STRUCTURE AND COMPOSITION
OF *CAPSICUM ANNUUM* SEEDS

LIGHT, ELECTRON MICROSCOPIC AND ELEMENT

ANALYSIS STUDIES OF *CAPSICUM ANNUUM* SEEDS

By

Ping Chen, B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

May 1991

MASTER OF SCIENCE (1991)   McMASTER UNIVERSITY
(Biology)   Hamilton, Ontario

TITLE: Light, Electron Microscopic and Element
          Analysis Studies of *Capsicum annuum* Seeds

AUTHOR: Ping Chen, B.Sc., M.Sc.
               (East China Normal University,
               Shanghai, P. R. China)

SUPERVISOR: Dr. J. N. A. Lott

NUMBER OF PAGES: xiv, 141

ABSTRACT

Using light microscopy, histochemical techniques and electron microscopy, the structure and composition of *Capsicum annuum* seeds were studied. The seed had mainly protein and lipid as storage reserves. Starch was not detected in either the embryo or the endosperm; however, the thickened cell walls of the endosperm likely act as a store of non-starch polysaccharide. Cells from both the embryo and the endosperm contained a large number of lipid vesicles, many protein bodies and a nucleus. In section, protein bodies were oval to round in shape and had protein crystalloids and globoid crystals as inclusions in the proteinaceous matrix. Protein bodies in embryo tissues varied greatly in size both inter- and intra-cellularly, while in the endosperm protein bodies were similar in size. Considerable size variation of globoid crystals was also found inter- and intra-cellularly in embryo tissues but not in the endosperm.

Using energy dispersive X-ray (EDX) analysis and neutron activation analysis (NAA), the elemental content of *C. annuum* seeds was studied. EDX analysis of globoid crystals of *C. annuum* seeds revealed the presence of P, K and Mg in all cases, a fact that is consistent with globoid crystals being phytin-rich. Fe, Zn, Mn, Ca, S and Cl were occasionally detected in globoid crystals. In both the cotyledon and the

hypocotyl-radicle axis, Fe was always detected in globoid crystals of protoderm cells, was occasionally detected in globoid crystals of ground meristem cells, and was never detected in provascular cell globoid crystals. NAA results showed that Mg, K, S and Cu were higher in the embryo than in the endosperm, while Cl, Na and Ca were higher in the endosperm. Mn had similar concentrations in both tissues.

Characteristics of protein bodies of *C. annuum* seeds were compared to those of other solanaceous species which have been studied, namely *Lycopersicon esculentum, Atropa belladonna* and *Datura stramonium.* Similarities in protein body characters were found among these four species, but more species in this family should be studied to determine whether protein bodies are a useful character for the study of taxonomic relationships. Because the previously reported studies of solanaceous seeds were not as detailed as this study, some difficulties were encountered in comparing size, shape and inclusions of protein bodies of *C. annuum* seeds to those found in other solanaceous seeds.

This thesis contains the results of the most comprehensive studies of the structure and the elemental content of *C. annuum* seeds. It is also the most detailed study to date dealing with seeds of any solanaceous species.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ATBO   -   acidic toluidine blue O

EDX   -   energy dispersive X-ray

IMIX   -   integrated microanalyzer for imaging and x-ray

LM   -   light microscope

LSD   -   least significant difference

PAS   -   periodic acid schiff

P/B   -   peak-to-background ratio, the ratio of peak minus
           background counts to background counts

SEM   -   scanning electron microscope

SE   -   standard error

STEM   -   scanning transmission electron microscope

TEM   -   transmission electron microscope

Chapter 1

INTRODUCTION

The biological and economic importance of seeds is enormous. The structural, physiological and biochemical features of the seed largely determine the time and place of germination and the vigour of the young seedling. For example, dormancy allows the species to survive harsher conditions than would otherwise be possible. Mechanisms to control germination allow growth of the seedling to occur at a time when successful seedling establishment is likely. The food reserves in the seed are available to sustain the growth and development of the seedling until it can establish itself as a photosynthesizing, autotrophic plant.

Mankind also depends heavily on seeds for food and many industrial products. Cereal grains alone contribute about 50% of the per capita energy intake on a world scale (Duffus and Slaughter 1980). Many oil seeds not only are valuable as human food but also are used in industry for the production of commodities such as paint and soap (Vaughan 1970).

Seeds also contain a great deal of information of taxonomic use. A skilled seed analyst can identify many genera

and species by studying only the seeds (Robbins *et al.* 1964).

Studies of seed structure and the nature of storage reserves have been done to a very limited extent on some species of the Solanaceae (Gassner 1955; Vaughan 1970; Spitzer and Lott 1980; Simola 1987; Friedman and Levin 1989). This thesis presents results of my studies of the structure and the storage reserves of sweet pepper (*Capsicum annuum*) seeds. This thesis includes four chapters. In the introductory chapter storage reserves and their sites of accumulation in seeds in general are reviewed, the major features of the family Solanaceae and known characteristics of *C. annuum* seeds are surveyed, and the objectives of my research are stated. Chapter 2 presents the results of microscopic studies of *C. annuum* seeds using light microscopy, transmission electron microscopy and scanning electron microscopy. Chapter 3 presents the results of elemental analysis studies of pepper seeds that were done with energy-dispersive X-ray (EDX) analysis and neutron activation analysis (NAA). The last chapter in the thesis presents conclusions and future studies.

1. STORAGE RESERVES IN SEEDS

a) Chemistry:

Seed storage reserves principally include carbohydrates, lipids and proteins. However, other minor but important reserves such as phytin and non-protein nitrogenous

substances such as alkaloids are also present. Among these reserves some alkaloids, amines and lectins are nutritionally undesirable or even toxic to mankind or domestic animals.

The content of storage reserves in seeds is determined ultimately by genetic factors and therefore varies greatly between species. The chemical composition of seeds can be modified by breeding; much effort has been and still is being expended by plant breeders to produce seeds with a more desirable nutritional content for humans and domestic animals. Agronomic practices or environmental conditions during seed development and maturation may also result in some modification of the chemical composition of seeds.

Table 1 lists the storage reserves of various seeds and grains. Carbohydrates, mainly starch, predominate in the cereals and other Graminaceous plants, though proteins and lipids are also stored. A large number of species, such as cotton, peanut and oil palm, store lipids as their main reserve in seeds, but substantial amounts of other reserves, especially proteins, are also present. A high amount of protein and starch are present in a third group of seeds which includes many legumes such as soybean, broad beans and peas. In addition to carbohydrates, lipids and proteins, phytin, a salt of *myo*-inositol hexaphosphoric acid, is frequently found as a reserve for phosphorus, carbohydrate and a variety of cations (Ashton and Williams 1958; Lui and Altschul 1967).

Carbohydrates: Carbohydrates are the major storage

reserve of most seeds cultivated for food (Table 1).
Carbohydrates that accumulate in seeds can be polysaccharides
or oligosaccharides. The principal and most widespread storage
polysaccharide is starch, although other polysaccharides such
as hemicelluloses, amyloids and galactomannans may be present
and in some cases may be the major storage carbohydrate.

Starch consists of two polymers of D-glucose, one linear
polymer called amylose and the other, a branched polymer,
called amylopectin (Baldwin 1957). Amylose has about 300-400
glucose units joined only by α-1,4 glucosidic linkages.
Amylopectin is much larger (10^2-10^3 times) than amylose and is
composed of many amylose chains joined via α-1,6 linkages
(Baldwin 1957). The proportions of amylose and amylopectin
vary among seeds of different species and even among different
varieties of the same species. Rice starch generally consists
of 15-37% amylose depending on the variety (Bewley and Black
1985). In the well known waxy varieties of cereals including
maize, barley and rice, the starch in the endosperm consists
entirely of amylopectin. In wrinkled peas, amylose occupies
66% or more of the starch while in smooth peas only about 35%
amylose is found (Badenhuizen 1969).

Hemicelluloses are the major form of storage
carbohydrates in some seeds such as the ivory nut (*Phytelephas
macrocarpa*), the date (*Phoenix dactylifera*) and coffee (*Coffea
arabica*) (Meier 1958; Keusch 1968; Wolfram *et al.* 1961). Such
seeds characteristically have endosperm or perisperm which is

very hard due to hemicelluloses deposited in very thick cell walls. Many of the hemicelluloses are mannans. The endosperm of date contains 58% galactomannan (Table 1). The glucomannans and galactomannans have been found as major storage components in iris (*Iris croleuca*) and *Gleditsia ferox* respectively (Andrews *et al.* 1953; Reid and Meier 1970). Xyloglucans are found as amyloids in seeds of tamarind (*Tamarindus*), *Annona* and several other species (Srivistava and Singh 1967; Kooiman 1967). The glucomannans, galactomannans and xyloglucans are laid down in the endosperm cell walls of these species.

Sucrose, raffinose and other oligosaccharides are commonly found as minor reserves in seeds. For example, about 20% of the dry weight of defatted wheat embryos is made up of sucrose and raffinose (Bewley and Black 1985). It is increasingly evident that these oligosaccharides are an important source of carbohydrates for respiration during germination and early seedling growth (Bewley and Black 1985).

Lipids: Lipids are a chemically heterogeneous group of substances which have in common their insolubility in water and solubility in a variety of organic solvents such as petroleum ether, chloroform or hexane. Some are called triglycerides or triacylglycerols.

The triglycerides are esters of glycerol and fatty acids:

$$
\begin{array}{l}
CH_2OOCR^1 \\
| \\
R^2COOCH \\
| \\
CH_2OOCR^3
\end{array}
$$
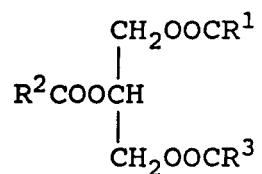
R^1, R^2 and R^3 represent hydrocarbon chains of fatty acids and the three may be identical but usually are different. Triglycerides which are solid at room temperature are called fats and have saturated fatty acids as their major fatty acids. Those which are liquid at room temperature are called oils and have unsaturated fatty acids as their major fatty acids (Stumpf 1965). In seed oils both saturated and unsaturated fatty acids are present, but unsaturated fatty acids are predominant. In saturated fatty acids, all the carbon atoms hold as many hydrogen atoms as possible. Unsaturated fatty acids contain carbon atoms joined by double bonds; such carbon atoms are able to form additional bonds with other atoms (Stumpf 1965). Fatty acid content in seeds of any given species is influenced by genetic and environmental factors. For example, one cultivar of rapeseed (*Brassica napus*) 'Regina' has erucic acid as the major fatty acid of seed oils, while another cultivar 'Oro' only has a trace of erucic acid but has oleic acid as the major fatty acid in seeds (Appelqvist 1975). The erucic acid fraction of the oil has special industrial applications, such as in the manufacture of plastics and the lubrication of jet engines,

while low erucic acid content is 'required' for human consumption (Vaughan 1970). Altering the temperature during seed development can also cause a change of fatty acid proportions, with lower temperatures favouring a more unsaturated fatty acid (Canvin 1965)

Most seed storage lipids are neutral fats or oils. Other storage lipids such as phospholipids and glycolipids are also present in some seeds. In *Briza spicata*, for example, 78% of the total lipids are glycolipids (Smith and Wolff 1966). A great number of species, such as most members of the Papaveraceae, Sapindaceae and Cucurbitaceae, have seeds with a high amount of storage lipid. Legumes, on the other hand, have members covering a wide range from low to high seed lipid content.

Proteins: According to Osborne's classification, seed proteins are defined into four groups based on their solubility: 1) albumins - soluble in water at neutral or slightly acid pHs and heat coagulatable; 2) globulins - soluble in salt solutions but insoluble in water and not as readily coagulatable upon heating as the albumins; 3) glutelins - soluble in dilute acidic or alkaline solutions but insoluble in water; 4) prolamins -soluble in aqueous alcohols (70-90%) but insoluble in water (Osborne 1924). The classification, as one can predict, is not ideal since it depends upon solubility.

Most investigations of these various groups of proteins

have been carried out on edible seeds of crop species, especially those of the legumes and Gramineae. Not all four groups of protein are necessarily present in seeds of any given species (Table 2). The levels of prolamins in the seeds of the Gramineae are frequently high, though all of the four types of proteins are found. Cereal grains are also rich in glutelins. Oat is different from other cereal grains in that 80% of its protein is globulin, a protein which is often low in cereals, but is generally found to be the major reserve protein in dicotyledonous seeds such as legumes (Table 2). Albumins constitute a widespread and neglected category of proteins in seeds. It had been considered that albumins were enzymes or metabolic proteins, following the work of Danielsson (1956). However, it has gradually become evident that enzymes in seed tissues are not confined to albumins (Murray 1984). Albumins in pea cotyledons actually serve as reserve proteins and account for 40% of extractable pea seed proteins (Table 2).

The amino acid composition of the total protein fraction is strongly influenced by the nature of the major storage protein. Characteristically, the prolamins have high levels of proline, glutamine and asparagine, and low levels in tryptophan, lysine and methionine. Globulins and albumins are high in glutamine, asparagine and arginine, deficient in the sulphur amino acids cysteine and methionine, and unlike prolamins have a level of lysine that is nutritionally

adequate for humans. Comparing the four types of seed proteins, albumin has the best amino acid balance for animal nutrition, followed by glutelin, globulin and prolamin. The amino acid composition of both cereal and legume seed proteins is not ideally suited for the diet of human beings or animals, as one or more essential amino acids such as lysine, methionine or tryptophan are deficient. Attempts have been made to modify the amino acid composition of prolamin-rich cereals such as barley and in particular to increase their lysine level. High-lysine mutants, such as barley Hiproly and corn Opaque 2, have been successfully obtained (Munck *et al.* 1971).

Phytin: Phytin is a salt of *myo*-inositol hexaphosphoric acid (Lui and Altschul 1967). It serves as a major reserve for inositol, phosphorus and other mineral nutrients for early seedling growth. Seeds from many species generally contain 1-3% of their dry weight as phytin (Graf 1986) and in some species the levels are even higher (Mukhamedova and Akremov 1977). The composition of phytin may vary among species and among different tissues in a species (Lott 1975; Maga 1982). K and Mg are the most common cationic constituents found in phytin, but other elements such as Mn, Fe, Zn, Ba and Na may also present depending on the tissue type, species and soil conditions (Lott 1985). In squash cotyledons Ca is absent in most globoid crystals (phytin-rich particles), but present in globoid crystals in the protoderm and most provascular regions

(Lott *et al.* 1979). The frequency of Ca presence in globoid crystals seems to be related to squash seed size (Lott and Vollmer 1979a; Ockenden and Lott 1990).

Phytin is recognized as nutritionally undesirable to human beings, since it is insoluble and can bind essential dietary minerals (Fulcher *et al.* 1981; Maga 1982; Bewley and Black 1985).

b) Subcellular Locations of Reserves:

Carbohydrates, lipids, proteins and mineral nutrients are stored within specific subcellular structures within seeds and are mobilized in response to the nutrient requirements of the developing seedlings.

Subcellular locations for carbohydrates: Not all seed storage carbohydrates are laid down in the same subcellular sites. Hemicelluloses, glucomannans, galactomannans and xyloglucans are stored in cell walls. Starch is deposited in discrete subcellular bodies called starch grains (or starch granules). Starch grains vary in shape and size among species and even in a given species (see review by Seidemann 1966). Starch grains in seeds or cereal grains are contained in amyloplasts, which like other plastids, are double membrane bound (Kirk and Tilney-Bassett 1967). Sizes of starch grains vary from approximately 2 μm to 100 μm in diameter in grains (Seidemann 1966). Many starch grains are polygonal, as occurs in corn and rice endosperm. Others, including those found in

in wheat and rye grains, have two distinct shapes; one large and lenticular, the other small and round. Some seeds, like those of wrinkled pea have compound starch grains that are aggregates of several individual grains. Some starch grains, such as those found in corn and mango seeds have indentations which are due to close packing with protein bodies in the cell (Wursch and Hood 1981; Robutt et al. 1974). Scanning electron micrographs of a number of starch grains suggest that the grain surface is smooth and without definition (Hood 1982). However, the results of freeze-etching studies suggest that for some starch grains the surface has a three-dimensional pattern (Chabot et al. 1978). The internal structure of starch grains is complex since the different techniques that applied have revealed different features. Starch grains that have been hydrolysed with acid have lamellar ring structures as studied by TEM (Buttrose 1963; Gallant et al. 1972). In addition to the ring structure, the central regions of starch grains appear to differ structurally from the outer regions (Allen et al. 1976; Hollinger and Marchessault 1975).

Lipid vesicles: Lipid vesicles are the subcellular organelles in which the lipid reserves are laid down. A number of other names such as oil bodies, spherosomes and oleosomes have also been used for these structures. Lipid vesicles range in size from 0.2 to 6 μm in diameter depending upon the species (Bewley and Black 1985). Whether the lipid vesicles are surrounded by membranes is controversial. No peripheral

membrane is seen in lipid vesicles of developing seeds of
Crambe and *Sinapis* (Smith 1974; Rest and Vaughan 1972). On the
other hand, freeze-fracture studies on barley aleurone cells
and biochemical analyses of peanut and walnut lipid vesicles
(Buttrose 1971; Bewley and Black 1985) support the idea that
lipid vesicles are bounded by half-unit membranes. Beside the
lipid reserves, enzyme protein may also occur in lipid
vesicles. Enzymes for fatty acid biosynthesis have been
reported in lipid vesicles of developing castor bean (Harwood
and Stumpf 1972).

Protein bodies: Seed proteins are usually deposited
within special subcellular bodies called protein bodies.
Protein bodies were first isolated by Hartig (1855) from
certain oil seeds. Since then studies of protein bodies have
been carried out on a large number of species. Protein bodies
are oval to circular in section and are surrounded by a lipo-
protein unit membrane (Dieckert and Dieckert 1972; Yatsu
1965). Protein bodies have been reported to range in diameter
from 0.1 to 25 μm (Bewley and Black 1985). They differ in
structural complexity from species to species, cell to cell
and even within one cell. Some protein bodies are structurally
simple and consist only of a proteinaceous matrix surrounded
by a limiting membrane, but most protein bodies contain
inclusions. Detailed classification of protein bodies based on
their structural complexity can been found in a review by Lott
(1980). The most common inclusions are protein crystalloids

and globoid crystals. Protein crystalloids are proteinaceous regions with a definite crystalline lattice arrangement and globoid crystals are electron dense regions which contain phytin. However, other inclusions such as soft globoids, druse crystals and niacin reserves have also been reported (Lott *et al* 1971; Lott and Buttrose 1978a; Spitzer and Lott 1982a; White and Lott 1983; Fulcher 1982). Protein crystalloid regions do not appear to be surrounded by membranes, but there are some reports which support the presence of a membrane around globoid crystals (Buttrose 1971; Swift and Buttrose 1972). Globoid crystals in protein bodies are naturally electron-dense regions and thus are ideal subjects for EDX analysis. EDX analysis studies of a number of seeds have shown that P, K and Mg are usually present in globoid crystals, and that other elements such as Ca, Cl, Fe, Mn, Si, Na and Ba may be present (Lott and Ockenden 1986).

2. THE SOLANACEAE

The family Solanaceae includes about 90 genera and 2300 species, some 1500 of which belong to the single genus, *Solanum* (Hickey and King 1981). The Solanaceae are distributed world wide, but the concentration of diversity lies in western and southern South America. About 59 genera in this family are native to South America (Langer and Hill 1982). Numerous members of this family are cultivated as food plants and for

use as ornamentals. There are also some poisonous species, weeds and several species that provide drugs used in medicine (Hickey and King 1981).

Habits and characteristics: Members of this family range from herbs through shrubs to small trees (Langer and Hill 1982). The characteristics of this family are as follows (see Robbins 1917 for a detailed description): a) leaves are usually alternate, but sometimes are present in pairs on the upper part of the plant; b) hermaphroditic flowers are solitary, or arranged in cymes; c) fruits are either berries or capsules and bear numerous seeds; d) seeds in this family are small, often flattened and contain endosperm which is either oily, as in tomato, or starchy, as in *Browallia.* The development of endosperm may be free nuclear, cellular, or helobial (Corner 1976). The helobial type seems to combine in a remarkable way the main features of free nuclear and cellular types. The first division of the primary endosperm nucleus is followed by a transverse wall dividing the primary endosperm cell unequally into two cells; free nuclear divisions then occur in each cell (Maheshwari 1950). Embryos are straight or curved, and are embedded in endosperm (Hickey and King 1981).

Food plants: Potato (*Solanum tuberosum*), which evolved in the Peru-Bolivia area, is the most important food crop in the

family and is a major source of food energy in many countries
(Dalton 1978). *S. tuberosum* is an annual that reproduces
either asexually by tubers or sexually by seeds. The food
value of potato tubers derives from their high content of
starch and their content of Vitamin C (Gray and Hughes 1979).
The tomato, which belongs to the genus *Lycopersicon*, is
another crop of considerable agricultural importance. The
consumption of *Lycopersicon* is largely limited to cultivars of
L. esculentum, but the wild species have great potential value
as a source of genes for resistance to diseases and for
improved fruit colour and quality. The pepper (*Capsicum
annuum*), which includes a vast number of horticultural
varieties, is used as a food, a spice and as an ornamental.
Capsaicin, a volatile phenolic compound which has a pungent
taste, gives *Capsicum* its value in the spice trade. Pungency
of different varieties of peppers varies greatly and some
varieties lack the pungent constituent entirely (Heiser, Jr.
1969). Eggplant (*Solanum melongena*) is commonly used as a
vegetable. Other widely grown food plants from this family
include Cayenne pepper (*Capsicum frutescens*), Cape gooseberry
(*Physalis peruviana*) and tree tomato or *Cyphomandra
crassifolia* (Hickey and King 1981). In addition to the food
plants mentioned above, the Solanaceae provides a number of
other plants used for food in tropical south and central
America. *Physalis ixocarpa* (husk tomato), which has been
cultivated in Mexico and Guatemala for a long period of time,

has fruits that are often eaten as a vegetable. The fruits of *Solanum quitoense*, which are produced in Colombia and Ecuador, are used to make a delicious beverage. Pepino (*Solanum muricatum*), another species of *Solanum*, that is hardly known outside South America, is used as a fruit from Colombia to Peru (Heiser, Jr. 1969).

Ornamental plants: A large number of species of Solanaceae are widely used as ornamentals (Hickey and King 1981; Hessayon 1985). Species such as winter cherry (*Solanum capsicastrum*), Jerusalem cherry (*Solanum pseudocapsicum*) and some varieties of *C. annuum* bear very colourful, cherry-sized fruits and are used as house plants. *Petunia, Nicotiana, Salpiglossis, Schizanthus* and *Browallia* are popular garden annuals, and the last three are also cultivated as flowering pot-plants. Genera such as *Brunfelsia, Solandra, Streptosolen, Lycium* (matrimony-vine), *Cestrum* and the woody species of *Datura* are sometimes grown as decorative flowering shrubs, and some of them such as *Solandra* and *Cestrum* are fragrant. *Nierembergia*, a creeping perennial herb, and *Physalis* (ground-cherry), a perennial herb, are also grown as ornamentals.

Drug and poisonous plants: A number of solanaceous species contain high concentrations of narcotics, especially alkaloids, and in many countries these are extracted for use in the pharmaceutical industry or in illegal pursuits. *Solanum*

laviculare and *Solanum laciniatum*, native plants of New Zealand, are currently under commercial cultivation in Hungary as they contain the alkaloid solasidine, which is a precursor in the production of corticosteroids used in the manufacture of cortisone and oral contraceptives (Langer and Hill 1982). *Datura stramonium* (Jimson weed), *Datura metaloides* (sacred datura), *Datura metel*, *Datura candida* (queen of the night), *Hyoscyamus niger* (black henbane), *Hyoscyamus albus* (white henbane) and *Hyoscyamus muticus* (Egyptian henbane) contain the alkaloids hyoscyamine, atropine, and hyoscine in all parts of the plant. A very small amount (4-5 g) of leaves or seeds from these plants can be fatal to a child. *Datura* and *Hyoscyamus* are important in modern medicine. Although *Datura* is sometimes used as an ornamental, *Hyoscyamus* is not recommended as an ornamental. *Nicotiana tabacum* (tobacco), an annual with great commercial value, has leaves that are used in the production of pipe tobacco, cigarettes and cigars. Tobacco leaves can cause severe poisoning when eaten as cooked greens or as a salad. Some other examples of poisonous solanaceous plants are *Atropa belladonna* (deadly nightshade), *Mandragora officinarum* (Mandrake), *Solanum dulcamara* (European bittersweet), *Solanum nigrum* (black nightshade), *Solanum carolinense* (horse nettle), *Solanum sodomeum* (apple of sodom) and *Solanum pseudocapsicum* (Jerusalem cherry). Poisoning from *Solanum* is mainly due to solanine, a glyco-alkaloid which is extremely toxic (Hardin and Arena 1974). Solanine is also present in high

concentrations in the sprouts and green outer portion of potato (*S. tuberosum*) tubers exposed to light (Liener 1969).

Weeds: Some of the species described above as poisonous plants, such as *D. stramonium, S. carolinense, S. dulcamara, S. nigrum,* and *S. triflorum,* are also widespread weeds. Other non-poisonous weeds in this family include *Nicandra physalodes, Physalis heterophylla* and *Physalis subglabrata* (Muenscher 1935). Weeds of Solanaceae are often seen in hay fields, meadows and gardens, and they also contaminate crop fields, often in the fields of potato, tomato, eggplant, cucumber and onion (Muenscher 1935).

3. CHARACTERISTICS OF *CAPSICUM ANNUUM* SEEDS

Capsicum annuum L. is the most widely used species in the genus *Capsicum,* a genus which includes four other domesticated species, namely, *Capsicum baccatum* var. *pendulum, Capsicum pubescens, Capsicum chinense,* and *Capsicum frutescens.* *C. annuum* is an annual herb. The fruit is a berry varying considerably in size (from 1 to 30 cm in length) and shape from variety to variety (Rylski 1986). The seed is small, flattened, light yellow in colour and up to 5 mm in diameter (Gassner 1955; Rylski 1986).

The literature related to *C. annuum* seeds covers a

variety of aspects, however most research has been carried out for agricultural reasons. For example, the correlation between fruit size and number of seeds of *C. annuum* under controlled conditions has been studied, and it has been shown that fruits containing the normal number of seeds developed normally whereas seed-deficient or seedless fruits are generally small and abnormally shaped (Rylski 1986). The conditions for seed germination (Perl and Feder 1981; Sachs *et al*. 1981, 1982) and methods for detecting and controlling seed borne fungi and viruses of *C. annuum* (Sharon *et al*. 1982; Bashan and Okon 1987; Sultana *et al*. 1988) have also been studied.

Past studies of the structure of *C. annuum* seeds are very limited. Available results of these studies will be discussed in Chapter 2.

4. THE OBJECTIVES OF MY RESEARCH

Sweet pepper is an important horticultural crop. The cultivation of this plant depends on seed germination. The seeds are not eaten deliberately as a food by humans, but are sometimes ingested. Studies of the seed structure and storage reserves of sweet pepper will provide more understanding of this important crop plant as well as to provide information of use in taxonomic comparisons.

The objects of my research are:

1) To study the structure of *C. annuum* seeds using light

microscopy, scanning electron microscopy and transmission electron microscopy (see Chapter 2).

2) To demonstrate the presence of storage reserves of *C. annuum* seeds using histochemical methods (see Chapter 2).

3) To analyze quantitatively the overall amount of selected elements in *C. annuum* seeds using NAA and to determine mineral nutrient reserves in the embryo and endosperm of *C. annuum* and their subcellular localization through the use of EDX analysis (see Chapter 3).

4) To discuss the relationship between protein body characteristics and taxonomic groupings (see Chapter 2, 3 and 4).

Table 1
Storage Reserves of Various Seeds and Grains

Species	Average percent composition of seeds or grains (dry wt.)			Major storage organ
	Protein	Fat	Carbohydrate	
Cereals:				
Corn (*Zea mays*)	11	5	75 (starch)	Endosperm
Barley (*Hordeum vulgare*)	12	3	76 (starch)	Endosperm
Oat (*Avena sativa*)	13	8	66 (starch)	Endosperm
Wheat (*Triticum aestivum*)	12	2	75 (starch)	Endosperm
Rye (*Secale cereale*)	12	2	76 (starch)	Endosperm
Legumes:				
Broad bean (*Vicia faba*)	23	1	56 (starch)	Cotyledon
Field pea (*Pisum arvense*)	24	6	56 (starch)	Cotyledon
Garden pea (*Pisum sativum*)	25	6	52 (starch)	Cotyledon
Peanut (*Arachis hypogaea*)	31	48	12 (starch)	Cotyledon
Soybean (*Glycine max*)	37	17	26 (starch)	Cotyledon
Others:				
Castor bean (*Ricinus communis*)	18	64	trace	Endosperm
Oil palm (*Elaeis guineensis*)	9	49	28	Endosperm
Pine (*Pinus pinea*)	35	48	6	Megagametophyte
Cotton (*Gossypium spp*)	39	33	15	Cotyledon
Date (*Phoenix dactylifera*)	6	9	58 (galactomannan)	Endosperm

Based on Crocker and Barton 1957, and Winton and Winton 1932.

Table 2
Protein Composition of Various Seeds and Grains

Species	percent of total protein			
	Albumin	Globulin	Glutelin	Prolamin
Oat (*Avena sativa*)	trace	80	5	15
Corn: IND 260 (*Zea mays*)	14	0	31	48
Corn: Opaque 2 (*Zea mays*)	25	0	39	24
Rice (*Oryza sativa*)	5	10	80	5
Garden pea (*Pisum sativum*)	40	60	0	0
Squash (*Cucurbita pepo*)	trace	92	trace	trace

After Beevers 1976.

Chapter 2

MICROSCOPIC STUDIES

<u>INTRODUCTION</u>

Structural studies of seeds from economically important members of families such as the Gramineae and Leguminosae, began almost two centuries ago, but little is known about seeds of the economically important family Solanaceae. The research presented in this chapter used both light and electron microscopy to study the structure of sweet pepper seeds. Moreover, histochemical stains were used to determine the chemical composition of storage reserves of sweet pepper seeds. The introduction to this chapter includes a review of past microscopic studies of solanaceous seeds and a general overview of relevant microscopic methods.

a) Past Microscopic Studies of Solanaceous Seeds:

Considering that this family contains so many species important to mankind, there is a paucity of information about the structure and storage reserves in seeds of solanaceous plants. Some information is available for *Lycopersicon esculentum, Nicotiana tabacum, Solanum tuberosum, Capsicum annuum, Solanum melongena, Physalis pubescens* and *Atropa*

23

belladonna (Winton 1916; Netolitzky 1926; Winton and Winton 1935; Hayward 1938; Gassner 1955; Spitzer and Lott 1980; Simola 1987). Results of past microscopic studies of these plants were described as follows:

L. esculentum (tomato): Seeds of this crop plant are oval in outline and very much flattened laterally. Seeds average 3-4 mm in length and 2-4 mm in breadth on the flat surface (Hayward 1938). The testa includes four layers: an outer epidermal layer which ultimately produces the hairs; an intermediate parenchymatous region which is subdivided into an outer and inner zone; and the innermost layer which is highly pigmented (Souèges 1907). Endosperm cells contain lipid vesicles and protein bodies. Endosperm protein bodies are 3-5 μm in diameter in sections. Cotyledon cells are smaller than those of the endosperm, but store similar reserves (Winton and Winton 1935; Gassner 1955). The protein body structure of tomato seeds was studied by Spitzer and Lott (1980). Protein bodies in all regions of the endosperm and embryo were oval to round in sections. Protein bodies in the protoderm cells of the embryo, in the ground meristem cells of the radicle and hypocotyl, in the mesophyll cells of the cotyledon and in the endosperm cells generally contained one or more globoid crystals of different sizes, a protein crystalloid and a proteinaceous matrix. Protein bodies in provascular regions of the radicle, hypocotyl and cotyledon often lacked a protein crystalloid (Spitzer and Lott 1980).

A. belladonna (deadly nightshade): Subcellular structures of the seeds of this plant have been studied by Simola (1987). Different parts of mature seeds of *A. belladonna* were reported to be structurally uniform, but only cotyledon cells were described in detail. Cotyledon cells contained protein bodies with protein crystalloids and globoid cavities. Protein crystalloids stained with both PAS and amido black stains which suggests that they are composed of glycoproteins. Globoid cavities may have contained phytate. The size and number of globoid cavities varied considerably from protein body to protein body, which was considered to be due to the level of transection. Cotyledon cells contained many lipid vesicles as well. No proplastids, mitochondria or dictyosomes were identified in cotyledon cells in the Simola (1987) study.

N. tabacum (tobacco): The brown seeds of this plant are minute, about 0.5 mm in length, and irregularly oval-shaped. The curved embryo is embedded in abundant endosperm. The seed testa consists of a layer of epidermal cells which are elongated tangentially in transverse section and two or three layers of thin walled parenchyma walls (Netolitzky 1926; Gassner 1955). Cells from the endosperm and cotyledon contain lipid vesicles and protein bodies (Gassner 1955). Inclusions in protein bodies have not been revealed.

S. tuberosum (potato): The yellowish-brown seeds are small, oval to kidney-shaped and flattened laterally. The U-shaped embryo lies embedded in the endosperm, with both the

tip of the radicle and those of the two cotyledons toward the micropyle (Hayward 1938). The testa consists of three layers: a single layer of epidermal cells; an intermediate zone with partially digested and crushed tissue; and an innermost layer of cells functioning as an haustorial zone. There are no true epidermal hairs, but the thickened portions of epidermal cell walls show hair-like projections (Souèges 1907). Very little is known about cell structures of the embryo and the endosperm.

C. annuum (pepper): Studies of seed structures by Gassner (1955) focused on the testa. The cells of the epidermis are clearly noticeable, the other layers of cells are at least partially collapsed. As is true for tomato and tobacco seeds, cells from both endosperm and embryo contain lipid vesicles and protein bodies. Endosperm cells have much thicker cell walls than do embryo cells (Gassner 1955). Inclusions of protein bodies in this species have not been reported.

S. melongena (eggplant): Seeds are flattened, kidney shaped, 3-4 mm in diameter and resemble most varieties of *C. annuum* (Winton & Winton 1935). Moderately thick-walled cells throughout the endosperm contain small protein bodies. Embryo cells also contain protein bodies and these protein bodies have been described as characterless based on light microscopy studies (Winton & Winton 1935). Since some structural details of protein bodies cannot be resolved with light microscopy, EM studies should be used to confirm the fine structure of

protein bodies.

P. pubescens (strawberry tomato): Endosperm cells have rigid cell walls and embryo cells have thin cell walls. Protein bodies were described as being similar to those of tomato by Winton and Winton (1935).

b) Relevant Microscopic Methods:

Bright field microscopy combined with histological and histochemical stains provide a very useful method for structural studies of seeds as well as for demonstrating storage reserves of seeds. Electron microscopes, due to their improved resolution, provide an additional means of studying subcellular structures. Thus, both light microscopy and electron microscopy were used to study sweet pepper seeds.

Major advantages of light microscopy are its simplicity, ease of maintenance and easier sample preparation than TEM (Meek 1976). When combined with histochemical methods, studies of the chemical and physical properties of cells can be made. Two basic criteria must be considered in order to carry out histochemical methods successfully (Gahan 1984). First of all it is necessary to have an understanding of the chemistry of a reaction to be performed and secondly the cells should be retained in a state that is as close as possible to that of the living state.

The electron microscope resembles the optical microscope

in many ways but employs radiation of a much shorter wavelength than that of light. Because of the extremely short wavelength and the focusability of electron beams, the resolving power of an electron microscope is much higher than that of a light microscope (Meek 1976).

Using a transmission electron microscope, the cell type, precise location of cells, and detailed cell ultrastructure can be determined. The major disadvantage of transmission electron microscopy is that tissues generally have to undergo chemical fixation, dehydration and embedding, processes that may introduce artifacts. In addition, the requirements for thinner and smaller specimens, makes sample preparation more difficult for TEM than for light microscopy. Due to the density of the cytoplasm in seed storage tissues and the presence of hard globoid crystals, preparation of seed tissues for TEM is particular difficult.

In addition to TEM, scanning electron microscopy (SEM) was used to determine seed structures of *C. annuum*. Because of their low water content, seeds can be prepared for viewing in the SEM with little processing. The study of seed coat structures by SEM has been very useful in certain plant groups (Tomb 1974; Brisson and Peterson 1976). However, internal structures of oilseeds are difficult to obtain with room temperature studies in a SEM due to the oil which flows over the surface of the seed when cells are cut or broken (Arnott and Webb 1983). Cryogenic preparation of samples for SEM

involves the quick freezing of untreated tissue samples and viewing of the sample in a frozen state (Beckett and Read 1986). With cryogenic preparation of samples for SEM, a specimen can be observed directly without being chemically fixed and dehydrated (Lott *et al*. 1985a). Specimens retain more normal volumes and shape than those that have been chemically fixed and dehydrated, and it is a quick method of specimen preparation (Beckett *et al*. 1984; Beckett and Read 1986). In this research, room temperature studies in a SEM was used to reveal the external surface character of *C. annuum* seeds, while cryogenic preparation of samples for SEM was used to study the internal structure of *C. annuum* seeds.

MATERIALS AND METHODS

Seeds of *C. annuum* (cv. California Wonder 300) were obtained from Tregunno Seeds Limited, Hamilton, Ontario. Seed coats were removed prior to sample preparation.

Light Microscopy and Histochemistry:

Preparation of fresh tissue: Sections of unfixed tissues, cut with a sharp razor blade, were used to demonstrate the existence of lipids in sweet pepper seeds.

Preparation of sections of embedded materials: Embryo and endosperm were separated. The two parts were fixed under different conditions because of their different characteristics. Small blocks of embryo were fixed at 4°C for

4 h in 5% glutaraldehyde in distilled water. Small pieces of endosperm were fixed at 4°C for 12 h in 5% glutaraldehyde in distilled water. Excess fixative was removed with three changes of distilled water over 0.5 h. Both embryo and endosperm samples were dehydrated at 24°C in 70% ethanol for 2 h, followed by 95% ethanol for 12 h. Embryo samples were infiltrated with catalyzed JB-4 monomer solution A (Polysciences, Inc., Warrington, PA) for 24 h. Endosperm samples were infiltrated for 3 d with catalyzed JB-4 monomer solution A with a change made every 24 h. The infiltrated samples were embedded in a mixture of catalyzed JB-4 monomers (solution A and solution B 25-40:1, V:V) in a plastic block moulding cup. Aluminum chucks were placed in the moulding cups and the space between the aluminum chuck and the moulding cup was sealed with paraffin wax. Polymerization was carried out at 24°C for 15 to 30 min.

Sections, 1-3 μm thick, were cut on a glass knife using a Sorvall JB-4 Porter-Blum microtome, were floated on a waterbath at 40-60°C, were picked up on glass slides, and were dried for 12 h at 40-60°C. Specimens used for lipid histochemistry were cut from OsO_4 post-fixed tissue prepared for transmission electron microscopy. Sections about 1 μm thick were cut on a Reichert ultramicrotome, picked up with a platinum loop, placed on a glass slide and dried at 40-60°C.

Staining and Microscopy: Stains applied to fresh tissues

included Nile blue sulphate, Sudan black B and the fluorochrome Nile blue A. General oversight stains such as safranin and fast green, or toluidine blue O were used to stain sections of embedded tissue for observation of endosperm and embryo structure. Histochemical stains for bright field microscopy and for fluorescence microscopy for proteins, carbohydrates, lipids and DNA were employed to identify the composition of reserves in *C. annuum* seeds. Detailed staining procedures are given in Appendices 1, 2 and 3.

Stained sections were examined with a Carl Zeiss RA-2 microscope equipped with bright field optics or with a Carl Zeiss standard 16 microscope equipped with IV FI epifluorescence condenser for fluorescence microscopy.

Scanning Electron Microscopy:

Room temperature sample preparation: Samples of whole dry seeds, or longitudinally sectioned seeds were mounted on aluminum stubs with silver glue and gold coated for a total of 180 s with a Polaron ES1005 Series II 'cool' Sputter Coater.

Cryogenic preparation of samples for scanning electron microscopy: Seed tissue blocks were mounted with Tissue Tek (Miles Laboratories, Inc., Naperville, IL.) in wells in an EMSCOPE specimen holder. The specimen holder with the tissue blocks in two wells was plunged into subcooled liquid nitrogen, and the frozen tissue blocks were transferred under vacuum to the EMSCOPE SP2000 cryogenic preparation chamber

held at -180 to -189°C. Frozen tissue blocks were fractured near the surface of the specimen holder, sputter coated with gold, and transferred under vacuum to a SEM cryostage which was kept below -163°C during observation.

Microscopy: Samples of both preparations were viewed at 15 kV with an ISI model DS-130 scanning electron microscope.

Transmission Electron Microscopy:

Preparation of sections: Endosperm tissue and embryo tissues including the hypocotyl-radicle axis and the cotyledons were dissected from dry seeds. Endosperm samples were fixed at 4°C for 5 h in 5% glutaraldehyde in distilled water and embryo samples were fixed for 3 h at 4°C in 5% glutaraldehyde in distilled water. Excess fixative was removed with three changes of distilled water over 0.5 h. Both endosperm and embryo samples were dehydrated in an ethanol series (10%, 20%, 30%, 50%, 70%, 80%, 90%) at 4°C for a 0.5 h period for each concentration. This dehydration procedure was completed by two changes of 100% ethanol for 1 h and 3 h at 24 °C, and 100% propylene oxide for 12 h at 24°C. Embryo samples were infiltrated with Spurr's resin:propylene oxide (1:3, 1:2, 1:1, 2:1, 3:1, 1:0, 1:0, 1:0, 1:0) each for 12 h periods at 24°C. Endosperm samples were infiltrated in the same way as embryo samples except that two additional 12 h periods of pure resin were used. Infiltrated samples were embedded in Spurr's resin in flat rubber embedding moulds and polymerized at 60°C

for 48 h. Ultrathin sections, 70-90 nm thick, were cut with either glass or diamond knives using a Reichert Om U2 ultramicrotome and placed onto Formvar and carbon coated copper grids.

Staining and Microscopy: Sections were stained with uranyl acetate saturated in 50% ethanol for 20 min, followed by Reynold's lead citrate for 60 s (Lewis and Knight 1977). Post-stained sections were viewed on a JEOL 1200 EX-II scanning transmission electron microscope (JEOL Ltd, Tokyo, Japan).

Some Measurements by Integrated Microanalysis for Imaging and X-ray (IMIX):

a) Size of globoid crystals: Sections used for image analysis of globoid crystals were almost anhydrously prepared (see Chapter 3), since globoid crystals were retained the best by this preparation. Grey tone digital images were collected from a STEM detector by the image collection program. The paint program was used to create a binary colour image by means of the grey level. Globoid crystals were painted red (or any other colour), and other cell constituents were kept black.

The areas and longest diameters of globoid crystals were obtained through the feature analysis program.

b) Seed size: One hundred *C. annuum* seeds were placed flat on a piece of black paper. Grey tone digital images of

the seeds were collected through a video camera to a television screen. After the brightness and the contrast were adjusted, the images were transferred to the IMIX system by the image collection program and analyzed as given above.

RESULTS

Light microscopy, SEM and TEM together provided complementary methods for studying the structure and composition of sweet pepper seeds. Room temperature preparation of seeds for SEM was useful for studies of the morphology of the seed coat surface but was not useful for studies of sectioned seeds since the cut surface was smeared with storage lipid. Thus cryogenic preparation for SEM was used for studying aspects of the internal structure of sweet pepper seeds. Light microscopy combined with histochemical staining was useful for determining the composition of storage reserves in the embryo and endosperm. For both light microscopy and TEM, it was found that the thick cell walls of the endosperm and the dense cytoplasm of both the embryo and the endosperm made it difficult to obtain adequate infiltration of resins, even when the infiltration was done extremely slowly.

Sweet pepper seeds were small, flattened with the thickness about 1 mm. The average longest-diameter of pepper seeds was 5.28 mm, the breadth was 4.34 mm and average area of

about half of a seed surface was 16.31 mm^2 (Table 3). The seed was kidney-shaped, and light yellow in colour. Inside the seed coat, the endosperm tissue surrounded the curved embryo, which consisted of two cotyledons and a hypocotyl-radicle axis (Fig. 1). The cotyledons were similar in length to the hypocotyl-radicle axis.

<u>Testa:</u>

Cell walls of seed coat stained with PAS reagent (Table 4). As seen in transverse section, the epidermal cells of the seed coat were higher on the edge region than on the flat side of the seed (Fig. 2). In surface view, the radial cell walls of epidermal cells of the seed coat were wavy (Fig. 3).

<u>Embryo and Endosperm:</u>

Embryo: The protoderm of the embryo was covered by a layer of cuticle which was stained by Sudan black B. The positions and cytological characteristics of dormant embryo tissues indicated their relationships to the mature tissues in the seedlings that would develop from the seed. These embryo tissues included protoderm, palisade ground meristem, spongy ground meristem and provascular tissues in the cotyledon (Fig. 4), and protoderm, ground meristem, and provascular tissues in the hypocotyl-radicle axis (Fig. 5). The terms palisade ground meristem and spongy ground meristem were used in the cotyledons because the cells had differentiated sufficiently to distinguish the two regions. There were distinctive

intercellular spaces in the ground meristem throughout the whole embryo (Fig. 6).

Cells in all embryo tissues had thin primary cell walls which stained with the PAS test for carbohydrate and also with Congo Red which stains for β-glucan residues (Figs. 7, 8 and Table 4). Carbohydrates which stained with the PAS reagent existed only in the cell walls. The negative results of the Lugol's iodine stain confirmed that starch was absent in cells of all embryo tissues of *C. annuum* (Table 4). The main storage reserves in cells of embryo tissues were lipid and protein. Positive staining for lipid was obtained with Nile blue sulphate, Nile blue A and Sudan black B (Figs. 9, 10 and Table 4). Positive staining for protein was obtained with Coomassie brilliant blue and Mercury-Bromophenol blue (Table 4 and Fig. 11). Storage lipids were stored in lipid vesicles, while protein was located in protein bodies. One of the protein body inclusions, believed to be globoid crystals, stained metachromatically with ATBO (Fig. 12).

Protein bodies were found in all tissues of the embryo. They were oval to round in shape in sections, generally contained one or more globoid crystals and contained one to three protein crystalloids embedded in the proteinaceous matrix (Fig. 13). Protein bodies varied in size both inter- and intra-cellularly (Fig. 14). Protein bodies in the protoderm and provascular cells of both the hypocotyl-radicle axis and the cotyledon were smaller than those in the ground

meristem (Fig. 14). In a given cell of any of these tissues the size of protein bodies still varied (Fig. 15)

Globoid crystals were naturally electron dense, but they were often fragmented or lost during sectioning creating electron transparent areas (Figs. 13 and 15). Globoid crystals were round or almost round in profile in sections and as revealed by SEM studies (Figs. 13 and 16). At the light microscopic level, globoid crystals stained metachromatically by ATBO (Fig. 12 and Table 4). The metachromasy of ATBO (at pH 1.0) indicates that either sulphate esters or highly phosphorylated compounds are present (Ashford *et al.* 1975). Acriflavine HCl, which was claimed by Fulcher (1982) to be a specific fluorescent stain for phytin in wheat, did not give a positive stain for globoid crystals in the embryos of the sweet pepper seeds. The size of globoid crystals in sections varied from tissue to tissue as well as in a given protein body (Fig. 17). Average longest diameter of globoid crystals in thin sections were highest in the ground meristem, and were lowest in provascular tissues (Table 5). The number of globoid crystals in a protein body varied from protein body to protein body. Commonly one to three globoid crystals were found in a protein body in sections. Infrequently a large number of various sized globoid crystals were present in a protein body in sections (Fig. 18).

Protein crystalloids sometimes had irregular shapes in section (Fig. 13). Usually one protein crystalloid was found

in a protein body in sections (Fig. 17). A few protein bodies were observed to contain two or three protein crystalloids (Fig. 13). This may be a sectioning effect with the two or three regions actually being part of a single irregularly shaped protein crystalloid. Protein bodies without protein crystalloids were also found (Figs. 15 and 18). This could be a sectioning effect, since thin sections were cut from a relatively big object. Quite a few numbers of protein bodies displayed this structural characteristic (Fig. 15) making a sectioning effect less likely.

Endosperm: Endosperm cells had thick cell walls which stained with PAS (Table 4 and Fig. 19). The cell walls of the endosperm were not stained by Congo red which stains for β-glucan residues (Fulcher 1982). The cells of the outermost layer of the endosperm next to the seed coat, had a layer of cuticle which was stained with Sudan black B. Endosperm cells next to the embryo were often empty and partially collapsed. The main storage reserves in the endosperm were protein and lipid (Figs. 20, 21 and 22), which were similar to those of embryo tissues (Table 4). One of the protein body inclusions, thought to be globoid crystals, stained metachromatically with ATBO (Fig. 23). Starch was not detected in endosperm cells (Table 4).

Protein bodies were more uniform in size in the endosperm than they were in embryo tissues (Fig. 24). Protein bodies contained one or more globoid crystals and usually a protein

crystalloid in the proteinaceous matrix (Figs. 25 and 26).

Globoid crystals in the endosperm were round or almost round in shape in sections and as revealed by SEM studies (Figs. 26 and 27). Globoid crystals were naturally electron dense, but they were often lost during sectioning leaving electron transparent areas (Fig. 25). Globoid crystals were less variable in size in the endosperm than in embryo tissues (Table 5). The average longest diameter of globoid crystals in the endosperm protein bodies was 0.54 μm (Table 5). Globoid crystals were never found in the protein crystalloid areas. Globoid crystals stained metachromatically by ATBO, but were not stained by acriflavine HCl (Table 4).

Protein crystalloids sometimes had irregular shapes in sections (Fig. 28). A few protein bodies were found which apparently contained more than one protein crystalloid (Fig. 29). This may be a sectioning effect with several regions actually being part of a single irregular protein crystalloid. Protein bodies without protein crystalloids were very rarely found, unlike the situation found in embryo tissues. In sections, the protein crystalloid in an endosperm protein body occupied a greater fraction of the protein body area in the endosperm compared to that which occurred in embryo tissues. (Figs. 20 and 29).

DISCUSSION

Structural studies of some Solanaceae seeds have been published. Most were done before the 1950's and the results were often presented as hand drawings or in written descriptions only. Very little cell ultrastructural information has been published about solanaceous seeds. Only the seeds of *Lycopersicon esculentum* and *Atropa belladonna* have been studied at the electron microscopic level. This thesis presents the most detailed study of the composition of storage reserves and cell ultrastructure of *Capsicum annuum* seeds to date and it is also the most detailed study to date dealing with seeds of any solanaceous species.

Difficulties Encountered in the Research:

In general, seed tissues are difficult to prepare for microscopic studies due to the dense cytoplasm filled with storage materials and the presence of hard globoid crystals (Lott 1980). *C. annuum* seeds were also difficult to prepare. Due to the marginal infiltration of epoxy resin, tears and chatter often occurred when seed tissues were thin-sectioned. The hard globoid crystals infiltrated least of all and thus were often pulled out during thin-sectioning leaving holes in the sections. Further complications of working with *C. annuum* seeds were found. The endosperm had very thick cell walls which impeded infiltration. The cells of both the outermost layer of the endosperm next to the seed coat and the protoderm cells of the embryo were covered by a layer of cuticle. The

cuticle is composed of the fatty material cutin and is impervious to water. The hydrophobic cuticle likely hindered access of the aqueous fixatives. As discussed by O'Brien and McCully (1981), it is difficult to obtain satisfactory polymerization of epoxy resins inside cuticle covered tissues. Epoxy resin polymerization is very sensitive to the balance between components and satisfactory polymerization will not occur if the components are not in the proper ratios (O'Brien and McCully 1981). One possibility was that cutin may act as an ultrafilter, altering the balance between the resin components during infiltration.

Storage Reserves:

The storage reserves in embryo tissues and the endosperm of *C. annuum* were very similar, and were mainly protein and lipid. Starch was not detected in either the embryo tissues or the endosperm. The thickened cell walls of the endosperm may act as a store of mannan-containing polysaccharides.

Pepper seeds contained similar storage reserves to other solanaceous species that have been reported. In tomato seeds, the major subcellular structures containing storage reserves are lipid vesicles and protein bodies as identified by TEM (Spitzer and Lott 1980). The embryo and endosperm cells of *A. belladonna* seeds are filled with protein bodies and small lipid vesicles as well (Simola 1987). *D. stramonium* seeds also have protein and lipid as the main storage reserves (Maldonado

and Lott, unpublished data).

The application of biochemical techniques to the study of plant cells has revealed considerable information concerning the composition of the storage reserves in seeds (Friedman and Levin 1989; Gifford 1987; Reddy and Sarojini 1987). However, biochemical techniques usually require the destruction of plant tissues. Thus, difficulties are encountered in attempting to relate data from a bulk sample to the situation in individual cells or tissues. Histochemistry thus serves as an excellent means to localize constituents within a particular tissue or cell.

With histochemical stains, proteins were identified in protein bodies of *C. annuum* seeds. Inside protein bodies, globoid crystals were found to contain highly phosphorylated compounds. However, there seems to be no specific histochemical stain for phytin. Fulcher (1982) stated that Acriflavine HCl provides improved specificity of detection of phytin. However, he has also stated that the chemical basis of the phytin/Acriflavine HCl interaction has not yet been determined. In this research, globoid crystals of *C. annuum* were not stained by Acriflavine HCl. Acriflavine HCl did not stain the commercial magnesium, potassium salt of phytic acid (Sigma, St. Louis, MO) in my experiment. Other scientists have also found that Alcriflavine HCl does not work (Moss; Maldonado and Lott, personal communication). Globoid crystals of *D. stramonium* seeds were likely phytin rich as studied with

EDX analysis, but were not stained by Acriflavine HCl
(Maldonado and Lott, unpublished data). From the evidence
stated above, Acriflavine seems not to be a reliable method
for detection of phytin. Globoid crystals of *C. annuum* were
stained metachromatically with ATBO (at pH 1.0). Toluidine
blue O at pH 1.0 stains metachromatically either sulphate
esters or highly phosphorylated compounds (Ashford *et al.*
1975). From the results of the EDX analysis of globoid
crystals (see Chapter 3), it was unlikely that sulphate esters
were present in the globoid crystals of *C. annuum*. It was
evident from the results of EDX analysis and the ATBO stain
that globoid crystals in *C. annuum* seeds likely were phytin-
rich particles. The specificity of Toluidine blue O depends on
its pH. At pH 4.4 the metachromatic reaction is not specific
for highly phosphorylated compounds; several other polyanions
such as those containing carboxyl or sulphate groups can also
give metachromatic reaction with Toluidine blue O (Ashford *et
al.* 1975). However, as the pH is lowered to 1.0, the
ionization of carboxyl groups is suppressed (Pearse 1980a) and
basic dyes will no longer bind to them. Thus the metachromasy
of Toluidine blue O at pH 1.0 is more specific, indicating
that either sulphate esters or highly phosphorylated compounds
are present.

With histochemical stains, lipids were located in the
subcellular structures called lipid vesicles. Lipid staining
was confirmed with fresh seed tissues. Lipid stains such as

Nile blue sulphate and Sudan black B would only stain the lipids of *C. annuum* seeds in fresh tissues or tissues with osmium tetroxide as a post-fixative. Glutaraldehyde is considered incapable of rendering most lipids insoluble in organic solvents (Hayat 1981). Fixation with OsO_4 results in at least partial retention of lipids (Hayat 1981). It has been demonstrated in zoological material that fixation with OsO_4 is indispensable for subsequent demonstration of lipid droplets with Sudan dyes (Coimbra and Lopes-Vaz 1971).

Sweet pepper seeds contained about 30% lipids as determined by extraction with hexane and a mixture of chloroform and methanol (see Chapter 3). However, the composition of the lipids of sweet pepper seeds was not studied in this research. Past studies of chili pepper (*C. annuum*) have shown that the seed oil is rich in unsaturated fatty acids with 73.2% linoleic acid, 7.9% oleic acid, 0.2% myristic acid, 15.9% palmitic acid and 2.9% stearic acid (Reddy and Sarojini 1987). The oil of chili pepper resembles most edible oils in its chemical and physical constants (Eckey 1954). Seeds of some other species of the Solanaceae also contain lipids similar to most edible oils. Fatty acids of tomato seed oil include 58% linoleic acid, 19.9% oleic acid, but 15.6% palmitic acid and 4.5% stearic acid (Rymal *et al.* 1974). The composition of fatty acids in tobacco seed oil have been found to be 71.2% linoleic acid, 15.7% oleic acid, 8.4% palmitic acid and 3.8% stearic acid (Sengupta and

Mazumder 1976).

Structural Aspects:

a) Testa:

In the family Solanaceae the most widely studied seed structural feature has been the testa. Testa patterns have been used by several authors to differentiate various *Solanum* species (Souèges 1907; Edmonds 1983; Whalen 1989). The seed coat patterns revealed by SEM studies were similar to those of the section *Solanum* as studied by Edmonds (1983). The difference was that the hair-like structures covering the surface of the seeds of section *Solanum* did not appear on the seed surface of *C. annuum*. The outer cell walls of testa epidermal cells of section *Solanum* were easily damaged, leaving the strands of thickening in the lateral walls protruding from the seed coat and appearing as hair-like structures (Edmonds 1983). The lateral cell walls of the epidermal cells of *C. annuum* testa were thickened as well, but they only protruded slightly from the seed coat and thus did not appear as hair-like structures.

b) Embryo and Endosperm:

Cells of endosperm and different embryo tissues of *C. annuum* contained a large number of lipid vesicles, many protein bodies and a nucleus. Some aspects of subcellular structures are discussed in the following sections:

Cell Walls: Embryo tissues of *C. annuum* have thin cell

walls in comparison to the thick cell walls of the endosperm. Thickened cell walls in seed tissues are often considered to be the storage sites of non-starch polysaccharides. *Lupinus* cotyledon parenchyma cells have thickened cell walls containing galactans (Meier and Reid 1982). *Trigonella* endosperm has extraordinarily thickened cell walls composed of galactomannans (Meier and Reid 1982). Endosperm cell walls of date (*Phoenix dactylifera*) are extremely thick, and they are rich in mannans (DeMason *et al.* 1989). The composition of endosperm cell walls of *C. annuum* has never been determined chemically. However, the sensitivity of the endosperm cell walls to endomannanase has shown that mannan-containing polysaccharides might be important structural components of the endosperm cell walls of *C. annuum* (Watkins *et al.* 1985).

Protein Bodies: Apparently two types of protein bodies were present in sections of the embryo tissues of *C. annuum*. One type of protein body had one or more globoid crystals and usually one protein crystalloid in the proteinaceous matrix. The other type of protein body contained numerous globoid crystals but no protein crystalloid in the proteinaceous matrix.

Protein bodies in the endosperm often contained a protein crystalloid and one or more globoid crystals in the proteinaceous matrix. Protein bodies in the endosperm were more homogeneous in size than in the embryo. Globoid crystals in protein bodies in the endosperm were more homogeneous in

size than in the embryo. In sections, the protein crystalloid in a protein body occupied a greater fraction of the protein body area in the endosperm than in the embryo. Reasons for the structural difference between protein bodies of the embryo and the endosperm are unknown.

It has been suggested that seed protein bodies may be a useful character for studies of taxonomic relationships between plant groups (Lott 1981). Similar tissues taken from equivalent organ regions should be compared from species to species for studies of taxonomic affiliation (Lott 1981). Often published information about protein bodies is incomplete and thus it is difficult to compare protein body characteristics from species to species. Seed protein bodies of only four species in the family Solanaceae have been studied structurally at the EM level, namely *L. esculentum*, *A. belladonna*, *D. stramonium* and *C. annuum*. In the studies of *A. belladonna* seeds, terms such as cotyledon cells or radicle cells were used (Simola 1987), and thus it was assumed that cells from different tissues were the same. Apparently the size of protein bodies varied inter- and intra-cellularly in *A. belladonna* (Simola 1987). It has been noticed in tomato embryos that protein bodies are larger in ground meristem cells than those in provascular cells (Spitzer and Lott 1980). In *C. annuum* seeds, the size of protein bodies in sections was smallest in provascular tissues and largest in the ground meristem. Protein bodies in different tissues of *D. stramonium*

seeds have been studied (Maldonado and Lott, unpublished data). Apparently, size and inclusions of protein bodies are very similar in a given embryo tissue. However, protein body size as well as globoid crystal size varied from tissue-to-tissue in the embryo of *D. stramonium*.

Table 3
Size of *Capsicum annuum* seeds
(n=100)

Measurement[a]	Longest Diameter (mm)	Breadth (mm)	Area (mm^2)[b]
Minimum	4.49	3.56	11.76
Maximum	6.21	4.98	20.86
Mean±SE	5.28±0.03	4.34±0.03	16.31±0.16

Note: a. Images of *C. annuum* seeds were collected through
a video camera. The measurement was carried
out with an Integrated Microanalyzer for Imaging
and X-ray (IMIX).
b. Area represents about half of the seed surface,
because seed images, which were collected by
projecting from above, represent only the top
surface of the seed.

Table 4
Reaction of *Capsicum annuum* seed tissues
to some histochemical stains

Stain/reagent	Colour	Items to be demonstrated	Structure stained
Bright field stains:			
PAS reaction	magenta	polysaccharides	cell walls including seed coat
Lugol's iodine	brown	starch	no stain
Feulgen	red	DNA	nuclei
Nile blue sulphate	blue	lipids	lipid vesicles
Sudan black B	black	lipids	lipid vesicles
Coomassie brilliant blue	blue	proteins	protein bodies
Mercury-Bromophenol blue	blue	proteins	protein bodies
ATBO	pink	highly-phosphorylated compounds or sulphate esters	globoid crystals
Fluorescent stains:			
Autofluorescence:			
-	blue	phenolic compounds	seed coats
-	green	proteins	protein bodies
Induced fluorescence:			
Nile blue A	yellow	lipids	lipid vesicles
Congo red	red	β-glucan residues	cell walls in all embryo tissues

Note: Unless otherwise stated, structures stained the same in all embryo tissues and endosperm.

Table 5
Longest diameter (μm) of globoid crystals[a] from different tissues of *Capsicum annuum* seeds[b]
(n=200)

	EMBRYO			ENDOSPERM
	Protoderm	Provascular tissue	Ground meristem	
Meane±SE	0.78±0.02	0.33±0.01	1.77±0.07	0.54±0.01
Range	0.37-1.21	0.12-0.61	0.46-3.39	0.33-0.71

Note: a. Globoid crystals measured were randomly selected.
b. The measurement was carried out on semi-thin sections (150 nm).

Figure 1.   <u>Scanning Electron Micrograph of</u> *C. annuum*
              <u>Embryo and Endosperm</u>

              Longitudinal section showing the arrangement of
              the embryo and the endosperm in the seed,
              hypocotyl-radicle axis (A), cotyledons (C) and
              endosperm (E). Scale represents 300 μm.

Figure 2.   <u>Scanning Electron Micrograph of</u> *C. annuum*
              <u>Seed Coat</u>

              Cryogenic prepared transverse section showing
              bigger epidermal cells of the seed coat on the
              edge region than on the flat side of the seed;
              endosperm (E), hypocotyl-radicle axis (A),
              epidermal cells of the seed coat (EC). Scale
              represents 50 μm.
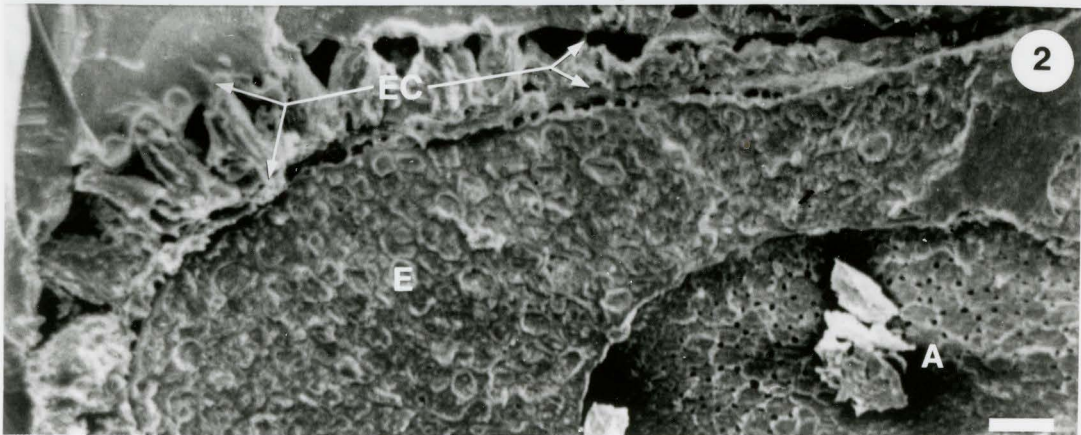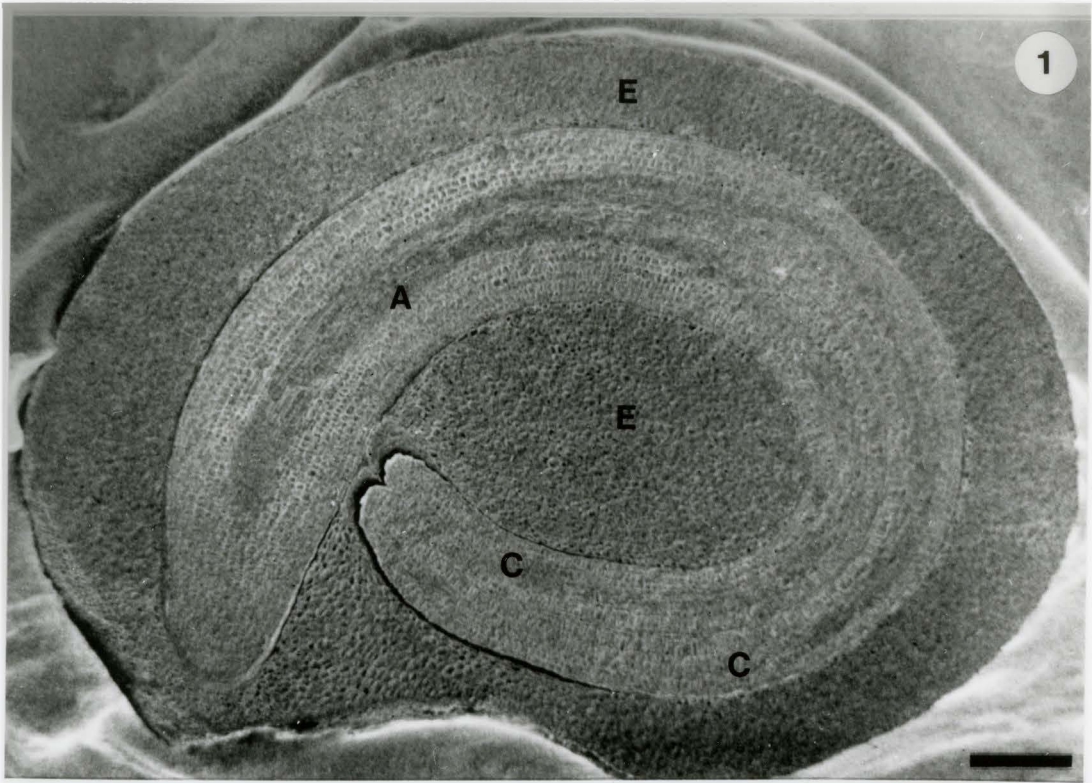
Figure 3.   **Scanning Electron Micrograph of the Seed Coat**
                  Surface

                  Room temperature prepared sample showing
                  the wavy radial cell walls of testa epidermal
                  cells. Arrows point to the radial cell wall of an
                  epidermal cell. Scale represents 50 μm.

Figure 4.   **Light Micrograph of *C. annuum* Cotyledons**

                  Longitudinal section showing different tissues of
                  the two cotyledons; protoderm (PT),
                  provascular tissue (PV), palisade ground meristem
                  (PGM), spongy ground meristem (SGM). Scale
                  represents 100 μm.

Figure 5.   **Light Micrograph of *C. annuum* Hypocotyl-**
                  radicle Axis

                  Longitudinal section showing different tissues of
                  the hypocotyl-radicle axis; protoderm (PT),
                  provascular tissue (PV), ground meristem (GM).
                  Scale represents 100 μm.

Figure 6   **Scanning Electron Micrograph of the Ground**
                  Meristem Tissue in a Hypocotyl-radicle Axis
                  Showing Intercellular Spaces

                  Cryogenic prepared transverse section showing
                  intercellular spaces (IS) and the wrinkled cell
                  walls in the ground meristem tissue of a
                  hypocotyl-radicle axis. Scale represents 5 μm.

Figure 7.   Histochemical Stain of Cotyledon Tissues: PAS

Light micrograph of cotyledon tissues of
C. annuum, stained with PAS. The cell walls
contained polysaccharides as indicated by the
magenta staining. Scale represents 30 μm.

Figure 8.   Histochemical Stain of Hypocotyl-radicle
Axis Tissues: Congo Red

Light micrograph of hypocotyl-radicle axis
tissues of *C. annuum*, stained with Congo red,
The cell walls contained β-glucan. Protein
crystalloids were autofluorescent. Scale
represents 15 μm.

Figure 9.   Histochemical Stain of Hypocotyl-radicle
Axis Tissues: Nile Blue A

Light micrograph of provascular tissue in a
hypocotyl-radicle axis of *C. annuum*. A hand cut
section of unfixed tissue was stained with Nile
blue A. The yellow stained regions indicated
that these cells contained lipid. Scale
represents 15 μm.
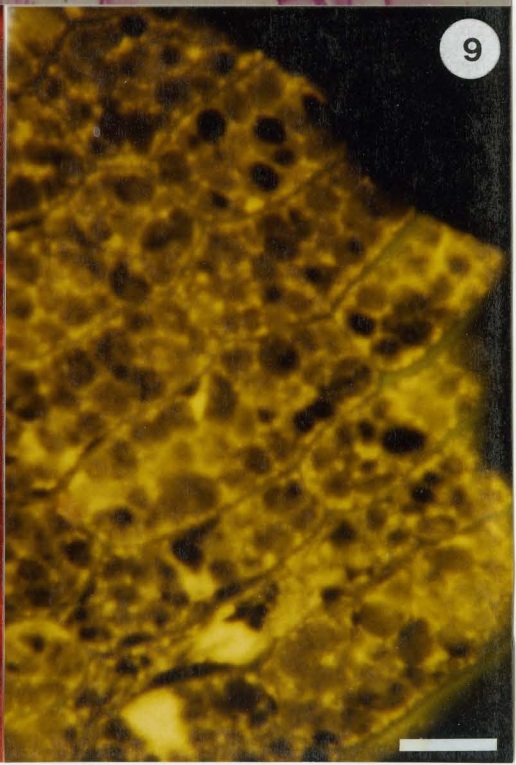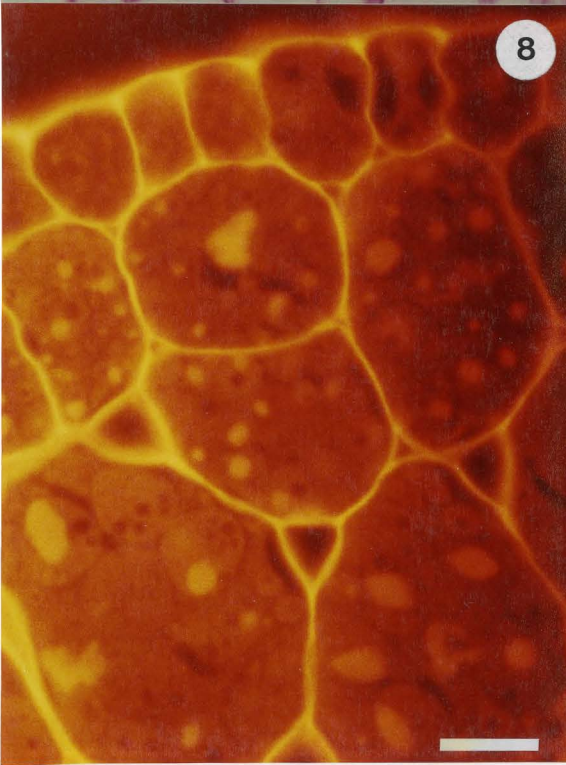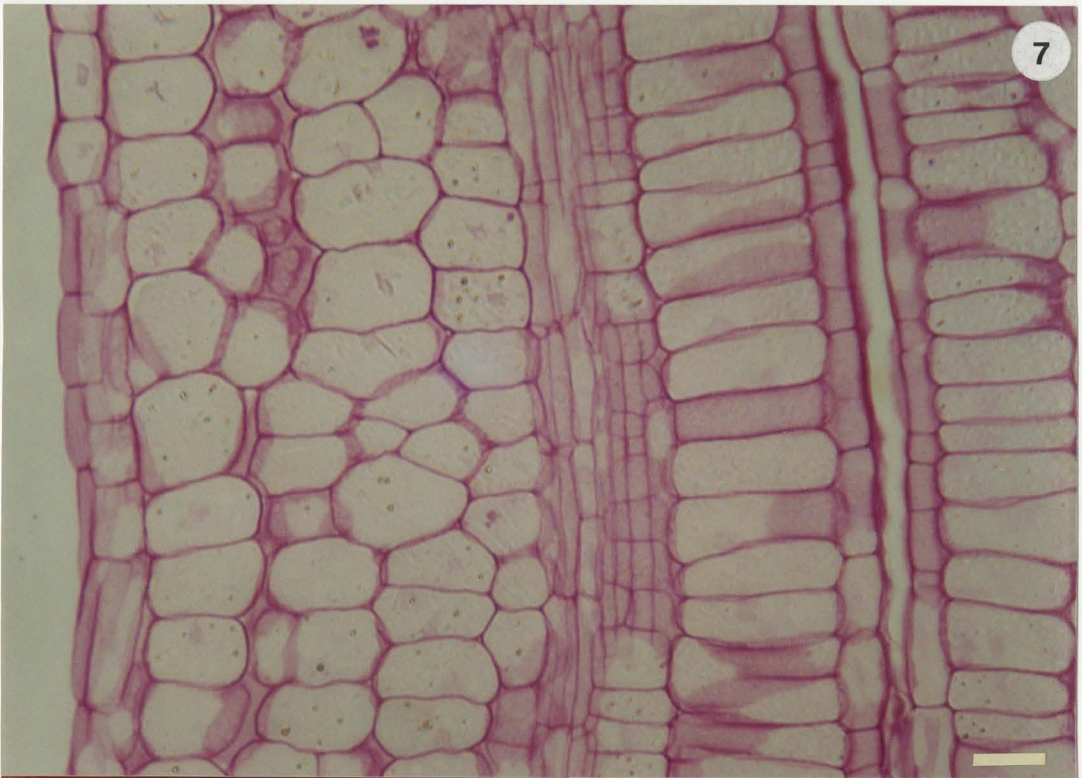
Figure 10.   Histochemical Stain of Hypocotyl-radicle
              Axis Tissues: Nile Blue Sulphate

              Light micrograph of hypocotyl-radicle axis
              tissues of *C. annuum*, stained with Nile
              blue sulphate. These cells contained lipid as
              indicated by blue staining. Scale represents
              15 μm.

Figure 11.   Histochemical Stain of Cotyledon Tissues:
              Mercury-Bromophenol Blue

              Light micrograph of cotyledon tissues of
              C. annuum, stained with mercury-
              bromophenol blue. The round blue-purple
              staining structures are protein bodies.
              Scale represents 30 μm.
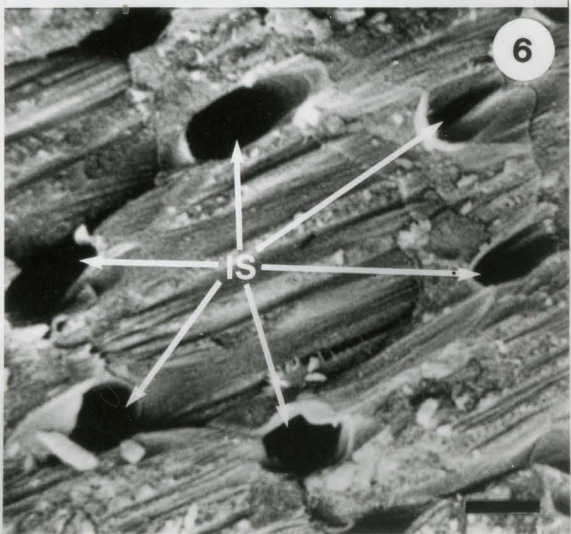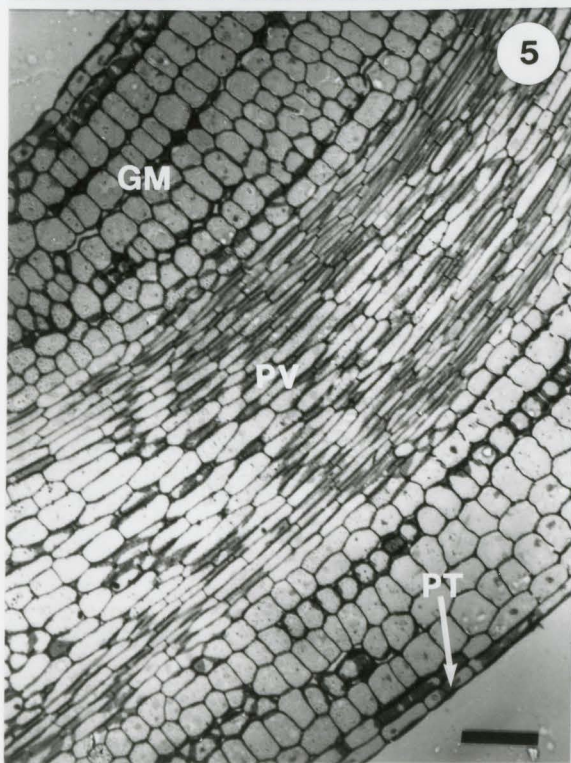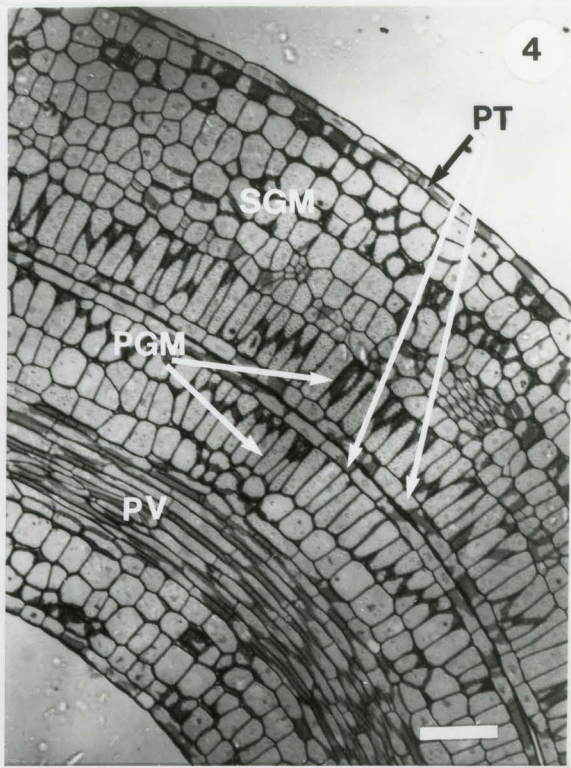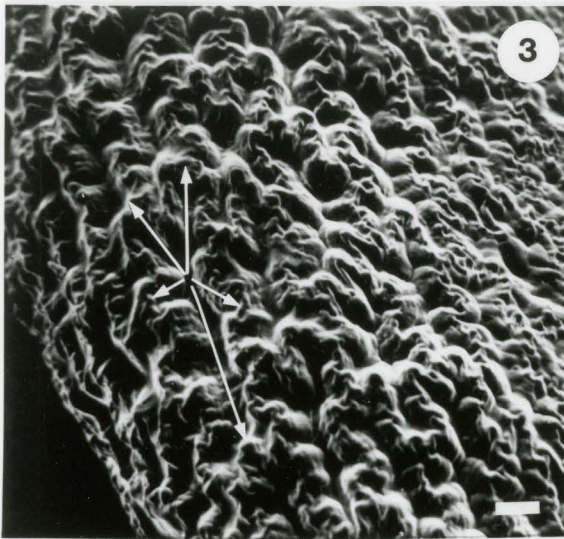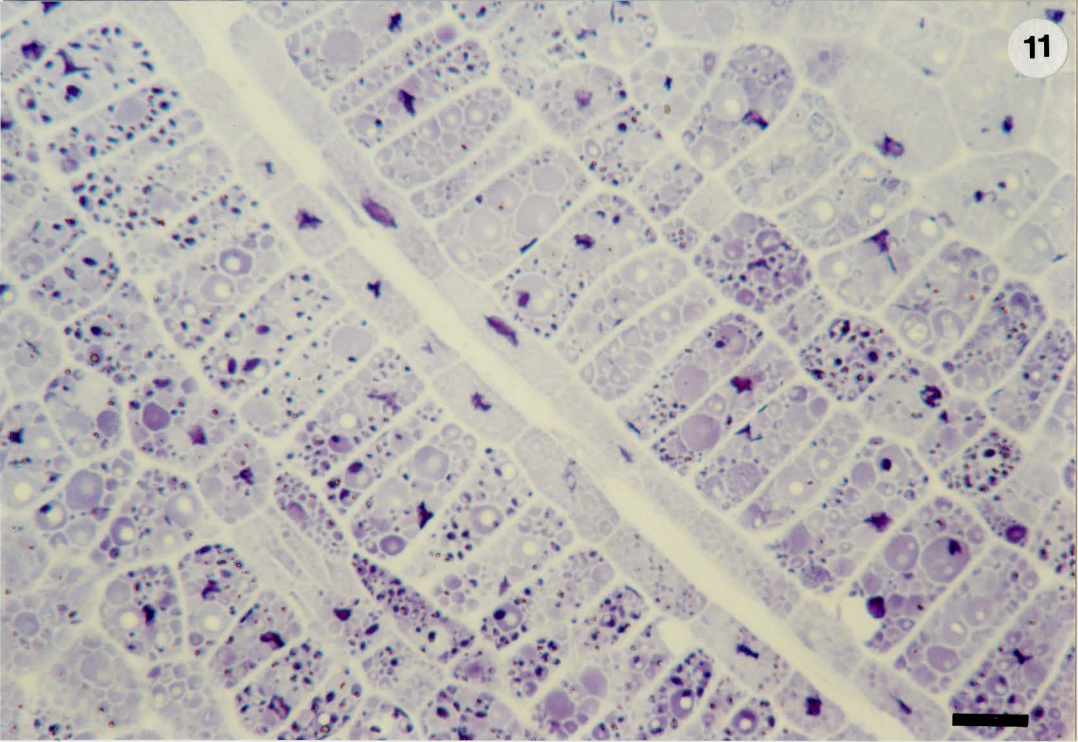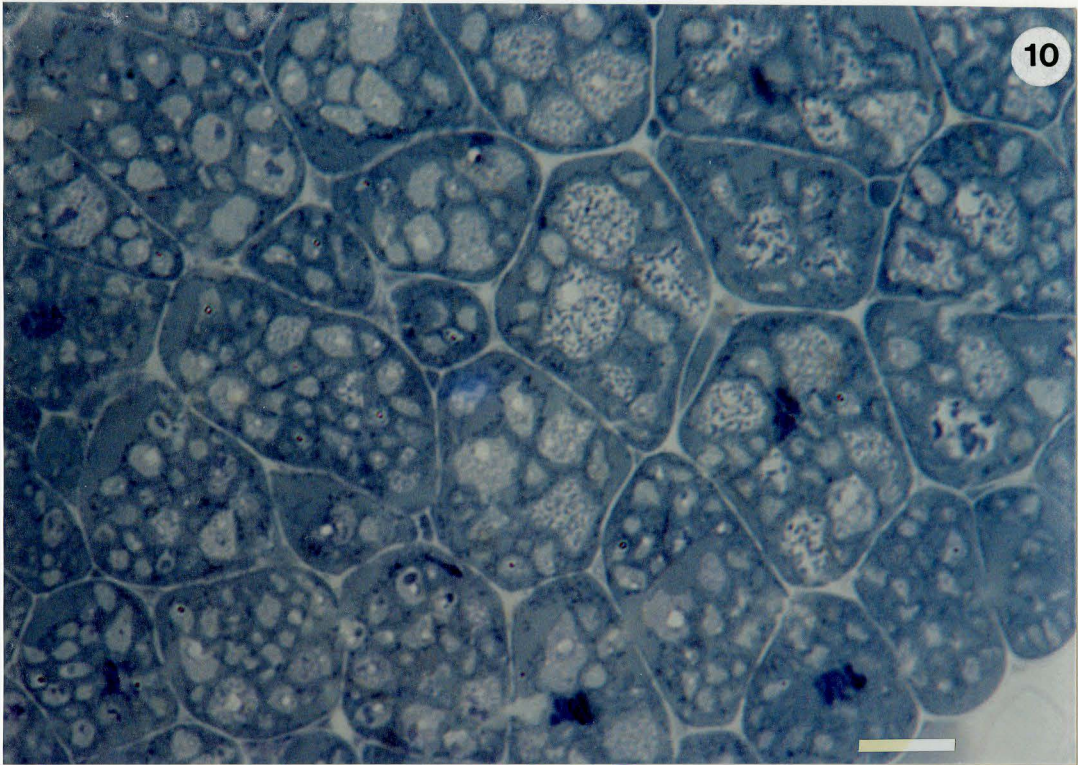

Figure 12.   Histochemical Stain of Cotyledon Tissues: ATBO

Light micrograph of cotyledon tissues of
C. annuum, stained with ATBO. These cells
contained highly phosphorylated compounds,
believed to be globoid crystals, as indicated
by arrows. Scale represents 30 μm.

Figure 19.   <u>Histochemical Stain of Endosperm Cells: PAS</u>

Light micrograph of endosperm cells of *C. annuum*, stained with PAS. The cell walls contained polysaccharides as indicated by the magenta staining. Scale represents 15 μm.

Figure 20.   <u>Histochemical Stain of Endosperm Cells: Mercury-Bromophenol Blue</u>

Light micrograph of endosperm cells of *C. annuum*, stained with mercury-bromophenol blue. The round blue-purple staining structures are protein bodies. Scale represents 15 μm.
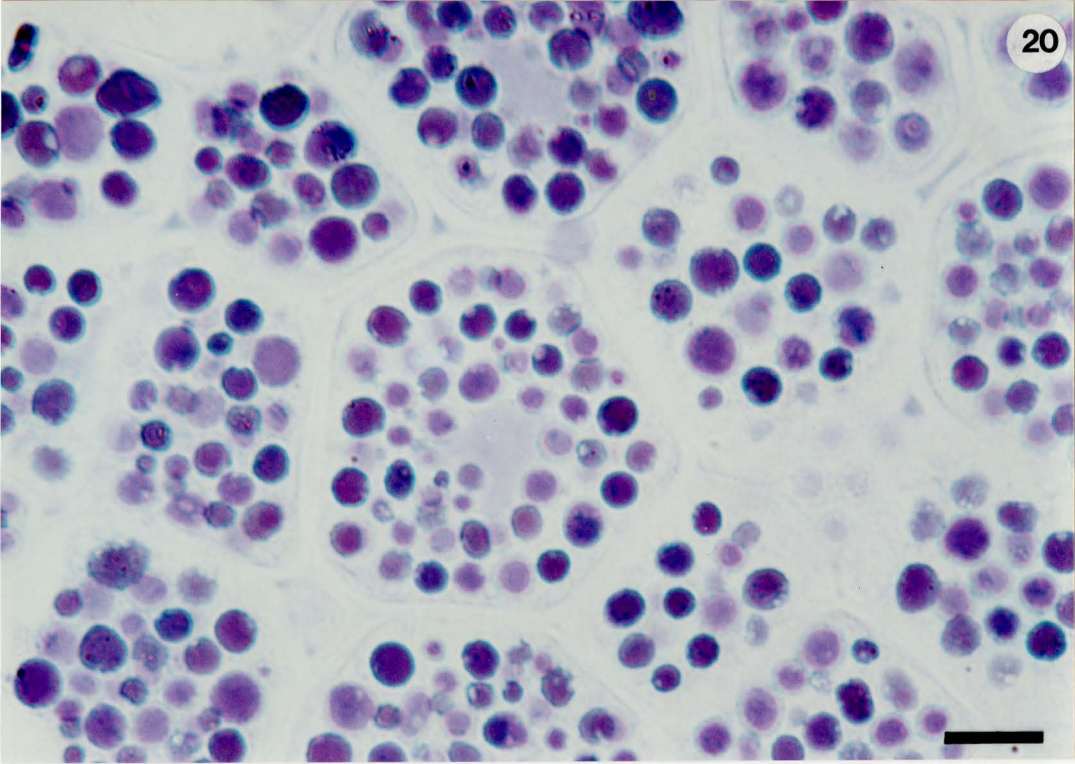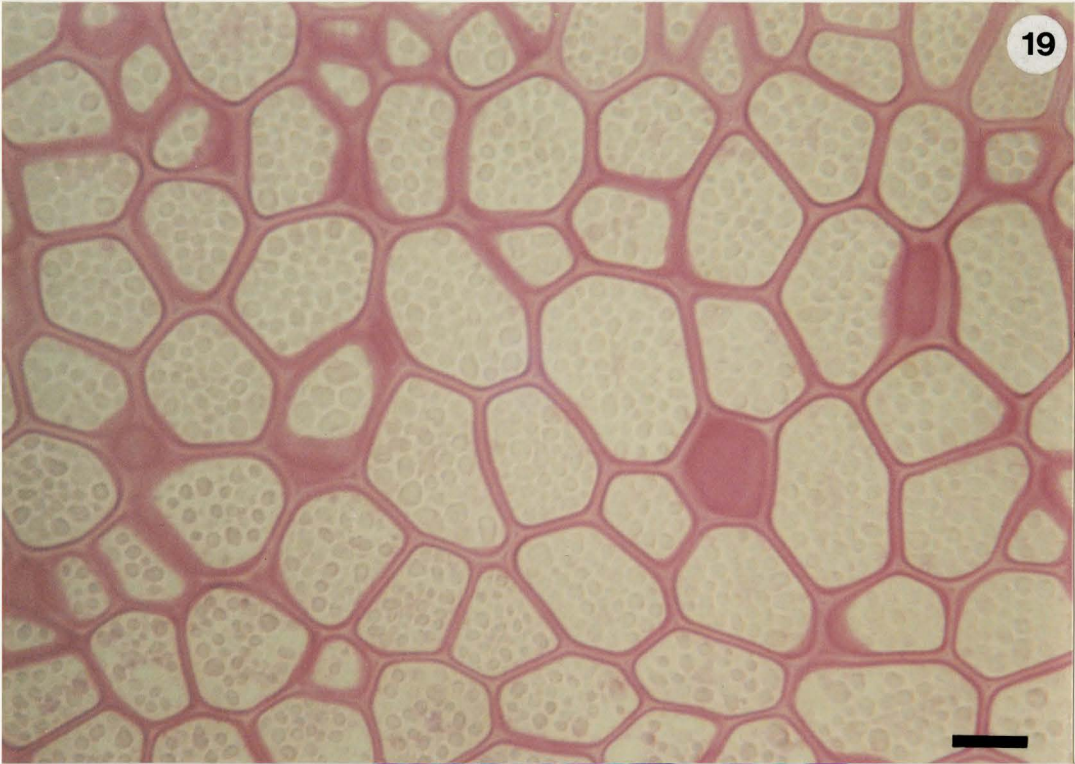
Figure 21.   Histochemical Stain of Endosperm Cells:
              Nile Blue Sulphate

              Light micrograph of endosperm cells of *C.
              annuum*, stained with Nile blue sulphate.
              Endosperm cells contained lipid as indicated by
              the blue staining. Scale represents 15 μm.

Figure 22.   Histochemical Stain of Endosperm Cells:
              Sudan Black B

              Light micrograph of endosperm cells of *C.
              annuum*, stained with Sudan black B. Endosperm
              cells contained lipid as indicated by the
              black staining. Scale represents 15 μm.
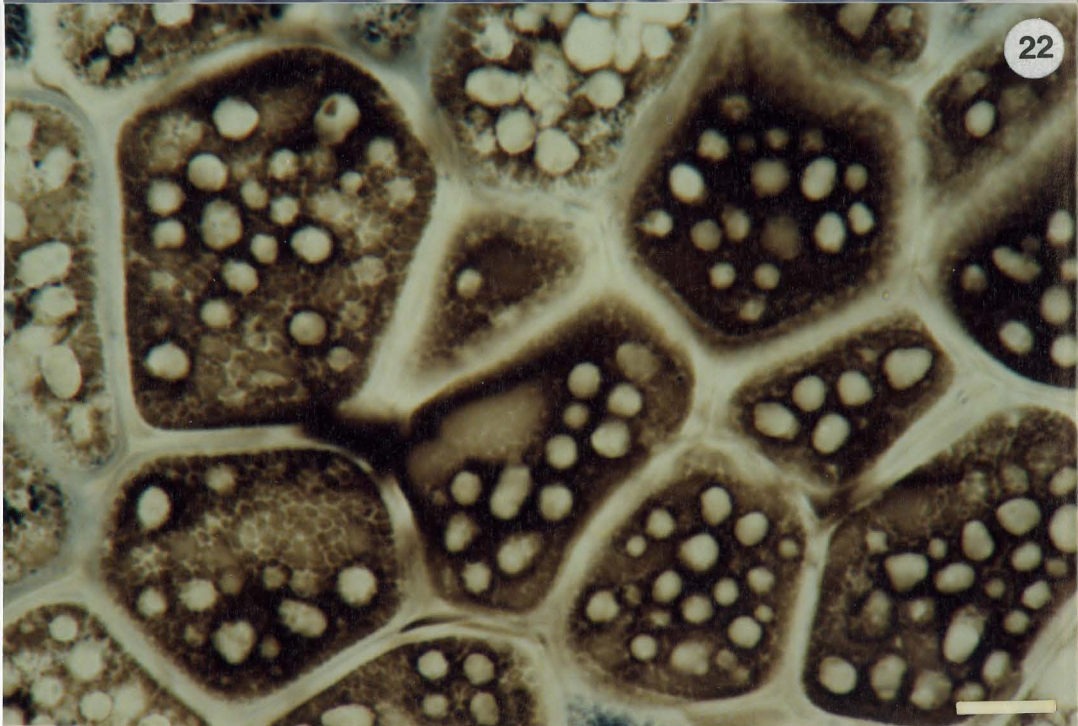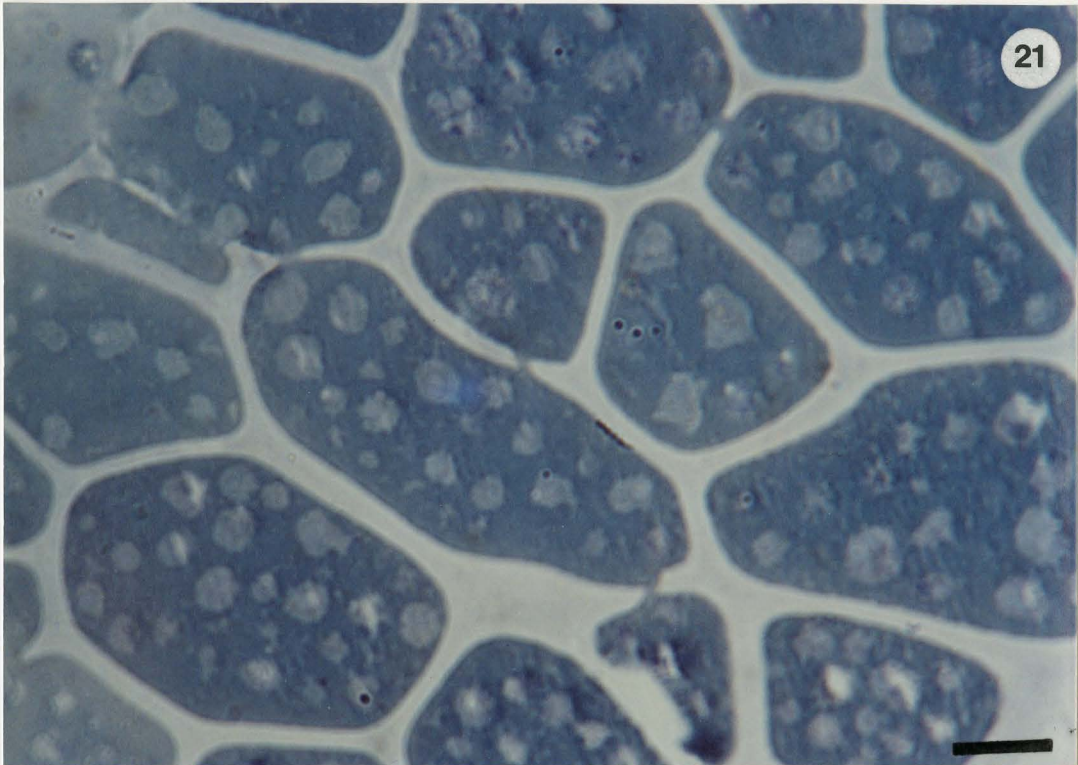
Figure 23.   <u>Histochemical Stain of Endosperm Cells: ATBO</u>

Light micrograph of endosperm cells of *C. annuum*, stained with ATBO. Endosperm cells contained highly phosphorylated compounds, believed to be globoid crystals, as indicated by arrows. Scale represents 15 μm.
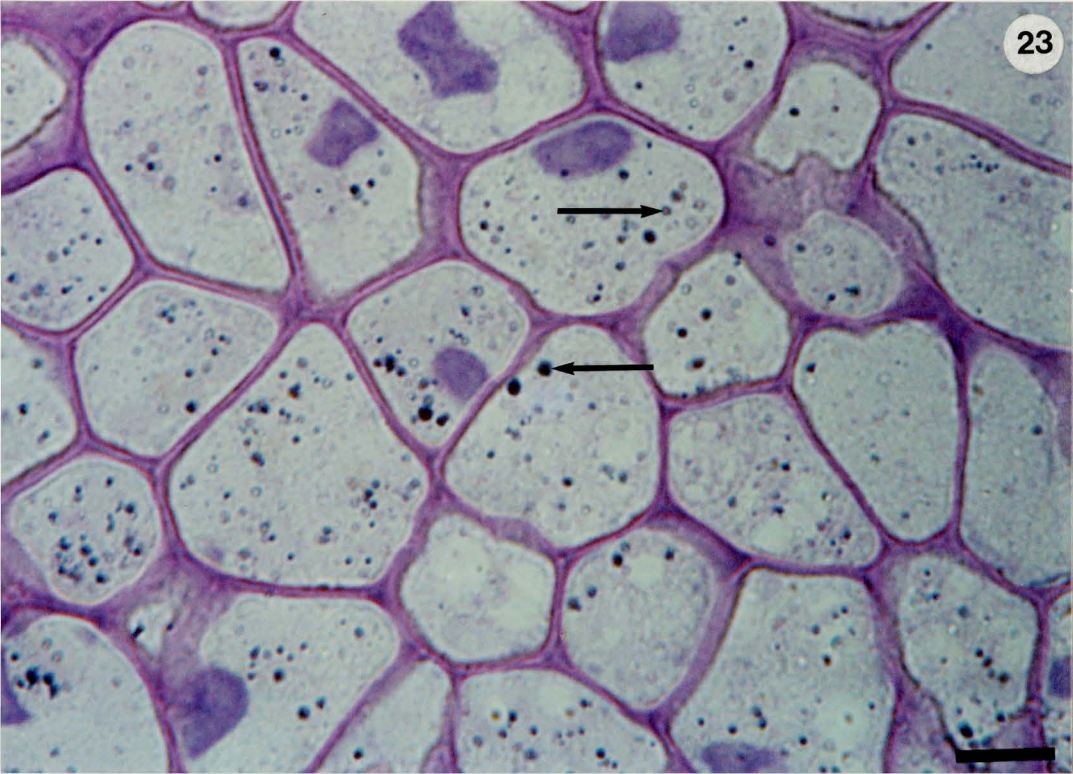
Figure 24.   <u>Light Micrograph of Endosperm Cells</u>

Protein bodies (PB) of similar size in endosperm
cells; cell wall (CW). Scale represents 10 μm.

Figure 25.   <u>Transmission Electron Micrograph of Endosperm</u>
<u>Protein Bodies</u>

Thin-section showing protein bodies (PB) in an
endosperm cell; globoid crystals (GC), globoid
crystal cavities (GCC), lipid vesicles (L), cell
wall (CW). Scale represents 1 μm.

Figure 26.   <u>Transmission Electron Micrograph of an</u>
<u>Endosperm Protein Body</u>

Thin section showing a protein body with globoid
crystals (GC), a protein crystalloid (PC) and
the proteinaceous matrix (PM). Scale represents
1 μm.

Figure 27.   <u>Scanning Electron Micrograph of Endosperm</u>
<u>Protein Bodies</u>

Cryogenically prepared sample showing the round
shaped globoid crystals (GC) and thick cell
wall (CW). Scale represents 5 μm.

Figure 28.   <u>Transmission Electron Micrograph of an</u>
<u>Endosperm Protein Body</u>

Thin-section showing a protein body with
an irregular shaped protein crystalloid (PC);
proteinaceous matrix (PM). Scale represents
1 μm.

Figure 29.   <u>Transmission Electron Micrograph of an</u>
<u>Endosperm Protein Body</u>

Thin-section showing a protein body with a
globoid crystal cavity (GCC), protein
crystalloids (PC) and the proteinaceous
matrix (PM). Scale represents 1 μm.
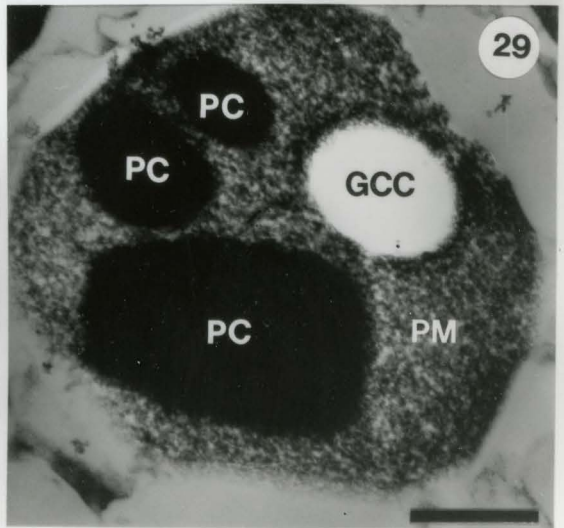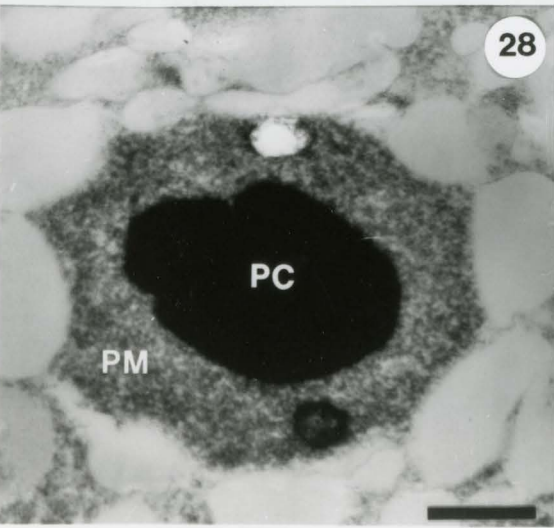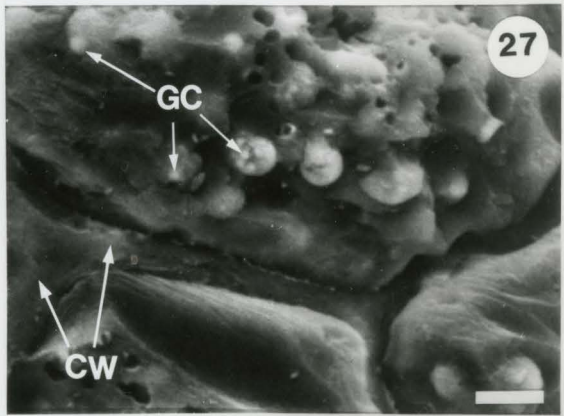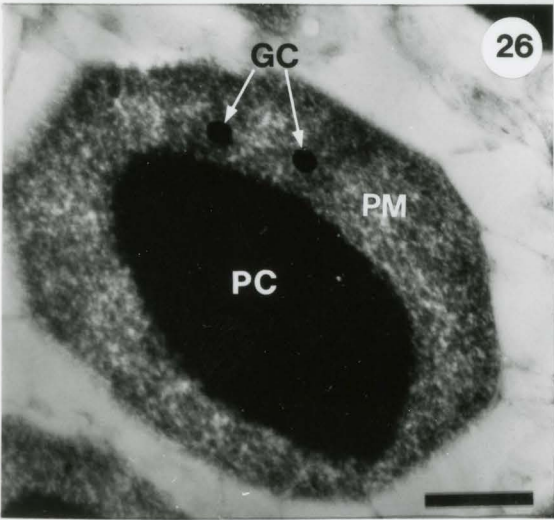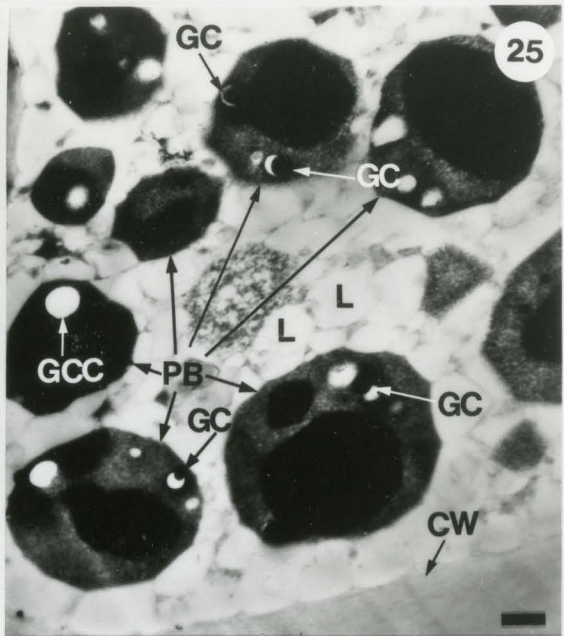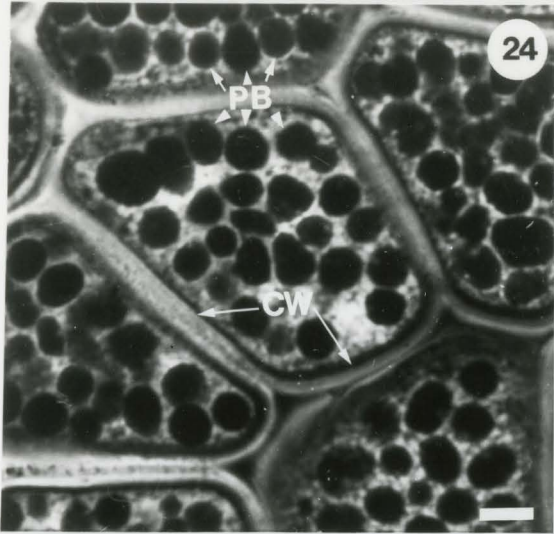
Chapter 3

ELEMENTAL ANALYSIS

INTRODUCTION

The present research used both energy dispersive X-ray (EDX) analysis and neutron activation analysis (NAA) to determine the composition of mineral nutrient reserves in sweet pepper seeds. EDX analysis of globoid crystals of various seeds has been carried out extensively by Lott, his co-workers and other scientists (Lott 1975, 1985; Lott *et al.* 1978, 1979, 1982, 1984; Lott and Buttrose 1978a, 1978b; Lott and Vollmer 1979a, b; Spitzer and Lott 1980, 1982b, 1982c; Spitzer *et al.* 1980, 1981; Stewart *et al.* 1988; Ockenden and Lott 1990; Buttrose 1978; Hofsten 1973; Sharma and Dieckert 1975). These studies have revealed that globoid crystals usually contain P, K and Mg. Occasionally other minor elements such as Ca, Fe, Zn, Mn, Ba, Na, Cl and S have also been detected.

The distribution of elements detected in globoid crystals has been found to vary from species-to-species, from organ-to-organ and from tissue-to-tissue. Environmental factors, such as soil conditions also influence the composition of

mineral reserves in globoid crystals. Many globoid crystals from Brazil nut embryos contained barium which has not been found in other seeds (Lott and Buttrose 1978b). In *Cucurbita maxima*, Ca was more frequently found in globoid crystals from the radicle and stem than in globoid crystals from the cotyledon (Lott *et al.* 1978, 1979). In *C. maxima* and *Cucurbita mixta* cotyledons, the globoid crystals containing some Ca were restricted to certain locations, such as upper and lower protoderm regions and most provascular cells (Lott *et al.* 1978, 1979). In *Lycopersicon esculentum* Mn and Fe were often found in globoid crystals from protoderm cells but rarely in other cell types (Spitzer and Lott 1980). Some variations in levels of Fe in *Lycopersicon* and *Capsella* seed globoid crystals, and Fe and Mn in wheat seed globoid crystals have been shown to be related to the availability of these elements in the soil (Spitzer *et al.* 1980, 1981; Lott 1985). In most species phytin is concentrated in the globoid crystals. Through the use of EDX analysis, mineral nutrients (in the form of phytin) have been detected in other subcellular sites other than globoid crystals as well. In soybean and pea cotyledons phytate is present in protein bodies, but frequently it does not form discrete globoid crystals (Lott and Buttrose 1978a; Lott *et al.* 1984). In some species, such as *Phaseolus*, most of the phytin is in a water soluble form (Lolas and Markakis 1975).

NAA studies of seed tissues also reveal interesting

findings. For example, in *Cucurbita* the Ca amount seems to be related to the seed size. Ca in the small sized *Cucurbita andreana* cotyledons is about three times higher than in *C. maxima*, which has much larger cotyledons (Ockenden and Lott 1988). However, NAA of *Cucurbita* pollen revealed that the concentration of Ca in pollen grains was not significantly different between *C. andreana* and *C. maxima* (Skilnyk 1990).

Energy Dispersive X-ray Analysis:

With energy dispersive X-ray (EDX) analysis equipment mounted on a STEM or a SEM, one can obtain elemental composition rapidly, efficiently and simultaneously from a small area of a biological sample.

An atom in a biological specimen has a nucleus of protons and neutrons surrounded by electrons in various energy states. These electrons occur in orbits or shells with well defined energies. Each element in the periodic table contains electrons in shells with particular discrete energy levels. When a high energy electron from the electron beam strikes the atom, an inner shell electron (of lower energy) may be ejected. In order to return the atom to energy stability, an outer shell electron (of greater energy) will fall to fill the vacancy in the inner energy shell. In doing so, an X-ray may be released, with an energy equal to the difference between the energy of the two electron orbitals. The X-ray energies are characteristic for each element. If a number of elements

in a biological sample are simultaneously irradiated by the electron beam, X-rays of a number of energies will be emitted. The energy dispersive X-ray analysis system is designed to measure the energies of X-rays entering the detector. The emission of X-rays from the specimen, which may be a bulk sample or a thin section, depends upon the energy of the electron probe. Electrons entering the specimen must have enough energy to induce ionization of the atom, if that element is to be detected. Thus the accelerating voltage is an important parameter since it directly affects X-ray yield. Spatial resolution of X-ray analysis in the sample is another important factor since it determines the precision with which the elements present can be localized in space. Thin specimens give better spatial resolution than bulk specimens. For a bulk specimen, one technique to improve spatial resolution is to use the accelerating voltage as low as possible to detect the element of interest. For a bulk specimen the two parameters, i.e. spatial resolution and the accelerating voltage should be considered very carefully to achieve the best conditions for X-ray analysis of particular elements. If an analysis is to be optimized for a single X-ray line, an accelerating voltage approximately three times the critical excitation voltage for that line is needed (Barbi 1979). For example, to measure Fe the $K_{\alpha\beta}$ line at 7.1 keV, one should use an accelerating voltage of 20 or 25 kV. When performing an analysis on a sample, it may be necessary to conduct the analysis at a least

two accelerating voltages to confirm the presence of light and heavy elements.

An EDX analysis detector has a lithium drifted silicon wafer (Marshall 1980). A thin beryllium window is normally placed in front of the detector crystal to maintain the positive pressure of gas inside the detector cylinder. The beryllium window also protects the crystal from contamination (Marshall 1980). The 7.5 μm thick beryllium window can effectively transmit X-rays down to Na (atomic number of 11). In order to detect elements of lower atomic number, windowless detectors or ultrathin windows like Formvar and cellulose nitrate on fine wire screens or unsupported stretched polypropylene films have been developed to reduce absorption of X-rays (Goldstein *et al.* 1981). But because of the internal absorption of the low energy X-rays in the specimen itself, light elements are difficult to detect in any event (Russ 1972a).

The EDX analysis system combined with a SEM or a STEM can be used to investigate elemental composition of seed tissues. With a STEM, one can precisely localize detectable elements in subcellular regions. Thus, elemental composition in globoid crystals can be compared from protein body to protein body within one cell, from cell-to-cell, from tissue-to-tissue and from organ-to-organ. However, with a STEM-mounted EDX analysis system one usually must use tissue that has been chemically fixed, dehydrated, embedded and sectioned. These types of

preparative procedures are well known to extract diffusible elements from biological tissue (Morgan 1980). One of the advantages with SEM for seed tissue is that it is possible to analyze the tissue directly without applying chemical fixatives. Due to poorer spatial resolution, SEM-mounted EDX analysis systems do not localize the elemental composition in seed tissue so precisely as in a STEM-mounted EDX analysis system. However, the two systems complement each other and for that reason both systems were used in this study.

Neutron Activation Analysis:

Although the first neutron activation analysis was reported by Hevesey and Levi in 1936, the technique was not widely used until the mid-1950s. The increased availability of research reactors and the development of small, low-cost neutron generators have accelerated the growth of this field. Increasingly NAA has gained acceptance as a very sensitive, accurate and fast analytical method for certain elements (Valkovic 1977).

The principles of NAA have been described in detail by Koch (1960). In general, the procedure involves the bombardment of a sample with a flux of neutrons which converts some atoms of elements in the sample to various radionuclides, and a measurement of characteristic radiation as these radionuclides decay to stable elements. Two parameters, the energy emission spectrum and the half life, allow the

unambiguous identification and quantitation of the elements that originally existed in the sample (Heydorn 1984).

Activation analysis with neutron generators can be used to provide a totally automated, nondestructive analytical procedure. Thus the elements in a biological sample can be measured directly without destroying their organic matrix. This method is highly accurate and precise as well. A relative standard deviation of 0.25% can be achieved when the analysis is carried out under optimum conditions (Valkovic 1977). NAA is well known for its sensitivity; it has a detection range of 10^{-6} to 10^{-12} g of an element (Wang *et al.* 1975).

MATERIALS AND METHODS

Sample Preparation for Energy Dispersive X-ray Analysis:

a) Crushed tissues: Endosperm and selected embryo regions were soaked with hexane, and then chloroform:methanol (2:1, V:V) for 24 h each to extract lipids. Defatted endosperm, cotyledons and hypocotyl-radicle axes of *Capsicum annuum* were crushed on double sided tape on aluminum stubs and then chromium coated. Samples of dry and unextracted cotyledons were crushed and coated as above to provide a check against extraction during the defatting procedure.

b) Almost anhydrously prepared tissue: Endosperm, cotyledon and hypocotyl-radicle axis regions of *C. annuum* were dissected into small blocks approximately 1 mm^3. Procedures of

fixation, dehydration and infiltration described below were modified from Sack *et al.* (1988) and Skilnyk (1990). Blocks of embryo were fixed with 2% paraformaldehyde in 100% glycerol for 4 h, and blocks of endosperm were fixed with the same fixative for 12 h. Samples were then given three 0.5 h washes in a 1:1 mixture of glycerol and 100% ethanol, were dehydrated with 95% ethanol for 1 h, 100% ethanol for 1 h, and 100% propylene oxide for 12 h. Samples were infiltrated with Spurr's epoxy resin:propylene oxide mixtures (1:3, 1:2, 1:1, 2:1, 3:1, 1:0, 1:0, 1:0, 1:0) for 12 h periods at 24°C. The infiltrated tissue was embedded in Spurr's resin in flat embedding moulds and polymerized at 60°C for 48 h. Semi-thin sections, 0.5 μm thick, were cut using a Reichert Om U2 ultramicrotome. Since water is known to extract elements from thin sections, the sections were cut dry (Lott *et al.* 1984; Skilnyk 1990), picked up with forceps, and placed onto 100 mesh Formvar and carbon coated copper grids. A drop of 100% acetone was placed onto the grid to flatten the sections.

Energy Dispersive X-ray Analysis:

EDX analyses of crushed and defatted tissues were carried out on five randomly selected seeds. About 20 globoid crystals from each of three regions from each seed were done. The three regions included the endosperm, the cotyledon and the hypocotyl-radicle axis. About 20 globoid crystals from dry, unextracted cotyledon tissue that was ground up and pooled

from five randomly selected seeds were also EDX analyzed.

EDX analyses of almost anhydrously fixed tissues were carried out on three randomly selected seeds. About 20 globoid crystals from each of seven different tissues were analyzed for each seed, and each globoid crystal was selected from a different cell. The seven tissues were endosperm, and the protoderm, ground meristem and provascular tissues of both the cotyledon and the hypocotyl-radicle axis.

Analyses for both preparations were carried out with an ISI model DS-130 scanning electron microscope and a PGT System 4 energy dispersive X-ray analyzer (Princeton Gamma Tech, Princeton, NJ). An accelerating voltage of 15 kV was used. The run time of each analysis was 60 s and the count rate was kept between 1000 and 2500 counts per second. X-ray counts for the measured elements were obtained by integrating peaks at the following window widths: Mg, 1.146 to 1.358 keV; P, 1.899 to 2.125 keV; S, 2.190 to 2.422 keV; K, 3.187 to 3.437 keV; Ca, 3.562 to 3.818 keV; and Cr, 5.269 to 5.551 keV. The background subtraction values were defined using a program modified from Stewart *et al.* (1987) which joins the following predetermined X-ray energy levels: 676, 1036, 1460, 2452, 2972, 6100, 7500, 9400, 11272, 13348, 14672, 15124, 16800, 18924 eV. The total number of counts in each window was determined before and after background subtraction, and was used to calculate peak-to-background ratios. The overlap of the K_β peak of K and the K_α peak of Ca was corrected by subtracting 9.26% of the total

x-ray counts in the potassium K_β peak from the total counts in the window that included calcium K_α and potassium K_β. The correction factor was derived from the analysis of potassium salts as suggested by Barbi (1979) and was carried out by Ockenden and Lott (1990).

The almost anhydrously prepared tissues were also analyzed at an accelerating voltage of 100 kV with a JEOL 1200 EX-II scanning transmission electron microscope (JEOL Ltd, Tokyo, Japan) connected to a PGT model IMIX-II microanalysis system (Princeton Gamma Tech, Princeton, NJ). This system was used to determine the presence of heavier elements such as Fe, Mn and Zn, and it also gave a better spatial resolution. At least 5 globoid crystals, each one from different cells of the selected tissues, were analyzed. Cell walls, lipid vesicles, and the proteinaceous regions of protein bodies were also analyzed. The count rate for all analyses of globoid crystals was kept between 1000 and 3000 counts per second.

Preparation for Neutron Activation Analysis:

Endosperm and embryos from about 400 *C. annuum* seeds were separated, cut into small pieces, oven dried at 50°C for 1.5 h, left in the desiccator for 48 h over anhydrous $CaSO_4$, and returned to the oven for 1 h before being weighed for neutron activation analysis.

Neutron Activation Analysis:

Preweighed seed pieces were sealed into polyethylene vials which previously had been washed, soaked in dilute nitric acid and rinsed with deionized water. Samples were then irradiated for 240 s at a thermal flux of 5×10^{12} neutrons $cm^{-2}s^{-1}$ in the McMaster University Nuclear Reactor. Following irradiation each sample was transferred into a clean preweighed vial prior to the start of counting (delay time 200 s, count time 900 s) Counting geometry was fixed at 6 cm from the face of a coaxial hyperpure germanium detector, with an efficiency of 19.8% and a resolution of 1.9 keV at 1332 keV (APTEC Engineering Limited, Downsview, Ontario). The detector was coupled to a PC based Nuclear Data AccuSpec acquisition interface board with 16 K channel memory and a Nuclear Data Model ND581 ADC with ND599 Loss Free Counting Module for pulse height analysis of the gamma ray spectra to quantitate Mg, K, Ca and S. The system is calibrated with in-house standards. Wheat flour (1567a), which was obtained from the National Bureau of Standards in Washington D. C., was used as certified reference material and was analyzed as unknowns with each run.

It was not possible to accurately measure P using NAA because of the possible interference with Si (Valkovic 1977), so such measurements were not attempted.

RESULTS

The lipid extracted from *Capsicum annuum* seeds by hexane followed by a mixture of chloroform and methanol (2:1, v:v) accounted for about 30% of the seed dry weight.

Energy Dispersive X-ray Analysis:

All globoid crystals analyzed contained P, K and Mg; some also contained traces of one or more other element such as Ca, Mn, Fe, S, Cl and Zn.

Elements such as Ca, Mn, Fe and Zn were detected in traces in globoid crystals in certain tissues of the embryo (Figs 30-37, 39-44), but were not detected in globoid crystals in the endosperm (Fig. 45). Fe was detected in globoid crystals throughout almost all tissues studied except for the provascular tissues in the embryo (Table 12, Figs. 30-44). Infrequently Ca was found in a very low concentration in globoid crystals in the protoderm of the hypocotyl-radicle axis and in the layer of ground meristem cells next to the provascular tissue of the cotyledon (Figs. 30 and 42). Mn was detected in globoid crystals in the protoderm of the hypocotyl-radicle axis and in the layer of ground meristem cells next to the provascular tissue of the cotyledon (Figs. 30 and 42). Zn, on the other hand, was identified occasionally in globoid crystals in the protoderm of both the hypocotyl-radicle axis and the cotyledon and in globoid crystals from the spongy ground meristem and provascular tissue of the

cotyledon (Figs. 30, 36, 37, 42, 43 and 44).

S, P, sometimes K, Ca and Cl were detected in the proteinaceous region of protein bodies (Figs. 46-48); K, and occasionally P and S were detected in cell walls throughout all tissues of the seed (Figs. 49-51). In lipid vesicles Cl was detected occasionally (Figs. 52-53). Cl appeared occasionally in the spectra of EDX analysis of globoid crystals and other regions (Figs. 30, 33, 35, 36, 42, 43, 44, 46, 48, 49 and 52), and it was always detected in sections of Spurr's epoxy resin where no tissue was present (Fig. 54). Cl was not detected in the Formvar support film (Fig. 55).

EDX analysis of globoid crystals from crushed, defatted tissues of the embryo and the endosperm showed that the peak-to-background ratios of globoid crystals for P were higher than those for K or Mg (Table 6). Also in the embryo and the endosperm the peak-to-background ratios for K from globoid crystals were higher than the ratios for Mg. Within the same element, the peak-to-background ratios of P, K and Mg from globoid crystals in the hypocotyl-radicle axis were not significantly different from those of the cotyledon. However, the peak-to-background ratios for endosperm globoid crystals were lower than those of the embryo (Table 6). The ratios of K/P for globoid crystals in the hypocotyl-radicle axis were not significantly different from those of the cotyledon, but were higher in the endosperm (Table 7). Mg to K ratios for globoid crystals in the hypocotyl-radicle axis were not

significantly different from those of the cotyledon, but were lower in the endosperm (Table 7). The (K+Mg)/P ratios for globoid crystals were not significantly different among the hypocotyl-radicle axis, the cotyledon and the endosperm (Table 7). Only the Mg/P ratios for globoid crystals showed significant differences among all three areas (Table 7). Comparison of the results of analysis of globoid crystals from defatted and untreated cotyledon tissue with those from untreated cotyledon tissue showed that the peak-to-background ratios of P and K and Mg were significantly different (Table 8). The untreated tissues showed values about 20 % less than those from defatted cotyledon tissues. The K/P, Mg/P, (K+Mg)/P and Mg/K values between the two tissues were not significantly different (Table 9).

Results of EDX analyses from almost anhydrously prepared tissues and statistical comparisons of these results are given in Tables 10 and 11. In both the cotyledon and hypocotyl-radicle axis, the peak-to-background ratios of P and K and Mg were higher for globoid crystals in the ground meristem tissue than for globoid crystals in the protoderm or the provascular tissues. In all tissues the peak-to-background ratios for P were much higher than the peak-to-background ratios for K or Mg. Also in all tissues the peak-to-background ratios of K from globoid crystals were higher than those of Mg. The peak-to-background ratios of Mg for globoid crystals in the endosperm were significantly different from the ratios

obtained from any tissues in the embryo except for those from the ground meristem of the hypocotyl-radicle axis (Table 10). The ratios of K/P for globoid crystals in the palisade ground meristem were lower than those of the ground meristem of the hypocotyl-radicle axis or those of the spongy ground meristem of the cotyledon. The ratios of K/P were much higher in the provascular tissues than any other tissues. The ratios of Mg/P for globoid crystals were slightly lower in the protoderm and provascular tissues of the hypocotyl-radicle axis and the cotyledon than in the rest of tissues. The (K+Mg)/P ratios for globoid crystals in the palisade ground meristem were lower than those of the ground meristem of the hypocotyl-radicle axis or those of the spongy ground meristem of the cotyledon. The average Mg/K ratios for globoid crystals were always lower than 1 in all tissues. The provascular tissues of both the hypocotyl-radicle axis and the cotyledon had lower average ratios of Mg/K than other tissues studied (Table 11).

Though the preparation methods and the EM systems used were different, the ratios of K/P, Mg/P, (K+Mg)/P and Mg/P for globoid crystals in endosperm tissues were very similar between the crushed, defatted samples and the almost anhydrously prepared samples (compare Table 7 with Table 11).

Neutron Activation Analysis:

NAA results showed the quantitative values for elements in the embryo and endosperm tissues of sweet pepper seeds (Table 13). Among the elements measured, Mg, K and S were present in much higher concentrations than Cl, Mn, Na, Ca and Cu in both the embryo and the endosperm. Iodine was not present in detectable amounts in either the embryo or the endosperm. Values of K, Mg, S and Cu were higher in the embryo than in the endosperm. The Mn value was very similar in both the embryo and endosperm, but values of Cl, Na and Ca were lower in the embryo than in the endosperm.

DISCUSSION

Both EDX analysis and NAA are useful methods for studying mineral nutrients in seeds. EDX analysis permits the localization of elements in subcellular regions of seed tissues. In this research the major subcellular structure of interest was the globoid crystal in protein bodies. NAA, on the other hand, is useful for measuring the overall element content of tissues.

Conventional sample preparation using chemical fixation, dehydration and infiltration will cause movement and extraction of diffusible elements. This loss of diffusible elements is well known and has been investigated in some detail (Morgan 1980). Several methods can be used to prevent

loss of diffusible elements from biological samples.

Cryogenic preparation was attempted for the EDX analysis of globoid crystals of sweet pepper seeds. This method, which involves the quick freezing of unfixed tissue samples and viewing in a frozen state, has some advantages over other methods, but also has limitations for highly hydrated samples (Lott *et al*. 1985a; Talmon 1982). Numerous cryogenic studies have shown the benefits of this preparation technique for EDX analysis (Marshall 1977; Echlin *et al*. 1980; Fuchs and Fuchs 1980; Yeo *et al*. 1977). The major advantage of cryogenic specimen preparation is the retention of diffusible components in their *in vivo* positions. Also it is a quick method of specimen preparation and a simple way of exposing the interior of large biological specimens (Beckett *et al*. 1984; Beckett and Read 1986; and Lott and Kerr 1986). This method was found to be unsuitable for EDX analysis of globoid crystals of sweet pepper seeds since samples suffered from beam damage and charged badly when exposed to the electron beam. The difference of physical texture of the endosperm and embryo of sweet pepper seeds made the irregular topography commonly found with cryo-fractured surfaces, even more severe. The irregular topography after fracturing made it difficult to control the geometrical conditions for each analysis. Difficulties with x-ray analysis of frozen-fractured bulk specimens have been discussed by Talmon *et al*. (1979), Beckett and Read (1986) and Marshall (1980). The conductivity of ice

decreases markedly when the temperature drops to below -70°C (Marshall 1980). Thus, cryo-prepared samples tend to build electrical charges under the relatively low accelerating voltage and high beam current required for x-ray analysis in a SEM (Marshall 1977). Charging may be remedied by adding a coating of an efficient electrical conductor such as chromium (Marshall 1977). The charging encountered with sweet pepper seeds was only sightly reduced by chromium coating and was not reduced enough to permit EDX analysis studies.

EDX analysis of freeze-dried seed tissue powders has been used as a method to study possible extraction of elements from globoid crystals during fixation, dehydration and embedding procedures needed for thin sections (Darley and Lott 1973; Lott 1975; Lott et al. 1978; Lott and Vollmer 1979a, 1979b; Lott and Spitzer 1980; Spitzer et al. 1981; Spitzer and Lott 1982b; and Lott et al. 1982). The naturally electron dense globoid crystals can be identified without the addition of a fixative. Because seed tissues are dry they can be viewed in a SEM without dehydration. Thus it is possible to obtain data on elemental composition of globoid crystals in an unfixed state through the use of dry tissue powders.

The procedure that I used to crush seed tissues differed from the method described by Lott and Vollmer (1979a), however the principles of avoiding chemical fixation and retaining the elemental composition of globoid crystals were similar. The method used by Lott and Vollmer (1979a) was not applicable to

the seed tissue of *Capsicum annuum* since the seed tissue was very hard and did not powder readily. The oily tissue powder of *C. annuum* seeds also caused severe charging problems during EDX analysis. The seeds were thus defatted before they were prepared for EDX analysis. Some untreated dry seed tissue was EDX analyzed to check for possible elemental extraction by the organic solvents used to defat the seed tissue. There were significant differences between the peak-to-background ratios for P, Mg and K in globoid crystals, with the untreated dry tissue always showing lower values than defatted tissue. The charging that occurred in untreated oily tissue may be responsible for the difference. Also the oily coating of the sample surface may reduce X-ray yield. However, the ratios of Mg to P and K to P were not significantly different between the defatted and untreated samples, which demonstrates that the organic solvents did not differentially extract elements from globoid crystals of sweet pepper seeds.

It has been well documented that osmium tetroxide solution can cause extraction or redistribution of elements (Lott and Spitzer 1980; Roos and Barnard 1985). The work of Lott *et al.* (1978) on *Cucurbita maxima* seeds reported that osmium fixation can cause considerable elemental extraction from globoid crystals. There has been also evidence that the presence of osmium interferes with the polymerization of the plastic, causing incomplete polymerization (Roos and Barnard 1985). A high concentration of osmium in the specimen may mask

out portions of peaks from P, since the osmium M line at 1.978
keV cannot be resolved from the phosphorus K_α line at 2.015
keV (Fowler and Parker 1973).

Even though osmium tetroxide solution was avoided,
aqueous aldehyde containing fixatives also can extract
monovalent and divalent cations (Roomans 1980). EDX analysis
of *Pisum sativum* embryos following aqueous aldehyde fixation
showed that there was very little retention of K in globoid
crystals but Mg, Ca and P were retained (Lott *et al.* 1984).
Loss of P and K also occurred in *Pisum* protein bodies where no
globoid crystals were present. About 30-40% of P, 60-80% of Mg
and 70-90% of K present in globoid crystals of anhydrously
fixed *Cucurbita* pollen were lost when blocks were sectioned
onto water filled microtome troughs compared to those cut on
ethanol filled microtome troughs (Skilnyk 1990).

Since the loss of elements from globoid crystals during
the conventional sample preparation is well documented, this
research did not focus on investigating factors which cause
the elemental extraction. However, efforts were made to avoid
loss of elements from thin sections or bulk samples. In this
research osmium tetroxide fixation was avoided. The
paraformaldehyde fixative was prepared anhydrously,
dehydration was almost anhydrous, and sections were cut dry.
It was found that microtome troughs filled with absolute
ethanol, as used by Skilnyk (1990), were almost impossible to
work with, due to the evaporation of absolute ethanol and the

tendency of sections to stick to the edge of the glass knife. Post stains were not applied since elements may be extracted during post staining. The advantage of this fixed and sectioned tissue over crushed tissue, was the ability to localize elements from specific subcellular regions of cells of known location.

To date EDX analysis studies of globoid crystals in seed protein bodies of solanaceous plants have been carried out only on two species, tomato (Spitzer and Lott 1980) and jimson weed (Maldonado and Lott, unpublished data). Statistical comparisons of peak-to-background ratios and ratios of Mg/P, K/P and Mg/K based on peak-to-background ratios have never been published for globoid crystals for any species in the family Solanaceae. This is the first report of such research.

EDX analyses of globoid crystals on a range of monocot and dicot species have shown that P, K and Mg are the most common elements detected, although other elements such as Fe, Ca, Mn, Ba and Na may be present (Lott 1980; Lott 1985). EDX analysis carried out on sweet pepper seeds supports the concept that phytate is present in globoid crystals. The phytates in sweet pepper seeds appear to be predominately a mixed magnesium and potassium salt of phytic acid, likely with the potassium amount higher than magnesium. One or more other elements such as Fe, Zn, Ca and Mn were also present in trace amounts in globoid crystals in some embryo tissues but not in the endosperm. Sometimes a relatively high amount of Fe was

detected in some tissues. In sweet pepper embryos, Fe and Zn were found more frequently than Ca and Mn. The same cell types in the hypocotyl-radicle axis and the cotyledon had similar traces of minor elements in globoid crystals. In the protoderm of both the hypocotyl-radicle axis and the cotyledon, the only difference was that Ca was detected occasionally in globoid crystals in the protoderm of the hypocotyl-radicle axis but not in those of the cotyledon. The distribution of minor elements in globoid crystals in the ground meristem tissues of both the hypocotyl-radicle axis and the cotyledon were similar except for the layer of cells immediately next to the provascular tissue which was very different from the rest of the ground meristem tissues, and different from each other. Frequency of detection of minor elements in globoid crystals in this layer of the cotyledon, where Ca, Mn, Zn and Fe were sometimes detected, was higher than it was in the hypocotyl-radicle axis, where only Ca traces were sometimes found. The distribution of minor elements in globoid crystals in protoderm and provascular tissues in the hypocotyl-radicle axis and the cotyledon was similar but not identical.

Fe was found in some globoid crystals throughout all sweet pepper embryo tissues except for the provascular tissues. Since Fe is a necessary mineral nutrient for plants, it is not surprising to find reserves of it in a seed. It has been reported previously that Fe is present in globoid crystals of some species such as *Avena* and *Casuarina* (Buttrose

1978), tomato (Spitzer and Lott 1980; Spitzer *et al.* 1980) and *Capsella* (Spitzer *et al.* 1980). Chemical analyses of wheat and barley grains have also shown the presence of Fe (Bassiri and Nahapetian 1977; Låg and Steinnes 1978; Nahapetian and Bassiri 1976).

Mn, another minor element, has been found in the phytin of oats (Ashton and Williams 1958) and in globoid crystals of *Arachis hypogaea* seeds (Sharma and Dieckert 1975), in globoid crystals in certain regions of the radicle of wheat (Lott and Spitzer 1980), in the globoid crystals of the mesocotyl of *Avena* and the cotyledon of some species of *Casuarina* (Buttrose 1978). In sweet pepper seeds, Mn was found occasionally in the protoderm of the hypocotyl-radicle axis and in the layer of the ground meristem cells immediately next to the provascular tissue in the cotyledon.

Ca has been found more frequently in globoid crystals in *Cucurbita* species with smaller seeds than in *Cucurbita* species with larger seeds (Lott and Vollmer 1979a). Such correlation does not seem applicable to some species. Sweet pepper seeds are smaller than the smallest of *Cucurbita* seeds that have been studied. In tomato, which has seeds of similar size to those of sweet pepper, Ca was present infrequently and randomly in globoid crystals in the embryo and the endosperm (Spitzer and Lott 1980). The Ca distribution in globoid crystals in seeds of wheat and some umbelliferous species is also seemingly random (Lott and Spitzer 1980; Spitzer and Lott

1982b, c).

Zn was detected in some embryo regions of sweet pepper (Table 12). The presence of Zn in globoid crystals has been reported before. Zn traces were detected in globoid crystals of mesophyll tissues of *Pisum sativum* (Lott *et al.* 1984) and in globoid crystals of aleurone cells of developing wheat grains (Batten and Lott 1986). Zn was also found in globoid crystals in aleurone cells of developing barley (Duffus and Rosie 1976).In developing rice, Zn was only detected in globoid crystals in aleurone cells at 7 to 10 days after flowering but not at 17 to 19 days after flowering (Ogawa *et al.* 1979).

S was sometimes detected in globoid crystals, but it might have been from the surrounding proteinaceous regions. Cl appeared occasionally in the EDX analysis spectra of globoid crystals; apparently as a contamination from Spurr's epoxy resin.

It has been suggested that composition of globoid crystals and the distribution of certain minor elements in globoid crystals in seed tissues might be related to taxonomic groups (Lott 1981). Therefore, the results from *C. annuum* seeds were compared to the available results of other solanaceous seeds, namely *Lycopersicon esculentum* and *Datura stramonium* (Table 15). The globoid crystals in seeds of these three plants are all likely to be formed predominately from a mixed magnesium and potassium salt of phytic acid.

The composition of the minor elements in globoid crystals in the endosperm was only available for tomato. It was reported that Mg, K, Ca and Mn but no Fe were detected in globoid crystals in the endosperm tissue of tomato (Spitzer *et al.* 1980), a composition which differed from pepper endosperm tissue. No other minor elements were detected in globoid crystals in the endosperm tissue of *C. annuum*.

In the embryos considerable similarity was found in the distribution of minor elements in the hypocotyl-radicle axis and the cotyledon of all three species, *L. esculentum, D. stramonium* and *C. annuum* (Table 15). Among these minor elements Fe was the most frequently detected element in the embryo globoid crystals of the three species. Fe was always detected in globoid crystals in the layer of ground meristem cells immediately next to the provascular tissues in seeds of tomato, jimson weed and pepper. Fe was found in globoid crystals in provascular tissues in seeds of tomato and jimson weed, but not in pepper. In jimson weed and pepper, Zn was the second most frequently detected minor element in embryo globoid crystals. However, information on Zn distribution was not available for tomato seed globoid crystals. The distribution of Zn in globoid crystals in the embryos of jimson weed and pepper was similar but not identical. The distribution of Mn in globoid crystals in tomato embryos was almost identical to that of jimson weed embryos but not to pepper embryos. The distribution of Ca in globoid crystals in

embryo tissues was the most distinctive difference between these three species. Ca was not detected in globoid crystals in embryo tissues of jimson weed (Maldonado and Lott, unpublished data), yet in tomato and pepper Ca was infrequently distributed in globoid crystals.

Since elements such as K and Mg presumably were present as counterions for the anionic phosphate groups on phytic acid, it is useful to present data in the form of ratios of these elements to phosphorus. K/P was commonly higher for globoid crystals in the provascular tissues than for globoid crystals in the rest of the embryo. The average ratios of Mg/K, based on the peak-to-background values for globoid crystals in *C. annuum* seeds, were lowest in provascular tissues where most globoid crystals were very small, and were highest in ground meristem tissues where globoid crystals were large. As observed by Lott *et al.* (1985) in different species, the higher the (Mg+Ca)/K ratio, the more likely a tissue is to have large globoid crystals in its protein bodies. The Ca concentration in embryo tissues of *C. annuum* was very low, thus Mg/K ratio almost represented the (Mg+Ca)/K ratio.

Phytate-rich particles in many seeds are not evenly distributed (Lott and Ockenden 1986). In some species, different organs or parts may be more or less important in phytate storage. In wheat (*Triticum aestivum*), globoid crystals commonly are present in protein bodies in the aleurone layer and the embryo, but are absent from most

protein bodies in the endosperm (Wada and Maeda 1980). In maize nearly 90% of the total grain phytate is in the embryo (O'Dell *et al* 1972). In sweet pepper seeds, globoid crystals in protein bodies were present in all cells of the embryo and endosperm, although the size and frequency varied.

The ratios of Mg/K obtained from NAA results was much lower than those obtained by EDX analysis (Table 14). A direct comparison of these ratios should not be made since NAA results are quantitative ones for concentrations of elements in the whole sample whereas EDX analysis results are not. The peak-to-background ratios should not be used as quantitative estimates since the number of X-rays produced is not the same for equal concentrations of elements. Since Mg is lighter than K, Mg is markedly underestimated by EDX analysis (Russ 1972b). Since K was often detected in cell walls and the proteinaceous regions but Mg was not, Mg/K ratios prepared by the two methods would be expected to differ.

Table 6

Mean (±SE) peak-to-background ratios from EDX analysis
of globoid crystals in crushed and defatted seed tissue
of *Capsicum annuum*

Sample	n	K P/B	Mg P/B	P P/B
Hypocotyl-radicle axis	103	3.20±0.08a	2.90±0.09a	7.24±0.23a
Cotyledon	103	3.25±0.07a	2.92±0.07a	7.54±0.18a
Endosperm	103	2.94±0.07b	2.22±0.07b	5.99±0.17b

Note: 1. EDX analysis was carried out in a SEM at an
         accelerating voltage of 15 kV.
      2. F-test and LSD (least significant difference)-test
         were used for statistical comparisons. Values
         followed by same letters within the same column are
         not significantly different at P>0.05.
      3. Five seeds were analyzed.

Table 7

Ratios of K to P, Mg to P, Mg plus K to P, and Mg to K
based on peak-to-background values from EDX analysis of
globoid crystals in crushed and defatted seed tissue of
Capsicum annuum

Sample	n	K/P	Mg/P	(K+Mg)/P	Mg/K
Hypocotyl-radicle axis	103	0.48±0.02 b	0.41±0.01 a	0.88±0.02 a	0.92±0.03 a
Cotyledon	103	0.42±0.01 b	0.39±0 b	0.84±0.02 a	0.93±0.03 a
Endosperm	103	0.53±0.02 a	0.37±0.01 c	0.90±0.02 a	0.78±0.03 b

Note: 1. EDX analysis was carried out in a SEM at an
         accelerating voltage of 15 kV.
      2. F-test and LSD-test were used for statistical
         comparisons. Values followed by same letters within
         the same column are not significantly different at
         P>0.05.
      3. Five seeds were analyzed.

Table 8

Mean (±SE) peak-to-background ratios of EDX analysis
of globoid crystals in crushed tissue of *Capsicum
annuum* cotyledons

Cotyledon	n	K P/B	Mg P/B	P P/B
Defatted	103	3.25±0.07a	2.92±0.07a	7.54±0.18a
Untreated	21	2.60±0.12b	2.34±0.13b	6.25±0.32b
% reduction		19.58%	19.75%	17.22%

Note: 1. EDX analysis was carried out in a SEM at
an accelerating voltage of 15 kV.
2. F-test was used for statistical comparisons. Values
followed by same letters within the same column are
not significantly different at P>0.05.
3. Five separated defatted cotyledons were analyzed.
4. Untreated cotyledon tissues were from five pooled
and ground seeds.

Table 9

Ratios of K to P, Mg to P, Mg plus K to P, and Mg to
K based on peak-to-background values from EDX analysis
of globoid crystals in crushed cotyledons of *Capsicum
annuum*

Cotyledon	n	K/P	Mg/P	(K+Mg)/P	Mg/K
Defatted	103	0.45±0.01a	0.39±0 a	0.84±0.02 a	0.93±0.03 a
Untreated	21	0.43±0.02a	0.38±0.01 a	0.80±0.02 a	0.93±0.06 a

Note: 1. EDX analysis was carried out in a SEM at
an accelerating voltage of 15 kV.
2. F-test was used for statistical comparisons. Values
followed by same letters within the same column are
not significantly different at P>0.05.
3. Five separated defatted cotyledons were analyzed.
4. Untreated cotyledon tissues were from five pooled
and ground seeds.

Table 10
Mean (±SE) peak-to-background ratios from EDX analysis
of globoid crystals in thin sections of different
tissues of *Capsicum annuum* seeds

Sample	n	K P/B	Mg P/B	P P/B
Hypocotyl-radicle axis:				
Protoderm	62	3.90±0.21d	2.80±0.12 cd	7.59±0.31ef
Ground meristem	62	4.38±0.10bc	3.45±0.12b	8.51±0.23c
Provascular tissue	62	4.16±0.15cd	2.71±0.10cd	7.18±0.24f
Cotyledon:				
Protoderm	62	4.20±0.15cd	2.90±0.12c	8.23±0.27cd
Palisade ground meristem	62	4.76±0.09ab	4.01±0.08a	10.55±0.16 a
Spongy ground meristem	62	5.01±0.10a	3.94±0.08a	9.83±0.17b
Provascular tissue	62	4.41±0.12bc	2.59±0.12d	7.86±0.22de
Endosperm	62	4.18±0.12cd	3.19±0.08b	8.23±0.17cd

Note: 1. EDX analysis was carried out in a STEM at an
      accelerating voltage of 15 kV.
    2. F-test and LSD-test were used for statistical
      comparisons. Values followed by same letters within
      the same column are not significantly different at
      $P > 0.05$.
    3. Three seeds were analyzed.
    4. Sections were almost anhydrously prepared.

Table 11
Ratios of K to P, Mg to P, Mg plus K to P, and Mg to
K based on peak-to-background values from EDX analysis
of globoid crystals in thin section of different
tissues of *Capsicum annuum* seeds

Sample	n	K/P	Mg/P	(K+Mg)/P	Mg/K	% of (Mg/K> 1)
Hypocotyl-radicle axis:						
Protoderm	62	0.52± 0.02b	0.37± 0.01cd	0.89± 0.02bc	0.76± 0.02bc	8.1
Ground meristem	62	0.53± 0.02b	0.40± 0.01a	0.94± 0.02ab	0.80± 0.03ab	21.5
Provascular tissue	62	0.59± 0.02a	0.38± 0.01c	0.97± 0.02a	0.68± 0.03d	8.1
Cotyledon:						
Protoderm	62	0.53± 0.02b	0.35± 0.01d	0.88± 0.02cd	0.71± 0.03cd	14.1
Palisade ground meristem	62	0.46± 0.01c	0.38± 0.01bc	0.84± 0.02d	0.86± 0.02a	15.6
Spongy ground meristem	62	0.52± 0.01b	0.40± 0.01ab	0.92± 0.02bc	0.80± 0.02ab	10.8
Provascular tissue	62	0.58± 0.02a	0.33± 0.01e	0.90± 0.02bc	0.60± 0.03	4.8
Endosperm	62	0.51± 0.01b	0.39± 0.01 abc	0.90± 0.02bc	0.79± 0.02b	12.3

Note: 1. EDX analysis was carried out in a STEM at
an accelerating voltage of 15 kV.
2. F-test and LSD-test were used for statistical
comparisons. Values followed by same letters within
the same column are not significantly different at
$P > 0.05$.
3. Three seeds were analyzed.
4. Sections were almost anhydrously prepared.

Table 12
The presence and absence of Ca, Mn, Fe and Zn by visual
study of EDX analysis spectra of globoid crystals in
thin sections from different tissues of *Capsicum annuum*
seeds

Tissue	Ca	Mn	Fe	Zn
Hypocotyl-radicle axis:				
Protoderm	-/+	-/+	+	-/+
Ground meristem	-	-	-/+	-
Ground meristem next to the protoderm	-	-	-/+	-
Ground meristem next to the provascular tissue	-	-	+	-
Provascular tissue	-	-	-	-
Cotyledon:				
Protoderm	-	-	+	-/+
Palisade ground meristem	-	-	-/+	-
Spongy ground meristem	-	-	-/+	-
Spongy ground meristem next to the protoderm	-	-	-/+	-
Spongy ground meristem next to the provascular tissue	-/+	-/+	-/+	-/+
Provascular tissue	-	-	-	-/+
Endosperm	-	-	-	-

Note: 1. EDX analysis was carried out in a STEM at
an accelerating voltage of 100 kV.
2. -: not detected; +: always detected; -/+: detected
in some cases.
3. Five seeds were analyzed.
4. Sections were almost anhydrously prepared.

Table 13
Selected elements (μgg^{-1}) from *Capsicum annuum* embryo and endosperm measured by neutron activation analysis (NAA)

Tissue	Mg	K	Cl	Mn	Na	Ca	I	S	Cu
Embryo (Mean±SE)	6225 ±205	11875 ±205	237 ±10	37 ±1	19 ±0	174 ±6	−	3150± 0	26± 1
Endosperm (Mean±SE)	2440 ±60	6275 ±135	341 ±1	36 ±2	144 ±2	490 ±5	−	2570± 0	12± 0

Note: − indicates that the amount of the element is below the detection limit.

Table 14
The ratio of Mg to K (Mean±SE) as measured by
NAA and EDX analysis of different preparations

Sample	NAA	EDX analysis of crushed tissue	EDX analysis of anhydrously fixed tissue
EMBRYO:	0.53±0.01		
Hypocotyl-radicle axis:		0.92±0.03	
Protoderm			0.76±0.02
Ground meristem			0.80±0.03
Provascular tissue			0.68±0.03
Cotyledon:		0.93±0.03	
Protoderm			0.71±0.03
Palisade mesophyll			0.86±0.02
Spongy mesophyll			0.80±0.02
Provascular tissue			0.60±0.03
ENDOSPERM	0.39±0.00	0.78±0.18	0.79±0.02

Table 15
Comparison of presence and absence of Ca, Mn, Fe and Zn by visual study of EDX analysis spectra of globoid crystals in thin section from different tissues of tomato (Lycopersicon esculentum)[a], jimson weed (Datura stramonium)[b] and pepper (Capsicum annuum)

Ca

	Tomato	Jimson weed	Pepper
Hypocotyl-radicle axis: Protoderm	-	-	-/+
Ground meristem	-	-	-
Ground meristem next to provascular tissue	-	-	-
Provascular tissue	-/+	-	-
Cotyledon: Protoderm	-	-	-
Palisade ground meristem	-	-	-
Spongy ground meristem	-/+	-	-
Ground meristem next to provascular tissue	-	-	-/+
Provascular tissue	-	-	-
Endosperm	-/+	*	-

Mn

	Tomato	Jimson weed	Pepper
Hypocotyl-radicle axis: Protoderm	-/+	+	-/+
Ground meristem	-	-	-
Ground meristem next to provascular tissue	-	-	-
Provascular	-	-	-
Cotyledon: Protoderm	-/+	+	-
Palisade ground meristem	-	-	-
Spongy ground meristem	-	-	-
Ground meristem next to provascular tissue	-	-	-/+
Provascular tissue	-	-	-
Endosperm	-	*	-

Table 15 continued

Table 15 continued from last page:

Fe

	Tomato	Jimson weed	Pepper
Hypocotyl-radicle axis: Protoderm	-/+	+	+
Ground meristem	-	-/+	-/+
Ground meristem next to provascular tissue	+	+	+
Provascular tissue	-/+	+	-
Cotyledon: Protoderm	-/+	+	+
Palisade ground meristem	-	+	-/+
Spongy ground meristem	-/+	-/+	-/+
Ground meristem next to provascular tissue	+	-/+	-/+
Provascular tissue	-/+	+	-
Endosperm	-	*	-

Zn

	Tomato	Jimson weed	Pepper
Hypocotyl-radicle axis: Protoderm	*	-/+	-/+
Ground meristem	*	-	-
Ground meristem next to provascular tissue	*	-	-
Provascular tissue	*	-/+	-
Cotyledon: Protoderm	*	+	-/+
Palisade ground meristem	*	-	-
Spongy ground meristem	*	-/+	-
Ground meristem next to provascular tissue	*	-	-/+
Provascular tissue	*	-/+	-/+
Endosperm	*	*	-

Note: 1. -: not detected; +: always detected; -/+ detected in some cases; *: information not available.
2. a: from Spitzer and Lott (1980);
b: from Maldonado and Lott (unpublished data).

Figs. 30 to 54. Tissues from hypocotyl-radicle axis, cotyledon and endosperm of dry pepper seeds were almost anhydrously prepared. Sections, 0.5 μm thick, were cut dry. EDX analysis was carried out in a STEM at an accelerating voltage of 100 kV. All spectra represent 60 s analysis. The principal emission lines of elements present are as follows: Mg 1.254 keV, K_α; P 2.015 keV, K_α; S 2.307 keV, K_α and 2.468 keV, K_β; Cl 2.622 keV, K_α; K 3.312 keV, K_α and 3.589 keV, K_β; Ca 3.690 keV, K_α; Mn 5.895 keV, K_α; Fe 6.400 keV, K_α; Zn 8.631 keV, K_α. Copper peaks are considered to be contaminated by copper grids used, thus are not identified on the spectra.

Fig. 30. EDX analysis spectrum of a protoderm cell globoid crystal in a hypocotyl-radicle axis.

Fig. 31. EDX analysis spectrum of a globoid crystal in a ground meristem cell next to the protoderm in a hypocotyl-radicle axis.

Fig. 32. EDX analysis spectrum of a ground meristem cell globoid crystal in a hypocotyl-radicle axis.

Figs. 33, 34. EDX analysis spectra of globoid crystals in ground meristem cells next to the provascular tissue in a hypocotyl-radicle axis.

Fig. 35. EDX analysis spectrum of a provascular cell globoid crystal in a hypocotyl-radicle axis.

Figs. 36, 37. EDX analysis spectra of protoderm cell globoid crystals in a cotyledon.
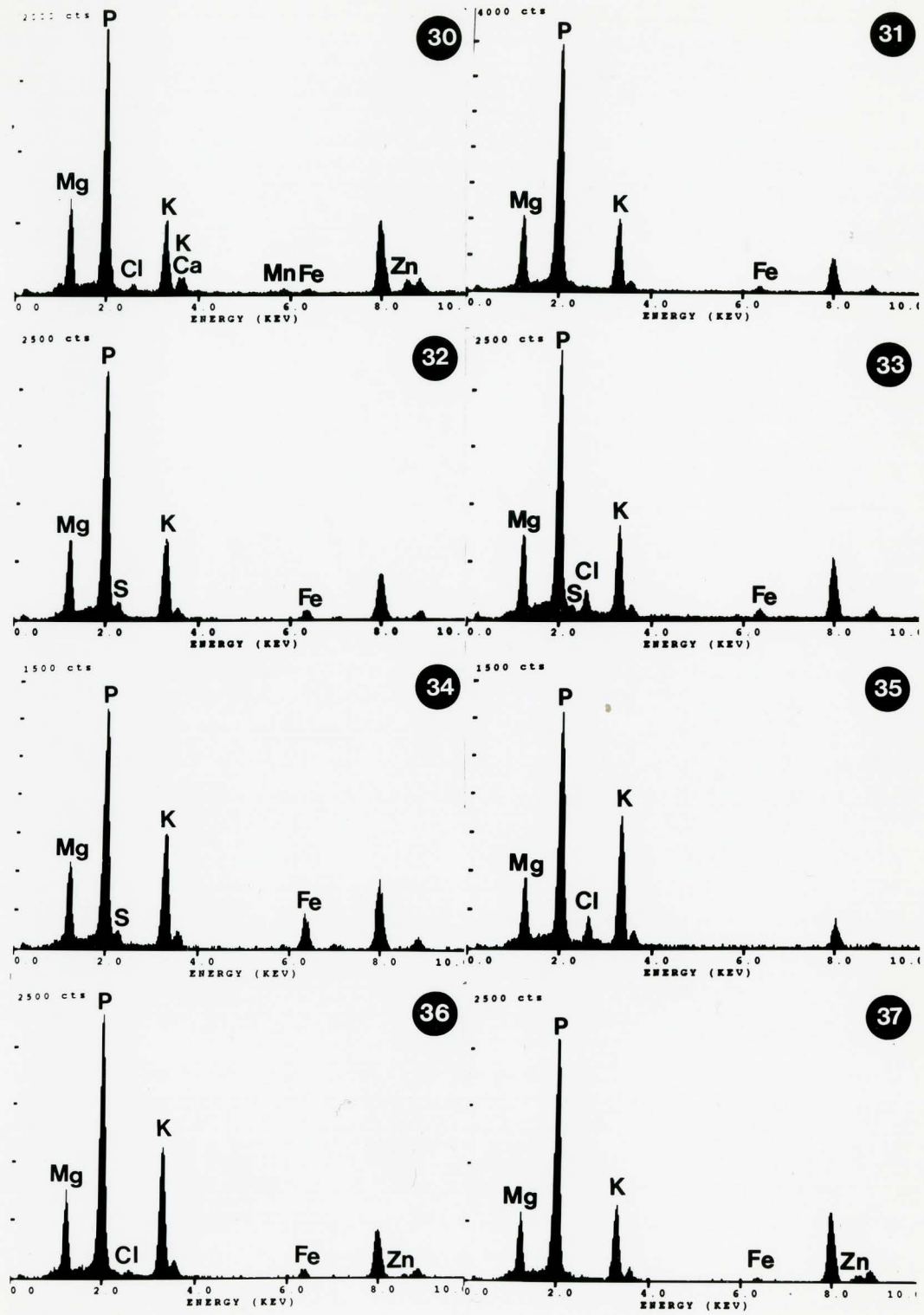
Fig. 38. EDX analysis spectrum of a globoid crystal in a palisade ground meristem cell in a cotyledon.

Fig. 39. EDX analysis spectrum of a globoid crystal in a spongy ground meristem cell in a cotyledon.

Figs. 40, 41. EDX analysis spectra of globoid crystals in spongy ground meristem cells next to the protoderm in a cotyledon.

Figs. 42, 43. EDX analysis spectra of globoid crystals in spongy ground meristem cells next to the provascular tissue in a cotyledon.

Fig. 44. EDX analysis spectrum of a provascular cell globoid crystal in a cotyledon.

Fig. 45. EDX analysis spectrum of a globoid crystal in an endosperm cell.
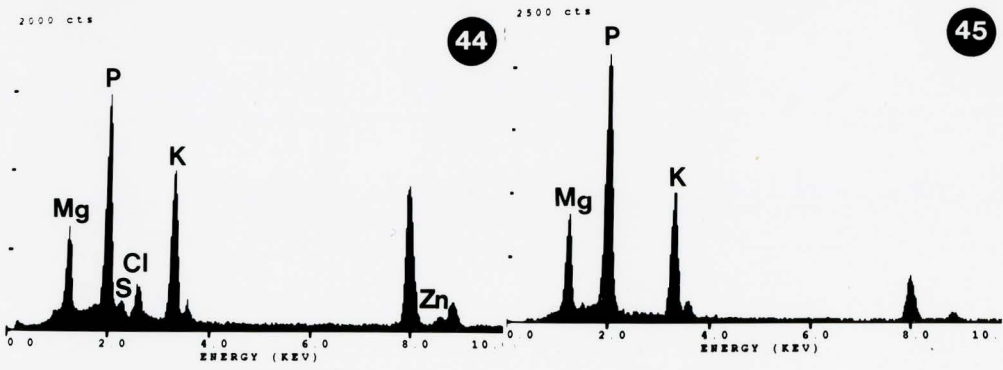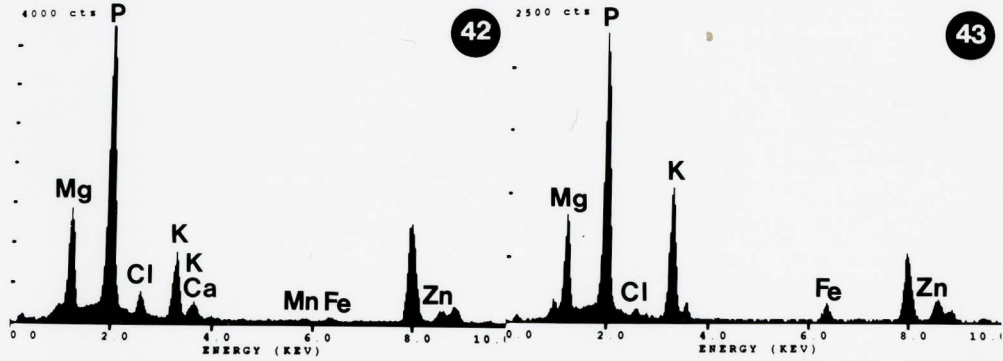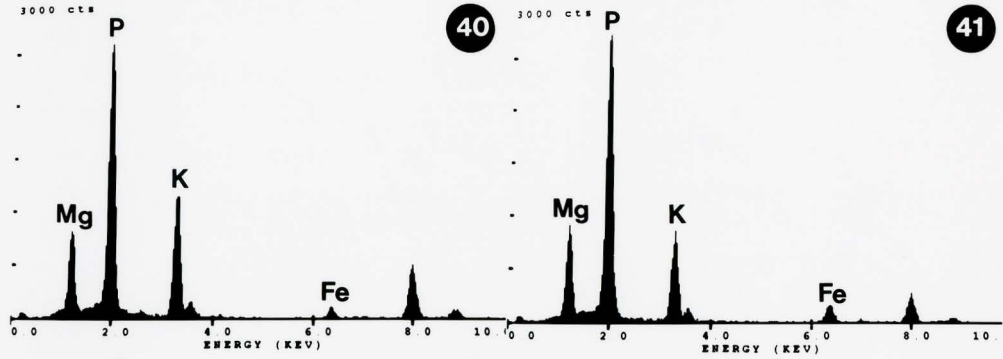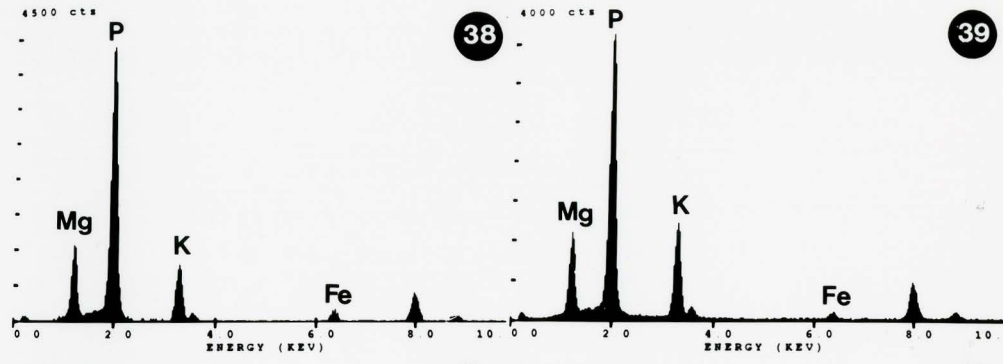
Fig. 46. EDX analysis spectrum of the proteinaceous region of a provascular cell protein body in a hypocotyl-radicle axis.

Fig. 47. EDX analysis spectrum of the proteinaceous region of a protein body in a spongy ground meristem cell in a cotyledon.

Fig. 48. EDX analysis spectrum of the proteinaceous region of an endosperm cell protein body.

Fig. 49. EDX analysis spectrum of the cell wall region of a ground meristem cell in a hypocotyl-radicle axis.

Fig. 50. EDX analysis spectrum of the cell wall region of a protoderm cell in a cotyledon.

Fig. 51. EDX analysis spectrum of the cell wall region of an endosperm cell.

Fig. 52. EDX analysis spectrum of a protoderm cell lipid vesicle in a hypocotyl-radicle axis.

Fig. 53. EDX analysis spectrum of an endosperm cell lipid vesicle.
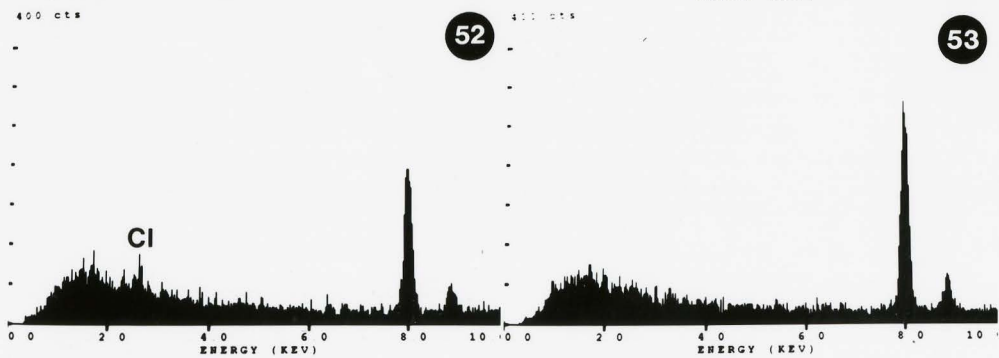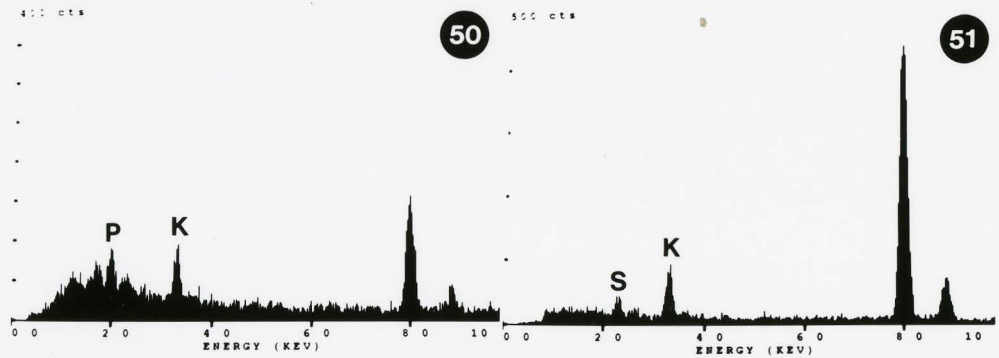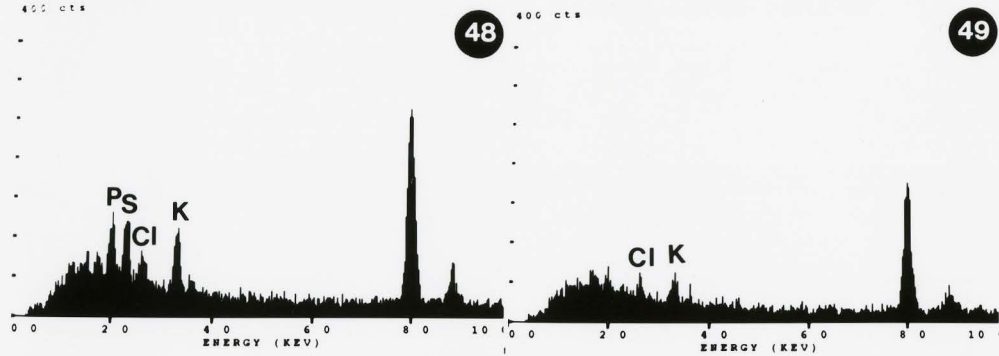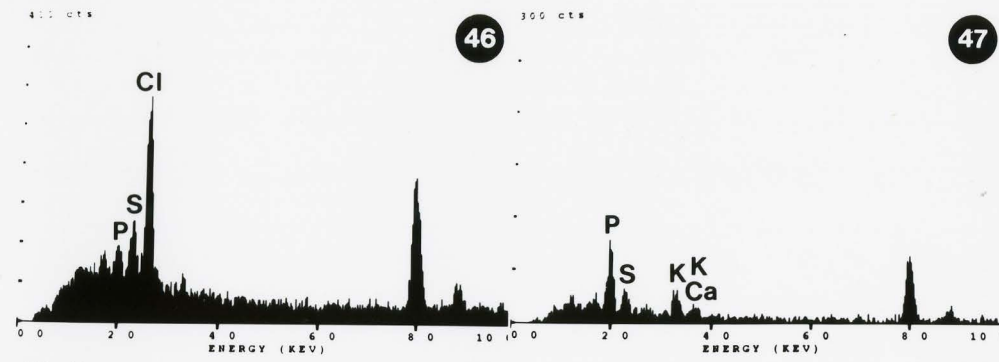
Fig. 54. EDX analysis spectrum of Spurr's epoxy resin where no specimen was present.

Fig. 55. EDX analysis spectrum of a Formvar support film. Analysis conditions were the same as used for previous figures.
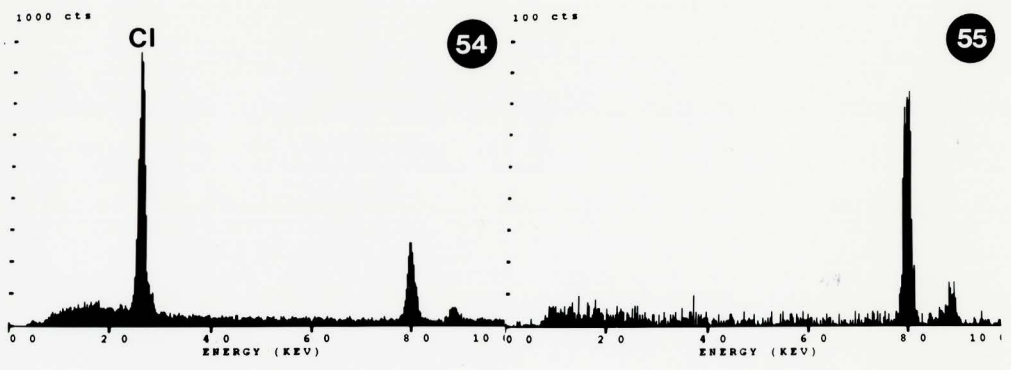
Chapter 4

CONCLUSIONS AND FUTURE STUDIES

CONCLUSIONS

The seed of *Capsicum annuum* consisted of a testa, an embryo and the endosperm. The endosperm tissue surrounded the curved embryo which had two cotyledons and a hypocotyl-radicle axis.

SEM studies of *C. annuum* testa revealed that the hair-like structures, which cover the seed surface of the section *Solanum* in family Solanaceae (Edmonds 1983), did not appear on the seed surface of *C. annuum*.

Light microscopy, electron microscopy and histochemical studies revealed that the main storage reserves in *C. annuum* seeds were protein and lipid. Storage protein and lipid were located in protein bodies and lipid vesicles respectively. Starch was not detected in either the embryo tissues or the endosperm of *C. annuum*, but the thickened cell walls of the endosperm may serve as a store of polysaccharide.

Protein bodies of *C. annuum* seeds were oval to round in shape in sections. Two types of protein bodies appeared to occur in sections of embryo tissues. One type had one or more

globoid crystals and usually one protein crystalloid as inclusions in the proteinaceous matrix. The other type of protein body in sections contained numerous globoid crystals but no protein crystalloids in the proteinaceous matrix. Endosperm protein bodies usually contained a protein crystalloid and one or more globoid crystals as inclusions in the proteinaceous matrix. In sections, protein bodies of *C. annuum* embryo tissues varied greatly in size both inter- and intra-cellularly whereas in the endosperm protein bodies were similar in size in sections.

Protein crystalloids in *C. annuum* seed protein bodies were sometimes irregular in shapes. Such irregular protein crystalloids appeared more frequently in the endosperm than in embryo tissues. Protein crystalloids occupied a greater fraction of area of protein bodies in sections of endosperm than in sections of embryo tissues.

Globoid crystals in *C. annuum* seed protein bodies were round or almost round in profile in sections and were similar in size in the endosperm. In embryo tissues, however, globoid crystals varied in size both inter- and intra-cellularly in sections. The average longest diameter of globoid crystals in semi-thin sections was highest in the ground meristem cells and lowest in provascular cells in both the hypocotyl-radicle axis and the cotyledon.

EDX analysis studies revealed that all globoid crystals in *C. annuum* seed protein bodies contained P, Mg and K, a fact

that is consistent with their being phytate-rich. In the embryo one or more other elements, such as Fe, Zn, Ca and Mn, were also present in trace amounts in globoid crystals in some cells. In endosperm cells, Fe, Zn, Ca and Mn were absent in globoid crystals.

Among the elements measured by NAA, Mg, K and S were present in much higher concentrations than Cl, Mn, Na, Ca and Cu in both the embryo and the endosperm. Values of Mg, K, S and Cu were higher in the embryo than in the endosperm, while Cl, Na and Ca were higher in the endosperm. Mn had similar concentrations in both the embryo and the endosperm.

The results of the size, shape and inclusions of protein bodies as well as the elemental composition of globoid crystals in *C. annuum* seeds were compared to those of other solanaceous plants which have been studied. Difficulties in comparison were encountered because of the lack of information. However, seed protein bodies of *Lycopersicon esculentum, Atropa belladonna, Datura stramonium* and *C. annuum* contained protein crystalloids and globoid crystals, but lacked druse crystals. Variations in the size of protein bodies, found to occur both inter- and intra-cellularly in the embryo tissues of *C. annuum*, have also been found in embryo tissues of *L. esculentum* and *A. belladonna* (Spitzer and Lott 1980; Simola 1987). Protein bodies in *D. stramonium* embryo varied in size from tissue-to-tissue, but in a given tissue protein bodies seemed to be similar in size (Maldonado and

Lott, unpublished data). The elemental composition of globoid crystals in protein bodies of a given organ and tissue were similar between embryos of *L. esculentum, D. stramonium* and *C. annuum*. The globoid crystals from embryo tissues of these three species were rich in P, Mg and K. One or more other elements, such as Ca, Mn, Fe and Zn, were also present in trace amounts in globoid crystals of some embryo tissues of these three plants. Fe was the most frequently detected trace element, and the distribution of Fe in globoid crystals in different embryo tissues of *L. esculentum, D. stramonium* and *C. annuum* were similar but not identical.

FUTURE STUDIES

Is the size of protein bodies related to the pathways of protein body origin? From this research, it was found that protein bodies in embryo tissues of *C. annuum* varied greatly in size both inter- and intra-cellularly while protein bodies in the endosperm were similar in size. Studies from several species support theories of vacuolar origin of the bounding membrane of seed protein deposits, while results from some other plants support the hypothesis that the bounding membrane of seed protein deposits may arise from endoplasmic reticulum or from a dictyosome (Murray 1984). There are also indications that protein bodies may be formed in different ways even in the same cell (Simola 1987). It was suggested from studies of

developing cotyledons of *Vigna* that cells form a large number of small protein bodies, most of which are formed by the expansion of cytoplasmic vesicles originated from dictyosomes (Harris and Boulter 1976). Examinations of the ultrastructure of *C. annuum* seeds during development could identify whether different paths are involved in protein body origin and determine whether pathways of protein body origin are related to protein body size.

Protein body shape, size and inclusions may be related to taxonomic groups (Lott 1981). However, seeds of only four species of the important family Solanaceae have been studied with electron microscopy. Taxonomically these four species, namely, *L. esculentum, D. Stramonium, A. belladonna* and *C. annuum* belong to four different genera. Comparisons made in this thesis among *L. esculentum, A. belladonna, D. stramonium* and *C. annuum* embryo protein bodies indicated that some features of protein bodies were similar among these species. It would be necessary for more species in this family to be studied to determine whether protein bodies are a useful character for the study of taxonomic relationships.

It was suggested that cell walls of *C. annuum* endosperm may be rich in mannan-containing polysaccharides (Watkins *et al.* 1985). Biochemical studies could be applied to identify the composition of cell walls of *C. annuum* endosperm. Moreover, structural, biochemical and histochemical studies during germination could determine whether cell wall

polysaccharides of *C. annuum* endosperm are mobilized as food reserves.

REFERENCES

ALLEN, J. E., HOOD, L. F., and PARTHASARATHY, M. V. 1976.
The ultrastructure of unmodified and chemically-
modified tapioca starch granules as revealed by the
freeze-etching technique. J. Food Technol. **11**: 537-541.

ANDREWS, P., HOUGH, L., and JONES, J. K. N. 1953. Mannose-
containing polysaccharides. Part III. The
polysaccharides in the seeds of *Iris ochroleuca* and *I.
sibirica*. J. Chem. Soc. 1186-1192.

APPELQVIST, L.-Å. 1975. Biochemical and structural aspects
of storage and membrane lipids in developing oil seeds.
In Recent Advances in the Chemistry and Biochemistry of
Plant Lipids. *Edited by* T. Galliard and E. I. Mercer.
Academic Press, London. Proc. Phytochem. Soc. **12**: 247-
286.

ARNOTT, H. J., and WEBB, M. A. 1983. Scanning electron
microscopy and histochemistry of oilseeds. *In* New
Frontiers In Food Microstructure. *Edited by* D. B.
Bechtel. The American Association of Cereal Chemists,
Inc. St. Paul, Minnesota. pp. 149-198.

ASHFORD, A. E., LING LEE, M., and CHILVERS, G. A. 1975.
Polyphosphate in eucalypt mycorrhizas: a cytochemical
demonstration. New Phytol. **74**: 447-453.

ASHTON, W. M., and WILLIAM, P. C. 1958. The phosphorus
compounds of oats. I. The content of phytate
phosphorus. J. Sci. Food Agric. **9**: 505-511.

BADENHUIZEN, N. P. 1969. The Biogenesis of Starch Granules
in Higher Plants. Appleton-Century-Crofts, New York.

BALDWIN, E. 1957. Dynamic Aspects of Biochemistry. Cambridge
Univ. Press, 3rd Ed. pp 86-87.

BARBI, N. C. 1979. Quantitative methods in biological X-ray
microanalysis. Scanning Electron Microscopy **1979/II**:
659-672.

BASHAN, Y., and OKON, Y. 1987. Internal and external infections of fruits and seeds of peppers by *Xanthomonas campestris* pathovar *Vesicatoria*. Can. J. Bot. **64:** 2865-2871.

BASSIRI, A., and NAHAPETIAN, A. 1977. Differences in concentrations and interrelationships of phytate, phosphorus, magnesium, calcium, zinc, and iron in wheat varieties grown under dry land and irrigated conditions. J. Agric. Food Chem. **25:** 1118-1122.

BATTEN. G. D., and LOTT, J. N. A. 1986. The influence of phosphorus nutrition on the appearance and composition of globoid crystals in wheat aleurone cells. Cereal Chem. **63:** 14-18.

BECKETT, A., PORTER, R., and READ, N. D. 1984. Low-temperature scanning electron microscopy of fungal material. J. Microsc. **125:** 193-199.

BECKETT, A., and READ, N. D. 1986. Low-temperature scanning electron microscopy. *In* Ultrastructure Techniques for Microorganisms. *Edited by* H. C. Aldrich and W. J. Todd. Plenum Publishing Corporation, New York. pp. 45-86.

BEEVERS, L. 1976. Nitrogen Metabolism in Plants. *Edited by* Arnold. London

BEWLEY, J. D., and BLACK, M. 1985. Seeds, Physiology of Development and Germination. Plenum Press, New York.

BRISSON, J. D., and PETERSON, R. L. 1976. A critical review of the use of scanning electron microscopy in the study of the seed coat. Scanning Electron Microscopy **1976/II:** 477-495.

BRONNER, R. 1975. Simultaneous demonstration of lipids and starch in plant tissues. Stain Technol. **50:** 1-4.

BUTTROSE, M. S. 1963. Electron-microscopy of acid-degraded starch granules. Stärke **3:** 85-92.

BUTTROSE, M. S. 1971. Ultrastructure of barley aleurone cells as shown by freeze-etching. Planta (Berl.) **96:**13-26.

BUTTROSE, M. S. 1978. Manganese and iron in globoid crystals of protein bodies from *Avena* and *Casuarina*. Aust. J. Plant Physiol. **5:** 631-639.

CANVIN, D. T. 1965. The effect of temperature on the oil content and fatty acid composition of the oils from

several oil seed crops. Can. J. Bot. **43:** 63-69.

CHABOT, J. F., ALLEN, J. E., and HOOD, L. F. 1978. Freeze-etch ultrastructure of waxy maize and acid hydrolyzed waxy maize starch granules. J. Food Sci. **43:** 727-734.

COIMBRA, A., and LOPES-VAZ, A. 1971. The presence of lipid droplets and the absence of stable Sudanophilia in osmium-fixed human leukocytes. J. Histochem. Cytochem. **19:** 551-557.

CORNER, E. J. H. 1976. The Seeds of Dicotyledons. Cambridge University Press, Cambridge. pp. 254-255.

CROCKER, W., and BARTON, L.V. 1957. Physiology of Seeds. Chronica Botanica, Waltham, Mass.

DALTON, G. E. 1978. Potato production in the context of world and farm economy. *In* The Potato Crop, the Scientific Basis for Improvement. *Edited by* P. M. Harris. Chapman and Hall, London. pp. 647-677.

DANIELSSON, C.E. 1956.Plant proteins. Annu. Rev. Plant Physiol. **7:** 215-236.

DARLEY, J. J., and LOTT, J. N. A. 1973. Low temperature freeze-drying for the scanning electron microscope using liquid nitrogen at low vacuum. Micron. **4:** 178-182.

DEMASON, D. A., SEKHAR, K. N. C., and HARRIS, M. 1989. Endosperm development in the date palm (*Phoenix dactylifera*). Amer. J. Bot. **76:** 1255-1265.

DIECKERT, J. W., and DIECKERT, M. C. 1972. The deposition of vacuolar proteins in oil seeds. *In* Symp. Seed Proteins. *Edited by* G. E. Inglett. AVI Publ., Westport, Conn. pp. 52-85.

DUFFUS, C. M., and ROSIE, R. 1976. Changes in trace element composition of developing barley grain. J. Agric. Sci. **87:** 75-79.

DUFFUS, C. M., and SLAUGHTER, J. C. 1980. Seeds and Their Uses. John Wiley and Sons, Chichester.

ECHLIN, P. LAI,, C. E. HAYES, T. L. and HOOK, G. 1980. Elemental analysis of frozen hydrated differentiating phloem parenchyma in roots of *Lemna minor* L. Scanning Electron Microscopy **1980/II:** 383-394.

ECKEY, E. W. 1954. Vegetable Fats and Oils. Reinhold
    Publishing Corp., New York.

EDMONDS, J. M. 1983. Seed coat structure and development in
    Solanum L. section *Solanum* (Solanaceae). Botanical
    Journal of the Linnean Society **87**: 229-246.

FOWLER, B. A., and PARKER, P. 1973. Observations on the
    preparation of epoxy embedded tissue samples for
    energy-dispersive X-ray analysis. Stain Technol. **48**:
    333-335.

FRIEDMAN, M., and LEVIN, C. E. 1989. Composition of jimson
    weed (*Datura stramonium*) seeds. J. Agric. Food Chem.
    37: 998-1005.

FUCHS, W., and FUCHS, H. 1980. The use of frozen-hydrated
    bulk specimens for X-ray microanalysis. Scanning
    Electron Microscopy **1980/II**: 371-382.

FULCHER, R. G. 1982. Fluorescence microscopy of cereals.
    Food Microstructure **1**: 67-175.

FULCHER, R. G., O'BRIEN, T. B. and WONG, S. I. 1981.
    Microchemical detection in cereal bran. Cereal Chem.
    58: 130-135.

GAHAN, P. B. 1984. Plant Histochemistry and Cytochemistry,
    an Introduction. Academic Press, London.

GALLANT, D., MERCIER, C., and GUILBOT, A. 1972. Electron
    microscopy of starch granules modified by bacterial
    alpha amylase. Cereal Chem. **49**: 354-364.

GASSNER, G. 1955. Microskopische Untersuchung Pflanzlicher
    Nahrungs und Genussmittel. Verlag von Gustav Fischer,
    Stuttgart.

GIFFORD, D. J. 1987. An electrophoretic analysis of the seed
    proteins from *Pinus monticola* and eight other species
    of pine. Can. J. Bot. **66**: 1808-1812.

GOLDSTEIN, J. I., NEWBURY, D. E., ECHLIN, P. JOY, D. C.,
    FIORI, C., and LIFSHIN, E. 1981. Scanning Electron
    Microscopy and X-ray Analysis: A Text for Biologists,
    Materials Scientists, and Geologists. Plenum Press, New
    York.

GRAF, E. 1986. Phytic Acid: Chemistry and Applications.
    Pilatus Press, Minneapolis.

GRAY, D., and HUGHES, J. C. 1979. Tuber quality. *In* The

Potato Crop, the Scientific Basis for Improvement. *Edited by* P. M. Harris. Chapman and Hall, London. pp. 504-544.

HARDIN, J. W., and ARENA, J. M. 1974. Human Poisoning from Native and Cultivated Plants. Duke University Press, Durham.

HARGIN, K. D., MORRISON, W. R. and FULCHER, R. G. 1980. Triglyceride deposits in the starchy endosperm of wheat. Cereal Chem. **57:** 320-325.

HARRIS, N. and BOULTER, D. 1976. Protein body formation in cotyledons of developing cowpea (*Vigna unguiculata*) seeds. Ann. Bot. **40:** 739-744.

HARTIG, T. 1855. Über das Klebermehl. Botan. Z. **13:** 881-882

HARWOOD, J. L., and STUMPF, P. K. 1972. Fat metabolism in higher plants. XLIX. Fatty acid biosynthesis by subcellular fractions of higher plants. Lipids **7:** 8-19.

HAYAT, M. A. 1981. Fixation for Electron Microscopy. Academic Press, New York.

HAYWARD, H. E. 1938. The Structure of Economic Plants. Macmillan, New York.

HEISER, Jr. C. H. 1969. Nightshades, the Paradoxical Plants. W. H. Freeman and Company, San Francisco.

HESSAYON, D. G. 1985. The Indoor Plant Spotter. Jarrold & Sons Ltd., Norwich.

HEVESEY, G., and LEVI, H. 1936. Action of neutrons on the rare earth elements. Mat.-fys. Medd. (K. Danske Videnske, Selsk.) **14(5):** 1-34.

HEYDORN, K. 1984. Trace Elemental Research, Vol. 1. CRC Press, Inc. Boca Raton, Florida.

HICKEY, M., and KING, C. 1981. 100 families of flowering plants. Cambridge University Press, Cambridge.

HOFSTEN, A. V. 1973. X-ray analysis of microelements in seeds of *Crambe abyssinica*. Physiol. Plantarum **29:** 76-81.

HOLLINGER, G., and MARCHESSAULT, R. H. 1975. Ultrastructure of acid- and enzyme-modified cross-linked potato starch. Biopolymers **14:** 265-276.

HOOD, L. F. 1982. Current concept of starch structure. *In* Food Carbohydrates. *Edited by* D. R. Linebeck and G. E. Inglett. Avi Publishing Co., Inc., Westport, Conn. pp. 217-236.

HUMASON, G. L. 1972. Animal Tissue Techniques. Third Edition. *Edited by* D. Kennedy and R. B. Park. W. H. Freeman and Company, San Francisco.

JOHANSEN, D. A. 1940. Plant Microtechnique. McGraw-Hill Book Company, Inc., New York.

KEUSCH, L. 1968. Die Mobilisierung des Reservemannans im keimenden Dattelsamen. Planta **78:** 321-350.

KIRK, J. T. O., and TILNEY-BASSETT, R. A. E. 1967. The nature of plastids. *In* The Plastids, Their Chemistry, Structure, Growth, and Inheritance. *Edited by* R. Emerson *et al*. W. H. Freeman and Company. London and San Francisco. pp. 1-91.

KOCH, R. C. 1960. Activation Analysis Handbook. Academic Press, New York.

KOOIMAN, P. 1967. The constitution of amyloid from seeds of *Annona muricata* L. Phytochemistry **6:** 1665-1673.

LAG, J., and STEINNES, E. 1978. Contents of some trace elements in barley and wheat grown in Norway. Meld. Nor. Landbrukshoegsk. **57:** 1-11.

LANGER, R. H. M., and HILL, G. D. 1982. Agricultural Plants. Cambridge University Press, Cambridge.

LEWIS, P. R., and KNIGHT, D. P. 1977. Staining Methods for Sectioned Material. *Edited by* A. M. Glauert. North-Holland Publishing Company, Amsterdam.

LIENER, I. E. 1969. Toxic Constituents of Plant Foodstuffs. Academic Press, New York.

LOLAS, G. M., and MARKAKIS, P. 1975. Phytic acid and other phosphorus compounds of beans (*Phaseolus vulgaris* L.). J. Agric. Food Chem. **23:** 13-15.

LOTT, J. N. A. 1975. Protein body composition in *Cucurbita maxima* cotyledons as determined by energy dispersive X-ray analysis. Plant Physiol. **55:** 913-916.

LOTT, J. N. A. 1980. Protein bodies. *In* The Biochemistry of Plants, A Comprehensive Theatise. *Edited by* N. E. Tolbert. Academic Press, New York. pp. 589-623.

LOTT, J. N. A. 1981. Protein bodies in seeds. Nord. J. Bot. 1: 421-432.

LOTT, J. N. A. 1985. Accumulation of seed reserves of phosphorus and other minerals. *In* Seed Physiology. Vol. 1. *Edited by* D. R. Murray. Academic Press, Sydney. pp. 139-166.

LOTT, J. N. A., and BUTTROSE, M. S. 1978a. Globoids in protein bodies of legume seed cotyledons. Aust. J. Plant Physiol. 5: 89-111.

LOTT, J. N. A., and BUTTROSE, M. S. 1978b. Thin sectioning, freeze fracturing, energy dispersive X-ray analysis, and chemical analysis in the study of inclusions in seed protein bodies: almond, Brazil nut, and quandong. Can. J. Bot. 56: 2050-2061.

LOTT, J. N. A., CARSON, J., and KERR, P. 1985a. Cryogenic preparation of samples for scanning electron microscopy. Microc. Soc. Can. Bull. 13: 5-9.

LOTT, J. N. A., GOODCHILD, D. J., and CRAIG, S. 1984. Studies of mineral reserves in pea (*Pisum sativum*) cotyledons using low-water-content procedure, Aust. J. Plant Physiol. 11: 459-469.

LOTT, J. N. A., GREENWOOD, J. S., and VOLLMER, C. M. 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 Physiol. 61: 984-988.

LOTT, J. N. A., GREENWOOD, J. S., and VOLLMER, C. M. 1982. Mineral reserves in castor beans: the dry seed. Plant Physiol. 69: 829-833.

LOTT, J. N. A., and KERR, P. 1986. Use of cryogenically prepared samples in the scanning electron microscopic study of dry-to-wet transitions. Scanning Electron Microscopy 1986/III: 979-986.

LOTT, J. N. A., LARSON, P. L., and DARLEY, J. J. 1971. Protein bodies from the cotyledons of *Cucurbita maxima*. Can. J. Bot. 49: 1777-1782.

LOTT, J. N. A., and OCKENDEN, I. 1986. The fine structure of phytate-rich particles in plants. *In* Phytic Acid: Chemistry and Applications. *Edited by* E. Graf. Pilatus Press, Minneapolis. pp 43-55.

LOTT, J. N. A., RANDALL, P. J., GOODCHILD, D. J., and CRAIG,

S. 1985b. 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. Ann. J. Plant Physiol. **12**:
341-353.

LOTT, J. N. A. and SPITZER, E. 1980. X-ray analysis studies
of elements stored in protein body globoid crystals of
Triticum grains. Plant Physiol. **66**: 494-499.

LOTT, J. N. A., SPITZER, E., and VOLLMER, C. M. 1979.
Calcium distribution in globoid crystals of *Cucurbita*
cotyledon protein bodies. Plant Physiol. **63**: 847-851.

LOTT, J. N. A. and VOLLMER, C. M. 1979a. Composition of
globoid crystals from embryo protein bodies in five
species of *Cucurbita*. Plant Physiol. **63**: 307-311.

LOTT, J. N. A. and VOLLMER, C. M. 1979b. An energy
dispersive X-ray analysis study of protein bodies in
the seeds of *Astragalus bisulcatus* and *Oxytropis
Lambertii*. Can. J. Bot. **57**: 987-992.

LUI, N. S. T., and ALTSCHUL, A. M. 1967. Isolation of
globoids from cotton seed aleurone grain. Arch.
Biochem. Biophys. **121**: 678-684.

MAGA, J. A. 1982. Phytate: its chemistry, occurrence, food
interactions, nutritional significance, and methods of
analysis. J. Agric. Food Chem. **30**: 1-9.

MAHESHWARI, P. 1950. An Introduction to the Embryology of
Angiosperms. McGraw-Hill Book Company, Inc., New York.

MARSHALL, A. T. 1977. Electron probe x-ray microanalysis of
frozen hydrated biological specimens. Microsc. Acta **79**:
254-266.

MARSHALL, A. T. 1980. Principles and instrumentation. *In* X-
ray Microanalysis in Biology. *Edited by* M. A. Hayat.
University Park Press, Baltimore. pp. 1-64.

MEEK, G. A. 1976. Practical Electron Microscopy for
Biologists. John Wiley & Sons, London.

MEIER, H. 1958. On the structure of cell walls and cell wall
mannans from ivory nuts and from dates. Biochem.
Biophys. Acta **28**: 229-240.

MEIER, H., and REID, J. S. G. 1982. Reserve polysaccharides
other than starch in higher plants. *In* Encyclopedia of
Plant Physiology, New Series, vol. 13A. Plant

Carbohydrates I. *Edited by* F. A. Loewus and W. Tanner. Springer-Verlag, Berlin. pp. 418-471.

MORGAN, A. J. 1980. Preparation of specimen. *In* X-ray Microanalysis in Biology. *Edited by* M. A. Hayat. University Park Press, Baltimore. pp. 65-166.

MUENSCHER, W. C. 1935. Weeds. The MacMillan Company, New York.

MUKHAMEDOVA, K. S., and AKRAMOV, S. T. 1977. Amounts of phospholipids and phytin in the seeds of various plants. Chem. Nat. Cmpd. **13**: 422-424.

MUNCK, L., KARLSSON, K-E., and HAGBERG, A. 1971. Selection and characterization of a high-protein, high-lysine variety from the world collection. Symposium: Barley Genetics. Washington Univ. Press, Pullman, Washington (1969).

MURRAY, D. R. 1984. Accumulation of seed reserves of nitrogen. *In* Seed Physiology. vol 1: Development. *Edited by* D. R. Murray. Academic Press, Sydney. pp. 83-137.

NAHAPETIAN, A., and BASSIRI, A. 1976. Variations in concentrations and interrelationships of phytate, phosphorus, magnesium, calcium, zinc, and iron in wheat varieties during two years. J. Agric. Food Chem. **24**: 947-950.

NETOLITZKY, F. 1926. Anatomie der Angiospermen-Samen. *In* Handbuch der Pflanzenanatomie. *Edited by* K. Linsbauer. Verlag von Gebrüder Borntraeger, Berlin. **10**: 279-283.

O'BRIEN, T. P., and MCCULLY, M. E. 1981. The Study of Plant Structure, Principles and Selected Methods. Termarcarphi Pty Ltd., South Melbourne.

OCKENDEN, I., and LOTT, J. N. A. 1988. Mineral storage in *Cucurbita* embryos. I. Calcium storage in relation to embryo size. Can. J. Bot. **66**: 1477-1481.

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. Can. J. Bot. **68**: 646-650.

O'DELL, B. L., deBOLAND, A. R., and KOIRTYOHANN, S. R. 1972. Distribution of phytate and nutritionally important elements among morphological components of cereal grains. J. Agri. Food Chem. **20**: 718-721.

OGAWA, M., TANAKA, K., and KASAI, Z. 1979. Energy dispersive x-ray analysis of phytin globoids in aleurone particles of developing rice grains. Soil Sci. Plant Nutr. 25: 437-448.

OSBORNE, T. B. 1924. The Vegetable Proteins. 2nd Ed. Longmans Green, London.

PEARSE, A. G. E. 1980a. Histochemistry, Theoretical and Applied. Vol. 1. Preparative and Optical Technology. Churchill Livingstone, Edinburgh.

PEARSE, A. G. E. 1980b. Histochemistry, Theoretical and Applied. Vol. 2. Analytical technology. Churchill Livingstone, Edinburgh.

PERL, M., and FEDER, Z. 1981. Improved seedling development of pepper seeds *Capsicum annuum* by seed treatment for pre-germination activities. Seed Sci. Technol. 9: 655-664.

REDDY, B. S., and SAROJINI, G. 1987. Chemical and nutritional evaluation of chili *Capsicum annuum* seed oil. J. Am. Oil Chem. Soc. 64: 1419-1422.

REID, J. S. G., and MEIER, H. 1970. Chemotaxonomic aspects of the reserve galactomannans in Leguminous seeds. Z. Pflanzenphysiol. 62: 89-92.

REST, J. A., and VAUGHAN, J. G. 1970. The development of protein and oil bodies in the seed of *Sinapis alba* L. Planta (Berl.) 105: 245-262.

ROBBINS, W. W. 1917. The Botany of Crop Plants, A Text and Reference Book. Blakiston's Son & Co., Philadelphia.

ROBBINS, W. W., WEIER, T. E. and STOCKING, C. R. 1964. Botany, An Introduction to Plant Science. 3rd Ed. John Wiley and Sons, New York.

ROBUTT, J. L., HOSNEY, R. C. and WASSON, C. E. 1974. Modified Opaque-2 corn endosperm II. Structure viewed with a scanning electron microscope. Cereal Chem. 51: 173-179.

ROOMANS, G. M. 1980. Localization of divalent cations in phosphate-rich cytoplasmic granules in yeast. Physiol. Plant. 48: 47-50.

ROOS, N., and BARNARD, T. 1985. A comparison of subcellular element concentrations in frozen-dried, plastic-embedded, dry-cut sections and frozen-dried

cryosections. Ultramicrosc. **17**: 335-344.

RUSS, J. C. 1972a. Elemental X-ray Analysis of Materials (EXAM) Methods. *Edited by* Charles J. Walsh. EDAX International, Inc., Prairie View, Illinois.

RUSS, J. C. 1972b. Obtaining quantitative X-ray analytical results from thin sections in the electron microscope. *In* Thin-section Microanalysis. *Edited by* J. C. Russ and B. J. Panessa. EDAX Laboratories, North Carolina.

RYLSKI, I. 1986. Pepper (*Capsicum*). *In* CRC Handbook of Fruit Set and Development. *Edited by* Monselise. CRC Press. Inc. Boca Raton, Florida. pp. 341-354.

RYMAL, K. S., SMIT, C. J. B., and NAKAYAMA, T. O. M. 1974. Fatty acid and amino acid composition of the seeds of three cultivars of the tomato, *Lycopersicon esculentum* L. J. Amer. Soc. Hort. Sci. **99**: 12-15.

SACHS, M., CANTLIFFE, D. J., and NELL, T. A. 1981. Germination studies of clay coated sweet pepper *Capsicum annuum* cultivar early-calwonder seeds. J. Am. Soc. Hortic. Sci. **106**: 385-389.

SACHS, M., CANTLIFFE, D. J., and NELL, T. A. 1982. Germination behaviour of sand coated pepper *Capsicum annuum* seed. J. Am. Soc. Hortic. Sci. **107**: 412-416.

SACK, F. D., LEOPOLD, A. C., and HOEKSTRA, F. A. 1988. Structural correlates of imbibitional injury in *Typha* pollen. Amer. J. Bot. **75**: 570-578.

SEIDEMANN, J. 1966. Stärke-Atlas. Paul Parey, Berlin-Hamburg.

SENGUPTA, A., and MAZUMDER, U. K. 1976. Triglyceride composition of tobacco seed oil. J. Am. Oil Chem. Soc. **53**: 680-683.

SHARMA, C. B., and DIECKERT, J. W. 1975. Isolation and partial characterization of globoids from aleurone grains of *Arachis hypogaea* seed. Physiol. Plant. **33**: 1-7.

SHARON, E., OKON, Y., BASHAN, Y., and HENIS, Y. 1982. Detached leaf enrichment, A method for detecting small numbers of pseudomonas-syringae pathovar tomato and xanthomonas-campestris pathovar vesicatoria in seed and symptomless leaves of tomato *Lycopersicon esculentum* cultivar VF-198 and pepper *Capsicum annuum* cultivar MA'OR. J. Appl. Bacteriol. **53**: 371-378.

SIMOLA, L. K. 1987. Developmental changes in the subcellular structure of the seeds of *Atropa belladonna*. Nord. J. Bot. **7**: 67-77.

SKILNYK, H. R. 1990. Structural Studies and Elemental Analysis of *Cucurbita* Pollen. M.Sc thesis, McMaster University, Hamilton, Ontario.

SMITH, C. G. 1974. The ultrastructural development of spherosomes and oil bodies in the developing embryo of *Crambe abyssinica*. Planta **119**: 125-142.

SMITH, C. R., Jr., and WOLFF, I. A. 1966. Glycolipids of *Briza spicata* seed. Lipids **1**: 123-127.

SOUÈGES, R. 1907. Développement et structure du tégument séminal chez les Solanacées. Ann. Sci. Nat. Bot. Ser. IX, **6**: 1-124.

SPITZER, E., and LOTT, J. N. A. 1980. Thin-section, freeze-fracture, and energy dispersive x-ray analysis studies of the protein bodies of tomato seeds. Can. J. Bot. **58**: 699-711.

SPITZER, E. and LOTT, J. N. A. 1982a. Protein bodies in umbelliferous seeds. I. Structure. Can. J. Bot. **60**: 1381-1391.

SPITZER, E. and LOTT, J. N. A. 1982b. Protein bodies in umbelliferous seeds. II. Elemental composition. Can. J. Bot. **60**: 1392-1398.

SPITZER, E., and LOTT, J. N. A. 1982c. Protein bodies in umbelliferous seeds. III. characterization of calcium-rich crystals. Can. J. Bot. **60**: 1399-1403.

SPITZER, E., LOTT, J. N. A., and VOLLMER, C. M. 1980. Studies of metal uptake, especially iron, into protein bodies of *Capsella* and *Lycopersicon* seeds. Can. J. Bot. **58**: 1244-1249.

SPITZER, E., WEBBER, M., and LOTT, J. N. A. 1981. Elemental composition of globoid crystals in protein bodies of wheat grain grown on soil treated with sewage sludge. Can. J. Bot. **59**: 403-409.

SRIVISTAVA, H. C., and SINGH, P. P. 1967. Structure of the polysaccharide from tamarind kernel. Carbohyd. Res. **4**: 326-342.

STEWART, A., NIELD, H. and LOTT, J. N. A. 1988. An investigation of the mineral content of barley grains

and seedling. Plant Physiol. **86:** 93-97.

STUMPF, P. K. 1965. Lipid metabolism. *In:* Plant
    Biochemistry. *Edited by* J. Bonner and J. E. Varner.
    Academic Press, New York. pp. 323-345.

SULTANA, N., KHAN, S. A., and KHANZADA, A. K. 1988. Studies
    on seed-borne mycoflora of chillies and control of
    fruit rot disease. Pak. J. Sci. Ind. Res. **31:** 365-368.

SWIFT, J. G. and BUTTROSE, M. S. 1973. Protein bodies, lipid
    layers and amyloplasts in freeze-etched pea cotyledons
    Planta **109:** 61-72.

TALMON, Y., 1982. Thermal and radiation damage to frozen
    hydrated specimens. J. Microsc. (Oxford) **125:** 227-237.

TALMON, Y., DAVIS, H. T., SCIVEN, L. E., and Thomas, E. L.,
    1979. Mass loss and etching of frozen hydrated
    specimens. J. Microsc. (Oxford) **117:** 321-332.

TOMB, A. S. 1974. SEM studies of small seeds. Scanning
    Electron Micorscopy **1974:** 375-380, 638.

VALKOVIC, V. 1977. Nuclear Microanalysis. Garland
    Publishing, Inc., New York.

VAUGHAN, J. G. 1970. The Structure and Utilization of Oil
    Seeds. Chapman and Hall, London.

WADA, T., and MAEDA, E. 1980. A cytological study on the
    phosphorus accumulating tissues in the gramineous
    seeds. Japanese J. Crop Sci.**49:** 475-481.

WANG, C. H., WILLIS, D. L., and LOVELAND, W. D. 1975.
    Radiotracer Methodology in Biological, Environmental
    and Physical Sciences. Prentice Hall, Inc., Englewood
    Cliffs, New Jersey.

WATKINS, J. T., CANTLIFFE, D. J., HUBER, H. B., and NELL, T.
    A. 1985. Gibberellic acid stimulated degradation of
    endosperm in pepper. J. Amer. Soc. Hort. Sci. **110:** 61-
    65.

WHALEN, M. D. 1979. Speciation in *Solanum*, section
    Androceras. *In* The Biology and Taxonomy of the
    Solanaceae. *Edited by* T. G. Hawkes. Academic Press,
    London. pp. 589-596.

WHITE, M. J., and LOTT, J. N. A. 1983. Protein body
    inclusions in seeds of *Eucalyptus maculata* and
    Eucalyptus erythrocorys. Can. J. Bot. **61:** 1911-1918.

WINTON, A. L. 1916. Microscopy of Vegetable Foods. John Wiley and Sons, New York.

WINTON, A. L., and WINTON, K. B. 1932. The Structure and Composition of Foods. John Wiley and Sons, New York.

WOLFROM, M. L., LAVER, M. L. and PATIN, D. L. 1961. Carbohydrates of the coffee bean II. Isolation and characterization of a mannan. J. Org. Chem. **26:** 4533-4535.

WOOD, P. J. and FULCHER, R. G. 1978. Interaction of some dyes with cereal β-glucans. Cereal Chem. **55:** 952-966.

WURSCH, P. and HOOD, L. F. 1981. Structure of starch from mango seed. Staerke **33:** 217-221.

YATSU, L. Y. 1965. The ultrastructure of cotyledonary tissue from *Gossypium hirsutum* L. seeds. J. Cell Biol. **25:** 193-199.

YEO, A. R., LÄUCHLI, A., KRAMER, D., and GULLASCH, J. 1977. Ion measurements by X-ray microanalysis in unfixed, frozen, hydrated plant cells of species differing in salt tolerance. Planta **134:** 35-38

APPENDICES

APPENDIX 1. GENERAL OVERSIGHT STAINS:

1) SAFRANIN AND FAST GREEN (Johansen, 1940)

      Preparation of solutions:
            Dilute solution of safranin: 2 ml of a stock 2%
            aqueous safranin to 1 liter of water.
            Ammonia alcohol: 4 to 5 drops ammonia in 100 ml of
            70% ethanol.
            Fast green: 0.5% fast green FCF in 70% ethanol.

      Staining procedure:
          a) Hydrate slides in distilled water.
          b) Stain samples in dilute safranin for 30 s.
          c) Rinse slides briefly in 70% picric acid
             alcohol.
          d) Rinse slides briefly in ammonia alcohol.
          e) Stain samples in fast green for 20 s.
          f) Rinse slides twice in absolute alcohol for 10 s
             each.
          g) Clear in xylene and mount slides with DPX
             mounting medium (BDH Ltd.).

2) TOLUIDINE BLUE O (O'Brien and McCurry, 1981)

      Staining procedure:
          a) Stain samples in 0.05% aqueous toluidine blue O
             for 5 min.
          b) Rinse slides in distilled water.
          c) Air dry and mount slides with DPX mounting
             medium.

APPENDIX 2. HISTOCHEMICAL STAINS:

1) PERIODIC ACID-SCHIFF (PAS) REACTION (O'Brien and McCully
   1981, and Humason 1972)

      Demonstration: PAS demonstrates polysaccharides with a
          magenta colour.

Preparation of solutions:
  Aldehyde blockade: Prepare saturated solution of
  dimedon (5,5-dimethycyclohexane-1,3-dione) in
  water.
  Periodic acid solution: 0.6 g of periodic acid,
  0.3 ml of concentrated nitric acid, and 100 ml of
  distilled water.
  Schiff's reagent: 1 g of basic fuchsin, 20 ml of 1
  N HCl, 1 g of sodium metabisulphite, 2 g activated
  charcoal, and 200 ml of distilled water.

Staining procedure:
  a) Place slides in saturated solution of dimedone
    overnight.
  b) Rinse slides thoroughly for 0.5 h in running
    tab water.
  c) Place slides in periodic acid for 10 min.
  d) Rinse slides in distilled water.
  e) Stain samples in Schiff's reagent for 20 min.
  f) Flush slides in running tab water for 10 min.
  g) Dehydrate and mount slides with DPX mounting
    medium.

2) LUGOL'S IODINE (Bronner, 1975)

Demonstration: Lugol's iodine tests for the presence of
  starch, which turns a yellow-brown color.

Preparation of solution:
  Lugol's reagent: 0.5 g of I_2, 1 g of KI, and 100
  ml of distilled water.

Staining procedure:
  a) Stain samples in Lugol's reagent for 5 s.
  b) Rinse slides with tap water for 2 min.
  c) Air dry and mount slides with DPX mounting
    medium.

3) FEULGEN AND FAST GREEN (Humason, 1972)

Demonstration: Schiff's reagent stains DNA a pink
  colour. Fast green is a counterstain for
  cytoplasm.

Preparation of solutions:
  Schiff's reagent: See PAS stain methods.
  Bleaching solution: 10 ml of 1 N HCl, 10 ml of
  sodium bisulphate, and 200 ml of distilled water.
  Fast green: 0.05% fast green FCF in 95% ethanol.

Staining procedure:
  a) Heat both the slides and the 1 N HCl to 60 °C.
  b) Place warm slides in warm 1 N HCl for 10 min at 60 °C.
  c) Rinse slides in 1 N HCl at room temperature for 10 s, then rinse slides in water.
  d) Stain samples in Schiff's reagent for 3 h in the dark.
  e) Place slides in 3 changes of bleaching solution each for 10 s.
  f) Rinse in running tap water for 10 min.
  g) Rinse in distilled water.
  h) Counterstain in fast green for 10 s.
  i) Blot slides dry and mount with DPX mounting medium.

4) SUDAN BLACK B (Bronner, 1975)

Demonstration: Sudan black B stains lipids black.

Preparation of the stain: Prepare a fresh saturated solution of Sudan black B in 70% ethanol. Keep this solution in a closed container for 12 h at 37 °C, filter and use within 24 h.

Staining procedure:
  a) Place Sudan black B solution in an oven at 60 °C for 30 min before staining.
  b) Place slides in 70% ethanol for 1-2 min.
  c) Stain samples in Sudan black B at 60 °C in an oven for 1 h.
  d) Rinse slides in 70% ethanol for 1 min, then rinse in tap water.
  e) Air dry and mount slides mount with DPX mounting medium.

5) NILE BLUE SULPHATE METHOD (Modified from Pearse, 1980b)

Demonstration: The Nile blue sulphate method demonstrates lipids. Unsaturated hydrophobic lipids are stained pink; free fatty acids are stained pink to blue and phospholipids are stained blue.

Preparation of the stain: 10 mg of Nile blue A (Nile blue sulphate) in 100 ml of 0.01% (V/V) aqueous sulphuric acid.

Staining procedure:

1) Stain samples with Nile blue sulphate at 60 °C for 12 h.
2) Differentiate sections in 1% acetic acid for 1 to 2 min.
3) Rinse thoroughly in tap water.
4) Air dry and mount slides with DPX mounting medium.

6) COOMASSIE BRILLIANT BLUE R250 (Gahan, 1984)

Demonstration: Coomassie brilliant blue stains all proteins blue.

Preparation of solutions:
Clarke's solution: 3 parts of absolute ethanol, and 1 part of 1% aqueous acetic acid (pH 2.0).
Coomassie brilliant blue solution: 0.02% Coomassie brilliant blue R250 in Clarke's solution (pH 2.0).

Staining procedure:
a) Stain samples in Coomassie brilliant blue for 24 h.
b) Rinse slides in Clarke's solution.
c) Destain samples in Clarke's solution for 20 min.
d) Dehydrate slides in 98% ethanol and absolute ethanol each for 5 min.
e) Mount slides with DPX mounting medium.

7) MERCURY-BROMOPHENOL BLUE (Pearse, 1980b)

Demonstration: This stain demonstrates proteins. Most proteins stained deep clear blue. Some reddish regions reflect the increase of protein concentration.

Preparation of solutions:
Mercury-bromophenol blue: Dissolve 0.4 g of $HgCl_2$ in 40 ml of 2% aqueous glacial acetic acid, and add 25 mg bromophenol blue sodium salt.
Sodium bicarbonate solution: a few crystals of sodium bicarbonate in distilled water.

Staining procedure:
a) Hydrate slides in distilled water.
b) Stain samples in mercury-bromophenol blue for 15 to 60 min.
c) Wash slides in two changes of 5% aqueous

glacial acetic acid for 5 min each.
d) Place slides in sodium bicarbonate solution until sections turn blue.
e) Rinse slides quickly in distilled water.
f) Air dry and mount slides with DPX mounting medium.

8) ACIDIC TOLUIDINE BLUE O (ATBO) (Ashford *et al.* 1975)

Demonstration: Polyphosphates or sulphate esters are stained metachromatically.

Preparation of solution:
ATBO solution: 0.05% of toluidine blue O in 0.1 N sodium-acetate buffer (pH 1.0).

Staining procedure:
a) Stain samples in acid toluidine blue O for 10 min.
b) Rinse slides in running tap water for 5 min.
c) Air dry and mount slides with DPX mounting medium.

APPENDIX 3. FLUORESCENT STAINS:

1) ACRIFLAVINE HCl (Fulcher, 1982)

Demonstration: Acriflavine HCl is supposed to stain phytin crystals fluorescent red. The chemical basis of the phytin/Acriflavine HCl interaction has not yet been determined.

Preparation of solution:
Acriflavine HCl solution: 0.01% (W/V) Acriflavine HCl in water. Adjust pH to 3.1 with HCl.

Staining procedure:
a) Stain samples in 0.01% Acriflavine HCl for 5-15 min.
b) Rinse slides in ethanol.
c) Air dry and mount slides under cover-glass in fluorescence-free immersion oil.

Examine sections with FC I, FC II, or FC III fluorescence filter combinations.

2) NILE BLUE A (Hargin *et al.* 1980)

Demonstration: Nile blue A is a specific fluorochrome for non-polar lipids, which appear intensely fluorescent yellow when stained with Nile blue A.

Staining procedure:
a) Stain samples in 0.01% (W/V) aqueous Nile Blue A for 30-60 s.
b) Rinse slides briefly in water and mount slides in water.

Examine sections immediately using fluorescence filter combination FC II.

3) CONGO RED (Wood and Fulcher 1978)

Demonstration: Congo red is useful in detecting β-glucan residues. It produces a fluorescent red product with β-glucan residues.

Staining procedure:
a) Stain samples in 0.01% (W/V) Congo red in distilled water for 15 min.
b) Rinse slides in distilled water.
c) Wash slides in running water for 2 min.
d) Air dry and mount samples with immersion oil.

Examine sections with fluorescence filter combination FC I.