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Composition of Globoid Crystals from Embryo Protein Bodies in Five Species of *Cucurbita*[1]

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ABSTRACT

Previous energy-dispersive x-ray analysis studies of globoid crystal composition in seed protein bodies gave an indication that there might be a correlation between seed size and the type of elements stored in globoid crystals. This possibility was tested by conducting energy-dispersive x-ray analysis studies of P, K, Mg, and Ca levels in globoid crystals of four embryo regions (radicle, stem, cotyledon center palisade mesophyll, cotyledon center spongy mesophyll) in each of five different *Cucurbita* species (*C. mixta*, *C. moschata*, *C. foetidissima*, *C. pepo*, and *C. andreana*). The species were chosen to provide a range of seed size and weight. Globoid crystals from all embryo regions in all five species contained P, K, and Mg. Some variations in the levels of these elements did occur but there was no consistent pattern with regard to area of the seed or with regard to seed size. Calcium distribution showed significant variations. In species with large seeds (*C. mixta*, *C. moschata*) Ca was mainly found in globoid crystals in the radicle. Globoid crystals in species with small seeds (*C. foetidissima*, *C. pepo*, *C. andreana*) contained Ca in all embryo regions tested. The results of this study support the concept that Ca distribution in globoid crystals can be correlated with seed weight.

The protein bodies in seeds of most plant species contain mineral reserves in addition to proteins. These mineral reserves are generally thought to occur mainly as phytin, a cation (K, Mg, Ca) salt of inositol hexaphosphoric acid (see review in 6). In many species the phytin reserves are concentrated into electron-dense regions called globoid crystals (6, 9, 10, 12). The bulk of a seed's available reserves of several important macronutrients are thus found in globoid crystals inside protein bodies.

Lott *et al.* (8) carried out the first studies in which elemental composition of globoid crystals from different parts of a dicot embryo was studied with energy-dispersive x-ray analysis. Globoid crystals from all embryo regions of *Cucurbita maxima* were found to contain P, K, and Mg. The distribution of Ca was of particular interest since there was a definite spatial arrangement. Globoid crystals of the radicle and stem regions contained considerable Ca while globoid crystals in much of the cotyledon contained little, if any, Ca.

Globoid crystals of protein bodies in the main storage organ of various oil seeds have been studied with EDX[2] analysis (2, 3, 6, 7). Lott and Buttrose (6) observed that where Ca distribution was concerned there seemed to be an effect not only due to area of the embryo in question but also due to seed size. Globoid crystals from large cotyledons (sunflower, cashew, pistachio, walnut, hazel nut, jojoba) were found to contain either no Ca or only traces of Ca while globoid crystals from cotyledons of small seeds (*Helichrysum*) or radicles (Brazil nut) were found to contain Ca.

The possible relationship between Ca content in globoid crystals and seed size has never been carefully investigated. The observation of a possible influence of seed size upon Ca distribution is based upon comparison of studies of one embryo region only in seeds of different genera and different families. In this paper we describe the results of detailed EDX analysis studies into the P, K, Mg, and Ca content in globoid crystals in different embryo regions of five *Cucurbita* species. Since size of the seeds of the five *Cucurbita* species chosen ranged from large to small, this should provide a good test of any relationship between seed size and elemental distribution.

MATERIALS AND METHODS

Seeds. Seeds of *Cucurbita andreana*, *Cucurbita foetidissima*, *Cucurbita mixta*, *Cucurbita moschata* cv. Seminole Pumpkin, and *Cucurbita pepo* cv. Egg Gourd were kindly provided by W. P. Bemis, Department of Plant Sciences, University of Arizona, Tucson.

Dry Weights. The seed coats of 25 seeds of each *Cucurbita* species were removed. Before weighing, the seed coat and embryo samples were placed in an oven at 40 C for 16 hr to ensure uniform dryness.

Sample Preparation for EDX Analysis. Radicle, stem, cotyledon center palisade mesophyll, and cotyledon center spongy mesophyll samples were dissected out of seeds from each of the five *Cucurbita* species. Samples were placed in small pouches made of folded aluminum foil and frozen in liquid N_2. A foil pouch containing a tissue sample was removed from the liquid N_2, quickly placed on a wooden block, and hit several times with a hammer. This process fractures the frozen tissue into small pieces. As quickly as possible the cold aluminum foil pouch was opened and the tissue fragments dropped into a Pyrex test tube that was partially immersed in liquid N_2. If a sample adhered to the foil pouch the entire pouch plus tissue was dropped into the precooled test tube. Tissue samples in test tubes were then low temperature freeze-dried according to the procedure of Darley and Lott (4). Following freeze-drying the tissue powder was dusted or pressed onto Formvar and carbon-coated grids.

EDX Analysis. EDX analyses of 25 globoid crystals from each of the four embryo regions of one seed of each *Cucurbita* species were conducted using 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 by EDAX International Inc. All analysis work took place at an accelerating voltage of 80 kv and each analysis was 1 min in duration. To reduce equipment-caused variation, the EDAX electronics were turned on at least 14 hr before use, the filament of the TEM was kept aligned and the count rate (in and out) was adjusted to between 1,300 and 2,000 counts/sec. In freeze-dried powders globoid crystals were identified strictly on the basis of their

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

electron density. Although it is possible that a small portion of nongloboid material was analyzed along with the globoid crystal we avoided cases where the globoid crystal was obviously encased in other cellular material.

Using the EDIT window system integrated counts under the Ca, K, Mg, and P peaks plus backgrounds were determined. Windows used were: 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. After peak plus background values were determined the spectra were then put through the EDIT background subtract program and the windows as listed above were reanalyzed. The major K_α peak for calcium, centered at 3.69 kev, is overlapped by the minor K_β peak of potassium, centered at 3.59 kev. From standards it is known that the K_β peak of potassium is 10% of the K_α peak of potassium. The true Ca value was thus determined by subtracting 10% of the value for the K_α peak of potassium from the initial Ca value.

From this data peak and background values were determined. Division of peak values by background values gave peak-to-background numbers for each element. For each of the four elements in each of the samples mean peak-to-background numbers and standard error ×2 values were calculated. In addition, per cent of total mean peak-to-background numbers were calculated for each of the samples by adding together the mean peak-to-background number for Ca, K, Mg, and P in a given tissue region in a given species and then determining the per cent of that total number represented by each element. Analysis of variance calculations for the cotyledon samples were carried out using a Hewlett-Packard 2000 computer. Duncan's new multiple range test (5) was used to determine whether or not differences between the treatment means were significant at the 99% level.

Table I. Mean Weights in Grams of Seeds, Embryos and Seed Coats in Six Cucurbita Species

Species	Mean* Seed Weight	Mean* Embryo Weight	Mean* Seed Coat Weight
C. mixta	0.271	0.206	0.065
C. maxima	0.267	0.213	0.054
C. moschata	0.112	0.080	0.032
C. foetidissima	0.041	0.025	0.016
C. pepo	0.038	0.027	0.011
C. andreana	0.019	0.014	0.005

* Mean of 25 seeds

In addition to the one seed of each species that was sampled extensively using the procedure outlined above, other seeds of a given species were checked on strictly qualitative grounds. In the additional seeds, five globoid crystals of each tissue region were EDX analyzed.

RESULTS

Mean seed weights, mean embryo weights, and mean seed coat weights for six *Cucurbita* species are presented in Table I. The species with the heaviest seed, *C. mixta*, thus has a seed over 14 times heavier than that of *C. andreana*, the species with the smallest seed. To facilitate comparisons with seed weight, in all subsequent tables the species are arranged in the same order as they appear in Table I. Results for *C. maxima*, a species not used in this study, are presented here to facilitate comparison of the results presented in this paper with the results of Lott *et al.* (8).

Mean peak-to-background numbers and standard error ×2 values for P, K, Mg, and Ca content in globoid crystals from various tissues of *Cucurbita* embryos are presented in Tables II through V. Results of a statistical comparison of values for selected embryo regions are presented in Table VI. From Tables II through IV it is evident that P, K, and Mg are commonly found in globoid crystals in all tissue regions investigated in all five *Cucurbita* species examined. Statistically significant variations in P, K, and Mg values between different embryo regions do occur but there does not seem to be either any consistent pattern common to all species or any pattern in relation to seed weight.

Compared to the distributions of P, K, and Mg, the distribution of Ca shows striking differences in the species studied (Table V). In the species with the two largest seeds, namely *C. mixta* and *C. moschata*, the Ca is located to a very large extent in radicle globoid crystals only. This distribution pattern contrasts with the widespread Ca distribution found in globoid crystals of the three species with smallest seed weights (*C. foetidissima, C. pepo, C. andreana*).

While the data presented in Tables II through VI are based upon extensive study of one seed, strictly qualitative studies of additional seeds of each *Cucurbita* species were generally consistent with the results presented here. Considerable variation in the Ca content of globoid crystals from spongy mesophyll cells of *C. andreana* was found but in all seeds tested some Ca was present in at least some of the spongy mesophyll globoid crystals.

The P, K, Mg, and Ca values, expressed as per cent of the total mean peak-to-background number for a given species and tissue region, are presented in Table VII. The fraction of the total obtained from P is remarkably constant within one species and

Table II. Mean Peak-to-background Numbers for Phosphorus and Standard Error x2 Values for Globoid Crystals from Various Tissues of Cucurbita Embryos.

All numbers presented are mean peak-to-background numbers based upon analysis of 25 globoid crystals in freeze-dried tissue powders. Standard error x2 values are in brackets.

SPECIES	EMBRYO REGION			
	RADICLE	STEM	COTYLEDON CENTER PALISADE	COTYLEDON CENTER SPONGY
C. mixta	2.86 (0.44)	2.93 (0.47)	1.93 (0.47)	3.11 (0.74)
C. moschata	2.97 (0.48)	3.47 (0.59)	3.81 (0.51)	3.59 (0.54)
C. foetidissima	3.58 (0.33)	1.89 (0.84)	3.13 (0.44)	4.27 (0.67)
C. pepo	2.09 (0.39)	3.11 (0.49)	2.68 (0.57)	2.84 (0.45)
C. andreana	2.22 (0.34)	1.94 (0.25)	2.80 (0.70)	3.37 (0.61)

Table III. Mean Peak-to-background Numbers for Potassium and Standard Error x2 Values for Globoid Crystals from Various Tissues of *Cucurbita* Embryos.

All numbers presented are mean peak-to-background numbers based upon analysis of 25 globoid crystals in freeze-dried tissue powders. Standard error x2 values are in brackets.

SPECIES	EMBRYO REGION			
	RADICLE	STEM	COTYLEDON CENTER PALISADE	COTYLEDON CENTER SPONGY
C. mixta	1.46 (0.30)	1.66 (0.31)	1.17 (0.28)	1.48 (0.28)
C. moschata	1.65 (0.22)	2.18 (0.44)	2.86 (0.52)	1.81 (0.29)
C. foetidissima	1.89 (0.20)	2.57 (0.38)	1.68 (0.33)	2.65 (0.67)
C. pepo	0.87 (0.19)	1.43 (0.25)	1.39 (0.24)	1.48 (0.20)
C. andreana	0.83 (0.13)	0.90 (0.11)	1.21 (0.22)	1.20 (0.22)

Table IV. Mean Peak-to-background Numbers for Magnesium and Standard Error x2 Values for Globoid Crystals from Various Tissues of *Cucurbita* Embryos.

All numbers presented are mean peak-to-background numbers based upon analysis of 25 globoid crystals in freeze-dried tissue powders. Standard error x2 values are in brackets.

SPECIES	EMBRYO REGION			
	RADICLE	STEM	COTYLEDON CENTER PALISADE	COTYLEDON CENTER SPONGY
C. mixta	0.62 (0.11)	0.95 (0.21)	0.51 (0.15)	0.97 (0.29)
C. moschata	1.30 (0.28)	1.30 (0.33)	1.62 (0.24)	1.60 (0.35)
C. foetidissima	1.45 (0.23)	1.54 (0.26)	1.00 (0.20)	1.33 (0.24)
C. pepo	0.40 (0.22)	1.02 (0.20)	0.94 (0.24)	0.93 (0.19)
C. andreana	0.69 (0.14)	0.67 (0.12)	0.98 (0.23)	1.14 (0.26)

Table V. Mean Peak-to-background Numbers for Calcium and Standard Error x2 Values for Globoid Crystals from Various Tissues of *Cucurbita* Embryos.

All numbers presented are mean peak-to-background numbers based upon analysis of 25 globoid crystals in freeze-dried tissue powders. Standard error x2 values are in brackets.

SPECIES	EMBRYO REGION			
	RADICLE	STEM	COTYLEDON CENTER PALISADE	COTYLEDON CENTER SPONGY
C. mixta	0.40 (0.08)	0 (0.03)	0 (0.00)	0 (0.00)
C. moschata	0.31 (0.10)	0 (0.00)	0 (0.00)	0.01 (0.01)
C. foetidissima	0.20 (0.05)	0.11 (0.07)	0.12 (0.05)	0.23 (0.07)
C. pepo	0.19 (0.03)	0.22 (0.11)	0.15 (0.08)	0.28 (0.12)
C. andreana	0.11 (0.03)	0.04 (0.03)	0.08 (0.06)	0.14 (0.06)

even between species. Since Ca levels vary, especially in *C. mixta* and *C. moschata*, this information can be used to access changes in the other cations (K, Mg). In *C. mixta* it seems likely that both K and Mg levels are decreased when Ca levels are elevated. In *C. moschata* the K level is lowered when Ca is elevated.

In species where there is fairly widespread distribution of Ca

Table VI. Statistical Comparisons of the Elemental Composition of Globoid Crystals of Various Tissues in *Cucurbita* Embryos.

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.

Species	Areas Compared	Elements			
		P	K	Mg	Ca
C. mixta	Radicle : Stem	NS	NS	NS	S
	Stem : Palisade	NS	NS	S	NS
	Stem : Spongy	NS	NS	NS	NS
	Palisade : Spongy	S	NS	S	NS
C. moschata	Radicle : Stem	NS	NS	NS	S
	Stem : Palisade	NS	NS	NS	NS
	Stem : Spongy	NS	NS	NS	NS
	Palisade : Spongy	NS	S	NS	NS
C. foetidissima	Radicle : Stem	NS	S	NS	NS
	Stem : Palisade	S	S	S	NS
	Stem : Spongy	NS	NS	NS	NS
	Palisade : Spongy	NS	S	S	NS
C. pepo	Radicle : Stem	S	S	S	NS
	Stem : Palisade	NS	NS	NS	NS
	Stem : Spongy	NS	NS	NS	NS
	Palisade : Spongy	NS	NS	NS	NS
C. andreana	Radicle : Stem	NS	NS	NS	NS
	Stem : Palisade	NS	NS	NS	NS
	Stem : Spongy	S	NS	S	S
	Palisade : Spongy	NS	NS	NS	NS

(*C. foetidissima, C. pepo, C. andreana*) not all globoid crystals in a sample of 25 contained Ca. The only exception to this was the *C. pepo* radicle sample where all 25 globoid crystals analyzed contained at least some Ca. It is thus evident that even when Ca is common there are some globoid crystals in a seed which are entirely lacking in this element.

DISCUSSION

Standardized data collection methods plus calculation of peak-to-background ratios result in data that are useful for the types of comparisons attempted in these studies. Reasons for using peak-to-background values and discussion of limitations in comparisons have been previously published (8). The values presented here are arbitrary units and thus no attempt should be made to consider these data in terms of quantitative values such as per cent dry weight or per cent of a globoid crystal. Since peak heights produced are not the same for equal concentrations of all elements, caution should be used when attempting comparisons of levels for the four elements studied here. From known *P. values* (11) it is clear that Ca and K would produce EDX peaks of very similar heights if the two elements were present in a sample in equal concentrations. *P values* also indicate that compared to Ca and K peaks the values for phosphorus are a slight underestimate and the values for Mg are a major underestimate.

In the Lott *et al.* (8) study of globoid crystal composition in different areas of *Cucurbita maxima* seeds, glutaraldehyde-fixed, dehydrated and embedded material was used. Fixation trials showed that there was not a major loss of elements from globoid crystals in *C. maxima*. Rather than investigate fixation effects for each of the five species used in the studies reported here, we chose to use freeze-dried tissue powders where no fixative extraction occurs. Development of the sample preparation method used in this paper allowed the use of much smaller samples than was previously possible when frozen tissue was ground in a mortar and pestle. Because of the differences in sample preparation procedures any direct comparison of values from this paper and Lott *et al.* (8) should be avoided.

Globoid crystals in the five *Cucurbita* species used in this study have not been previously EDX-analyzed. While EDX analysis does not provide any exact information on the compounds contained within a region, our results are consistent with the general concept that globoid crystals are rich in phytin. EDX analysis of phytin would detect P from the hexaphosphoric acid part of the molecule and K, Mg, and Ca from the associated cations.

The results presented here plus those of Lott *et al.* (8) support the hypothesis that there can be a correlation between seed size and Ca distribution in the globoid crystals of mature embryos. *Cucurbita* species with small seeds (*C. foetidissima, C. pepo, C. andreana*) have Ca in globoid crystals of radicle, stem, and cotyledon. *Cucurbita* species with large seeds have Ca largely concentrated into globoid crystals of the radicle (*C. mixta, C. moschata*) or entire root-shoot regions (*C. maxima*).

Small embryos are likely to have a more widespread Ca distribution in globoid crystals in storage cells than are large embryos. The reason that seed size and Ca distribution in globoid crystals are related is unclear. It seems unlikely that this difference is due strictly to genetic differences between the species studied. In this regard it is interesting to note that *C. maxima*, which has a large seed, and *C. andreana*, which has a small seed, are considered to have a very close genetic relationship (1). These two species are in fact interfertile when crossed (13).

Table VII. P, K, Mg and Ca Percentages of the Total Mean Peak-to-background Numbers for the Globoid Crystals of Various Tissue Regions of Five *Cucurbita* Species.

Species	Tissue Region	% of Total Mean Peak-to-background numbers*			
		P	K	Mg	Ca
C. mixta	Radicle	53.56	27.34	11.61	7.49
	Stem	52.89	29.96	17.15	0
	Cotyledon Center Palisade	53.46	32.41	14.13	0
	Cotyledon Center Spongy	55.94	26.62	17.44	0
C. moschata	Radicle	47.67	26.48	20.87	4.98
	Stem	49.93	31.37	18.70	0
	Cotyledon Center Palisade	45.96	34.50	19.54	0
	Cotyledon Center Spongy	51.21	25.82	22.82	0.15
C. foetidissima	Radicle	50.28	26.54	20.37	2.81
	Stem	51.55	29.51	17.68	1.26
	Cotyledon Center Palisade	52.78	28.33	16.86	2.03
	Cotyledon Center Spongy	50.35	31.25	15.69	2.71
C. pepo	Radicle	58.87	24.51	11.27	5.35
	Stem	53.81	24.74	17.65	3.80
	Cotyledon Center Palisade	51.94	26.94	18.21	2.91
	Cotyledon Center Spongy	51.36	26.76	16.82	5.06
C. andreana	Radicle	57.66	21.56	17.92	2.86
	Stem	54.65	25.35	18.87	1.13
	Cotyledon Center Palisade	55.23	23.86	19.33	1.58
	Cotyledon Center Spongy	57.61	20.51	19.49	2.39

* Calculated by adding together the mean peak-to-background numbers for each of the elements P, K, Mg and Ca in a given tissue region of a given species and then determining the % of that total number represented by each element. This % figure must not be confused with actual % of the element in the globoid crystals.

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