Seed Science Research (2001) **11**, 35–44 DOI: 10.1079/SSR200058

Changes in the element composition of globoids and whole embryos in developing seeds of *Cucurbita maxima*

Irene Ockenden, Marcia West, Jon Domingues and John N.A. Lott*

Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

Abstract

Cucurbita maxima Duch. cv. Warted Hubbard, which produces large fruits with many seeds, was used as a test system to study changes in several mineral nutrients during seed formation. Developing embryos increased markedly in weight, size and oil content. Energy dispersive X-ray (EDX) analysis was carried out on mineral nutrient storage globoids in developing *Cucurbita* cotyledons from 4 to 9 weeks after pollination. Phosphorus peak-to-background (p/b) and potassium p/b ratios increased slightly after the fourth week, while magnesium p/b ratios remained unchanged. The calcium p/b ratio was statistically higher at 4 weeks than in the subsequent weeks. Traces of iron and zinc were found in most of the globoids at all fruit ages. The zinc p/b ratio was higher in globoids at 4 weeks than at 9 weeks. Traces of manganese were detectable in only 18% of the globoids at 4 weeks and not detectable in any globoids at 9 weeks. The frequency of the small globoids of 0.25 µm in diameter was the highest at 4 weeks and was zero at 9 weeks. The frequency of the larger globoids increased progressively from 4 to 9 weeks. As the spherical globoids increased in size and hence in volume, there was a 6.4-fold increase in the embryo phytic acid, which is the main component of globoids. On a whole embryo basis, P content increased 4.5-fold, K 3.3-fold, Mg 5.4-fold and Ca 3.2-fold from the fourth to the ninth week.

Keywords: *Cucurbita maxima*, developing seeds, globoids, energy dispersive X-ray microanalysis, element composition

*Correspondence
Tel: (905) 525–9140 ext. 24589
Fax: (905) 522–6066
Email: lott@mcmaster.ca

Introduction

Studies on germinating seeds far outnumber studies on developing seeds, largely due to the ease of studying seedling growth as opposed to seed development. Within the seed development studies few deal with the storage of mineral nutrients, and those that do deal with acquisition and storage of mineral elements generally are based upon whole seeds. The stage in seed development when mineral nutrient storage structures (globoids) appear, and element compositional changes that occur in these globoids as development progresses, have been studied even less frequently.

Globoids are spherical, electron-dense inclusions within the protein bodies of seeds. They are composed of phytate (Lott *et al.*, 1995), a K, Mg and Ca salt of phytic acid which is *myo*-inositol 1,2,3,4,5,6-hexakis[dihydrogen phosphate] (IUPAC and IUPAC-IUB, 1968). Globoids may also contain lower amounts of elements such as Fe, Mn and Zn. Based on their analysis of developing castor bean endosperm, Greenwood and Bewley (1984) proposed that phytate was synthesized in the cytoplasm as small electron-dense particles. The particles were transported into the vacuoles developing into protein bodies, and there the particles condensed into globoids. How phytic acid and the associated cations are actually packaged within the globoids has not been defined, other than the many observations that globoids occur as spheres of various sizes.

Cucurbita maxima Duch. cv. Warted Hubbard, which produces large squash fruits, was selected as a test system for studying mineral storage during development for a number of reasons. The fruits contain many seeds, so that there is enough embryo tissue for both energy dispersive X-ray analysis (EDX analysis) of globoids and whole embryo analysis by chemical procedures. The seeds have simple embryos and lack endosperm at maturity (Jacks, 1990). The embryo cells generally contain one large globoid per

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press

protein body (Lott *et al*., 1978), facilitating the selection of globoids for EDX analysis and sizing. In addition, substantial background data exist from previous research, such as studies of total mineral reserves in mature squash seeds (Ockenden and Lott, 1988b) and the composition of globoids in mature *Cucurbita* seeds (Lott *et al*., 1978; Ockenden and Lott, 1990). Previous work has shown that the globoids in spongy ground meristem cells, which make up much of the cotyledon in squash, are relatively similar in content (Lott *et al*., 1978). This fact aided the selection of the sampling protocol used in this paper. Changes in elemental composition during growth of *Cucurbita* seedlings have been documented in both whole seedlings (Ockenden and Lott, 1988a) and globoids (Beecroft and Lott, 1996). Based on work of Beecroft and Lott (1996), there are dynamic changes within the globoids during germination and seedling development. For example, K decreases markedly while Mg and Ca increase in the globoids. The current study examines the seed development portion of this test system.

There are no studies of changes in mineral nutrient stores in developing seeds within large fruits such as squash. Based on EDX analysis of globoids within the aleurone of developing rice grains (Ogawa *et al*., 1979a) and the developing endosperm of castor bean seeds (Greenwood, 1983), some changes in the globoid composition were expected as development progresses. Both studies found that K was absent or in low amounts in the globoids in the early stages and rose towards maturity. Since aqueous fixation was used to prepare seeds of both species, there may have been some loss of K (Lott *et al*., 1984); however, there was an increase in K at maturity over that in the initial stage. Other changes in globoid composition were more varied in the two species. Calcium decreased in the castor bean and became undetectable in rice. Traces of Zn were detected in the globoids of rice kernels only in the earliest stage. Magnesium increased in the castor bean globoids but remained relatively constant in rice globoids. There was an increase in the peak-to-background (p/b) values for P in castor bean but, when reported as a percentage of total p/b values for K, Mg, Ca and P, the P was lower at maturity than in the early stages. No p/b values were given for rice globoids. However, the percentage that P represented, of the total net counts collected for all the elements measured, was lower at maturity than in the earlier stages.

In this paper EDX analysis of globoids within *Cucurbita maxima* embryos from developing fruits at six stages of development is presented. Phosphorus, K, Mg, Ca and trace elements Fe, Mn, and Zn in globoids were studied to determine if any changes occurred as embryo development progressed. Whole embryo content of P, K, Mg and Ca and phytic acid are presented for the initial and final stages.

Materials and methods

Plant material

Seeds of *Cucurbita maxima* Duch. cv. Warted Hubbard were obtained from Stokes Seeds Ltd., St. Catharines, Ontario, Canada and were grown in the McMaster University greenhouse. Each plant was grown in a container with about 15 litres of a loam soil/peat/perlite mixture and fertilized with dilute NPK fertilizer (15:15:30) each week. The plants were grown in the summer months, received natural light and were exposed to temperature fluctuations similar to field conditions. Flowers were hand-pollinated and only one fruit was allowed to grow per plant. Fruits were harvested at 3, 4, 5, 6, 7, 8 and 9 weeks after pollination, with four fruits harvested for each week. At 3 weeks after pollination, the embryos were very immature and contained few globoids. Thus, the 3-week fruits were discarded. At 9 weeks the seeds were mature. The seeds were removed from the fruits, and some were frozen immediately to ensure that the elements remained *in situ,* while some were dried with a stream of air. The frozen seeds were stored at –20°C until prepared for study.

As the fruits were opened, it was evident that the degree of seed maturity varied somewhat from fruit to fruit within a given week of maturation and from seed to seed within a single fruit. These differences are commonly found in squash and are likely due to inherent differences in growth rates, the timing of fertilization and differences in environmental factors such as temperature and light to which each fruit was exposed at a given stage of development. To determine the status of the seeds at each stage of development, 10 air-dried embryos from each of the 24 fruits were weighed individually. For each of the seeds, the embryo thickness was measured with Mitutoyo calipers in the centre of the flat cotyledon pair (surface to surface). The oil in whole embryos was extracted three times in *n*-hexane for 72 hours for each extraction (Bair and Snyder, 1980). The hexane was evaporated and the oil weighed.

Preparation of seed tissue for microscopy and EDX analysis

After eliminating obviously underdeveloped or aborted seeds, three seeds were selected randomly from each of the batches of frozen seeds of the 24 fruits and about 1 mm blocks of frozen tissue were removed from the cotyledon centre. No tissue from the cotyledon sides, base or tip was used. The blocks of tissue were placed into cryogenic vials filled with 100% ethanol (cooled to –50°C) which were then placed in a –80°C freezer for 15 d. This freeze-substitution procedure with a non-aqueous medium

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press

was used to ensure that there was no movement of the water-soluble elements. Next the vials were placed into a –20°C freezer for 24 h, and then the tissue blocks were placed into micro test-tubes filled with propylene oxide for 24 h at room temperature. The 100% propylene oxide was replaced with 2:1 (v/v) propylene oxide:Spurr's resin, then 1:1, 1:2 and two changes of 100% Spurr's resin with 24 h in each solution. The resin was polymerized at 70°C for 12 h. To avoid extraction of elements from sections cut into water-filled microtome boats (Skilnyk and Lott, 1992), 1–2 μm thick sections were cut dry with glass knives and placed on formvar-coated copper grids. The sections were flattened with a drop of absolute ethanol. EDX analysis was performed in the scanning-transmission mode of a JEOL-TEMSCAN microscope at 80 kV with a PGT model IMIX-II microanalysis system (Princeton Gamma Tech., Princeton, New Jersey, USA). Detector distance, aperture, spot size and tilt were kept the same for all analyses. Ten spectra were collected for each of the three seeds from each of the four fruits per week. Each spectrum was obtained for a globoid from a different spongy ground meristem cell. Spongy ground meristem cells in different parts of the section were sampled. Effort was made to avoid the smaller cells of the provascular and protodermal areas. The 2–3 cell layers of palisade ground meristem near the upper protoderm were evident at all ages, and globoids in these cells were avoided for EDX analysis. Only intact globoids were selected for analysis. The size of each globoid analysed was measured using a scale bar on the microscope screen.

X-ray counts were obtained for each of the elements by integrating peaks at the window widths described in Beecroft and Lott (1996). Background-subtraction procedures were derived from Stewart *et al.* (1988) and were modified as necessary for the current analyses. Corrections for peak overlaps for Ca, Fe and Zn were derived from Beecroft and Lott (1996). The results for X-ray analysis are presented as peak-to-background ratios.

It is a characteristic of the X-ray analysis system, with its necessary background-subtraction corrections, that elements present in trace amounts (e.g. Mn, Fe and Zn in our samples) may show slightly negative as well as slightly positive ratios. To ensure that such elements were genuinely present, we started by selecting the lowest negative p/b value for each element present in trace amounts. Next, we assumed that any positive value up to the same magnitude could also be background. The positive equivalents of these negative values were called cut-off limits, below which the element was not considered to be present. These cut-off limits were different for each of the elements at each stage of development (Beecroft and Lott, 1996).

In addition, 2–3 μm thick sections of the embedded tissue blocks were placed on glass microscope slides, dried, stained with toluidine blue O, washed and dried. Some freehand sections were also made of central cotyledon tissue from dry seeds and stained as described above. Sections were examined with a Zeiss RA light microscope. Approximate numbers of cells from upper protoderm to lower protoderm were counted, and the approximate degree of vacuolation was noted.

Statistical differences were determined using analysis of variance with Minitab (Version 7.1) followed by Tukey's test (Zar, 1984) to separate the means.

Tissue preparation and chemical analysis for P, K, Mg, Ca and phytic acid

Frozen seeds were placed on a block of dry ice, and a razor blade was used to quickly remove the outer seed coats from 30 embryos for the 4-week-old seeds and 20 embryos for the 9-week-old seeds. The thin, membranous, yellow–green layer surrounding the cucurbit embryos (Singh, 1953) was difficult to remove in the frozen state; hence, the word 'embryos' is used here to refer to embryos plus the adhering layer. Although the green layer represents about 2% of the mature embryo weight, it represents about 1.5% of the total P, K or Mg, and has somewhat higher Ca (about 15%). We believe that the green layer does not significantly alter the results presented here (Ockenden and Lott, 1988b; Nield and Lott, 1989).

The frozen embryos were placed in small canisters (five per canister) with a large slit in the lid and were dipped into liquid nitrogen. The samples were freeze-dried in a Labconco Freeze Dry System for 48 h at –53°C. The freeze-dried embryos were stored in a desiccator. The embryos were ground with a pestle and mortar and used for analysis of P, K, Mg and Ca and for the extraction of phytic acid. The results are all based on dry weights determined after drying the ground tissues for 2 h at 105°C as recommended by Roberts and Roberts (1972).

For total P analysis, the ground embryo samples (about 0.1 g) were charred, ashed at 500°C, and the ash was acid treated as described in Ockenden *et al.* (1997). Total P analysis was carried out on ashed tissue using the molybdenum blue method derived from the Association of Official Analytic Chemists (AOAC) (1990), with modifications described in Ockenden *et al.* (1997). The procedure for phytic acid extraction and analysis was also derived from AOAC (1990), with a few modifications as described in Ockenden *et al.* (1997). Phytic acid was extracted into 2.4% HCl and separated on an anion exchange resin AG 1-X8 (200–400) mesh. The eluate was digested with nitric and sulphuric acids, and the released

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press

inorganic phosphorus was determined using the molybdenum blue method. The concentration of inorganic phosphorus from phytate was converted to phytic acid concentration by multiplying the phosphorus value by 3.55 (Raboy and Dickinson, 1984).

Total K, Mg and Ca, in ashed ground embryos, were measured by atomic absorption spectroscopy (Varian AA-1275) with parameter settings and standards as outlined in the Varian Manual. Details of the procedure are found in Reid *et al.* (1999). Sample sizes for ashing were about 0.1 g for 9-week embryos and 0.05 g for 4-week embryos. For Mg and Ca the acid-treated ash was taken up in 1% HCl containing 1000 p.p.m. of lanthanum. For K analysis, the ash was taken up in water containing 1100 p.p.m. caesium.

The results for all elements are expressed on a per embryo rather than a per cent weight basis because the increase in the storage lipid, which is essentially devoid of the elements measured, obscures the true increases in the element composition during development. Extraction of phytic acid was carried out on embryo tissue which had not been defatted, since our preliminary work indicated that the oil in the small samples used did not interfere with the extraction.

Results

Cucurbita maxima fruits varied considerably in size and contained up to several hundred seeds. The seeds, even at 4 weeks, appeared mature in size, but the embryos inside the seed coats were immature, which is characteristic of cucurbit seed growth (Singh, 1953). Three-week cotyledons were small and contained so few globoids that they were not used in the detailed sampling. At 4 weeks, cotyledons were very thin, and some were shorter than the length of the cavity within the seed coats. The space around the small embryos was filled with jelly-like endosperm. As the seeds matured, the cotyledons thickened and lengthened until the whole space within the seed coat was occupied and no endosperm remained.

Light microscopy showed that the upper and lower protoderms, the palisade ground meristem and the provascular tissue could be distinguished by position and cell form at all weeks of development. The approximate number of cells from the upper protoderm to the lower protoderm in the centre of the cotyledon was similar for the 4- and 9-week stages. In sections, the 4-week cotyledons were several times thinner than the 9-week cotyledons, and the ground meristem cells showed a major expansion in size during embryo development. The protein bodies were easily identified in later stages of maturity. At 4 weeks there were many small vacuoles containing some storage reserves.

To determine the progress of seed development within the fruits, the embryo (air-dried) weight, thickness and oil content were measured (Fig. 1). Embryo weights and thicknesses increased markedly between the fourth and the sixth week. The mean measurements for the sixth and seventh weeks were not significantly different from each other, but were different from earlier and later weeks of development. The percentage oil present at 4 weeks was already quite substantial (30%) and reached 44% at 9 weeks. While oil contents of the fourth and fifth weeks were not significantly different from each other, they were different from the eighth and ninth weeks. Based on these results, which indicate that sixth and seventh week embryos were similar and that those of the eighth and ninth weeks were also quite similar, the p/b ratios from the EDX analysis are presented as values for each week and as pooled data for early, mid and late stages of development.

Table 1 shows the mean peak-to-background (p/b) ratios for K, Mg, Ca and P in globoids of 4–9-week embryos. Potassium was lowest in the fifth week. Magnesium did not vary significantly from 4 to 9 weeks. Calcium was present in very low amounts but was significantly higher in the fourth week than in the subsequent weeks. Phosphorus was the most abundant element, with the lowest level at 4 and 5 weeks. If the results were pooled to give three developmental stages of early (4 and 5 weeks), mid (6 and 7 weeks) and late (8 and 9 weeks), there was a significantly lower amount of K and P at the early stage. The amount of Mg remained the same, and a higher amount of Ca was present in the early stage than in the mid and late stages. This suggested that changes in the elemental composition of the globoids in *Cucurbita* embryos occurred early in development and that the changes in the globoid composition were small.

Table 2 shows the percentage of the globoids that had detectable traces of Mn, Fe and Zn for each week of development. Calcium values are shown for comparison. Most of the globoids analysed for each week contained some Ca. Manganese was present in only a small percentage of globoids but showed a definite downward trend from the fourth week, where 18% of the globoids contained some detectable Mn, to the lack of detectable Mn in globoids by the ninth week. Iron and Zn had essentially the same frequency in globoids from 4 to 9 weeks. The mean p/b ratios for Mn, Fe and Zn for 4 weeks were 0.15 ± 0.19, 0.24 ± 0.29 and 0.80 ± 0.64, respectively, and for 9 weeks were –0.03 ± 0.07, 0.42 ± 0.41 and 0.33 ± 0.25, respectively. Thus, even at 4 weeks Mn is present in very small amounts. Zinc had a significantly higher p/b ratio at 4 weeks than at 9 weeks, suggesting that, like Ca, Zn was present in higher amounts in the globoids at the early stage of development and then

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press

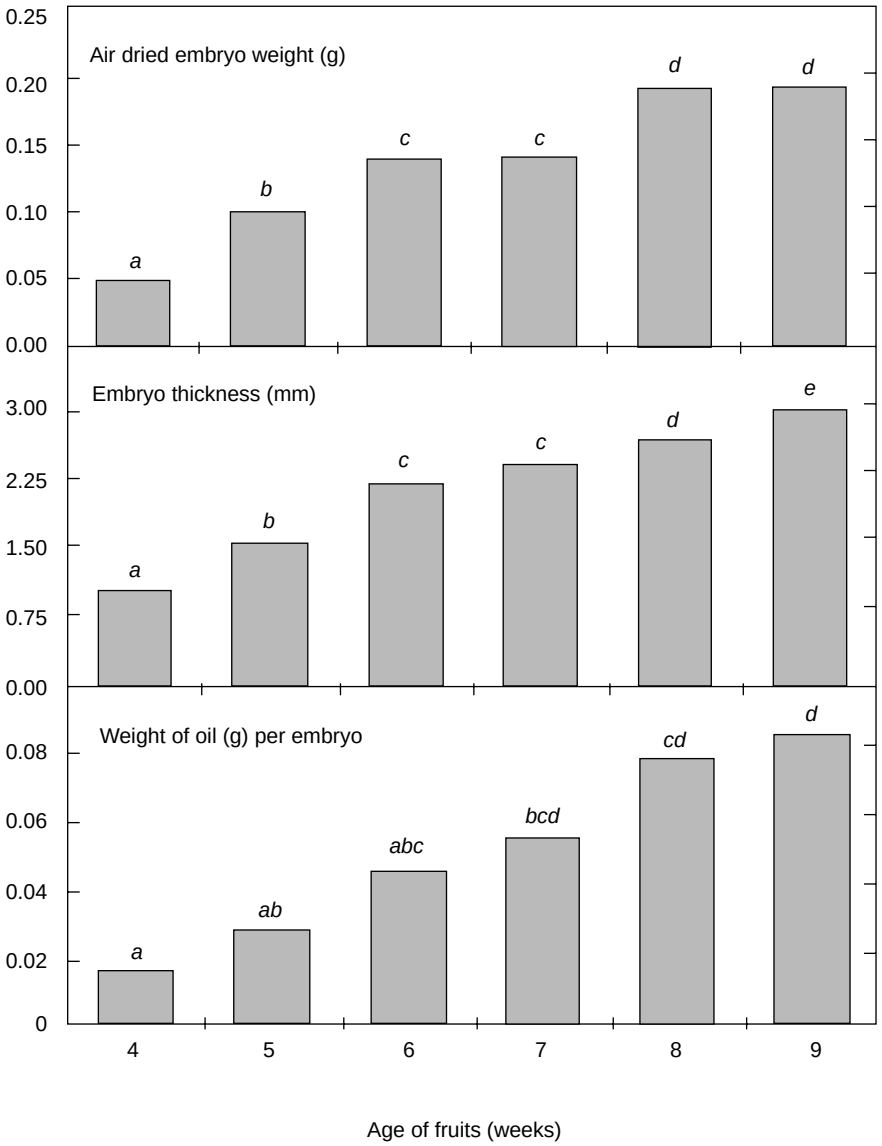


Figure 1. Changes in *C. maxima* embryo air-dried weight, thickness and oil content during seed development from 4 to 9 weeks. $N = 40$ embryos for each week (10 embryos per fruit). Embryo thickness was measured in the centre of the cotyledons. Where the same letters occur on two or more columns, there was no significant difference between the values at $P = 0.05$.

decreased. Also like Ca, Zn remained at a detectable level in the globoids during the 6 weeks of growth.

Table 3 shows the range of globoid diameters measured for the 120 globoids analysed for each week of development and the frequency with which globoids were found in each size range. The percentage of globoids in the smaller ranges was markedly higher at 4 and 5 weeks than in the later weeks. Thus, there was an increase in individual globoid size during embryo development. By the ninth week there were no globoids with diameters of 0.75 μm or smaller, while the frequency of globoids with diameters of 3 μm and larger had increased. The mean globoid diameters for each stage from 4 to 9 weeks were 0.9 ± 0.5, 1.2 ± 0.7, 1.7 ± 0.7, 1.8 ± 0.7, 2.0 ± 0.8 and 2.4 ± 0.7 μm, respectively. All were significantly different from each other except the means for the sixth and seventh weeks. Table 3 also shows the volumes calculated for the given ranges of diameters of the globoid spheres. At 4 weeks the 64 globoids in the smallest size range (53% of the 120) had a combined volume of 5.5 μm^3 while the single 3 μm diameter globoid at 4 weeks had a volume of 14.1 μm^3.

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press

Table 1. Peak-to-background ratios (mean ± SD) of K, Mg, Ca and P in globoids of developing embryos of *C. maxima* from: (A) 4–9 weeks after pollination and (B) pooled into early, mid and late stages of development

	Maturation period	K p/b	Mg p/b	Ca p/b	P p/b
A	4 weeks	5.74 ± 1.65a	4.38 ± 1.06a	1.94 ± 1.60a	10.12 ± 1.96a
	5 weeks	5.05 ± 1.57b	4.71 ± 1.20a	0.56 ± 0.44bc	10.53 ± 1.97a
	6 weeks	5.93 ± 2.03a	4.26 ± 1.30a	0.76 ± 0.70bc	11.50 ± 2.02b
	7 weeks	5.87 ± 1.76a	4.44 ± 1.15a	0.89 ± 1.14b	11.72 ± 1.86b
	8 weeks	6.32 ± 1.85a	4.52 ± 1.16a	0.51 ± 0.50c	11.44 ± 1.86b
	9 weeks	6.04 ± 1.70a	4.44 ± 1.08a	0.57 ± 0.46bc	11.17 ± 1.85ab
B	Early (4–5 weeks)	5.40 ± 1.65d	4.55 ± 1.14d	1.25 ± 1.36d	10.33 ± 1.97d
	Mid (6–7 weeks)	5.90 ± 1.90e	4.35 ± 1.23d	0.82 ± 0.94e	11.61 ± 1.94e
	Late (8–9 weeks)	6.18 ± 1.78e	4.48 ± 1.12d	0.54 ± 0.48f	11.31 ± 1.85e

Analysis was on four fruits per week, with three seeds analysed per fruit and 10 globoids analysed per seed, for a total of 120 spectra per week. For columns within either part A or B, values followed by the same letter are not significantly different at $P = 0.05$.

Table 2. Percentage of spectra (120 per week) in which Ca, Mn, Fe and Zn were present in the globoids of developing *C. maxima* embryos

Week	Ca	Mn	Fe	Zn
4	100	18	99	93
5	94	2	79	82
6	99	2	97	88
7	100	4	100	72
8	99	3	100	100
9	100	0	100	100

The elements were considered present if their p/b ratios were above the cut-off point, which represented the above zero level equivalent to the lowest below zero p/b found for each of the elements, e.g. if for an element the lowest p/b was −0.32, then +0.32 would be the cut-off point below which the element would be considered as absent.

Measurements of total embryo K, Mg, Ca, P and phytic-acid phosphorus were carried out on embryos at 4 and 9 weeks of age (Table 4). As seen in the ranges, there was some fruit-to-fruit variation in both weights of the embryos and the amounts of the elements. However, over the 5-week period the mean values for embryo weight increased fourfold while the mean amounts of K, Mg, Ca and P per embryo increased 3.3-, 5.4-, 3.2- and 4.5-fold, respectively. As seen for globoids, total Ca was also present in lower amounts than the other three elements. The K level was higher than that of Mg, with the difference more marked at 4 weeks than at 9 weeks. Phosphorus was present in the highest amount, and its increase was mainly due to the accumulation of phytic acid in the globoids. While phosphorus increased 4.5-fold, the phytic acid increased 6.4-fold. At 9 weeks the percentage phytic acid, based on dry weight, reached 4.1%. At 4 weeks, on average, 63% of the phosphorus was bound up in phytic acid, while at 9 weeks this percentage, on average, had risen to 89%. Calculated on a per cent dry weight basis, there was little difference in the amounts of K, Mg, Ca and P at 4 and 9 weeks. For example, %P was 1.16 ± 0.09 at 4 weeks when the oil content was 30% and 1.29 ± 0.06 at 9 weeks when the oil content was 44%. The percentage of phytic acid, however, increased 1.6-fold, indicating that a higher portion of the embryo P had been incorporated into phytic acid.

Table 3. Frequency of globoid sizes in each week of development ($N = 120$ per week). Globoids were measured in 0.25 μm intervals. The smallest globoids were 0.25 μm in diameter. Only one globoid of 5.50 μm was found (at 8 weeks)

Globoid diameter (μm)	Per cent of globoids in each size range at each week of development						Globoid volume range (μm^3)
	4	5	6	7	8	9	
0.25–0.75	53.3	32.5	11.7	10.0	3.3	0.0	0.01–0.2
1.00–1.75	40.8	55.8	43.3	40.8	33.3	20.0	0.5–2.8
2.00–2.75	5.0	9.2	38.3	41.7	46.7	50.8	4.1–10.9
3.00–3.75	0.8	0.0	6.7	6.7	14.2	26.7	14.1–27.6
4.00–5.50	0.0	2.5	0.0	0.8	2.5	2.5	33.5–87.1

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press

Table 4. The ranges and means per embryo content of K, Mg, Ca, P, phytic acid phosphorus (PA-P) and phytic acid (PA-P × 3.55) in embryos of 4- and 9-week fruits of *Cucurbita maxima*

	Range of values in embryos from four fruits (μg embryo^{-1})		Mean ± SD (μg embryo^{-1})	
	4-week	9-week	4-week	9-week
K	314–379	887–1382	342 ± 28	1130 ± 276
Mg	150–205	706–1197	178 ± 27	956 ± 251
Ca	15–25	50–80	20 ± 4	63 ± 13
P	482–571	1854–2971	522 ± 44	2347 ± 543
PA-P	260–376	1543–2786	328 ± 55	2098 ± 549
PA	923–1335	5478–9890	1163 ± 197	7450 ± 1947
PA-P as %P	53–69	83–94	63 ± 7	89 ± 5
Embryo dry weight (g)	0.039–0.049	0.136–0.227	0.046 ± 0.004	0.182 ± 0.047

Weight per embryo was based on $N = 30$ for 4-week fruits and $N = 20$ for 9-week fruits.

Discussion

While there are no values from previous work to compare with the week 4 EDX analysis results, the week 9 values compared well with two previous studies of mature squash seed globoids (Ockenden and Lott, 1990; Lott *et al.*, 1994). Considerable variability in the p/b ratios of globoids can occur, an example being the complex embryo of rice kernels where variation in composition was found between different cell types, organs and occasionally even between globoids in the same cell (Wada and Lott, 1997). Squash has a relatively simple embryo and has been the subject of many EDX analyses in this laboratory. In the present study, selection of tissue from only the centre of the cotyledon and sampling of globoids in only the spongy ground meristem cells helped to minimize variability.

From examination of samples in this study of developing squash cotyledons, it is clear that considerable electron-dense material, presumably phytate, forms early as protein bodies develop. In early stages (weeks 4 and 5) it is common to find globoids in vacuoles with only some clumps of protein. Because most protein bodies in squash have only one globoid in mid and later stages of seed development, and these are mostly large in size, we believe we are tracking the same population of globoids. Globoid size data are consistent with the formation of small globoids that grow in diameter and volume as the embryo develops. In species where protein bodies contain numerous globoids of different sizes, the possibility of different populations of globoids produced at different times of development would be a more difficult problem and would require much more extensive sampling. We reduced variability by eliminating from the study cells of the two protoderm layers, the provascular regions and the palisade ground meristem. Globoids in these tissues differ from those in the spongy ground meristem (Lott *et al.*, 1979). Lott *et al.* (1978) showed that globoids from spongy ground meristem cells were not significantly different in P, K, Mg and Ca content in tissue from the base, sides and centre of squash cotyledons. Globoids from the cotyledon tip region did differ in K levels from those in the centre and side regions, even though P, Mg and Ca did not. To reduce variability only tissue from the cotyledon centre was used.

Based on previous studies (Ogawa *et al.*, 1979a; Greenwood, 1983), we expected to find an increase in K p/b ratios during development, but the increase in cucurbit K p/b ratio was slight compared to that of rice aleurone and castor bean endosperm globoids. However, direct comparisons may be tenuous since endosperm was used for both rice and castor bean studies, whereas the study reported here was carried out on cotyledon tissue. The increase in the cucurbit globoid P p/b ratio was small compared to that found in castor bean globoids. The P value for rice was not reported as a p/b ratio. In EDX studies of globoids in both rice and castor bean, P decreased as a percentage of the total counts or as the sum of all the p/b ratios. Our globoid Mg p/b ratios remained essentially the same during development, as was found for rice aleurone globoids, rather than increasing as it did in castor bean endosperm globoids. Higher globoid Ca p/b ratios in early stages of development were found in all three studies.

While Ca has many functions during plant development (Marschner, 1995), globoid Ca may have been sequestered to reduce its precipitation with phosphorus in the cytoplasm (Bush, 1995). Alternatively, high Ca concentrations in the early seed development may be necessary for the formation of the globoids. Calcium phytate is virtually insoluble in water (Martin and Evans, 1986), and Ca as a divalent cation can cross-link phytic acid molecules, leading to the formation of globoids. Ogawa *et al.* (1979a) suggested that at the early milky stage in rice, when

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press

the K p/b ratio was at the lowest level, the phytic acid was neutralized by cations of such elements as Ca, Zn, Mn and Fe. Rice aleurone has many globoids per protein body (Wada and Lott, 1997). Castor bean endosperm, like the cucurbit embryos, tends to have only one large globoid per protein body (Greenwood and Bewley, 1984). Whether the difference in size and number of the globoids influences the globoid composition during development is not yet known.

The importance of the micronutrients Mn, Fe and Zn are many, including their roles in enzyme functions (Marschner, 1995), but there is no direct information on their functions in developing globoids. One can speculate that Fe may participate in globoid formation, but it is not present in higher amounts initially as was Ca. The Zn p/b ratio is significantly higher at 4 weeks than later in development and may be needed for the formation of globoids (Ogawa *et al.*, 1979a). Manganese, which can be toxic in high amounts (Marschner, 1995), may need to be temporarily sequestered in the globoids during the early stages of development.

In rice aleurone and scutellum the globoids appeared early in grain formation and then increased in number and size with time (Wada and Maeda, 1987). In developing cucurbit cotyledons, globoids were scarce at 3 weeks, but were small but plentiful at 4 weeks. The frequency of large globoids in *Cucurbita* increased progressively during development. Counting number of globoids per unit area is difficult in non-aqueously prepared and dry cut, thick sections, but qualitatively there was no obvious increase in the number of globoids. Cucurbit globoids can be lost from thin sections and sometimes can shatter into irregular shards. We cannot rule out that some of the globoid diameters may have been measured on peripheral sections of a globoid and thus did not represent the maximum diameter. However, the large number of globoids measured and the very distinct differences seen in the distribution of various-sized globoids in each week, attests to the fact that the measurements were valid. If many globoids were sectioned near their edge, we should have seen more small globoids in the later stages of development.

The measured increase in the thickness of the embryos reflects an increase in the storage reserves, especially protein and lipid. Light microscopy results suggest that by 4 weeks most of the cell division had been completed in the cotyledon centre. The observed increases in cell size account for increases in cotyledon thickness during embryo development. The observation of many small vacuoles in spongy ground meristem cells at 4 weeks indicates that many of the protein-storing vacuoles/protein bodies have also been formed by 4 weeks. Phytate deposition, while very significant for the plant, contributes relatively little to the total cotyledon volume. Our results show that the mature embryo phytic acid accounts for about 4.08% of the dry weight in squash.

The 6.4-fold increase in phytic acid at 9 weeks over that at 4 weeks was also accompanied by an increase in the percentage total phosphorus that was bound up in phytate, from 63 to 89%. Again, there is no comparative value for the week 4 embryos, but Splittstoesser (1982) reported that 85% of the total phosphorus may be present as phytate in mature cucurbit embryos. There are several reports of rapid accumulation of phytate in the initial stages of seed/grain development, followed by a slower increase as maturation progresses (Saio, 1964; Makower, 1969; Ogawa *et al.*, 1979b), and this may be the reason that considerable amounts of phytate and numerous globoids were already present at 4 weeks. Since the globoid volume increases exponentially with increases in globoid diameter, the capacity to store phytic acid is very different in the small and large globoids. The volume calculated for the diameters emphasizes that the volumes of the progressively larger globoids could account for much of the increasing phytic acid concentrations observed here.

Developing soybeans, which have a simple seed with large cotyledons, showed an increase in P, K, Mg and Ca on a whole seed basis (Sale and Campbell, 1980). Lazlo (1990) found that there was a variation in the pattern of Mg and Ca accumulation in seeds of six varieties of soybeans. Greenwood *et al.* (1984) found that P and phytic acid increased in both the embryo and endosperm tissues of developing castor beans. Ogawa *et al.* (1979b) detected an increase in P, K and Mg in developing rice kernels. As in the above studies, chemical analysis of whole cucurbit embryos showed that P, K, Mg and Ca all increased with embryo development. The amounts detected in the 9-week embryos agreed well with values from the neutron activation analysis of mature dry embryos from the same cucurbit cultivar (Ockenden and Lott, 1988b). The reader should be aware that elements such as P, K, Mg and Ca occur in many compartments, including nuclei, cytoplasm, cell walls and globoids. Thus, globoid composition and whole embryo composition are not expected to be parallel throughout embryo development.

By increasing the divalent (Ca^{2+}, Mg^{2+}) to monovalent (K^{+}) cation ratio experimentally, Lott *et al.* (1985) were able to produce globoids in pea cotyledons, which usually have soluble phytate and no globoids. Lott *et al.* (1985) proposed that seed tissues with higher (Ca + Mg)/K ratios produced larger globoids. Lott *et al.* (1994) tested the hypothesis further by inducing the formation of many small globoids, instead of a single large globoid in *Cucurbita maxima,* by injecting the developing fruits with K to

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press

decrease the (Ca + Mg)/K ratio. The current study is consistent with the above relationship of globoid size and the ratio of divalent to monovalent cations. The mean ratio of (Ca + Mg)/K, based on quantitation of these elements in whole embryos, was 0.58 at 4 weeks and 0.90 at 9 weeks. The 4-week and 9-week embryos had mean globoid diameters of 0.9 μm and 2.4 μm, respectively.

The studies of Ogawa *et al.* (1979a), Greenwood (1983) and our study found some changes in the element composition in the developing globoids, but these changes differed in the three species. This supports the statement of Lott *et al.* (1985) that variations in the mineral storage may vary within or between tissues of one species as well as between species. How much variation exists within the large *Cucurbitaceae* family remains unknown. The current work is a good start on detailed characterization of changes in globoid composition during development, but many more studies are needed to define the changes in globoids in seeds of various species. This important but little-studied developmental process is of global significance since crop seeds/fruits produce about 50 million metric tonnes of phytate each year (Lott *et al.*, 2000).

Acknowledgements

This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada. We wish to thank Art Yeas for his assistance with the growth of the test plants and Klaus Schultes, Daryl Reid and Derrick Flannigan for advice on electron microscopy.

References

Association of Official Analytical Chemists (1990) *AOAC official methods of analysis* (15th edition), pp. 56, 800–801. Arlington, VA, AOAC International.

Bair, C.W. and Snyder, H.E. (1980) Electron microscopy of soybean lipid bodies. *Journal of the American Oil Chemists Society* **57**, 279–282.

Beecroft, P. and Lott, J.N.A. (1996) Changes in the element composition of globoids from *Cucurbita maxima* and *Cucurbita andreana* cotyledons during early seedling growth. *Canadian Journal of Botany* **74**, 838–847.

Bush, D.S. (1995) Calcium regulation in plant cells and its role in signaling. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**, 95–122.

Greenwood, J.S. (1983) Phytin deposition within the protein bodies of the developing endosperm of castor bean (*Ricinus communis* cv. Hale). PhD Thesis, University of Calgary.

Greenwood, J.S. and Bewley, J.D. (1984) Subcellular distribution of phytin in the endosperm of developing castor bean: a possibility for its synthesis in the cytoplasm prior to deposition within protein bodies. *Planta* **160**, 113–120.

Greenwood, J.S., Gifford, D.J. and Bewley, J.D. (1984) Seed development in *Ricinus communis* cv. Hale (castor bean). II. Accumulation of phytic acid in the developing endosperm and embryo in relation to the deposition of lipid, protein, and phosphorus. *Canadian Journal of Botany* **62**, 255–261.

IUPAC and IUPAC-IUB (1968) The nomenclature of cyclitols. *European Journal of Biochemistry* **5**, 1–12.

Jacks, T.J. (1990) Cucurbit seeds: Cytological, physiochemical, and nutritional characterizations. pp. 356–363 *in* Bates, D.M.; Robinson, R.W.; Jeffrey, C. (Eds) *Biology and utilization of the Cucurbitaceae.* Ithaca, Cornell University Press.

Lazlo, J.A. (1990) Mineral contents of soybean seed coats and embryos during development. *Journal of Plant Nutrition* **13**, 231–248.

Lott, J.N.A., Greenwood, J.S., Vollmer, C.M. and Buttrose, M.S. (1978) Energy-dispersive x-ray analysis of phosphorus, potassium, magnesium, and calcium in globoid crystals in protein bodies from different regions of *Cucurbita maxima* embryos. *Plant Physiology* **61**, 984–988.

Lott, J.N.A., Spitzer, E. and Vollmer, C.M. (1979) Calcium distribution in globoid crystals of *Cucurbita* cotyledon protein bodies. *Plant Physiology* **63**, 847–851.

Lott, J.N.A., Goodchild, D.J. and Craig, S. (1984) Studies of mineral reserves in pea (*Pisum sativum*) cotyledons using low-water-content procedures. *Australian Journal of Plant Physiology* **11**, 459–469.

Lott, J.N.A., Randall, P.J., Goodchild, D.J. and Craig, S. (1985) Occurrence of globoid crystals in cotyledonary protein bodies of *Pisum sativum* as influenced by experimentally induced changes in Mg, Ca and K contents of seeds. *Australian Journal of Plant Physiology* **12**, 341–353.

Lott, J.N.A., Ockenden, I., Kerr, P., West, M., Leech, T. and Skilnyk, H. (1994) The influence of experimentally induced changes in the (Mg + Ca):K balance on protein bodies formed in developing *Cucurbita* seeds. *Canadian Journal of Botany* **72**, 364–369.

Lott, J.N.A., Greenwood, J.S. and Batten, G.D. (1995) Mechanisms and regulation of mineral nutrient storage during seed development. pp. 215–235 *in* Kigel, J.; Galili, G. (Eds) *Seed development and germination.* New York, Marcel Dekker.

Lott, J.N.A., Ockenden, I., Raboy, V. and Batten, G.D. (2000) Phytic acid and phosphorus in crop seeds and fruits: a global estimate. *Seed Science Research* **10**, 11–33.

Makower, R.U. (1969) Changes in phytic acid and acid-soluble phosphorus in maturing pinto beans. *Journal of the Science of Food and Agriculture* **20**, 82–84.

Marschner, H. (1995) *Mineral nutrition of higher plants.* London, Academic Press.

Martin, C.J. and Evans, W.J. (1986) Phytic acid–metal ion interactions. II. The effect of pH on Ca(II) binding. *Journal of Inorganic Biochemistry* **27**, 17–30.

Nield, H. and Lott, J.N.A. (1989) Distribution of minerals within different regions of *Cucurbita maxima* fruits. *Communications in Soil Science and Plant Analysis* **20**, 1085–1100.

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press

Ockenden, I. and Lott, J.N.A. (1988a) Changes in the distribution of magnesium, potassium, calcium and phosphorus during growth of *Cucurbita* seedlings. *Journal of Experimental Botany* **39**, 973–980.

Ockenden, I. and Lott, J.N.A. (1988b) Mineral storage in *Cucurbita* embryos. III. Calcium storage as compared with storage of magnesium, potassium, and phosphorus. *Canadian Journal of Botany* **66**, 1486–1489.

Ockenden, I. and Lott, J.N.A. (1990) Elemental storage in *Cucurbita* embryos: X-ray microanalysis of magnesium, potassium, calcium, and phosphorus within globoid crystals. *Canadian Journal of Botany* **68**, 646–650.

Ockenden, I., Falk, D.E. and Lott, J.N.A. (1997) Stability of phytate in barley and beans during storage. *Journal of Agricultural and Food Chemistry* **45**, 1673–1677.

Ogawa, M., Tanaka, K. and Kasai, Z. (1979a) Energy-dispersive x-ray analysis of phytin globoids in aleurone particles of developing rice grains. *Soil Science and Plant Nutrition* **25**, 437–448.

Ogawa, M., Tanaka, K. and Kasai, Z. (1979b) Accumulation of phosphorus, magnesium and potassium in developing rice grains: Followed by electron microprobe x-ray analysis focusing on the aleurone layer. *Plant and Cell Physiology* **20**, 19–27.

Raboy, V. and Dickinson, D.B. (1984) Effect of phosphorus and zinc nutrition on soybean seed phytic acid and zinc. *Plant Physiology* **75**, 1094–1098.

Reid, D.A., Lott, J.N.A., Attree, S.M. and Fowke, L.C. (1999) Mineral nutrition in white spruce (*Picea glauca* [Moench] Voss) seeds and somatic embryos. I. Phosphorus, phytic acid, potassium, magnesium, calcium, iron and zinc. *Plant Science* **141**, 11–18.

Roberts, E.H. and Roberts, D.L. (1972) Moisture content of seeds. pp. 424–429 *in* Roberts, E. H. (Ed.) *Viability of seeds*. Syracuse, NY, Syracuse University Press.

Saio, K. (1964) The change in inositol phosphates during the ripening of rice grains. *Plant and Cell Physiology* **5**, 393–400.

Sale, P.W.G. and Campbell, L.C. (1980) Patterns of mineral nutrient accumulation in soybean seed. *Field Crops Research* **3**, 157–163.

Singh, B. (1953) Studies on the structure and development of seeds of Cucurbitaceae. *Phytomorphology* **3**, 224–239.

Skilnyk, H.R. and Lott, J.N.A. (1992) Mineral analyses of storage reserves of *Cucurbita maxima* and *Cucurbita andreana* pollen. *Canadian Journal of Botany* **70**, 491–495.

Splittstoesser, W.E. (1982) The appearance of phytase and the changes in phytate and inorganic phosphorus during germination and early seedling growth of pumpkin (*Cucurbita moschata* Poir.). *HortScience* **17**, 402–403.

Stewart, A., Nield, H. and Lott, J.N.A. (1988) An investigation of the mineral content of barley grains and seedlings. *Plant Physiology* **86**, 93–97.

Wada, T. and Lott, J.N.A. (1997) Light and electron microscopic and energy dispersive x-ray microanalysis studies of globoids in protein bodies of embryo tissues and the aleurone layer of rice (*Oryza sativa* L.) grains. *Canadian Journal of Botany* **75**, 1137–1147.

Wada, T. and Maeda, E. (1987) Cyto-histological studies on phytin-containing particles in developing rice grains. *Japanese Journal of Crop Science* **56**, 503–511.

Zar, J.H. (1984) *Biostatistical analysis* (2nd edition) Englewood Cliffs, NJ, Prentice-Hall.

Received 18 May 2000
accepted after revision 5 November 2000
© CAB *International*, 2001

https://doi.org/10.1079/SSR200058 Published online by Cambridge University Press