STRUCTURAL STUDIES AND ELEMENTAL ANALYSES OF *CUCURBITA* POLLEN

LIGHT MICROSCOPY, ELECTRON MICROSCOPY AND ELEMENTAL ANALYSES OF *CUCURBITA MAXIMA* AND *CUCURBITA ANDREANA* POLLEN.

By

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ABSTRACT

Cucurbita maxima and Cucurbita andreana are closely related species; however, the calcium phytate levels of the Cucurbita seeds differ significantly. Hybridization of the two Cucurbita species is difficult to achieve. The aim of my research was to extensively study the structure of C. maxima and C. andreana pollen and determine whether the pollen of the species differed in their elemental content.

This thesis entails the most comprehensive study of the structure, chemical composition and elemental content of the pollen from a dicot plant. This study of Cucurbita pollen required the use of various light microscopy, electron microscopy and elemental analyses techniques.

Histochemical stains revealed information about the chemical structure of pollen and pollen storage reserves. The primary storage reserve of Cucurbita pollen consisted of starch. Additional storage reserves consisted of protein, small amounts of lipids and mineral reserves.

Additional studies of the external and internal structures of the Cucurbita pollen were carried out with transmission electron microscopy and scanning electron microscopy respectively. Preparation of sections of pollen for electron microscopy was difficult. Sectioning problems arose due to the thick pollen wall, the density of pollen cytoplasm, and inadequate infiltration of epoxy resin into

pollen during processing. C. maxima and C. andreana pollen grains were fixed anhydrously because water-soluble phytates such as potassium phytate are extracted by aqueous fixatives. The cytoplasm of both species contained many small electron-dense globoid particles. Globoid particles were observed in sections of pollen and in samples of ground pollen.

Elemental analysis of globoid particles from Cucurbita pollen was carried out, for the first time, with energy dispersive X-ray (EDX) analysis. EDX analysis spectra of globoid particles from C. maxima and C. andreana pollen were compared. Spectra of Cucurbita pollen globoid particles were similar to spectra from phytate deposits in seeds. EDX analysis of globoid particles of pollen revealed that the globoid particles contained mainly Mg, P and K. EDX analysis spectra of globoid particles of sections of Cucurbita pollen revealed that water soluble materials, likely K phytate, were extracted from sections cut onto water-filled microtome boats. Pollen sections cut onto absolute ethanol-filled microtome boats experienced less extraction of water soluble materials from the sections. Neutron activation analysis of whole pollen grains revealed that the individual concentrations of Mg, K, Ca and P were not significantly different from species to species. The elemental analyses results strongly suggest that phytate is stored in globoid particles of Cucurbita pollen.

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ABBREVIATIONS

EDX - energy dispersive x-ray analysis

FAA - formalin-acetic acid-alcohol

NAA - neutron activation analysis

NBS - National Bureau of Standards

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

PAS - periodic acid Schiff

SEM - scanning electron microscope

TEM - transmission electron microscope

Chapter 1

INTRODUCTION

The Objectives Of This Study :

This thesis reports the results of studies of the structure and elemental content of *Cucurbita* pollen. This is the first detailed ultrastructural study of the cell walls and cytoplasmic contents of *Cucurbita maxima* and *Cucurbita andreana* pollen undertaken using both light microscopy and electron microscopy. Furthermore, this is the first detailed ultrastructural study of pollen from a dicot plant. The objectives of this study are: 1) To carry out structural studies of *C. maxima* and *C. andreana* pollen using the light microscope.

2) To carry out ultrastructural studies of *C. maxima* and *C. andreana* pollen using the scanning electron microscope (SEM) and the transmission electron microscope (TEM).

3) To use energy dispersive X-ray (EDX) analysis and neutron activation analysis (NAA) to analyze the elemental content of *C. maxima* and *C. andreana* pollen and to determine the subcellular localization of mineral nutrient reserves.

4) To compare the EDX analysis spectra of electron dense particles in *C. maxima* and *C. andreana* pollen to EDX

analysis spectra of phytate-containing globoid crystals of C. maxima and C. andreana seeds.

Phytate functions as one of the storage reserves in Cucurbita seeds and other species of seeds. Phytate is a salt which consists mainly of phytic acid and cations. Phytic acid serves several important physiological functions in plants and is the major phosphorous reserve in seeds (Graf, 1986).

History and Chemistry of Phytic Acid :

Pfeffer encountered phytate over a century ago, in 1872. Pfeffer's chemical studies revealed that phytate was an "inositol hexaphosphate" but he was uncertain of its exact structure (Pfeffer, 1872; Cosgrove, 1966; Oberleas, 1973). The structure of phytic acid was first determined by Anderson in 1914, almost 50 years after phytic acid was discovered (Graf, 1986). It was not until 20 years ago, that the exact structure of phytic acid was established by Johnson and Tate (1969). Johnson and Tate, (1969) used ^{31}P NMR to identify the structure of phytic acid; their studies confirmed the myo-inositol hexaorthophosphate structure proposed by Anderson in 1914.

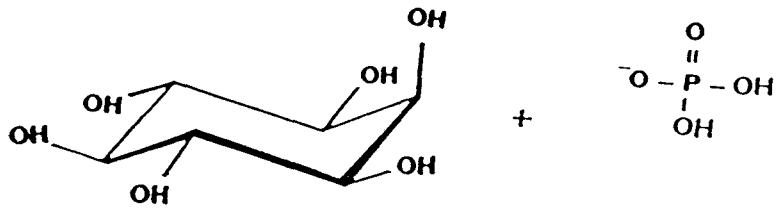
Phytic acid known as myo-inositol hexaphosphoric acid is the esterification product of myo-inositol and phosphate. Phytic acid favours the chair conformation (Posternak, 1965). As a result of this conformation, it is strongly negatively charged and has a tremendous potential

for complexing with positively charged ions and proteins (Figure 1.1). Cations such as Mg^{+2}, K^{+1}, Ca^{+2}, Zn^{+2}, and proteins can commonly bind to form insoluble salts called phytin or phytate. The salt is also referred to as myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) (IUPAC-IUB, 1968; Lolas et al, 1976; Graf, 1986). Mn, Fe, Cu, and Na have been found in some sources of phytate; however, they are not so common (Bewley and Black, 1985). Binding of the positively charged ions and proteins occurs due to the ionization of the phosphate hydrogen of phytic acid (Salisbury and Ross, 1985).

Salts of myo-inositol hexaphosphate can be obtained from the naturally occurring storage reserves of seeds and grains or they can be prepared synthetically, as was demonstrated by Posternak (Posternak, 1965; Cosgrove, 1966). Phytate can occur with various cation combinations.

Occurrence of Phytic Acid :

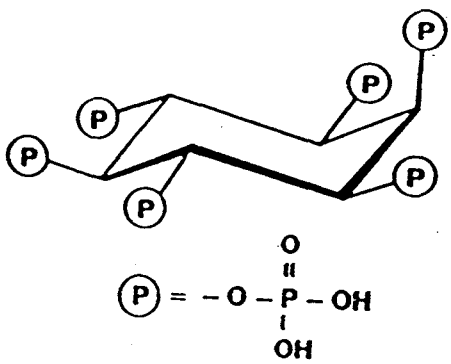
1-3% of the dry weight of all nuts, cereals, oil seeds, spores and pollen consists of phytic acid (Graf, 1986). In a seed there are four groups of phosphorus-containing compounds: 1) phytic acid, 2) nucleic acids, 3) lipids and 4) proteins. Phytic acid, myo-inositol hexaphosphoric acid, serves as one of the major phosphate reserves in seeds of various species. In many species the proportion of seed phosphorus associated with phytic acid can be 80% (Dalling and Bhalla, 1984). Phytin is an



MYO-INOSITOL PHOSPHATE

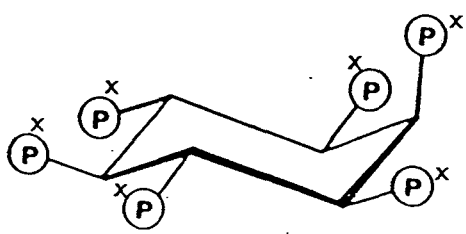
1. Esterification:

PHYTIC ACID



2. Ionization of phosphate H:

$H = H^+ + e^-$ $+ Ca^{2+}, Mg^+, K^+ = x$



PHYTIN or PHYTATE

(Graf, 1986)

Figure 1.1 Structure of Phytic Acid

Phytic acid, known as myo-inositol hexaphosphoric acid is the esterification product of myo-inositol and phosphate. Phytate favours the chair conformation. It is strongly negatively charged and has a tremendous potential for complexing with cations. Ionization of the phosphate hydrogen allows cations to bind and form salts called phytin or phytate.

insoluble salt recognized as being nutritionally undesirable for humans because it can bind essential dietary minerals (Fulcher et al, 1981; Maga, 1982; Serraino and Thompson, 1984; Bewley and Black, 1985; Graf, 1986). However, its occurrence in dietary fiber is believed to reduce colon cancer (Graf and Eaton, 1985). Phytic acid serves as a powerful inhibitor of iron-mediated production of hydroxyl radicals, a hazardous oxidant. Thus phytate effectively blocks the generation of dangerous oxygen species (Graf and Eaton, 1985).

Phytin acts as a storage compound for myo-inositol, phosphorus and cations. Phytic acid serves several important physiological functions in plants such as antioxidant protection during dormancy, storage of phytate, storage of cations, and storage of cell wall precursors. Thus phytic acid is a valuable seed reserve which is degraded during seed germination to help nourish the developing seedling.

Phytate is found in globoid crystals within protein bodies. In cereals, generally 1% of the grain weight is phytin which is localized in globoid inclusions within protein bodies of vacuolar origin (Dalling and Bhalla, 1984). The composition of phytate may vary with tissue type and with species (Lott, 1975; Maga, 1982). It is generally agreed that very little is understood with regard to the biosynthesis and degradation of phytate (Greenwood and Bewley, 1984; Graf, 1986). However, it is known that: (1)

phytin biosynthesis occurs in the seed tissue in which it accumulates; (2) phytin degradation is highly regulated and influenced at transcriptional, translational and catalytic levels (Scott and Loewus, 1986).

Currently there are two theories for phytin biosynthesis. It has been proposed by some workers that protein bodies are the sites of phytic acid biosynthesis while others favour biosynthesis in association with the endoplasmic reticulum. There is some evidence that phosphorylation of myo-inositol can occur in isolated protein bodies (Loewus, 1983). However, studies of the castor bean by Greenwood and Bewley (1984) suggest that the initial biosynthesis of phytin occurs in the cytoplasm, possibly in association with the cisternal endoplasmic reticulum. Phytin is then transported to the protein bodies where it will condense to form globoid crystals.

Phytic Acid in Cucurbits :

Research on phytic acid in cucurbits was first carried out on the seed tissues of C. digitata, C. palmata and C. foetidissima, which are wild gourds (Bolley and McCormack, 1952). It is believed that the phytin in these cucurbits is stored in the proteinaceous matrix of the protein bodies such that protein-phytin complexes exist in these seeds (Bolley and McCormack, 1952).

Early seed research on cucurbits, by Lott et al (1971), showed that C. maxima cotyledons contained protein

bodies which consisted of proteinaceous matrix, protein crystalloid, soft globoid, and globoid crystal regions. Further research on C. maxima cotyledons has been carried out using transmission electron microscopy and scanning electron microscopy combined with energy dispersive X-ray (EDX) analysis. Mineral reserves usually occur in naturally electron-dense globoid crystals and are generally considered to be largely phytate. EDX analysis has often been used to analyze the mineral compositions of the globoid crystals of cucurbit seeds (Lott et al, 1971; Lott, 1975; Lott et al, 1978a; Lott et al, 1978b; Lott and Vollmer, 1979; Lott et al, 1979; Lott, 1981; Lott et al, 1982; Lott, 1983; Lott and Ockenden, 1986). EDX analysis of cucurbit cotyledons revealed that globoid crystals usually contain P, K, Mg and trace amounts of Ca.

Domestic C. maxima seeds are three times longer than the seeds of the wild species, C. andreana. Mineral analyses have shown that C. andreana embryos contain about three times more Ca than their counterpart (Ockenden, 1987). These species can be hybridized by pollinating the C. andreana stigma with C. maxima pollen; however, the reverse pollination is more difficult to achieve. This may be due to differences in the pollen grains or due to incompatibility between the species (Mulcahy et al, 1985).

Phytic Acid in Pollen :

Seeds and pollen possess similar properties such as a high storage reserve content, capacity to withstand an interruption in the growth cycle known as dormancy, the ability to resume growth through germination when conditions are favourable, the ability to grow for some time with little if any nutrients, and they both undergo dehydration during maturation followed by subsequent hydration. However, seeds and pollen exhibit enough differences in their structures and functions that analogies are avoided (Scott and Loewus, 1986b).

Pollen contains the male gametes which are produced in anthers of flowering plants. The function of pollen is to transfer genetic information from the male parent to the offspring during fertilization. Pollen generally consists of a large vegetative cell within which a small generative cell is found. Sizes of pollen grains range from 10 to 200 μm in diameter but the average size is 30 μm. The shapes of pollen vary from spherical, oval, cubic, hexahedral and fibrous to other shapes (Echlin, 1968; Iwanami et al, 1988). Pollen from most species is shed at the binucleate stage prior to sperm formation; however, 30% of pollen is shed at the trinucleate stage following sperm production (Brewbaker, 1967).

EDX analysis has been used extensively on seed tissues to determine their mineral compositions. Recent

studies of mineral reserves in pollen grains have discussed the presence of phytate and phytases (Jackson et al, 1982; Jackson and Linskens, 1982; Helsper et al, 1984; Hara et al, 1985; Dickinson and Lin, 1986; Graf, 1986; Scott and Loewus, 1986a; Scott and Loewus, 1986b; Baldi et al, 1987; Baldi et al, 1988).

Baldi et al (1987), used EDX analysis to show that the spectra from electron-dense particles of Lilium longiflorum pollen resembled spectra from seed phytate. Lines of evidence supporting the occurrence of phytate in Lilium longiflorum pollen are: 1) electron-dense inclusions enclosed by a single membrane; 2) EDX spectra of these inclusions show much higher P, Mg, Ca and K relative to the cytosol; and 3) degradation of the inclusions during germination occurs and this may be due to the utilization of stored materials as happens in seed globoids (Larson, 1965; Crang and Miles, 1969; Sanger and Jackson, 1971a; Southworth and Dickinson, 1981; Cresti et al, 1985a; Dickinson and Lin, 1986; Scott and Loewus, 1986b; Baldi et al, 1987).

Phytic acid is the phosphorus reserve in lily pollen. However, like seeds, not all pollen contains measurable amounts of phytate (Scott et al, 1986b). The amount of phytate in pollen of various gymnosperms and angiosperms has been studied (Jackson et al, 1982). Phytate accumulation occurs late in pollen development well after tetrad formation (Scott et al, 1986b). When pollen grains land on

the stigma they give rise to pollen tubes which will grow down into the style. Once a pollen tube reaches the ovule, it penetrates the ovule and discharges its sperm cells into the ovule (Figure 1.2). It is thought that the amount of phytate present in a pollen grain is related to the length of the style that must be penetrated by the pollen tube and the need for pollen tube wall formation (Jackson *et al*, 1982).

It is because there are studies that support the occurrence of phytin in *Lilium longiflorum* and pollen of other species of plants, as well as the fact that detailed studies have investigated phytate in *Cucurbita* seeds, that the aim of this study is to determine whether *Cucurbita* pollen grains contain phytate reserves. *Cucurbita* pollen serves as a good system to investigate because little work has been done on dicot pollen but a lot of phytate research has been carried out with *Cucurbita* seeds. Thus *Cucurbita* pollen serves as a good system to test for phytate. The research for this thesis forms not only the first detailed study of the fine structure of *Cucurbita* pollen, but also provides the most rigorous study to date of mineral nutrient storage in dicot pollen. The structures of *C. maxima* and *C. andreana* pollen were investigated by light microscopy and electron microscopy. The storage reserves of the pollen were investigated using a scanning electron microscope equipped with an EDX analysis system.

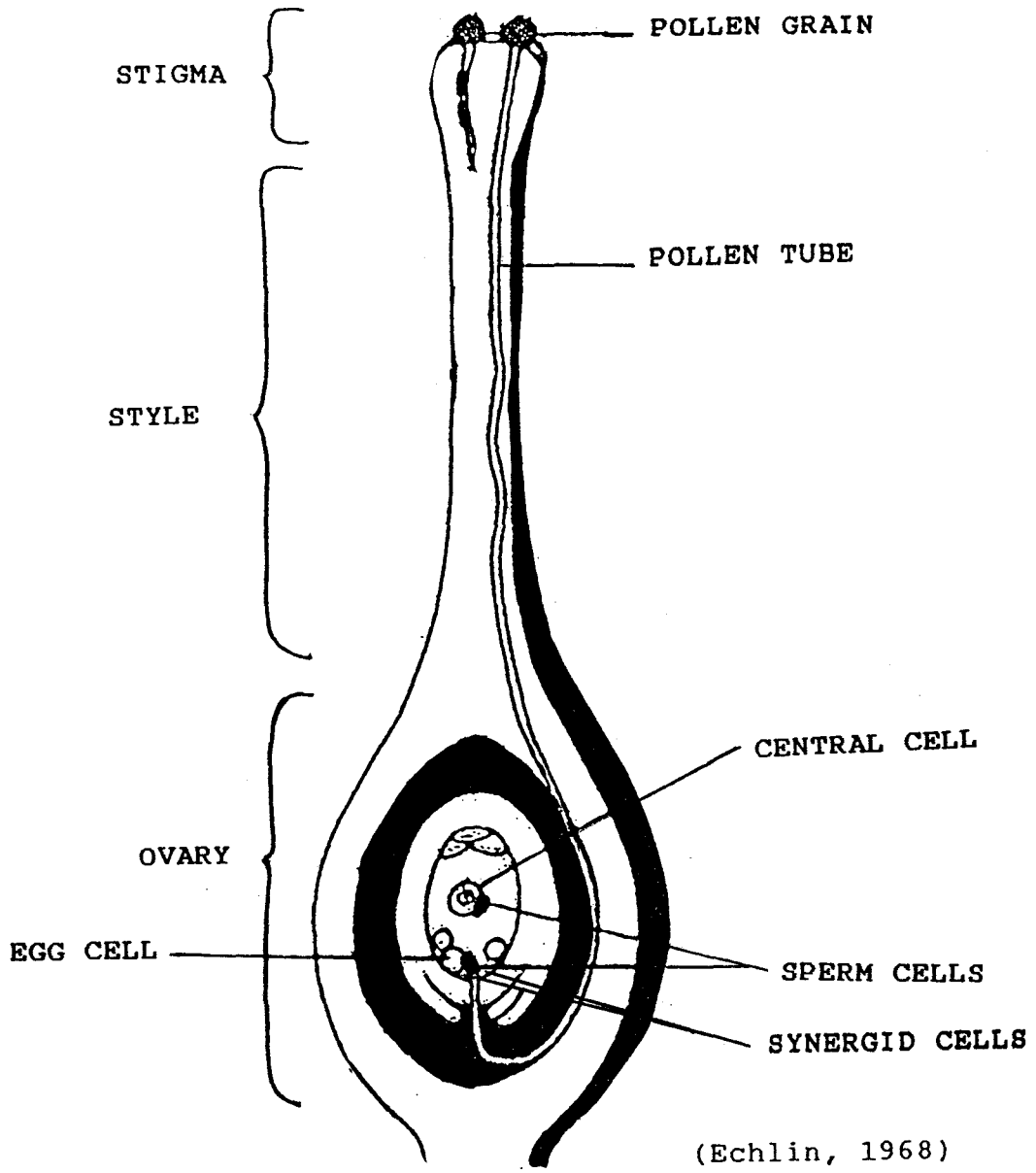


Figure 1.2 Germination of Pollen
Mature pollen grains germinate upon making contact with the stigma. If the pollen is compatible, a pollen tube will form and penetrate the stigma. The pollen tube grows down the style towards the ovary. The generative cell travels dowm the pollen tube. Two sperm cells, formed by the division of the generative cell, are released upon reaching the synergid cells and result in double fertilization.

Conclusion:

Phytate has been found in the pollen of Lilium longiflorum and other species. The storage of phytic acid in lily pollen has been established. However there have been no studies which document the presence of phytic acid in C. maxima or C. andreana pollen. It is of interest to determine whether the pollen grains of these particular Cucurbita species also store phytate.

Phytate is an important phosphorus, carbohydrate, and cation reserve in seeds. Its occurrence in Cucurbita seeds has been well documented. Phytate can occur with various cation combinations. Generally phytate is stored in globoid crystals within protein bodies. C. maxima and C. andreana possess seeds of different sizes. Recent studies have shown that the smaller embryos of C. andreana store more Ca than the larger C. maxima embryos. Hybridization of these species can occur; however, fertilization of the C. andreana embryo sac by C. maxima sperm occurs more frequently than the reciprocal fertilization. The difficulty in achieving the reciprocal fertilization may be due to differences in the pollen grains so studies reported here were undertaken to see if the pollen grains of these two Cucurbita species had differences in mineral storage.

This thesis is organized as follows: Chapter 2: Light Microscopy and Histochemistry, Chapter 3: Electron Microscopy, Chapter 4: Elemental Analyses, Chapter 5:

Discussion. Chapters 2, 3, and 4 include introduction, methods and results sections. The discussions for these three chapters are presented in Chapter 5.

Chapter 2

LIGHT MICROSCOPY AND HISTOCHEMISTRY.

INTRODUCTION

Light microscopical and histochemical studies serve as an excellent means of demonstrating the morphology and chemical composition of both botanical and zoological specimens. However, more often than not, botanical techniques were borrowed from zoological techniques. It was not until botanical scientists stopped borrowing methods from clinical laboratories, that botanical methods became more refined (Johansen, 1940). In most cases, each plant species must be treated individually to ensure that the specimens are prepared in a manner which best suits their preservation. Suitable specimen preservation is often determined by trial-and-error; upon achieving a good level of preservation, various structural and histochemical studies can be undertaken.

Historical Background :

The first cellular structures to be studied by the light microscope were cork cells observed by Hooke in 1655. However, interest in the chemical structure as opposed to morphology of the cell did not commence until the early 1800's. The earliest known publication of a histochemical study was written by a botanist named Raspail in 1825

(Gahan, 1984). Histochemical investigations were primarily botanical until studies on animal tissues commenced in 1856 (Pearse, 1985a).

By the end of the eighteenth century histochemistry was divided into physiology and biological chemistry (Pearse, 1985a). However, it has only been over the last two decades that plant histochemistry has really made an impact on the study of cell and tissue chemistry (Gahan, 1984). Although advances have been made in plant histochemistry, very few histochemical studies have been carried out on pollen. As a result of neglect in this area, the number of structural studies of pollen cytoplasm available, let alone the number of histochemical studies, is limited (Johri and Vasil, 1961; Hoefert, 1967; Mulcahy et al, 1985; Vasil, 1987).

Why Histochemistry ? :

Histochemistry serves as a means of determining the chemical composition of cellular components. Histochemistry is the study of the chemical properties and physical properties of animal and plant cells, or their structural components (Thompson, 1966). It mostly involves the application of stains which localize constituents within the cell. Stains not only outline the general morphology of the tissue being studied but enable one to determine the chemical structure of the tissue of interest. In my studies the specificity of histochemical stains has allowed me to

determine the kinds of storage reserves that are present inside pollen grains.

General oversight stains differ from histochemical stains in that they are applied to sections to determine the basic structure of cells. For example, the Giemsa stain usually stains cells indiscriminately and has little or no selective differentiation (Johansen, 1940). The application of histochemical stains and general oversight stains to plastic sections for light microscopy can yield a lot of information regarding the chemical composition of the cell as well as structural information respectively.

There have been very few histochemical or structural studies of pollen cytoplasm. One of the main and most obvious reasons for the lack of studies of pollen may be the difficulty in preserving pollen for light microscopy and electron microscopy. Heslop-Harrison (1968a) described the problems encountered with preparation of pollen for structural studies. Pollen is difficult to fix and often there is loss of material from the specimen during fixation, embedding and sectioning.

Mature pollen grains readily take up water *in vitro* and *in vivo* (Vasil, 1960; Stanley and Linskens, 1965; Heslop-Harrison, 1986; Vasil, 1987). This uptake of water is essential for the initiation of pollen germination, however pollen grains of most species do not germinate well in water alone and the pollen tubes formed are rather short (Vasil,

1987). Water enters the pollen grain via the apertures or pores on the surface of the pollen grain (Heslop-Harrison, 1986). When the water potential of the germination medium or stigma exudate is greater than that of the vegetative cell, the hydrostatic pressure builds up in the grain and this pressure is relieved by either bursting or emergence of the pollen tube (Heslop-Harrison, 1986). Frequent bursting of pollen grains and pollen tubes, due to the uptake of water, is a common annoyance and perplexity in research on the culture of pollen as well as in preparation of pollen grains for microscopical studies (Heslop-Harrison, 1986; Vasil, 1987).

Formalin-acetic-alcohol (FAA) was used by Vasil (1960) to fix Cucurbita pollen, and by Raghavan (1988) to fix rice, Oryza sativa, pollen and anthers. FAA is an aqueous fixative but it penetrates the specimen quickly. Since it is an aqueous fixative, water soluble materials such as K-phytate would be extracted from the cytoplasm, as has been observed with other aqueous fixatives (Lott et al, 1984).

FAA is a coagulant fixative (O'Brien and McCully, 1981). Coagulant fixatives usually contain ethanol and acetic acid which may denature albumins, forming a precipitate of fibrous protein strands. An advantage to preparing specimens with coagulative fixatives is that cells prepared with these fixatives take up the histochemical stains better than cells that have been fixed in

glutaraldehyde (O'Brien and McCully, 1981).

In this study, various general and histochemical stains were used to observe the morphology and chemical structure of Cucurbita maxima and Cucurbita andreana pollen. Emphasis was placed on determining the nature of storage reserves of Cucurbita pollen.

MATERIALS AND METHODS

Source of Pollen Samples :

Pollen of Cucurbita maxima and Cucurbita andreana flowers was studied. Cucurbita maxima and Cucurbita andreana plants were grown in the McMaster University greenhouse at approximately 24°C with natural light combined with artificial light from the beginning of June to the end of October. Pollen was collected from the anthers of well developed flowers, air-dried at room temperature for 3 days and stored in Chromerge-washed glass vials at -20°C until use.

Preparation for Light Microscopy :

The light microscopy fixation procedure entailed a standard aqueous fixation using formalin-acetic-alcohol (FAA), such preparations were stained with various histochemical and general oversight stains.

Preparation of Pollen Sections :

Fixation: Dried C. andreana and C. maxima pollen samples were fixed in formalin-acetic acid-alcohol (FAA) using the Lillie method (Humason, 1972). The FAA fixative was prepared from 100 ml of 70% ethanol, 5 ml of glacial acetic acid, and 5 ml of concentrated formalin. Formalin is a formaldehyde solution containing 37% formaldehyde gas in water (w/v) with 10-15% methanol added to prevent polymerization. Pollen was fixed for 4.5 h at room

temperature in 1 ml of fixative in Eppendorf tubes.

Dehydration: Excess fixative was washed off with 70% ethanol for 1 h. The first dehydration step with 70% ethanol for 1.5 h at room temperature was followed by 95% ethanol for 1.5 h. Samples were left overnight at room temperature in 95% ethanol.

Infiltration: The dehydrated pollen samples were infiltrated following the directions of the JB-4 Embedding Kit (Polysciences, Inc.). The pollen was infiltrated with Solution A which consisted of catalyzed JB-4 formulated water-soluble monomer. The catalyzed JB-4 solution was changed every 24 hours for a total of 7 days.

Embedding: The infiltrated tissue samples were embedded in a mixture of catalyzed Solution A and the activator, Solution B, in plastic Eppendorf tubes. The embedded samples were centrifuged for 2 minutes and sealed with paraffin wax. Then, polymerization was carried out at room temperature for 30 minutes. The polymerized sample pellets were sliced longitudinally and placed in a plastic block molding cup. Aluminum chucks were placed in the molding cups containing the embedding mixture and the pollen samples. The samples were polymerized in the same manner as described above.

Sectioning: The blocks were sectioned on a Sorvall JB-4 Porter-Blum microtome with a glass knife. Section

thickness ranged from 2 to 5 μm. The sections were floated on a waterbath. The sections were dried on glass slides at 40 - 60°C using a Lipshaw microscope slide warming table.

Use of Light Microscopy Stains :

A series of general oversight stains and histochemical stains was applied to the light microscopy sections. The general oversight stains that were used included a polychrome stain, safranin-fast green and Giemsa stain. These stains were employed to show the general structure of the mature pollen grains. Various histochemical stains were used to stain C. andreana and C. maxima pollen sections for the presence of storage reserves such as polysaccharides, proteins, and lipids. Also, stains were used to identify structures of the cytoplasm and cell walls of the pollen grains. Preparation of the stains and staining methods that were applied are outlined below. Light micrographs were taken with a Zeiss RA microscope equipped with a Zeiss MC 63 photomicrographic camera for 35 mm film. The film type used for the light micrographs was Kodacolor Gold 200.

Preparation of Pollen Smears :

Smears of ground pollen were used to confirm the histochemical tests for proteins and lipids. These extra tests were performed on unfixed Cucurbita pollen because lipids and possibly proteins can be extracted during

fixation and dehydration. Oven-dried Cucurbita pollen was ground with a mortar and pestle and passed through two brass sieves. The first sieve was 140 mesh (140 bars per inch) and the second sieve was 300 mesh (300 bars per inch). The ground pollen was stored in acid-washed glass vials in a desiccator until ready for use. Pollen samples were placed in Eppendorf tubes and stained using the procedures outlined below. After staining the pollen samples were centrifuged using a Canlab Biofuge A and excess stain was drawn off with a pipette. Samples were destained by resuspension of pollen and pelleted by centrifugation until all excess stain was removed. Stained pollen was smeared onto a glass slide and air dried. The histochemical tests that were confirmed with pollen smears were the protein stains Coomassie brilliant blue and mercury-bromophenol blue, and the lipid stains Nile blue sulfate and Sudan black B.

1) General Oversight Stains:

i) **Polychrome Stain** (Conn et al, 1965):

Solutions: 5% tannic acid, 1.5% iron alum, orange G in tannic acid 0.5%, 2% zinc chloride, and dilute safranin

1) Hydrate slides with distilled water.
2) Place slides in tannic acid for 10 minutes. Rinse slides in distilled water.
3) Place slides in iron alum for 15 s until a blue-grey precipitate forms. Rinse quickly in distilled water.
4) Stain slides with orange G in tannic acid for 30 min.
5) Rinse slides 2 - 3 times in distilled water.
6) Place slides in $ZnCl_2$ for 2 min.
7) Rinse slides in distilled water.
8) Stain samples in dilute safranin for 4 min and rinse slides in distilled water.

9) Air dry and mount slides.

Site of Action: Polychrome acts as a staining agent for lignin, suberin, cellulose, starch, and cytoplasm.

ii) **Safranin and Fast Green** (O'Brien and McCully, 1981):

Solutions: dilute safranin, dilute fast green

i) safranin - 1 % aqueous safranin O solution.

ii) fast green - 0.05% fast green FCF in 95% ethanol.

1) Hydrate slides with distilled water.
2) Stain slides with safranin for 30 minutes.
3) Rinse slides in water until no safranin bleeds from the sections.
4) Dehydrate the sections through 30, 50, 70 to 95% ethanol for 1 minute each.
5) Counterstain sections in fast green for 10 seconds.
6) Dehydrate through three changes of absolute ethanol.
7) Transfer to xylene, and mount slides.

Site of Action: Safranin stains cutin, tannin deposits and chromatin red and fast green serves as a counterstain for the other cell constituents.

iii) **Modified Jenner-Giemsa Stain** (Humason, 1972):

Solutions: Jenner solution, Giemsa stain, and buffered rinse

i) stock Giemsa stain - Giemsa stain 1 g, 66 ml of methanol, and 66 ml of glycerol.

ii) working Jenner stain - Jenner stain 0.2 g and 100 ml of methanol.

iii) working Giemsa stain - Distilled water 100 ml, 3 ml of methanol, and 2.5 ml of Giemsa stock solution.

1) Stain slides in Jenner stain for 6 minutes.
2) Transfer directly to Giemsa stain for 13 minutes.

3) Rinse for 2 minutes in a buffered rinse.

Site of Action: Giemsa stain is a staining agent for:
nuclear material.
DNA - purple
RNA - blue
Acidophilic Protein - pink

2) Histochemical Stains:

i) **Periodic Acid Schiff (PAS) and Hematoxylin** (Humason, 1972):

Solutions: periodic acid, Schiff's reagent, hematoxylin, acid alcohol, and saturated lithium carbonate

i) periodic acid - Periodic acid 0.6 g, 100 ml of distilled water, and 0.3 ml of nitric acid, concentrated.

ii) Schiff's reagent - Basic fuchsin 1 g, 20 ml of 1 N HCl, 1 g of sodium metabisulphite, 2 g of activated charcoal, and 200 ml of distilled water.

iii) acid alcohol - 10 ml of concentrated HCl in 1000 ml of 70% ethanol

1) Hydrate slides with distilled water.
2) Place slides in periodic acid for 10 min and rinse in water.
3) Put slides in Schiff's reagent for 20 min.
4) Flush slides with running water for 10 min.
5) Stain slides with hematoxylin for 10 minutes.
6) Quickly dip slides in acid alcohol.
7) Place slides in saturated lithium carbonate until the sections turn blue. Rinse in distilled water.
8) Dehydrate and mount slides.

Site of Action: Starch and some polysaccharides of the cell wall stain magenta. Callose and cellulose are not stained. Hematoxylin is a counterstain.

ii) **Lugol's Iodine** (Humason, 1972):

Solution: iodine crystals and potassium iodide in distilled water

1) Stain sections for 5 s in Lugol's solution.
2) Rinse with tap water.
3) Air dry sections and mount slide.

Site of Action: Lugol's iodine is a staining agent which tests for starch.

iii) **Feulgen Reaction** (Humason, 1972)

Solutions: Schiff's reagent, bleaching solution, fast green

i) Schiff's reagent - See PAS stain methods.

ii) bleaching solution - 10 ml of 1 N HCl, 10 ml of sodium bisulfite, and 200 ml of distilled water.

iii) fast green - see safranin and fast green stain methods.

1) Heat slides and 1 N HCl to 60°C.
2) Place slides in 1 N HCl for 10 minutes at 60°C.
3) Rinse slides in cold 1 N HCl for 10 s, then rinse slides in water.
4) Stain in Schiff's reagent for 3 h in the dark.
5) Place in 3 changes of bleaching solution for 10 s.
6) Rinse in running water for 10 minutes.
7) Rinse in distilled water.
8) Counterstain in fast green for 10 s.
9) Blot slides dry and mount.

Site of Action: Schiff's reagent stains DNA containing material pink and fast green is a counterstain for the cytoplasm. Staining of DNA may be faint unless coagulant fixatives have been used (O'Brien and McCully, 1981).

iv) **Nile Blue Method** (Humason, 1972):

Solution: Nile blue sulfate (Nile Blue A), sulfuric acid

i) Nile blue sulfate - 5 mg of Nile blue sulfate in 0.01% sulfuric acid.

1) Hydrate slides in distilled water.
2) Stain in Nile blue A solution for 5 s.
3) Differentiate sections in 1% acetic acid for 2 minutes.
4) Rinse well in distilled water.
5) Air dry and mount slides.

Site of Action: Nile blue sulphate stains unsaturated hydrophobic lipids, free fatty acids and phospholipids blue.

v) **Sudan Black B** (O'Brien and McCully, 1981):

Solutions: Sudan black B and ethanol

i) Sudan black B - Sudan black B in a saturated solution of 70% aqueous ethanol.

1) Stain sections in Sudan black B for 15 minutes.
2) Rinse in 70% aqueous ethanol.
3) Air dry and mount slides.

Site of Action: This method is preferable for methacrylate embedded sections because it is the most sensitive and lipid concentration is likely to be low in most cells. Lipid-containing components appear dark blue to black.

vi) **Plasmal Reaction** (Pearse, 1985b):

Solutions: Schiff's reagent, bisulphite water and mercuric chloride

i) Schiff's reagent - see PAS stain methods.

ii) mercuric chloride - 1% mercuric chloride in water.

iii) bisulphite water - 5 ml 10% bisulphite, 5 ml of 1 N HCl and 100 ml of distilled water.

1) Hydrate sections in distilled water.
2) Place sections in 1% aqueous mercuric chloride for 2 min.
3) Place sections in Schiff's Reagent for 5 min.
4) Rinse in three changes of bisulphite water.
5) Rinse sections in water, dehydrate in ethanol and mount sections onto slides.

Site of Action: Phospholipids appear magenta.

vii) **Coomassie Brilliant Blue R 250** (Gahan, 1984):

Solutions: Coomassie brilliant blue R 250, Clarke's solution

i) Coomassie blue - 0.02% in Clarke's solution (pH 2).

ii) Clarke's (1851) solution - 3 parts of ethanol, 1 part of 1% acetic acid (pH 2.0).

1) Stain sections for 24 h in Coomassie brilliant blue.
2) Rinse in Clarke's solution.
3) Destain in fresh Clarke's solution for 20 minutes.
4) Dehydrate in 98% and absolute ethanol for 5 minutes and mount slides.

Site of Action: Coomassie brilliant blue stains all proteins blue.

viii) **Mercury-Bromophenol Blue** (Pearse, 1985a):

Solutions: Mercuric chloride, glacial acetic acid, bromophenol blue and sodium bicarbonate

i) mercury-bromophenol blue - Dissolve 0.4 g of mercuric chloride in 40 ml of 2% glacial acetic acid and add 25 mg of bromophenol blue.

1) Hydrate sections in water.
2) Stain sections with mercury-bromophenol blue for 15 to 60 minutes at room temperature.
3) Wash in two changes of 0.5% glacial acetic acid for 5 minutes.
4) Place sections in sodium bicarbonate solution until they turn blue.
5) Rinse sections quickly in distilled water.
6) Air dry sections and mount slides.

Site of Action: Most proteins appear deep blue in colour or greyish-blue. In some regions a range of reddish colours may be seen. This reflects the dichromatic property of the dye.

RESULTS

1) GENERAL OVERSIGHT STAINS :

Cucurbita pollen is entomophilous and coloured bright yellow. The mature pollen grains of *Cucurbita maxima* and *Cucurbita andreana* were spherical in shape and porate with several distinct apertures. The pollen of the two *Cucurbita* species studied had approximately the same diameter of 105 µm (Figure 2.1). The pollen cytoplasm was well preserved. The good preservation of the pollen can be attributed to the penetrating power of FAA (Humason, 1972). FAA helped maintain the integrity of the pollen grain by preventing it from bursting during the fixation process.

The pollen sections, stained with the general oversight stains, such as polychrome stain, safranin-fast green, or Giemsa stain, illustrate the overall structure of the pollen grains. A summary of the results of the staining procedures is presented in Table 2.1. Pollen of the two *Cucurbita* species was similar with respect to morphology and structure.

The cell wall of *Cucurbita* pollen was very thick and consisted of two distinguishable layers. These layers were, the inner wall layer, called the intine, and the outer wall layer, called the exine (Figures 2.1, 2.2, 2.3). The wall material in the intine demonstrated complexity because several different components were present. It was much

Table 2.1

Reaction of Cucurbita maxima and Cucurbita andreana Pollen to Various General Oversight and Histochemical stains.

STAIN	SITE OF ACTION	CYTOPLASMIC &/OR NUCLEAR COMPONENTS	INTINE	EXINE
GENERAL OVERSIGHT STAINS :				
Polychrome	cellulose	-	-	-
	suberin & lignin	-	red	-
Safranin-Fast green	cutin	-	red	-
	general	green	-	-
Giemsa	proteins	pink	pink	-
	DNA	purple	-	-
HISTOCHEMICAL STAINS :				
PAS and Hematoxylin	polysac-charides	magenta	magenta	-
Lugol's Iodine	starch	brown-black	-	-
Feulgen & Fast green	sites of DNA	pink	-	-
Nile Blue Sulphate	lipids	blue*	blue	_
Sudan Black B	lipids	black*	_	black
Plasmal Reaction	phospho-lipids	-	magenta	-
Coomassie Brilliant Blue	proteins	blue	-	-
Mercury-Bromophenol Blue	proteins	blue	-	-

* Staining results were confirmed with pollen smears

Figure 2.1 **Mature Cucurbita Pollen Grain**

Light micrograph of mature *Cucurbita andreana* pollen fixed with FAA and stained with Giemsa Stain. *Cucurbita* pollen has a thick wall (W) which consists of two layers, the intine and the exine. Black arrows indicate the apertures, which are the sites from which pollen tubes will emerge during germination. The vegetative nucleus (N) is surrounded by storage material which is comprised mostly of starch. Starch grains are the unstained bodies surrounded by stain. Scale represents 10 μm.

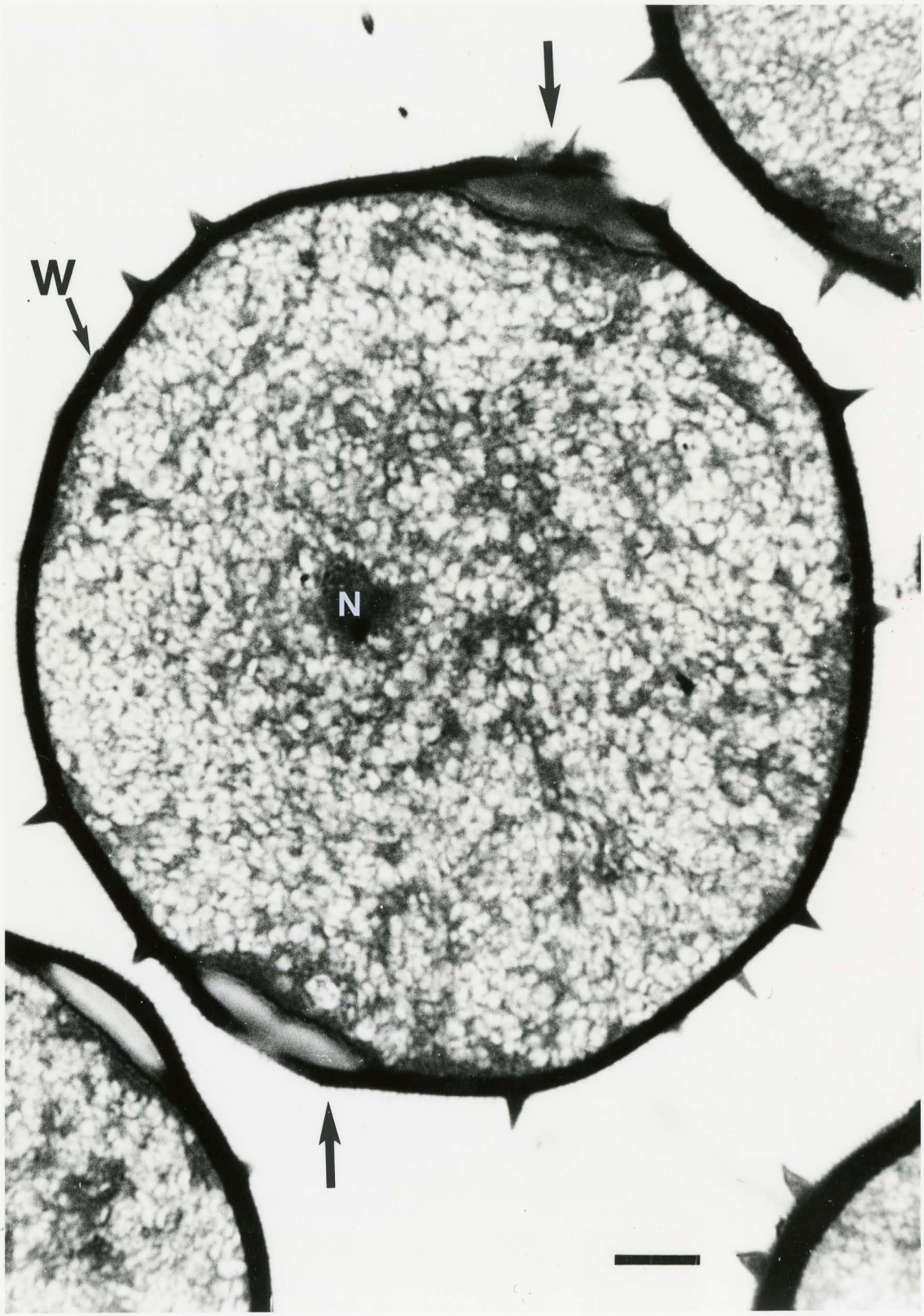
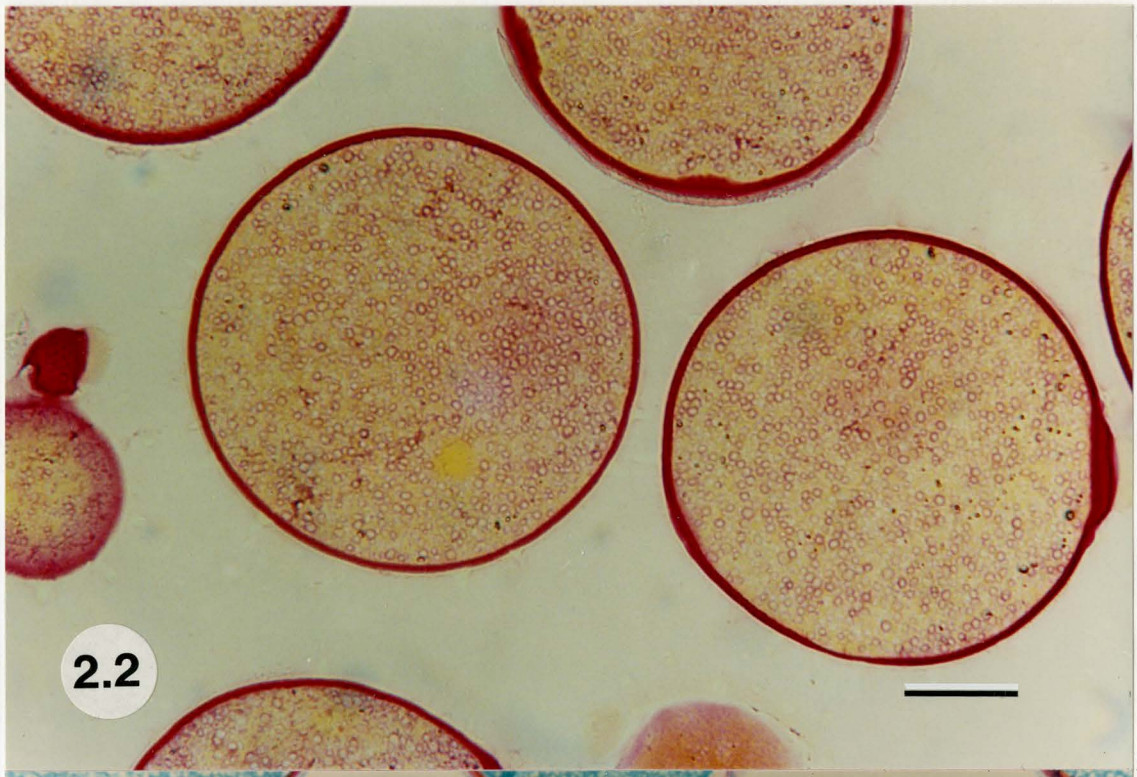
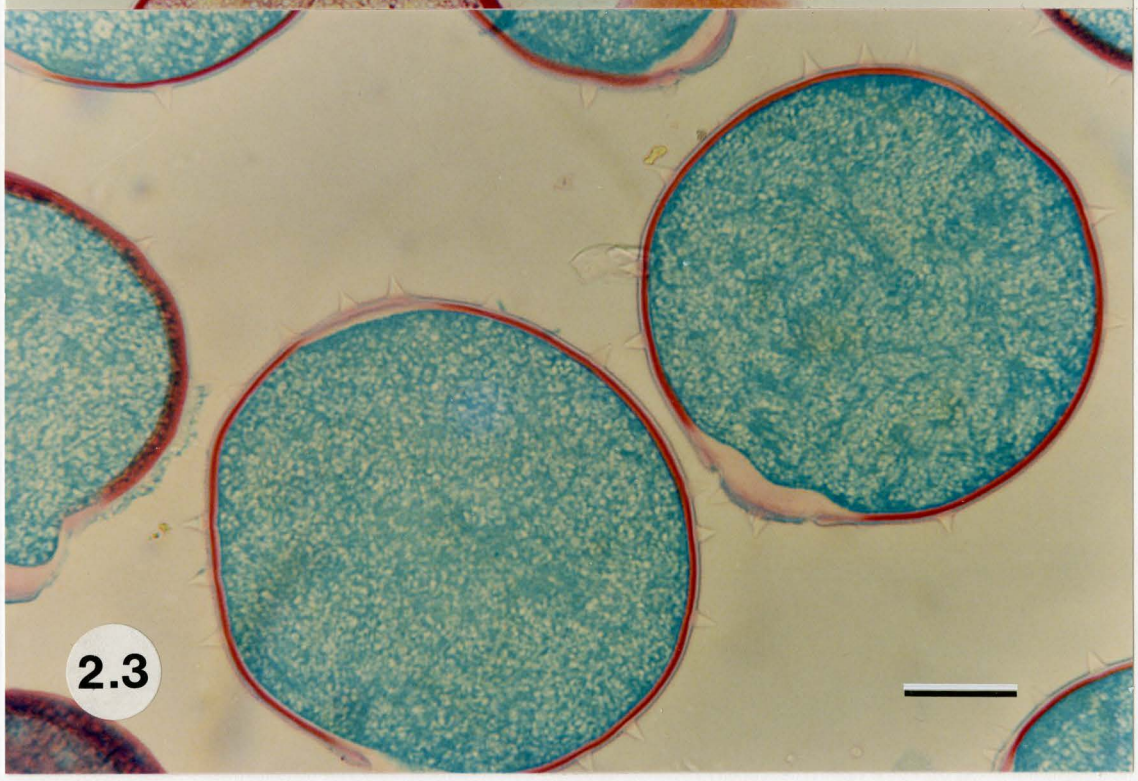

W
N

Figure 2.2 **General Oversight Stain: Polychrome Stain**
Light micrograph of mature C. andreana pollen fixed with FAA and stained with Polychrome stain. The Cucurbita pollen intine contained suberin and/or lignin/or cutin (red staining region). The intine was thickened at the apertures and also stained positively. Scale represents 25 μm.

Figure 2.3 **General Oversight Stain: Safranin-Fast Green**
Light micrograph of mature Cucurbita maxima pollen fixed with FAA and stained with Safranin-Fast Green stain. Cucurbita pollen intine contained cutin (red staining region). However very little cutin was present in the aperture regions. The Fast Green counterstained the cytoplasm green. Scale represents 25 μm.



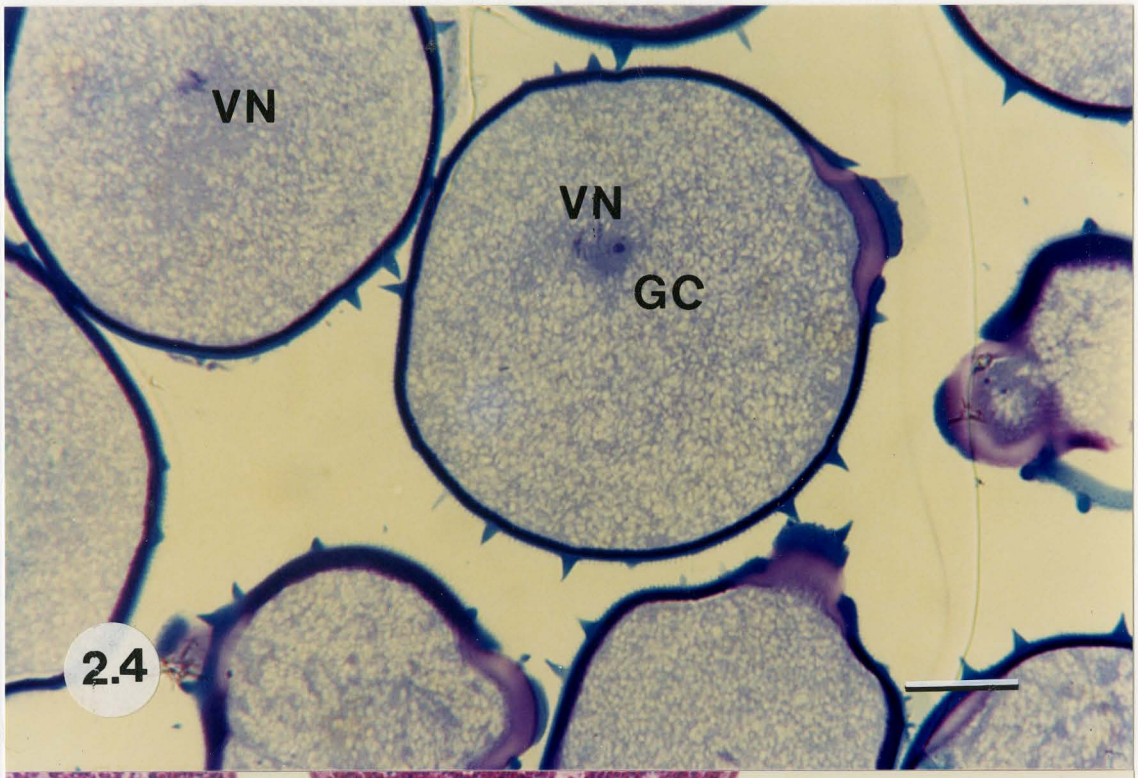
2.2



2.3

Figure 2.4 **General Oversight Stain: Giemsa Stain**
Light micrograph of mature *C. maxima* pollen fixed with FAA and stained with Giemsa stain. *Cucurbita* pollen vegetative nucleus (VN) and nucleus of the generative cell (GC) stained purple. The generative cell contains a regular shaped nucleus. The vegetative nucleus appears as strands of dense material. Scale represents 25 μm.

Figure 2.5 **Histochemical Stain: PAS and Hematoxylin**
Light micrograph of mature *C. andreana* pollen fixed with FAA and stained with PAS and hematoxylin. *Cucurbita* pollen intine and cytoplasm contained polysaccharides (magenta staining region). Polysaccharides surrounded the generative cell (GC) and vegetative nucleus (VN). Scale represents 25 μm.


VN
VN
GC
2.4

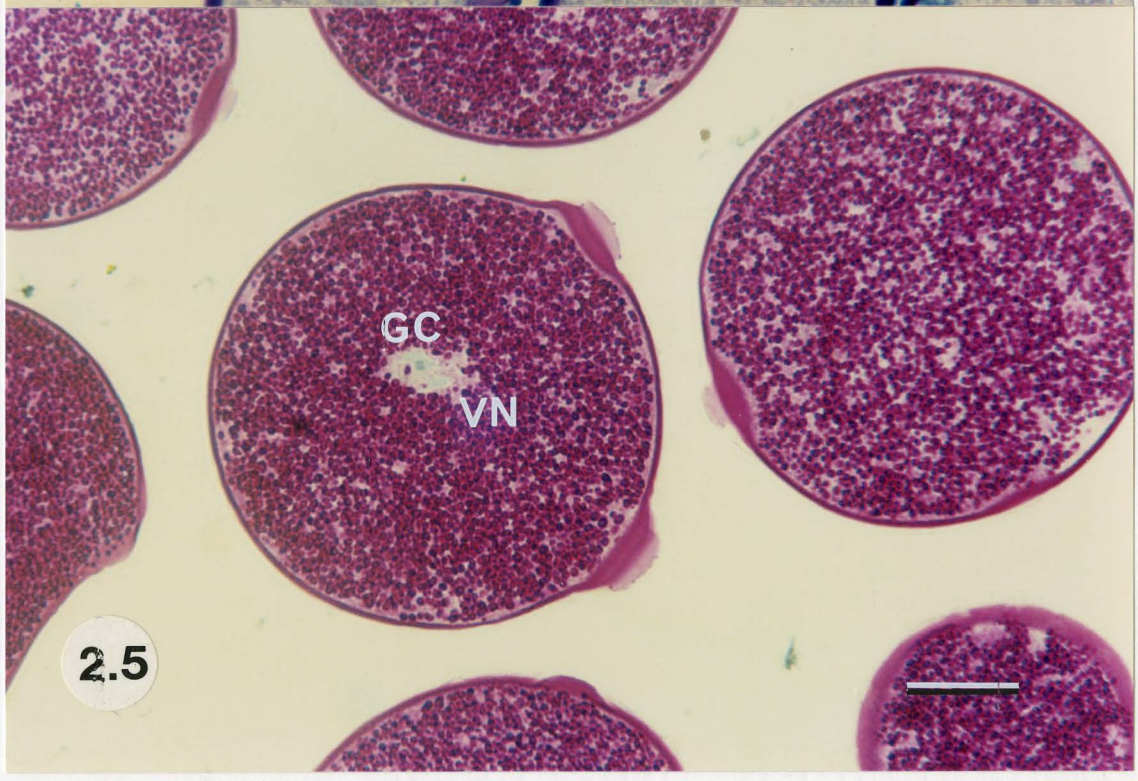
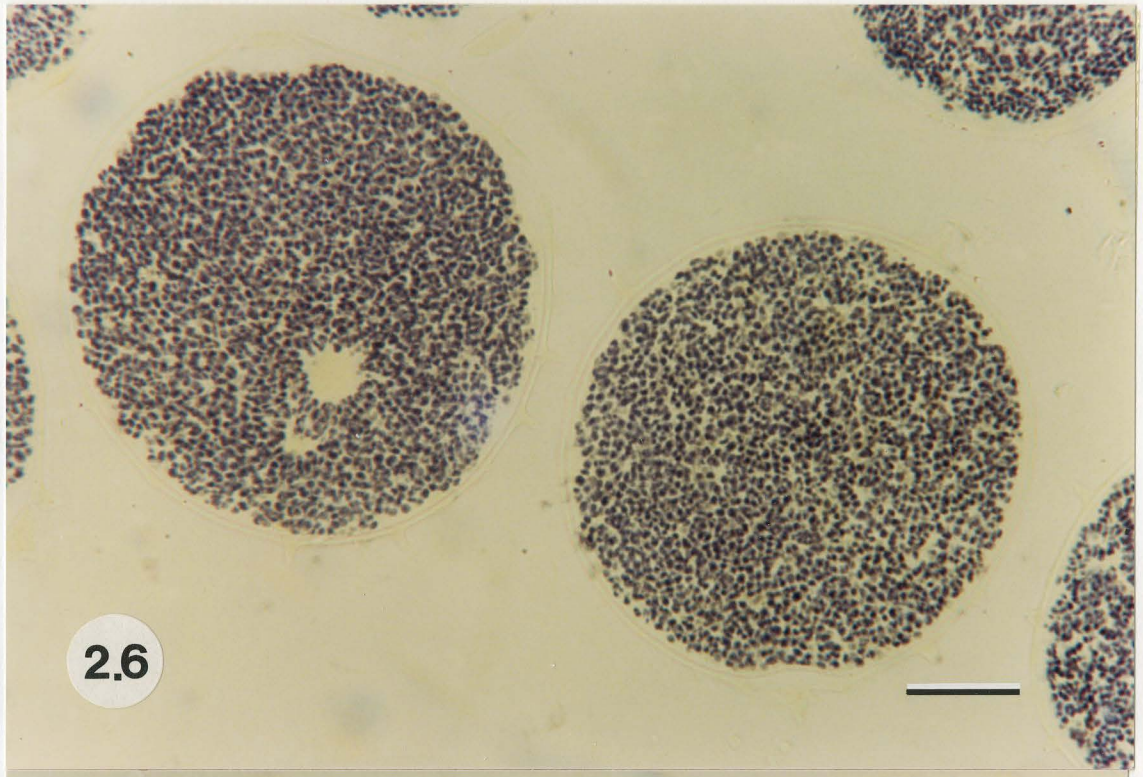
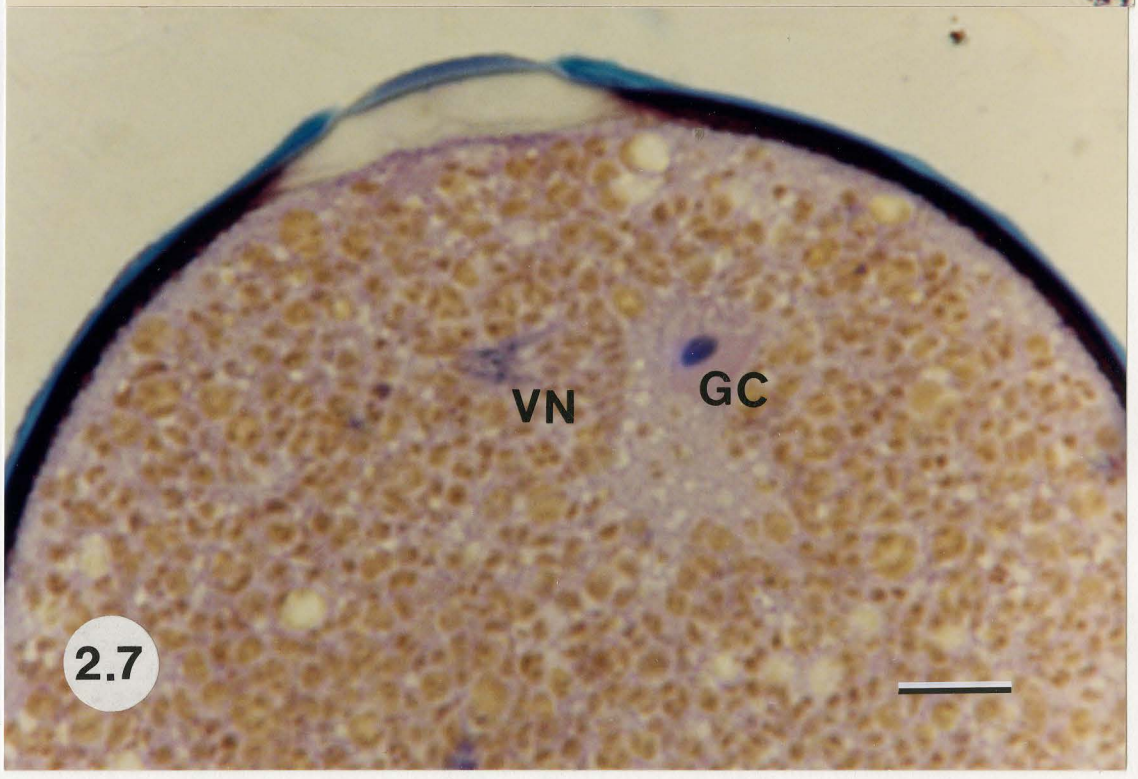

GC
VN
2.5

Figure 2.6 **Histochemical Stain: Lugol's Iodine Stain**
Light micrograph of mature C. maxima pollen fixed with FAA and stained with Lugol's iodine. Cucurbita pollen storage reserves consisted primarily of starch (brown-black staining region). The intine contained polysaccharides but they did not consist of starch. Scale represents 25 μm.

Figure 2.7 **Giemsa Stain and Lugol's Iodine**
Light micrograph of mature C. maxima pollen fixed with FAA and stained with Giemsa and Lugol's iodine. The two stains reacted differently in the presence of each other stain compared to use by themselves. However the stains showed the abundance of starch grains within the Cucurbita pollen cytoplasm and showed the distribution of starch around the vegetative nucleus (VN) and generative cell (GC). Scale represents 10 μm.



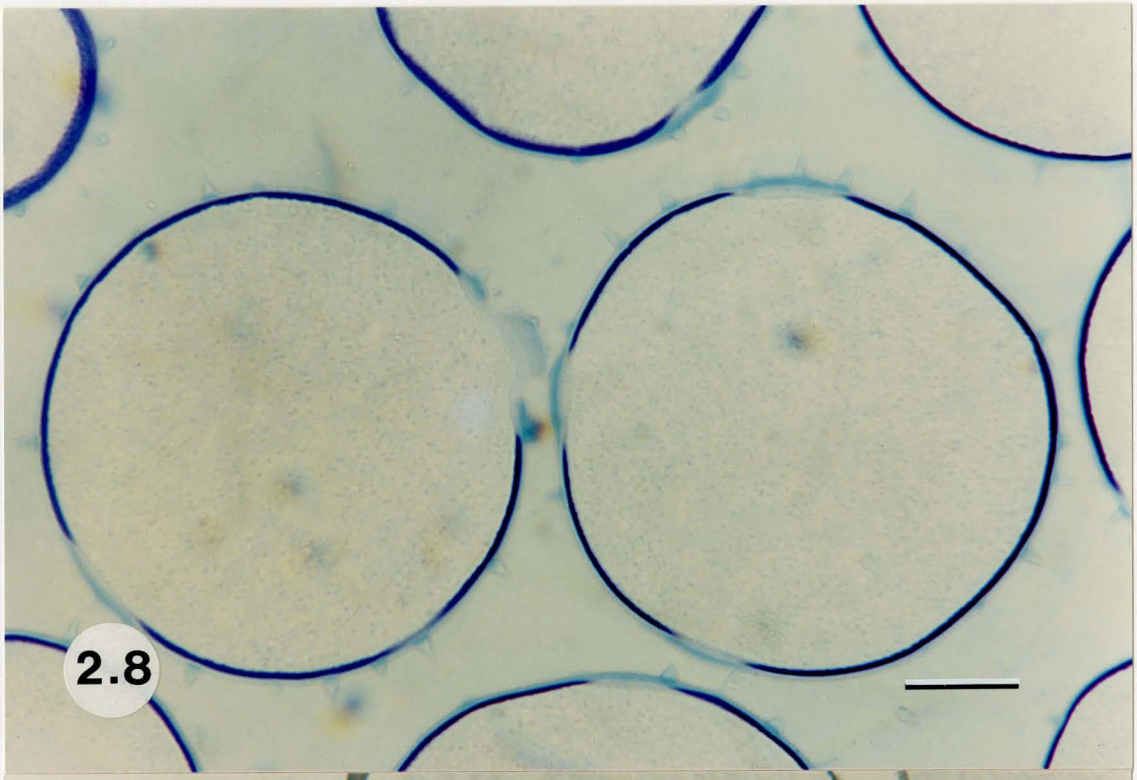
2.6



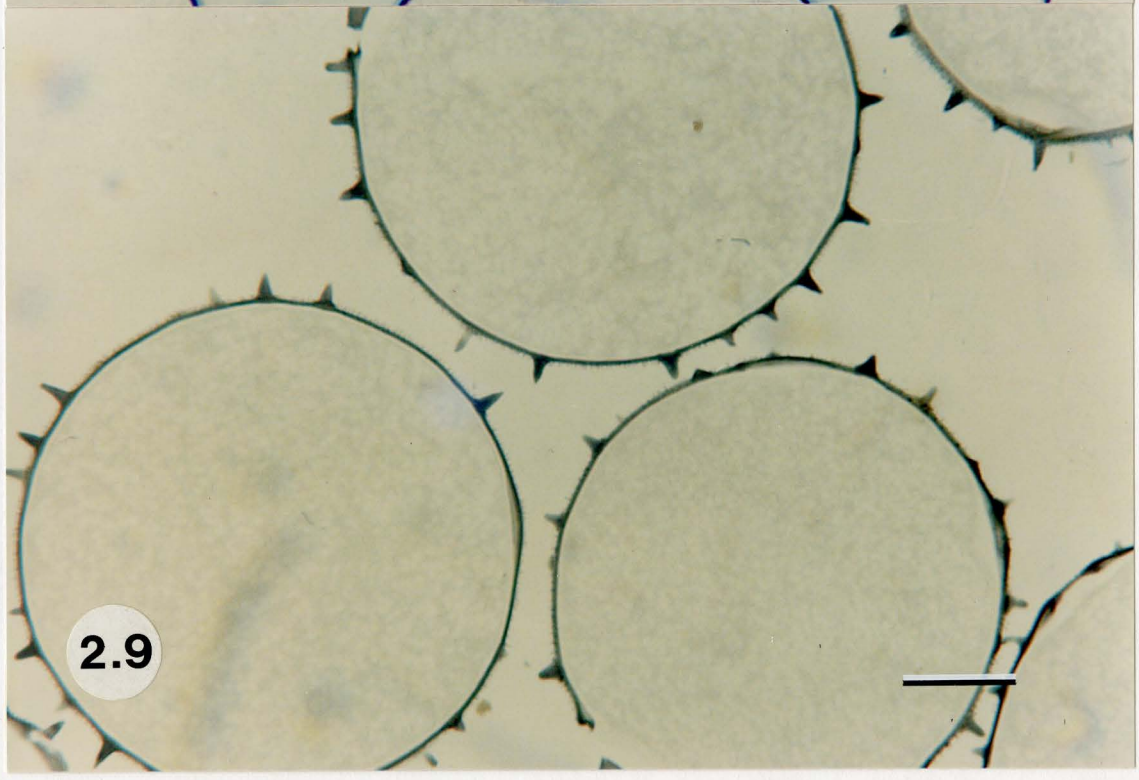
2.7

Figure 2.8 **Histochemical Stain: Nile Blue Sulphate**
Light micrograph of mature *C. andreana* pollen fixed with FAA and stained with Nile blue sulphate. The *Cucurbita* pollen intine contained hydrophobic lipids (blue staining region). There was less lipid material in the aperture region. Scale represents 25 μm.

Figure 2.9 **Histochemical Stain: Sudan Black B**
Light micrograph of mature *C. maxima* pollen fixed with FAA and stained with Sudan black B. The exine contained hydrophilic lipids (grey staining region). The cap region of the aperture contained lipids, however, the intine region did not. Scale represents 25 μm.



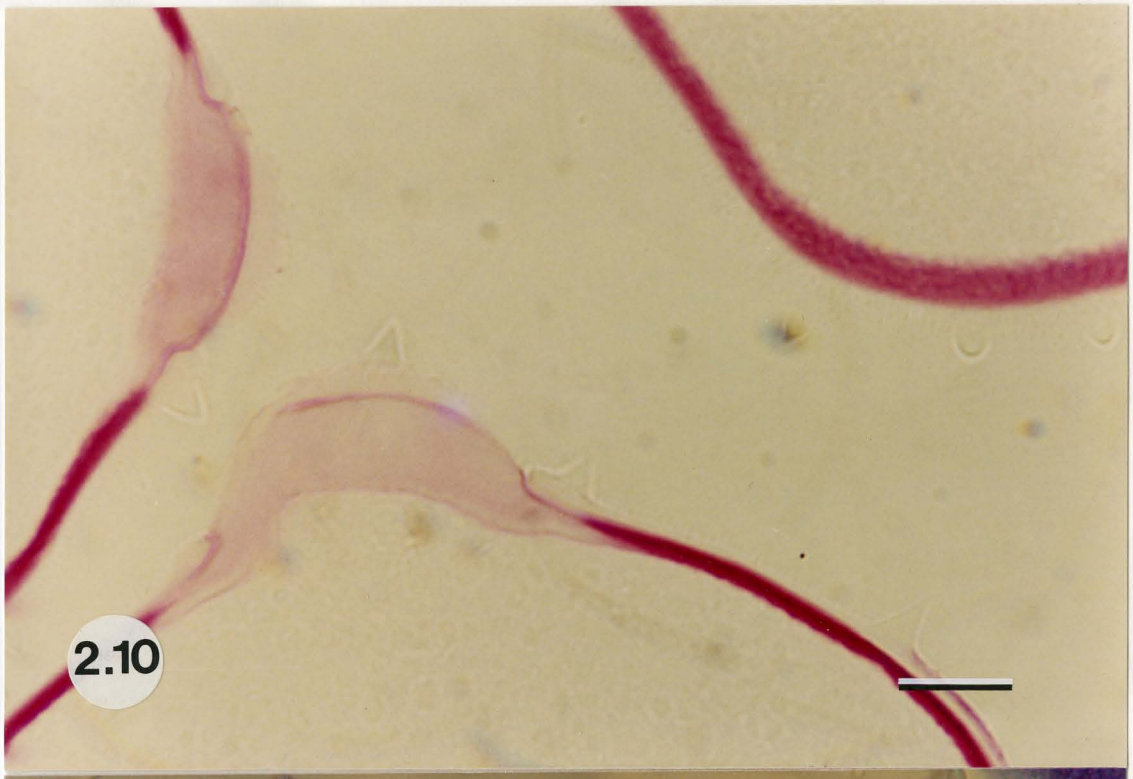
2.8



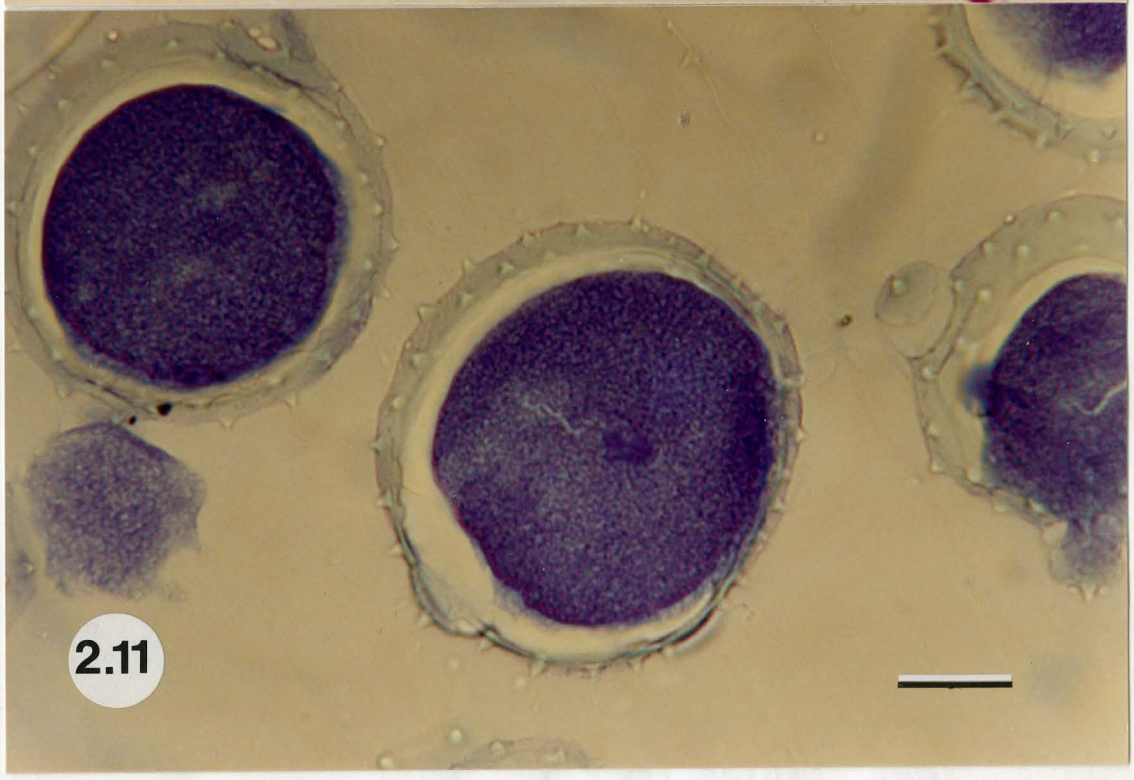
2.9

Figure 2.10 **Histochemical Stain: Plasmal Reaction**
Light micrograph of mature *C. andreana* pollen fixed with FAA and stained using the plasmal reaction. The *Cucurbita* pollen intine contained phospholipids (magenta staining region). There were less phospholipids in the aperture region. Scale represents 10 μm.

Figure 2.11 **Histochemical Stain: Mercury-Bromophenol Blue**
Light micrograph of mature *C. maxima* pollen fixed with FAA and stained with mercury-bromophenol blue. Proteins were finely dispersed throughout the pollen cytoplasm (purplish blue staining region). The wall region did not appear to contain any proteins. Scale represents 25 μm.



2.10



2.11

thicker at each of the apertures. (Figure 2.1). The apertures are the areas from which the pollen tubes will emerge upon pollen germination. Cucurbita exine had several apertures and was covered with spines. The exine was thickened at the aperture to form a cap-like region (Figures 2.1, 2.3). The presence of the red stain in the intine, from both the polychrome stain (Figure 2.2) and the safranin-fast green stain (Figure 2.3), demonstrated that the intine layer contains lignin and/or suberin, and/or cutin containing compounds respectively. However the exine reacted negatively to these stains and thus did not appear to contain these compounds.

Large vacuoles were not present in the mature Cucurbita pollen cytoplasm. The presence of the generative cell and vegetative nucleus in the cytoplasm of the tube cell was demonstrated well by the purple colour produced by the Giemsa stain (Figure 2.4). The generative cell consisted of a nucleus enclosed in a small amount of cytoplasm. The vegetative nucleus was larger than the generative cell and was usually found in close proximity to the generative cell. Other cytoplasmic organelles such as mitochondria, Golgi apparatus and the endoplasmic reticulum were not discernible with the light microscope. The presence of storage reserves in Cucurbita pollen was demonstrated by histochemical stains.

2) HISTOCHEMICAL STAINS :

The specificity of the histochemical stains proved to be very useful in identifying the nature of the storage reserves in Cucurbita pollen sections. Pollen grains were well preserved by FAA and took up adequate amounts of stain, thus making identification of the stained storage reserves an easy process. A summary of results is presented in Table 2.1.

The Cucurbita tube cell cytoplasm was densely packed with storage material. The main storage reserve of Cucurbita maxima and Cucurbita andreana pollen was starch. Both the PAS procedure which demonstrates polysaccharides (Figure 2.5) and Lugol's iodine which specifically stains starch (Figure 2.6), gave positive results. Starch is a polysaccharide which is composed of thousands of branched and unbranched D-glucose units (Salisbury and Ross, 1985). The starch grains in the pollen were round and were located in the cytoplasm. It was not possible to determine if the starch grains were localized in amyloplasts. The intine which stained positively with PAS, was negative with Lugol's iodine stain, indicating that the wall did not contain starch but was composed of another type of polysaccharide (Figure 2.6). Pollen stained by Lugol's iodine and Giemsa demonstrated the location of the starch grains with respect to nuclear material (Figure 2.7).

The Feulgen reaction, which stains for DNA,

demonstrated the presence of nuclear DNA and suggested the possibility of cytoplasmic DNA in the pollen grain. The faint staining of the cytoplasm by the Feulgen stain may have been indicative of plastid DNA but this could not be proved.

The lipid stains, Nile blue sulfate and Sudan black B, stained the pollen wall of Cucurbita sections blue (Figure 2.8) and dark grey (Figure 2.9) respectively. The positive staining results confirmed that the pollen wall contained some lipid material. The plasmal reaction, which stains for phospholipids, revealed that part of the wall composition consisted of phospholipids (Figure 2.10). The cytoplasm of the pollen gave negative results when stained with the lipid stains. It was suspected that the cytoplasmic lipids were extracted by ethanol during the fixation and dehydration processes and these results were false negatives.

Histochemical tests for lipids, carried out on the pollen smears revealed that the cytoplasm contained some lipid material. The use of the pollen smears prevented determination of the relative proportion of cytoplasmic lipids to the rest of the storage materials. However, if the cytoplasm contained a lot of lipids, it would not be expected that all of the lipids would be extracted from every pollen grain in the sections.

The protein stains, Coomassie brilliant blue, and

mercury-bromophenol blue, demonstrated that Cucurbita pollen proteins were finely dispersed throughout the cytoplasm (Figure 2.11). Protein stains were tried on sections of Begonia seeds which are known to contain large protein bodies. The Begonia results confirmed that the protein stains were working properly. The pollen smears also stained positively for proteins confirming the stained pollen section results.

From the observations of the polysaccharide stains and the other histochemical stain results, it appeared that starch was the predominant storage reserve found in Cucurbita pollen. Ultrastructural observations, presented in Chapter 3, will help reveal more detailed information about the pollen cytoplasm structure.

Chapter 3

ELECTRON MICROSCOPY.

INTRODUCTION

Electron Microscopy :

The first practical electron microscope was invented in 1938 by Ruska and von Borries (Wolfe, 1972). The transmission electron microscope resembles the light microscope in terms of its basic principles; however, it differs from the light microscope in three ways. The first difference is that electrons instead of light are required for viewing the object. The second difference is the electron microscope uses magnetic or electrostatic fields to focus the electrons, instead of glass lenses. The third major difference is that the electron microscope column must be under vacuum in order not to alter the electron's path of travel.

The principal advantage to electron microscopy is that it enables the observation of specimens at higher resolution than is possible with a light microscope. The resolution is better with an electron microscope because electrons have shorter wavelengths than light. The resolution of an electron microscope is at least 100 times better than the resolution of the light microscope but depends on the type of electron microscope and the specimen preparation.

There are two basic kinds of electron microscopy widely used in life science research, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM serves as a useful means of observing the three-dimensional detail of a specimen surface. While constantly being improved, most SEM's have a lower resolving power and lower magnification range than TEM's. However the SEM has a greater depth of field than TEM's (Hayat, 1978). SEM enables the study of larger and thicker specimens than TEM.

TEM is capable of resolving structures at 0.2 to 0.3 nm spacings (Hayat, 1978). Thus it has practical applications in examining cellular structures at very high resolution. In order to benefit from the great resolving power of the TEM, it is necessary to use techniques such as thin sectioning to prepare the specimen. By producing thin sections for the TEM, the ultrastructure of the specimen can be examined. However, in order to produce thin sections of tissue for TEM, it is necessary for the tissue to undergo chemical fixation and embedding before thin sectioning.

Advances in electron microscopy have resulted in the development of a scanning transmission electron microscope (STEM) which possesses a resolution comparable to that obtained with the TEM (Hayat, 1978). In order to use the STEM, thin sections, such as those required for TEM, must be prepared.

Electron Microscopy of Pollen :

The number of published ultrastructural studies of vegetative and generative cell cytoplasms and nuclei is very limited (Chambers and Godwin, 1961; Rosen et al, 1964; Sassen, 1964). Over the last 30 years ultrastructural studies of pollen have concentrated on three different areas of research (Mulcahy et al, 1985). In the 1960's pollen studies mainly dealt with pollen wall development. In the 1970's fine structural studies focussed on pollen-stigma interactions. In the last 10 years there has been a rediscovery of ultrastructural studies pertaining to the cytoplasm of pollen. Since 1978, more than 50% of pollen structural studies have been carried out using electron microscopy (Rowley et al, 1988). SEM studies constituted over half of these electron microscope studies.

In this study electron microscopy served as a means of obtaining valuable structural information about Cucurbita pollen. Both SEM and TEM were applied in this study to gain more information about the internal and external structure of Cucurbita pollen. These fine-structural studies supplemented light microscopic studies presented in the preceding chapter.

SEM was used in this study to examine the surface detail and general morphology of whole Cucurbita maxima, Cucurbita andreana and Lilium longiflorum pollen samples. For TEM, Cucurbita and Lilium pollen was prepared with

chemical fixatives and embedded in order to obtain thin sections of well preserved material. In this study the structure of aqueously prepared C. maxima pollen and anhydrously prepared C. maxima, C. andreana and L. longiflorum pollen was examined. Over the last 15 years, anhydrous fixations have been used as means of preparing dehydrated plant tissues (Hallam, 1976; Öpik, 1985). Seeds, spores and pollen are examples of plant structures which normally undergo dehydration as part of their development. These structures are frequently found with a low water content. In order to fully understand the structure of dehydrated seeds and pollen, anhydrous fixation preparations must be employed (Hallam, 1976).

MATERIALS AND METHODS

Preparation for Scanning Electron Microscopy :

Air-dried Cucurbita maxima and Cucurbita andreana pollen grains were mounted to aluminum stubs with double-sided sticky tape. These samples were coated with gold for a total of 2 minutes using a Polaron ES1005 Series II 'cool' Sputter Coater. The pollen grains were viewed from 10 to 15 kV with an ISI model DS-130 scanning electron microscope.

Preparation for Transmission Electron Microscopy :

Aqueous Fixations :

Cucurbita maxima pollen samples were fixed using one of two different aqueous fixatives. Approximately 100 mg of pollen from each species was placed in Eppendorf tubes and used for each fixation. The samples were rotated continuously throughout the fixation process.

The first aqueous fixative was prepared using the method of Moffat and Sommerville (1988). The fixative contained 2% (v/v) glutaraldehyde in 200 mM sodium cacodylate buffer pH 7.2 (pH adjusted with HCl). The pollen was postfixed with 1% OsO_4 in cacodylate buffer.

The second aqueous fixative was prepared using the method of Baldi et al (1987). The fixative contained 2.5% glutaraldehyde and 2% paraformaldehyde in 25 mM PIPES buffer pH 7.2 (pH adjusted using NaOH). The pollen was postfixed with 1% OsO_4 in PIPES buffer.

All pollen samples were fixed in the primary fixatives for 5 h at room temperature and postfixed for 2 h at room temperature. Each fixation step was followed by a 1 h wash in the appropriate buffer solutions. Pollen samples were dehydrated sequentially through an ethanol series consisting of 70%, 80%, 95%, 100%, 100% and propylene oxide at room temperature for 2 h per step. Each sample was infiltrated through a propylene oxide:Spurr's epoxy resin series consisting of 3:1, 2:1, 1:1, 1:2, 1:3, 100%, 100%, 100%, 100%, and 100% resin at room temperature for 12 h per step. Samples were then placed in Beem capsules and polymerized for 60 h at 60°C. Thin sections in the range of 60 to 120 nm thick, were cut on a Reichert ultramicrotome using glass knives, and were picked up onto Formvar-coated 100 mesh copper grids. Each section contained about 10 pollen grains. Some of the sections were post-stained with uranyl acetate saturated in 50% methanol for 15 minutes followed by Reynold's lead citrate for 2 minutes. Sections were viewed on a Philips EM 300 transmission electron microscope operating at 60 kV.

Anhydrous Fixations :

Aqueously fixed samples become hydrated and are not desirable for structural studies of dehydrated seeds or pollen. Furthermore, water soluble materials, which may be present in the cells, tend to be extracted. Two anhydrous

fixatives were used for fixing the Cucurbita and Lilium pollen samples. In each fixation, the samples were rotated continuously.

The first anhydrous fixative was prepared using a modified version of the method of Sack et al (1988). Air-dried pollen samples were fixed for 4 h at room temperature in 2% paraformaldehyde dissolved in pure glycerol (w/v). The samples were postfixed for 2 h in 1% OsO_4 dissolved in glycerol (w/v). Samples were washed for 1.5 h in a (1:1) glycerol:100% ethanol mixture after each fixation step. Dehydration began with 95% ethanol because glycerol is hygroscopic and not completely anhydrous. The infiltration was carried out beginning with propylene oxide followed by a series of Spurr's resin:propylene oxide mixtures which consisted of 1:3, 1:2, 1:1, 2:1, 3:1, and 100%, 100%, 100% resin.

The second anhydrous fixative was prepared using the modified method of Öpik (1985). This fixative consisted of osmium tetroxide vapour. Approximately 100 mg of air-dried C. andreana and C. maxima pollen was put into separate glass vials and placed in a sealed jar containing 1 g of solid OsO_4. After fixation for 30 days at room temperature in the OsO_4 vapour, the pollen was removed and infiltrated with Spurr's resin or with a series of Spurr's resin:propylene oxide mixtures as described above.

In both anhydrous preparations, samples were

infiltrated for a period of 1 week at room temperature with solution changes every 12 h. Samples were placed in Beem capsules and polymerized for 60 h at 60°C. Thin sections were cut, stained and viewed as described under "Aqueous Fixations".

RESULTS

1) SCANNING ELECTRON MICROSCOPY :

Cucurbita maxima and Cucurbita andreana pollen grains were not difficult to prepare for SEM. However, they were not stable in the SEM. When individual pollen grains were directly exposed to the electron beam for longer than 15 minutes, they began to suffer from beam damage. The pollen grains lost their shape due to mass loss and the areas of exine around the apertures began to crack.

SEM was useful in revealing information about the morphology of the external surface of Cucurbita maxima and Cucurbita andreana pollen. The pollen grains of both species were similar; they were spherical and had a diameter of about 105 μm (Figure 3.1). The surface of the pollen was covered with numerous small, uniformly arranged spines called spinules. "Micro-bumps" also covered the entire pollen surface. A number of apertures projected from the exine; each aperture was about 15 μm in diameter (Figure 3.1).

The Lilium longiflorum pollen was elliptical in shape and was about 130 μm in length and 55 μm in width. The exine surface was covered with sculptured polygonal patterns. There were no apertures or spinules protruding from the exine of the L. longiflorum pollen; however, there was a colpus region which ran the length of the grain.

Figure 3.1 Scanning Electron Micrographs of C. andreana and C. maxima Pollen

Mature Cucurbita pollen was air-dried and gold-coated.

A) External structure of C. andreana pollen. Numerous spinules and several apertures project from the pollen surface. Scale represents 20 μm. 657X

B) Higher magnification of the aperture region of C. andreana pollen. An arrow points to the aperture (A). Spinules (Sp) are distinct. A series of smaller bumps (m) covers the surface of the pollen. Scale represents 3.5 μm. 1,480X

C) External structure of C. maxima pollen. Numerous spinules and several apertures project from the pollen surface. Scale represents 20 μm. 655X

D) Higher magnification of the aperture region of C. maxima pollen. An arrow points to the aperture (A). Spinules (Sp) are distinct. A series of smaller bumps (m) covers the whole surface of the pollen. Scale represents 2.5 μm. 2,475X

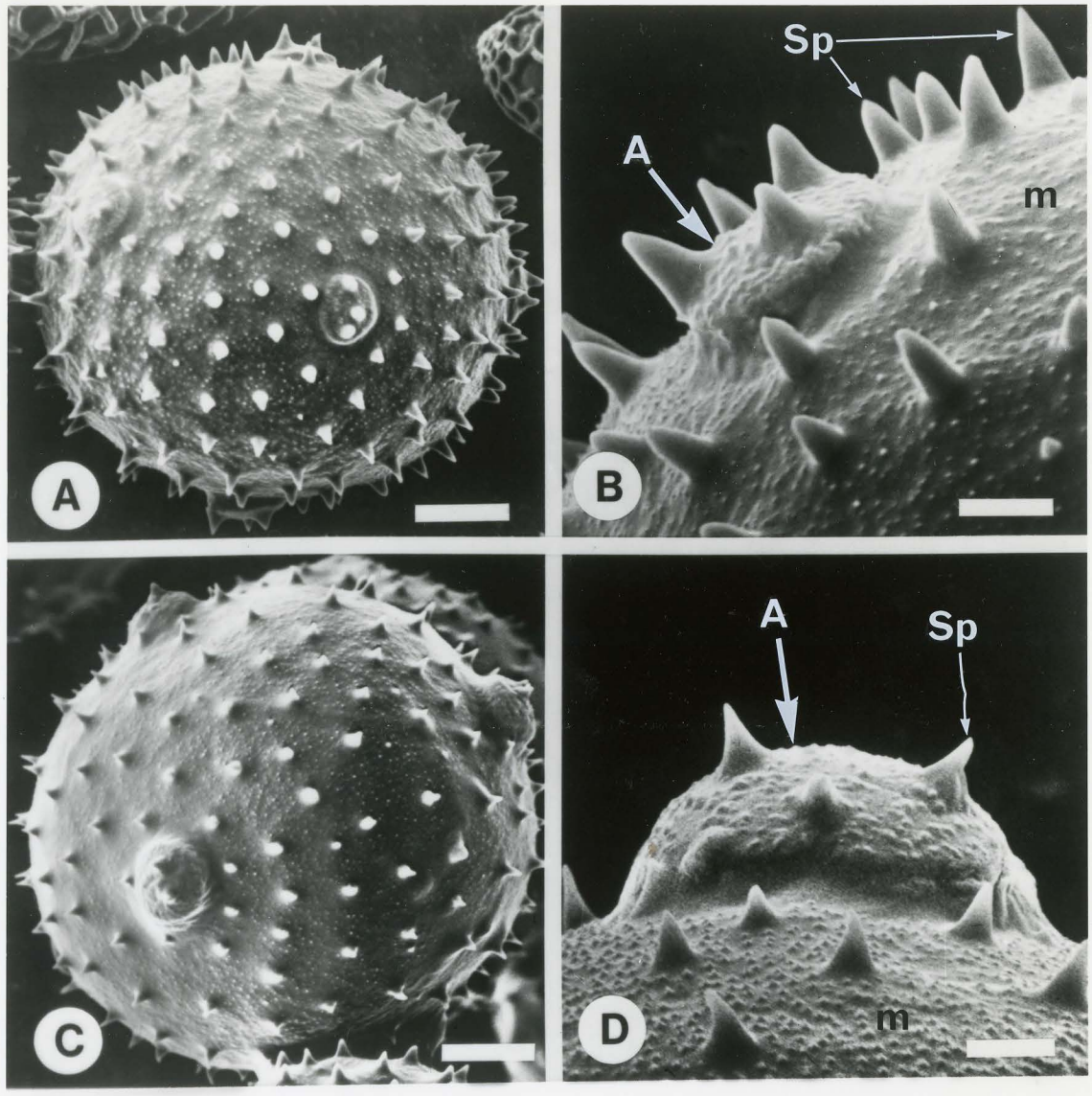

A
Sp
A
m
B
C
A
Sp
m
D

Figure 3.2 **Transmission Electron Micrographs of *C. maxima* and *C. andreana* Pollen**

Mature *Cucurbita* pollen was fixed with anhydrous paraformaldehyde and pure glycerol.

A) Internal structure of *C. maxima* pollen wall. The wall consisted of two distinct layers, the exine (Ex) and the intine (In). The exine consisted of two regions, the outer region stained uniformly. However, the inner region had a granular appearance. The intine was non-uniform and consisted of fibrous material. Scale represents 0.5 μm. 21,300X. Stained with uranyl acetate and lead citrate.

B) The aperture (Ap) of *C. andreana* pollen. The intine (In) layer was thickened at the aperture. The cap of the aperture originated from the exine layer. Scale represents 5 μm. 3,160X. Stained with uranyl acetate.

C) The vegetative nucleus (Nu) of *C. andreana* pollen. The nucleus was lobed and organelles, such as mitochondria, were located in the cytoplasm near the projections of the nucleus. Scale represents 1.5 μm. 6,000X. Stained with uranyl acetate and lead citrate.

D) Generative cell (Gen) and generative nucleus (Nu) of *C. andreana* pollen. The generative cell did not appear to contain any plastids. Very little cytoplasm surrounded the generative cell nucleus. The wall of the generative cell was electron-transparent. Scale represents 1 μm. 15,000X. Stained with uranyl acetate and lead citrate.

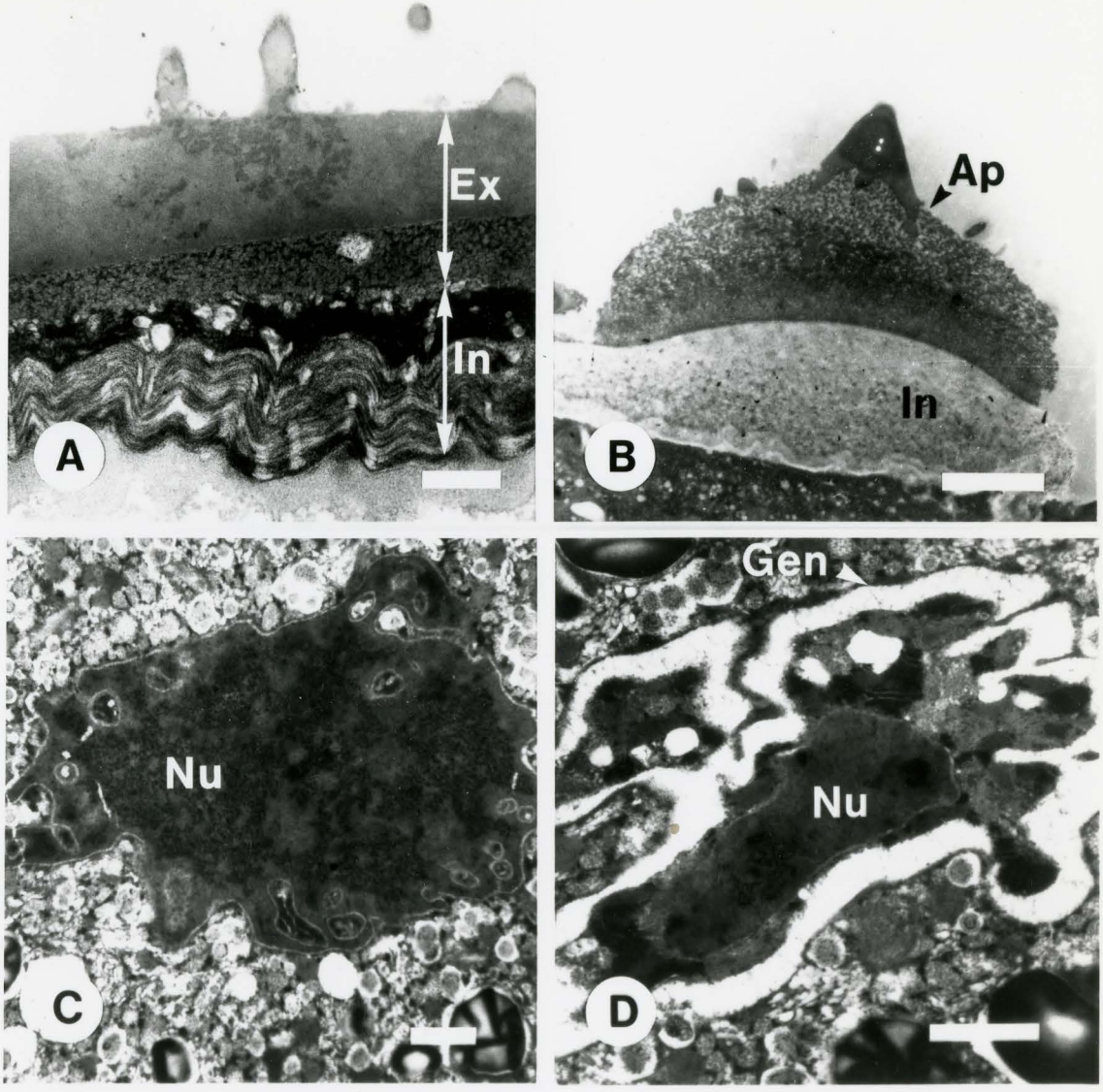

Ex
In
A
Ap
In
B
Nu
C
Gen
Nu
D

Figure 3.3 Mature Cucurbita Pollen Cytoplasm

Mature C. maxima pollen was fixed with anhydrous paraformaldehyde and pure glycerol. Cucurbita cytoplasm was dense and contained many starch grains (S). An abundance of fibrillar bodies (FB) and endoplasmic reticulum (ER) associated with oval-shaped bodies (o) were present in the Cucurbita cytoplasm. Large vacuoles were not present in the cytoplasm. Scale represents 2 μm. 7,100X. Stained with uranyl acetate and lead citrate.

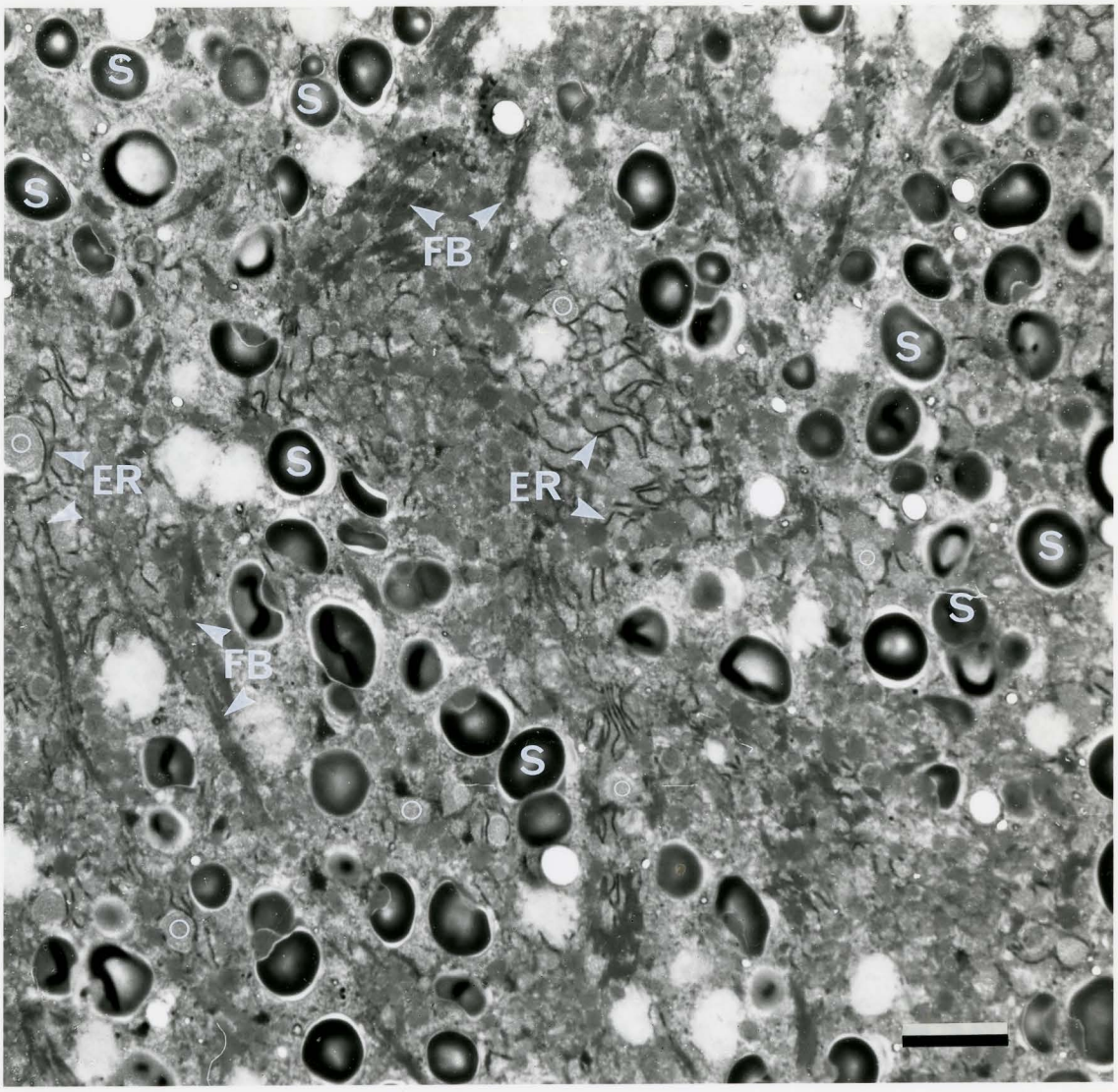

S
S
S
FB
S
ER
S
ER
S
S
FB
S

Figure 3.4 Transmission Electron Micrographs of C. maxima and C. andreana Pollen

A) Structure of C. maxima pollen cytoplasm fixed aqueously with glutaraldehyde in cacodylate buffer. White arrows indicate oval-shaped bodies which are found throughout the pollen cytoplasm. Scale represents 1 μm. 11,600X. Stained with uranyl acetate.

B) Structure of C. andreana pollen cytoplasm fixed anhydrously and post-fixed with osmium tetroxide. The star indicates an oval-shaped body. These bodies were distributed throughout the cytoplasm. The electron-translucent centre of the body was very regular in shape. The centre of the body appeared to contain regular-shaped particles which were arranged in rings. Starch grains (S) were also commonly found in the cytoplasm. Scale represents 1 μm. 16,500X. Stained with uranyl acetate and lead citrate.

C) Structure of C. andreana pollen cytoplasm fixed anhydrously and post-fixed with osmium tetroxide. The endoplasmic reticulum (ER) was rough and often layered. Several electron-dense particles were present (indicated by white arrows). Scale represents 0.5 μm. 29,000X. Stained with uranyl acetate and lead citrate.

D) Structure of C. maxima pollen cytoplasm fixed anhydrously. Electron-dense particles and the holes from which electron-dense particles were removed during sectioning or preparation of the pollen are indicated by black arrows. The particles were scattered among starch grains (S). Scale represents 1 μm. 18,500X. Stained with uranyl acetate and lead citrate.

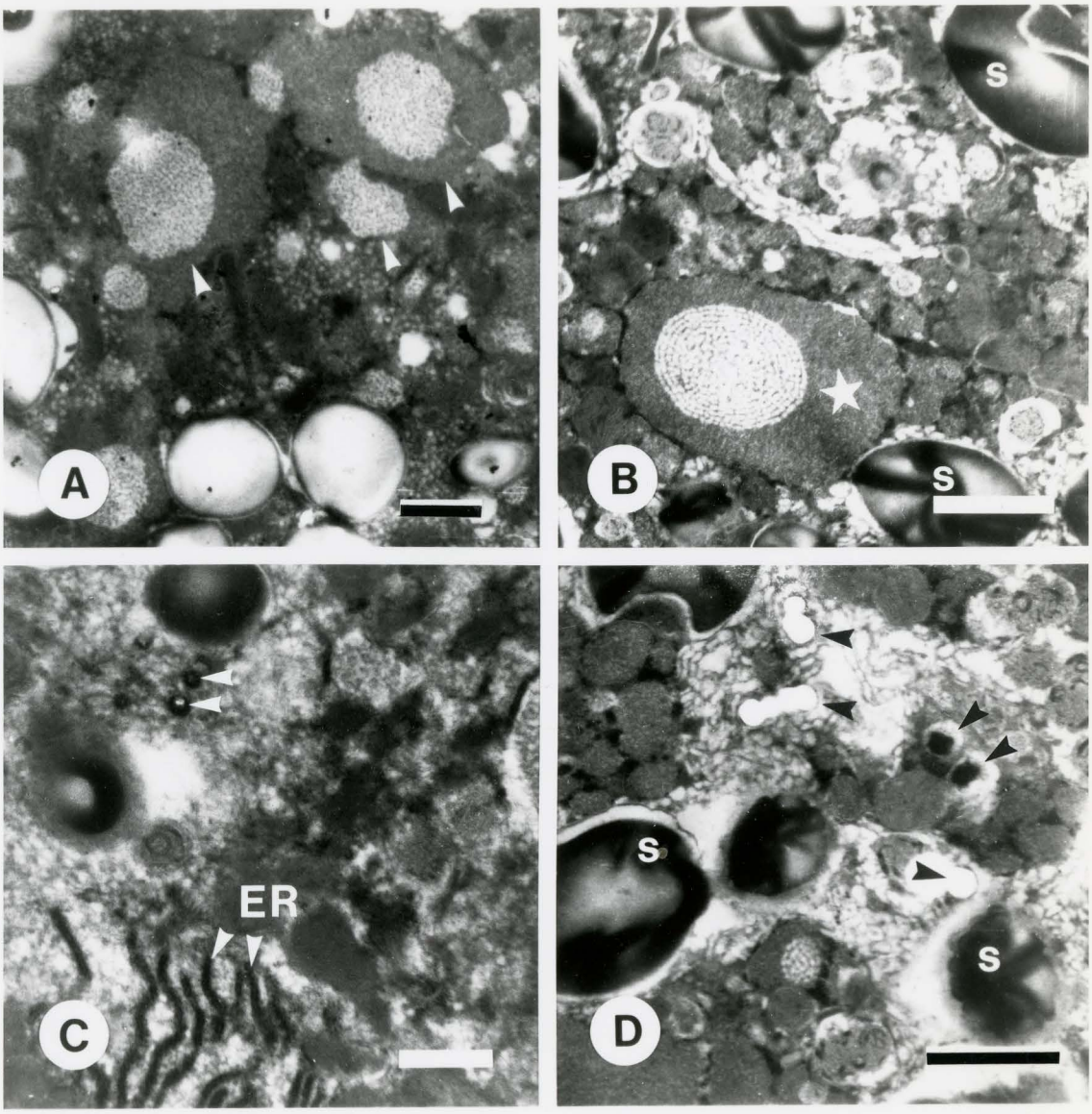

A
B
S
S
C
ER
D
S
S

Figure 3.5 Transmission Electron Micrographs of *C. maxima* Pollen

A) Structure of *C. maxima* pollen cytoplasm fixed anhydrously. The black arrow indicates the central portion of an oval-shaped body. The white arrow indicates the rough endoplasmic reticulum which is found in close proximity to the oval-shaped body. Scale represents 0.25 μm. 53,500X. Stained with uranyl acetate and lead citrate.

B) Structure of *C. maxima* pollen cytoplasm fixed anhydrously. The rough endoplasmic reticulum (RER) was found in close proximity to the oval-shaped bodies and to material that had the same electron density as the material found in the oval-shaped bodies (indicated with white arrows). Scale represents 0.25 μm. 53,500X. Stained with uranyl acetate and lead citrate.

C) Structure of *C. maxima* pollen cytoplasm fixed aqueously in glutaraldehyde and PIPES buffer and post fixed with osmium tetroxide. Electron-dense particles (indicated by white arrows) were abundant in the pollen cytoplasm and usually were found in groups. Scale represents 1 μm. 18,600X. Stained with uranyl acetate.

D) Structure of *C. maxima* pollen cytoplasm fixed aqueously with glutaraldehyde and cacodylate buffer. Electron-dense particles (indicated by white arrows) were distributed throughout the pollen cytoplasm and usually found in groups. Scale represents 0.5 μm. 22,700X. Stained with uranyl acetate and lead citrate.

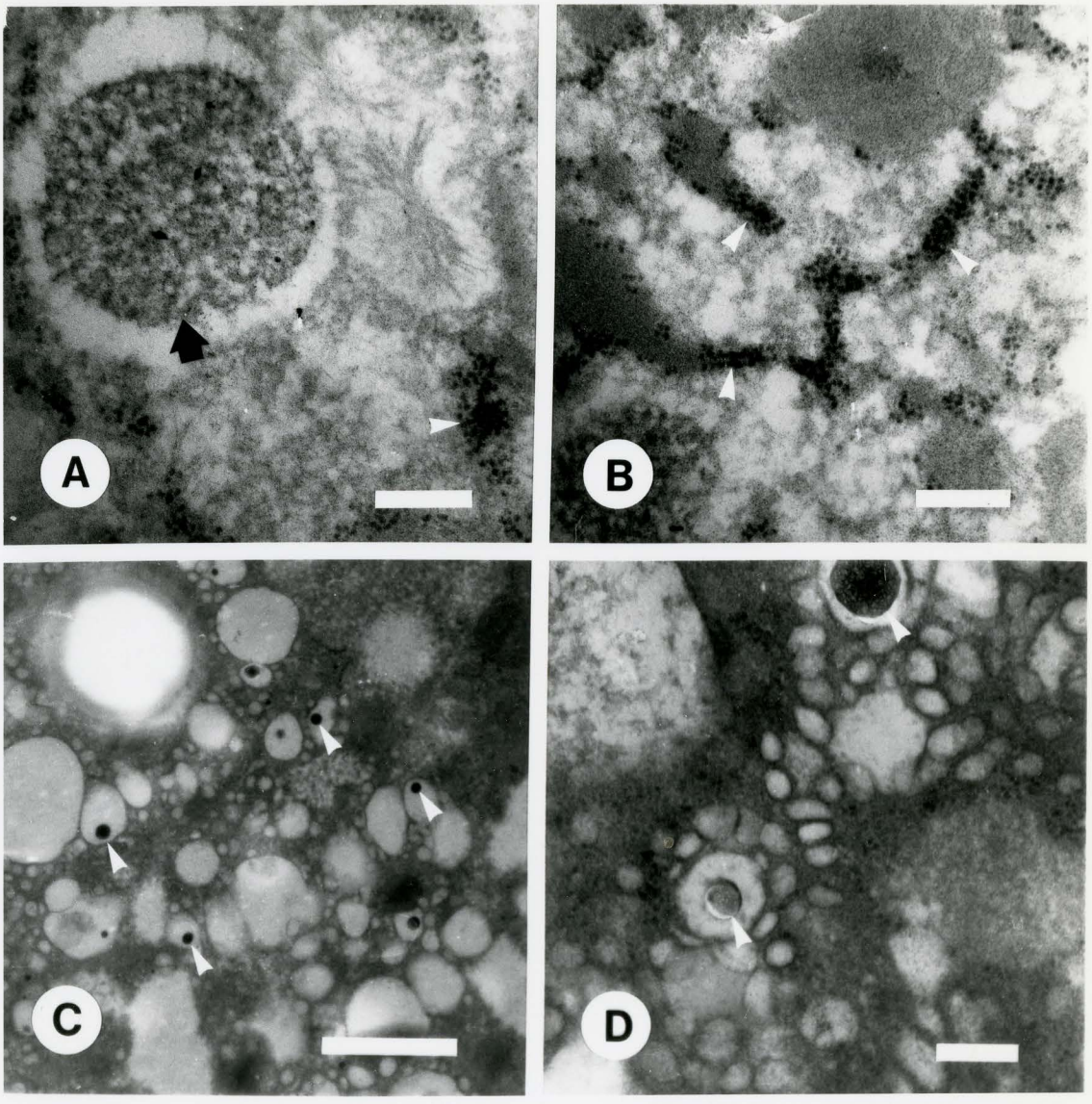

A
B
C
D

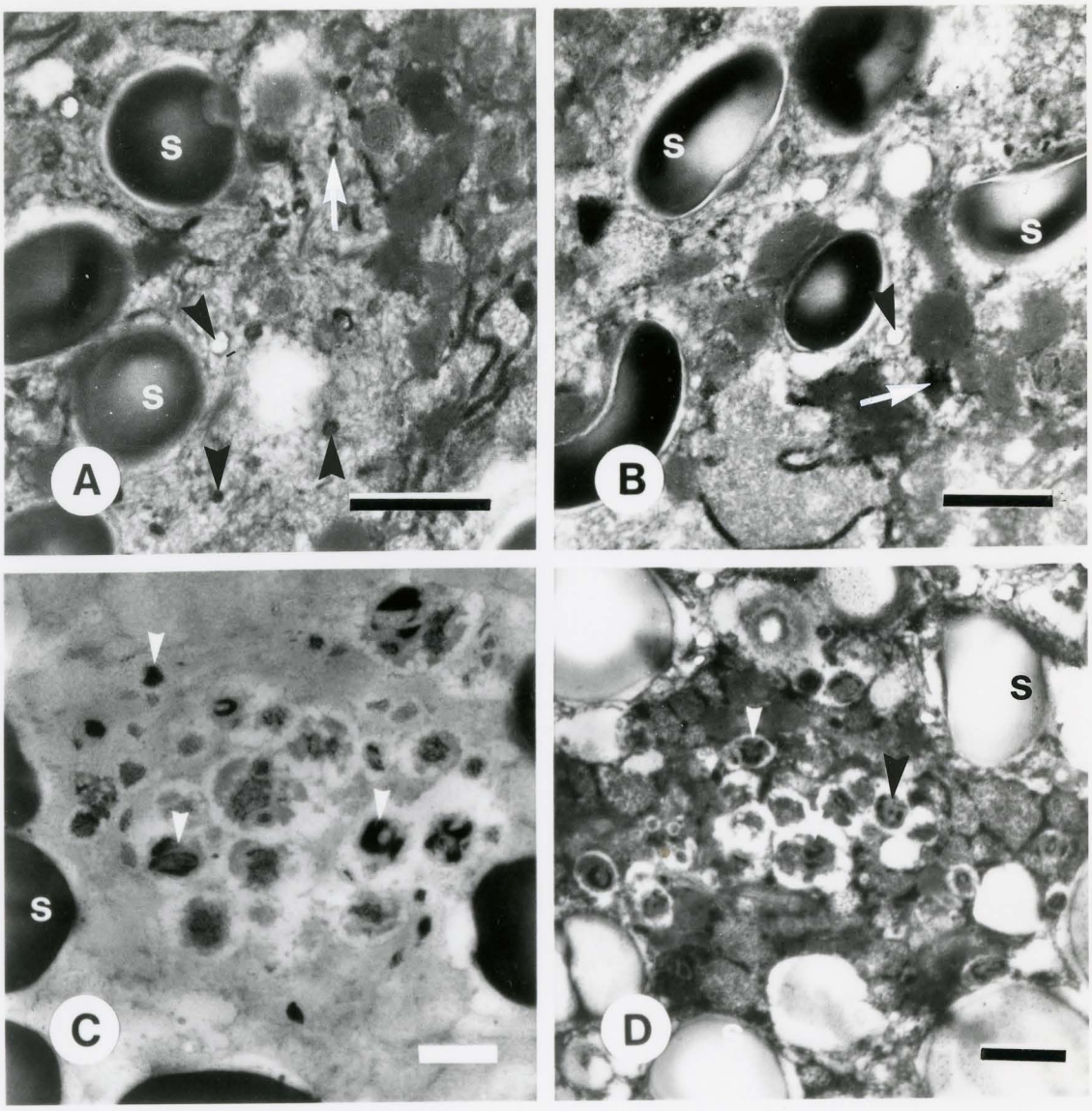
Figure 3.6 **Transmission Electron Micrographs of *C. maxima* and *C. andreana* Pollen**

A) Structure of *C. maxima* pollen cytoplasm fixed anhydrously. The black and white arrows indicate the distribution of the electron-dense particles in the cytoplasm. Starch grains (S). Scale represents 1 μm. 18,600X. Stained with uranyl acetate and lead citrate.

B) Structure of *C. andreana* pollen cytoplasm fixed anhydrously. The white arrow indicates an electron-dense particle in the cytoplasm next to starch grains (S). The black arrow indicates a hole where a particle was removed during sectioning. Scale represents 0.5 μm. 20,600X. Stained with uranyl acetate and lead citrate.

C) Structure of *C. maxima* pollen cytoplasm fixed anhydrously. Electron-dense particles (indicated by white arrows) were found throughout the pollen cytoplasm and usually found in groups in the cytoplasm near starch grains (S). Scale represents 0.5 μm. 21,400X. Stained with uranyl acetate and lead citrate.

D) Structure of *C. andreana* pollen cytoplasm fixed anhydrously. Electron-dense particles (indicated by white and black arrows) were found throughout the pollen cytoplasm and usually found in groups in the cytoplasm near starch grains (S). Scale represents 0.5 μm. 21,400X. Stained with uranyl acetate.


S
S
A
S
S
B
S
C
S
D

2) TRANSMISSION ELECTRON MICROSCOPY :

Aqueous Fixation

Cucurbita maxima pollen was difficult to section because the pollen grains were very dense and were poorly infiltrated in spite of a prolonged infiltration schedule. Thin sections from both aqueous fixations were studied. The pollen was reasonably well preserved despite the problems with infiltration.

Anhydrous Fixation

Ultra-thin sections of C. maxima, C. andreana and L. longiflorum pollen, fixed with glycerol and paraformaldehyde, were more difficult to section than blocks of the aqueously fixed pollen. L. longiflorum pollen was very poorly preserved using the paraformaldehyde and glycerol fixation and little structural detail could be observed.

Thin sections of osmium tetroxide vapour fixed pollen could not be used for structural studies. The blocks of osmium tetroxide vapour fixed pollen were soft, very poorly infiltrated and the pollen grains tended to pop out of the block face upon sectioning. Thus paraformaldehyde and glycerol fixed C. maxima and C. andreana samples were used for studying the fine structure.

Pollen Structure

Upon studying the fine structure of pollen from both types of fixations, it was determined that the basic

structure of Cucurbita pollen did not differ, regardless of whether the pollen was prepared by an aqueous or anhydrous fixative. However it appeared that the anhydrously fixed pollen was better preserved than the aqueously fixed pollen because more detail of the anhydrously fixed cytoplasm could be observed. It was also determined that the C. maxima pollen and C. andreana pollen were structurally similar.

The Cucurbita pollen wall consisted of two distinct layers, the exine and the intine (Figure 3.2). The thickness of the whole wall structure exclusive of the apertures was about 2 μm. The exine was divided into two regions (Figure 3.2). The outer region of the exine was composed of dense material which stained uniformly. The spines which projected from the pollen surface appeared to be composed of different material as they stained lighter. The inner region of the exine had a granular appearance and was not as thick as the outer region.

The intine was very dense and its structure was distinct (Figure 3.2). The intine appeared to be non-uniform and was composed of an electron-dense layer containing fibrous material. The electron-density of the intine layer varied. At the apertures the intine layer was thickened penetrating into the aperture as a plug (Figure 3.2). The outer surface of the aperture appeared to be comprised of granular material. The spike portion of the aperture was coated with an electron-dense material which may contain

pollenkitt.

The vegetative or tube cell nucleus was large, about 10 um in diameter, electron dense and located in the central portion of the pollen grain. The nucleus had several lobes projecting from it (Figure 3.2). Often a number of organelles, such as mitochondria, were located in the cytoplasm near the convolutions of the nucleus. The generative cell was found in close proximity to the vegetative nucleus.

The entire generative cell was almost half the size of the vegetative nucleus. The generative cell differed substantially from the vegetative cell. The generative cell was amoeboid in shape and possessed an electron translucent wall (Figure 3.2). Furthermore, very little cytoplasm surrounded the generative nucleus and the cytoplasm of the generative cell did not contain any starch grains (Figure 3.2).

The C. maxima and C. andreana pollen vegetative cell cytoplasm contained various organelles; however, it did not contain any large vacuoles. An abundance of starch likely to be in amyloplasts was present. In sections the profiles of starch grains were of different sizes and their shapes varied from kidney-shaped to spherical (Figure 3.3). Starch grains were dispersed throughout the cytoplasm. Mitochondria, fibrillar bodies, dictyosomes and rough endoplasmic reticulum were commonly found dispersed in the

pollen cytoplasm (Figure 3.3). Mitochondria were abundant and varied in shape. Numerous dictyosomes were scattered through the cytoplasm. The endoplasmic reticulum was frequently observed and in some cases it was stacked (Figures 3.3 and 3.4).

Bodies not previously described in pollen structural studies were present in the cytoplasm. There were plenty of oval-shaped bodies which contained spherical-shaped crystal-like structures. It is suspected that these bodies contain proteins, since the histochemical tests for proteins revealed that proteins were finely dispersed throughout the pollen cytoplasm.

Many electron-dense particles ranging from 0.2 to 2.0 μm in diameter were present in Cucurbita pollen (Figures 3.4, 3.5 and 3.6). The electron-dense particles were often round or oval in shape and were frequently found in groups throughout the pollen cytoplasm (Figures 3.5 and 3.6). These particles were also present in Lilium longiflorum pollen. Differences in the amount and size of the particles were observed between the aqueous and anhydrous fixations. Fewer and smaller electron-dense particles were found in the aqueously fixed Cucurbita maxima pollen than in the anhydrously fixed Cucurbita pollen. Observations of Cucurbita and Lilium sections that had not been fixed with OsO_4 and had not been post-stained, revealed that these particles were naturally electron-dense (Figures 4.1). The

composition and density of the electron-dense particles were similar to globoid crystals found in seeds. Seed globoid crystals act as storage reserves for phytate. It is believed that the electron-dense particles observed in the vegetative cytoplasm represent the type of globoid particles found in pollen. From this point on, the term globoid particles will be used to refer to these electron-dense particles. Elemental analysis of the pollen globoid particles, as discussed in Chapter 4, is required to determine whether the mineral reserves of pollen are similar to the mineral reserves of seed globoid crystals..

Starch appeared to be the main storage reserve of Cucurbita pollen. Although the histochemical tests for lipids indicated that lipids were present in the cytoplasm, few lipid bodies were observed. It was not determined whether the oval-shaped bodies were proteins or not.

Chapter 4

ELEMENTAL ANALYSES.

INTRODUCTION

In this thesis both energy dispersive X-ray (EDX) analysis and neutron activation analysis (NAA) were used to help understand the elemental composition of Cucurbita maxima and Cucurbita andreana pollen.

An EDX analysis system combined with a SEM or TEM allows one to investigate the elemental composition of biological samples. Lott and co-workers have applied EDX analysis extensively to analyze the elemental content of seed tissues. Elemental analysis of Cucurbita maxima and Cucurbita andreana seeds has been carried out using EDX analysis (Lott, 1975; Lott et al, 1978a; Lott et al, 1979; Lott and Vollmer, 1979; Lott, 1981; Lott, 1983; Lott, 1984; Ockenden and Lott, 1988; Ockenden and Lott, 1989). Elemental analyses of these seeds, using atomic absorption spectroscopy and NAA, showed that C. andreana embryos contained about three times more Ca than the C. maxima embryos. It is because there is this difference in the mineral composition of these two species that it is of interest to determine whether C. maxima and C. andreana pollen also differ in their storage of Ca and other elements.

Energy Dispersive X-ray (EDX) Analysis :

In order to carry out qualitative analysis of the mineral nutrient reserves, biological samples were prepared in several different ways. Conventional fixation methods, which use aldehyde containing fixatives, are an insufficient means of preparing tissues for EDX analysis since they extract monovalent and divalent cations (Roomans, 1980). Other less conventional means of preparing samples for EDX analysis include the use of freeze-dried powders, cryogenic preparation, and anhydrous and partially anhydrous fixations (Roomans, 1980; Lott et al, 1984; Lott et al, 1985a; 1985b; Ockenden and Lott, 1989). In this study, various preparation techniques that involved fixed and unfixed pollen were tried. Sections of pollen prepared with aqueous or anhydrous fixations were analyzed. Unfixed preparations involving cryo-fractured pollen and ground pollen samples were also analyzed.

Most biological tissues are composed mainly of water. This feature poses problems for microscopists who want to observe and analyze unfixed tissues. When a hydrated sample is placed in the SEM, it begins to desiccate rapidly, which leads to distortion of the sample structure as well as some loss of vacuum (Beckett et al, 1984). One way to circumvent this problem is to quick freeze the hydrated sample (Lott et al, 1985b).

Quick freezing enables preparation and preservation

of tissues in a state that closely approximates the natural condition of the tissues, without introducing foreign fixatives (Blackmore and Barnes, 1984). Other advantages of quick freezing reviewed by Lott et al, (1985b), are: 1) frozen-hydrated samples are more likely to hold their volume and shape than those that have been chemically dehydrated; 2) extraction or movement of soluble materials is minimized, thus making it a suitable method to combine with EDX analysis; 3) it is possible to observe internal surfaces and structures by fracturing frozen material; 4) it is possible to study the internal spaces within tissues and determine whether spaces are normally air filled, water filled or filled with mucilaginous materials; 5) it is possible to carry out comparisons of dry and hydrated material; 6) it is possible to study liquids and semi-solids; and 7) preparation time is approximately 15 minutes.

The quick freezing and cryo-fracturing procedure involves three main operational phases (Beckett and Read, 1986). In the first phase the hydrated samples are mounted and rapidly frozen in sub-cooled nitrogen, yielding fully frozen samples. In the case of seeds, samples are dehydrated to begin with; however the average cryo-sample tends to be hydrated. The other two phases are carried out under vacuum or in a dry argon gas atmosphere to prevent frosting of the surface. In the second phase, the sample is fractured, etched or both to reveal the surface features. The fractured

surface is coated with chromium. Chromium is used for several reasons; it is a good electrical conductor, it does not produce X-ray lines that interfere with measurement of elements of interest, it does not excessively absorb X-rays produced within the sample or prevent electron penetration of the sample, and it can be applied without excessive heating of the sample (Marshall, 1977). In the third phase, the frozen bulk sample is transferred under vacuum to a temperature controlled stage, called a cryo-stage, in the SEM. Here the specimen can be observed without noticeable deterioration. Another method of preparing dry samples for EDX analysis which does not involve chemical fixation is using ground samples. This method of sample preparation was first employed by Lott (1975), to prepare cotyledon tissue from dry seeds.

Neutron Activation Analysis (NAA) :

NAA has been used for the analysis of trace elements in various environmental and biological materials. NAA is a very sensitive method, having a detection range of 10^{-6} to 10^{-12}g of an element (Wang et al, 1975). With NAA it is possible to carry out quantitative multi-element analysis with ease, accuracy, and without destroying the sample. Provided one has access to a source of neutrons, such as a nuclear reactor, a detector and analyzer, NAA can be readily carried out.

The basic principle of NAA is that stable isotopes, when irradiated by neutrons, can undergo a nuclear transformation to produce radionuclides that can be determined quantitatively (Heydorn, 1984). The most important reaction in trace element analysis is neutron-gamma; in this reaction the radionuclides produced decay to stable elements, usually by emitting a beta particle followed immediately by a gamma ray (Corliss, 1964). By measuring the energy, half-life, and relative intensities of the emitted radiation it is possible to identify the nuclides in the sample and thus the elements in the sample (Heydorn, 1984).

In order to carry out elemental analysis of P, two separate irradiations of the pollen samples were necessary because Al and P produce the same irradiation product ^{28}Al. Hence, concentrations of Al and P in the sample must be determined using two separate irradiations. The first type of irradiation involved thermal neutrons, or slow neutrons, which react with the nuclei of most of the elements emitting gamma-rays (Corliss, 1964). The second type of irradiation involved epithermal neutrons, or fast neutrons, which are found in lower concentrations within the reactor flux. Epithermal neutrons react in a complex manner with the nuclei, emitting gamma-rays as well as neutrons, protons and alpha particles (Corliss, 1964). Epithermal irradiation can be achieved by placing the sample shielded with cadmium in a

reactor flux. The cadmium allows only epithermal neutrons to react with the atomic nuclei of the sample. The Al and P concentrations can be determined mathematically using the results from the separate irradiations and the formulae outlined by Gatschke and Gawlik (1980).

In this study EDX analysis was used primarily to analyze the elements localized in specific structures of pollen; however, it is a qualitative analysis. Quantitative NAA was used to determine some of the elements present in whole pollen samples. The elemental composition of pollen can be measured without destroying the organic matrix. Since McMaster University has a nuclear reactor on campus this procedure was used to analyze the elemental composition of C. maxima, C. andreana and L. longiflorum pollen.

MATERIALS AND METHODS

Energy Dispersive X-ray (EDX) Analysis :

EDX analysis was the method of elemental analysis chosen to analyze the elemental composition of small areas of pollen grains. Pollen samples were prepared for EDX analysis using three different methods. The three methods involved the use of sections of fixed pollen, cryo-fractured pollen, and ground pollen samples. Most of the analyses were carried out on sections of pollen. All samples were studied with an ISI model DS-130 SEM operating at 15 kV. EDX analysis studies used a PGT system 4 Energy Dispersive X-Ray Analysis System. All samples were analyzed for 100 s. The count rates ranged between 1000 and 3000 counts per s. The low take-off angle, the angle that the X-rays, emerging from the sample surface, travel in the direction of the detector was 23^{o}. A minimum of 75 spectra were analyzed for the control and anhydrous samples. A minimum of 30 spectra were analyzed for the aqueous samples and anhydrous samples sectioned with microtome knives filled with water. X-ray counts for Mg, P, K, Ca and S were calculated by integrating peaks at the following window widths: Mg = 1.146 to 1.358, P = 1.899 to 2.125, S = 2.190 to 2.422, K = 3.187 to 3.437, and Ca = 3.562 to 3.818 keV. The total number of counts was determined before and after background subtraction. The K_{β} peak of K overlapped with the K_{α} peak of Ca, this overlap was corrected. The correction of the overlap involves

subtracting 9.26% of the total X-ray counts in the potassium K_β peak from the total counts in the Ca window. The correction factor was derived from the analysis of potassium salts as suggested by Barbi (1979). The backgrounds were subtracted using a program that joins a series of predetermined points. The eV values used in the background subtraction program were as follows: 536, 660, 724, 888, 972, 1416, 1568, 2448, 2852, 2996, 4424, 5100, 6100, 9400, 11272. Peak-to-background ratios for each element and simple ratios to P such as, Mg/P, K/P and Ca/P were calculated. Combined element peak-to-background ratios such as (Mg + Ca), (Mg + K), and (Ca + K) were ratioed to the peak-to-background ratio of P because phytate is a compound containing hexaphosphate. The P of hexaphosphate can be used as an internal standard to check the possible substitutions of different cations. The ratio (Mg + Ca)/K was also calculated for each sample set, because this ratio is commonly used as a means of determining the cation ratio in phytate containing materials.

Preparation for Energy Dispersive X-ray Analysis :

1) Fixed Pollen :

i) Aqueous Fixations

Two aqueous fixatives were prepared using the methods of Moffat and Sommerville (1988), and Baldi et al (1987) as outlined under "Preparation For Transmission

Electron Microscopy" in Chapter 3. Pollen grains were fixed and prepared for EDX analysis as for TEM except that OsO_4 was omitted. Osmium tetroxide was omitted to avoid the extraction of cations from phytin and to avoid osmium contribution to the phosphorus peak (Lott, 1978; Sicko-Goad et al, 1975). Sections of Cucurbita pollen which were cut using glass microtome knives were not post-stained.

ii) Anhydrous Fixations

Anhydrous fixatives were used in order to reduce the amount of extraction of water soluble materials from the cytoplasm. Two anhydrous fixatives were prepared for fixing the Cucurbita and Lilium pollen samples. The fixatives were prepared using the methods of Sack et al (1988), and Öpik (1985) as outlined under "Preparation for Transmission Electron Microscopy".

Osmium tetroxide vapour fixation was used to determine whether or not the globoid particles, observed and analyzed by EDX analysis, were the same as the globoid particles observed and analyzed in the paraformaldehyde and glycerol fixed pollen grains. Sections were cut and floated onto microtome boats filled with either distilled water or absolute ethanol. Absolute ethanol was used to prevent extraction of water-soluble materials from the sections while they were in the boat. Sections were not post-stained.

2) Unfixed Pollen Samples :

i) Cryo-fractured Material

The Cucurbita and Lilium pollen samples were cryogenically prepared using an EMscope SP2000 cryo-unit (Bio-Rad House, Hempstead, England). Air-dried C. maxima, C. andreana and Lilium pollen grains were mixed with approximately 0.1 ml of an adhesive called Tissue-tek O.C.T. compound (Miles Scientific), to form a dense glue-like mixture. The pollen and Tissue-tek mixture were placed into the specimen holder wells and allowed to overfill each well. The specimen and the holder was frozen by plunging into sub-cooled nitrogen. Slushed nitrogen was prepared by placing liquid nitrogen under vacuum and sub-cooling it to below its boiling point. The specimen holder was transferred, under vacuum, to the work chamber of the EMscope SP2000. In the work chamber, the specimen surfaces were fractured and coated with chromium. Chromium was evaporated onto the surfaces of the frozen specimens for 10 to 30 s at 10 mA. Once coated, the specimens were transferred under vacuum to the EMscope cryostage in an ISI model DS-130 SEM. Samples were viewed and analyzed at an accelerating voltage of 15 kV and at about -162^{o}C.

ii) Ground Pollen Samples

Oven-dried C. maxima, C. andreana, and L. longiflorum pollen samples were ground with a mortar and pestle and passed through two brass sieves. The first sieve

was 140 mesh (140 bars per inch) and the second sieve was 300 mesh (300 bars per inch). The ground pollen was stored in chromerge-washed glass vials in a desiccator until ready for use. Pollen dust was smeared onto formvar coated 100 and 50 mesh Cu grids; loose particles were shaken off. Each grid was carbon coated prior to carrying out EDX analysis.

Neutron Activation Analysis (NAA) :

Sample Preparation for Neutron Activation Analysis:

Fresh pollen was dried in a Blue M oven for 2 h at 100 to 110°C. The percent moisture content of the Cucurbita pollen was approximately 52%. The moisture content of L. longiflorum pollen was not determined. In order to carry out NAA, 40 mg pollen samples were placed in polyethylene vials that had been washed in 10% HNO_3 and dried in a Blue M oven at 100°C for 2 to 3 h. Each sample was placed in a plastic "rabbit" carrier which pneumatically transported it into the core of the McMaster Nuclear Reactor for irradiation. Ultrapure P, NBS Wheat Flour (SRM 1567a) and NBS Rice Flour (SRM 1568a) of the National Bureau of Standards, Washington, DC, were used as standards for NAA. One gram sulfur standards were used regularly to monitor the neutron flux.

NAA Procedures :

i) Neutron Activation Analysis of Mg, K & Ca

In order to determine the concentration of Mg, K, and Ca each pollen sample was irradiated thermally for 240 s and transferred into a non-radioactive vial. Delay times were

200 s and samples were counted for 900 s at a distance of 1 cm from the snout of an Aptec Engineering Limited (Downsview, Ontario) coaxial hyperpure germanium detector with a 19.8% efficiency and a resolution of 1.9 keV FWHM at 1333 keV. The detector contained a Nuclear Data Accuspec interface board with 16K channel memory and a Nuclear Data Model ND581 8K ADC and ND599 Loss Free Counting module for pulse height analysis.

ii) Neutron Activation Analysis of P

The method of Gatschke and Gawlik (1980) was used to determine the P concentration. Samples were irradiated epithermally for 240 s and transferred into non-radioactive polyethylene vials. Delay times were 200 s, samples were counted for 900 s in the same manner as the thermally irradiated samples.

The elements Mg, K, Ca, and P were studied in each of the pollen samples. Analysis was based on the energy intensities of the 1014.1 keV line of ^{27}Mg, the 1542.7 keV line of ^{24}K, the 1778.9 keV line of ^{28}P, and the 3083.0 keV line of ^{49}Ca. For actual analysis of the sample, a multielement standard such as NBS Wheat Flour (SRM 1567a) was used. The standard was activated under the same conditions as the sample. The purpose of the standard was to provide a conversion factor from counts to percent under conditions identical to those under which the sample was irradiated and counted.

Statistical Analysis :

In order for the differences in data to be meaningful, the difference in the means of samples should be large relative to the variability within each sample. Two-sample t tests were used at a 5% level of significance to compare the means calculated from the EDX-analysis spectra data and from the NAA data. Two-sample t tests analyze the differences between two means of independent samples with unequal and unknown variances; the sample sizes do not have to be equal.

The mean, standard deviation, and degrees of freedom formulae were identical to those used for the Minitab program called TWOSAMPLE-T (Ryan et al, 1985). The null hypothesis is: how different are the means of two populations (Ho: $\mu_1=\mu_2$)? For this statistical analysis the t test statistic was the value from a t-table corresponding to a 95% confidence and degrees of freedom (Ryan et al, 1985).

RESULTS

1) ENERGY DISPERSIVE X-RAY ANALYSIS :

The globoid particles varied in size; they ranged from 0.13 to 1.5 μm in diameter (Figures 4.1 and 4.2). Different sizes of globoid particles were analyzed by EDX analysis. The globoid crystals were very electron-dense and easy to locate in the sections of pollen using the STEM mode (Figure 4.1).

EDX analysis spectra of globoid particles, from the anhydrous paraformaldehyde and glycerol fixation, sectioned by microtome knives filled with water and by microtome knives filled with ethanol were significantly different (Figure 4.3; Table 4.1 and Table 4.2). There was a large difference in the vertical scale values for the globoid particles analyzed (figure 4.3). The globoid particles analyzed from sections cut by microtome knives filled with water, had vertical scale values of 800, whereas the globoid particles analyzed, from sections cut by microtome knives filled with ethanol and from ground pollen samples, had vertical scale values of 12,500 (Figure 4.3).

The anhydrous sections cut by microtome knives filled with water had lower Mg, K and P peak-to-background ratios than sections cut by microtome knives filled with ethanol (Table 4.1). The Ca peak-to-background ratios for the anhydrous sections cut by microtome knives filled with

Figure 4.1 **Scanning Transmission Electron Micrographs of C. andreana and C. maxima Pollen**

Cucurbita pollen was fixed with paraformaldehyde and glycerol. The pollen was not post-fixed or post-stained. The sections were cut using an ethanol filled microtome boat.

A) C. andreana pollen cytoplasm. The white arrows indicate the distribution of the globoid particles that were analyzed by EDX analysis. Scale represents 1.0 μm. 7,200X.

B) C. andreana pollen cytoplasm. The white arrows indicate the globoid particles that were analyzed by EDX analysis. Scale represents 0.5 μm. 13,500X.

C) C. maxima pollen cytoplasm. Globoid particles (indicated by white arrows) were found throughout the pollen cytoplasm and were analyzed by EDX analysis. Scale represents 1.0 μm. 10,200X.

D) C. maxima pollen cytoplasm. Globoid particles of different sizes (indicated by white arrows) were found throughout the pollen cytoplasm and analyzed by EDX analysis. Scale represents 0.5 μm. 26,000X.

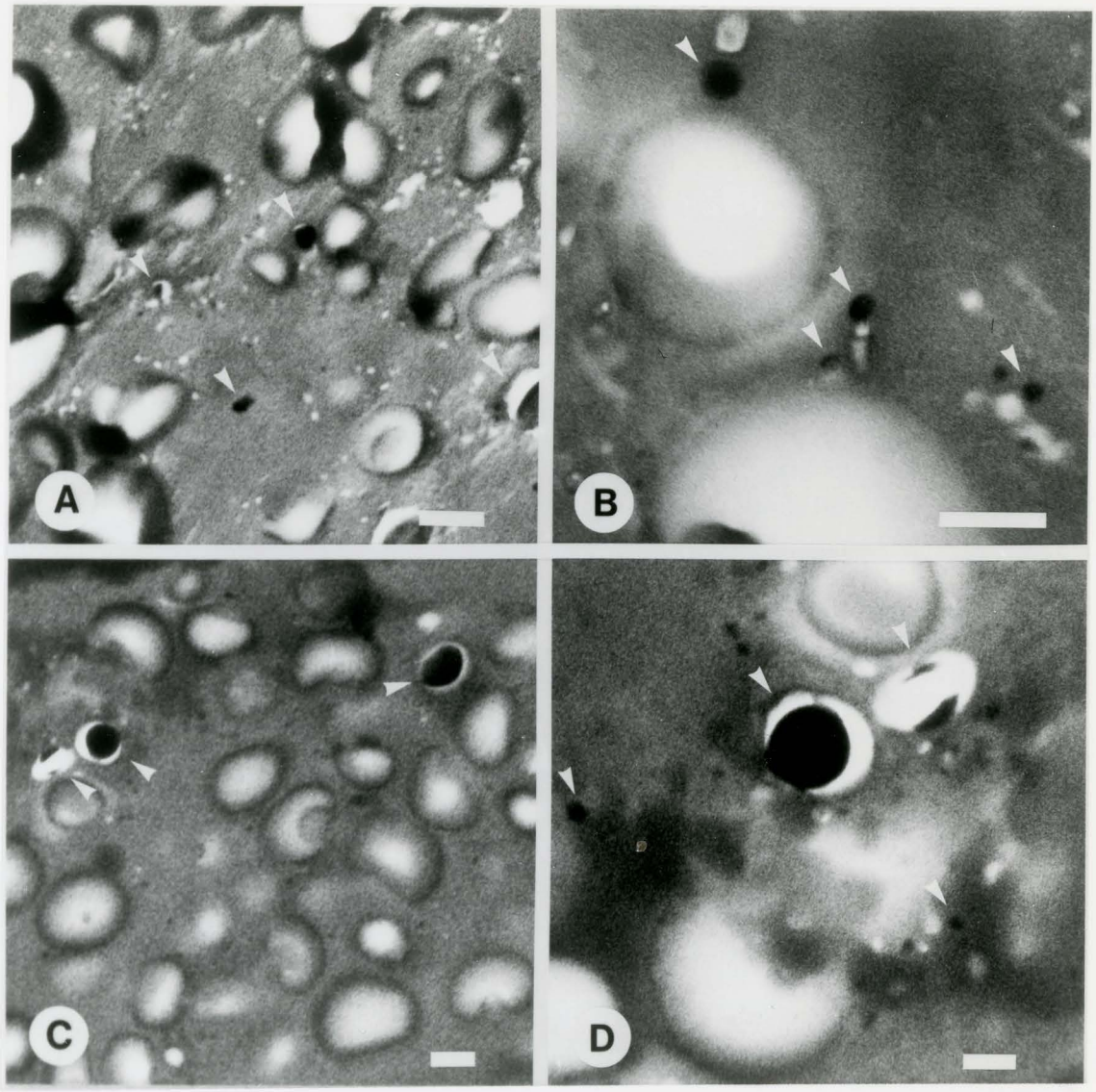

A
B
C
D

Figure 4.2 **Scanning Electron Micrographs of *C. andreana* and *C. maxima* Pollen**

A) Secondary electron image of *C. andreana* pollen cytoplasm fixed anhydrously with osmium tetroxide. The white arrows indicate globoid particles in the cytoplasm that were analyzed by EDX analysis. Scale represents 2.0 μm. 4,500X.

B) Secondary electron image of *C. andreana* pollen cytoplasm fixed anhydrously with osmium tetroxide at higher magnification. The white arrows indicate globoid particles in the cytoplasm that were analyzed by EDX analysis. Scale represents 1.0 μm. 14,200X.

C) Secondary electron image of cryo-fractured *C. maxima* pollen. The pollen possessed a thick wall and was difficult to fracture. Scale represents 10.0 μm. 730X.

D) Secondary electron image of cryo-fractured *C. maxima* pollen cytoplasm. The pollen was difficult to fracture. The pollen could not be analyzed by EDX analysis because problems with beam damage were encountered. Scale represents 10.0 μm. 1000X.

E) Secondary electron image of ground *C. maxima* pollen. The white arrows indicate the globoid particles. EDX analysis was used to analyze the mineral content of the globoid particles. Scale represents 0.25 μm. 52,300X.

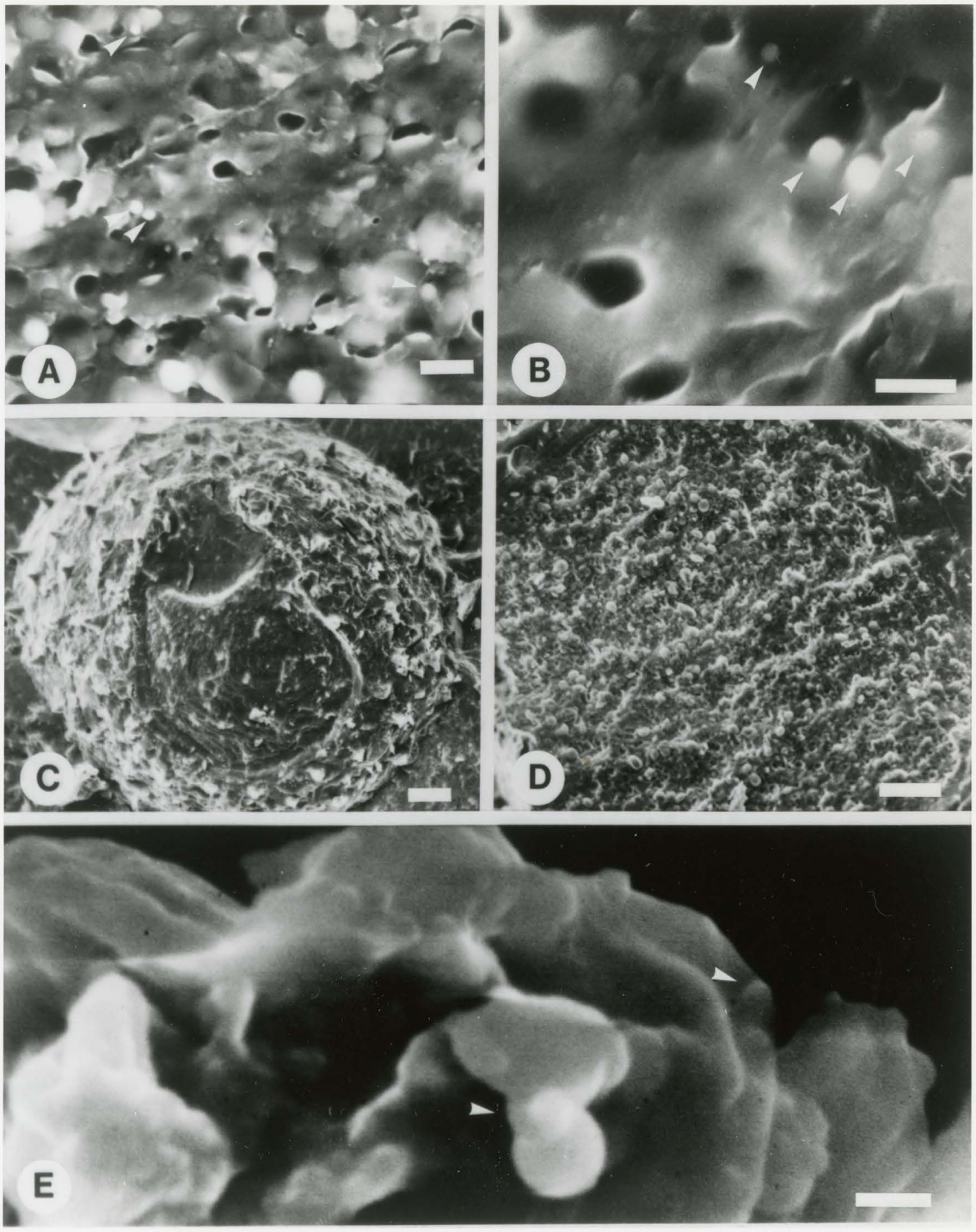

A
B
C
D
E

Figure 4.3 **EDX Analysis Spectra of Globoid Particles from C. andreana, C. maxima and L. longiflorum Pollen Sections**

The energy lines for each element analyzed are as follows: Mg = 1.252, P = 2.012, S = 2.306, Cl = 2.622, K = 3.312, and Ca = 3.690 keV.

The globoid particles in the ground pollen were easy to distinguish because they were round glowing bodies.

A) EDX analysis spectrum of a globoid particle from C. andreana pollen. The pollen was fixed anhydrously in glycerol and paraformaldehyde, and sectioned onto a water filled microtome boat. The water extracted K and Mg from the section of the pollen grain. P, S, Cl and some Mg were present in the globoid particle. (Vertical scale: 800).

B) EDX analysis spectrum of a globoid particle from C. maxima pollen. The pollen was fixed anhydrously in glycerol and paraformaldehyde, and sectioned onto a water filled microtome boat. Mg, P, K, Ca, Cl and S were present in the globoid particle. (Vertical scale: 800).

C) EDX analysis spectrum of a globoid particle from L. longiflorum pollen. The pollen was fixed anhydrously in glycerol and paraformaldehyde, and sectioned onto a water filled microtome boat. P, K, Ca and Cl were present in the globoid particle. (Vertical scale: 800).

D) EDX analysis spectrum of a globoid particle from C. andreana pollen. The pollen was fixed anhydrously in glycerol and paraformaldehyde, and sectioned onto an ethanol filled microtome boat. Mg, P, K, S and Cl were present in the globoid particle. (Vertical scale:12,500).

E) EDX analysis spectrum of a globoid particle from C. maxima pollen. The pollen was fixed anhydrously in glycerol and paraformaldehyde, and sectioned onto an ethanol filled microtome boat. Mg, P, K, S and Cl were present in the globoid particle. (Vertical scale:12,500).

Figure 4.3 **EDX Analysis Spectra of Globoid Particles from Ground and Sieved C. andreana, C. maxima and L. longiflorum Pollen**

The energy lines for each element analyzed are as follows: Mg = 1.252, P = 2.012, S = 2.306, Cl = 2.622, K = 3.312, and Ca = 3.690 keV.

The globoid particles in the ground pollen were easy to distinguish because they were round glowing bodies.

F) EDX analysis spectrum of a globoid particle from L. longiflorum pollen. The pollen was fixed anhydrously in glycerol and paraformaldehyde, and sectioned onto an ethanol filled microtome boat. Mg, P, K, S and Cl were present in the globoid particle. (Vertical scale:12,500).

G) EDX analysis spectrum of a globoid particle from C. andreana pollen. The pollen was ground and passed through two sieves. Mg, P, K, S and Cl were present in the globoid particle. (Vertical scale: 12,500).

H) EDX analysis spectrum of a globoid particle from C. maxima pollen. The pollen was ground and passed through two sieves. Mg, P, K, S and Cl were present in the globoid particle. (Vertical scale: 12,500).

I) EDX analysis spectrum of a globoid particle from L. longiflorum pollen. The pollen was ground and passed through two sieves. Mg, P, K, S and Cl were present in the globoid particle. (Vertical scale: 12,500).

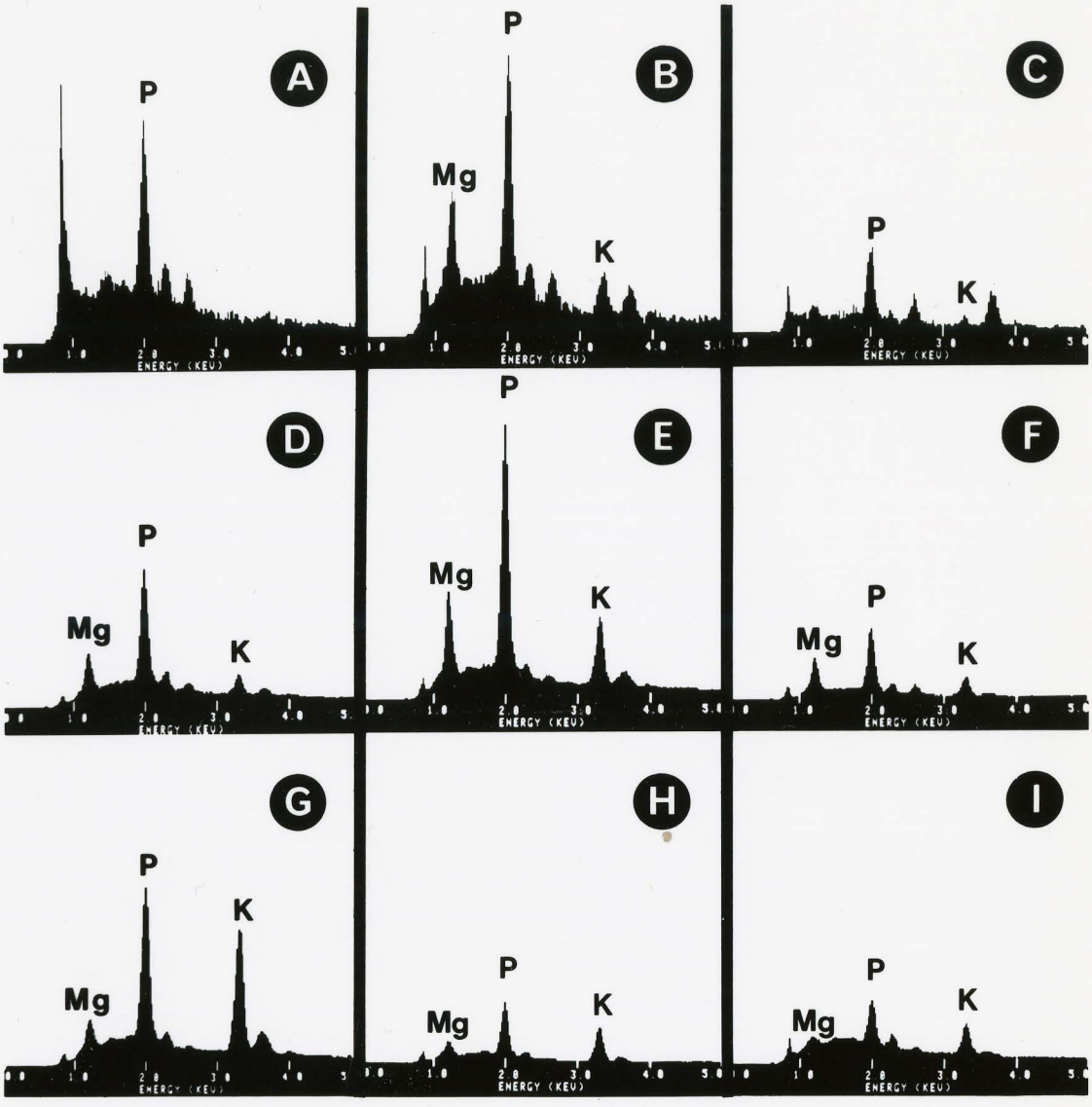

A
P
B
P
Mg
K
C
P
K
D
P
Mg
K
E
P
Mg
K
F
P
Mg
K
G
P
K
Mg
H
P
K
Mg
I
P
K
Mg
ENERGY (KEV)

Figure 4.4 **EDX Analysis Spectra of Globoid Particles from C. maxima and C. andreana Pollen Sections**

The energy lines for each element analyzed are as follows: Mg = 1.252, P = 2.012, S = 2.306, Cl = 2.622, K = 3.312, and Ca = 3.690 keV.

A) EDX analysis spectrum of a globoid particle from C. maxima pollen. The pollen was fixed aqueously with glutaraldehyde in sodium cacodylate buffer, and sectioned onto a water filled microtome boat. The combination of the aqueous fixation and water filled microtome boat extracted K and Mg from the pollen. P and some Ca were present in the globoid particle. (Vertical scale: 2000).

B) EDX analysis spectrum of a globoid particle from C. andreana pollen. The pollen was fixed anhydrously in glycerol and paraformaldehyde, and sectioned onto a water filled microtome boat. The combination of the anhydrous fixation and water filled microtome boat extracted K and Mg from the pollen. Mg, P, and K were present in the globoid particle. (Vertical scale: 2000).

C) EDX analysis spectrum of a globoid particle from C. maxima pollen. The pollen was fixed anhydrously in glycerol and paraformaldehyde, and sectioned onto a water filled microtome boat. The water extracted K and Mg. P, some Ca, S and Cl were present in the globoid particle. (Vertical scale: 2000).

D) EDX analysis spectrum of a globoid particle from C. maxima pollen. The pollen was fixed anhydrously in osmium tetroxide vapour and sectioned onto a water filled microtome boat. The water extracted K and Mg. P and some S were present in the globoid particle. (Vertical scale: 2,000).

E) EDX analysis spectrum of a globoid particle from C. maxima pollen. The pollen was fixed anhydrously in osmium tetroxide vapour and sectioned onto an ethanol filled microtome boat. P, K, some S and Cl were present in the globoid particle. (Vertical scale: 6,000).

F) EDX analysis spectrum of a globoid particle from C. andreana pollen. The pollen was fixed anhydrously in osmium tetroxide vapour and sectioned onto an ethanol filled microtome boat. Mg, P, K, some S and Cl were present in the globoid particle. (Vertical scale: 6,000).

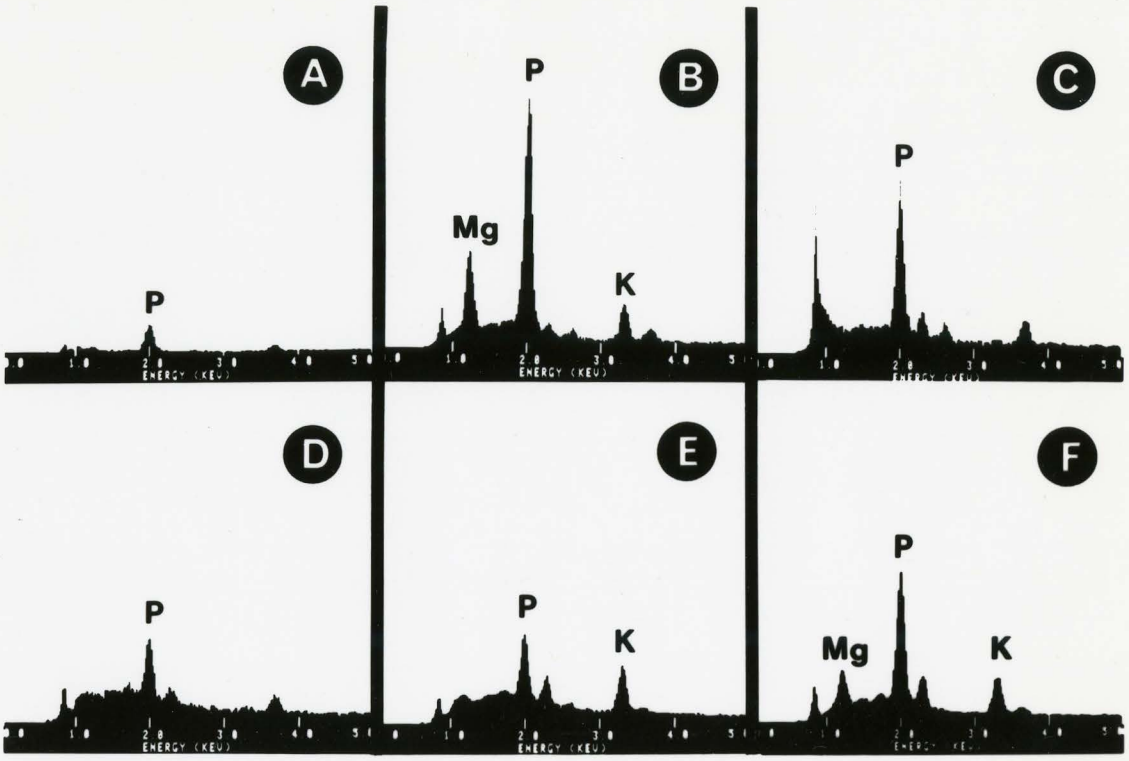

A
P
B
P
Mg
K
C
P
D
P
E
P
K
F
P
Mg
K
ENERGY (KEV)

Table 4.1

Energy Dispersive X-Ray Analysis Peak to Background Ratios of Globoid Particles from Fixed and Unfixed *Cucurbita* and *Lilium* Pollen

Sample	n*	Element Peak to Background Ratios Mg P/B	K P/B	P P/B	Ca P/B
FIXED : Paraformaldehyde and Glycerol (Water Boat)					
C. maxima	28	0.257	0.119	2.406 b	0.892 e
C. andreana	26	0.681	0.442	2.842 b	0.703 e
L. longiflorum	25	0.544	0.639	1.542	2.664
FIXED : Paraformaldehyde and Glycerol (Ethanol Boat)					
C. maxima	95	1.221	0.948	3.404 c	0.256 f
C. andreana	83	1.749	1.373	4.646 c	0.152 f
L. longiflorum	23	2.189	1.512	4.421 c	0.012 g
UNFIXED : Ground and Sieved Pollen					
C. maxima	68	0.844 a	3.589	3.873 d	0.139 g
C. andreana	73	0.849 a	2.834	3.210 d	0.055 g
L. longiflorum	80	0.165	2.229	1.129	0.136 g

* - n represents the number of samples that were analyzed

Values for numbers in each column that are followed by the same letter (example a) are not significantly different. Values for numbers in each column that are not followed by a letter are significantly different.

Table 4.2

Energy Dispersive X-Ray Analysis Peak to Background Ratios of Globoid Particles from Fixed and Unfixed Cucurbita and Lilium Pollen

Sample	n*	Element Peak to Background Ratios (Mg+K)/P	(Mg+Ca)/P	(K+Ca)/P	(Mg+Ca)/K
FIXED :	**Paraformaldehyde and Glycerol (Water Boat)**				
C. maxima	28	0.189 a	0.646 e	0.575 g	9.656 +
C. andreana	26	0.351 a	0.515 e	0.419 g	3.131 +
L. longiflorum	25	0.866 b	2.166	2.226	5.061 +
FIXED :	**Paraformaldehyde and Glycerol (Ethanol Boat)**				
C. maxima	95	0.679 c	0.449 e	0.367 h	1.288 j
C. andreana	83	0.746 c	0.457 e	0.379 h	1.385 j
L. longiflorum	23	0.845 b	0.500 e	0.359 h	1.455 j
UNFIXED :	**Ground and Sieved Pollen**				
C. maxima	68	1.359 d	0.245 f	1.191 i	0.285 k
C. andreana	73	1.370 d	0.299 f	0.883 i	0.299 k
L. longiflorum	80	2.289	0.393 f	2.368	0.135

* - n represents the number of samples that were analyzed

Values for numbers in each column that are followed by the same letter (example a) are not significantly different. Values for numbers in each column that are not followed by a letter are significantly different.

+ - values are large due to small K P/B

Table 4.3

Energy Dispersive X-Ray Analysis Element to P Peak to Background Ratios of Globoid Particles from Fixed and Unfixed *Cucurbita* and *Lilium* Pollen

		Element Peak to Background Ratios		
Sample	n*	Mg/P	K/P	Ca/P
FIXED :	**Glutaraldehyde and Sodium Cacodylate**			
C. maxima	25	0.195 a	0.099 d	0.416 g
FIXED :	**Paraformaldehyde and Glycerol (Water Boat)**			
C. maxima	28	0.130 a	0.059 d	0.516 g
C. andreana	26	0.224 a	0.127 d	0.292 g
L. longiflorum	25	0.403	0.463	0.003
FIXED :	**Paraformaldehyde and Glycerol (Ethanol Boat)**			
C. maxima	95	0.325 b	0.218 e	0.081 h
C. andreana	83	0.408 b	0.339 e	0.041 h
L. longiflorum	23	0.493	0.351 e	0.007 h
UNFIXED :	**Ground and Sieved Pollen**			
C. maxima	68	0.207 c	1.153 f	0.038 i
C. andreana	73	0.225 c	1.145 f	0.018 i
L. longiflorum	80	0.156	2.132	0.236

* - n represents the number of samples that were analyzed

Values for numbers in each column that are followed by the same letter (example a) are not significantly different. Values for numbers in each column that are not followed by a letter are significantly different.

Table 4.4

Energy Dispersive X-Ray Analysis Peak to Background Ratios of Globoid Particles from Aqueously and Anhydrously Fixed *Cucurbita* Pollen

Sample	n*	Element Peak to Background Ratios Mg P/B	K P/B	P P/B	Ca P/B
AQUEOUS : Glutaraldehyde and Sodium Cacodylate					
C. maxima	25	0.248 a	0.094 b	2.239 d	0.979 f
AQUEOUS : Glutaraldehyde, Paraformaldehyde and PIPES					
C. maxima	25	0.177 a	0.052 b	1.081	0.212
ANHYDROUS : Paraformaldehyde and Glycerol (Water Boat)					
C. maxima	28	0.257 a	0.119 b	2.406 d	0.892 f
C. andreana	26	0.681 a	0.442	2.842 d	0.703 f
ANHYDROUS : Osmium Tetroxide Vapour (Water Boat)					
C. maxima	30	0.015	0.492	1.385 e	0.690 f
ANHYDROUS : Osmium Tetroxide Vapour (Ethanol Boat)					
C. maxima	59	0.192	1.761 c	1.333 e	0.552 f
C. andreana	54	1.398	2.020 c	5.805	0.918 f

* - n represents the number of samples that were analyzed

Values for numbers in each column that are followed by the same letter (example a) are not significantly different. Values for numbers in each column that are not followed by a letter are significantly different.

Table 4.5

Energy Dispersive X-Ray Analysis Peak to Background Ratios of Globoid Particles from Aqueously and Anhydrously Fixed *Cucurbita* Pollen

Sample	n*	Element Peak to Background Ratios (Mg+K)/P	(Mg+Ca)/P	(K+Ca)/P	(Mg+Ca)/K
AQUEOUS :	**Glutaraldehyde and Sodium Cacodylate**				
C. maxima	25	0.293 a	0.611 b	0.515 d	13.19 +
AQUEOUS :	**Glutaraldehyde, Paraformaldehyde and PIPES**				
C. maxima	25	0.331 a	0.578 b	0.320 d	7.48 +
ANHYDROUS :	**Paraformaldehyde and Glycerol (Water Boat)**				
C. maxima	28	0.189 a	0.646 b	0.575 d	9.66 +
C. andreana	26	0.351 a	0.515 b	0.419 d	3.13 +
ANHYDROUS :	**Osmium Tetroxide Vapour (Water Boat)**				
C. maxima	30	0.295 a	0.534 b	0.812 e	1.43
ANHYDROUS :	**Osmium Tetroxide Vapour (Ethanol Boat)**				
C. maxima	59	1.584	0.354 c	1.673	0.195
C. andreana	54	0.824	0.389 c	0.751 e	1.147

* - n represents the number of samples that were analyzed

Values for numbers in each column that are followed by the same letter (example a) are not significantly different. Values for numbers in each column that are not followed by a letter are significantly different.

+ - values are large due to small K P/B

Table 4.6

Neutron Activation Analysis Values and National Bureau of Standards Values for Standard Reference Material.

Concentration of element as percent dry weight

Sample	Mg	K	Ca	P
NAA Sample Values* :				
Wheat Flour	0.042	0.140	0.019	0.135
Wheat Flour	0.039	0.140	0.019	0.135
Rice Flour	0.058	0.120	0.012	0.150
Rice Flour	0.056	0.110	0.013	0.158
NBS Certified Values :				
Wheat Flour	0.040	0.133	0.019	0.134
Rice Flour	0.056	0.128	0.011	0.153

* - duplicates of wheat flour and rice flour were analyzed

Rice flour standards were used to calculate the concentrations of wheat flour samples. Wheat flour standards were used to calculate the concentrations of rice flour samples.

Table 4.7

Concentrations of Mg, K, Ca and P in Pollen Samples as Determined by Neutron Activation Analysis.

Sample	n*	Concentration of element as percent dry weight Mg	K	Ca	P
Cucurbita maxima	3	0.128 a	1.320 b	0.084 c	0.532 d
Cucurbita andreana	2	0.178 a	1.300 b	0.117 c	0.710 d
Lilium longiflorum	1	0.098	1.260	0.036	0.420

* - n represents the number of samples that were analyzed

Values for numbers in each column that are followed by the same letter (example a) are not significantly different. Values for numbers in each column that are not followed by the same letter are significantly different.

Table 4.8

Concentrations of Elements Ratioed to P in Pollen as Determined by Neutron Activation Analysis.

Concentration of element as percent dry weight				
Sample	n	Mg/P	K/P	Ca/P
C. maxima	3	0.241 a	2.481 b	0.158 c
C. andreana	2	0.251 a	1.831 b	0.165 c
L. longiflorum	1	0.233	3.000	0.086

n - represents the number of samples that were analyzed

Values for numbers in each column that are followed by the same letter (example a) are not significantly different. Values for numbers in each column that are not followed by the same letter are significantly different.

Table 4.9

Concentrations of Elements Ratioed to P and K in Pollen as Determined by Neutron Activation Analysis.

Concentration of element as percent dry weight								
Sample	n*	(Mg+K)/P		(Mg+Ca)/P		(K+Ca)/P		(Mg+Ca)/K	
C. maxima	3	3.499	a	0.536	b	3.384	c	0.161	d
C. andreana	2	2.198	a	0.449	b	2.645	c	0.227	d
L. longiflorum	1	3.233		0.319		3.086		0.106	

* - n represents the number of samples that were analyzed

Values for numbers in each column that are followed by the same letter (example a) are not significantly different. Values for numbers in each column that are not followed by the same letter are significantly different.

water were larger than the Ca peak-to-background ratios for the anhydrous sections cut by microtome knives filled with ethanol (Table 4.1).

Mg, K and P were extracted from the anhydrously fixed pollen when the sections were cut by microtome knives filled with water (Figure 4.3). When the microtome boat was filled with absolute ethanol, there was less extraction of the water soluble materials from the sections (Table 4.1 and Figure 4.3). C. maxima and C. andreana P and Ca peak-to-background ratios were not significantly different from species to species (Table 4.1). L. longiflorum and Cucurbita P peak-to-background ratios were not significantly different from species to species (Table 4.1).

Globoid particles were localized and analyzed in ground Lilium and Cucurbita pollen samples. The globoid particles were spherical in shape and appeared as glowing white bodies (Figure 4.2). The EDX analysis spectra revealed the presence of Mg, K, and P in the pollen samples (Figure 4.3). The Mg, K and P peak-to-background ratios for ground pollen and the anhydrously fixed pollen were significantly different from each other (Table 4.1). The ground pollen K and P peak-to-background ratios were larger than the ratios for the anhydrous pollen samples. C. maxima and C. andreana ground pollen Mg, P and Ca peak-to-background ratios were not significantly different from species to species (Table 4.1). Cucurbita and L. longiflorum Ca peak-to-background

ratios were not significantly different from species to species (Table 4.1).

The combined element ratios to P and the single element ratios to P for the anhydrously fixed samples and the ground pollen samples were significantly different from sample to sample (Table 4.2 and Table 4.3). For each anhydrous fixation and for the ground pollen samples, the combined element and single element ratios for C. maxima and C.andreana did not significantly differ from species to species (Table 4.2 and Table 4.3). Cucurbita and L. longiflorum (Mg + Ca)/P, (K + Ca)/P, (Mg + Ca)/K, K/P, and Ca/P ratios, of anhydrously fixed pollen sectioned by microtome knives filled with ethanol, did not significantly differ from species to species. However, Cucurbita and L. longiflorum (Mg + K)/P, (K + Ca)/P, (Mg + Ca)/K, Mg/P, K/P, and Ca/P ratios, of ground pollen samples, were significantly different from species to species.

The EDX analysis spectra of globoid particles from aqueously fixed C. maxima pollen and anhydrously fixed C. maxima pollen sectioned by microtome knives filled with water were similar (Figure 4.4). The K and Mg peak-to-background ratios for the aqueous fixatives did not significantly differ from species to species and revealed that K and Mg had been extracted (Table 4.4).

The Mg/P, K/P, Ca/P, Mg, and K peak-to-background ratios, for the C. maxima aqueous and anhydrous fixations

that were cut by microtome knives filled with water, were not significantly different from fixation to fixation (Table 4.3 and Table 4.4). C. maxima and C. andreana pollen Mg/P, K/P, Ca/P, P and Ca peak-to-background ratios, of the anhydrous fixation that were cut by microtome knives filled with water, were not significantly different from species to species (Table 4.3 and Table 4.4). Significant differences, from species to species, were observed in the Mg and K peak-to-background ratios of C. maxima and C. andreana pollen (Table 4.4). The Ca peak-to-background ratios, for the aqueous or anhydrous fixations, were not significantly different from fixation to fixation (Table 4.4).

Osmium tetroxide vapour fixed pollen was very difficult to section. Globoid particles were spherical and were easy to detect in the sections due to their glowing appearance (Figure 4.2). EDX spectra of globoid particles, from the osmium tetroxide vapour fixation and cut by microtome knives filled with water, revealed that K and Mg were extracted from the pollen section (Figure 4.4). The Mg and K peak-to-background ratios, for the C. maxima osmium tetroxide vapour fixations cut by microtome knives filled with water and ethanol, were significantly different from section to section (Table 4.4). C. andreana and C. maxima osmium tetroxide vapour fixations cut by microtome knives filled with ethanol, had Ca and K peak-to-background ratios that were not significantly different from species to

species. However Mg and P peak-to-background ratios of C. maxima and C. andreana osmium tetroxide vapour fixed pollen cut by microtome knives filled with ethanol, were significantly different from species to species (Table 4.4).

The combined element ratios to P, for the aqueous fixations and the paraformaldehyde and glycerol fixations cut by microtome knives filled with water, were not significantly different from fixation to fixation (Table 4.5). The (Mg + K)/P and (Mg + Ca)/P ratios, for both anhydrous fixations cut by microtome knives filled with water and the aqueous fixations were not significantly different from fixation to fixation (Table 4.5). The combined element ratios to P, for the osmium tetroxide vapour fixations cut by microtome knives filled with water and by microtome knives filled with ethanol, were significantly different from section to section. C. maxima and C. andreana (Mg + Ca)/P ratios, for the osmium tetroxide vapour fixations cut by microtome knives filled with ethanol, were not significantly different from species to species (Table 4.5).

After trying the cryo-fracturing procedure with unfixed pollen, it was soon discovered that Cucurbita and Lilium pollen were very difficult to prepare for EDX analysis. It was possible to obtain a clean fracture through the Cucurbita and Lilium pollen using cryogenic procedures, but the samples were unstable under the electron beam

(Figure 4.2). EDX analysis could not be carried out on the pollen samples for two reasons. Globoid particles could not be localized due to the poor resolution of the system. Cryo-fractured pollen suffered from severe beam damage and large holes formed where the electron beam was focussed on the cytoplasm.

2) NEUTRON ACTIVATION ANALYSIS :

Neutron activation analysis (NAA) is known for its sensitivity and can be applied to measuring a wide range of elements. The reproducibility of NAA was demonstrated with the NBS standards (Table 4.6). The NAA results showed that the wheat flour and rice flour samples had Mg, K, P and Ca concentrations that were similar to the concentrations of the respective NBS certified standards. It was possible to obtain elemental concentrations for the standards by measuring each sample with respect to the other standard.

NAA of pooled samples of whole C. maxima and C. andreana pollen grains showed that the concentrations of Mg, K, P, and Ca were not significantly different from species to species (Table 4.7). Mg/P, K/P, and Ca/P ratios for pooled samples of whole C. maxima and C. andreana pollen grains were not significantly different from species to species (Table 4.8). Cucurbita pollen contained more K than P, the pollen contained approximately 1.3 % K and 0.5 - 0.7 % P (Table 4.7). L. longiflorum pollen had concentrations of Mg, K, P and Ca, similar to those of Cucurbita pollen and

also had similar Mg/P, K/P, Ca/P ratios. However t tests could not be used to analyze this data because only one Lilium pollen sample was analyzed (Table 4.7 and Table 4.8).

The combined element ratios to P for C. maxima and C. andreana pollen were not significantly different from species to species (Table 4.9). L. longiflorum and Cucurbita pollen combined element ratios to P were similar from species to species (Table 4.9). The Cucurbita NAA (Mg + K)/P, (Mg + Ca)/P, (K + Ca)/P, Mg/P, and K/P ratios were roughly two times greater than the respective ratios of the ground Cucurbita pollen samples measured with EDX analysis (Table 4.2 and Table 4.9; Table 4.3 and Table 4.8). The Cucurbita NAA (Mg + Ca)/K ratio was approximately one half the size of the respective ratio of the ground Cucurbita pollen sample measured with EDX analysis (Table 4.2 and Table 4.9).

Chapter 5

GENERAL DISCUSSION.

LIGHT MICROSCOPY AND HISTOCHEMISTRY :

Pollen from both species of Cucurbita were similar with regard to their colour, general morphology, internal structure and storage reserves. Botanical descriptions of the morphology of Cucurbita maxima and Cucurbita andreana flowers stated that the flowers of these species were similar; however, they differed in size by a factor of two (Bailey, 1948; Whitaker, 1951). Unlike the seeds and flowers of the aforementioned Cucurbita species, which differed in size, the pollen grains were about the same size.

The coagulative fixative, FAA, preserved the Cucurbita pollen well enough to carry out staining with various general oversight stains and histochemical stains. This was because coagulative fixatives were capable of producing rapid fixation as fast as they penetrated the cells (Johansen, 1940; Thompson, 1966; Humason, 1972; Gahan, 1984). Standard glutaraldehyde solutions often do not penetrate the walls of specimens which possess thick cuticles and waxes, while fixatives containing formaldehyde or mixtures of formaldehyde with other fixatives do pass through such walls (O'Brien and McCully, 1981).

Cucurbita pollen was not covered by a cuticle.

However, it possessed a very thick and resistant tube cell wall composed of polysaccharides, lignin, and lipids which hindered the fixation and infiltration processes. Fortunately Cucurbita pollen specimens infiltrated well and were relatively easy to section. The pollen grains were easy to section considering the abundance of storage material, primarily starch, present in the cytoplasm and the thickness of the tube cell wall. Generally the presence of starch grains, protein bodies or crystals in a specimen has been found to slow or impede infiltration such that very little plastic penetrates the starch and cell walls (O'Brien and McCully, 1981).

A further complication of working with pollen was maintaining the pollen grain in osmotic balance with the fixative. Maintaining the correct osmotic balance is necessary to prevent pollen grains from bursting (Stanley and Linskens, 1965; Vasil, 1987). Vasil (1960) showed that Cucurbita pollen burst in fixatives of low osmotic pressure. Frequent bursting of C. maxima and C. andreana pollen grains was also encountered when I was preparing pollen for light microscopy studies.

Heslop-Harrison (1986) extensively researched the role of water in pollen tube germination and the hydrodynamics of germination. This study revealed that during the final stage of pollen maturation, the metabolic rate decreased and pollen underwent dehydration.

Reactivation of pollen occurred at the stigma surface with the uptake of water. The apertures served as the preferred pathway of water gain and water loss. Water stretched the exine causing changes in the shape of the apertures resulting in an increase in the area of exposed intine. Exposure of the intine to water lessened the hydraulic resistance to further inflow of water, thus allowing more water to flow into the grain. The influx of water created a build up of hydrostatic pressure in the grain. The build up of pressure was relieved by bursting or by emergence of the pollen tube. The findings from Heslop-Harrison's hydrodynamic studies explain why problems were often encountered during the fixation process of Cucurbita and pollen of other species of plants and support the necessity of the fast-penetrating fixatives employed in this study.

The bulk of structural studies of pollen have focused on the pollen grain wall. Generally the surface of mature pollen of entomophilous species, such as cucurbit pollen, is coated superficially with pollenkitt. Pollenkitt is restricted to angiosperm species, has a yellow-orange colour, and consists mainly of carotenoids, flavonoids and lipids (Heslop-Harrison, 1968a; Heslop-Harrison, 1968b; Wiermann and Vieth, 1983; Chapman, 1987; Dobson, 1988). This coating serves functions such as sealing the exine and retarding water loss, acting as a screen protecting the generative nucleus from UV irradiation, providing olfactory

cues to pollinators, and cementing pollen grains together during dispersal (Linskens, 1967; Heslop-Harrison, 1968a; Heslop-Harrison, 1968b, Dobson, 1988).

Details of the Cucurbita pollen wall and cytoplasm were demonstrated by the general oversight stains. The histochemical stains revealed the chemical structure of the pollen grain. The pollen wall consisted of two layers, the intine and the exine. The staining results demonstrated that the pollen walls of Cucurbita were structurally and chemically similar to pollen walls of other species.

Studies of pollen walls of other species revealed that the exine and intine were structurally adapted to accommodate volume changes associated with dehydration and hydration (Heslop-Harrison, 1986). The apertures were areas where the exine was reduced or virtually absent and functioned in pollen tube germination. The exine is generally composed of sporopollenin which contains 10-25% lignin-like material and 75-90% lipid, and is very resistant to chemical and physical damage (Shaw and Yeadon, 1966; Heslop-Harrison, 1968b; Knox and Heslop-Harrison, 1969; Guilford et al, 1988).

Extensive studies of pollen development in various species have shown that the exine is formed during the tetrad period within a callosic wall (Brooks and Shaw, 1968; Takahashi and Kouchi, 1988). Generally the wall structure of pollen is similar for different species. The exine has two

strata, the outer sculptured sexine and the inner non-sculptured nexine. The sculptured sexine is formed from sporopollenin which is laid down in a range of patterns that are distinctive enough that they allow identification of genera and even species (Chapman, 1987).

The histochemistry of Cucurbita pollen was similar to the histochemistry of pollen from other species. The intine of Cucurbita and the intine of pollen from other species of plants generally is thicker than the exine and consists of a pectocellulosic layer which is permeable to water (Knox and Heslop-Harrison, 1969; Chapman, 1987). The polychrome stain did not demonstrate the presence of cellulose in the Cucurbita pollen. This may be due to an error in the sensitivity of the polychrome stain. Studies of plant cell walls have shown that cellulose fibers are interconnected by pectic-type polysaccharides (Sassen, 1964; Tu et al, 1988). The intine is easily distinguished from the exine by the intine's positive response to PAS (Pickett-Heaps, 1968; Knox and Heslop-Harrison, 1969). Generally the intine is thickened in the vicinity of each aperture, forming a plug, and the apertures are capped with sporopollenin.

Enzyme proteins have been found in the intine and were concentrated near the apertures (Knox and Heslop-Harrison, 1969). These proteins were readily leached out during the germination process and may function in incompatibility reactions (Stanley and Linskens, 1965;

Chapman, 1987; Vasil, 1987).

The number of published histochemistry studies over the last 30 years, which pertain to pollen cytoplasm and storage reserves, is limited. Published histochemical studies of mature pollen from several species including Cucurbita have shown that the cytoplasm is compact and contains storage material (Vasil, 1960; Larson and Lewis, 1962; Bouveng, 1963; Rosen et al, 1964; Sassen, 1964; Larson, 1965; Hoefert, 1969; Sanger and Jackson, 1971a; Cass, 1973; Opute, 1975; Baker and Baker, 1979; Vasil, 1987; Iwata et al, 1988; Jewell et al, 1988; Raghavan, 1988; Rodriguez-Rosales and Donaire, 1988).

The cytoplasm of viable pollen has been described as being non-vacuolate, granular and possessing many plastids which may or may not contain starch (Vasil, 1987). Reserve food materials such as starch, lipids and proteins, are required for pollen tube growth. Pollen grains germinate on a stigma with the production of a pollen tube which then penetrates the style and the ovule in order to release male gametes into the embryo sac. The growth of the pollen tube requires energy which can be derived from the pollen storage reserves.

Histochemical studies of the storage materials of C. maxima and C. andreana pollen demonstrated that the primary storage reserve was starch. Cucurbita pollen cytoplasm was very dense and contained many starch grains. The presence of

starch was confirmed by Lugol's iodine. PAS served as a good basic histochemical stain to localize polysaccharide materials in plant cells. Cell walls and starch grains are typically stained with PAS (Pickett-Heaps, 1968). The presence of polysaccharide materials in the Cucurbita pollen intine and cytoplasm were demonstrated with PAS.

The occurrence of starch in pollen varies from species to species but it constitutes the major carbohydrate store in most angiosperm pollen (Bouveng, 1963). Starch has been observed in mature pollen of Ranunculus macranthus, Petunia, Beta, Haemanthus katherinae, Typha latifolia, Oryza sativa, and Hordeum vulgare (Larson and Lewis, 1962; Sassen, 1964; Hoefert, 1969; Sanger and Jackson, 1971a; Vasil, 1987; Iwata et al, 1988; Raghavan, 1988; Jewell et al, 1988). The mature pollen of Lilium longiflorum and Lilium regale does not contain starch (Rosen et al, 1964). Baker and Baker (1979) surveyed mature pollen grains of 1000 species and found that there are two classes of pollen, starchy and starchless. The Liliaceae family is starchless. It is believed that all pollen contains some storage lipids. The lipid content of starchless pollen grains is elevated (Baker and Baker, 1979).

Reasons for starch storage in pollen have been outlined by Baker and Baker (1979). It is metabolically less complicated to turn sugars into starch than into oil. It is also less complicated to break starch into sugars used for

respiration, wall building, and callose production in the pollen tube. However, starch is less compact than oil. Thus if the size of the pollen grain is a limiting factor, it may be necessary to store oil. This phenomenon has been observed in small seeds (Baker and Baker, 1979).

The presence of starch in pollen is most often characteristic of primitive angiosperm families. Generally the flowers of families containing starchy pollen are polypetalous, actinomorphic, syncarpous but hypogynous (Baker and Baker, 1979). In general the pollen grains that contain large amounts of starch have a large diameter of approximately 100 μm. Baker and Baker (1979) found that as the diameter of the pollen increases the amount of starch present in the grain increases. They also found that pollen size is related to length of style. Generally pollen maturation is associated with the accumulation of starch grains (Raghavan, 1988). Starch accumulation occurs during the latter stages of pollen maturation (Larson, 1965). In all families studied, starch was stored within amyloplasts in the pollen grains (Baker and Baker, 1979).

Most histochemical studies of lipids and proteins in pollen refer to the structure of the cell wall. Baker and Baker (1979) stated that all pollen contains some lipids in the cytoplasm but the amount varies with species and with the size of the pollen grain. The C. maxima and C. andreana pollen smears demonstrated that small amounts of lipids were

present in the cytoplasm. It is believed that the lipids were extracted during the fixation and dehydration process. It was also possible that the lipid tests lacked sensitivity because Begonia seeds which contain lipids responded negatively to the lipid stains. Generally it is found that in pollen where starch is absent, the lipid content is increased. Studies of mature lily pollen have shown that the main storage reserve was lipid (Rosen et al, 1964; Sassen, 1964).

Other species of plants which contain lipids as their main reserve in pollen are Parkinsonia aculeata, Elacis guineensis, Petunia, and Olea europaea (Larson, 1965; Opute, 1975; Rodriguez-Rosales and Donaire, 1988). The types of lipids found in the pollen cytoplasm are neutral and polar lipids (Opute,1975). The lipids serve as energy reserves, and in the synthesis of membranes and cell walls (Rodriguez-Rosales and Donaire, 1988).

There have been very few histochemical studies which focus on localizing proteins in pollen cytoplasm. Most protein studies involve cytochemical localization of enzymes in the wall of the pollen grain (Knox and Heslop-Harrison, 1969; Knox, 1971; Knox and Heslop-Harrison, 1971). The protein stain, Coomassie brilliant blue, allows precise localization of proteins and protein-containing bodies such as mitochondria, plastids and nuclei in embryo sac tissue (Fisher, 1968; Gahan, 1984). Coomassie brilliant blue stains

proteins without interference from cations such as potassium or sodium, nor from carbohydrates such as sucrose (Bradford, 1976). Cucurbita pollen smears and sections gave positive results to Coomassie brilliant blue as well as to mercury-bromophenol blue. The pollen cytoplasm contained finely dispersed proteins which may have been indicative of the presence of plastids and other protein-containing bodies.

The combination of general oversight stains and histochemical stains has revealed information about the chemical nature of Cucurbita pollen wall and cytoplasm. The stains have also given insight into the structure of Cucurbita pollen cytoplasm, which has received little attention. The results have shown that Cucurbita pollen was similar structurally and chemically to pollen of many other species. Cucurbita pollen grains were large and stored mainly starch. The storage of starch is characteristic of species which possess large pollen grains. Proteins and lipids were also found in Cucurbita pollen; however, their presence in the cytoplasm was less prominent than the starch reserves.

ELECTRON MICROSCOPY :

Scanning electron microscopy was a useful means of visualizing Cucurbita and Lilium pollen for structural studies of the external surface of the pollen grains. However, individual Cucurbita pollen grains could not be

studied for too long because the pollen apertures were sensitive to the electron beam.

It is generally agreed that one of the major drawbacks of working with pollen, and the main reason that investigations of the fine structure of pollen have been retarded, is that there has been great difficulty in cutting ultra-thin sections of the relatively hard pollen grains (Chambers and Godwin, 1961, Rosen et al, 1964; Sassen, 1964; Gullvag, 1966; Vasil, 1960; Dumas et al, 1985; Vasil, 1987). Hayat (1981a) determined that compact or dense tissues permitted slow penetration of fixatives. Also the presence of thick coatings of water-proof substances, such as cutin on the tissue surface, hindered penetration of the fixative. In general, pollen is dense and possesses a thick wall protected by an oily coating of pollenkitt (Dobson ,1988). Loss of materials from Tilia platyphyllos and Lilium pollen during fixation, embedding and sectioning has been reported (Chambers and Godwin, 1961; Heslop-Harrison, 1968a) .

Aqueous fixation of dry seeds and other dry tissues did not provide an accurate representation of the air-dry state since partial hydration of the cellular components caused some modification of structure and even damage (Swift and Buttrose, 1973; Hallam, 1976; Webb and Arnott, 1982; Öpik, 1985). Aqueous fixatives caused swelling of dry peanut tissues and anhydrous organic solvents did not. When organic solvents were used the cell walls were wrinkled (Yatsu,

1983).

Water-soluble materials such as K-phytate tend to be extracted by aqueous fixation (Lott et al, 1985b). Manston and Katchburian (1984) reported interference with the matrix/mineral relationships as well as with the physical state of the mineral itself with aqueous fixation. Another problem with aqueous fixation of pollen is that the presence of water or high amounts of moisture tends to initiate germination or rupture (Vasil, 1960; Heslop-Harrison, 1986; Vasil, 1987). Thus results obtained from aqueously fixed dry tissues must be interpreted cautiously (Öpik, 1985). Structural studies of seed and pollen structure in the air-dry state should be observed by completely anhydrous fixation methods. Considering the number of seed ultrastructural studies, very few structural studies of anhydrously prepared tissues have been carried out (Öpik, 1985).

It is generally agreed that anhydrous preparation of dry tissues for thin sectioning presents great practical difficulties (Hallam, 1976; Yatsu, 1983; Öpik, 1985). Penetration of chemicals into dry tissues is very slow (Öpik, 1985). Often imperfect infiltration of the resin into the tissues occurred (Yatsu, 1983). In my research, imperfect fixation and infiltration was observed for the OsO_4 vapour fixed pollen. After the fixation of pollen with osmium tetroxide vapour was carried out, pollen was

embedded. However, the blocks of pollen could not be used for thin sectioning because they were too soft. The soft blocks produced by the OsO_4 vapour fixation of tissue have been reported before (Humason, 1972; Sack et al, 1988).

Thomson (1979) used various fixatives with glycerol to develop an anhydrous fixative. He determined that a glycerol and paraformaldehyde fixative did not cause swelling of the cells and he achieved good overall preservation of cellular organization and organelles of dry peanut seeds. Plastic embedding of the dry seed tissue was imperfect but tolerable. Thomson (1979) strongly recommended the use of anhydrous paraformaldehyde and glycerol fixation for other dry material.

Cucurbita and Lilium pollen fixed anhydrously with paraformaldehyde and glycerol was not perfectly infiltrated and embedded. However, it was possible to obtain ultra-thin sections with a diamond knife. The tube cell cytoplasm appeared to be well preserved, with good preservation of the organelles. Sack et al (1988) used paraformaldehyde and glycerol to fix Typha pollen and found that the pollen grains appeared undamaged. In Typha pollen, membranes and mitochondria were intact, the plasmalemma was flush with the intine, and large starch grains and lipid bodies were present.

The electron microscopic studies revealed a lot of information about Cucurbita pollen structure. Unlike C.

maxima and C. andreana seeds, which differed in length by a factor of 3, the C. maxima and C. andreana pollen were about the same size. Echlin (1968) found that the pollen of pumpkin, which belongs to Cucurbitaceae, was among the largest grains. Generally the standard size of pollen grains has been measured to be about 50 μm in diameter (Iwanami et al, 1988). However the diameter of pollen varies over a wide range of sizes and is related directly to the means of pollen dispersal (Echlin, 1968). Pollen which is transported by insects, such as Cucurbita pollen, tends to be larger than 60 μm or less than 20 μm in diameter (Echlin, 1968).

Cucurbita pollen was spherical in shape and covered with uniformly arranged spinules. Apertures were also present on the Cucurbita pollen surface. There never appeared to be more than 10 apertures on one grain. The apertures appeared as large projections from the exine and are the places from which the pollen tube would emerge during germination.

The structure of the Cucurbita pollen wall was similar to the structure of the pollen walls of other species. The Cucurbita pollen grain wall was thick and consisted of two layers. It has generally been agreed that pollen walls have a thickness of greater than 1 μm and that the wall structure consists of two distinct layers (Rowley, 1960; Rosen et al, 1964; Sassen, 1964; Mepham and Lane, 1968; Echlin and Godwin, 1969; Knox and Heslop-Harrison,

1969; Knox and Heslop-Harrison, 1970; Knox and Heslop-Harrison, 1971; Cresti et al, 1985a; Cresti et al, 1985b; Van Went and Gori, 1989). The interface between the two layers had no sharp boundary (Knox and Heslop-Harrison, 1970). Cresti et al, (1985a) found that the pollen wall was thick and similar to an ordinary plant cell wall. Observations of pollen grain wall structure revealed that the Cucurbita wall consisted of two main layers, the exine and the intine. The exine, the outer wall layer, was divided into the outer and inner regions called the ektexine and endexine respectively (Rowley, 1960). Each region of the Cucurbita exine was structurally uniform. The exine formed caps of sproropollenin at each aperture site. The intine, the inner wall layer, had a fibrillar appearance and was thickened at each of the aperture sites.

In terms of the chemical composition of the tube cell walls, it has been determined that the exine consists of acid resistant material which originates from precursor materials synthesized in the tapetum (Heslop-Harrison, 1962; Heslop-Harrison, 1968a; De Vries and Ie, 1970; Chapman, 1987). Burgess (1985) attributed the stability of the exine to the presence of sporopollenin. Sporopollenin is composed of a polymer of carotenoid derivatives and is synthesized on the surface layer of the tapetum (Brooks and Shaw, 1968; Heslop-Harrison, 1968a; De Vries and Ie, 1970; Knox and Heslop-Harrison, 1970; Burgess, 1985; Cresti et al, 1985a;

Van Went and Gori, 1989).

Heslop-Harrison (1968a) found that deposition of the cellulose, which makes up part of the intine, first began beneath the apertures during development of the angiosperm microspore. Synthesis of the intine then continued radially outward from the aperture between the plasmalemma and the inner face of the exine. The intine was thickened at each of the apertures whereas the exine was reduced (Knox and Heslop-Harrison, 1969; Knox and Heslop-Harrison, 1970). In some cases microtubules and coated vesicles were found in close proximity to the intine. Often the microtubules were involved in the growth of intine and the deposition of cellulose (Heslop-Harrison, 1968a). In Gladiolus the presence of vesicles was associated with the occurrence of enzymes and antigens in the intine (Knox, 1971).

Enzymes such as acid phosphatase have been localized in the apertures of 50 angiosperm species (Stanley and Linskens, 1965; Knox and Heslop-Harrison, 1969; Knox and Heslop-Harrison, 1970; Knox, 1971; Knox and Heslop-Harrison, 1971; Vasil, 1987; Tu et al, 1988). These enzymes were readily leachable from the apertures and were believed to be factors involved in allergenicity and pollen/pistil interactions (Stanley and Linskens, 1965; Knox and Heslop-Harrison, 1969; Knox and Heslop-Harrison, 1970; Knox and Heslop-Harrison, 1971; Jackson and Kamboj, 1985; Vasil, 1987).

Tu et al (1988) studied the composition of cell walls and found that most cell walls were composed of cellulose fibers interconnected by pectic-type polysaccharides. These walls usually contained several different types of proteins such as glycoproteins and enzymes. The glycoproteins were responsible for cell recognition and the enzymes were responsible for cell wall metabolism, nutrient transfer, species recognition and disease resistance (Tu et al, 1988). Acid phosphatase, an enzyme commonly found in the cell wall, was important in hydrolyzing and solubilizing organic phosphate-containing molecules.

Antigens have also been found in the intine; it is believed that they act as recognition substances that are involved in inter- and intra-specific incompatibility reactions (Knox, 1971).

Cucurbita pollen was binucleate and bicellular which meant that two cells and two nuclei were present in the mature grain. Hence the pollen was shed before sperm formation (Brewbaker, 1967). Thus when the fine structure of Cucurbita pollen was examined one expected to find a vegetative nucleus and a generative cell with its nucleus. The ultrastructure of Cucurbita pollen was characteristic of the ultrastructure of pollen of many plant species.

In Cucurbita pollen the vegetative nucleus was found to be highly lobed. Examples where lobed nuclei have been found in the pollen of other species include Petunia,

Parkinsonia aculeata, Hymenocallis occidentalis, Hippeastrum belladonna, Ranunculus macranthus, Nicotiana alata, Brassica oleraceae, Hippeastrum vitatum, Zea mays, Acacia retinodes, Brassica campestris, Typha latifolia, Petunia hybrida and, Capparis spinosa (Sassen, 1964; Larson, 1965; Cresti et al, 1985a; Dumas et al, 1985; McConchie and Knox, 1985; Mogensen, 1986; Cass and Fabi, 1987; McCoy and Knox, 1988; Sack et al, 1988; Wagner and Mogensen, 1988; Van Went and Gori, 1989). Often mitochondria, dictyosomes, and rough endoplasmic reticulum were found in close proximity to the lobes of the tube cell nucleus (Dumas et al, 1985; Mogensen, 1986). The chromatin of the vegetative nucleus usually is highly dispersed and only small areas of condensed heterochromatin are found (Cresti et al, 1985a; Dumas et al, 1985; Van Went and Gori, 1989).

In Cucurbita pollen sections the vegetative nucleus appeared to be larger than the generative cell and they were located in close proximity to one another. Generally, the generative cell is smaller than the vegetative nucleus (Mogensen, 1986). The vegetative nucleus was generally found in close proximity to the generative cell (Sanger and Jackson, 1971a; Mogensen, 1986; McCoy and Knox, 1988; Sack et al, 1988; Wagner and Mogensen, 1988). The association of the vegetative nucleus and generative cell may be an essential prerequisite for germination and ensuring that they enter the pollen tube together (McCoy and Knox, 1988).

The generative cell wall appears to have little structural rigidity, and the overall shape of the generative cell has been described as amoeboid or elongated (Sassen, 1964; Sanger and Jackson, 1971b; Cresti et al, 1984; Burgess, 1985; Mogensen, 1986; McCoy and Knox, 1988; Sack et al, 1988; Wagner and Mogensen, 1988). The Cucurbita generative cell has an electron transparent wall and was amoeboid shaped. The elongated shape of the generative cell is believed to facilitate its movement down the pollen tube (Sanger and Jackson, 1971b; Cresti et al, 1984).

The generative cell wall of Cucurbita was similar to the generative cell wall of other species; it was not electron-dense (Sassen, 1964; Sanger and Jackson, 1971a, Cresti et al, 1985b; Mogensen, 1986; McCoy and Knox, 1988; Sack et al, 1988; Wagner and Mogensen, 1988). Generally, the generative cell wall contains cellulose and pectin and develops through fusion of pectin vesicles (Gullvåg, 1966).

The generative cell composition of C. maxima and C. andreana consisted of a nucleus and a small amount of cytoplasm. The shape of the Cucurbita generative cell was elongated and the cell wall was thick and electron transparent. C. maxima and C. andreana generative cell cytoplasm did not appear to contain any plastids. It is generally agreed that the generative cell cytoplasm is devoid of plastids (Sassen, 1964; Larson, 1965; Gullvåg, 1966; Hoefert, 1969; Sanger and Jackson, 1971a; Sanger and

Jackson, 1971b; Cresti et al, 1984; Dumas et al, 1985; Feijo and Pais, 1988; Wagner and Mogensen, 1988; Van Went and Gori, 1989). However microtubules and mitochondria have been found in the cytoplasm of some species. Microtubules were found in close proximity to the generative cell wall and likely functioned in maintaining the elongated shape of the cell (Sanger and Jackson, 1971b; Cresti et al, 1984; Mogensen, 1986; Sack et al, 1988; Van Went and Gori, 1989). Many mitochondria were present in the cytoplasm (Gullvåg, 1966; Hoefert, 1969; Sanger and Jackson, 1971a; Wagner and Mogensen, 1988; Van Went and Gori, 1989). Experiments carried out by Sanger and Jackson (1971b) showed that the microtubules were necessary for the maintenance of asymmetric shape of the generative cells. Addition of colchicine to the generative cell destroyed the microtubules and caused the generative cell to become spheroid shaped (Sanger and Jackson, 1971b).

The vegetative cytoplasm of Cucurbita pollen and other species contained numerous Golgi bodies, dictyosomes, fibrillar bodies, small vacuoles, mitochondria, abundant rough endoplasmic reticulum (RER), storage reserves such as starch, lipids and proteins, and in some cases, unidentified electron-dense particles. Variations were observed in the number of ribosomes, the morphology of the endoplasmic reticulum, number and morphology of dictyosomes, and the amount and nature of cytoplasmic reserves (Gullvåg, 1966;

Hoefert, 1969; Sanger and Jackson, 1971b; Van Went and Gori, 1989). In pollen from Cucurbita and other species, the vegetative cytoplasm filled the tube cell and no large vacuoles were present (Van Went and Gori, 1989). Starch in amyloplasts was the primary form of storage reserve found in the vegetative cell cytoplasm of C. maxima and C. andreana pollen. The Lugol's iodine and PAS stains, carried out on light microscopy sections, confirmed the presence of starch grains observed in the electron micrographs. Starch was found in the vegetative cell cytoplasm of pollen of many other plant species (Hoefert, 1969; De Vries and Ie, 1970; Sanger and Jackson, 1971a; Cass, 1973; Baker and Baker, 1979; Feijo and Pais, 1988; Raghavan, 1988; Sack et al, 1988). Many studies of the Golgi bodies or dictyosomes of germinated pollen agreed that they are involved in carbohydrate condensation and transport required for pollen tube wall formation (Larson and Lewis, 1962; Sassen, 1964; Dashek and Rosen, 1965; Larson, 1965; Gullvåg, 1966; Rosen and Thomas, 1970; Sanger and Jackson, 1971c; Vasil, 1987).

The fibrillar bodies observed in the Cucurbita pollen cytoplasm have been observed in Nicotiana pollen. Crystalline-fibrillar bodies were randomly scattered throughout the vegetative cytoplasm of Nicotiana pollen and were believed to be crystalline forms of proteins (Cresti et al, 1985b).

RER was abundant in the cytoplasm of pollen of many

species of plants including Cucurbita pollen (Larson and Lewis, 1962; Sassen, 1964; Larson, 1965; Crang and Miles, 1969; Hoefert, 1969; Sanger and Jackson, 1971c; Cass, 1973; Cresti et al, 1985b; Dumas et al, 1985; Mogensen, 1986; Cass and Fabi, 1987). The RER of pollen was responsible for the formation of bodies which acted as reservoirs for storage products. The storage products were limited by a single membrane (Gullvåg, 1966). In pollen of Beta and other species the RER was highly organized and was found throughout the vegetative cytoplasm (Hoefert, 1969). It is believed that the RER functioned in assisting protein synthesis or in transferring processed materials from the nucleus to the cytoplasm or the reverse. Areas with high concentrations in polyribosomes are where synthesis of proteins occurs (Sanger and Jackson, 1971c; Wolfe, 1972; Van Went and Gori, 1989). Van Went and Gori (1989) determined that the abundance of ribosomes and extensive RER present in the pollen cytoplasm were indicative of protein-synthesizing machinery. Cass and Fabi (1987) found protein bodies associated with the RER in the vegetative cytoplasm of Zea mays pollen.

Electron-dense particles have been observed in the cytoplasm of C. maxima, C. andreana, L. longiflorum and several other species. Mature pollen grains of Tilia platyphyllos, Parkinsonia aculeata, Hymenocallis occidentalis, Ranunculus macranthus, Hippeastrum belladonna,

Lychnis alba, Lilium longiflorum contained electron-dense particles similar to the globoid particles observed in Cucurbita pollen (Chambers and Godwin, 1961; Larson, 1965; Crang and Miles, 1969; Southworth and Dickinson, 1981; Baldi et al, 1987).

The fact that no osmium tetroxide or post-stains were used, in the anhydrous fixation of Cucurbita and Lilium, eliminated the possibility that the electron-dense particles were osmiophilic structures or staining artifacts. Furthermore, phytic acid has been found in the pollen of several families such as Fagaceae, Juglandaceae, Malvaceae, Anacardiaceae, Onagraceae, Myrtaceae, Araceae, Scrophulariaceae, Orchidaceae, Poaceae, Musaceae, Solanaceae and Liliaceae (Jackson and Linskens, 1982; Jackson et al, 1982; Helsper et al, 1984; Baldi et al, 1987). The energy dispersive X-ray analysis results of Cucurbita pollen globoid crystals will be discussed later in this chapter.

The vegetative cell of Cucurbita and other species most likely functions as a storage site of nutritive material necessary for tube growth (Cresti et al, 1985a; Van Went and Gori, 1989). Ultrastructural studies of Cucurbita and other species have shown that vegetative cells generally contain food reserves, usually in the form of starch or sugars, or lipids and proteins.

Pollen grains contain reserve materials at the time of shedding which are used during the initial autotrophic

phase of germination (Vasil, 1960; Vasil, 1987). To continue germination successfully, the pollen switches to a heterotrophic phase to support the enormous growth of the pollen tube which involves the synthesis of large amounts of cell wall material (Vasil, 1987).

MINERAL ANALYSES :

Electron microscopy studies combined with EDX analysis studies of Cucurbita and Lilium pollen revealed the presence of particles that I have decided to call globoid particles. Globoid particles were observed in sections of pollen and in ground pollen samples. The EDX analysis spectra of the globoid particles varied with respect to the type of fixation that was used and with respect to how the pollen was sectioned.

The EDX analysis results showed that aqueous fixatives that were used to prepare C. maxima pollen, extracted K and Mg from the sections. These results agreed with findings of other studies, in which aqueous aldehyde containing fixatives extracted monovalent and divalent cations (Roomans, 1980). EDX analysis studies of yeast cells showed that divalent cations were extracted from the cells as a result of using an aqueous fixation (Roomans, 1980). EDX analysis of aqueously prepared Pisum sativum seeds revealed that there was very little retention of K in globoid particles but P, Mg and Ca were retained (Lott et al, 1984). Loss of P and K also occurred in Pisum protein bodies which

lacked globoid particles. Thus conventional aqueous fixation methods, which use aldehyde fixatives, were an insufficient means of preparing pollen and other biological materials for EDX analysis of phytate deposits. Anhydrous preparation of biological materials was necessary for EDX analysis studies.

The EDX analysis results of Cucurbita globoid particles revealed that large amounts of water soluble materials were extracted from sections of anhydrously fixed pollen which were cut onto water filled microtome boats. However when the boat was filled with absolute ethanol, there was less extraction of the water soluble materials from the sections. The fine structural studies and mineral analyses of anhydrously fixed Cucurbita pollen revealed that globoid particles were present in the vegetative cell cytoplasm. Electron-dense globoid particles were present in C. maxima, C. andreana and Lilium pollen. The pollen globoid particles tended to fracture during sectioning due to incomplete infiltration. Difficulty in cutting sections of pollen grains was noted by Chambers and Godwin, (1961). Most studies of dry tissues using anhydrous procedures have had difficulty obtaining adequate infiltration of epoxy resin through the walls of cells (Webb and Arnott, 1982; Yatsu, 1983; Sack et al, 1988). Although the Cucurbita pollen was not infiltrated perfectly, it was infiltrated well enough to carry out structural studies and mineral analysis.

EDX analysis of globoid particles, from C. maxima, C.

andreana and L. longiflorum anhydrously fixed and ground pollen samples, revealed that the peak to background ratios were significantly different from sample to sample. Differences in peak to background ratios were due to differences in the number of counts for each element. Combined element to P and single element to P ratios for C. maxima and C. andreana ground and anhydrously fixed pollen samples were not significantly different from sample to sample. This meant that even though the number of counts differed, the proportions of the elements in C. maxima and C. andreana pollen did not differ from species to species. Most of the combined element to P and single element to P ratios for Cucurbita and L. longiflorum anhydrously fixed pollen samples were not significantly different from species to species. However the combined element to P and single element to P ratios for Cucurbita and L. longiflorum ground pollen samples were significantly different from species to species.

The K peak to background ratios of globoid particles from ground pollen samples were significantly larger than K peak to background ratios of globoid particles from anhydrous pollen samples. Differences in the K peak to background ratios for anhydrously fixed epoxy embedded sections and ground pollen samples may reflect movement of K within the sample during the embedding process (Fowler and Parker, 1973).

The EDX analysis spectra of pollen globoid particles, from anhydrous and ground samples, resembled the spectra of seed globoid crystals. The globoid crystals of Cucurbita seeds and seeds of other species are electron-dense regions that are rich in phytate.

Although globoid particles have never been identified in Cucurbita pollen before, pollen from many species contain electron-dense particles which resemble globoid particles. Electron-dense particles were observed in the pollen cytoplasm of several species such as Tilia platyphyllos, Parkinsonia aculeata, Hymenocallis occidentalis, Ranunculus macranthus, Hippeastrum belladonna, Lynchnis alba, Hippeastrum vitatum and Lilium longiflorum (Chambers and Godwin, 1961; Sassen, 1964; Larson, 1965; Crang and Miles, 1969; Southworth and Dickinson, 1981; Cass and Fabi, 1987; Baldi et al, 1987). Electron-dense particles found in Lilium pollen by Southworth and Dickinson, (1981) were similar to globoid particles in appearance. The particles in Lilium were bounded by a single membrane.

Mineral analysis of pollen using EDX analysis has been previously carried out by Baldi et al, (1987). EDX analysis of electron-dense particles of aqueously fixed Lilium longiflorum pollen revealed that the spectra of the particles were similar to the spectra of seed phytate. During germination of L. longiflorum pollen, the electron-dense particles lost their rounded appearance and appeared

to be reduced in size. It is believed that the decrease in size was due to the use of storage material for pollen tube wall synthesis (Southworth and Dickinson, 1981; Baldi et al, 1987).

EDX analysis of globoid particles of pollen and various seeds revealed that the globoid particles contained mainly Mg, P and K. Electron microscopy observations and EDX analysis spectra suggested that the globoid crystals in pollen are composed of phytate. Cucurbita pollen globoid crystal spectra were very similar to spectra of globoid crystals of cotyledons of C. maxima, soybean, C. mixta, Ricinus communis and tomato (Lott and Vollmer, 1973; Lott, 1975; Lott and Buttrose, 1978; Lott et al, 1982; Lott et al, 1982; Lott, 1983; Spitzer and Lott, 1980). The ground meristem of C. andreana cotyledons more commonly contain Ca in their globoid crystals than does C. maxima (Lott et al, 1979). Other smaller Cucurbita seeds such as C. foetidissima and C. pepo also contained Ca in globoid crystals more frequently than in species of Cucurbita with larger seeds (Lott and Vollmer, 1979b).

EDX analysis is appropriate for identifying globoid particles because it enables determination of the location of P and cations (Lott and Buttrose, 1978). Due to the electron density of globoid particles, it serves as an ideal material for EDX analysis (Greenwood et al, 1978). EDX analysis serves as an ideal method of determining the

mineral composition of fixed samples provided that some basic rules are followed. Osmium tetroxide should not be used to prepare the samples as it may cause differential extraction of elements or redistribution artifacts (Lott et al, 1978b; Lott and Spitzer, 1980; Lott, 1984; Roos and Barnard, 1985). Osmium tetroxide fixation resulted in a major loss of P, Mg, K and Ca from radicle globoid particles. Despite the loss of elements, the OsO_4-fixed globoid particles were still electron-dense (Lott et al, 1978). There is also some evidence that the presence of osmium interferes with the polymerization process of the plastic, leading to incomplete polymerization and a higher loss of elements during EDX analysis (Roos and Barnard, 1985). Another problem with OsO_4 is that the osmium M spectral line (1.978 keV) cannot be resolved from the phosphorous K spectral lines (K_α, 2.015 and K_β, 2.016 keV). A high concentration of osmium in the sample, may cause its characteristic x-rays to overflow the detector channels and mask out portions of peaks from P (Fowler and Parker, 1973). This osmium contribution to the P peak results in a shoulder, thus making analysis difficult (Sicko-Goad et al, 1975). Roos and Barnard, (1985) reported that osmium tetroxide fixation led to lower concentrations of almost all elements detected. Osmium tetroxide fixation prior to embedding led to surprisingly low concentration values except for P. In this study, the use of OsO_4 made sectioning

of the pollen samples very difficult. In some pollen samples, OsO_4 contributed to the P peak results; thus the OsO_4 results could be misleading.

Ideally, the best way to prepare biological material for EDX analysis is to use no fixative at all. A means of preparing biological samples without fixation is by using cryogenically prepared samples. Numerous studies of cryogenically prepared samples have demonstrated the benefits of this technique. Cryo-fixed specimens retain more normal volume and shape than samples that have been chemically dehydrated (Beckett et al, 1984; Read and Beckett, 1986; Lott and Kerr, 1986). Cryo-fracturing enables the study of considerable sub-cellular detail and is a simple way of exposing the interior of large biological specimens (Echlin, 1977).

Although cryo-preparation methods were developed to limit loss or movement of diffusible elements, pollen samples prepared for cryo-fracturing could not be used for EDX analysis because they suffered from severe charging and beam damage. Usually sample charging is remedied by chromium coating the sample (Marshall, 1977). However, the charging encountered with Cucurbita pollen could not be reduced by chromium coating. Marshall, (1982) experienced beam damage in cryo-protected samples; this type of sample damage is called thermal damage. Thermal damage is thought to result

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caution of using such slightly thicker sections is the possible problem with artifact signals from nearby structures which can lead one to falsely attribute to the globoid particle.

The value of activation analysis as an important research tool was recognized in this study. Neutron activation analysis (NAA) is known for its sensitivity and can be applied to measuring a wide range of elements. The sensitivity of NAA was demonstrated by comparing the NAA concentration values for the NBS standards to the NBS certified concentration values. Thus NAA served as a useful means of carrying out quantitative analysis. The elements Mg, K, Ca, and P were measured quantitatively without combining NAA with another preparative technique. NAA of pooled whole pollen samples revealed that the concentrations for each element, Mg, K, Ca and P in C. maxima and C. andreana pollen were not significantly different from species to species. The same observations were made for the combined element to P, and single element to P ratios. Greater than 1% of Cucurbita pollen dry weight was K and between 0.5 - 0.7% dry weight was P. In L. longiflorum pollen, Mg, K, Ca and P concentrations were similar to Cucurbita pollen element concentrations. NAA, whole pollen Mg/P and K/P values were similar to Mg/P and K/P values of ground and sieved Cucurbita pollen measured by x-ray analysis. However, the whole pollen NAA combined element to

P ratio values were twice the EDX analysis values for the ground Cucurbita pollen samples.

The elemental analyses strongly suggest that phytate is stored in C. maxima, C. andreana and L. longiflorum pollen. Since it is not uncommon to find phytate stored in pollen from other species, it would not come as a surprise if the presence of phytate in Cucurbita pollen was confirmed. Jackson et al (1982) confirmed the presence of phytic acid in Petunia hybrida pollen using paper electrophoresis. It is believed that stored phytate is required for pollen tube formation.

Phytate accumulation occurs well after tetrad formation in pollen development (Helsper et al, 1984). In germinating Petunia pollen, phytate is broken down into myo-inositol, minerals and phosphate (Jackson and Linskens; 1982).

The process of phytate degradation is highly regulated (Scott and Loewus, 1986). Tube elongation of germinating Petunia pollen is retarded by the presence of phosphate in concentrations inhibitory to pollen phytase. Other research suggested that myo-inositol is an essential precursor of wall components during pollen tube development (Scott and Loewus, 1986). It is thought that myo-inositol is converted by an oxidation pathway to UDP-glucuronic acid (Jackson et al, 1982). UDP-glucuronate is an important precursor for uronosyl and pentosyl derivatives of plant cell wall

polysaccharides and is synthesized via the inositol oxidation pathway (Rosenfield et al 1978; Hara et al, 1985). Loewus et al, (1978) demonstrated the utilization of myo-inositol for pollen tube wall polysaccharides in Lilium longiflorum.

Phytases have been found in the pollen of Petunia hybrida, Typha latifolia, Lilium longiflorum, (Jackson and Linskens, 1982; Hara et al, 1985; Scott and Loewus, 1986a; Baldi et al, 1988). Pollen phytases are required to supply the raw materials for pollen tube wall growth (Jackson and Linskens, 1982). Labelling experiments on developing pollen tubes with myo-[2-^{14}C]inositol and myo-[2-^{3}H]inositol revealed that myo-inositol was a precursor for pollen tube wall material and that the tip region, where extension occurs, is made up of a microfibrillar pectin (Dashek and Rosen, 1965; Kroh and Scott, 1968; Rosen and Thomas, 1970; Rosenfield et al, 1978; Heslop-Harrison, 1983; Helsper et al, 1984). Myo-inositol was used for phosphatidyl-inositol and pectic polysaccharide biosynthesis in pollen (Helsper et al, 1984).

The process of pollen tube wall formation involves Golgi-derived vesicles (Larson, 1965). Golgi vesicles have been observed throughout germination of Ranunculus macranthus and Brassica oleracea pollen (Larson and Lewis, 1962; Dumas et al, 1985). Golgi vesicles accumulate at the tip of the pollen tube and mobilize and secrete wall

polysaccharides which are incorporated into the developing pollen tube wall (Sassen, 1964; Larson, 1965; Gullväg, 1966; Southworth and Dickinson, 1981). In Lilium pollen, it is believed that pectin synthesis occurs in the Golgi-derived vesicles (Dashek and Rosen, 1965).

Jackson et al, (1982) suggested that the length of the style may be the most important factor in determining whether or not phytic acid is stored in significant amounts in pollen. During early stages of pollen tube development, biosynthesis of myo-inositol from D-glucose-6-P seems to have an insignificant role as a source of myo-inositol for oxidative conversion to uronosyl and pentosyl constituents of pollen tube wall polysaccharides (Jackson, 1982). In the initial phase of germination, pollen is autotrophic and relies on its reserves for initiating tube growth (Vasil, 1987). Once the reserves are used up, the pollen becomes heterotrophic and relies on the exudates of the stigma and styles for further nourishment (Chen and Loewus, 1977; Maiti et al, 1978; Vasil, 1987). Boron enhances formation of pectic substances for the growing tube wall (Loewus, et al, 1962, Stanley and Loewus, 1964; Vasil, 1987). Pollen is generally deficient in B (Vasil, 1960). The amount of B in the pistil may be a factor involved in incompatibility reactions.

SUMMARY

Light microscopy and histochemical studies of Cucurbita pollen revealed that C. maxima and C. andreana pollen were not easy to prepare for light microscopy studies due to problems with bursting. Light microscopic studies revealed that C. maxima and C. andreana pollen grains were spherical and the same size. Like pollen from other species, Cucurbita pollen has a very thick tube cell wall which consisted of two distinct layers, the exine and the intine. Numerous apertures projected out from the surface of the pollen grain.

Cucurbita pollen was found to be structurally and chemically similar to pollen of many other species. Cucurbita pollen was binucleate and bicellular. The pollen consisted of a vegetative cell which housed the vegetative nucleus and the generative cell which contained the generative nucleus. Storage reserves of Cucurbita pollen primarily consisted of starch. Additional storage reserves of Cucurbita pollen consisted of protein, small amounts of lipids and minerals.

Electron microscopic studies of Cucurbita pollen revealed information about the fine structure. In general, preparation of pollen sections for electron microscopy was difficult due to problems with sectioning. Sectioning problems arose due to the thick pollen wall, the density of cytoplasm of the pollen, and inadequate infiltration of the

pollen. Anhydrous fixation gave better preservation of pollen contents and less extraction of water soluble materials than aqueous fixatives.

The fine structure of Cucurbita pollen was similar to the structure of pollen of many other species. Starch was the primary storage reserve in Cucurbita pollen. Globoid crystals served as mineral reserves of Cucurbita and Lilium pollen. Globoid crystals were present in the vegetative cytoplasm and were detectable in the absence of stains and OsO_4. The Cucurbita pollen globoid crystals were analyzed by EDX analysis.

EDX analysis results demonstrated that aqueous fixatives tended to extract water soluble materials, likely K-phytate, from sections of Cucurbita pollen. EDX analysis spectra of globoid crystals from anhydrous fixation, sectioned onto water filled boats and onto ethanol filled boats were significantly different. This study showed that, to prevent extraction of water soluble materials from pollen sections, it was not only important to use an anhydrous fixation and embedding protocol but just as important to section material onto an ethanol filled boat. EDX analysis spectra of globoid crystals from Cucurbita pollen were similar to spectra of seed phytate. C. maxima and C. andreana combined element to P ratios, and single element to P ratios were not significantly different from species to species. Ground pollen ratios differed from anhydrously

fixed pollen ratios suggesting that some movement of or extraction of elements may not have been totally alleviated by the anhydrous fixation.

NAA of whole pollen revealed that in C. maxima and C. andreana the individual concentrations of Mg, K, Ca and P were not significantly different from species to species. Cucurbita pollen K and P concentrations were 1.3% and 0.5-0.7% respectively. Combined element to P, and single element to P ratios of whole Cucurbita pollen were two times higher than respective ratios of ground pollen analyzed by EDX analysis.

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