascular region (Fig. 8). Occasional cases were discovered where there was Ca in globoid crystals of both the provascular cells (Fig. 9) and the first layer of mesophyll cells (Fig. 10) around the provascular region. In such cases globoid crystals in the second layer of mesophyll cells away from the provascular region contained no Ca.

Palisade mesophyll cells from the cotyledon tip (Fig. 11), center (Fig. 12), and base (Fig. 13) regions contained globoid crystals with no Ca. The globoid crystal in Figure 11 was located immediately below the epidermal cell illustrated in Figure 1. This provides another illustration of globoid crystals in adjacent cells differing greatly in their Ca content. Occasional globoid crystals which had higher than normal K levels and no Mg were found (Fig. 13).

In general globoid crystals from spongy mesophyll cells of the cotyledon tip (Fig. 14), center (Fig. 15), and base (Fig. 16) regions contained no detectable Ca. Out of the approximately 200 spongy mesophyll globoid crystals that were analyzed, only one Ca-con-

and 2.137 K_β (4% of K_{α1,2} peak); potassium 3.312 K_{α1,2} and 3.589 K_β (10% of K_{α1,2} peak); calcium 3.690 K_{α1,2} and 4.012 K_β (10% of K_{α1,2} peak); chromium 5.411 K_{α1,2}; copper 0.930 L_α and 8.040 K_{α1,2}. Elements are identified on each spectra. Energy levels in keV are shown on each abscissa, and the vertical scale (VS) is shown above each spectrum. When present, the copper peaks are artifacts of copper support grid usage. Chromium peaks are also thought to be artifacts since Cr is present in the aperture.

FIG. 1. *C. maxima*, cotyledon tip; upper epidermis. Due to the small size of the globoid crystal, some of the surrounding proteinaceous material may have been analyzed with the globoid crystal.

FIG. 2. *C. maxima*, cotyledon tip; lower epidermis.

FIG. 3. *C. maxima*, cotyledon base; upper epidermis. Due to the small size of the globoid crystal, some of the surrounding proteinaceous material may have been analyzed with the globoid crystal.

FIG. 4. *C. maxima*, cotyledon tip; small provascular region. Due to the small size of the globoid crystal, some of the surrounding proteinaceous material may have been analyzed with the globoid crystal.

FIG. 5. *C. maxima*, cotyledon tip; large provascular region.

FIG. 6. *C. maxima*, cotyledon base; large provascular region.

FIG. 7. *C. maxima*, cotyledon center; large provascular region.

FIG. 8. *C. maxima*, cotyledon center; first mesophyll cell outside large provascular region illustrated by Figure 7.

FIG. 9. *C. maxima*, cotyledon center; center of mid-sized provascular region.

FIG. 10. *C. maxima*, cotyledon center; first mesophyll cell outside the mid-sized provascular region illustrated by Figure 9.

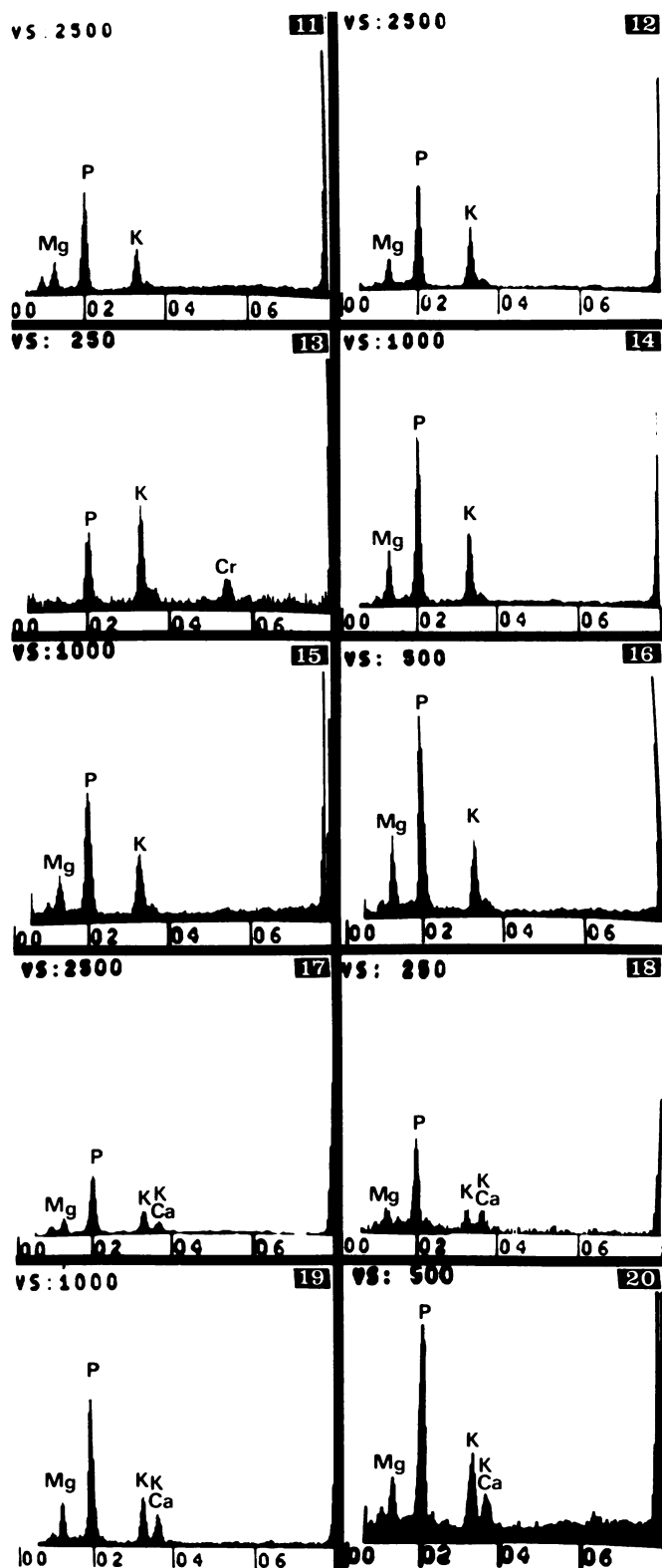


FIG. 11. *C. maxima*, cotyledon tip; palisade.
 FIG. 12. *C. maxima*, cotyledon center; palisade.
 FIG. 13. *C. maxima*, cotyledon base; palisade.
 FIG. 14. *C. maxima*, cotyledon tip; spongy mesophyll.
 FIG. 15. *C. maxima*, cotyledon center; spongy mesophyll cell apparently distant from any provascular regions.
 FIG. 16. *C. maxima*, cotyledon base; spongy mesophyll.
 FIG. 17. *C. maxima*, cotyledon center; spongy mesophyll, apparently distant from any provascular regions. This cell was beside the cell containing the globoid crystal analyzed in Figure 15.

taining globoid crystal was discovered (Fig. 17). This unusual globoid crystal was in a cell that was smaller than most spongy mesophyll cells.

Investigations of Globoid Crystals in *C. mixta* Cotyledons. Cells of both the upper and lower epidermis of *C. mixta* cotyledons contained some protein bodies with small globoid crystals. EDX analysis of these globoid crystals revealed the presence of Ca (Fig. 18).

In our studies the smallest provascular region found consisted of approximately 10 cells in cross section. In a given section from the region few cells contained globoid crystals and those that did had very small globoid crystals. EDX analysis of these globoid crystals plus some surrounding proteinaceous material revealed the presence of Ca (Fig. 19). The first layer of large mesophyll cells surrounding this small provascular region had large globoid crystals containing Ca. Within one of these surrounding mesophyll cells the amount of Ca present varied from trace amounts to reasonably high levels. The second and subsequent layers of mesophyll cells surrounding this small provascular region contained globoid crystals with no Ca content.

In all of the medium and large sized provascular areas studied, protein bodies with globoid crystals were common. No identifiable mature xylem or phloem was detected. The globoid crystals of medium and large sized provascular regions contained Ca irrespective of position (Figs. 20 and 21).

In all cases examined, the globoid crystals of mesophyll cells that surrounded large sized provascular areas contained no Ca (Fig. 22). Generally palisade (Fig. 23) and spongy (Fig. 24) mesophyll cells contained globoid crystals with no traces of Ca. One globoid crystal was discovered which contained no Ca and no Mg (Fig. 25).

Rarely an isolated provascular or spongy mesophyll cell was discovered which contained what appeared to be normal electron-dense globoid crystals. In these rare cells no P, K, Ca, or Mg peaks appeared upon EDX analysis of the globoid crystals.

Investigations of Globoid Crystals in *C. andreana* Cotyledons. In some epidermal cells, very small globoid crystals were discovered. Ca was commonly present in such crystals and the K levels were low (Fig. 26).

No large provascular regions the size of the largest ones in *C. maxima* and *C. mixta* were discovered. This difference may be related to seed size since *C. andreana* has a much smaller seed than the other two species studied. In all examined provascular regions no mature xylem or phloem cells were detected. Cells in small and medium sized provascular regions contained protein bodies with globoid crystals. These globoid crystals contained Ca (Fig. 27).

Ca was commonly found in the globoid crystals of palisade mesophyll cells (Fig. 28). Most spongy mesophyll cells also contained globoid crystals with Ca (Fig. 29). In the spongy mesophyll samples considerable variation was noted between seeds and between different blocks from a given seed. Globoid crystals in spongy mesophyll cells near the lower epidermis often lacked Ca (Fig. 30) whereas globoid crystals from cells further away from the lower epidermis contained Ca (Fig. 29). In a section of a given cell all globoid crystals tended either to have Ca or to be completely lacking in Ca. Although variation existed, all seeds examined had Ca in globoid crystals from at least some spongy mesophyll cells.

DISCUSSION

In the early stages of seedling growth, reserves stored in globoid crystals are mobilized to supply necessary macronutrients. Of the

FIG. 18. *C. mixta*, cotyledon center; upper epidermis.
 FIG. 19. *C. mixta*, cotyledon center; very small provascular region.
 FIG. 20. *C. mixta*, cotyledon center; center of provascular region.

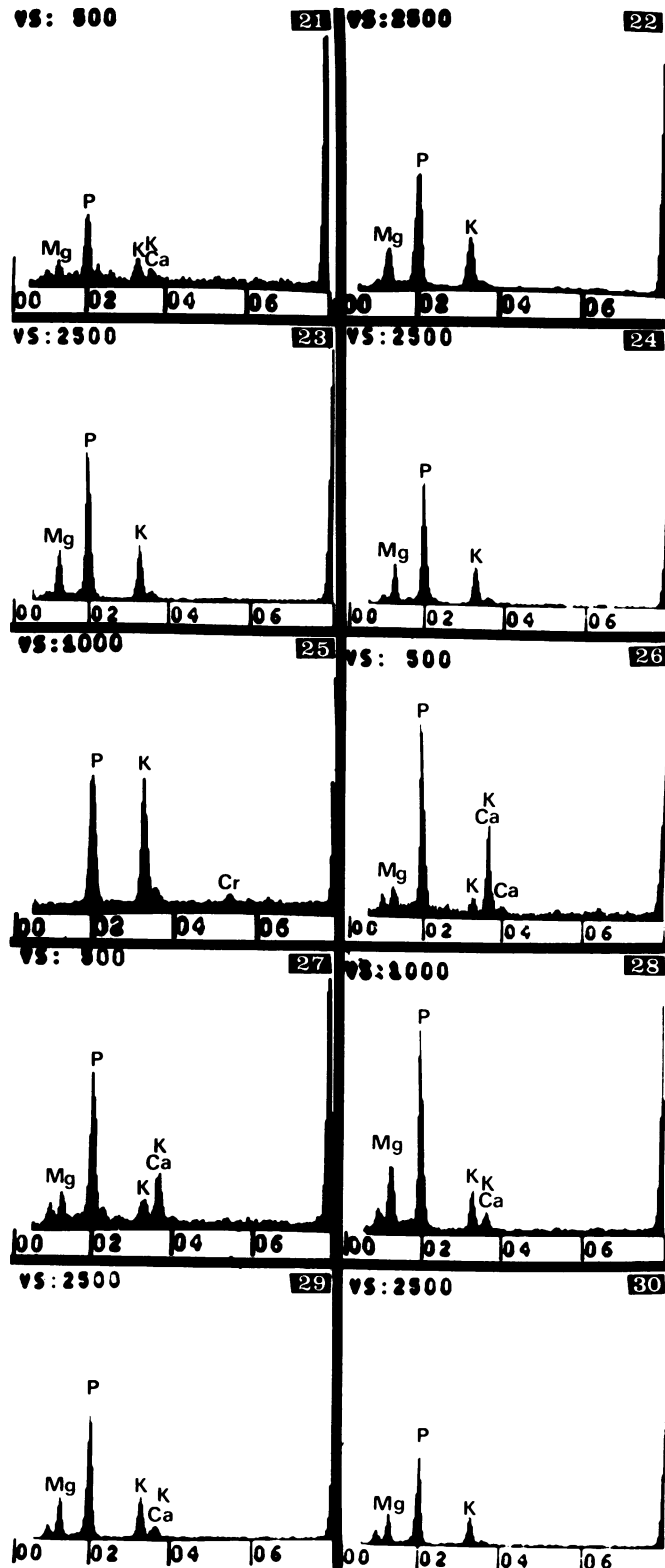


FIG. 21. *C. mixta*, cotyledon center; outer layer of provascular region. Due to the small size of the globoid crystal, some of the surrounding proteinaceous material may have been analyzed with the globoid crystal.

FIG. 22. *C. mixta*, cotyledon center; first cell outside provascular region illustrated by Figure 21.

FIG. 23. *C. mixta*, cotyledon center; palisade.

FIG. 24. *C. mixta*, cotyledon center; spongy mesophyll apparently well clear of any provascular region.

FIG. 25. *C. mixta*, cotyledon center; spongy mesophyll.

FIG. 26. *C. andreana*, cotyledon center; lower epidermis.

four macronutrients commonly found in globoid crystals (P, K, Mg, and Ca) it is the Ca distribution that is of special interest for two reasons. During seedling growth Ca often becomes limiting first and thus Ca content may be more critical for seedling establishment than P, K, and Mg content. Second, Ca shows the most interesting spatial relationships. From the results of Lott *et al.* (7) and Lott and Vollmer (10) it is clear that different regions of an embryo may differ in Ca content and that seed size can be an important factor. In *Cucurbita* species with large seeds, Ca was found mainly in storage tissues of the radicle (*C. mixta*, *C. moschata*) or root-shoot regions (*C. maxima*). In species with small seeds (*C. foetidissima*, *C. pepo*, *C. andreana*) Ca was found in globoid crystals in all embryo regions tested. The results of Lott and Vollmer (10) plus the more circumstantial evidence outlined in Lott and Buttrose (4) support the concept that Ca distribution in globoid crystals can be correlated with seed weight. The results presented here provide further support for some aspects of the Lott and Vollmer (10) study and extend our knowledge of cell-to-cell variations in Ca content of globoid crystals.

Globoid crystals in protein bodies of epidermal cells were usually small compared to those of adjacent mesophyll cell protein bodies. Ca was commonly found in the epidermal cell globoid crystals in the three species studied. No consistent difference in Ca content was evident between globoid crystals from the upper and lower epidermis. Since globoid crystals were found in a series of adjacent epidermal cells it seems unlikely that the presence of Ca reserves is related to eventual formation of such epidermal modifications as guard cells.

Ca was commonly present in globoid crystals in provascular regions of all three *Cucurbita* species analyzed. An exception was the occurrence of globoid crystals lacking Ca in the very small provascular regions of *C. maxima*. Within a provascular region the elemental content of all globoid crystals was similar. There was no differential element storage between cells that would mature into xylem and those that would become phloem. In *C. mixta* and *C. maxima*, where the mesophyll cell globoid crystals usually lack Ca, there was a pronounced change in Ca storage between the provascular cells and the first layer of mesophyll cells. In some cases, especially with small to medium sized provascular regions, there was Ca storage not only in provascular region globoid crystals but also in globoid crystals of the first layer of mesophyll cells surrounding the provascular region. In such cases there was a pronounced change in Ca content of globoid crystals between the first and second layer of mesophyll cells around a provascular region.

Why Ca should be localized in provascular regions is uncertain although several possibilities can be proposed. In cucurbit cotyledons it is more likely that cell divisions will take place in developing veins than in the mesophyll cells which enlarge during seedling development but divide little, if at all. A supply of Ca may also be necessary for development of provascular regions into functioning veins since the development of mature vascular tissue, especially xylem, requires synthesis and deposition of considerable cell wall material. It could also be that storage of Ca in provascular regions facilitates early movement of Ca from the cotyledon to the developing root-shoot regions.

In *C. mixta* and *C. maxima* the globoid crystals of palisade and spongy mesophyll cells from the central cotyledon region generally lacked Ca. These findings are consistent with earlier studies (7, 10). In both species, cases were discovered where mesophyll cells

FIG. 27. *C. andreana*, cotyledon center; medium sized provascular region.

FIG. 28. *C. andreana*, cotyledon center; palisade.

FIG. 29. *C. andreana*, cotyledon center; spongy mesophyll cell positioned at least five cells above lower epidermis.

FIG. 30. *C. andreana*, cotyledon center; spongy mesophyll, first cell above lower epidermis.

located immediately next to medium or small sized provascular regions contained Ca in their globoid crystals. The observation of a Ca-rich globoid crystal in a *C. maxima* spongy mesophyll cell does not allow us to rule out the possibility of randomly occurring mesophyll cells having globoid crystals with high Ca content. It is possible that a cell which appears to be surrounded only by other mesophyll cells may actually be next to a provascular region situated either above or below the plane of section.

Since only large globoid crystals were analyzed in the Lott *et al.* (7) study it seems unlikely that globoid crystals from epidermal cells or most provascular cells would have been chosen because of their small size. It seems probable that the occasional globoid crystal with high Ca content reported by Lott *et al.* (7) in *C. maxima* cotyledons was due to analysis of cells near provascular regions. Higher mean Ca values in such regions as the cotyledon base may be a reflection of the increased concentration of provascular tissue in such regions.

In *C. andreana*, globoid crystals of palisade and spongy mesophyll cells often contained at least some Ca, but considerable variation occurred within and between the seeds studied. Spongy mesophyll cells near the lower epidermis often lacked Ca-containing globoid crystals. The findings are consistent with the results of Lott and Vollmer (10) who commonly found Ca present in globoid crystals from freeze-dried tissue powders of *C. andreana* central cotyledon tissue.

The results suggest that Ca content and size of a globoid crystal are not directly related. Although it is true that globoid crystals from both epidermal cells and provascular cells are small and Ca-containing, it is also possible to have Ca-containing large globoid crystals in some mesophyll cells. Results indicate that Ca and K values may be related since Ca peaks were highest when the K peaks were lowest.

Some of the available EDX analysis studies of globoid crystal composition in cotyledons have noted the presence of Ca-containing and Ca-lacking globoid crystals. Such differences have been reported to occur in cotyledons of almond (5), macadamia (4), and jojoba (1). From the results of this study it seems likely that differences in globoid crystal composition in almond, macadamia, and jojoba cotyledons depend upon positional differences in the cotyledon.

The reason that occasional cells of *C. mixta* have electron-dense globoid crystals lacking P, K, Mg, and Ca is unknown. That globoid crystals have electron density based on something other than the presence of mineral reserves is suggested by this obser-

vation and supported by earlier work (6) which demonstrated that OsO₄-extracted globoid crystals were still electron-dense. Extraction is not considered to be the likely cause of the observation reported here since neighboring cells in the same tissue type contained globoid crystals with high elemental content.

From the results it is clear that a great deal of selectivity takes place when globoid crystals are deposited in developing seeds. Striking differences in the Ca content of globoid crystals from adjacent cells were found. Position of the cell within the cotyledon is clearly of greater importance in determining the elemental content of globoid crystals than is the cell type *per se*. For example, provascular cells in the cotyledons of either *C. maxima* or *C. mixta* may contain globoid crystals with Ca or without, depending upon whether the cell is in a very small provascular region or a larger one.

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