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Energy-dispersive x-Ray Analysis of Phosphorus, Potassium, Magnesium, and Calcium in Globoid Crystals in Protein Bodies from Different Regions of *Cucurbita maxima* Embryos[1]

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ABSTRACT

The seeds of *Cucurbita maxima* contain protein bodies with electron-dense globoid crystals. Because of their density globoid crystals are ideal material for energy-dispersive x-ray (EDX) analysis studies of elemental composition. Fixation trials were carried out to test globoid crystal extraction during glutaraldehyde fixation, water washing, and ethanol dehydration. Glutaraldehyde fixation without subsequent washing or dehydration alone produced no significant changes in elemental composition of cotyledon globoid crystals. If glutaraldehyde fixation was followed by water washes or ethanol dehydration there was some loss of the major globoid crystal elements but the relative percentages of the elements P, K, Ca, and Mg remained relatively unchanged. In this paper results of a study of the P, K, Mg, and Ca content of globoid crystals in different tissues of squash embryos are presented. The globoid crystals in the radicle were found to be the least dense in the embryo. Globoid crystals from all embryo regions contained P, K, and Mg. In the various embryo regions P and Mg maintained relatively constant proportions of the globoid crystal composition while K and Ca varied. Of particular significance is the distribution of Ca which is generally an immobile element. Calcium was found in highest amounts in the globoid crystals of the radicle and stem regions while globoid crystals in much of the cotyledon contained little, if any, Ca. The Ca storage thus seems to be spatially arranged in a manner that would aid early growth of the root-shoot axis.

In order for us to understand the nutritional state of embryo cells during the critical early stages of seedling growth we need to understand what elements are available and where they are located in the cell and embryo. Research from a number of laboratories has indicated that much of the seed's store of elements such as phosphorous is in an organic form, particularly in the form of phytin (5, 7, 10, 16). Phytin is a cation (Mg, K, Ca) salt of inositol hexaphosphoric acid (1, 2, 5, 15). Evidence from investigation of a number of seeds indicates that phytin is located in the protein bodies mainly in electron-dense regions called globoid crystals (11, 15, 17, 21). In *Cucurbita maxima* the seed protein bodies often are structurally complex and contain four regions, namely the protein crystalloid, the proteinaceous matrix, the soft globoid, and the globoid crystal (14). A study of globoid crystal elemental composition throughout the embryo will provide information on the distribution of much of the seed's store of elements such as P, K, Ca, and Mg. We have undertaken such a study through the use of energy dispersive x-ray analysis, a method that allows one to obtain specific information on the elemental composition of selected regions within a cell.

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MATERIALS AND METHODS

PREPARATION OF COTYLEDON TISSUE TO STUDY FIXATION EFFECTS

Five similar size seeds of *C. maxima* Duch. var. Warted Hubbard were chosen from the seeds of a fruit that was grown at McMaster University. One seed was apportioned to each of the five treatments and only central cotyledon tissue was used. Treatments were as follows.

a. Control. Tissue, without fixation, was cut into small pieces with a razor blade and frozen in liquid N_2. The frozen pieces were ground with a mortar and pestle precooled with liquid N_2. The ground tissue, still frozen, was placed in a cold storage vial and kept frozen in liquid N_2 until all samples were ready for freeze-drying.

b. Glutaraldehyde. Tissue was cut into small pieces and fixed for 1.5 hr at 1 C with 5% glutaraldehyde in distilled H_2O. The fixative was adjusted to pH 7.1 with NaOH. At the end of the fixation period the tissue was frozen, ground, and stored as described for control samples.

c. Glutaraldehyde, Ethanol. Tissue was fixed as described above, then rinsed with distilled H_2O and placed into 100% ethanol for 18 hr at 20 C. After the ethanol treatment the tissue was frozen, ground, and stored as described for the control.

d. Glutaraldehyde, 4-hr Water Wash. Tissue pieces were fixed as described in b above and then given a 4-hr wash in distilled H_2O. During fixation and washing the samples were kept on ice. Following the water wash the tissue pieces were frozen, ground, and stored as described for the control sample.

e. Glutaraldehyde, 18-hr Water Wash. Treatment as in d above except that the water wash was for 18 hr.

When all cotyledon samples were available as frozen powders they were low temperature freeze-dried according to the method of Darley and Lott (3). The dry powder was dusted on Formvar and carbon-coated grids.

PREPARATION OF TISSUE FOR THE STUDY OF DIFFERENT EMBRYO REGIONS

Squash seeds used were *C. maxima* Duch. var. Warted Hubbard, purchased from Stoke's Seeds Ltd., St. Catharines, Ontario and *Cucurbita maxima* Duch. var. Green Warted Hubbard ob-

tained from a single fruit grown at CSIRO, Adelaide, South Australia.

Two seeds of similar size from the Stoke's seed supply (seeds 1 and 2) and two seeds from a fruit grown at CSIRO (seeds 3 and 4) were selected for EDX[2] analysis. The seed coats were removed and tissue was sampled from the radicle, stem, cotyledon base, cotyledon center, cotyledon side, and cotyledon tip of each seed. Since the embryo axis of squash is very short, the stem sample was selected as the portion of the axis to which the cotyledons were attached. The cotyledon base sample was that portion of the cotyledon immediately adjacent to the cotyledonary node. A 3-mm^2 region at the center of the cotyledon was used as the cotyledon center sample. The cotyledon side sample came from a region (2 × 5 mm) at the edge of the cotyledon, midway between the cotyledon base and the cotyledon tip. For the cotyledon tip sample a 2-mm^2 region of tissue was taken from the edge of the cotyledon in the region furthest removed from the cotyledonary node.

Tissues from the cotyledon and stem regions were sliced 0.5 to 1 mm thick prior to fixation. The samples were prepared for EDX analysis according to the method of Lott (11). Tissues from the dry seeds were fixed in 5% glutaraldehyde in distilled H_2O, dehydrated, infiltrated with Spurr's low viscosity epoxy resin, and polymerized. Sections, 150 to 170 nm thick, were mounted on copper grids having a carbon-coated Formvar support film and were given an additional thin carbon coating prior to analysis.

EDX ANALYSIS

EDX analyses were performed with a Philips 300 TEM fitted with a model 606 x-ray spectrometer, a model 707A energy-dispersive analysis of x-ray system, and an EDIT data improvement system (EDAX International Inc.). All analysis work took place at an accelerating voltage of 80 kv. All analyses were 1 min in duration. To reduce variation due to the equipment, EDAX electronics were turned on at least 14 hr before use, the filament of the TEM was aligned before use, and the count rate (in and out) was adjusted to between 1,300 and 2,000 cps.

Globoid crystals were easily identifiable due to their electron density. Since the globoid crystals of squash are relatively large it was always possible to use a spot size that did not exceed the size of the globoid crystals. In sectioned material the palisade parenchyma and spongy mesophyll were distinguishable in each of the four cotyledon regions and were treated as separate tissues. Twenty-five globoid crystals from each fixation trial and from each tissue region of seed 1 were analyzed using EDX. At least five globoid crystals from radicle, stem, cotyledon base palisade, cotyledon base spongy, cotyledon tip palisade, and cotyledon tip spongy regions from each of the remaining three seeds were EDX-analyzed.

Integrated counts under the major P, K, Ca, and Mg peaks plus backgrounds were determined using the EDIT window program. Windows were chosen to allow inclusion of all counts in a peak. Windows used were as follows: calcium K_α from 3.50 to 3.74 kev; potassium K_α from 3.16 to 3.50 kev; magnesium K_α from 1.14 to 1.42 kev; phosphorus K_α from 1.86 to 2.20 kev. The spectra were then put through the EDIT background subtract program and the windows as listed above were reanalyzed. In this way the peak and the background values could be determined. For Ca where the major K_α peak, centered at 3.69 kev, is overlapped by the minor K_β peak of potassium, centered at 3.59 kev, the Ca value was determined by subtracting 10% of the value for the K_α peak of potassium from the Ca value. It is known from standards that the K_β peak of potassium is 10% of the K_α peak of potassium.

Peak-to-background numbers were calculated for each of the four major elements of each globoid crystal by dividing the peak-minus-background value by the background value. The mean peak-to-background numbers and standard deviations were calculated for each element of the globoid crystals of each tissue. The total mean peak-to-background numbers for the elements were calculated for each fixation trial or for each tissue by adding together the P, K, Ca, and Mg mean peak-to-background numbers. The percentage of this total for each element was calculated. An analysis of variance calculation was performed using a Hewlett-Packard 2000 computer. Duncan's new multiple range test (4) was employed to determine which differences between the mean peak-to-background numbers for each element were significant at the 99% level.

MINERAL ANALYSIS

In addition to EDX analysis studies of two seeds of the CSIRO-grown fruit, a sample of cotyledon tissue from other seeds of the same fruit was analyzed by The Australian Mineral Development Laboratories, Frewville, South Australia. A 10-g sample of cotyledon tissue was ground, dried at 100 C, and dissolved in $HClO_4$. P was determined by the colorimetric method with ammonium molybdate blue reagent. K, Ca, and Mg were determined by standard atomic absorption spectrophotometric techniques.

RESULTS

Studies into Elemental Loss during Fixation. Data presented show the effects of various fixation procedures on the elemental composition of globoid crystals from protein bodies in the central cotyledon regions. The procedures chosen test the effect of glutaraldehyde fixation alone, water washes after glutaraldehyde fixation, and ethanol dehydration after glutaraldehyde fixation. The fixation trials thus test the major treatments where extraction could occur during a regular EDX analysis fixation procedure as used by Lott (11). The effect of epoxy resins was not tested due to technical difficulties with grinding and freeze-drying tissue in liquid epoxy resins.

Numerical results from cotyledon tissue are presented in a manner that allows: (*a*) the study of fixation-induced changes in the levels of the elements P, K, Ca, and Mg (Table I); the study of the total levels of all four elements combined and the study of any differential extraction of elements (Table II); and the study of statistical significance of various differences (Table III).

From Table I it is clear that Ca was present in trace amounts only in some of the globoid crystals in cotyledon tissue. All

Table I. Effects of Various Fixation and Dehydration Treatments on P, K, Mg and Ca Levels in Globoid Crystals from the Center Cotyledon Protein Bodies of Squash.

EDX analyses of twenty-five globoid crystals per treatment were carried out and the mean peak-to-background numbers plus standard error x 2 were calculated for the P, K, Mg and Ca peaks.

Element	Treatment	Mean Peak-to-Background Number	Standard Error x2
P	Control	4.32	0.69
	Glutaraldehyde	5.17	0.73
	Glut. → EtOH (18 hr)	3.11	0.41
	Glut. → Wash (4 hr)	3.14	0.62
	Glut. → Wash (18 hr)	2.52	0.32
K	Control	2.79	0.45
	Glutaraldehyde	2.58	0.37
	Glut. → EtOH (18 hr)	1.73	0.24
	Glut. → Wash (4 hr)	1.80	0.40
	Glut. → Wash (18 hr)	1.35	0.23
Ca	Control	0.013	0.01
	Glutaraldehyde	0.000	0.00
	Glut. → EtOH (18 hr)	0.009	0.02
	Glut. → Wash (4 hr)	0.006	0.01
	Glut. → Wash (18 hr)	0.000	0.00
Mg	Control	1.67	0.23
	Glutaraldehyde	2.01	0.30
	Glut. → EtOH (18 hr)	1.36	0.21
	Glut. → Wash (4 hr)	1.31	0.27
	Glut. → Wash (18 hr)	1.19	0.21

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

treatments had a number of globoids with no detectable Ca. No significant differences in Ca levels were observed as a result of the different fixation and dehydration treatments (Table III).

Glutaraldehyde fixation alone did not result in any significant changes in the levels of the elements P, K, Mg, and Ca in the globoid crystals of cotyledon tissue (Table III). Fixation treatments involving more prolonged periods of time in water or in ethanol did cause a significant reduction in mean peak-to-background numbers for the elements P and K (Table III). While there was definite extraction with prolonged water washes or during dehydration, as can be seen from the reduction in total mean peak-to-background values in Table II, the per cent of the total mean peak-to-background numbers (Table II) is remarkably constant. Of the four elements measured, K would seem to be the most readily extracted element.

Studies of Different Regions of the Embryo. EDX analysis results from a detailed study of one Stoke's seed are presented in Tables IV to VI and Figures 1 to 3. Findings of the detailed study of one seed were confirmed by EDX analysis of selected tissue regions from three other seeds. One of these additional seeds, called seed 2, was of North American origin while seeds 3 and 4 (Figs. 4–6) were obtained in Australia.

From a study of P, K, Mg, and Ca in 10 different tissue regions (Table IV) it is clear that the major EDX-analyzable elements usually present in squash globoid crystals were P, K, and Mg. These three elements were routinely found in globoid crystals from all tissue regions. Ca was found in trace amounts, if at all, in most cotyledon globoid crystals but was present at higher levels in the radicle and stem.

The total mean peak-to-background numbers (Table V) give a rough estimate of how much of the elements P, K, Ca, and Mg combined were present in the globoid crystals. The results of this table show that the globoid crystals from the root region were the least dense in the squash seed.

P and Mg form a relatively constant proportion of the total counts in all tissue regions while the K and Ca values show major changes with respect to position in the embryo (Table V). Globoid crystals from protein bodies in much of the cotyledon lacked or had only traces of Ca. The radicle and stem had major amounts of Ca in their globoid crystals. The differences in Ca content in radicle, stem, and cotyledon tip can be seen by visual comparison of Figures 1 to 3. The relatively high content of Ca in the radicle and stem was associated with a relatively low content of K. The center cotyledon palisade value for Ca, which is higher than the other cotyledon values, was a result of two very high samples in the 25. If these two high values are removed the mean peak-to-background number drops from 0.23 to 0.15, a value identical to the cotyledon base samples.

There were relatively few statistically significant differences between the various cotyledon tissues and regions, but differences in Ca between the stem and the cotyledon base tissues were significant (Table VI). Similarly, P, K, and Mg were significantly different between the radicle and the stem.

The bulk of our EDX data was obtained from a detailed study of one seed. To see if the trends observed in seed 1 represented a

Table II. Effects of Various Fixation and Dehydration Treatments on the Total Mean Peak-to-background Numbers and Percentage of the Total Mean Peak-to-background Number for the Elements P, K, Mg and Ca.

These data allow one to estimate the relative amounts of the four elements in center cotyledon globoid crystals after various treatments and whether any differential extraction of elements occurs.

Treatment	Total Mean Peak-to-background Numbers*	% of Total Mean Peak-to-background Number† P	K	Ca	Mg
Control	8.79	49.15	31.74	0.15	19.00
Glutaraldehyde	9.76	52.97	26.43	0.00	20.59
Glut. EtOH (18 hr)	6.21	50.08	27.86	0.15	21.90
Glut. Wash (4 hr)	6.23	50.40	28.89	0.10	21.03
Glut. Wash (18 hr)	5.06	49.80	26.68	0.00	23.52

* The sum of the mean peak-to-background numbers for the elements P, K, Ca and Mg.

† Calculated by determining the % of the total peak-to-background number represented by the mean peak-to-background number for each of the elements P, Ca, K and MG. This % figure must not be confused with actual % of the element in the globoid crystal (see discussion section).

Table III. Statistical Comparison of Control and Various Fixation and Dehydratio Treatments of Center Cotyledon Tissue.

Analysis of variance calculations were conducted and a Duncan's new multiple range test was used to determine if differences were significant (S) or not significant (NS) at the 99% level.

Comparison		Element P	K	Ca	Mg
Control	: Glut.	NS	NS	NS	NS
Control	: Glut. → Wash (4 hr)	S	S	NS	NS
Control	: Glut. → Wash (18 hr)	S	S	NS	NS
Control	: Glut. → EtOH	S	S	NS	NS
Glut.	: Glut. → Wash (4 hr)	S	S	NS	S
Glut.	: Glut. → Wash (18 hr)	S	S	NS	S
Glut.	: Glut. → EtOH	S	S	NS	S
Glut. → Wash (4 hr)	: Glut. → Wash (18 hr)	NS	NS	NS	NS

Table IV. The P, K, Ca and Mg Levels from Globoid Crystals of Various Tissues of One Cucurbita maxima Duch. var. Warted Hubbard Embryo.

Twenty-five globoid crystals per tissue were analyzed using EDX analysis and the mean peak-to-background numbers and standard error x 2 were calculated for the major P, K, Ca and Mg peaks.

Element	Tissue Regions	Mean Peak-to-Background number	Standard Error x 2
Phosphorous	Radicle	2.80	0.75
	Stem	5.67	0.71
	Cotyledon Base Palisade	5.20	0.76
	Cotyledon Base Spongy	5.58	0.95
	Cotyledon Center Palisade	5.12	0.69
	Cotyledon Center Spongy	6.22	0.96
	Cotyledon Side Palisade	3.60	0.72
	Cotyledon Side Spongy	6.00	0.81
	Cotyledon Tip Palisade	4.62	0.89
	Cotyledon Tip Spongy	4.61	1.05
Potassium	Radicle	0.39	0.12
	Stem	1.14	0.20
	Cotyledon Base Palisade	0.94	0.15
	Cotyledon Base Spongy	1.67	0.33
	Cotyledon Center Palisade	1.38	0.33
	Cotyledon Center Spongy	1.87	0.35
	Cotyledon Side Palisade	1.13	0.28
	Cotyledon Side Spongy	2.23	0.41
	Cotyledon Tip Palisade	1.38	0.32
	Cotyledon Tip Spongy	1.20	0.36
Calcium	Radicle	0.38	0.11
	Stem	0.45	0.19
	Cotyledon Base Palisade	0.15	0.07
	Cotyledon Base Spongy	0.15	0.07
	Cotyledon Center Palisade	0.23	0.14
	Cotyledon Center Spongy	0.06	0.05
	Cotyledon Side Palisade	0.04	0.04
	Cotyledon Side Spongy	0.07	0.07
	Cotyledon Tip Palisade	0.03	0.03
	Cotyledon Tip Spongy	0.05	0.04
Magnesium	Radicle	0.89	0.21
	Stem	1.90	0.28
	Cotyledon Base Palisade	2.00	0.25
	Cotyledon Base Spongy	1.84	0.27
	Cotyledon Center Palisade	1.86	0.40
	Cotyledon Center Spongy	2.41	0.48
	Cotyledon Side Palisade	1.35	0.19
	Cotyledon Side Spongy	2.29	0.32
	Cotyledon Tip Palisade	1.66	0.30
	Cotyledon Tip Spongy	1.70	0.36

Table V. The Total Mean Peak-to-background Numbers and Percentages of the Total Mean Peak-to-background Numbers for P, K, Ca and Mg in the Globoid Crystals of Various Tissue Regions in One Cucurbita maxima Embryo.

Tissue Region	Total Mean Peak-to-background numbers*	% of Total Mean Peak-to-background Numbers† P	K	Ca	Mg
Radicle	4.46	62.78	8.74	8.52	19.96
Stem	9.16	51.40	12.43	4.41	29.76
Cotyledon Base Palisade	8.29	62.73	11.34	1.81	24.12
Cotyledon Base Spongy	9.24	60.39	18.08	1.62	19.91
Cotyledon Center Palisade	8.59	59.60	16.07	2.68	21.65
Cotyledon Center Spongy	10.56	58.40	17.71	0.57	23.82
Cotyledon Side Palisade	9.12	58.52	14.46	0.65	26.37
Cotyledon Side Spongy	10.39	56.66	21.06	0.66	21.62
Cotyledon Tip Palisade	7.69	60.08	17.95	0.39	21.58
Cotyledon Tip Spongy	7.56	60.98	15.87	0.66	22.49

* The sum of the mean peak-to-background numbers for each of the elements P, Ca, K, Mg.

† Calculated by determining the % of the total peak-to-background number represented by the mean peak-to-background number for each of the elements P, Ca, K and Mg. This % figure must not be confused with actual % of the element in the globoid crystal (see discussion section).

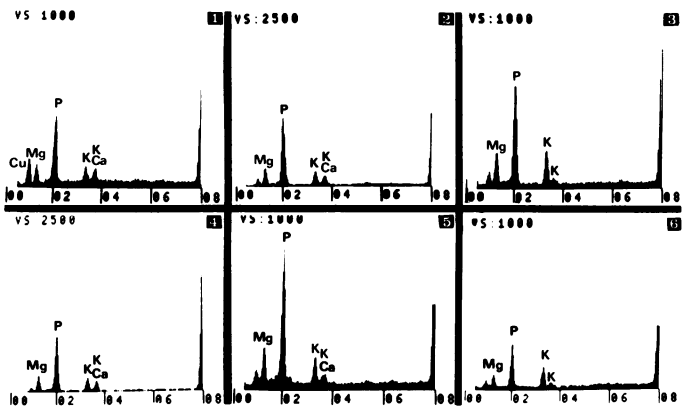
general phenomenon we compared the results with those obtained from three other seeds. In all four seeds, Ca was present in trace amounts, if at all, in the globoid crystals of the cotyledon tip. In all four seeds the globoid crystals of the radicle and stem contained major amounts of Ca. The results of EDX analysis of cotyledon base tissue were more variable. No Ca was detected for either the palisade or spongy mesophyll cotyledon base tissue for seed 3. In the other seeds traces of Ca, to somewhat greater amounts, were detected in the globoid crystals of the cotyledon base tissues.

Mineral Analysis. The results of chemical analysis of whole cotyledons confirm that Ca levels in the cotyledon were indeed low and that P was present in the cotyledons in amounts at least twice that of K or Mg. The P, K, Ca, and Mg contents of squash cotyledons, as per cent of dry wt, were 1.20, 0.59, 0.02, and 0.49, respectively. The chemical analysis results have a direct reference to the EDX results from seeds 3 and 4 since all seeds concerned come from one fruit.

Table VI. Statistical Comparisons of the Elemental Composition of Globoid Crystals of Various Tissues in a Cucurbita maxima Embryo.

Analysis of variance calculations were conducted and Duncan's new multiple range test was used to determine if differences were significant (S) or not significant (NS) at the 99% level.

Comparison		P	K	Ca	Mg
Radicle	: Stem	S	S	NS	S
Stem	: Cotyl. Base Palisade	NS	NS	S	NS
Stem	: Cotyl. Base Spongy	NS	NS	S	NS
Cotyl. Base Spongy	: Cotyl. Base Palisade	NS	S	NS	NS
Cotyl. Centre Spongy	: Cotyl. Centre Palisade	NS	NS	NS	NS
Cotyl. Side Spongy	: Cotyl. Side Palisade	S	S	NS	S
Cotyl. Tip Spongy	: Cotyl. Tip Palisade	NS	NS	NS	NS
Cotyl. Base Spongy	: Cotyl. Centre Spongy	NS	NS	NS	NS
Cotyl. Base Palisade	: Cotyl. Centre Palisade	NS	NS	NS	NS
Cotyl. Base Spongy	: Cotyl. Side Spongy	NS	NS	NS	NS
Cotyl. Base Palisade	: Cotyl. Side Palisade	NS	NS	NS	NS
Cotyl. Centre Palisade	: Cotyl. Side Palisade	NS	NS	NS	NS
Cotyl. Centre Spongy	: Cotyl. Side Spongy	NS	NS	NS	NS
Cotyl. Centre Spongy	: Coty. Tip Spongy	NS	S	NS	NS
Cotyl. Centre Palisade	: Cotyl. Tip Palisade	NS	NS	S	NS
Cotyl. Side Spongy	: Cotyl. Tip Spongy	NS	S	NS	NS
Cotyl. Side Palisade	: Cotyl. Tip Palisade	NS	NS	NS	NS
Cotyl. Base Spongy	: Cotyl. Tip Spongy	NS	NS	NS	NS
Cotyl. Base Palisade	: Cotyl. Tip Palisade	NS	NS	NS	NS



FIGS. 1-6 EDX spectra of globoid crystals from tissues of two *C. maxima* seeds. Squash seed 1 (var. Warted Hubbard) was obtained from Stokes Seeds Ltd. Seed 4 (var. Green Warted Hubbard) was obtained from a single fruit grown at CSIRO. Tissues were fixed in 5% glutaraldehyde in distilled H_2O, dehydrated, embedded in Spurr's epoxy resin, and sectioned prior to analysis. Elements present, energy levels in kev, and principle emission lines are as follows: phosphorous 2.013, $K_{\alpha 1,2}$ and 2.028, $K_{\alpha 4}$ (10% of $K_{\alpha 1,2}$ peak) and 2.137 K (4% of $K_{\alpha 1,2}$ peak); potassium 3.312, $K_{\alpha 1,2}$ and 3.589, K_β (10% of $K_{\alpha 1,2}$ peak); calcium 3.690, $K_{\alpha 1,2}$; magnesium 1.253, K_α; copper 0.930, L_α and 8.040, $K_{\alpha 1,2}$. Elements are identified on each spectrum, energy levels in kev are shown on each abscissa and the vertical scale (V) is shown above each spectrum. Analysis time for each globoid crystal was 60 sec. The copper peaks when present are due to the copper supporting grids used. The spectra shown best illustrate the mean values obtained for each element in each tissue.

FIG. 1. Radicle tissue from seed 1.
FIG. 2. Stem tissue from seed 1.
FIG. 3. Cotyledon tip spongy mesophyll tissue from seed 1.
FIG. 4. Radicle tissue from seed 4.
FIG. 5. Stem tissue from seed 4.
FIG. 6. Cotyledon tip spongy mesophyll tissue from seed 4.

DISCUSSION

When combined with standardized data collection methods the calculation of peak-to-background values is useful for comparisons of the type attempted in this paper. The peak-minus-background to background calculation compensates for variations in sample thickness and differences in sample density (J. Russ, personal communication). We have thus chosen, throughout this paper, to use peak-to-background values rather than peak values alone. The window program used to obtain our data gives a total count for the entire peak and for the entire background. The values are in arbitrary units, however, and thus although the data can be used for some comparisons no attempt should be made to relate the peak-to-background value directly to quantitative values such as per cent dry wt or per cent of the globoid crystal.

Since the number of x-rays produced and hence the peak height are not the same for equal concentrations of all elements one should use caution in the comparison of levels for the four elements of interest here. P values, which give the relative intensity of x-rays from each element at the same concentration (18), have been calculated from known standards (18). No attempt has been made to use P values in the calculations for this paper because we are not sure that the elements in globoid crystals are uniformly distributed. Nevertheless, the P values do provide a useful guide in interpretation of the data. Ca and K have P values (80-kv accelerating voltage) of 0.94 and 0.93, respectively for the K_α emission lines (18). The similarity of these P values means that the EDX peak heights and total counts would be very similar if Ca and K were present in equal concentrations. The P values (80-kv accelerating voltage) for the K_α emission lines of P and Mg are 0.75 and 0.47, respectively (18). Thus, compared to Ca and K the peak-to-background values for P are a slight underestimate and the peak-to-background numbers for Mg are a major underestimate. This information should be kept in mind when considering the results. Because of differing P values for the elements P, K, Ca, and Mg the total mean peak-to-background numbers as presented in Tables II and V should be considered a rough guide only. Fortunately, the per cent of the total mean peak-to-background numbers as presented in Table V show a relatively constant proportion of P and Mg. The elements which varied most, namely K and Ca, were also the elements with very similar P values. For this reason we feel that the type of comparison made in Table V is valid. We caution readers however not to confuse the per cent of total mean peak-to-background numbers with, for example, actual per cent of the element in the globoid.

EDX analyses used to obtain data on globoid crystal composition in different embryo regions were obtained from glutaraldehyde-fixed, dehydrated, embedded and sectioned tissue. Although it is undoubtedly true that the preparative procedure removes some material from the globoids it also seems that in *Cucurbita* the common fixation solutions examined did not greatly change the proportion of the elements. Comparisons that are strictly qualitative will not detect much if any difference between glutaraldehyde-fixed squash cotyledon tissue and unfixed tissue. Although the use of freeze-dried tissue powders provides a check on fixation effects, the use of sectioned material does have advantages that, in our opinion, counterbalance the loss of precision due to fixation effects. The advantages of fixed and sectioned material over freeze-dried tissue powders include: a more uniform thickness and hence somewhat less variability in the EDX analysis; the ability to determine accurately what cell type a globoid crystal is located in; and the ability to analyze only a globoid crystal without interference from surrounding protein or tissue. For the study of globoid crystal composition in different embryo regions we used

fixed and sectioned tissue rather than freeze-dried tissue powders. In view of the different seeds and the different preparative methods used one should not compare too directly the results presented in Tables I to III with the results in Tables IV to VI. A strictly qualitative study using freeze-dried tissue powders from center cotyledon and radicle tissues (results not presented) does, however, provide confirmation of the findings that there is considerable Ca in radicle tissue globoid crystals.

Very few EDX analysis or microprobe studies of seed tissues have been published. Of those which have, species include *Crambe abyssinica* (8), *Oryza sativa* (22, 23), *Triticum aestivum* (23), *Cucurbita maxima* (11), *Hordeum vulgare* (9), and *Protea compacta* (24). To our knowledge there have been no published x-ray analysis studies of globoid crystal composition in protein bodies from different regions of the same embryo or fruit.

The studies of Ferguson and Bollard (6) of the Ca, K, Mg, and P content of pea seeds and the movement of these elements from cotyledon to root-shoot axis in pea seedlings are of interest to this study. These workers found that pea seeds contained less Ca than P, K, or Mg and that over half of the Ca was located in the testa. Whereas P, Mg, and K were readily exported from the cotyledon to the axis during seedling growth, little Ca was mobilized. Those workers also found that for optimum seedling growth they had to supply Ca long before any other macronutrients. Thus, it is clear that Ca is a relatively immobile element which is in short supply in the pea seed. A review of literature on pea seed protein bodies can be found in Lott and Buttrose (12) and a review of mineral transport in pea seedlings can be found in Sutcliffe (20).

Our finding that Ca is concentrated in globoid crystals in the root-shoot axis of squash is of potentially great importance in considerations of seedling nutrition. The Ca supply from the globoid crystals, while it may be a small part of the total seed Ca content, is spatially arranged in the areas where rapid growth occurs upon germination. The globoid crystals, being part of the protein body system, are presumably capable of mobilization to meet the demands of the growing axis. It is known that globoid crystal digestion is one of the earliest observable changes in squash cotyledon protein bodies during germination (13). A consideration of gross Ca analysis in whole seeds or fruits may be misleading in that much of the Ca may be in a bound form, such as in Ca oxalate or in cell walls. Chemical methods, even where globoid crystals are isolated, result in a pooling of material from different cells, tissues, and often organs. Isolated globoid crystals from the embryos of peanut (19) and cottonseed (15) have been analyzed. Ca was present in globoid crystals of both seeds but since whole embryos were used in these studies the distribution of the globoid crystal Ca within the embryo remains undetermined.

In a previous paper Lott (11) reported on the globoid crystal composition in protein bodies from seeds grown in four different regions of North America. These central cotyledon samples were all found to contain P, Mg, and K with occasional traces of Ca. On the basis of these results Lott (11) proposed that the globoid elemental composition was specific and not dependent upon environmental conditions such as soil type. The results of this study, in which two seeds from North America and two seeds from Australia were investigated, provide further support for specific elemental composition in globoid crystals. We must be very careful to consider the embryo region involved since, although relatively constant seed to seed for one embryo region, the composition does differ between regions such as the axis and the cotyledon. The reason that occasional globoid crystals have greatly different elemental compositions from the norm also remains unknown.

Any future EDX analysis work on globoid crystal composition must give a precise tissue location for the samples. Chemical analysis work should also be done with a specific tissue rather than whole seeds.

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