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Protein Body Composition in *Cucurbita maxima* Cotyledons as Determined by Energy Dispersive X-Ray Analysis[1]

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ABSTRACT

Energy dispersive x-ray analysis was used to study the composition of certain protein body components in *Cucurbita maxima* cotyledons. The globoid crystal was rich in phosphorus, potassium, and magnesium. This elemental composition provides further evidence that the globoid crystal in squash cotyledon protein bodies is composed of phytin, a myoinositol hexaphosphoric acid salt of potassium and magnesium. Calcium, a common component of phytin in many species, was absent or present in only trace amounts in the globoid crystals of squash. Results of analyses of globoid crystals from seeds produced in different parts of North America suggest that there is definite specificity for the cations used in phytin deposition. Variations in soil types and other environmental factors seem not to have influenced the type of cation stored. Energy dispersive x-ray analysis of the proteinaceous regions revealed the presence of phosphorus, sulfur, and a trace of chlorine. Sulfur was expected, due to the presence of some sulfur containing amino acids in the protein.

In *Cucurbita maxima* the structure of the protein bodies in cotyledon tissues of dormant and germinating seeds has been investigated with a variety of electron microscopic techniques (8–10). Cotyledon mesophyll cell protein bodies were found to have at least four components which are the proteinaceous matrix, the protein crystalloid, the soft globoid, and the globoid crystal. Due to the electron density of the globoid crystal, it was proposed that this region is rich in phytin (8).

The energy dispersive x-ray analysis system makes possible an elemental analysis of chosen tissue regions (3). All elements of atomic number 11 (sodium) and higher in the periodic table can be detected and the detection sensitivity of 10^{-17} to 10^{-18} g (18) makes EDX[2] analysis very useful for biological studies. This paper reports the results of an EDX study of protein bodies from squash cotyledons. To study possible environmental influences upon the composition of certain protein body components, seeds grown in different parts of North America were tested.

MATERIALS AND METHODS

Squash seeds used were as follows: (*a*) *Cucurbita maxima* Duch. var. Chicago Warted Hubbard, obtained from Northrup King and Co., Minneapolis, whose seed was grown in Oregon; (*b*) *Cucurbita maxima* Duch. var. Warted Hubbard, obtained from Stokes Seeds Ltd., St. Catharines, Ontario, whose seed was grown at Rocky Ford, Colorado: (*c*) *Cucurbita maxima* var. Warted Hubbard, grown at McMaster University, Hamilton, Ontario, from the Stokes seed listed above; (*d*) *Cucurbita maxima* var. Warted Hubbard, grown at Summerland, B.C. from the Stokes seed listed in *b* above. Northrup King and Co. did not specify where in Oregon their seeds were grown.

Samples of cotyledon tissues from dry seeds were fixed for 1.5 hr at 1 C in 5% glutaraldehyde in distilled H_2O, washed several times in distilled H_2O, and dehydrated in a graded ethanol series (30 min each in 10%, 20%, 30%, 50%, 70%, 80%, and 95% at 1 C, 3 hr in 100% at 20 C). Following dehydration for 12 hr in propylene oxide, the tissue was infiltrated with Spurr's low viscosity epoxy resin (22) and polymerized. Sections of 150 to 170 nm thickness were cut on a Porter-Blum MT-1 ultramicrotome and mounted on copper grids. No electron stain was applied prior to analysis.

To check on the possibility that the fixation and dehydration procedure used above could have extracted materials from the globoid crystals, cotyledon tissue from dry seeds was sliced 1 mm thick, quick frozen in liquid nitrogen, ground in a mortar and pestle, and freeze-dried at low temperature using the method of Darley and Lott (2). The freeze-dried powder was dusted onto Formvar-coated grids prior to analysis. The electron-dense globoids could be readily identified.

Analysis was conducted with a Philips 300 transmission electron microscope fitted with a Model 606 x-ray spectrometer, a Model 707A energy dispersive analysis of x-rays system and an EDIT system for EDAX International Inc. Samples at maximum distance from the grid bars were chosen for analysis.

RESULTS

Fixed and sectioned cotyledon tissue from seeds grown in several different places in North America was analyzed with EDX. The EDX analysis showed that magnesium, phosphorus, and potassium were the only major elements present in the globoid crystals from inside protein bodies of seeds grown at Rocky Ford, Colorado (Fig. 1); Oregon (Fig. 5); Summerland, British Columbia (Fig. 6); and Hamilton, Ontario (Fig. 7). The copper peaks present in Figures 3, 4, and 6 are due to x-rays originating from the copper support grids.

EDX analysis of a section of a protein crystalloid surrounded by the proteinaceous matrix region revealed the presence of phosphorus, sulfur, and traces of chlorine (Figs. 2 and 3). In contrast, the cytoplasm, excluding protein bodies, contained a greater variety of elements including magnesium, silicon, phosphorus, sulfur, chlorine, and calcium.

EDX analysis of globoids from freeze-dried powdered coty-

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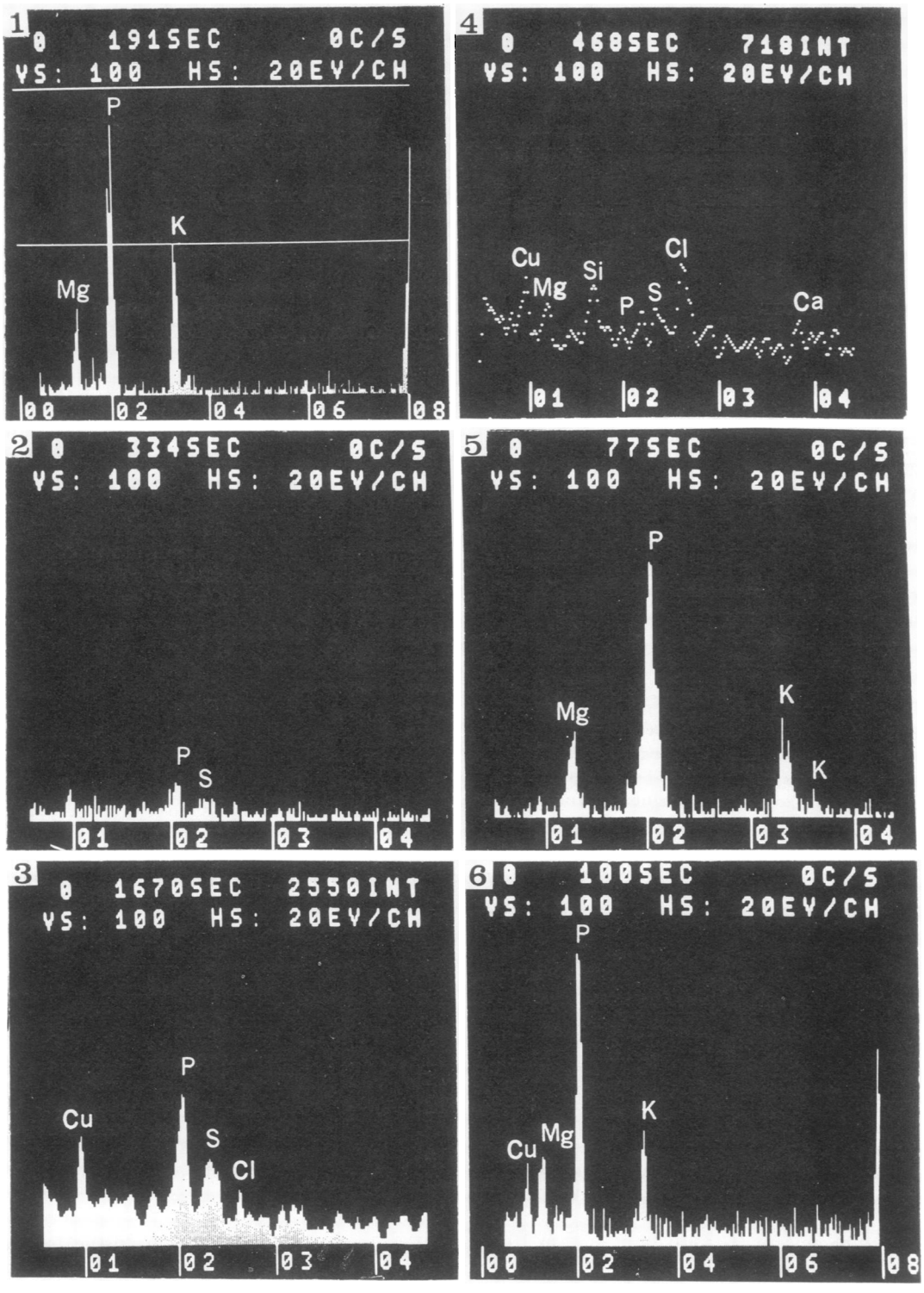
[2] Abbreviation: EDX: energy dispersive x-ray.



FIGS. 1 to 6

Cotyledon tissue from dry squash seeds was fixed in glutaraldehyde in distilled H_2O, dehydrated, embedded in Spurr's low viscosity resin, and sectioned, prior to EDX analysis. Elements present, energy levels in keV and principal emission lines are as follows: calcium 3.690; $K\alpha_{1,2}$; chlorine 2.621, $K\alpha_{1,2}$ and 2.631, $K\alpha_4$ (10% of $K\alpha_{1,2}$ peak); copper 0.930 $L\alpha$; magnesium 1.253, $K\alpha$; phosphorus 2.013, $K\alpha_{1,2}$ and 2.028, $K\alpha_4$ (10% of $K\alpha_{1,2}$ peak) and 2.137 K_β (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); silicon 1.739, $K\alpha_{1,2}$; sulfur 2.307, $K\alpha_{1,2}$ and 2.322 $K\alpha_4$ (50% of $K\alpha_{1,2}$) and 2.465 K_β (7% of $K\alpha_{1,2}$). Elements are identified on each spectrum. Energy levels in keV are shown on each abscissa. Vertical scale (VS) and analysis time in seconds are shown above each spectrum. The copper peak, when present, is due to x-rays from the copper support grid.

FIG. 1. EDX spectrum of a globoid crystal section from a cotyledon protein body. Seed was grown at Rocky Ford, Colo.

FIG. 2. EDX spectrum of a section of the protein crystalloid and proteinaceous matrix region of a cotyledon protein body. Squash seed was grown at Rocky Ford, Colo.

FIG. 3. EDX spectrum from the previous figure, 5× magnified and smoothed with the EDIT system. Sulfur-containing amino acids such as methionine and cystine may be responsible for the sulfur peak.

FIG. 4. EDX spectrum (5× enlarged and smoothed with the EDIT system) of the cytoplasm of the cell containing the protein body analyzed in the first three figures. No protein bodies were included in this analysis.

FIG. 5. EDX spectrum of a globoid crystal section from a cotyledon protein body. Seed was grown in Oregon.

FIG. 6. EDX spectrum as in Fig. 5, except that the seed was grown in Summerland, B. C.

ledons revealed the presence of phosphorus, magnesium, and potassium (Figs. 8 and 9) in approximately the same amounts and ratios as was found in globoid crystals in fixed and sectioned materials (Figs. 1, 5, 6 and 7). In one small-sized globoid crystal a trace of calcium was detectable (Fig. 9).

DISCUSSION

Phosphorus, which plays an important role in seedling growth, is stored in the seed almost entirely in organic forms (12). Of the total phosphorus content of dry seeds a large percentage is often in the form of phytin. For example, in wheat and oats phytic acid represents 53% of the total seed phosphorus (6, 26) and in cotton embryos more than 80% of the phosphorus is in the form of phytin (4).

During germination of the seed, phosphorus is rapidly mobilized (4). Phytase, the enzyme responsible for phytin breakdown, has been shown to occur in a number of seedlings, including squash (5, 14, 15, 24). In the seed, phytin may be regarded as a reserve form of phosphate (12, 26), and a metallic ion store (20, 26). Other functions besides phosphate storage have been proposed but are in some doubt (see review 26).

Phytin is generally reported to be a calcium and magnesium salt of myoinositol hexaphosphoric acid (12, 15), but potassium has also been reported to be present in phytin along with calcium and magnesium (4, 6, 11, 16). The calcium and magnesium content of the phytin molecule is variable between species and even between varieties of the same species (1). For example, wheat phytin contains 1.5% magnesium to 12% calcium, whereas oat phytin contains 15% magnesium, 8.3% calcium, and 5.7% manganese (12).

The protein bodies from seeds of different plant species can be classified into one of three protein body types as follows: protein bodies with no inclusions, protein bodies with a globoid, and protein bodies with both a globoid and a protein crystalloid inclusion (17). The protein bodies of squash contain both a globoid crystal and a protein crystalloid inclusion (8).

The location of phytin inside seeds, in cases where it has been investigated, generally seems to be in the globoids of the protein bodies (7, 11, 23). Chemical analyses of cottonseed globoids showed the globoids to be 60% phytic acid, and also showed that this phytin accounted for 97.5% of the total phosphorus present (11). In castor beans, chemical analyses showed that the globoids were 77.5% phytin on a dry weight basis (21). X-ray analysis of the globoids in seeds of *Crambe abyssinica* showed the presence of high amounts of phosphorus and calcium and lesser amounts of sulfur, magnesium, potassium, and sodium (7). The x-ray study of Hofsten (7) was the first work that I know of in which EDX analysis was used to investigate seed tissue components.

The EDX analyses conducted here provide good evidence that the globoid crystals in protein bodies in squash seeds are the location of the phytic acid deposits. These results are in keeping with earlier observations on the electron density and hardness of this globoid crystal (8) and the knowledge that squash protein bodies contain urea-insoluble particles which have a high phosphorus concentration (25).

From known standards, Russ (19) has calculated *P* values for EDX analyzable elements. These *P* values, which give the relative intensity of x-rays from each element from the same concentration (19), can be used in interpretation of peak heights. For an 80-kv accelerating voltage used in obtaining the analyses reported here, the following are the *P* values for the Kα emission line of elements of importance in this study: calcium 0.94, potassium 0.93, chlorine 0.87, sulfur 0.82, phosphorus 0.75, magnesium 0.47. The similarity of the calcium and potassium *P*

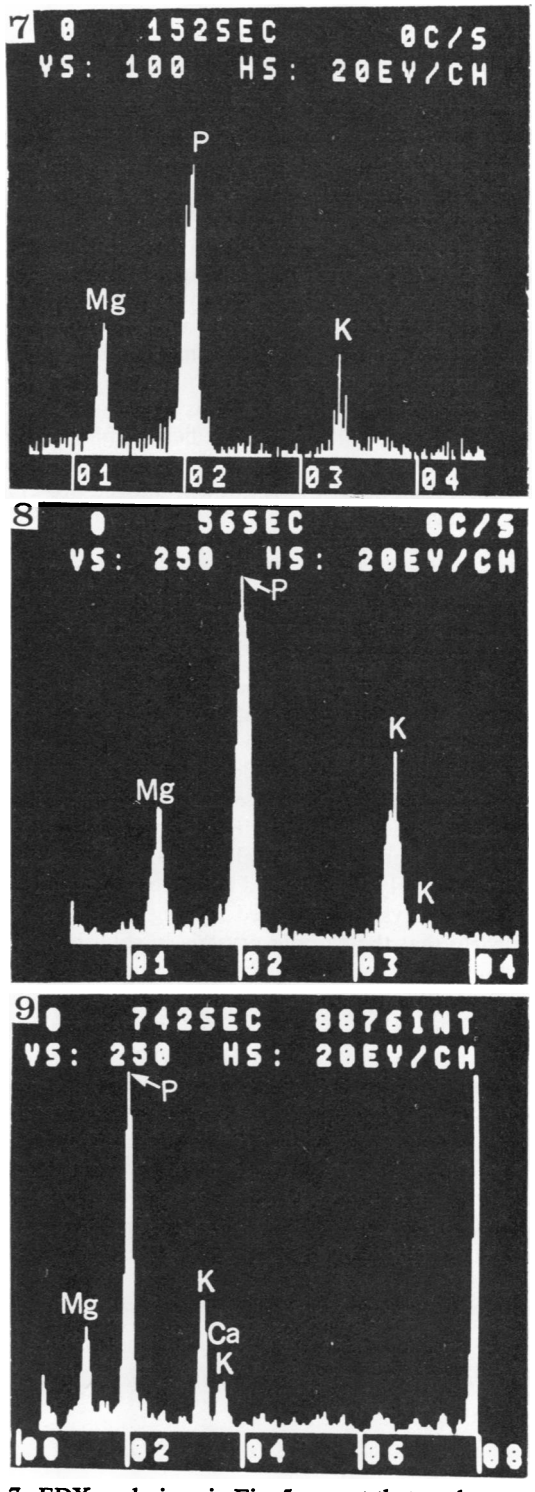


FIG. 7. EDX analysis as in Fig. 5, except that seed was grown in Hamilton, Ontario.

FIGS. 8 and 9. Seed was grown at Rocky Ford, Colo. Cotyledon tissue from a dormant seed was quick frozen in liquid nitrogen, ground, freeze-dried at low temperature, and dusted onto a coated grid. Energy levels in keV and principal emission lines for the elements present are listed above. Fig. 8: EDX spectrum of a large sized globoid crystal. Electron dense globoids could be readily identified for analysis. Fig. 9: EDX spectrum of a small sized globoid crystal. The K_β peak of potassium at 3.589 keV overlaps the $K\alpha_{1,2}$ peak for calcium at 3.690 keV. The peak height is much more than 10% of the potassium $K\alpha_{1,2}$ peak at 3.312 keV and thus a small amount of calcium is present in this globoid crystal.

values indicates that the peak height would be very similar if these two elements were present in equal amounts. Thus the trace of calcium detected in Figure 9 is, in fact, only a trace by comparison to the amount of potassium present.

The EDX analysis of the proteinaceous part of a protein body demonstrated the presence of phosphorus, sulfur, and a trace of chlorine. The analysis presented in Figures 2 and 3 was conducted on a section which had the globoid crystal from Figure 1 nearby and thus the possibility of the P peak coming from the dense globoid nearby could not be ruled out. However, analyses of proteinaceous regions of protein bodies sectioned so that no globoid was present also produced peaks for potassium and sulfur of about the same heights. The phosphorus present in the proteinaceous region of the protein body could be in one or more forms such as inorganic-P, protein-P, or as phytin, and the methods used here cannot answer this question. The presence of a protein-P fraction has been found in cotton embryos (4). Also it is known that the protein bodies of barley, which lack highly organized globoid crystals, contain a phytase enzyme (13). That the phosphorus present in the proteinaceous region is a fixation artifact is possible but is considered unlikely in view of the insolubility of phytin (21) and the much lower phosphorus levels found in cytoplasm analyses. The presence of sulfur in the proteinaceous parts of squash protein bodies was expected since the amino acid analyses of Wiley (25) showed the presence of the sulfur-containing amino acids methionine and half-cystine in squash protein bodies isolated from dormant seeds. The detection of sulfur provides a good test of the sensitivity of the EDX analysis system since out of 17 amino acids measured by Wiley (25) methionine and half-cystine were present in small amounts and ranked 13th and 17th respectively, with regard to amount present.

The EDX analyses conducted during this study demonstrated clearly that the globoid crystals in squash protein bodies are similar in seeds from various sources. Calcium was either undetectable or present in only trace amounts in the globoid crystals, whereas magnesium, potassium, and phosphorus were always present. This work suggests that the cations which make up phytin are actively selected by the developing cotyledon mesophyll cells. Variations in soil types and other environmental factors seem not to have influenced the type of cation stored. Some variation in the cation amounts relative to the amount of phosphate present was noted however. Larger and denser globoid crystals had more cation per unit of phosphorus than did smaller, less dense globoid crystals.

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