A STUDY OF INCOMPATIBILITY WITHIN THE GENUS *ZANTEDESCHIA*

by

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CHAPTER 5 EMBRYO CULTURE OF *ZANTEDESCHIA* INTERSPECIFIC HYBRIDS

ABSTRACT

Zantedeschia includes many important varieties for New Zealand cut flower and pot plant production. The genus is divided into two sections. The first section consists of a single species *Z.aethiopica*, the common white calla, and a few selections such as the dwarf 'Childsiana'. The second section includes the remaining five species whose spathes range in colour from ivory, yellow and pink to maroon. Interspecific hybridization following hand pollination readily occurs between species of Section II and all of the coloured *Zantedeschia* cultivars have been derived from this section. *Z.aethiopica*, however, has never been successfully crossed with any of the species from Section II. *Z.aethiopica* has a different flowering period, is more floriferous and more tolerant of *Erwinia* (a soft rot of tubers) than the other species. The ability to produce hybrids from the two sections would greatly increase the potential for improved cultivars. This project was initiated to explore interspecific incompatibility by examining pollen physiology, embryogenesis of compatible and incompatible crosses, and rescue of embryos from incompatible crosses using embryo culture techniques.

Z.aethiopica 'Childsiana' pollen was similar in morphology to pollen from the coloured species of Section II. Pollen from all species examined were oval in shape with a light-yellow, waxy pollen coat. The exine was smooth with no aperture, while the cytoplasm was full of starch grains. The pollen of *Zantedeschia* is trinucleate. In contrast to other trinucleate pollen lower concentrations of sucrose (5-10%) were preferable for pollen germination and high concentration of sucrose (30%) sharply reduced germination. Pollen germination of *Zantedeschia* was unaffected by agar concentration and pollen even germinated well in liquid media. The *in vitro* medium containing sucrose (100 gl^{-1}) , boric acid (50 mgl^{-1}) , calcium chloride (100 mgl^{-1}) and agar (10 gl^{-1}) with pH 6 was optimal and was selected for further pollen germination studies.

The mature megagametophyte of *Zantedeschia* was a typical seven-celled embryo sac, with a three celled egg apparatus, three antipodal cells and a secondary nucleus formed by the two polar nuclei. The secondary nucleus was present at the chalazal end of the embryo sac before fertilization. Ovules were anatropus i.e. inverted, and had an inner and outer-integument. The apical cells of the nucellar epidermis divided periclinally to form a nucellar cap (or epistase) 4 - 5 cells in thickness.

Control ovaries of unpollinated (Section II) *Zantedeschia* hybrids 'Best Gold' and 'Chromatella' grew for 2 weeks and then started to shrink and turn yellow. These ovaries eventually died 8 to 10 weeks after anthesis. Incompatible crosses had far less ovule development compared with compatible crosses. In incompatible crosses ovary size was much smaller than compatible crosses from four weeks after pollination.

The embryos of compatible crosses had a steady growth rate up to 12 weeks. The embryo was globular at 4 weeks but cotyledon, radicle and a leaf primordium were differentiated by 6 weeks. In the incompatible crosses globular embryos were also found at 4 weeks, but their growth rate was much slower thereafter. Most of the incompatible embryos remained at a globular stage of development. A small percentage of incompatible embryos continued development but the endosperm surrounding these embryos often became necrotic. By 8 weeks after pollination 1-2% of embryos from the *Z.'Chromatella'* X *Z.aethiopica* 'Childsiana' were larger than 0.7 mm with distinct cotyledon development. Compatible embryos at this time were about 2 mm long.

The endosperm in compatible crosses did not contain starch during the first two weeks after pollination, but small starch grains were seen in the outer endosperm from week 4 and starch levels increased throughout the endosperm to week 12. In the incompatible crosses starch levels were always lower and starch grains were confined to the outer region of the endosperm; the central region was poorly developed. In the incompatible crosses necrosis of the endosperm and embryo was clearly seen in most ovules from week 6. Even in embryo sacs with well developed embryos, the endosperm had shrunk and the seeds dried by week 8. The arrest of growth of embryos from incompatible crosses appeared to be related to degeneration of the endosperm and failure of nutrient metabolism and transport.

Young seeds were removed from ovaries at various stages (2 to 12 weeks after pollination) and cultured *in vitro*. The embryos from compatible crosses taken at 6 weeks or later grew well *in vitro* on Murashige and Skoog (1962) medium with sucrose (30 gl^{-1}). At this concentration of sucrose most embryos produced shoots, roots and cotyledonary haustorium tissue within one month of culture. High concentrations of sucrose (90 gl^{-1}) delayed growth of most of the cultured embryos.

Most embryos from incompatible crosses (dissected 6 to 10 weeks after pollination) were small (0.1-0.2 mm) and remained globular in culture. Higher concentrations of sucrose (60-90 gl^{-1}) did not improve embryo growth irrespective of the age of the embryos. Some incompatible embryos were larger (0.7 mm) when dissected, and in culture these eventually produced shoots but all tissues were albino.

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CHAPTER 1

GENUS OF *ZANTEDESCHIA* REVIEW

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INTRODUCTION

The genus *Zantedeschia* was named by Sprengel in honour of a professor Giavnni Zantedeschi a botanist of Breschia (Traub, 1948) and plants within the genus are commonly known as Arum or Calla lilies.

Zantedeschia is a relatively small genus and all species are native to Africa. Since Sprengel established the genus *Zantedeschia* in 1826, other names have been put forward such as *Arodes*, *Richardia* and *Calla* but the name *Zantedeschia aethiopica* (L.) was upheld by Engler in 1915. From that time *Zantedeschia* has been conserved against later generic names. Traub (1948) revised the genus on the same lines as Engler, and recognized eight species (Table 1).

In a monograph on the genus Letty (1973) has traced the history of the nomenclature and suggests that *Zantedeschia* must be considered the correct designation. Letty (1973) recognised six species with three subspecies (Table 1). She criticized Traub's lack of knowledge of plants in the field and indicated that the following entities should be included within *Zantedeschia albomaculata*: *Z. oculata* (Engl,1859), *Z. hastata* (Hook.f,1883), *Z. melanolecuca* (Hook. f,1869), *Z. angustiloba* (Schott,Engl,1883), *Z. macrocarpa* (Engl,1883) *Z. melanoleuca* var *tropicalis* (Brown.N.E,1901), *Z. chloroleuca* (Engl,1903) and *Z. melanolenca* var *concolor* (Davy.B,1924).

Table 1. *Zantedeschia* species and subspecies recognised by Letty (1973) and Traub (1948).

Traub (1948)	Letty (1973)
Z. aethiopica	*Z. aethiopica*
Z. elliottiana	*Z. elliottiana*
Z. rehmannii	*Z. rehmannii*
Z. hastata	*Z. pentlandii*
Z. melanoleuca	*Z. jucunda*
Z. angustiloba	*Z. albomaculata*
Z. albomaculata	Subspecies:
	Z. albomaculata subsp. *albomaculata*
	Z. albomaculata subsp. *mocrocarpa*
	Z. albomaculata subsp. *valida*

At present Letty,s (1973) work is accepted.

The species of *Z. albomaculata* show great variation in size, shape and maculation (spotting) of the leaf, the size, shape and colouring of the spathe; and the size of the berries and number of ovules (Letty, 1973).

The *Zantedeschia* species fall into two distinct sections:

(I) Section I contains one species *Zantedeschia aethiopica*, the white calla lily of florists, which is common on the humid meadows around Capetown and generally in southwest Cape province, South Africa. The natural habitat of the species extends eastward to Natal. The essential characters of *Z. aethiopica* are that it is a robust, evergreen plant up to 60 cm high without a dark purple area inside of the base of the spathe. The spathe is milk-white, and the anthers are bright yellow; the green, unspotted leaves are cordate or hastate (Letty, 1973). The plant is evergreen and does not die down in winter nor does it require a resting period. Flowering occurs in winter from June to November in New Zealand. The female flowers in the lower part of spadix are interspersed with staminate flowers. Following fertilization the fruits enlarge and later turn orange, become soft and then mucilaginous at maturity. The root stock of *Z. aethiopica* is a rhizome (D. Cohen, pers. comm., 1988).

Hybrids of *Z. aethiopica* are rarely found in the wild state (Letty, 1973). White flower spotted forms exist but

the genealogy is unknown (Pers.comm. E. Welsh, 1989, Massey University Horticulture Science, Palmerston North). New (1986) suggests that the different times of maturity of the male and female flowers result in the failure of self fertilization in white calla. Within *Z. aethiopica* manual hybridization and selection has produced several varieties differing in size, scent and chlorophyll content of spathes (e.g. Childsiana, Green Goddess, Gigantea and Fragrans).

(II) Section II consists of the remaining five species, all of which have several unifying characteristics. These species flower during summer. There are no staminate flowers among female flowers on the spadix. The fruit remain firm and green until they finally wither and rupture or decompose on the ground. They have compact corms which require a resting period (Letty 1973). The species from sections I and II can be separated using several characteristics (Table 2).

Z. aethiopica is the easiest species to grow within the genus and it is well suited to New Zealand environmental conditions (Tjia and Funnell, 1985). It has many advantages for cut flower or pot plant production. Unfortunately *Z. aethiopica* is only available in milk white, or in some cultivars, white with green spathes. In Europe and North America in particular they are sold as funeral flowers which restricts sale as a cut flower and pot plant (T.E.Welsh, pers. comm., 1989).

In contrast, species within section II can be hybridized with each other. In New Zealand, Harrison and his associates have produced an outstanding collection of *Zantedeschia* hybrids. The great range of colour, size and form of Harrison strains for cut flowers, small pot plant and garden plants has attracted considerable overseas attention (Amos, 1984)

Zantedeschia cultivars have a very exciting future as a cut flower export crop and are recommended by Action Flower Export Co Ltd.(1987) as one of the more profitable flower export crops for growers at NZ $ 1/stem for coloured flowers. The total annual export value reached NZ $ 1,127,599 in 1989 (The Orchardist, 1989).

Zantedeschia are remarkably tolerant and relatively free from most pests and diseases. The major problem affecting the rhizomes or tubers is a soft rot caused by *Erwinia* and *Pseudomonas*. *Erwinia* is thought to be the predominant pathogen (Action Export Co Ltd.1987). Sensitivity to soft rot disease has led to severe losses from disease either in storage, shipment or production. These problems greatly increase production cost per corm/rhizome. *Z. aethiopica* has many advantages for cut flower or pot plant production. For example it is more resistant to soft rot disease than section II hybrids (e.g. Best Gold, Pink Persuasion, Black Magic, Aztec Gold, Chrohatella, et) (Pers. Comm, D. Cohen,1988, DSIR Fruit and Trees, Palmerston North; Pers. Comm. P. Long, 1989, Massy university Plant Health. Palmerston North).

Table 2. Key to *Zantedeschia* species.

(after Letty ,1973)

Species	EG[1]	Flowers			Leaves	
		DT[2]	Colour	FL[3]	Maculate	Shape
Z. aethiopica	yes	No	White	June-Nov	No	Varied ovate-cordate
Z. rehmannii	No	No	White, pink, Dark-maroon	Sep-Feb	No	Lanceolate
Z. jucunda	No	Yes	Golden-yellow	Nov-Jan	Densely	Triangle-hastate
Z. elliottiana	No	No	Golden-yellow	Nov-Feb	Yes	Oblong-hastate
Z. pentlandii	No	Yes	Lemon, chrome	Nov-Dec	Seldom	Oblong-hastate
Z. albomaculata	No	Yes	White, ivory, Pale-yellow,	Oct-Feb	Rare	Triangle-orbicular
Z. albomaculata sub *albomaculata*	No	Yes	Coral-pink	Nov-Apr	Rare	Oblong-hastate
Z. albomaculata sub *macrocarpa*	No	Yes	Pale-yellow, Coral-pink	Oct-Feb	Sparsely	Triangle-hastate
Z. albomaculata sub *valida*	No	Yes	Cream, Ivory-cream	Oct-Mar	No	Ovate-cordate

1, evergreen; 2, dark throat; 3, flowering

The two different sections have complementary characteristics which are attractive for breeding purpose. It would be a great advantage if the genetic characteristics of these two sections could be combined. New coloured cultivars with greater disease resistance and an increased flowering period would give New Zealand the leading edge in this industry.

The barriers between *Z. aethiopica* and the coloured hybrids are unclear. Traub (1948) suggests there may be a distinct gap from the standpoint of gene exchange. Chromosome analysis of *Zantedeschia* species and hybrids (*Z. aethiopica*. *Z. pentlandii*, *Z. rehmannii*, *Z. elliottiana* hybrid, Best gold and *Z. albonaculata*) showed that all have 32 chromosomes. *Z. aethiopica* var. *childsiana* is distinct from other species in only having two sets of metacentric chromosomes the others were subtelecentric. In comparison, most of the chromosomes in the others species (*Z. pentlandii*, *Z. rehmanni*, *Z. elliottiana* hybrid, Best Gold and *Z. albomaculata*) metacentric to submetacentric(Pers. Comm. Jai-Long, 1989, DSIR Fruit and Trees, Palmerston North).

Preliminally experiments had been carried out by Cohen (1988) who crossed a range of summer flowering colour hybrids with *Z.aethiopica* and found that incompatibility was expressed in all crosses. In earlier crosses (Traub, 1948) embryos developed indicating that pollination and fertilization probably occurred. By dissecting these embryo and transferring them to *in vitro* conditions some shoots were successfully obtained. All shoots rescued by embryo culture were albino and died without producing roots (Cohen, 1988). There is limited information on interspecific incompatibility but it is apparent that it is more diverse and much less understood compared with intraspecific incompatibility (Shivanna, 1982). Prefertilization incompatibility is seen as a pollen-pistil rejection response, expressed at number of possible levels in the pistil, from the stigma surface to penetrated embryo sac. It involves reduced pollen germination,slow pollen tube growth or distortion and bursting of pollen tubes (Chen and Gibson, 1974; Kazimierska, 1978a; Heslop-Harrison, 1982).

Postfertilization barriers are common features of interspecific crosses. The genome of the parent species may be physiologically incompatible leading to hybrid seedling fatality, physiological abnormality and sterility (Chen et al., 1983; Shill et al., 1982). More commonly the endosperm aborts resulting in starvation and death of potentially viable embryos (White and Willam 1976). The physiological basis of hybrid endosperm abortion is unknown, but it may be related to disturbance of nutrient transfer patterns among the diploid maternal tissue, the diploid hybrid embryo and the triploid hybrid endosperm (Evans, 1962; Williams and White, 1976).

Recently, Takahashi (1974) and Hogenboom (1975) suggested that interpopulational incompatibility is controlled by incongruity. Incongruity is due to the lack of genetic information in one partner about some relevant aspect of the other. It may also be concerned with a post-fertilization barrier. Thus incongruity represents a passive reject where as incompatibility involves active rejection as a result of S gene action.

The hypotheses explaining interspecific incompatibility are based largely on genetic studies and very little work has been done on the physiological and biochemical aspects.

In this study four aspects will be examined

(1) Pollen physiology of *Zantedeschia*

Several researchers indicate that the two Sections within *Zantedeschia* have incompatible crosses (Traub, 1948; Letty, 1973; Cohen, 1989) but there has been no study of the

differences in pollen structure between these two sections. Pollen may be different in size, exine or internal organization between the two sections. This was investigated by dissecting pollen and using TEM and SEM to examine pollen structure.

The two Sections have different flowering times. It would be a great advantage if pollen could be stored. The pollen grains of *Zantedeschia* are 3-celled (Davis, 1966; Brebaker, 1967). Tricellular pollen is notoriously short-lived whatever the conditions (Heslop-Harrison and Shivanna, 1977). *in vitro* germination of pollen grains is a easy and reliable technique to examine pollen germination ability. To obtain a suitable pollen germination medium different concentrations of sucrose, boric acid, calcium chloride, agar, and various pH and temperature regimes were examined.

(2) Crosses between the two sections of *Zantedeschia* species in the field

Hybridization is possible among all the *Zantedeschia* species with the exception of *Z.aethiopica* (Traub, 1948; Shibuya, 1956). Difficulties in hybridization between the two sections of *Zantedeschia* are compounded by the different flowering times. *Z. aethiopica* naturally flowers in New Zealand during winter from June to December and the coloured callas flower during December and January.
Artificial pollinations between the two Sections of *Zantedeschia* were performed. The growth of ovary, ovule, embryo sac and embryo size was measured following dissection of the ovary and ovule every two weeks after pollination.

(3) Comparative embryo development of incompatible and compatible crosses

The detailed morphological study of embryo growth or failure in *Zantedeschia* hybrids is essential in a programme directed towards crop improvement in this genus. The arrested embryo growth and degeneration of the endosperm was clearly observed by using the dissection and stain technique. The processes of megagametogenesis and post-fertilization events in compatible crosses and incompatible crosses within *Zantedeschia* are unknown. The present study, forms a part of a more general post-fertilization embryo and embryo sac development survey. It concerns compatible and incompatible crosses and the development of embryo sacs leading to an explanation of failure in incompatible crosses.

(4) Embryo culture.

Abortive embryos can be rescued by using embryo culture techniques in many interspecies crosses.(Williams 1978. Neal, et al 1983) Embryo abortion occurs as either a pre- or post-fertilization event in interspecific crosses, but in *Zantedeschia* post-zygotic abortion was most common. *in vitro* culture of globular and early-stage embryos has proven to be difficult since these heterotrophic stages have complex nutritional and hormonal requirements (Neal, 1983). Most of *Zantedeschia* embryos from incompatible crosses remained in a globular stage. Osmotic pressure is obviously an important factor for growth of the immature embryo (Neal, 1983;Cook, 1988). This study aimed to rescue embryos from incompatible crosses by evaluating levels of sucrose supplemented in the culture media.

CHAPTER 2

POLLEN PHYSIOLOGY OF *ZANTEDESCHIA*

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INTRODUCTION

Species within the two Sections of *Zantedeschia* have different flowering times. *Z. aethiopica* naturally flowers in New Zealand during winter from June to December and the coloured callas flower during December and January. Because of this difference in flowering time attempts to hybridize the two sections would be assisted if pollen could be stored. It would be simplest if pollen of *Zantedeschia* could be stored for up to two months to cover the natural flowering period of the white callas.

Favourable conditions for the maintenance of pollen viability and vigour during storage have been investigated for many agronomic and horticultural crops (Johri and Vasil, 1961; Linskens, 1964; Lee et. al, 1985). Generally, low temperatures (4 - 20 °C) combined with low humidity lengthen the storage life of pollen. The optimum moisture requirement for maximum longevity of pollen under low temperature conditions varies greatly and is dependant on plant species.

Brewbaker (1967) surveyed 2,000 species of angiosperms. Seventy percent shed their pollen in the binucleate stage. In some families in which both binucleate and trinucleate pollen occurs. The remaining families are trinucleate. Generally all species within a genus produced either binucleate or trinucleate pollen, although 10 genera were found producing both pollen types. of the 265 families studied, only 32 families produced

both types of pollen. The family *Araceae* was one of these. Two species of *Zantedeschia* were accorded as having trinucleate pollen but the identification of the species was not given (Brewbaker, 1967). The significance of this difference in the structure of the hereditary materials of the pollen grain becomes apparent when the pollen germinates. In trinucleate types, the generative cell divides prior to dehiscence, and during germination the pollen tube grows very rapidly (Heslop-Harrison,1979a; 1979b). In contrast, in binucleate types, cell division take place during the early phase of germination and pollen tube growth is generally slower (Mascarenhas, 1975).

In general trinucleate pollen are difficult to germinate *in vitro*. They often require water-restricting conditions (Bar-Shalom and Mattsson, 1977) or high concentrations of sucrose. Often pollen tubes are very short. Pollen grains are also generally difficult to store (Heslop-Harrison and Shivanna,19-77). Pollen culture *in vitro* provides a simple experimental system for the determination of pollen viability, pollen germination and tube growth. Nutrients and environmental factors can also be readily manipulated in an *in vitro* system.

Some basic techniques are available for pollen culture. These involve the use of germination media containing mixtures of simple mineral salts. Calcium and boron concentrations in particular are often important (Brewbaker and Kwack, 1963; Vasil, 1960; Scott and Loewus, 1986). Normally sucrose is included as an osmoticum and an energy source (Johri and

Vasild, 1961; Kelley, 1955).

There is no information on suitable pollen germination medium specifically for *Zantedeschia* or other members of *Araceae*. The purpose of this study was to examine various media components commonly used in pollen germination studies. Different concentrations of boric acid, calcium, sucrose, agar, and levels of pH were examined to determine a suitable germination medium for *Zantedeschia* pollen. The best medium would then be used to monitor the viability of *Z. aethiopica* var *childsiana* pollen after storage in various controlled environments.

In recent years attempts have been made to store pollen in organic solvents (Jain and Shivana, 1988; Agarwal 1953; Iwanami, 1972; 1975). Generally polar organic solvents are toxic, but pollen viability can sometimes be maintained when the pollen is stored in non-polar solvents. Storage in organic solvents is simpler and more convenient than other methods such as cryopreservation. Jain and Shivana (1988) demonstrated that pollen gains of *Crotalaria saltiana* stored in n-hexane had high rates of germinability. The present study investigated the effect of this non-polar solvent on the longevity of pollen from *Z. aethiopica* var *childsiana*.

MATERIALS AND METHODS

(A) The Morphology of *Zantedeschia* Pollen Grains

(a) Sample Preparation

The *Z. aethiopica* var *childsiana* pollen grains were collected from plants grown outside at the Plant Growth Unit, Massey University. Pollen was collected in December, 1988. The mean max daily temperature at that time was 25 °C. Pollen from *Zantedeschia* hybrid "Best Gold" was collected at the same time from plants grown in a greenhouse where temperature was controlled (25 ± 2 °C ;Plant Growth Unit, Massey University).

(b) Electron Microscopy

Pollen grains were examined using both scanning and transmission electron microscopes at the Electron Microscope Unit, D.S.I.R. Palmerston North.

(c) Scanning Electron Microscopy (SEM)

Specimen preparation

Fresh anthers of *Z. aethiopica* var *childsiana* and *Zantedeschia* hybrid "Best Gold", were sliced and fixed in the Kanovsky's fixative (3% glutaraldehyde, 2% formaldehyde, 0.1 M phosphate buffer pH 7.2) for 1-2 days at room temperature (20 ± 2 °C). They were washed three times in phosphate buffer and post fixed in 1% OsO_4 in phosphate buffer for 2-3 hours at room temperature,then rinsed (3 times) in phosphate buffer before

dehydration in a graded acetone/water series up to 100% acetone.

Specimens were critical point dried using liquid CO_2 as the critical-point fluid in a Polaron critical-point drier specimens were then mounted on aluminium stubs using conductive silver paint, gold - coated (using a Polaron E 5100 cold stage sputter coater) and examined in a Cambridge 250 MK III scanning electron microscope.

(d) Transmission Electron Microscopy (TEM)

Specimen preparation

Fresh anthers *Z. aethiopica* var *childsiana* and *Zanted-eschia* hybrid " Best Gold" were sliced in Karnovsky's fixative in a petri dish and transferred to glass vials for vacuum infiltration. The fixative was replaced and specimens were fixed for 2 hours at room temperature, then washed (3 times) in phosphate buffer followed by post fixation in 1% OsO_4 in phosphate buffer for 1/2 hour at room temperature. Specimens were then dehydrated through a graded acetone/water series with two changes of 100% acetone. The specimens were infiltrated with an acetone/Polarbed 812 resin mixture (50/50) followed by 100% resin (8 hours). Specimens were embedded using fresh resin in silicone rubber moulds and cured for 48 hours at 60 °C.

The sections were cut using a diamond knife and a Reichert Ultracut E microtome, collected on copper grids, double stained

with ethanolic uranyl acetate followed by lead citrate (Venable and Coggeshall) and examined in a Phillips 201 C TEM.

(B) *in vitro* Germination of *Zantedeschia* Pollen

Pollen of *Z. aethiopica* var *childsiana* was collected from plants grown outside at the Plant Growth Unit, Massey University in July 1988 when the mean max daily temperature was 10 °C. Pollen from the coloured calla hybrids Pink Persuasion and Aztec Gold was collected at the same time from a greenhouse (24 ± 2 °C) at DSIR,Fruit and Trees, Palmerston North.

Before pollen shed scapes were cut 20 cm below the spathe and placed in distilled water at 21 ± 2 °C. At pollen shed fresh pollen was taken directly from the spadix using a small brush. Pollen grains from a single inflorescence were collected for each treatment.

Pollen Germination Media

In efforts to optimize the composition of the medium for germination of *Zantedeschia* pollen, sucrose (0 - 300 gl^{-1}), boric acid H_3BO_3 (0 - 200 mgl^{-1}), agar (0 - 40 gl^{-1}) and calcium chloride concentration ($CaCl_2.2H_2O$, 0 - 1000 mgl^{-1}) and arrange of pH (4 - 8) were examined. A medium containing 100 gl^{-1} sucrose, 10 mgl^{-1} boric acid, 10 gl^{-1} Davis agar at pH 7 was used as a basal medium from which all modifications originated. For example , pollen germination media used to test

sucrose effects had a variation in sucrose concentration (0%, 10%, 15%, and 30%) with all other basal ingredients constant.

All pollen germination media were prepared using distilled water and were autoclaved at 121 °C for 15 minutes. Media (1-2 ml) were spread onto a glass slide and allowed to solidify at room temperature in a lamina flow cabinet to prevent contamination.

Zantedeschia pollen is coated with a light-yellow waxy substance which makes the pollen sticky. To spread the pollen grains on the germination media, fresh pollen was collected and rolled-onto the slide with a plastic rod. Pollen slides were incubated at 21 °C. To determine the percentage germination, the number of germinated pollen grains in the field of view (160x magnification) was divided by the total number of grains in the field. Four measurements (50 - 150 grains/field) were taken randomly from each slide. Two replicates of the same inflorescence were performed for each treatment. Measurements were made at various times up to 40 hours, but since no increase in percentage germination was found after 20 hours, this was adopted as the standard time for measurements. The results of these experiments were analyzed using ANOVA package on the DSIR Vax computer. L.s.d. (P=0.05) following an analysis of variance.

(C) Pollen Germination and Tube Elongation

The *Z. aethiopica* var *childsiana* pollen grains were collected from plants grown in the field at the Plant Growth Unit, Massey University. Fresh pollen was collected with a needle and was put on to a glass slide in two drops of culture medium. The slide was placed on wet tissue in a glass jar covered with a tight lid to avoid drying out of the medium. Pollen germination was examined after 20 hours using a light microscope (160 - 500x magnification).

(D) Pollen Storage Experiments

Z. aethiopica var *childsiana* pollen was collected (as above) during November 1988 for pollen storage experiments when the mean max daily temperature was 20 °C. The germination rate of fresh pollen grains was checked using the pollen culture medium (100 gl^{-1} sucrose, 50 mgl^{-1} boric acid, 100 mgl^{-1} calcium, 10 gl^{-1} agar at pH 6). To determine the influence of humidity on pollen viability fresh pollen was placed in either an uncovered glass jar or a sealed glass jar with silica gel at 21±1, 4±1, or -9±1 °C.

The effect of store pollen in n-hexin (50mg pollen/ 1 ml n-hexin) at 21±1, 4±1, or -9±1 °C for 24 hours was also tested. Pollen was transferred to filter paper and after the n-hexane had evaporated its viability was determinated using the standard germination test.

RESULTS

(A) The Morphology of *Zantedeschia* Pollen Grains

The pollen grains of both the *Zantedeschia* Section II hybrid "Best Gold" and the Section I variety *Z.aethiopica* var *childsiana*,were ovoid in shape, with an average size of 38+2 µm in length (Plate 1) The exine was smooth and there was no aperture.

There were no obvious morphological differences between the pollen of Childsiana and Best Gold (Plate 1). Pollen grains observed using TEM had two distinct layers; the exine and intine. Pollen from the two varieties had similar exine structure which was divisible into three distinct layers; tectum, sculpture and endexine. The outer tectum or pollen coat was a thick layer with a continuous smooth surface. A thin columellae with many rods (sculpture) lay below the tectum. Between the intine and tectum was a non-sculptured nexine (endexine) where an electron dense lamellate was present. The inner-most pollen wall was a smooth layer of intine (Plate 2). The cytoplasm was full of rod-shaped crystalline starch grains.

The pollen of *Zantedeschia* is trinuclear (Davis, 1966; Brewbacker, 1967). The sperm nuclei and the vegetative nucleus can be clearly seen in (Plate 2). A prominent feature is the large amount of starch found in the pollen grains.

(B) Factors Affecting *in vitro* Germination and Storage of *Zantedeschia* Pollen

Pollen germination was observed on based media supplemented with various compounds.

(a) Sucrose and Boric Acid Interaction

Pollen germination was very poor in distilled water. The optimum sucrose concentration for pollen germination was between 50-100 gl^{-1}. The highest concentration of sucrose (300 gl^{-1}) sharply reduced pollen germination. Boric acid in lower concentrations (10-100 mgl^{-1}) promoted pollen germination but totally inhibited pollen germination at high concentration (200 mgl^{-1}).

Table 3 The sucrose and boric acid interaction on pollen germination of the *Zantedeschia* hybrid "Pink Persuasion"

		Sucrose[1] (gl^{-1})				
		0	50	100	150	300
Boric acid[2] (mgl^{-1})	0	0.9	2.6	2.5	1.4	0.4
	10	4.5	8.8	14.5	7.6	2.0
	50	3.1	7.5	9.8	2.1	0.8
	100	4.3	5.5	5.7	2.2	1.6
	200	0.0	0.0	0.0	0.0	0.0

1, L.S.D. 5% = 4.20; 2, L.S.D. 5% = 3.76

Table 4 The sucrose and boric acid interaction on pollen germination of the *Zantedeschia* hybrid "Aztec Gold"

		Sucrose[1] (gl^{-1})				
		0	50	100	150	300
Boric acid[2] (mgl^{-1})	0	3.7	2.2	6.0	3.3	0.8
	10	5.7	4.6	5.3	1.3	0.5
	50	6.6	12.8	9.7	2.9	1.2
	100	1.5	2.7	2.1	1.9	1.7
	200	0.0	0.0	0.0	0.0	0.0

1, L.S.D. 5% = 2.86; 2, L.S.D. 5% = 3.19

Table 5 The sucrose and boric acid interaction on pollen germination of the *Zantedeschia* aethiopica var childsiana

		Sucrose[1] (gl^{-1})				
		0	50	100	150	300
Boric acid[2] (mgl^{-1})	0	1.5	1.5	2.6	1.1	0.0
	10	12.0	17.9	27.3	12.0	4.4
	50	5.7	21.0	58.0	11.0	1.2
	100	10.8	18.3	7.7	12.3	7.8
	200	0.0	0.0	0.0	0.0	0.0

1, L.S.D. 5% = 4.33; 2, L.S.D. 5% = 3.87

Sucrose and boric acid had a synergistic effect on promotion pollen germination and this varied with genotype (Table 3,4,5). The best germination rate for " Aztec Gold " was 50 gl^{-1} sucrose + 50 mgl^{-1} boric acid, "Pink Persuasian" was 100 gl^{-1} sucrose + 10 mgl^{-1} boric acid and *Z.aethiopica* var *childsiana* was 100 gl^{-1} sucrose + 50 mgl^{-1} boric acid. Thus the optimal levels for sucrose and boric varied between cultivars. The medium containing 100 gl^{-1} sucrose and boric acid 50 mgl^{-1} was selected for further pollen germination studies for *Z.aethiopica* var *childsiana*.

(b) Calcium Chloride

Table 6 The influence of calcium on pollen germination of *Z.aethiopica* var *childsiana*

		Pollen germination* (%)
Calcium chloride (mgl^{-1})	0	39.1
	10	41.5
	100	52.2
	1000	8.5

*, L.S.D. 5% = 4.17

Medium levels 100 mgl^{-1} of calcium increased pollen germination of "Childsiana" when added to the basic medium (100 gl^{-1} sucrose, 50 mgl^{-1} boric acid with 10 gl^{-1} agar) lower calcium levels (10 mgl^{-1}) had little effect,but higher concentrations (1000 mgl^{-1}) greatly reduced pollen germination (Table 6).

(c) pH Level

Table 7 The effect of pH on pollen germination of *Z.aethiopica* var *childsiana*

		Pollen germination* (%)
pH	4	62.1
	5	64.6
	6	71.1
	7	67.3
	8	39.1

*, L.S.D. 5% = 4.36

The pollen of *Z.aethiopica* var *childsiana* germinated satisfactorily in basal media with a pH from 4 to 7 with an optimum at pH6. At pH8 pollen germination was markedly reduced (Table 7).

(d) Agar Concentration

Table 8 The effect of agar concentrations on the pollen germination of *Z.aethiopica* var *childsiana*

		Pollen germination* (%)
Agar (gl^{-1})	0	57.3
	10	62.6
	20	60.0
	30	59.1
	40	60.1

*, L.S.D. 5% = 2.79

Pollen germination of *Z.aethiopica* var *childsiana* was not affected by the agar concentration in the range 10-40 gl^{-1} and liquid medium (no agar) only slightly reduced germination rates (Table 8).

(e) Temperature

Table 9 The effect of temperature on pollen germination of *Z.aethiopica* var *childsiana*

		Pollen germination* (%)
Temperature (°C)	15	22.3
	20	74.3
	25	65.3

*, L.S.D. 5% = 4.83

The greatest percentage germination of *Z. aethiopica* var *childsiana* pollen was found at 20°C. At a lower temperatures (15°C) pollen germination was greatly reduced and at 25°C germination was also significantly reduced (Table 9).

(f) Variation Between Inforescences

Table 10 The effect of different inflorescence on pollen germination of *Z.aethiopica* var *childsiana*

		Pollen germination* (%)
Inflorescence (No)	1	65.7
	2	54.7
	3	45.6
	4	3.0
	5	71.6
	6	60.2

*, L.S.D. 5% = 4.87

Pollen collected from different inflorescence showed great variation in the percentage of viable pollen (Table 10). Because of this, pollen germination was tested for pollen samples from each inflorescence used for hybridization experiments.

(C) Pollen Germination and Tube Elongation

Zantedeschia pollen grains dehydrated and shrank very soon after anthesis. When grains were placed on stigmas or artificial media, they absorbed water and swelled prior to germination, this process occurred after 2-3 hours at 20-25 °C.

Pollen tubes elongate as a result of tube wall expansion of the tip upon supplementation of tube wall materials.(Iwanami et al., 1988) Protoplasmic streaming was observed in the germinating pollen under the light microscope and most of the protoplasm was located in the terminal area of the germinated pollen tube. When the tube elongated further, large vacuoles

developed in the grain or basal end of the tube which were interspaced with plugs (Plate 3c). The first plugs appeared at the basal end of the tube, and then several plugs were sequentially formed distally so that in a fully developed tube many plugs partitioned the tube into separate chambers (Plate 3c).

(D) *Z.aethiopica* var *childsiana* Pollen Storage

(a) Standard Freezer (-9±1 °C)

All pollen was shrunken and germinative capacity was lost within 2 days of being stored in the freezer.

(b) Room Temperature (21±2 °C)

Pollen lost its germination capacity gradually over 5 days in an unsealed container or in a sealed jar (Table 11).

Table 11 Germination capacity of pollen stored in either sealed or unsealed containers at room temperature (21±2 °C)

		Pollen germination (%)	
		unsealed[1]	sealed[2]
Day	1	71.7	68.7
	2	70.8	68.2
	3	47.9	54.3
	4	33.6	38.9
	5	0.0	3.7

1, L.S.D. 5% = 4.39; 2, L.S.D. 5% = 4.13

(c) Refrigerator

Table 12 Germination capacity of pollen stored in a refrigerator (4±1 °C)

		Pollen germination (%)	
		sealed[1]	unsealed[2]
Weeks	0	70.1	68.7
	1	71.3	35.3
	2	10.3	3.7
	3	1.1	0.0
	4	0.0	0.0

1, L.S.D. 5% = 4.08; 2, L.S.D. 5% = 3.78

Pollen was maintained with a good germination capacity for one week when it was placed in a sealed jar and stored in a refrigerator. The germinative capacity was greatly reduced after two weeks storage (Table 12).

In an unsealed container the germinative capacity had decreased 50% after one week of storage and was totally lost by the third week (Table 12).

(d) Organic Solvent (n-Hexane)

Pollen totally lost it germinative capacity using this treatment at all temperatures tested.

DISCUSSION:

Z.aethiopica var *childsiana* pollen was similar in morphology to pollen from the coloured cultivars. They have light yellow waxy pollen coat. The exine was smooth with no

aperture and the cytoplasm was full of starch grains (Plate 1,2). Pigment which colour pollen grains may protect them from UV radiation (Knox, 1984b). Particular pollen colours are associated with different pollination vectors, for example, yellow for insects; and blue, brown or black for birds. In general, wind-vectored pollen is spherical in shape with a smooth surface and starch is the main energy reserve. These pollen grains have a relatively thin wall and powdery non-sticky surface. In contrast, in animal-vectored pollen, the surface is usually heavily ornamentated to assist grain adhesion both to the pollinating insect and to the stigma surface. The main energy reserve in animal vectors pollen is lipid (Baker and Baker, 1983). *Zantedeschia* pollen mostly transferred by gravity, when shed the pollen dropped and the pearl string, sticky pollen grains hung on the stigma.

Zantedeschia pollen initiated germination within a short period of time (2-3 hours) when cultured *in vitro* and placed in temperature (21±1°C). Protoplasmic streaming occurred in the germinating pollen. Most of the protoplasm was located in the terminal area of the tube in germinating pollen. Large vacuoles formed in the grain or basal area of the tube. When plasmolysis was induced, the protoplasm separated into one or more regions in the pollen cell, but the streaming was independently active in each unit. This has been be observed in other plants (Iwanami, 1988) and it has been suggested that, unlike the blood stream in animals, protoplasmic streaming in pollen cells is self-regulating. When *Zantedeschia* pollen tubes elongated

plugs were formed which partitioned the fully developed tube into separate chamber (Plate3 a). This partition may function to prevent the back-flow of cytoplasm and nuclei in the long pollen tube (Iwanami, 1988). In some species plugs appear to block the actively growing protoplast in the tip from the empty tube above. This provides a means for maintaining turgor pressure in the living tip region which contains the male germ unit (Knox). In general, promotion of pollen growth is observed in pollen-pistil combinations between related or compatible genotypes.

The pollen of self-pollinating species is often starchy (Baker and Baker, 1979). The greater frequency of starch grains in these species may be related to the fact that these plants are not called upon to satisfy the nutritional needs or preferences of pollen collecting insects. *Zantedeschia* pollen was full of starch grains (Plate 2 a, c) this indicated that *Zantedeschia* pollen not satisfied the pollen collecting insects needs. This was observed that *Z.aethiopica* have bright yellow spadices which would attract visiting insects, although the abundant starchy grains within the pollen may not totally satisfy the nutritional needs of pollen collecting insects. *Z.aethiopica* was visited by bees and flies during this experiments.

Zantedeschia pollen is surrounded by a complex wall. Two distinct layers, the exine and the intine occurred within the pollen wall. The exine is the external ornamented layer

composed of sporopollenin, a polymer resistant to biodegradation. The composition of sporopollenin is still not understood, but it is thought to be produced by the oxidative polymerization of carotenoid pigments and their esters (Brook and Shaw, 1978). In addition to the role of exine in protection and dispersal of the male gametophyte the pollen exine acts as a repository for all the biochemical substances of sporophytic origin which operate in the recognition systems between the anther and pollen, and between the pollen and stigma (Heslop-Harrison, 1975a).

The similar exine and intine structure of *Zantedeschia* two different Sections pollen (Plate 2 b, d) (Childsiana and Best Gold) may indicated that the similar pollen walls possibly can be recognized between the anther and pollen between this two Sections, but the recognizable systems are far more complex.

For most species the outer wall contains one or more apertures which are designated areas for pollen germination and tube growth (Knox, 1984a). *Zantedeschia* pollen walls had no apertures (Plate 1 b, d) which is common in some families of tropical monocots (Knox, 1984b). In apertures pollen the intine proteins are principally concentrated in the region of the germpore (Heslop-Harrison et al., 1973); in non-aperture monocotyledonous pollen they are distributed throughout the intine (Knox, 1971; Heslop-Harrison, 1975a).

Approximately 70% of species release pollen in binucleate stage while the others are in trinucleate stage (Brewbaker,

1967). *Araceae* genera have both binucleate and trinucleate pollen, and *Zantedeschia* was trinucleate (Plate 2 c). Trinucleate cells incorporate the twin sperm cells and a vegetative nucleus. The sperm are commonly spindle shape or ellipsoidal (Brewbaker 1967). In ultrastructure studies of pollen *Plumbago zeylanica* differences between the pair of sperm cells have been observed (Russell 1980; Russell and Cass 1983). One sperm cell consistently is attached to the vegetative cell by a projection while the other is linked to the first by plasmodesmatal connections. *Zantedeschia* had two distinctive types of nucleus were observed in thin sections using a T E M. The ellipsoidal nucleus with a high density of heterochromatin was presumed to be the sperm nucleus. The big and irregular nucleus that had less heterochromatin was presumed to be the vegetative nucleus.

Binucleate and trinucleate pollen types have a distinct physiological differences which are reflected in their viability *in vitro* and storage longevity (Brewbaker, 1957; Brewbaker and Majumder, 1961). In general, binucleate pollen is easier to culture *in vitro*. Mature binucleate pollen has entered a sort of temporary dormancy, a quiescent state that is favourable for storage of pollen grains. In contrast, trinucleate pollen are short lived whatever the conditions (Marie-Therese et al., 1986). This indicated that the difficult of storage the pollen of *Zantedeschia* was due to its trinucleate pollen.

The most influential factors on germination of *Zantedeschia* were the concentration of boric acid and sucrose, and

the temperature. The culture medium recommended for *Z. aethiopica* var *childsiana* pollen contained 100 g1 sucrose. 50 mgl^{-1} boric acid, and 100 mgl^{-1} calcium chloride. The agar concentrations did not affected the pollen germination rates. Generally liquid or semi-liquid media are used in pollen germination of binucleate pollen, whereas, trinucleate pollen needs more solid media (Heslop-Harrison, 1977). Trinucleate species tend to have dry stigma and binucleate species often have wet stigmas. The binucleate pollen of wet-stigma types is presumably adapted for germination in a liquid media, while trinucleate pollen is commonly adapted for germination on dry stigma surface, where hydration is slow and controlled. To promote pollen germination the situation encountered on the stigma surface has to be simulated.

Stigmas can be divided into either the "dry" type which has little or no surface secretion at maturity, and the "wet" type, where a distinct surface secretion is present, with a free fluid surface (Shivana, 1977). A simple rapid test for characterization of stigmas as "wet" or "dry" is if in the receptive, state the secretion can be printed clearly onto a dry non-absorbent surface then the stigma is wet. This principal is not clear enough, however to separate all "dry" and "wet" stigma. The physiological of the stigma pointed out by Heslop-Harrison (1975), all sporophytic systems operate with a dry stigma where there can be a direct interaction between single pollen grains and single papillae. In contrast, with the exception of Gramineae, all gametophytic systems are

correlated with wet stigma surfaces on which there can be no intimate direct interaction between one pollen grain and one surface cell since germination takes place in a common fluid medium.Result, *Zantedeschia* stigma as had a little surface secretion which was printed clearly onto a dry plastic sheet. Pollen germinated even in a liquid media, which indicated that this was a "wet" stigma. Although Shivana (1977) concluded that all trinucleate forms within the monocotyledons have dry stigma, *Zantedeschia* appears to be an exceptions. This result suggested that there may be an intermediate type of stigma between "dry" and "wet".

Trinucleate pollen grains of *Compositae* and *Gramineae* respire 2 to 3 times as much as binucleate pollen grains in humid air and this is associated with a rapid loss of viability (Hoekstra and Bruinsma, 1975b). The physiologically and genetically advanced trinucleate pollen is equipped with fully developed mitochondria at dehiscence enabling it to germinate more rapidly *in vitro* and on the stigma (Hoekstra and Bruinsma, 1978). Differences in the rate of respiration in humid air between bi- and trinucleate pollen are determined by the different number and location of their mitochondria. *Zantedeschia* pollen when it was dried with silica gel and sealed, it could store longer then unsealed pollen. This indicated that the humid air associated with a rapid loos of pollen viability and caused the shortage of the pollen store length when it compared with sealed pollen.

Trinucleate pollen are notoriously short lived whatever the conditions and often pollen tube are short and need high sugar concentration for germination (Heslop-Harrison and Shivanna, 1977). The various characteristics of trinucleate pollen indicated that, in contrast to binucleate pollen, trinucleate pollen is difficult to germinate *in vitro* often demands special conditions or high (10-25 %) sucrose concentration (Bar-Shalom and Mattsson, 1977).

The pollen germination of *Z. aethiopica* var *childsiana* does not fit the general picture of trinucleate pollen. Pollen grains of *Zantedeschia* prefer low concentrations of sucrose (5-10%), and a high sucrose concentration (30%) sharply reduced the pollen germination ratio. Pollen was unaffected by agar concentration, and they germinated even in liquid media. This is different to *Jojoba* pollen where germination increases progressively as the agar concentration increases from 0.5% to 5% (Lee et al., 1985). It was expected that *Z. aethiopica* var *childsiana* pollen would germinated well in lower temperature (15 °C) compared with higher temperature (20-25 °C) because plants flowering in winter when average temperature are below 15 C. But in this study it was found that the optimum temperature for *Z. aethiopica* var *childsiana* pollen germination was 20 °C.

CONCLUSION

Z. aethiopica var childsiana pollen was similar in morphology to pollen from the coloured cultivars. The exine was smooth with no aperture and the cytoplasm was full of starch grains. When pollen tubes elongated plugs were formed which partitioned the fully tube into separate chambers.

Zantedeschia was trinucleate, two distinctive types of nucleus were observed. The ellipsoidal nucleus with a high density of heterochromatin was presumed to be the sperm nucleus. The big and irregular nucleus that had less heterochromatin was presumed to be the vegetative nucleus. *Zantedeschia* was wet stigma. The pollen germination of *Z.aethiopica* var *childsiana* does not fit the general picture of trinucleate pollen. Pollen grains of *Zantedeschia* preferred low concentrations of sucrose (5-10%), and a high sucrose concentration (30%) sharply reduced the pollen germination ratio. Pollen was unaffected by agar concentration, and they germinated well even in liquid media. Z.aethiopica var childsiana pollen germination well at high temperature (20-25 °C) rather then low temperature (15 °C).

CHAPTER 3

FIELD HYBRIDIZATION

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INTRODUCTION

The *Zantedeschia* species fall into two distinct sections (Table 1). Section I contains one species *Z.aethiopica*, the white calla lily of florists which is common in wet area near streams. Flowering occurs in winter from June to November in New Zealand. The essential characters of *Z.aethiopica* are that it is a robust, evergreen plant up to 60 cm high, without a dark purple area inside of base of the spathe (Traub, 1948; Letty, 1973).

Section II consists of the remaining five species, all of which have several unifying characteristics. These species flower during summer and have a wide range of colours. The plants are dormant in winter and have a tuberous storage organ (Letty 1973).

If the genetic characteristics of these two sections could be combined the resultant plants might have a number of interesting features. These might include new colours, improved growth habit, improved disease resistance and extended flowering. Such hybrids would give New Zealand the leading edge in the calla industry.

Hybridization is possible among all the *Zantedschia* species with the exception of *Z.aethiopica* (Traub, 1948; Shibuya, 1956; New, 1964; Letty, 1973; Cohen, 1988). Although seed can develop in crosses involving *Z.aethiopica* the seed is

not viable (Traub, 1948; Cohen, 1988).

It appears that seed development is initiated but beyond some point embryos are not viable. No information is available on the reasons for embryo abortion in these *Zantedeschia* hybrids.. This study aims to:

(a) attempt interspecific hybridization between and within the two Sections of *Zantedeschia*.
(b) to study embryo development in compatible crosses
(c) to study embryo development in incompatible crosses
(d) to attempt to culture interspecific embryos in vitro this aspect will be discussed in a later chapter.

MATERIALS AND METHODS

Pollination

The pollen of *Z.aethiopica* var *childsiana* used in this study was collected from inflorescence removed from plants grown at the Plant Growth Unit, Massy University and held in jars of water in a room at 20± 1 °C.

Two cultivars were used as female parents *Zantedeschia* hybrid "Best Gold" and *Z.chromatella* grown on the property of J.M. Wilson, Bonness Road, Fielding (15 kilometres north of Palmerston North). *Zantedeschia* "Best Gold" is a golden yellow selection made by J. M. Wilson which grows almost true from seed. *Zantedeschia* hybrid "Chromatella" is a commercial cream

selection of a *Z.albomaculata* type. Pollination was carried out in the field on Mr. Wilson's farm, from 21 November, 1988.

Table 13 Attempted hybridizations (after Letty, 1973 and Traub, 1948)

Treatment	Expected result
(1) Best Gold Selfed	Compatible cross
(2) Best Gold (♀) x Chromatella (♂)	Compatible cross
(3) Best Gold (♀) x Pink (♂)	Compatible cross
(4) Best Gold (♀) x Childsiana (♂)	Incompatible cross
(5) Best Gold unpollinated	
(6) Chromatella (♀) x Best Gold (♂)	Compatible cross
(7) Chromatella (♀) x Childsiana (♂)	Incompatible cross
(8) Chromatella unpollinated	

In addition, the pollen of a pink *Zantedeschia* hybrid was also used in one treatment. The origin of parent species was unclear but it is possibly derived from *Z.albomaculata* (Table 1, J. Wilson Pers. comm. 1988)

Emasculation was achieved before dehiscence by removing the male portion of the spadix including the region where staminate flowers were present near pistillate flowers.

Pollination was performed immediately following emasculation, using a fine brush to apply pollen to the stigmatic surfaces. To avoid pollen contamination the brush was

dipped in acetone 70% between different pollinations. The acetone 70% killed pollen immediately.

Selfed inflorescences were not emasculated. But the stigmas were pollinated with pollen from the same inflorescence. To prevent accidental pollination all inflorescence were covered with a paper envelope tied with a clip.

Ten inflorescence were pollinated for each type of pollen transfer (Table 3). Pollination was carried out on three occasions, 21, 23 and 26 December, 1988 to ensure pollination. The stage of pistil development was recorded for each female parent used. Stage 1: the stigma was light yellow and the ovary was flat and light green; stage 2: the stigma was yellow and appeared wet and the ovaries were deep green.

Every two weeks, samples of about 3-5 ovaries were collected from the field from each hybridization treatment to enable measurements of ovary, ovule, embryo sac and embryo development. Collections were made every two weeks from 2 to 12 weeks period pollination. The number of ovules developing and the size of the ovules , embryo sac and embryos were determined. To measure the length of ovule, embryo sac and embryo, ovaries were dissected under a stereo microscope. Fresh weights rather than dry weights were taken as some of the embryos dissected in this part of the study were used in vitro embryo culture experiments (Chapter 5).

RESULTS

(A) Field Pollination Trial

Pollination time (different pistil stage) did not affect ovary growth in all treatments (Table 13, Plate 5,6,7,8). Spikes grew well within all treatments, except for crosses with *Z.aethiopica* var childsiana. In crosses with Childsiana the spike grew weakly and spikes were readily infected by wilt disease (*Erwinia*). Nearly half of the spikes that crosses with Childsiana were wilted by the tenth week after pollination.

Table 14 The effect of pollination stage and different crosses on spike growth and condition

Crosses	No.	Stage	Health	Weak	Wilt	Compatible
Best Gold selfed	3	1	3	0	0	Yes
	5	2	4	0	1	
Best Gold (♀) x Chromatella (♂)	4	1	4	0	0	Yes
	6	2	6	0	0	
Best Gold (♀) x Pink (♂)	8	1	7	1	0	Yes
	2	2	2	0	0	
Chromatella (♀) x Best Gold (♂)	3	1	2	0	1	Yes
	2	2	2	0	0	
Best Gold (♀) x Childsiana (♂)	7	1	3	0	4	No
	3	2	3	0	0	
Chromatella (♀) x Childsiana (♂)	5	1	4	0	1	No
	5	2	1	0	4	
Chromatella unpollinated	8	1	0	0	8 (Dry)	
	2	2	0	1	2 (Dry)	
"Best Gold" unpollinated	6	1	0	0	6 (Dry)	
	4	2	0	1	4 (Dry)	

Note:

No: Total spikes that be measured
Health: Spikes grew strongly and ovaries were well developed.
Weak: Spikes grew weakly.
Wilt: Spike infected by *Erwinia* disease.
Dry: Unpollinated spikes withered and dead. This is distinct from wilted.

By 10 weeks the base of the peduncle area in a number of inflorescences was collapsing with *Erwinia* soft rot and all inflorescence were harvested. The rotten portion of the peduncle was cut off and the inflorescences were brought back to the laboratory.

Unpollinated inflorescences of *Zantedeschia* hybrid " Best Gold" and "chromatella" were chlorotic after 4-5 weeks and turned dry and died after 8-10 weeks (Plate 4d,h). In some cases, a few ovaries developed on unpollinated inflorescences especially on the top whorl of ovaries. This ovary development probably resulted from pollination prior to emasculation, since it was only found on inflorescences at stage 2 (the time of emasculation).

(B) Ovary and Ovule Development

The ovaries of unpollinated "Chromatella" and "Best Gold" ovaries grew for four weeks and then started to shrink and turn yellow. They eventually went dry and died 8 to 10 weeks after pollination (Table 14, Plate 4d,h).

Far fewer ovules developed in incompatible crosses compared with the compatible crosses (Table 15). The ovaries of compatible crosses and self-pollinated flowers developed quickly for the first 6 weeks and then growth plateaued for the remaining 6 weeks (Fig 1). In the incompatible crosses (between Chromatella X Childsiana and Best Gold X Childsiana) ovary size

was much smaller than other treatments (Fig 1). Ovary size was quite variable but was related to ovule size and number per ovary.

Table 15 The average number of ovaries and ovules within different crosses

Crosses and selfed	Ovaries per spike	Ovules per ovary	Compatible
Best Gold selfed	20	2.3	Yes
Best Gold (♀) x Chromatella (♂)	11.5	2.0	Yes
Best Gold (♀) x Pink (♂)	21	2.1	Yes
Chromatella selfed	46	3.0	Yes
Chromatella (♀) x Best Gold (♂)	34	2.7	Yes
Best Gold (♀) x Childsiana (♂)	24	0.8	No
Chromatella (♀) x Childsiana (♂)	42	0.7	No
Chromatella unpollinated	44		
"Best Gold" unpollinated	21		

Ovule growth during the first two weeks was not different between crossed and selfed ovules but growth rates diverged after 4 weeks growth (Fig 2). Ovules from compatible crosses and self-pollinated flowers (Table 14) had the same rate of growth (Fig 2). This was fast during the first 6 weeks and then slowed down for the remaining 6 weeks. Ovule from incompatible crosses were smaller in size (Fig 2, Plate 5a,b). They stopped to growing 6 weeks after pollination and then started to degenerate.

The fresh weights of ovules from all treatments, separated into two groups. Ovule weight was similar in the first group

of compatible and self-pollinated treatments The weight of ovules from incompatible crosses decreased from 8 weeks after pollination and resulted in ovules 3-4 times lighter than the first group by the 12 weeks (Fig 3).

(C) The Embryo Development

Compatible crosses and selfed embryos had steady growth rate up to 12 weeks. The growth rate of embryos from incompatible crosses was much slower. Embryos could not be seen until 4 weeks after pollination for compatible crosses, when they were approx. 0.1-0.2 mm in diameter. At this stage embryos from incompatible crosses were less than 0.1 mm (Fig 4). Embryos were not detectable under the dissection microscope during this stage because the liquid embryo sac and small size of the embryo. Those embryos could been seen after Dissected and stained with Toludine Blue O.

Embryos from all treatments including incompatible crosses were at the globular stage 2 weeks after pollination. Most embryos from selfed and compatible crosses reached the torpedo stage 4 weeks after pollination. In embryos from incompatible crosses growth was quite variable. Most embryos stayed in the globular stage and grew no further. Some of these embryos became brown and died 10 weeks after pollination.

(D) Embryo Sac Development

The development of the embryo sac reflected ovule development in all treatments (Fig 5). In compatible and selfed treatments (Table 15) growth of the embryo sac was very fast during the first 6 weeks, especially from 2 to 6 weeks (Fig 5). Two weeks after pollination the endosperm started to develop. The endosperm of the compatible crosses in the embryo sac was soft and juicy during the early stage (2-6 weeks), but grew more firm in the later stage (6-8 weeks after pollination).

Shrunken of the embryo sacs from the incompatible crosses were observed from 6 weeks after pollination. The endosperm was filled with liquid material during the early stages but by 8 weeks after pollination, this liquid dried and embryo sacs were shrunken.

DISCUSSION

Different times of maturity of the male and female flowers in the inflorescence, was suggested by New (1964) as the most probable cause of lack of self-pollination of *Z.aethiopica* but this may be the case for the others species in Section II, *Z.albomaculata*, and *Z.elliottiana*, *Z. rehmanni*. Pollen was shed when ovaries were receptive. In this study *Zantedeschia* hybrids Best Gold and Chromatella were used as the female parents and *Z.aethiopica* var *childsiana* was used as the pollen parent. The time of pollination did not affect the proportion

of seed set in all treatments. This indicated that the different time to maturity of the male and female flowers possibly only occurs in the *Z.aethiopica* and not the others species.

To avoid pollen contamination from the same inflorescence, careful emasculation was required including the removal of some of the female portion of the spadix where staminode flowers are present among the upper female flowers.

Incompatible crosses within *Zantedeschia* have fewer ovules develop per ovary compared with selfed and compatible crosses. The peduncle of incompatible crosses appeared to be more susceptible to attack by *Erwinia*. Nearly half of the incompatible crosses spikes collapsed within 10 weeks of pollination. This greater susceptibility to *Erwinia* disease was possibly related to the collapse of the embryo sac. Developing ovules act as "sink" tissues which can draw nutrients from the other parts of the plant which allows for rapid growth. Commonly faster growing tissue is more resistant to disease attack (Nitsch, 1952). When embryo sacs from incompatible crosses collapsed, the ovule tissue lost its "sink" strength and at this stage the spike was less resistant to attack by the disease.

The ovary provides protection for the ovules and tissues of the style and ovary provide guidance for pollen tube growth to the ovules for fertilization. Incompatible crosses of

Zantedeschia produced small ovules but the ovaries had the same growth patten as compatible crosses. The tissues of ovaries in incompatible crosses were very soft and succulent whereas the tissues of ovaries from compatible crosses were firm. The softness of ovaries was due to the liquid state of the endosperm and the small, shrunken presence of only a few ovules. Nitsch (1970) performed several experiments with melons and pumpkins, showing that the application of ground pollen, even of a different species was able to cause at least the first symptoms of fruit set. The type of growth substances by which pollen stimulates fruit development include auxins and gibberellins. Hormones regulate increased pod fill or abscision depending on whether then stimulate ovary hypertrophy or parthenocarpy (Esau, 1977). Although pollination may stimulate the initial swelling of the ovary or fruit, most species require the presence of developing seeds with viable embryos to allow the continuation of ovary or fruit growth (Wareing and Philips, 1981).

Ovule are the starting point for the development of seeds. *Zantedeschia* ovules are anatropous (French, 1986). Here pattern of ovary, ovule and embryo sac development was similar in all selfed and compatible treatments. The most obvious difference between the development of ovaries of compatible and incompatible crosses was that although the ovaries of incompatible crosses continued to grow up to 12 weeks the ovules and embryo sacs had started to degenerate 8 weeks after pollination.

Ovules from incompatible crosses had considerable variation in size. Most of the ovules shrank and stopped to growing when the embryo sacs collapsed. In a few case the ovules continued to grow even though the embryo sac had already collapsed. The growth of the ovules was due to the continued growth of outer integuments. The swelling of the ovules was easily misunderstood to be a well developed ovule. Traub (1948) stated that the seed set in crosses involving *Z.aethiopica* are not capable of germination and eventually rot after planting in the soil. It is now clear that the collapsed embryo sac leading to a degeneration of the seed is likely cause of seed rot. The degeneration of embryo and embryo sac will be further discussed in Chapter 4.

CONCLUSION

The different time to maturity of the male and female flower possibly only occurs in Z.aethiopica and not others species. Incompatible crosses within *Zantedeschia* have fewer ovules develop per ovary compared with selfed and compatible crosses. The peduncle of incompatible crosses appeared to be more susceptible to attack by *Erwinia*. The most obvious difference between the development of ovaries of incompatible crosses was that, although the ovaries of incompatible crosses continued to grow up to 10-12 weeks the ovules and embryo sacs had started to degenerate 6-8 weeks after pollination which was reflected in collapsed embryo sac.

Fig. 1-5 Ovary, ovule, embryo sac and embryo development after 2 to 12 weeks pollination within the genus *Zantedeschia* in compatible and incompatible crosses.

Bars represent ±SE for compatible crosses (A) and incompatible crosses (B).

BG x Chi, □ ; BG x P, + ; BG x Chr, ◇ ; BG x BG, △ ;

Chr x Chi, × ; Chr x BG, ▽

BG: *Zantedeschia* hybrid "Best Gold".

Chi: *Z. aethiopica* var. *childsiana*.

Chr: *Zantedeschia* hybrid "Chromatella"

P: *Zantedeschia* hybrid "Pink"

Fig 1

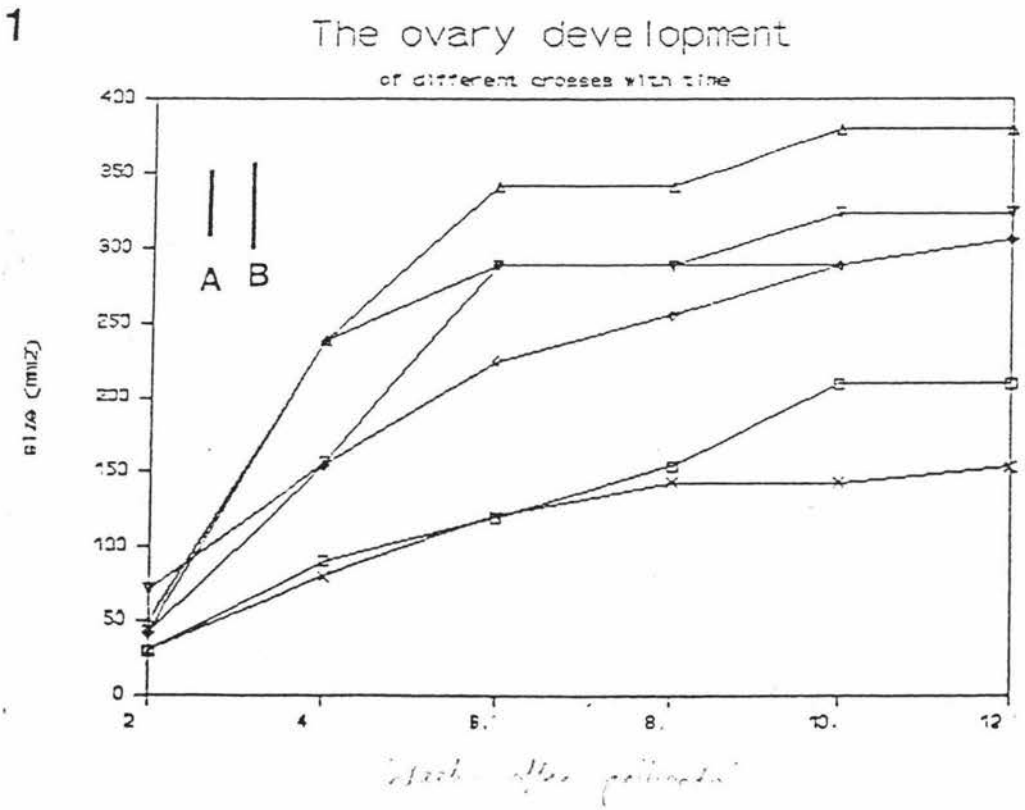


Fig 2

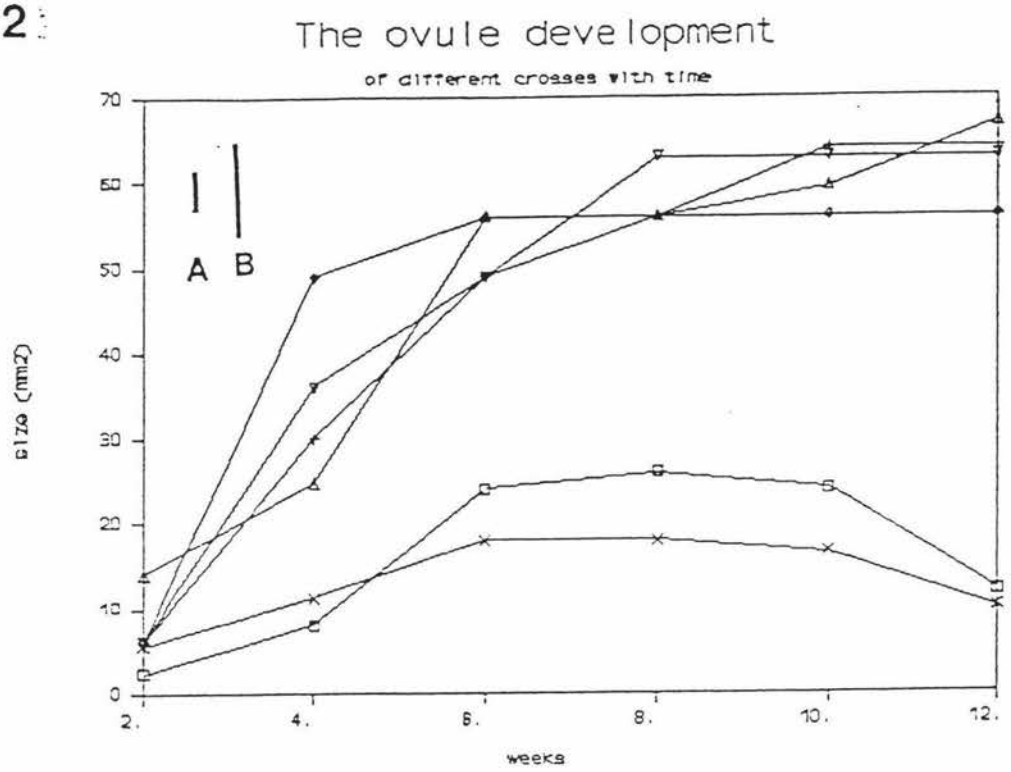

The ovule development
of different crosses with time
A B
size (mm2)
weeks
0
10
20
30
40
50
60
70
2.
4.
6.
8.
10.
12.

Fig 3

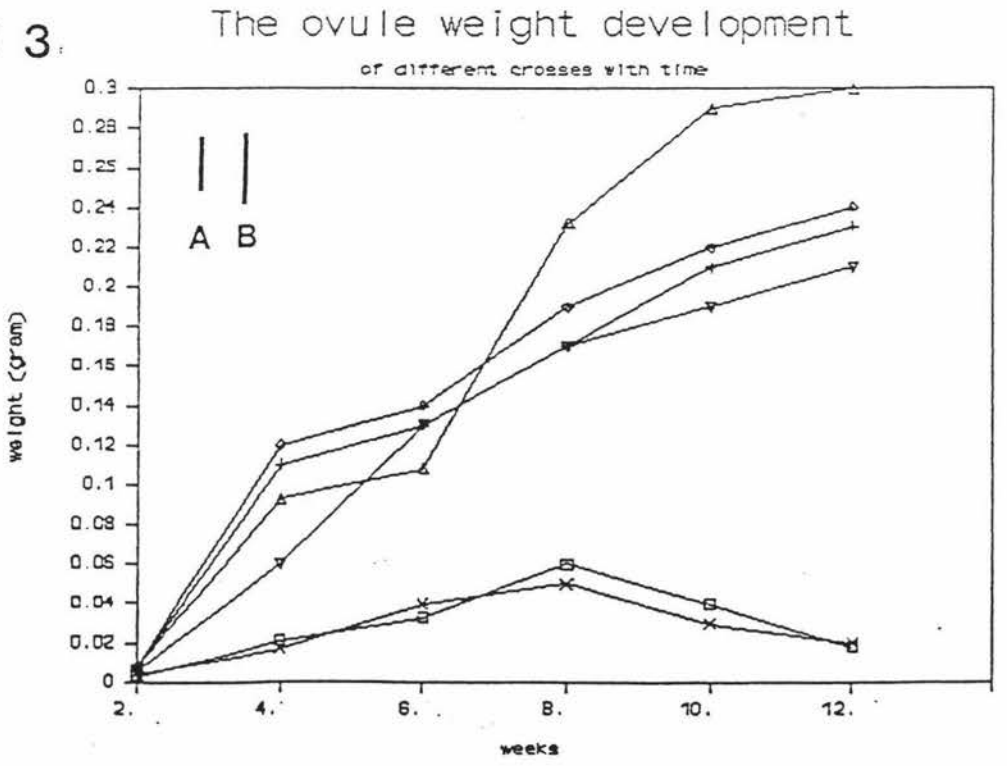

The ovule weight development
of different crosses with time
A B
weight (gram)
weeks
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
0.18
0.2
0.22
0.24
0.26
0.28
0.3
2.
4.
6.
8.
10.
12.

Fig 4

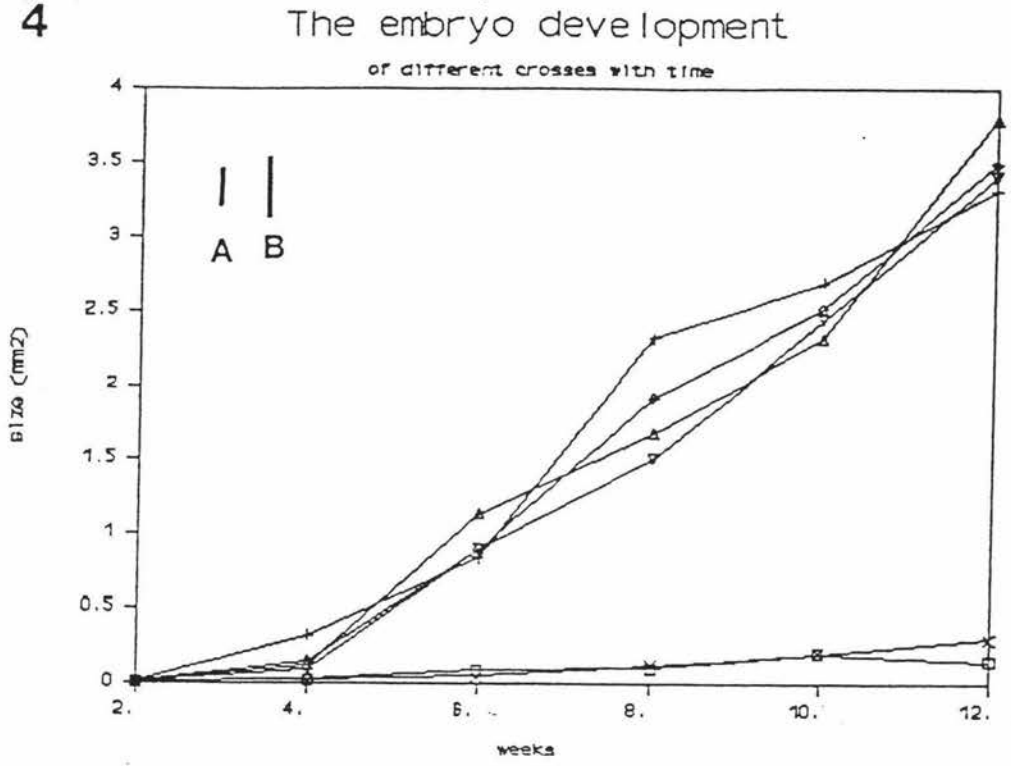
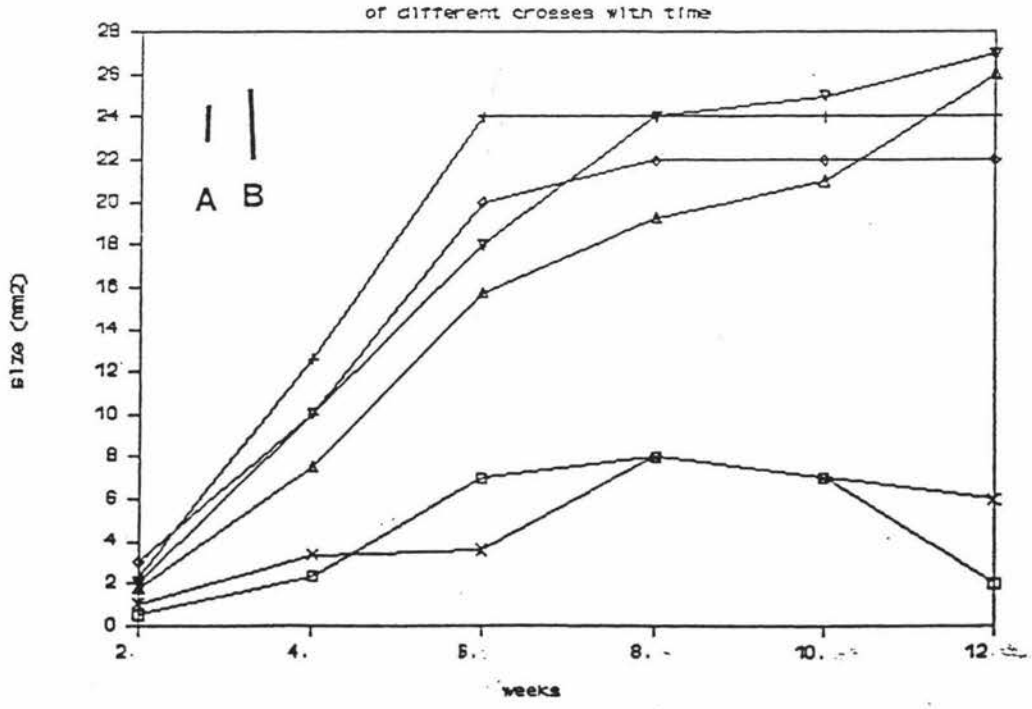

The embryo development
of different crosses with time
A B
size (mm2)
weeks
0
0.5
1
1.5
2
2.5
3
3.5
4
2.
4.
6.
8.
10.
12.

Fig 5


The embryo sac development
of different crosses with time
A B
size (mm2)
weeks
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
2.
4.
6.
8.
10.
12.

CHAPTER 4

EMBRYOGENESIS OF *ZANTEDESCHIA* COMPATIBLE AND INCOMPATIBLE CROSSES

Table of contents

INTRODUCTION

Detailed study of embryo development in *Zantedeschia* crosses is essential for the programme of interspecific hybridization directed toward crop improvement in this genus.

Previous attempts to produce hybrids between *Zantedeschia aethiopica* and other *Zantedeschia* species have been unsuccessful (Traub, 1948; Cohen, 1988). Some hybrids were maintained briefly using embryo culture but all plants were albino (Cohen, 1988). The details of hybrid failure is unknown for some interspecific crosses. Failure may occur in radically different ways in different species and embryo abortion can occur at any stage of development (e.g pollination, pre-fertilization and after-pollination).

Using a clearing technique it was found that the endosperm in hybrids with *Zantedeschia aethiopica* did not contain starch disappeared after four weeks from pollination. This indicated that the hybrid endosperm failed to accumulate starch grains to nourish embryo development and caused embryo death.

Pollination and fertilization occurred when a range of summer-flowering colour hybrids were crossed with *Z. aethiopica* (Cohen, 1988; Cohen, pers. comm. 1989) and suggested that the incompatible barrier between the two Sections of *Zantedeschia* appeared at post-fertilization seed development.

Postzygotic seed abortion is a common outcome of interspecific crosses. The genomes of parent species may be physiologically incompatible leading to chromosome elimination (Davies, 1974; Subrahmanyam, 1977) or embryo death. In post-fertilization seed failure with interspecific hybrids of *Trifolium* species (Williams and White, 1976) endosperm failure appears to be a major factor. Abnormal endosperm development also was responsible for seed failure in *Medicago* (Cooper and Brink, 1940) and Lycopersicon (Cooper and Brick, 1942). The physiological basis of hybrid endosperm abortion is unknown, but it may be related to disturbance of nutrient transfer patterns among the diploid maternal tissue, the diploid hybrid embryo, and the triploid hybrid endosperm (Williams and White, 1976).

Problems in other tissues besides the endosperm may also lead to embryo abortion. Delayed breakdown of starch and lipid in the integumentary tapetum and nucellus is a probable factor in reduced development of coenocytic endosperm in an annual x perennial *Medicago* species cross (Sangduen, et al 1983)

The processes of megagametogenesis and post-fertilization events in the compatible crosses and incompatible crosses (Table 3) is unknown. There are no reports that reveal ultrastructure details of megagametogenesis and post-fertilization embryogenesis in *Zantedeschia*. The present study, forming a part of a more general post-fertilization embryo and embryo sac development survey. It concerns compatible and incompatible

crosses and the development of embryo sac leading to an explanation of failure in incompatible crosses.

MATERIALS AND METHODS

Ovaries from compatible and incompatible crosses (Table 3) were collected every two weeks after pollination from the experimental plot and were carried in plastic bags to the laboratory. The ovules were dissected and fixed overnight at room temperature (21±1 °C) with (3% v/v glutaraldehyde, 2% v/v formaldehyde, in 0.1 M phosphate buffer at pH 7.2). The ovules were then washed three times in 0.1 M phosphate buffer (pH 7.2) over a 6 hour period.

Ovules were placed directly in ice cold Methyl Cellosolve for dehydration. The Methylcellosolve was replaced after 3 hours and the tissue were then left overnight at 4 °C. To improve dehydration large ovules harvested from week 6 onwards were sliced longitudinally.

After dehydration, ovules were transferred to Technovit (7100) Resin prepared according to the manufacturer recommendations for 3 hours with 2 subsequent changes of resin (after 12 hours and again after 4 days). Tissue was then examined to see if clearing was complete.

Resin with hardener was prepared and pipetted into plastic molds and tissues were embedded for 12 hours in an incubator

(50±2 °C). Sections 2-2.5 µm thick were cut with glass knives using a microtome (C. Reichert, Austria; OMU2), and placed on a glass slide with a drop of distilled water. Slides were gently heated on a hot plate (60-70 °C) to spread any folded regions the section out of and fine needles were also used to carefully stretch the sections. The slides were then dried. Sections were generally stained with Toluidine Blue O, and a range of other dyes were also used (after Feder and O'Brien, 1968).

(A) Staining with Toluidine Blue O

Stock Solution 0.05% in benzoate buffer (benzoic acid, 0,25 g sodium benzoate, 0.29 g; water, 200 ml) at pH 4.4

Procedure Toluidine Blue O was applied to sections for 30 sec they were then washed with distilled water and dried on a hot plate (60-70 °C). Sections were observed using a Zeis Photomicroscope II and photographed.

Result RNA stained purple; DNA stained blue or blue-green; polyphosphates, polysulfates, and polycarboxylic acids including alginic acid and pectic acid stained red or reddish purple and polyphexnols and lignin stained green or blue-green.

(B) Staining with Acid Fuchsin

Stock Solution (1% w/v) acid fuchsin in water

Procedure Sections were stained for 4-5 min in aqueous acid fuchsin (1% w/v) rinsed with water till the resin sections were free from stain, air dried or put on hot plate, and then mounted in immersion oil.

Result Most cellular components take up this stain but starch, the polysaccharides of the cell wall, and lignin are usually unstained. Mitochondria, plastids, and the nucleolus are usually stained very strongly, especially if sections are stained with warm dye solution on a hot plate and then rinsed thoroughly.

(C) Staining with Iodine/Potassium

Stock Solution Iodine 0.2 g dissolved in KI solution

Procedure Sections were stained with Iodine stain for 4-5 minutes, rinsed in water, air dried or put on a hot plate, and mounted.

Result Not only stains starch blue to black, but imparts a superb phase contrast effect to many cell components.

(D) Staining with Acid Fuchsin and Toluidine Blue O

Stock Solution As for each stain used separately

Procedure Staining procedure (A) and (B) were performed in sequence. This method strongly stains for each dye separately.

Result Interpretation of colours must be treated with caution since some structures take up both dyes. Otherwise results are as for each dye separately.

(E) Stain with Acid Fuchsin, Toluidine Blue O and Iodine

As for each staining procedure (A) (B) and (C), but the Iodine solution (C) stock strength was reduced to (1% v/v). If the I/KI stain was used at full strength the stains of Fuchsin and Toluidine Blue O were faded. If it was diluted to 1% the original concentration stock was still stained and in addition cuticle was stained blue-green.

(F) Sudan Black B:

Stock Solution A saturated solution of Sudan Black B in ethanol (70% v/v) was made fresh daily, filtered through Whatman No 1 paper and Millipore filtered.

Procedure Slides were placed on wet filter paper in a large petri dish, flooded with staining sol-

ution and left covered for one hour. Stain was removed and replaced with ethanol 70% v/v until excess Sudan Black was removed.

Result — Lipid-containing components appear dark blue to black. Cuticle and suberized walls are black, other walls and starch stained red.

RESULTS

The development of hybrid ovules, embryo sacs and embryos resulting from *Z.aethiopica* var *childsiana* crosses was slow compared with compatible crosses. All embryos obtained from hybrid crosses only developed to a stage comparable with the globe or pre-torpedo stage of selfed embryos. There was considerable variation (0.2-1.0 mm) in the length of the hybrid embryos at the time of ovule dissection. All embryos were chlorophyll deficient and had abnormal growth. Ovule and embryo sac started to degenerate six weeks after pollination.

(A) Typical development of the megagametophyte and ovule in a *Zantedeschia* species using hybrid "Best Gold" as an example

The mature megagametophyte was a typical seven-celled embryo sac with a three-celled egg apparatus,three antipodal cells and a secondary nucleus formed by the fusion of the polar nuclei. The secondary nucleus migrated to the chalazal end of the embryo sac before fertilization. The egg cell was large

with two synergids cells located at the micropylar pole of the embryo sac. These three cells showed a triangular arrangement and shared common surfaces, only the synergids directly contacted the micropyle (Plate 7). The secondary nucleus (fused polar nuclei) which migrated to the chalazal end before fertilization was the largest nuclei observed in the embryo sac.

The three antipodal cells were triangular in shape and were located in the chalazal end just below the secondary nucleus (Plate 7). The antipodal began to degenerate in the mature embryo sac (Plate 7).

The ovules of *Zantedeschia* was anatropus. They had an inner-integument and outer-integument (bitegmic), with the micropyle formed by the inner integument (Plate 6).

The apical cells of the nucellar epidermis divided periclinally to form a nucellar cap (or epistases) which was 4-5 cells in thickness. The outer-integument was comprised of eight to ten layers large cells and the inner integument had two distinct layers. The innermost epidermis was radially elongated to form a integumentary tapetum also called an endothelium (Bhojwani, 1974; Plate 6), around the embryo sac and this extended to the nucellar cap. The inner integumentary cells adjacent to the micropylar end of the embryo sac and micropylar canal contained some elongated cells (Plate 6).

(B) Development 2 Weeks After Pollination

(a) Combatable crosses:

Zantedeschia hybrid "Best Gold" selfed

A globular embryo had developed by 2 weeks after pollination. The primary endosperm nucleus formed two chambers. The micropylar chamber was large and was accompanied by radially elongated net-shaped endosperm cells which surrounded the embryo (Plate 8,11). The lower part of micropylar chamber had big lobar shaped cells.

The chalazal chamber degenerated and had a indistinct mass of irregular cells containing some scattered large nuclei. The inner-integument and integumentary tapetum shrank and degenerated but it still contained some small starch grains (Plate 11c). The nucellar cap (epistases) started to degenerate and shrink. No starch grains were found in epistases cells or endosperm cells two weeks after pollination (Plate 11c, 11e).

The outer-integument had expanded rapidly and produced a large specialized seed coat which contained starch grains. Crystal cells developed within the outer-integument which surrounded the embryo sac (plate 8a; 11a).

(b) Compatible Crosses:

Zantedeschia hybrid "Best Gold" crossed with *Zantedeschia* hybrid "Chromatella" and the reciprocal cross.

A globular embryos developed by 2 weeks after pollination. The micropylar chamber and chalazal chamber were similar to selfed embryos. Inner integument, integumentary tapetum, micropyle and nucellar cap (epistases) could still be clearly distinguished (Plate 9a, b, c). Some small starch grains were found in both cell layers of the inner integument but not in the nucellar cup (epistases) nor endosperm cells. The outer-integument had the same structure and similar starch grains and crystal cells that had developed in the Best Gold selfed ovule (Plate 9a).

(C) Incompatible Crosses:

Zantedeschia hybrid "Best Gold" crossed with *Z. aethiopica* var *childsiana*.

The development of embryo, endosperm, inner-integument and integumentary tapetum cells was quite similar to compatible crosses at this stage (Plate 10 a,b,c; 9 a,b,c).

The most distinction difference between compatible and incompatible crosses at this stage was with the nucellar cap (epistases). The nucellar cap on embryos of this incompatible cross was filled with small starch grains (Plate 12b). The

outer-integument (seed coat) was smaller and thinner compared with compatible crosses (Plate 10 a). Cuticle was secreted between the inner integument and outer integument layer (Plate 12d,e).

(C) Development 4 Weeks After Pollination:

(a) Compatible Crosses:

Zantedeschia hybrid "Best Gold" selfed.

Zantedeschia hybrid "Best Gold" crossed with *Zantedeschia* hybrid "Chrometella" and the reciprocal cross.

The inner-integument and integumentary tapetum degenerated into a thick lignified layer with concave structure (Plate 13 a; 14 a,b). The lignified layer was about 15-20 µm in thickness in the micropylar region, but it became thicker and had a width of about 50-60 µm in the chalazal end. Few starch grains could be found within the lignified layer which was filled with discontinuous bean-like lignified material (Plate 14 a,b). The lignified layer surrounded the whole embryo sac. A thick (10 µm) cuticle was found on the proximal surface of the inner lignified filament and a thin (5 µm) cuticle was secreted from the outer lignified filament (Plate 14a,b). This cuticle could be easily seen when stained with Suden Black B (black in colour) and was blue-green when stained with Fuchsin, Toluidine Blue O and Iodine.

Two layers of aleurone-like cells were formed on the

outermoster layers of the endosperm which stained densely with Toludine Blue O (Plate 13a). The endosperm cell had dense cytoplasm, smaller cells and thin cell wall (14a,b). Endosperm was well developed and filled with starch grains and protein bodies. Starch grains and protein bodies accumulated at the outer edge of the endosperm, rather than in the central region (Plate 14 a,b).

Embryos grew very fast during weeks 2-4. They developed from the proembryo stage (30-40 μm in length) to the torpedo embryo stage (1.2-1.4 mm in the length), and started differentiation (Plate 20 a). Small/dividing cells on the outer layer of the embryo where it stained strongly with Toluidine Blue O (Plate 13 a,d). The cells of the procambium and meristematic region were more vacuolate than those of the protoderm. An this stage the central cylinder of cotyledon and epicotyl (plumule) also developed. Within the embryo no starch grains were found (Plate 20 a). Outer-integument (seed coat) was still expanding.

(b) Incompatible Crosses

Zantedeschia hybrid "Chromatella" crossed with *Zantedeschia aethiopica* var *childsiana*.

The inner-integument and integumentary tapetum degenerated to a thick zig-zag like lignified layer with a vacuolate concave structure (Plate 14 c,d). The lignified layer was thin (20 μm) in the micropylar region and became thick (100 μm) at the chalazal end (Plate 15 a,c). The chalazal end produced

a lignified tissue that was strongly stained (green colour) by Toluidine Blue O (Plate 15 b) then degenerated and collapsed. A thick cuticle (10 µm) occurred on the surface of the inner lignified layer surrounding the embryo sac and a thin cuticle (5 µm) was secreted on the outer surface of the layer (Plate 15a).

Endosperm cells started to degenerate. Aleurone-like layers found in compatible crosses (14a) did not develop in the outermost layers of this incompatible cross. In contrast with the compatible crosses (14a) big starch grains and thick cell walls were produced by this layer (14c,d) which was less densely stained (13b arrow). Abnormal protein bodies were also found (14c).

The epistase still remained and embryos grew very slowly. Embryos were 150-200 µm in diameter and most remained in the globular stage and did not differentiate (Plate 13 b,c). Big vacuoles separated within the embryo and no starch grains were found in the embryo (Plate 13 c). Lignified cells were also observed at the chalazal end of the endosperm which caused browning of the chalazal end (Plate 15b). Some degeneration of cells also occurred. During dissection of embryos, browning of the chalazal end of the endosperm was also observed under the light microscope.

(D) Development Six Weeks After Pollination

(a) Compatible Crosses:

Zantedeschia hybrid "Best Gold" selfed

Zantedeschia hybrid "Best Gold" Crossed with *Zantedeschia* hybrid "Chromatella" and reciprocal cross.

The lignified inner-integument had now collapsed to form a thin layer, with a thick cuticle secreted on the inner surface and a thin cuticle on the outer surface (Plate 16a 17d). This structure later constituted the seed testa.

The endosperm was well developed (Plate 16a, 17d). The two outer-most layers of the endosperm were smaller and had thick cell walls (aleurone-ike layers) which coloured purple with Toluidine Blue O. There were no starch grains in these cells (Plate 17f). The remainder of the endosperm was densely filled with starch.

The embryo was well differentiated. The first and second leaf primordia had developed from the piliferous layer (Plate 20 b, c). The radical was in the process of forming but root-cap cells had not yet developed (Plate 20c).

(b) Incompatible Crosses:

Zantedeschia hybrid "Best Gold" crossed with *Z. aethiopica* var *childsiana* and *Zantedeschia* hybrid "Chromatella" crossed with *Z.aethiopica* var *childsiana*.

The lignified layer retained its thick zig-zag structure and contained some starch grains (Plate 17a,b,c). Embryos still remained globular with very little increase in size (most of the embryos were 150-250 μm in the length) (Plate 18 a, d).

Two different types of endosperm and embryo were found. In the first type the endosperm was well developed (Plate 18d) and the outer layers of the endosperm were similar to compatible crosses. The endosperm contained a small number of large starch grains (Plate 17c) and degenerated protein bodies (Plate 17 b). The embryos within this kind of endosperm were small (150-250 μm in length) and globular in shape. These embryos had cells with thick cell walls and large nuclei (Plate 19 a, b). There were no starch grains in embryo (Plate 19 c).

The second type of endosperm was shrunken and degenerated (Plat 18a). There were aggregates of callus-like cells with thick cell walls. Those callus-like cells which accumulated near the embryo and contained large starch grains (Plate 18b) and abnormal protein bodies (18 c), the other endosperm cells were vacuolated and contained little cytoplasm (18 a). Some individual cells with thickened walls appeared to be going into division (Plate 18 a, c).The embryos within this degenerating endosperm had large starch grains throughout the entire embryo (Plate 19 f). Embryos with this kind of endosperm were small, globular, and full of large starch grains (Plate 19 d,e,f).

(E) Development Eight Weeks After Pollination

(a) Compatible Crosses:

Zantedeschia hybrid "Best Gold" selfed and *Zantedeschia* hybrid "Best Gold" crossed with *Zantedeschia* hybrid "Chromatella" and reciprocal crosses.

The outer-integument continued to grow and produce massive cells which contained large quantities of storage products. Endosperm cells were well developed, they were more isodiametric and contained large quantitative of starch grains and protein bodies.

The outermost layer of endosperm contained few starch grains compared with the central region of endosperm cells. The inner starchy endosperm cells contained numerous large starch grains and it was difficult to obtain unblemished thin sections.

The embryo was well developed. Small starch grains were visible in the cotyledons.

(b) Incompatible crosses:

Zantedeschia hybrid "Best Gold" crossed with *Z.aethiopica* var *childsiana*

The degenerated inner-integument still retained its thick lignified filamentous structure. There were very few changes

in embryo size from the appearance at four weeks after pollination (Plate 21a).

The ovule continued to grow, particularly the outer-integument. The cells of outer-integument contained a large quantity of storage products (starch grains and crystal cells).

In some ovules embryo sac shrank and degenerated to a thick layer of cells around the outer edge (Plate 21 a,b). The endosperm contained few starch grains and these accumulated at the periphery (Plate 21 a,b; 22 a). Heart-shaped and abnormal growing embryos were found at this stage (Plate 22 a,b,c,d). Most of the embryos from this incompatible cross aborted 6 weeks after pollination. Very few embryos continued to grow until 8 weeks after pollination. These were usually abnormal and started to degenerate without differentiation. The embryos contained necrotic tissues which produced lignified material, in the centre many starch grains were also present (Plate 22 b, c, d). Cell division could still can be seen in some embryos (plate 22 b,c). In other embryos starch grains started accumulated at the coleorhiza of the embryo and these embryos lacked any normal differentiation (Plate 23 a,b).

(F) Development Ten to Twelve Weeks After Pollination

(a) Compatible crosses :

Zantedeschia hybrid "Best Gold" selfed

The inner starchy endosperm cells contained numerous

starch grains and it was difficult to obtain unblemished thin sections.

The cotyledon of the embryo had elongated cells and vascular tissue was differentiating (Plate 24 a,b). Raphide containing cells were seen at the base of the cotyledon. The crystal cells (Plate a,c) normally found in these plants first formed 10 weeks after pollination in compatible crosses. Small starch grains filled the entire embryo (Plate 24 a,b,c).

(b) Incompatible crosses:

Zantedeschia hybrid "Chromatella" crossed with *Z.aethiopica* var *childsiana*

The ovule started to shrink well before 10 weeks after pollination due to the collapsed embryo sac (Plate 25 a). Most of the embryos were brown or dead by this time. Those embryos that developed further had not accumulated large starch deposits.

One of these advanced embryos was triangular in shape and about 1.2 mm in length. It showed early embryo differentiation with a primary central cotyledon and proliferating layer with some starch grains building up in the cotyledon region (Plate 25 b,c). Crushed endosperm cells accumulated around the embryo sac which was filled with large starch grains. The outermost 4-5 layers of cells of the endosperm had thick cell walls with small starch grains. The centre of the endosperm contained vacuolate cells (Plate 25 d,e).

DISCUSSION

Incompatible embryo development was generally similar to that of compatible crosses during the first two weeks. However there were some differences in that embryos at incompatible crosses were irregular in shape and starch was present in the epistases. Where embryo development occurred with the incompatible crosses it was slow and did not commence immediately. An abnormally large nucellar cap (epistases) and an embryo proper arrested growth at the preglobular stage of development. The precise time of developmental arrest in the embryo proper could not be exactly determined but it probably occurred 2-3 weeks after fertilization. At this time (2-3 weeks after pollination) embryos from incompatible crosses were at an equivalent stage of development compared with compatible crosses.

In other abortive embryos such as the recessive embryo-lethal mutant of *Arabidopsis thaliana* embryos contained an abnormally large suspensor and growth of the embryo proper was arrested at a preglobular stage of development (Marsden and Meink, 1985). Continued growth of the suspensor during normal development appears to be inhibited by the developing embryo proper.

In this study the significance of the starch granules which accumulated in epistases cells of incompatible crosses during the period of pro-embryo development needs to be considered. The occurrence of starch in the epistases and not

in the adjacent endosperm cells may indicate that carbohydrates were immobilized within the epistases perhaps as a mechanism regulating carbohydrate flow to the embryo proper. In times of high carbohydrate demand the starch could undergo degradation to soluble compounds which could then be translocated to the embryo proper. It is likely that starch was normally utilized by the developing embryo proper and may have simply accumulated when growth of the embryo proper slowed or stopped.

Inner-integument and integumentary tapetum could be clearly identified before anthesis. The inner-integument had more starch grains than the integumentary tapetum. The inner-integument and integumentary tapetum may facilitate the diffusion of soluble substances from vascular tissue which ends in the chalazal region of the integument. Such materials could presumably pass freely through the antipodal region to the lower chamber of the embryo sac. An alternative path would be via the integumentary tapetum cells which are still the only cytoplasm-rich cells near the embryo sac.

During the pre-torpedo stage of development in embryos from compatible crosses the inner-integumentary tapetum showed some signs of degeneration which may release material to the developing sporophyte via the endosperm. Schulz and Jensen (1969), and Marinos (1970) suggested that the entire surface of the embryo sac is capable of absorption, hence solutes possibly pass through the epistases to the embryo until the globular stage. Senescence of the epistases occurred after the

endosperm was already well developed. While in general, attempts to culture early globular stage embryos are unsuccessful (Raghavan, 1976) torpedo stage embryos grow on relative simple media. The epistases tissue may be capable of providing the particular factors necessary for the early stages of embryo differentiation that are difficult to define for in vitro culture.

The incompatible embryos rarely grew much larger than normal pre-torpedo stage embryos of compatible crosses and most of them remained globular. At the later heart stages of embryo development dicotyosomes and endoplasmic reticulum are relatively inactive in hybrid ovules (Sangduen et al., 1983). Failure of nutrient metabolism and transport in all nutritive tissue including the nucellus, suspensor, integumentary tapetum and endosperm may have been responsible for this lack of continued growth.

Protein bodies and starch grains largely accumulated in the endosperm cells four weeks after pollination in compatible crosses. Degeneration of endosperm cells was found in incompatible crosses. The unusually large starch grains and ruptured protein bodies were clearly identified using iodine and acid fuchsin stains. protein bodies are bound by a single membrane (Khoo and Wolf, 1970). Krishnan et al. (1989) suggested that protein bodies are formed by various mechanisms which involve the participation of rough endoplasmic reticulum, vacuoles and dicotyoesomes. The main constituents of protein bodies are

proteins and phyton, in addition, they also contain hydrolase, lectin lipids and carbohydrates. In wheat endosperm the protein bodies are localized in vacuoles and with progressive developm-ent, these bodies occupy most of the expanding endosperm cells (Graham et al., 1962). The abnormal protein bodies observed here in the early stages of embryo development indicated that endosperm cells failed to store the food to supply the growing embryo at later stages.

Total sugar concentration was significant higher in the cob of aborting maize kernels than of non-aborting kernels from 8 days after pollination (Reed and Singletary, 1989). Sugar and sucrose accumulated during the early stage of embryo develop-ment and transfer of sugars from cob to kernels was impaired leading to the abortion. Aborted kernels are able to synthesize starch from sugars.The authors suggested that abortion is not the result of lose of kernel metabolic competency, but rather due to the termination of sugar supply. The unusually large starch grains sparsely arranged in the incompatible crosses of *Zantedeschia* endosperm cells looked like starch grains merged together which were not be absorbed by the embryo. This may disturbed sucrose/starch to nutrition needs of embryo.

The early necrosis at the chalazal end of embryo sac in the *Zantedeschia* incompatible crosses may be responsible for the degeneration of the endosperm tissues. In autoradiographic studies by Coe (1954) on *Zephyraonthes* the chalazal portion of the ovule and vascular tissue is quickly labelled at the stage

of the mature embryo sac while accumulation of the label occurs around the embryo sac. This indicates a preferential transport towards the chalazal end of the embryo sac and the tissue surrounding it.

In models of a possible nutrition pathway in the ovule of Spinach, Wilms (1980) suggested a shift in the pathway during development from young to mature embryo sac and post-fertilization stages. In this model, the chalazal tissue proliferates and the embryo sac does not receive any more nutrition after the young stage before fertilization. After fertilization these nucellus and original chalazal tissues, as well as degeneration products supply nutrients to the new activated embryo sac.

In *Zantedeschia*, the chalaza lies directly between the vascular bundle and the embryo sac and is continuous with the inner and outer integuments, It appears play an important role in sucrose transport to the endosperm. When the chalazal tissue is linglified or crushed, this presumably terminates sucrose flow, and causes the degeneration of endosperm tissue. Development of tannin-filled vacuoles in the chalaza and seed coat of barley were dependent on, and prevented by, early chalazal necrosis in the Seg I mutant (Felker et al., 1984). It was suggested that the basis of the seg I phenotype may be abnormal compartmentalization of tannin causing precipitation of cytoplasmic proteins and early death of chalazal cells.

Retarded growth of the endosperm in developing seeds has

been reported in many plant species (Brink and Cooper, 1940) Several kinds of hereditary alterations of the balance between endosperm, embryo, and maternal tissue can lead to embryo abortion. A physiological imbalance between the endosperm and hybrid embryos may produce substances which cause cessation of endosperm and/or embryo growth. Both embryo and endosperm compete for essential substances which promote growth in *Gossypium* (Weaver, 1957). In a reciprocal cross between *Medicago sativa* (4x) with *M. falcata* (2x) the difference in seed development is due to the rate of physiological activity initiated in the endosperm at the time of fertilization (Ledinghaw, 1940).

Williams and White (1976) concluded that seed abortion from the interspecific cross *Trifolium ambiguum* (4x) with *T. repense* (4x) may be attributed to starvation of the embryo, resulting from the inability of the physiologically disturbed endosperm to absorb nutrients from maternal tissue.

Sangduen et al. (1983) in annual x perennial *Medicago* species crosses concluded that maternal and embryonic tissue of ovules fail to carry out a timely sequence of metabolism involving lipid starch and nucellar cells. Delayed breakdown of starch and lipid in the integumentary tapetum and nucellus is a probable factor in reduced development of the coenocytic endosperm.

Some of these conclusions appear to be valid for *Zantedeschia* within the general context of disturbed development

leading to hybrid failure. The crushed necrotic chalazal tissue probably terminated sugar flow and caused the degeneration of endosperm tissue. Embryo growth was also suppressed or terminated near the globular stage possible because of disturbed endosperm development.

In this study failure of nutrient metabolism and transport in all nutritive tissues including the nucellar cap, chalaza, inner-integument, integumentary tapetum and endosperm of embryo, all related to the abortion of incompatible embryos. Early embryo development in *Zantedeschia* hybrids appeared to be a complex phenomenon requiring a high degree of coordination between anabolism and catabolism in both maternal and embryonic tissues.

CONCLUSION

Embryos development occurred with the incompatible crosses was generally similar to that of compatible crosses during the first two weeks. However there were some differences in that embryos at incompatible crosses were irregular in shape and starch was present in the epistases. It is likely that the epistase starch was normally utilized by the developing embryo proper and may have simply accumulated when growth of the embryo proper slowed or stopped. The inner-integument and integumentary tapetum may facilitate the diffusion of soluble substance from vascular tissue which ends in the chalazal region of the integument. In *Zantedeschia*, the chalaza lies directly between the vascular bundle and the embryo sac is continuous with the inner and outer integuments. It appears to

play an important role in sucrose transport to the endosperm. when the chalazal tissue is linglified or crushed, this presumably terminates sucrose flow, and causes the degeneration of endosperm tissue. The ruptured protein bodies and unusually large starch grains sparsely arranged in the endosperm cells looked like protein bodies and starch grains merged together which were not be absorbed by the embryo. Embryo growth was also suppressed or terminated near the globular stage possibly because of disturbed endosperm development. The failure of nutrient metabolism and transport in all nutritive tissue including epistases, chalaza, inner-integument, integumentary tapetum and endosperm of embryos, all related to the abortion of incompatible embryos.

CHAPTER 5

EMBRYO CULTURE OF *ZANTEDESCHIA* INTERSPECIFIC HYBRIDS

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INTRODUCTION

Previous attempts to incorporate new traits into white calla through interspecific hybridization were unsuccessful (Traub, 1948; Cohen, 1988). Embryo culture techniques have been used by plant breeders to obtain interspecific crosses of many plants (Pandy et al., 1987; Neal et al., 1983; Williams, 1987; Pattee et al., 1988; Phillips et al., 1982). Embryo abortion occurs as either a pre- or post-fertilization event in interspecific crosses, but in *Zantedeschia* post-zygotic abortion was most common (Chapter 4)).

in vitro culture of globular and early stage embryos has proven to be difficult since these heterotrophic stages have complex nutritional and hormonal requirement (Neal, 1983). The exact requirement have not be defined for most species. An analysis of seed development in *Pisum sativm* (Cook, 1988) demonstrated that increasing the osmotic pressure favours the growth of immature pea embryos. High osmotic pressure is frequently important in delaying precocious germination (Neal, 1983; Cook, 1988). One simple technique which allows the production of immature embryos was developed by Freyssiwet and Freyssiwet (1988). They used a higher concentration of sucrose (90 gl^{-1}) added to M.S. medium (Murshige and Skoog, 1960) with 0.1 mgl^{-1} BA for enlarged zygotic embryos of sunflowers which were cultured for two weeks in a dark environment then transferred to a lower sucrose (30 gl^{-1}) medium to produce shoots and roots. They claimed plantlet production from

immature embryos of fertile plants in 4 months.

Most of the *Zantedeschia* embryos that from incompatible crosses remained in a globular stage (Chapter 4). Osmotic pressure is obviously an important factor for growth at the immature embryo (Neal, 1983; Cook, 1988; Freyssiwet and Freyssiwet, 1988). This study aimed to rescue embryos from incompatible crosses by evaluating levels of sucrose supplemented in the culture media. It was hypothesized that if higher osmotic pressure favoured immature embryo growth *in vitro* then the system could be adapted to the culture of embryos from the incompatible crosses.

MATERIALS AND METHODS:

Ovaries of all treatments (Table 13) were harvested as above (Chapter 3; Materials and Methods). Embryos of compatible and incompatible crosses (Table 13) were excised on the same day the ovaries were removed from field grown plants. The ovaries were removed 6 weeks after pollination and thereafter every 7 days until 10 weeks after pollination. Ovaries from self-pollinated and compatible crosses were used as controls. In interspecific incompatible crosses (Table 13), the few self-fertilized ovules were easily identified by their advanced embryo development and presence of normal endosperm. These were omitted from further experimentation and analysis. The growth of embryos from incompatible crosses was disturbed and the endosperm failed to develop.

Embryo excision was performed under sterile conditions. Ovaries were surface-sterilized for 15 minutes in a 5% (w/v) sodium hypochlorite solution followed by rinses (3 x; 5 min) in sterilized distilled water. Ovules were placed on the bottom of a petri dish and embryos were carefully dissected using a stereo microscope at 16x or 25x magnification. Individual embryos were transferred to dishes containing sterilized growth media (Table 16). Four to Five dissected embryos were placed on each plate and plate were sealed with strips of plastic film. Each treatment included 4-5 embryos placed in a petri dish and there were two replicates. Embryos were cultured in the DSIR, Fruit and Trees division tissue culture room (Palmerston North), which was maintained at 26=1 C with 16 hour light duration at 30 µmoles m^2sec^1.

Dissected embryos were observed every week for 10 weeks, length of embryos and relative shoot, root, and haustorium tissue formation were recorded every two days for the first two weeks and then every week. The haustorium developed from the cells at the tip of the cotyledon.

Table 16 *Zantedeschia* embryo culture media

Murashige and Skoog medium (1960)	1 liter Standard medium
Sucrose:	30 gl^{-1}--------Treatment 1 60 gl^{-1}--------Treatment 2 90 gl^{-1}--------Treatment 3
Benzyladenine (BA):	0.0 mgl^{-1}------Basal 0.1 mgl^{-1}----- Initiation 3.0 mgl^{-1}- Multiplication
Davis Agar	7 gl^{-1}
Final pH	5.8

RESULTS

Normal *Zantedeschia* embryos from selfed or compatible crosses were easily to grow, even in a basal MS medium without any hormones when excised 6-7 weeks after pollination. Most of them produced haustorium tissue when culture in the MS medium. The size of the haustorium varied depending on the species. For example: selfed "Chromatella" produced a great amount of haustorium tissue (Plate 27 b,c,d) compared with selfed "Best Gold" (Plate 28 a,b).

In the incompatible crosses (Table 13), fertilization occurred and the seed began to develop but aborted before maturation (Chapter 4). Ovules found generally shrunken. Only a few embryos from incompatible crosses survived and enlarged for up to 10 weeks *in situ*. Most of the embryos remained in the globular stage, and only a few of these were successfully cultured.

From several hundred dissected ovules only about 100 embryos larger than 0.2 mm were found of these only about 20 embryos showed any growth in culture. Most embryos were abnormal or died and did not produce any shoots or roots. Transferring surviving embryos to higher concentration of BA (3 mgl^{-1}), resulted in only three which produced large amounts of haustorium tissue, shoots and/or roots (No 37,43,47) but all tissues were albinos (Plate 26 a-h). Many haustorium buds were produced when haustorium tissue was dissected and transferred

to a higher concentration of BA (3 mgl^{-1}).

Table 17 Development of embryos *in vitro* from the *Zantedeschia* hybrid "Best Gold" following growth on various treatment media for 7 weeks.

Treatment	No	Length (mm)	No. of embryos with Leaves	Roots	Haustorium	Weight (gram)
1	20	1.8-2.2	19	19	20	0.35
2	20	1.8-2.2	15	15	20	0.41
3	20	1.8-2.2	3	3	15	0.12

Table 18 Development of embryos *in vitro* from the *Zantedeschia* hybrid "Best Gold" X *Zantedeschia* hybrid "Chromatella" following growth on various treatment media for 7 weeks.

Treatment	No	Length (mm)	No. of embryos with Leaves	Roots	Haustorium	Weight (gram)
1	15	1.8-2.1	12	12	15	0.49
2	15	1.8-2.1	11	11	15	0.33
3	15	1.8-2.1	2	2	12	0.087

Table 19 Development of embryos *in vitro* from the *Zantedeschia* hybrid "Best Gold" X *Zantedeschia* hybrid "Pink" following growth on various treatment media for 7 weeks

Treatment	No	Length (mm)	No. of embryos with Leaves	Roots	Haustorium	Weight (gram)
1	10	1.8-2.0	9	9	10	0.27
2	8	1.8-2.0	8	8	8	0.39
3	8	1.8-2.0	6	6	8	0.21

Table 20 Development of embryos *in vitro* from the *Zantedeschia* hybrid "Chromatella" X *Zantedeschia* hybrid "Best Gold" fulling growth on various treatment media for 7 weeks.

Treatment	No	Length (mm)	No. of embryos with Leaves	Roots	Haustorium	Weight (gram)
1	13	1.8-2.2	9	10	13	0.53
2	13	1.8-2.2	6	12	13	0.27
3	13	1.8-2.2	9	11	13	0.22

Note:

Length: The initial embryo length.
Haustorium: It is developed from the cells at the tip of the cotyledon that produce callus and sometimes produce buds. Refer to scutellum of grasses (Hodman and Robbins, 1955), Haustorium is a structure which lies in contact with the endosperm and digests and absorbs food from the endosperm which it passes on to the growing parts of the embryo.

Embryos from all crosses grew better with low concentrations of sucrose (30 gl^{-1}) (Tables 17, 18, 19, 20). At the low concentration of sucrose most of the embryos produced shoots and roots and haustorium tissue within one month culture (Tables 17, 18, 19, 20) (Plates 27 a, b, c, d; 28 a, b, c). The different crosses had different responses to higher concentrations of sucrose. The *Zantedeschia* hybrid "Best Gold" was more sensitive to higher concentration of sucrose than embryos of Chromatella (Tables 17, 20). At the high concentration of sucrose (90 gl^{-1}),most of the cultured embryos had delayed growth and did not develop any chlorophyll. Especially in the embryo culture of selfed *Zantedeschia* hybrid "Best Gold" and *Zantedeschia* hybrid "Best Gold" x *Zantedeschia* hybrid "Chromatella", shoot and root growth was delayed and most embryos grew very weakly (Table 17,18). The embryo ceased to grow after 2-3 weeks culture and most did not produce any shoots or roots.

Incompatible cross:

Table 21 *Zantedeschia* hybrid "Chromatella" X *Z.aethiopica* var *childsiana*. Development at embryos in culture.

Treatment	No	Length (mm)	No of embryos with Leaves	Roots	Necrosis	Growth
						(6 weeks)
1	10	0.2	0	0	2	2
2	12	0.2	0	0	1	0
3	15	0.2	0	0	3	1
						(7 weeks)
1	4	0.2-0.4	0	0	0	0
2	4	0.3-1.4	0	0	0	4
3	4	0.2-0.8	0	0	2	2
						(8 weeks)
1	10	0.2-0.8	0	0	2	1
2	14	0.2-2.5	2	1	0	6
3	10	0.2-0.6	0	0	0	3
						(10 weeks)
1	10	0.6-2.0	3	0	1	4
2	10	0.2-1.3	0	0	2	2
3	6	0.2-1.3	0	0	1	1

Table 22 *Zantedeschia* hybrid "Best Gold" X *Z.aethiopica* var *chidsiana* Development at embryos in culture.

Treatment	No	Length (mm)	No of embryos with Leaves	Roots	Necrosis	Growth
						(6 weeks)
1	7	0.15	0	0	2	1
2	8	0.15	0	0	1	2
3	8	0.15	0	0	1	1
						(8 weeks)
1	4	2.5-3.0	3	3	0	4
2	4	2.5-3.0	2	2	0	4
3	1	0.8	0	0	0	1

Note

Necrosis: Browning, dead embryo;
growth: Embryos had advanced growth;

All embryos from incompatible crosses which were dissected 6 weeks after pollination were small (0.15-0.2mm). They were all globular. The different concentrations of sucrose had no effect on embryo growth (Table 21,22). Most of the embryos that had further growth were abnormal (Plate 26a,b,c). Three large embryos (No 37,43 and 47 were more then 0.7mm in length) eventually (2-3 months after culture) these embryos produced haustorium tissue, shoots and roots (Plate 26 d,f) but all

these tissues were albino (Plate 26 g,h). All of these three embryos came from "Chromatella" crosses with *Z.aethiopica* var *childsiana* (Table 21, No 37 from 8 weeks after pollination, treatment 2 and No 43,47 from 10 weeks after pollination, treatment 1). Dissected the haustorium tissue and transfer to the higher concentration of BA (3 mgl^{-1}) eventually produced vegetative buds (Plate 26e). Some of the embryos dissected reached the torpedo stage (length 2.0-3.0mm) (Table 21,22). These embryos turned green and produced shoots and roots. The normal growth of these embryos was possibly due to their being selfed.

DISCUSSION

Hybrid embryos larger than the "pre-torpedo" stage were rare in all incompatible crosses between *Z. aethiopica* var *childsiana* and *Zantedeschia* var *chromatella* or *Zantedeschia* hybrid "Best Gold". The invariably abnormal differentiation was possibly as a result of abnormal patterns of nutrient transfer in the absence of an endosperm (William & White, 1976, 1977).

Increasing sucrose concentration to 60 gl^{-1} and 90 gl^{-1}, did not increase immature embryo size. All immature embryos below 0.3 mm in length remained in the globular stage and no enlargement was seen with any treatment. Only a few embryos, which were originally greater than 0.5 mm long, increased in size after transfer to the culture medium. This indicated that the larger embryos were perhaps beyond the globular stage and

could respond to the culture medium. Three embryos that eventually produced shoots and roots were from originally large embryos (0.7-1.1 mm), This indicated that the original size of embryos was an important factor in embryo rescue. All of the three embryos came from the crosses of "Chromatella" x *Z.aethiopica* var *childsiana* rather then "Best Gold" x *Z.aethiopica* var *childsiana*. Dissection of embryos from the cross of "Best Gold" x *Z.aethiopica* var *childsiana* 10 weeks after pollination indicated all embryos were brown and dead none survived. This was possibly related to the early collapse of the endosperm which starved the embryo and led to the death of the embryo (Plate 18a).

Earlier embryogenesis studies (Chapter 4) indicate the differentiation of embryos from incompatible crosses is normally detectable 9-10 weeks after pollination. Large embryos were rare in experiment here and these appeared to be abnormal. Small abnormal embryos (about 1.5-2 mm long) had the potential to be rescued by embryo culture. The other small embryos (0.2-0.5 mm) were difficult to rescue. These embryos may require maintenance of the gradient of nutrients and other growth factors by the parent tissue for proper polarized differentiation. All the dissected embryos were necrotic and dead 12 weeks after pollination.

From this embryogenesis study it was suggested that the shrunken endosperm caused embryo starvation and death. The best time to rescue embryos from incompatible crosses was 8-10 weeks

after pollination. Embryos from earlier stages (6 week after pollination) were too small (0.2-0.3 mm) and remained in the globular stage. These embryos rarely survived in culture. Embryos removed later (10-12 weeks after pollination), were necrotic and dead. Hundreds of shoots proliferated from the three surviving embryos from incompatible crosses but all of them were albinos. None transferred to the soil. Some roots were produced but all of these roots growth weakly. Shoots could only be maintained in higher concentrations of BA (1-3 mgl^{-1}). When the concentration of BA was reduced (0.1-0.3 mgl^{-1}) the shoots turned pale and shrank.

Albino seedings with chlorophyll deficiencies have been observed in *Zantedeschia* interspecific crosses between *Z.albomaculata* x *Z.rehmannii* and *Z.albomaculata* x *Z.rehmannii* (Shibuya, 1956; New, 1967). In general albino *Zantedeschia* ~~albino~~ plants occurred in one cross~~es~~ whereas reciprocal crosses usually produced normal green plants (New, 1967). The hybrids between *Z.albomaculata* and *Z.rehmannii* are a good example of such unilateral abnormality. When *Z.albomaculata* was used as a female plant the resulting progeny contained various chlorophyll deficiencies. In the reciprocal crosses, hybrid plants were green with some chimaeras (New, 1967). Albino seedings have also been observed in interspecific crosses between a number of annual and perennial *Trifolium* species (Pendey et al., 1987; Przywara et al., 1989). In examining the interspecific hybridization of *Trifolium repens* with *T. hybridum* it was found that plastid development of leaf tissue

corresponded with increased greening of tissue. Mature chloroplast with developed internal membrane structure were restricted to those cells corresponding to the small green sectors of albino leaves. The same structure of plastids was also found in the albino leaves of *Zantedeschia* hybrids *Z.aethiopica* var *childsiana* (M) x *Z.albomaculata* var *chromatella* (F) small groups of plastid granules were aggregated beside the cell membrane, when examined under an electron microscope (Pers. comm. D. Cohen, 1990). At present the reasons which caused the shoot albino still unclear but could be related to unmatched (metacentric to submetacentric) chromosome pairing.

CONCLUSION

The invariably abnormaL of *Zantedeschia* incompatible crosses were possibly a result of abnormal patterns of nutrient transfer in the absence of an endosperm. Increasing sucrose concentration to 60 gl^{-1} or 90 gl^{-1}, did not increase immature embryo size. The original incompatible crosses embryos size was an important factor in embryo rescue and the best time to rescue embryos was 8-10 weeks after pollination.Embryos from earlier stages (6 weeks after pollination) were too small and rarely survived in culture. Embryos removed later (10-12 weeks), were necrotic and dead. Surviving embryo from incompatible crosses were albino. In the albino leaves small group of plastid granules were aggregated beside the cell membrane. The reasons to causing the shoot albino still unclear but could be related to unmatched chromosome pairing.

Explanation of plate 1-28

Note: All light microscope photos were measured (Bar=100 μm) excepts plate 4 and 5 (Bar=1 cm).

Plate 1: SEM of *Zantedeschia* pollen grains. (a,c bar=40 μm. b,d bar=10 μm)
a,b: *Z.aethiopica* var *childsiana*, c,d: *Zantedeschia* hybrid "Best Gold".

Plate 2: TEM of *Zantedeschia* pollen wall and nucleus.
a: x 1950 BG, Showed pollen starch and vegetative nucleus. b: x 18,000 BG, Showed pollen wall (Exine and Intine). c: x 2890 Chi, Showed pollen starch, vegetative nucleus and sperm nucleus. d: x 18,000 Chi, Showed pollen wall (Exine and Intine).

Plate 3: *Zantedeschia* pollen elongation. (Bar=100 μm)
a: Longitudinal sections of ovary. b: Pollen lay on stigma. c: Pollen tube elongation (same magnification with 3b).

Plate 4: Development of ovaries from different crosses of *Zantedeschia* 10 weeks after pollination.(Bar=1 cm)
a: BG selfed. b: BG x Chr. c: BG x Chi. d: BG unpollened. e: Chr selfed. f: Chr x BG (M). g: Chr x Chi. h: Chr unpollened.

Plate 5: Development of ovaries from different crosses of *Zantedeschia*. (Bar=1 cm)
a: The different crosses ovule development from 2, 4, 6, 8, 10 weeks after pollination. a1: BG x Chi. a2: BG selfed. a3: BG x Chr. a4 BG x Pink. a5: Chr x Chi a6: Chr x BG. a7: Chr selfed. b: Top, Chr selfed 10 weeks after pollination. Bottom, Chr x Chi 10 weeks after pollination. c: Chr x Chi 10 weeks after pollination (The necrosis of embryo sac).

Plate 6: Light microscopy of longitudinal sections through ovules of *Zantedeschia* hybrid "Best Gold".(Bar=100 μm) Showed outer-integument, inner-integument, integumentary tapetum (endothelium), epistase, micropyle, chalazal and funiculus.

Plate 7: Light microscopy of longitudinal sections through megagametrophyte of *Zantedeschia* hybrid "Best Gold".(Bar=100 μm)
Showed epistase, synergid cells, egg cell, secondary nucleus and antipodal cells.

Plate 8: Light microscopy of longitudinal sections through ovules of selfed BG 2 weeks after pollination.

(Bar=100 μm)
a: Showed distinguished chalazal chamber and micropyle chamber. Epistase and inner integument started to degenerated. b: Chalazal chamber. c: Showed embryo and epistase ,suspensor is probably the vacuolated cell between the embryo and epistase (arrow).

Plate 9: Light microscopy of longitudinal sections through ovules of BG x Chr 2 weeks after pollination. (Bar=100 μm)
a: Showed globular stage of embryo, chalazal chamber, micropyle chamber and remained epistase and inner integument. b: Chalazal chamber. c: Embryo and epistase.

Plate 10: Light microscopy of longitudinal sections through ovules of BG x Chi 2 weeks after pollination.
(bar=100 μm)
a: Showed remained epistase and inner-integument. b: Showed globular embryo and epistase, the epistase had thick cell wall. c: Chalazal chamber showed remained big nucleus.

Plate 11: Light microscopy of longitudinal sections through ovules of selfed BG 2 weeks after pollination using different stains. (Bar=100 μm)
a: Ovule section stained with Toluidine Blue O and Acid Fuchsin. b: Degenerated inner integument stained with Acid Fuchsia. c: Degenerated inner-integument stained with Iodine showed small starch grains. d: Embryo and epistase stained with Acid Fuchsin. e: Embryo and epistase stained with Iodine showed starch grains remained in epistase.

Plate 12: Light microscopy of longitudinal sections through ovules of BG x Chi 2 weeks after pollination using different stains.(Bar=100 μm)
a: Stained with Toluidine Blue O show remained integumentary tapetum, inner integument and epistase. b: Embryo stained with Iodine the epistase showed the starch grains that could not be found in plate 11e. c: Embryo and epistase stained with Acid Fuchsin showed thick cell wall (arrow). d: Integument tapetum and inner integument stained with Toluidine Blue O and Iodine showed the starch grains and cuticle developed between inner-integument and outer-integument. e: Integumentary tapetum and inner integument stained with Toluidine Blue O and Acid Fuchsin.

Plate 13: Comparison of the ovule development of Chr x BG and Chr x Chi 4 weeks after pollination with different stains. (Bar=100 μm)

a: Longitudinal sections through ovules of Chr x BG 4 weeks after pollination stained with Toluidine Blue O and Acid Fuchsin showed well developed embryo and endosperm. The layer of small no vacuolated cells in endosperm periphery (arrow) were densely stained, b: Longitudinal sections through ovules of Chr x Chi 4 weeks after pollination stained with Toluidine Blue O and Acid Fuchsin showed slowly development embryo and degenerated endosperm and chalazal end tissue. The epistase still remained, chalazal end and inner integument was lignified (showed green colour when stained with Toluidine Blue O). The layer cells in endosperm periphery are not well stained. c: Embryo of Chr x Chi 4 weeks after pollination stained with Toluidine Blue O and Acid Fuchsin showed vacuoles in the embryo and the embryo had no differentiation. d: Embryo of Chr x BG 4 weeks after pollination stained with Toluidine Blue O showed central cotyledon and the embryo started to differentiation.

Plate 14: Comparison of the endosperm and inner integument development of Chr x BG and Chr x Chi 4 weeks after pollination using different stains. (Bar=100 µm)
a: Endosperm and inner integument of Chr x BG 4 weeks after pollination stained with Toluidine Blue O and Acid Fuchsin showed well developed endosperm cells and protein bodies. The aleurone like cells produced on the outermost endosperm cells and two layer of cuticle developed, one of cuticle produced between inner-integument and endosperm another was produced between inner integument and outer-integument. b: Endosperm and inner-integument of Chr x BG 4 weeks after pollination stained with Toluidine Blue O and Acid Fuchsin and Iodine showed Protein bodies and starch grains. The aleurone like cells have not contained starch grains. c: Endosperm and inner integument of Chr x Chi 4 weeks after pollination stained with Toluidine Blue O and Acid Fuchsin showed degenerated endosperm cells. The big cells and thick cell walls and abnormal protein bodies that produced at the outermost endosperm was very distinguished different when compared with compatible crosses chr x BG (a,b). They had not produced aleurone like cells layer. d: Endosperm and inner-integument of Chr x Chi 4 weeks after pollination stained with Toluidine Blue O, Acid Fuchsin and Iodine showed big starch grains and degenerated inner-integument. 14a,b showed dense cytoplasm, smaller cells and thin cell walls compared with 14c,d.

Plate 15: Light microscopy of longitudinal sections through ovules of Chr x Chi 4 weeks after pollination using different

stains. (Bar=100 µm)
a: Inner integument stained with Sudan Black B (It is particular to stain cuticle) showed degenerated inner-integument and cuticle (Black in colour). b: Ovule sections stained with Toluidine Blue O and Iodine showed degenerated chalazal end and inner-integument Starch grains aggregated at the outer-layer of ovule and outer-layer of endosperm. Embryo had not contained starch grains c: Ovule sections stained with Sudan Black B showed two layers of cuticle surrounded embryo sac and degenerated chalazal end and inner integument.

Plate 16: Light microscopy of longitudinal sections through ovules of selfed Chr 6 weeks after pollination using different stains.(Bar=100 µm)
a: Stained with Toluidine Blue O showed well development embryo and endosperm. The outermost endosperm showed aleurone like cells. b: Stained with Toluidine Blue O showed leaf and central cotyledon. c: Embryo stained with Iodine showed small starch grains development in the embryo.

Plate 17: Comparison of the endosperm and inner integument development of Chr x Chi and Selfed Chr 6 weeks after pollination with different stains. (Bar=100 µm)
a, b, c, Chr x Chi endosperm and inner integument sections. a: stained with Toluidine Blue O showed degenerated inner integument and the outermost layers of the endosperm had thick cell wall (arrow). b: Stained with Toluidine Blue O and Acid Fuchsin showed abnormal protein bodies and thick endosperm cell wall. c: Stained with Toluidine Blue O, Acid Fuchsin and Iodine showed big starch grains in the endosperm cells and small starch grains in the remained inner-integument. d, e, f, Sections through endosperm and inner-integument of Chr selfed 6 weeks after pollination. d: Stained with Toluidine Blue O showed well development endosperm cells with the outermost layers of the endosperm had thick cell wall and densely stained . e: Stained with Toluidine Blue O and Acid Fuchsin showed abandoned protein bodies. The most outer- layer endosperm cells was densely stained with Acid Fuchsin. f: Stained with Toluidine Blue O, Acid Fuchsin and Iodine showed density starch grains in endosperm. The aleurone like cells did not contained starch grains.

Plate 18: Longitudinal sections through ovules from incompatible crosses of BG x Chi (a,b,c) and Chr x Chi (d), 6 weeks after pollination with different stains.(Bar=100 µm)
a: BG x Chi stained with Toluidine Blue O showed degenerated endosperm cells. The callus-like cells

aggregated on the embryo area, the individual cells with thickened walls appear to be going into division (arrow). The others endosperm cells were vacuolated. b: Endosperm cells (Showed with an arrow mark at plate 18a) stained with Toluidine Blue O, Acid Fuchsin and Iodine showed dig starch grains. c: Endosperm cells stained with Toluidine Blue O and Acid Fuchsin showed abnormal protein bodies and thick cell wall with vacuoles (arrow). d: ovules of Chr x Chi 6 weeks after pollination stained with Toluidine Blue O showed slowly growth of embryo. The outer layers of the endosperm look like a compatible crosses endosperm 13a.

Plate 19: Comparison of the embryo development from incompatible crosses of Chr x Chi and BG x Chi 6 weeks after pollination with different stains. (Bar=100 µm)
a, b, c, Embryo from sections Chr x Chi 6 weeks after pollination. a: Stained with Toluidine Blue O showed big nuclei. b: Stained with Acid Fuchsin showed big nuclei. c: Stained with Iodine showed no starch grains in embryo. d. e, f, Embryo sections from BG x Chi 6 weeks after pollination. d: Stained with Toluidine Blue O showed big nuclei. e: Stained with Acid Fuchsian showed big nuclei. f: Stained with Iodine showed big starch grains in embryo (The vacuole cells that present at plate 19d,e (arrow) was starch grains).

Plate 20: Embryo development of Chr x BG and selfed Chr 4-6 weeks after pollination. (Bar=100 µm)
a: Embryo from Chr x BG 4 weeks after pollination showed central cotyledon and plumule. b: Embryo from Chr x BG 6 weeks after pollination showed 1st and 2nd leaves. c: Embryo from Chr selfed 6 weeks after pollination showed the radical is forming and leaf and piliferous layer was appeared. The root cap has not yet developed.

Plate 21: Embryo sac development of BG x Chi 8 weeks after pollination with different stains. (Bar=100 µm)
a,b,d: Stained with Toluidine Blue O, Acid Fuchsin and Iodine. c Stained with Toluidine Blue O and Acid Fuchsin. a: Sectioned embryo sac showed degenerated endosperm cells, most of the endosperm cells aggregated at the outer layer of the embryo sac and embryo still remained in globular stage. b: Endosperm cells showed thick cell wall and starch grains. c: showed big nuclei. d Showed starch grains after stained with Iodin (The vacuole cells that presented at plate 21c (arrow) was starch grain).

Plate 22: Ovule development of BG x Chi 8 weeks after pollination with different stains. (Bar=100 µm)
a:Ovules sections stained with Toluidine Blue O, Acid

fuchsin and Iodine showed necrosis embryo and degenerated endosperm. b; embryo stained with Acid Fuchsin and Toluidine Blue O showed the necroses in the embryo and cell division. c: Embryo stained with Toluidine Blue O showed necrosis of embryo and cell division. d: Embryo stained with Toluidine Blue O, Acid Fuchsin and Iodin showed starch grains in the embryo. e: Endosperm cells (Plate 22a arrow) stained with Toluidine Blue O, Acid Fuchsin and Iodine showed big starch grains and abnormal protein bodies.

Plate 23: Embryo development of Chr x Chi 8 weeks after pollination with different stains. (Bar=100 µm)
a; Stained with Acid Fuchsin showed abnormal embryo. b: Stained with Toluidine Blue O, Acid Fuchsin and Iodine showed starch grains accumulated at the base of the embryo (coleorhiza). Epistase still remained and the embryo lack of differentiation it almost same size as 13a (4 weeks).

Plate 24: Embryo development of BG x Chr 10 weeks after pollination with different stains. (Bar=100 µm)
a,b,c, Stained with Toluidine Blue O, Acid Fuchsin and Iodine. a: Showed whole embryo the actual size is 2.6 mm, with crystal cells produced at the basal of the embryo. The embryo have well development vascular tissue and the small starch grains is appeared in the embryo. The central cotyledon started to differentiation. b: Central cotyledon cells and development vascular tissue. c: Crystal cell and starch grains, the crystal cells is that are normally found in plant and that they first form within 10 weeks of compatible crosses.

Plate 25: Ovule development of Chr x Chi 10 weeks after pollination with different stains. (Bar=100 µm)
a: Stained with Toluidine Blue O,Acid Fuchsin and Iodine showed crashed endosperm cells and abnormal embryo. The endosperm cells aggregated on the outer layer of embryo sac, within the central embryo sac the cells were vacuolated. b: Embryo stained with Toluidine Blue O, Acid Fuchsin and Iodine showed abnormal embryo it showed central cotyledon like structure and starch grains appeared. c: Embryo stained with Acid Fuchsin. d: most outer-layer of endosperm cells stained with Toluidine Blue O, Acid Fuchsin and Iodine showed thick cell wall and contained with small starch grains (arrow). Abnormal protein bodies and big starch grains appeared at the inner layers of endosperm. e: most outer- layer of endosperm cells stained with Acid Fuchsin showed thick cell wall (arrow).

Plate 26: Embryo culture from ncompatible : Chr x Chi.

a,b,c, Abnormal embryo. d: Albino embryo with shoot development. e: Development albino haustorium shoot buds. f: Albino embryo development with cotyledon, haustorium and small root. g: Albino shoot development. h: Albino shoot multiple in vitro.

Plate 27: Embryo culture from compatible crosses (selfed Chr). a: Shoots produced from haustorium. b: Haustorium. c:Haustorium buds. d: Haustorium shoot buds.

Plate 28: Embryo culture from compatible crosses : Chr x BG. a,b: Embryo produced haustorium, cotyledon and leaves. c: One month after transplant to greenhouse.

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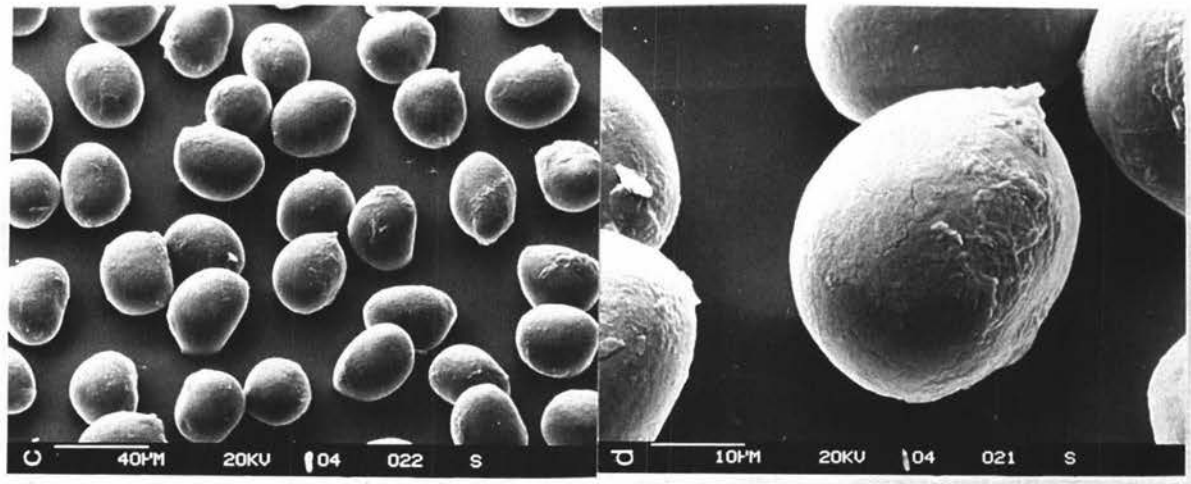
Zantedeschia "Best Gold"



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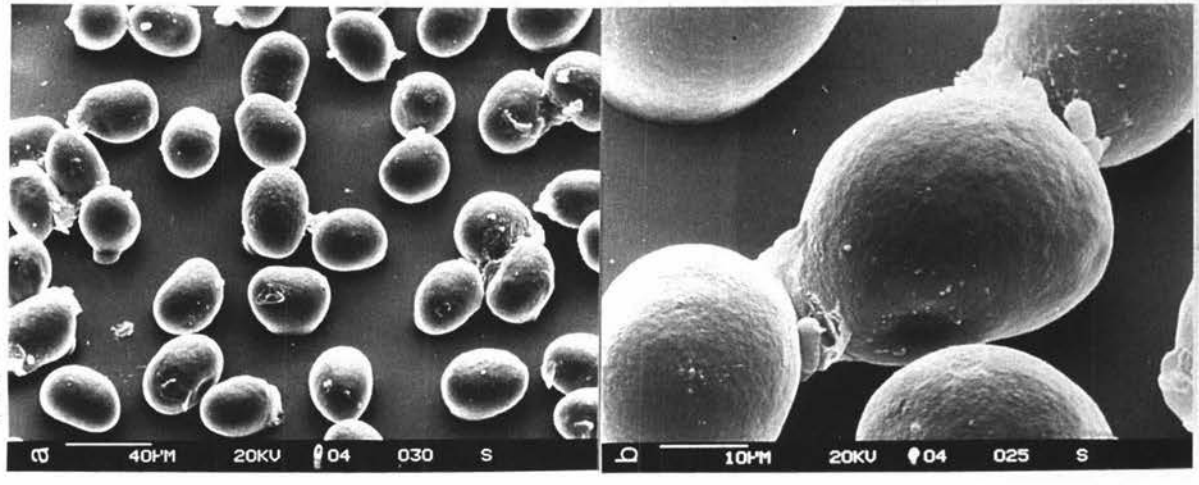
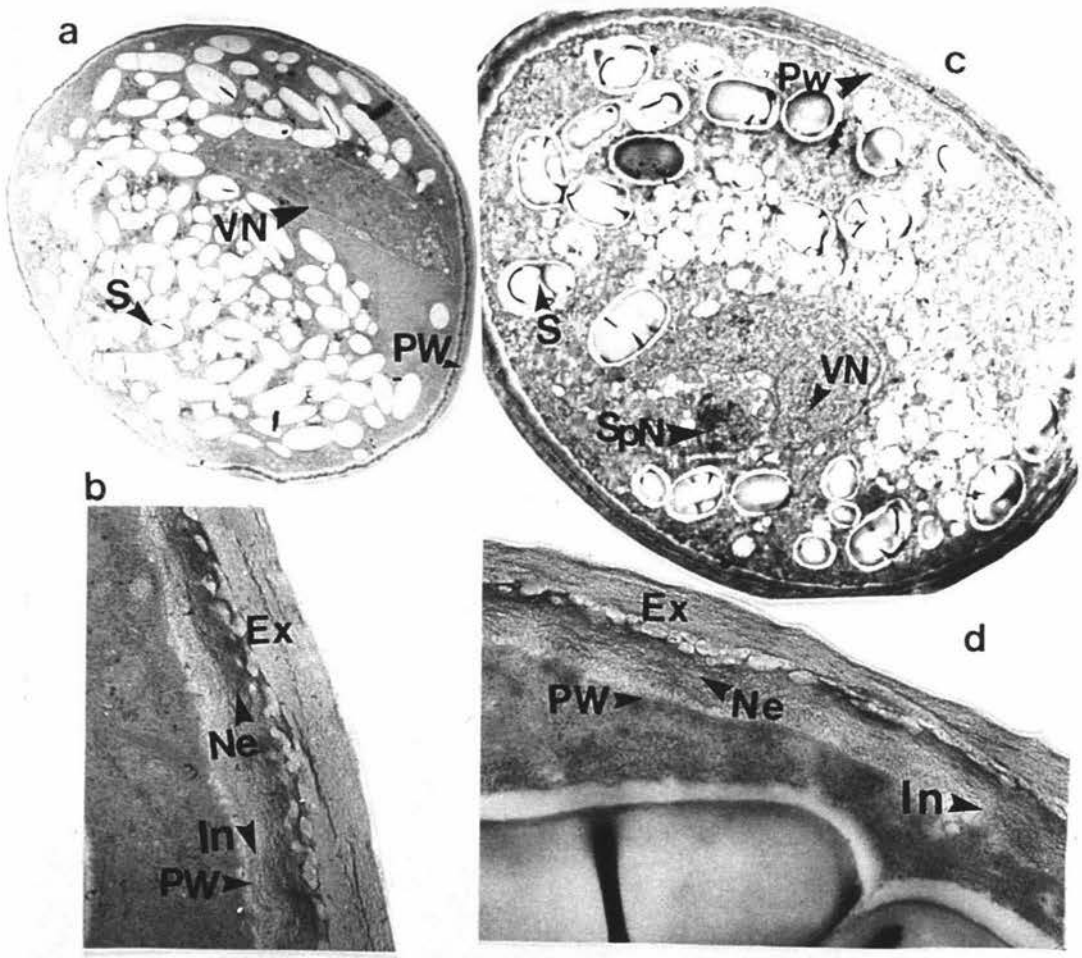


Plate 1

Plate 2



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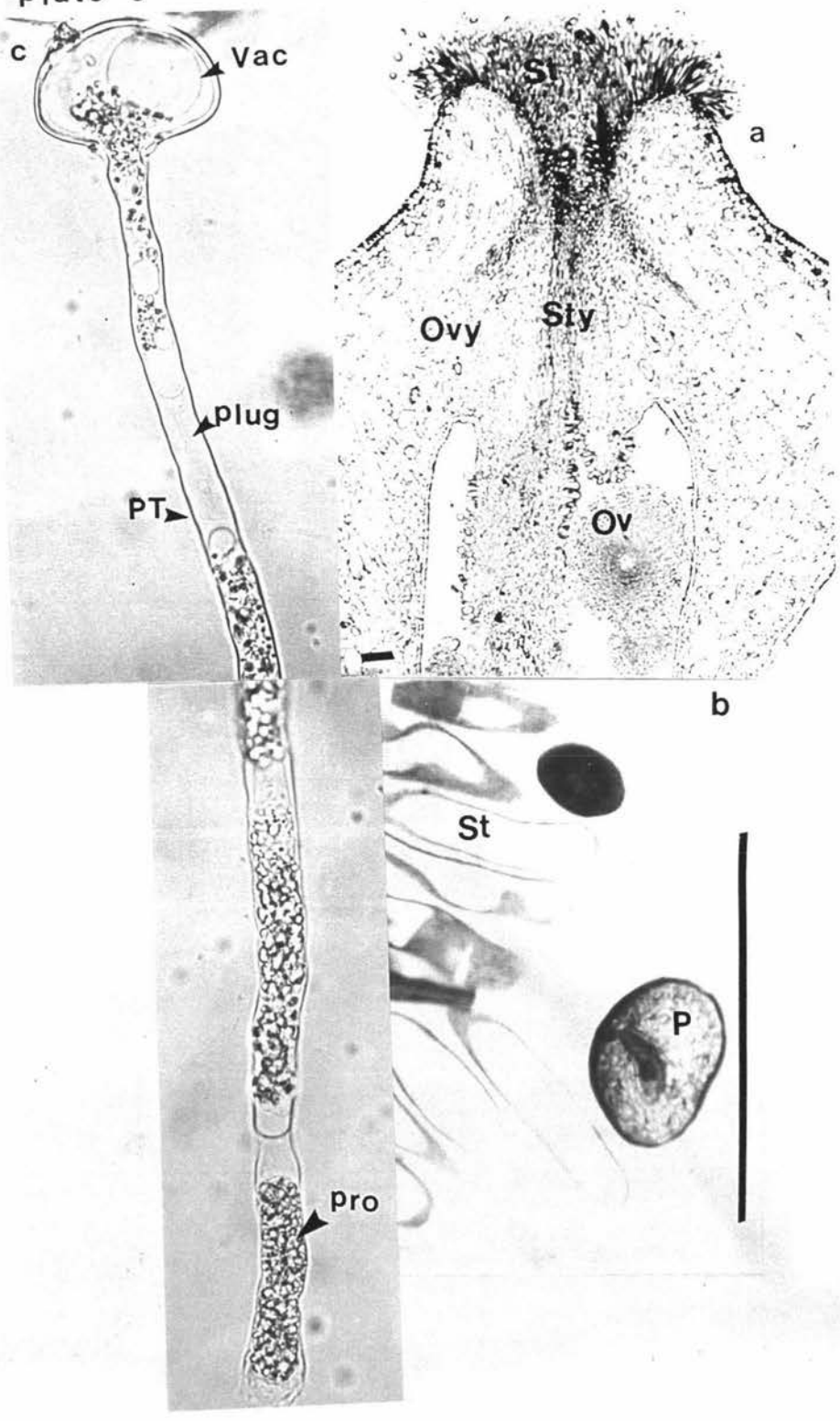

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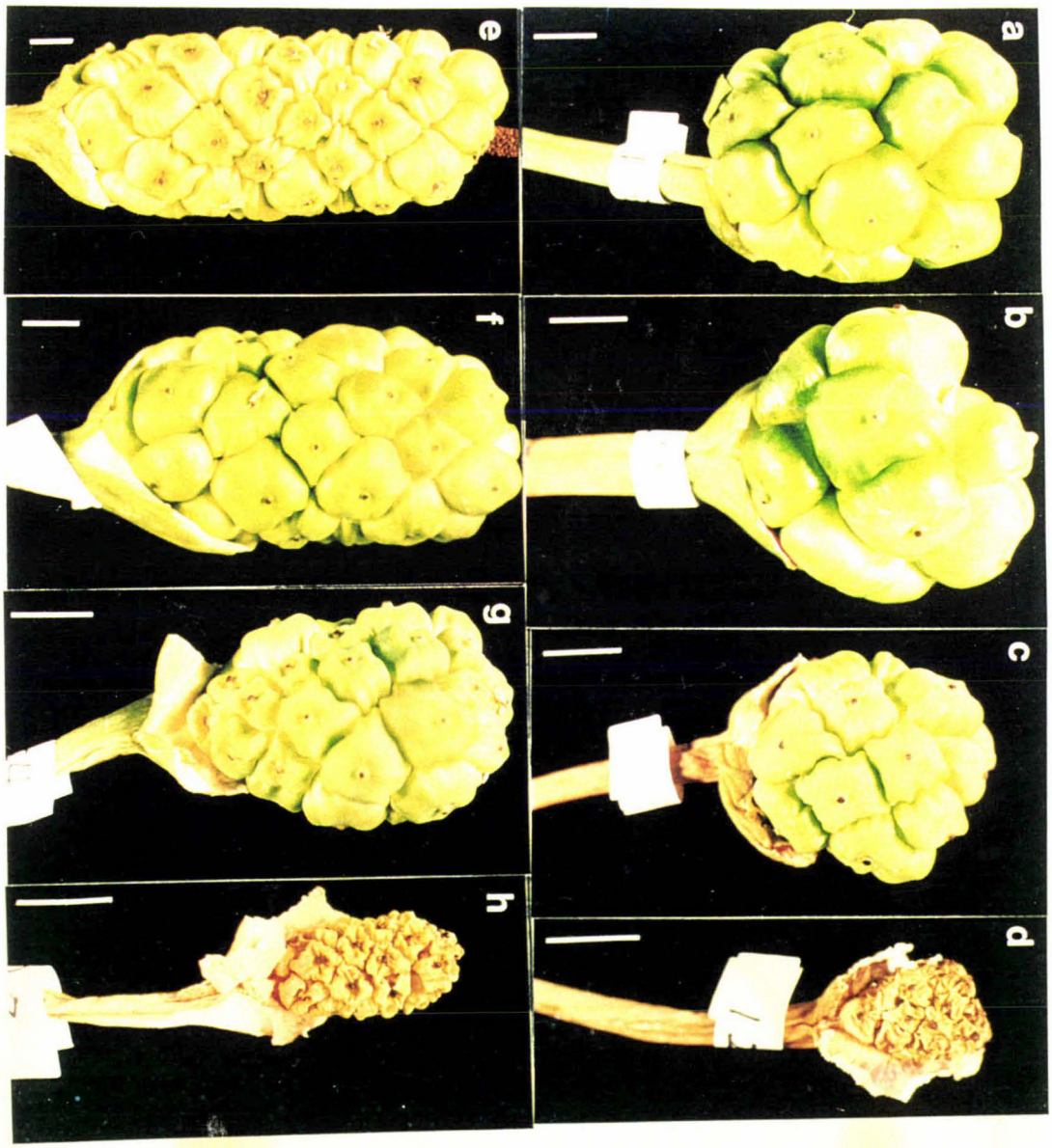

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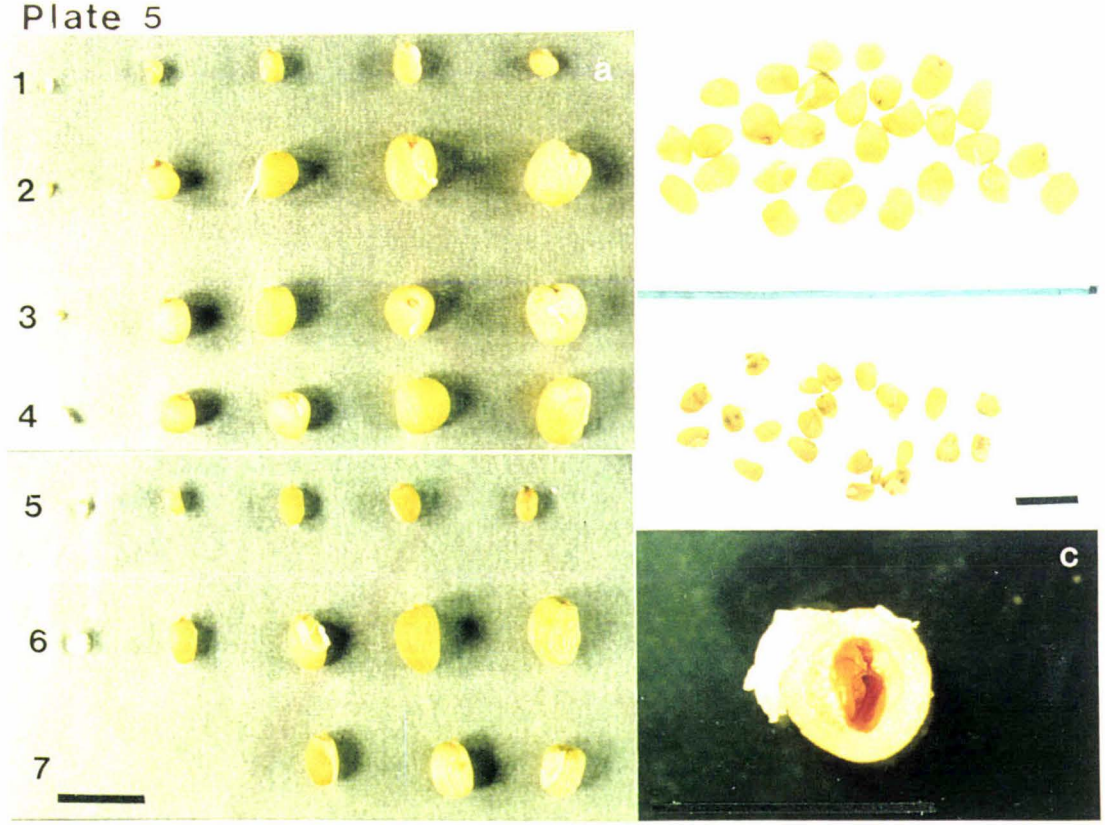

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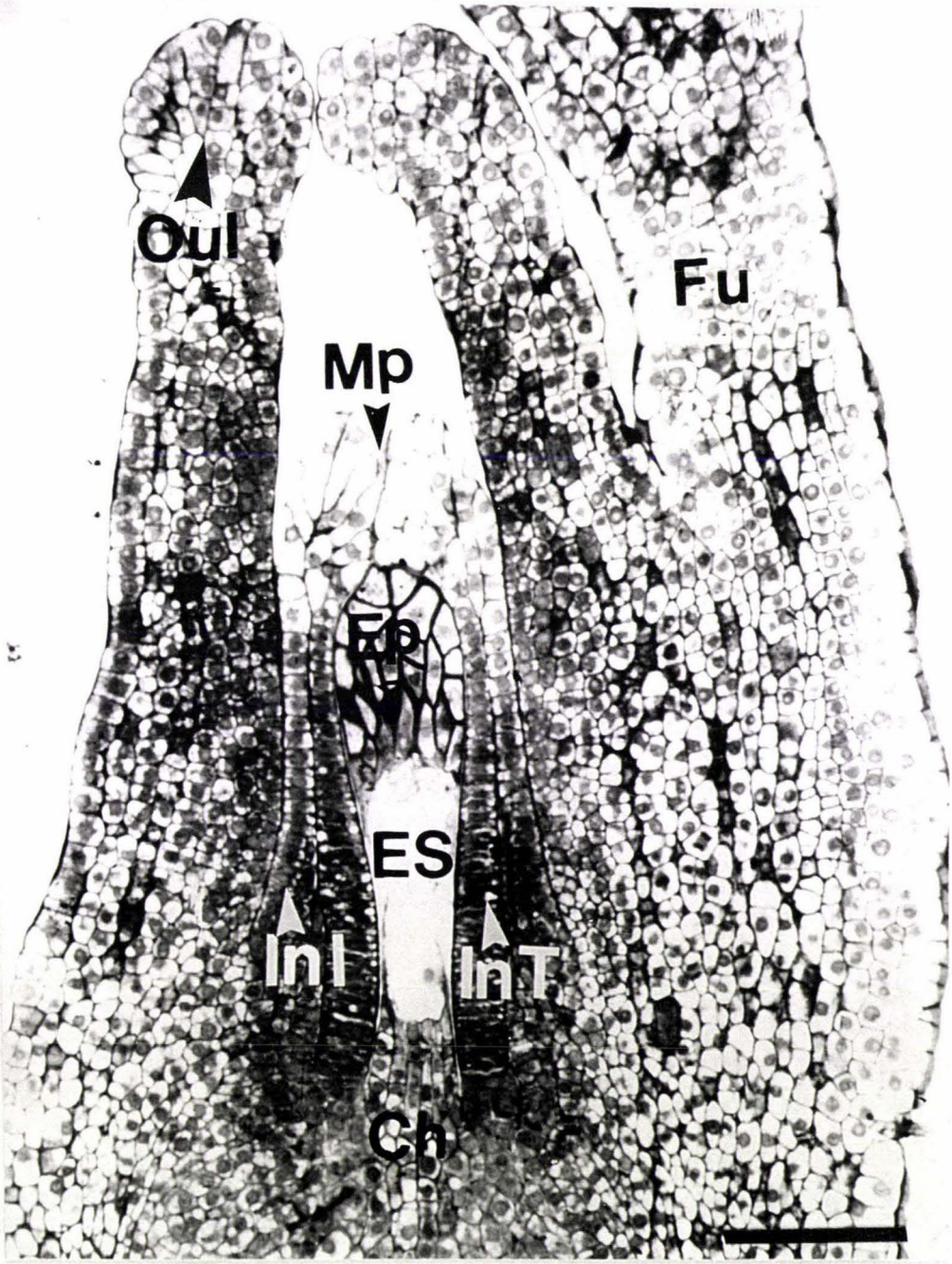
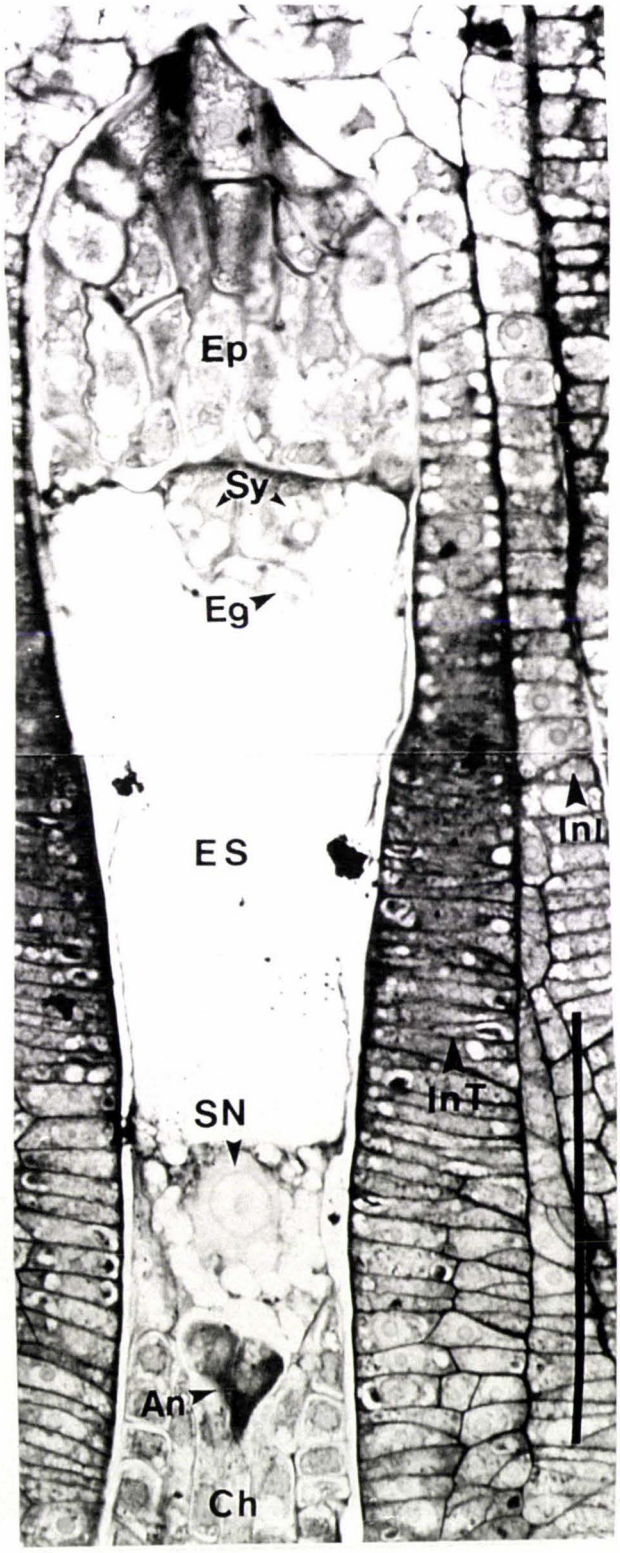


Plate 7



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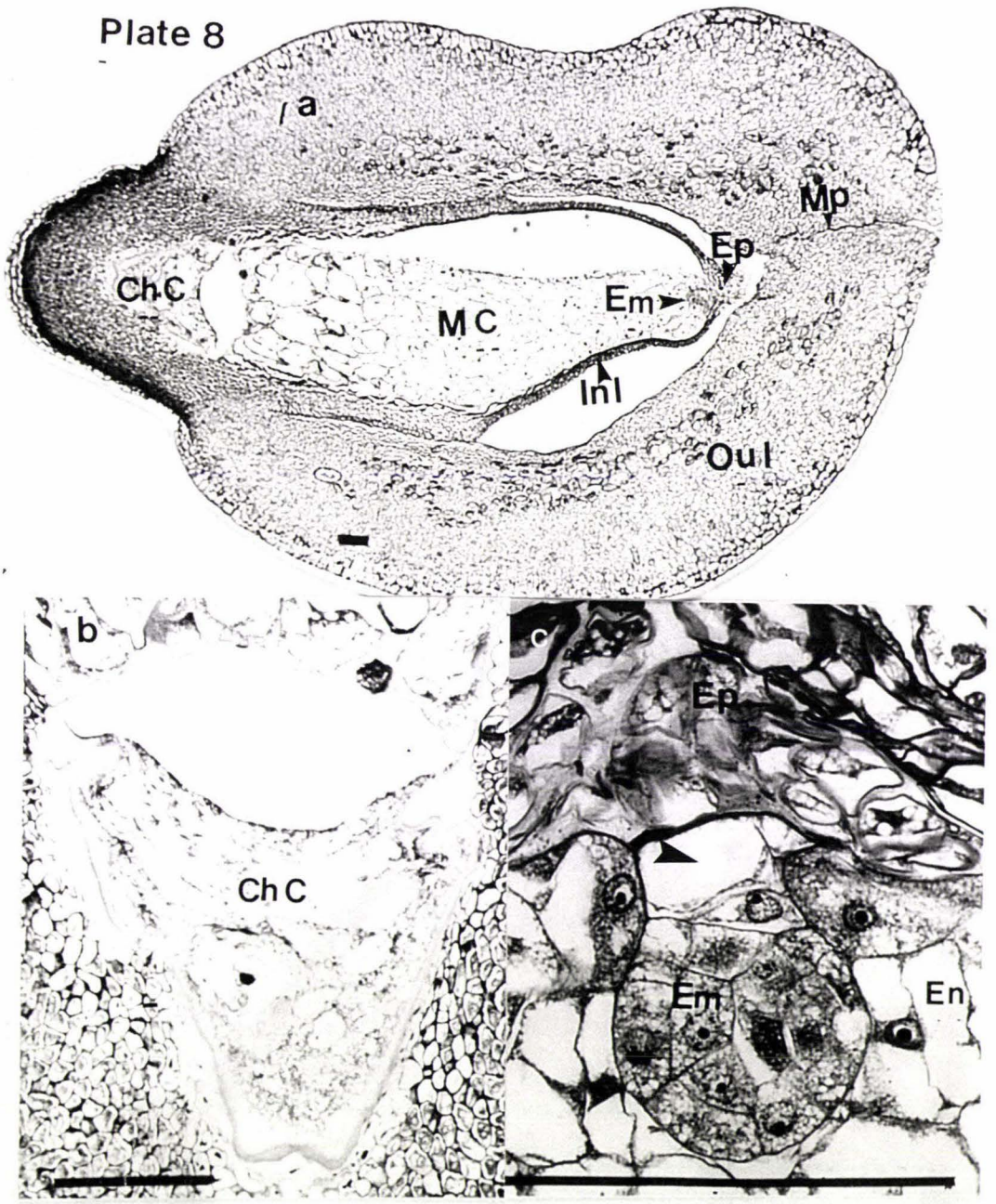
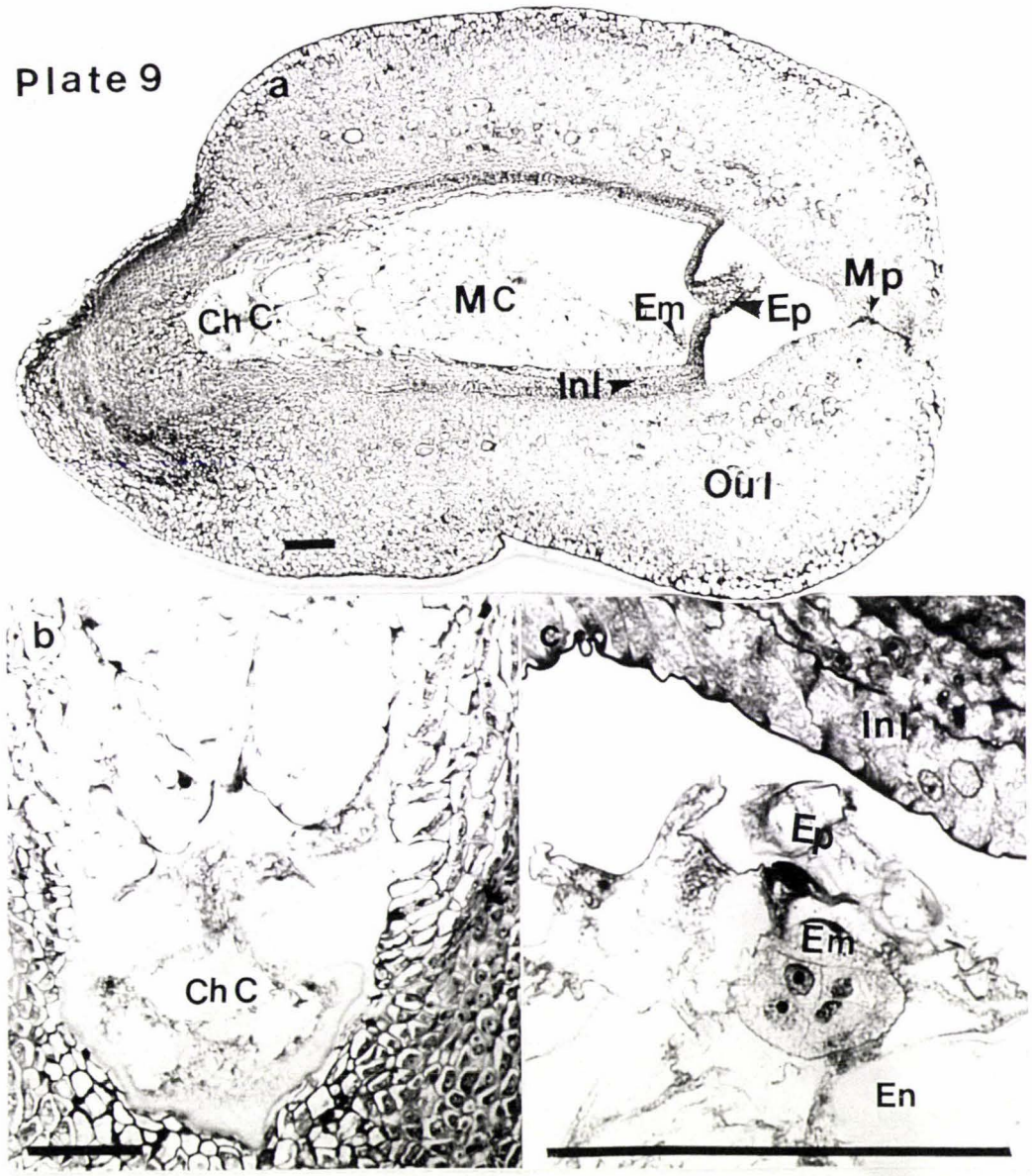


Plate 9



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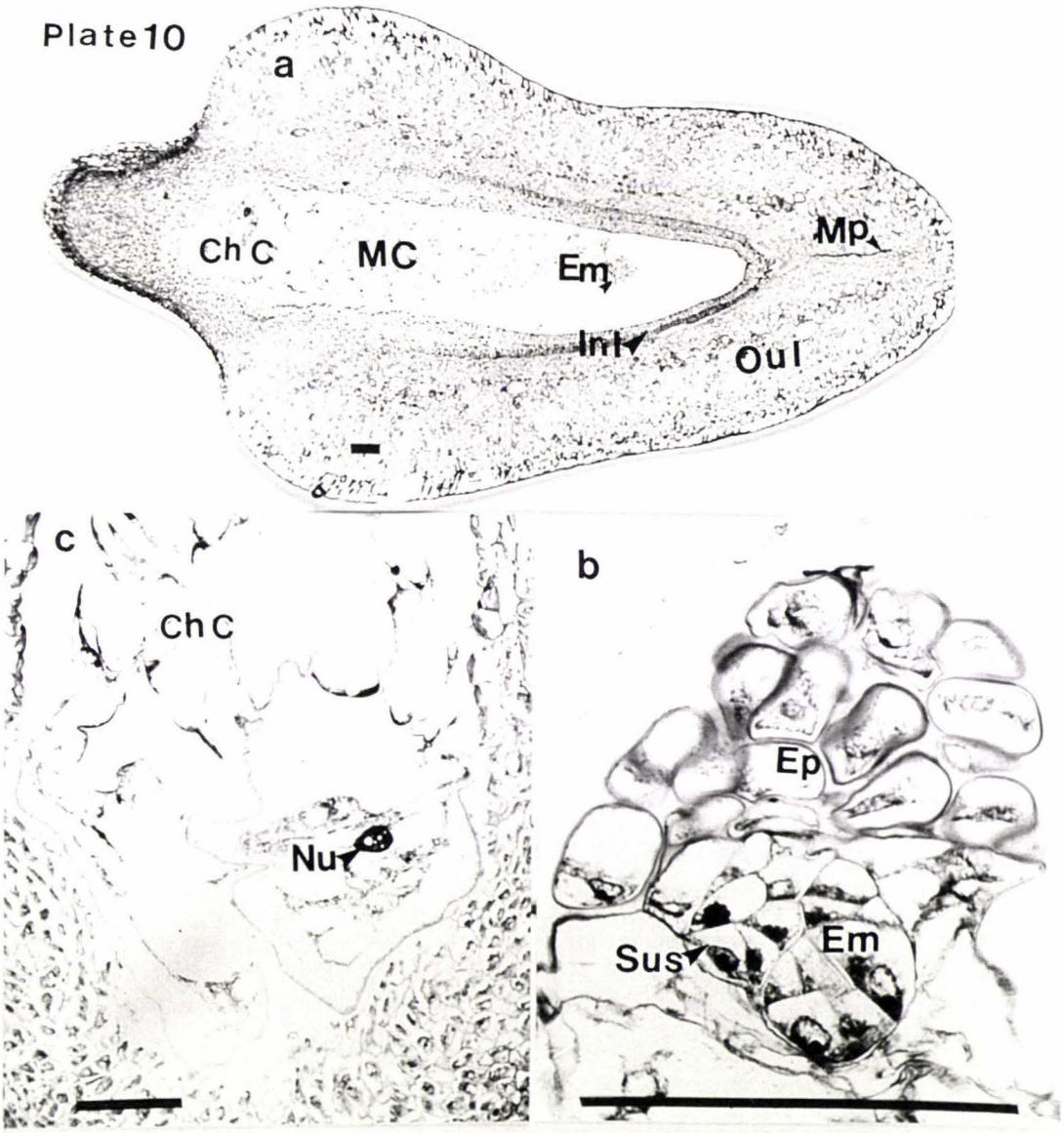

Plate 11

Plate 12

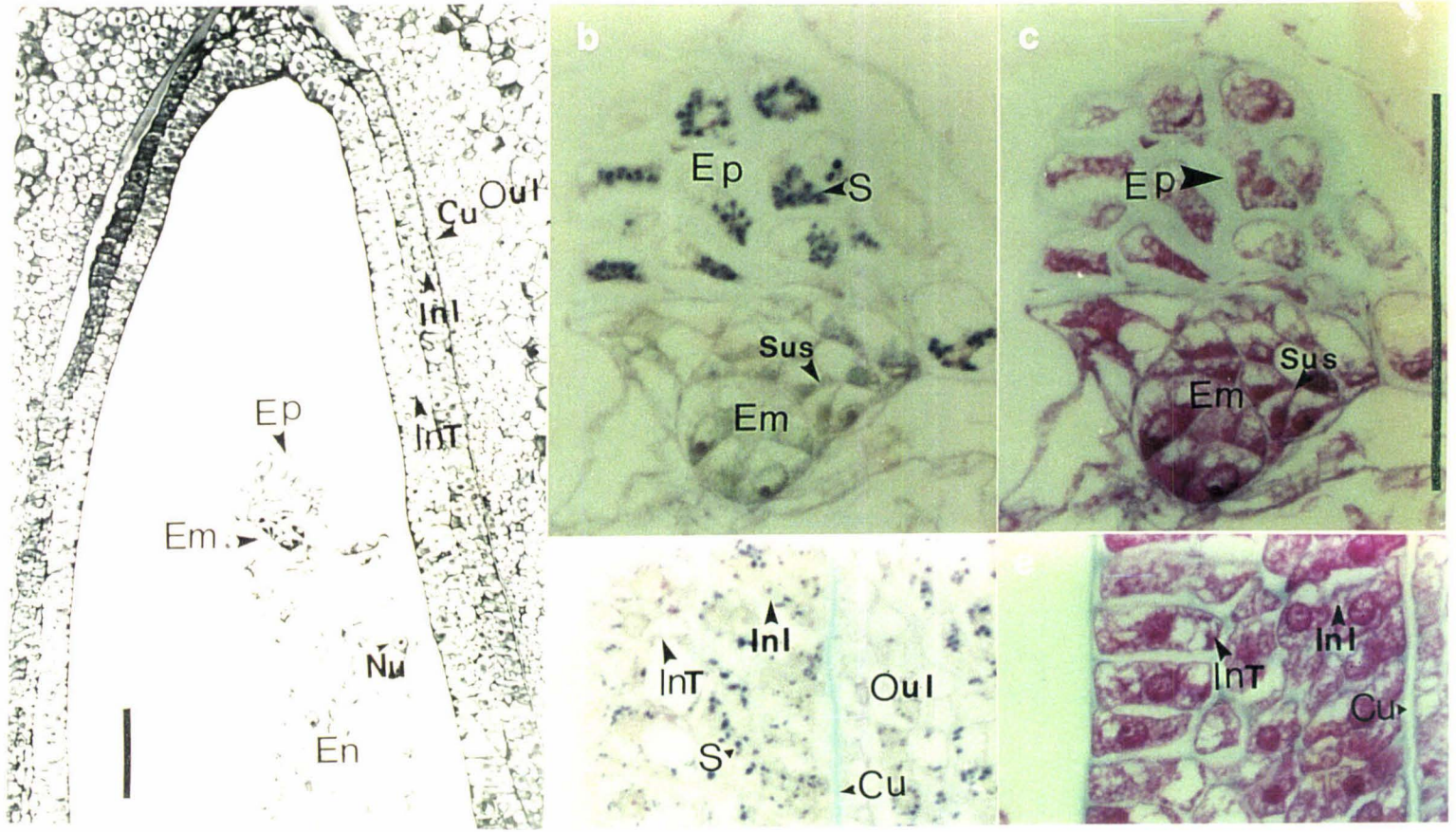
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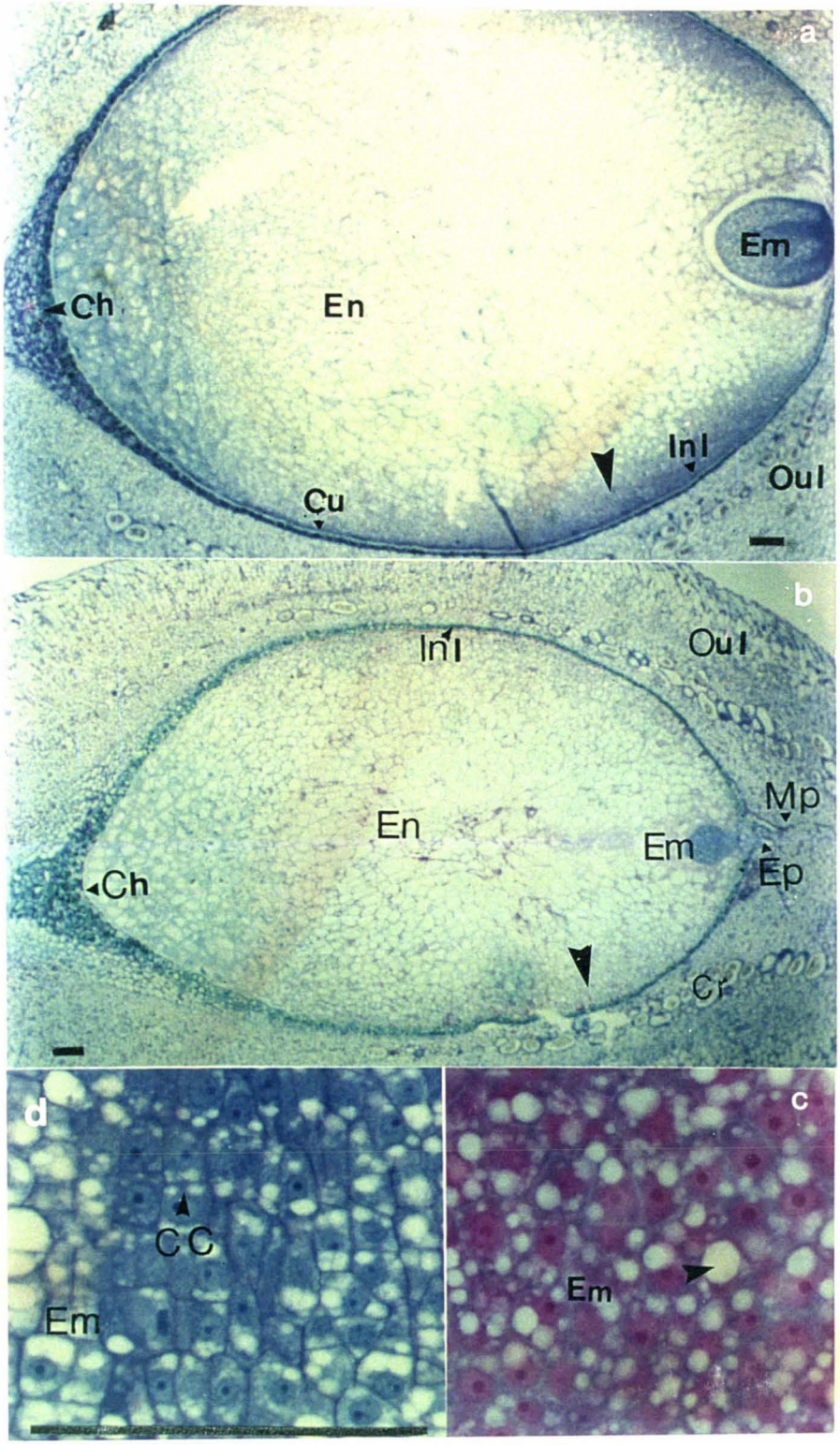
Plate 13



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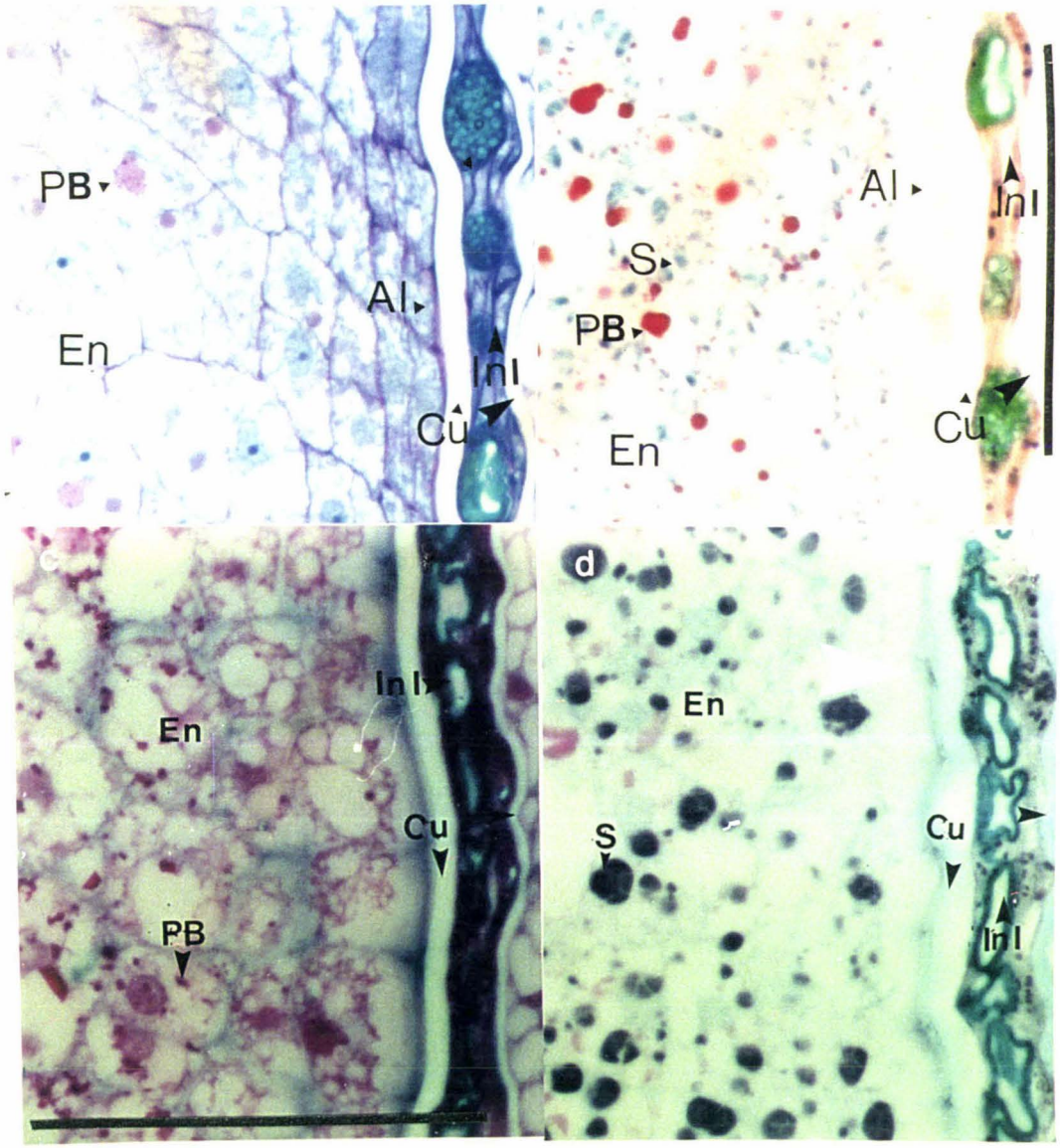

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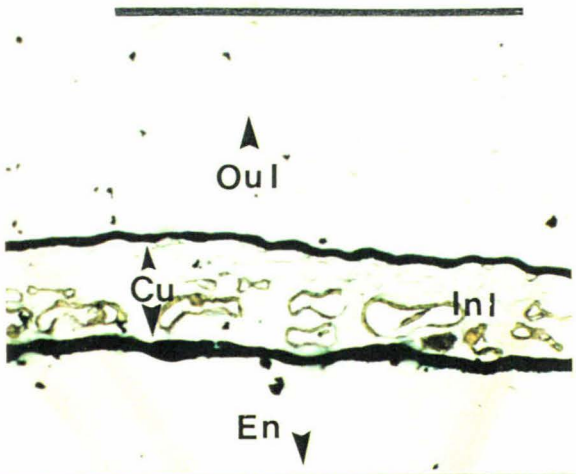

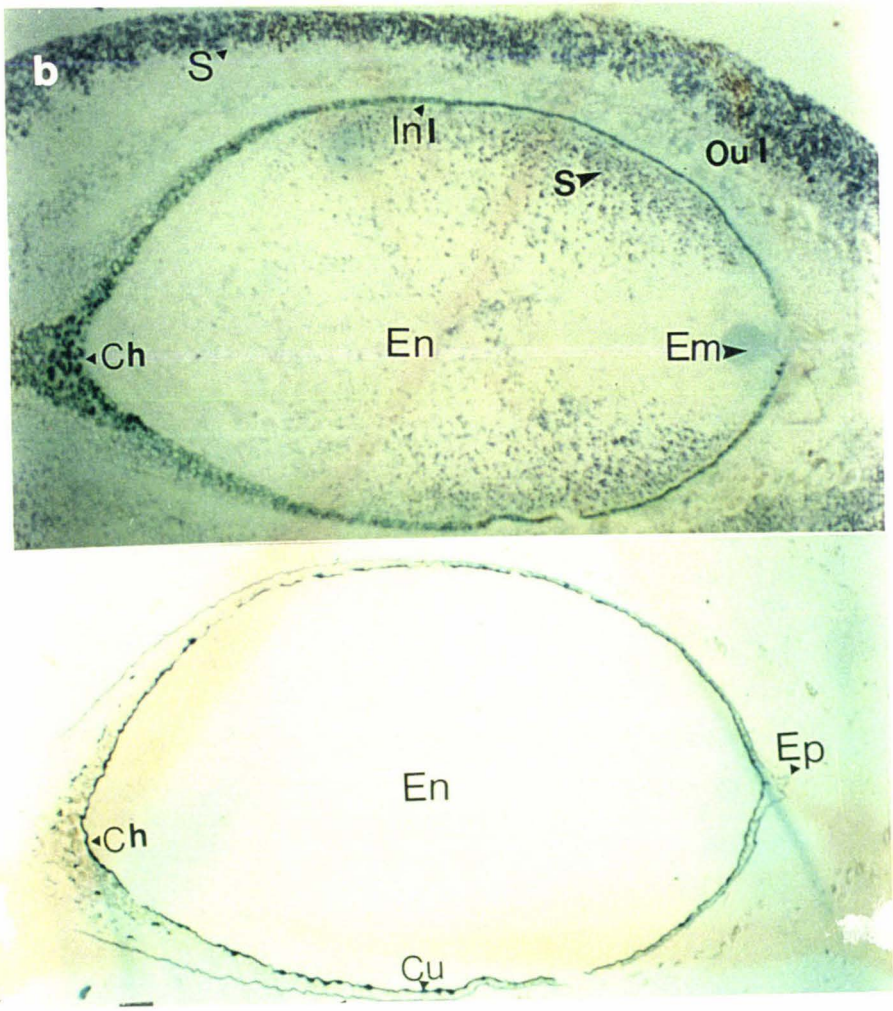

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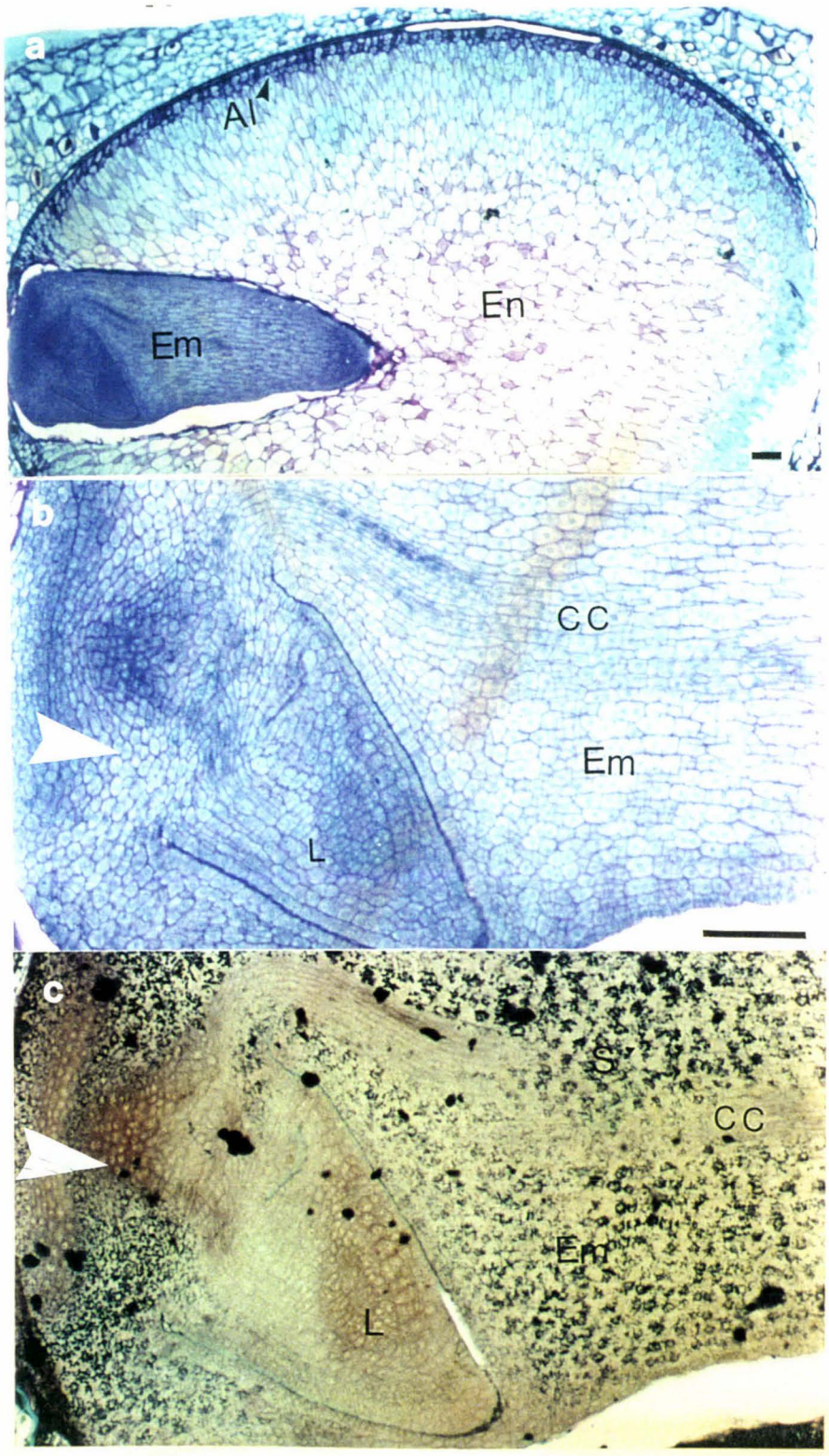

Plate 17

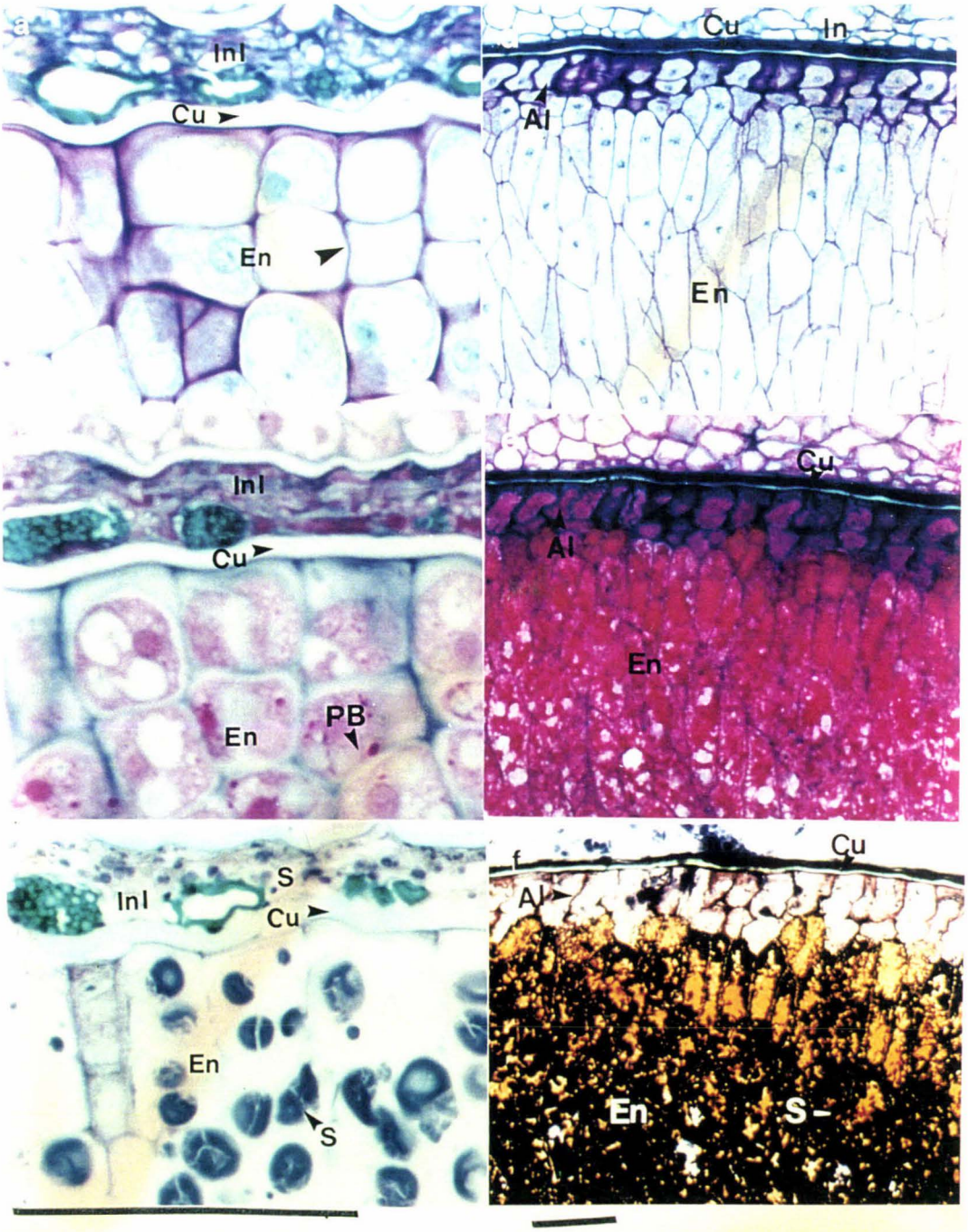
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Plate 10

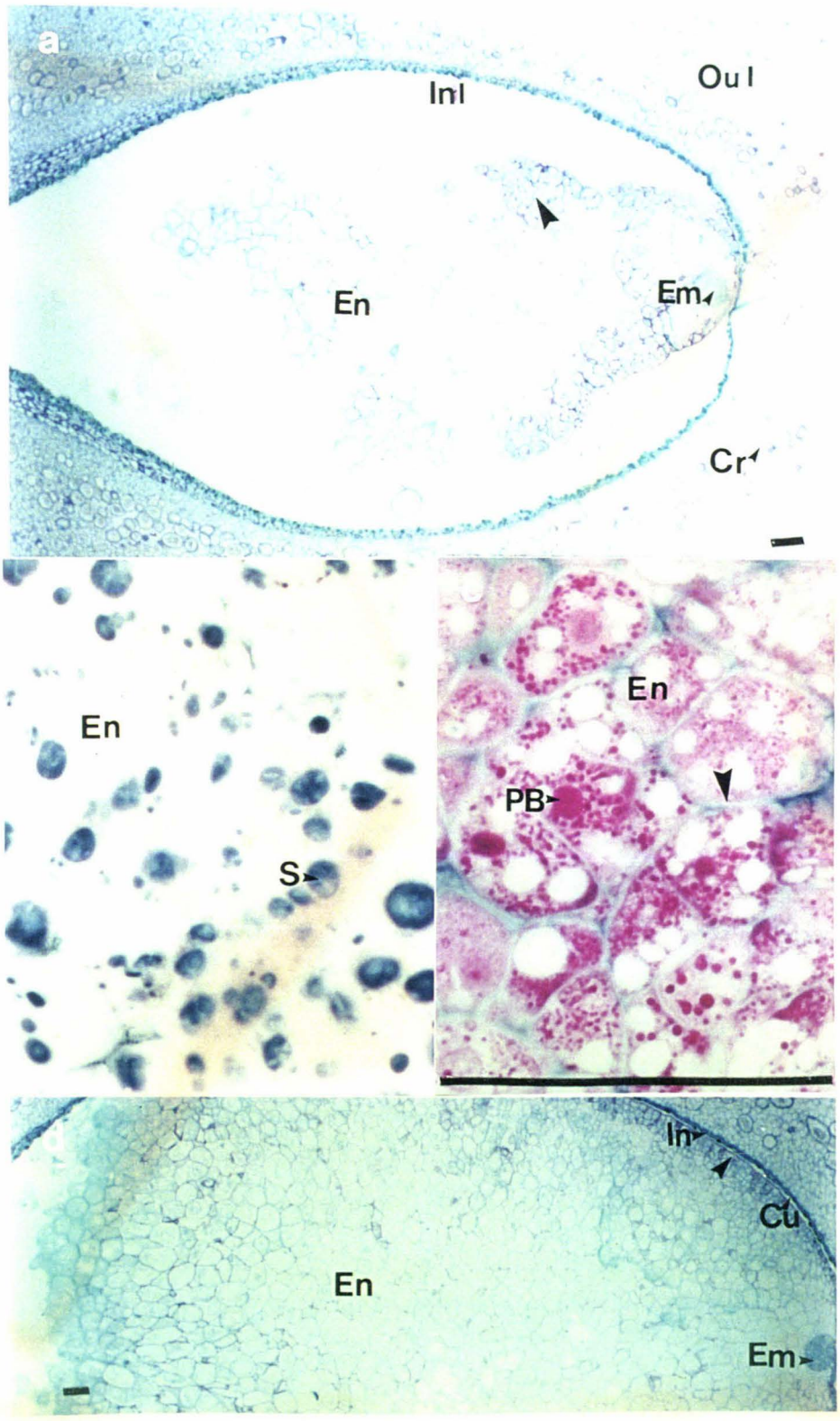

Plate 19

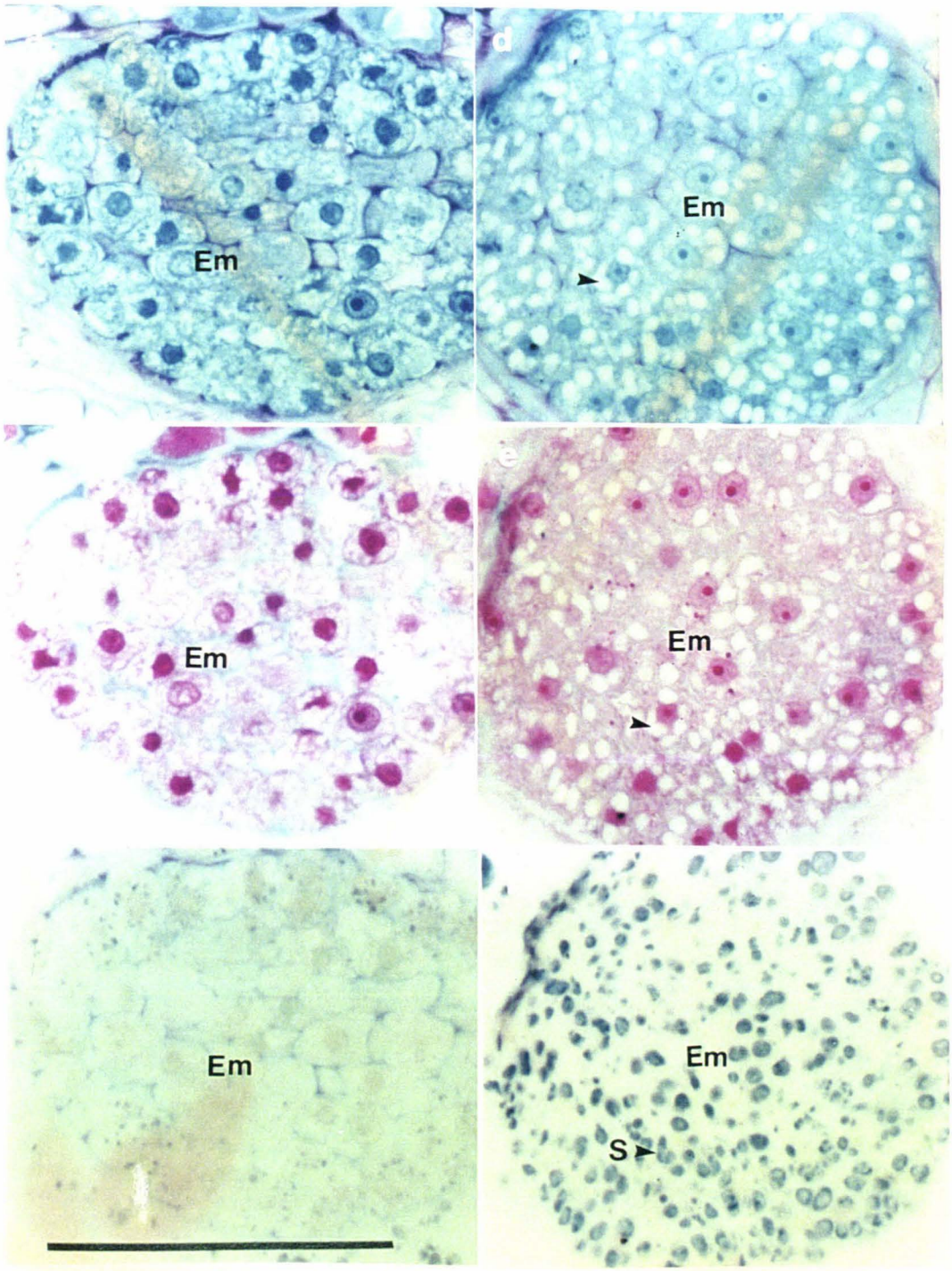

Plate 20

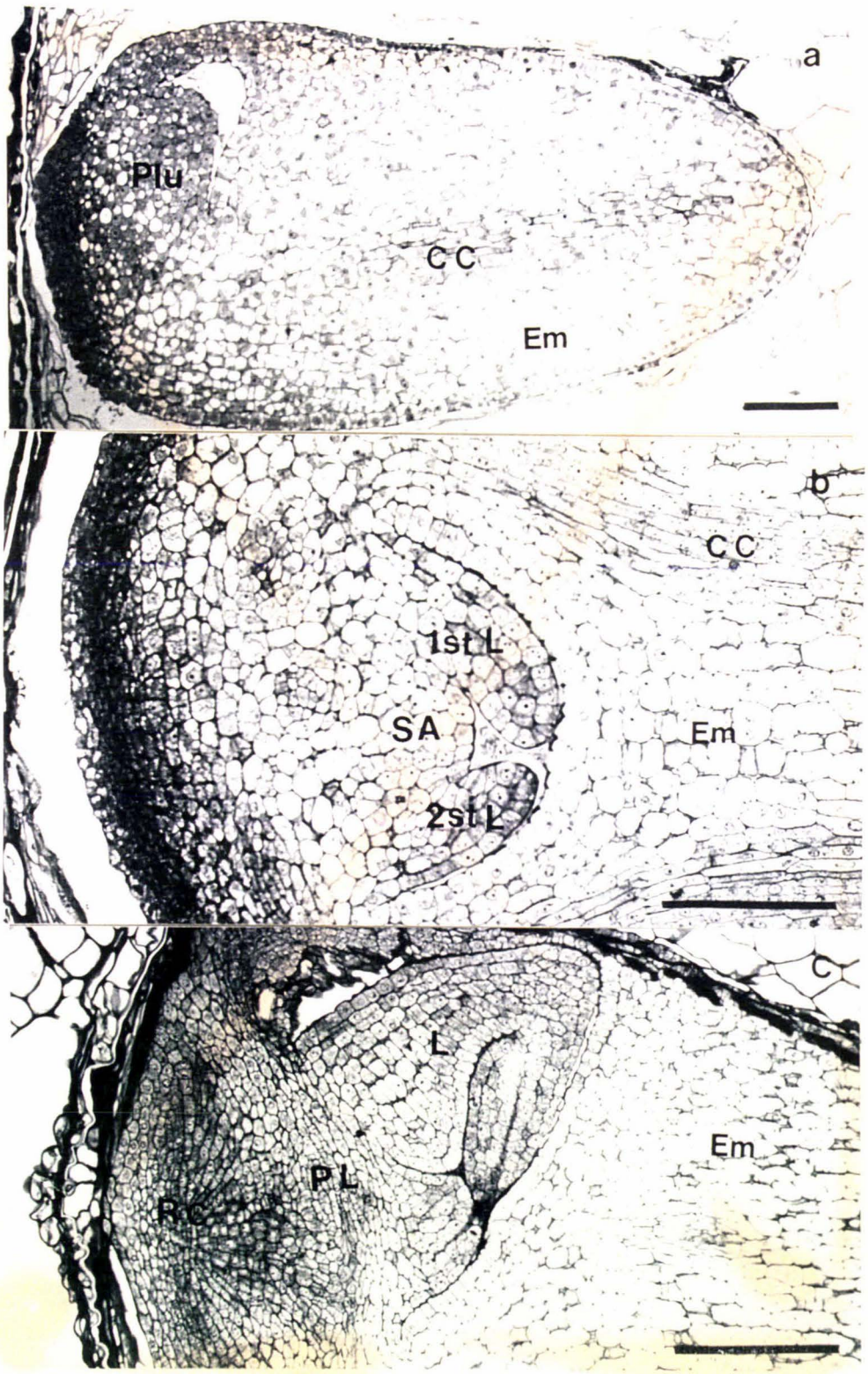

Plate 21

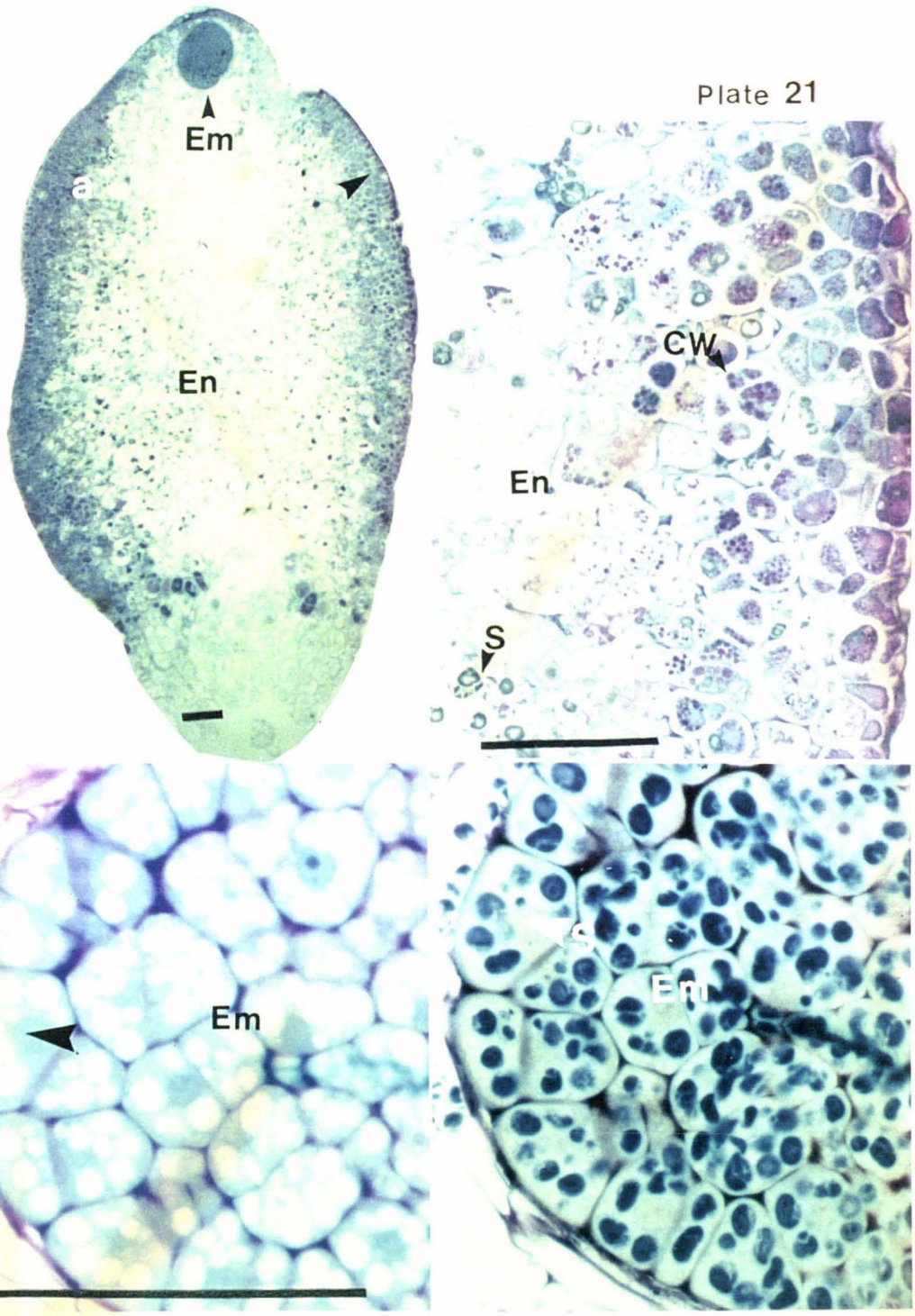

Plate 22

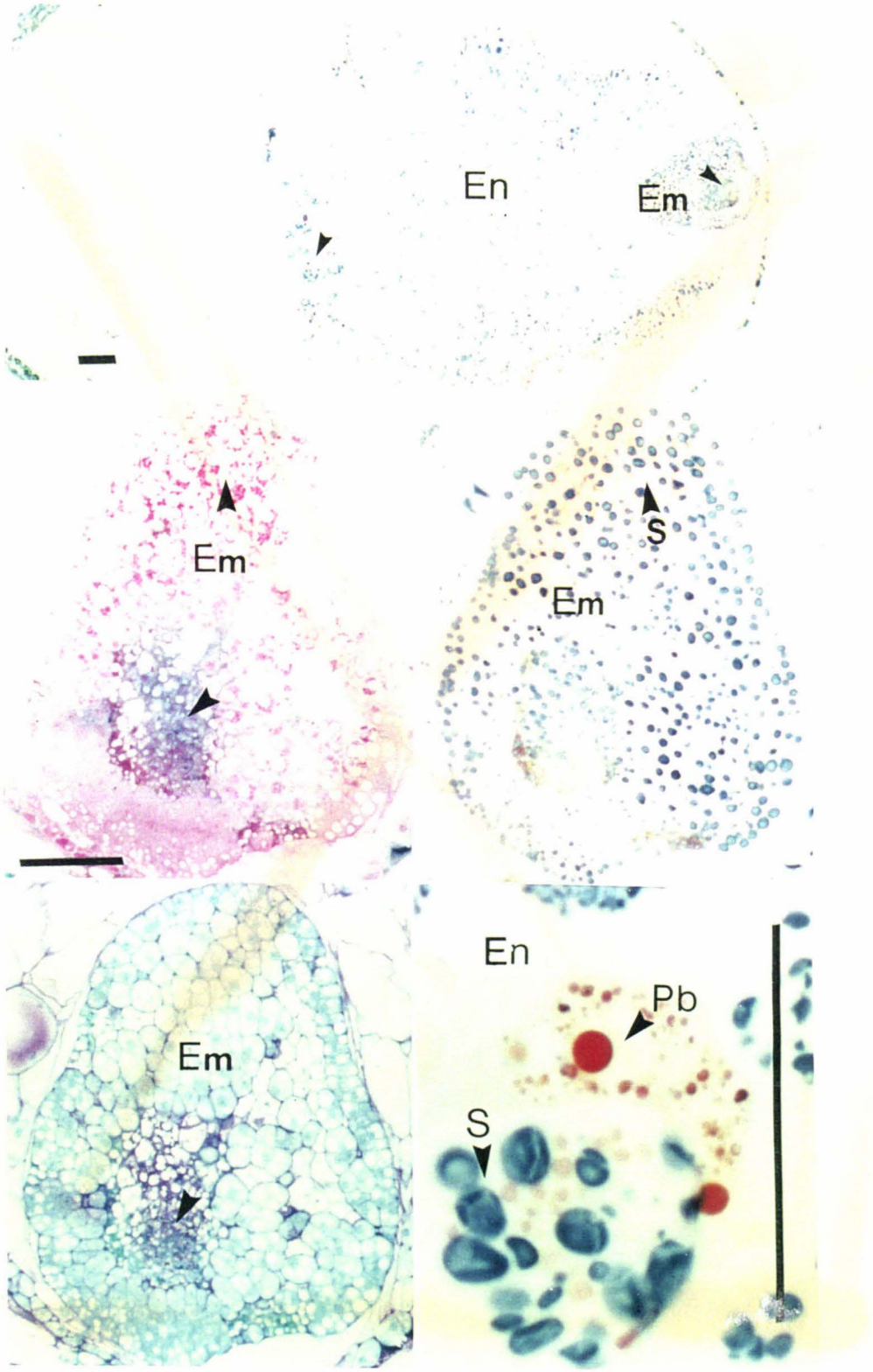

Plate 23

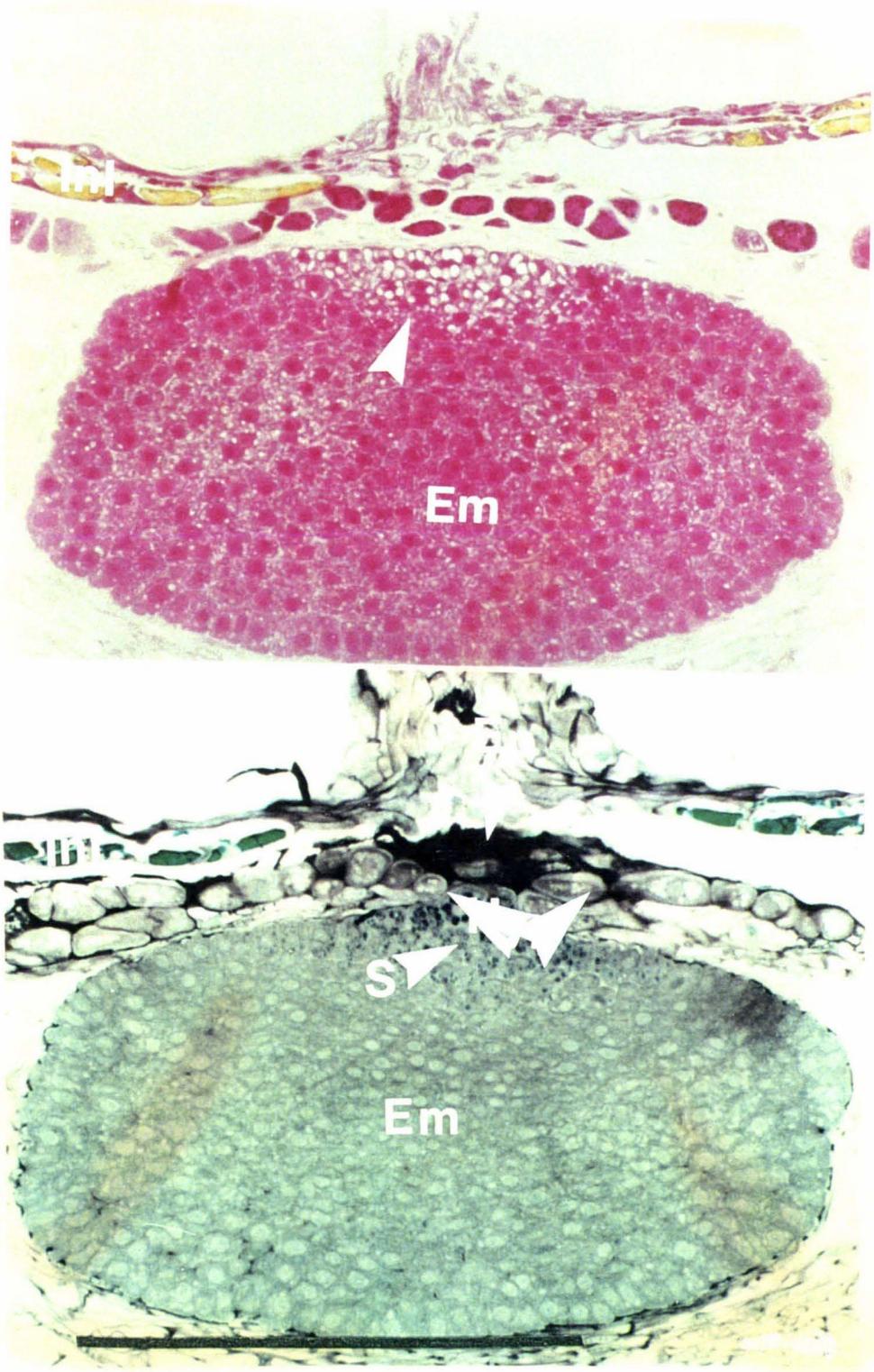

Plate 24

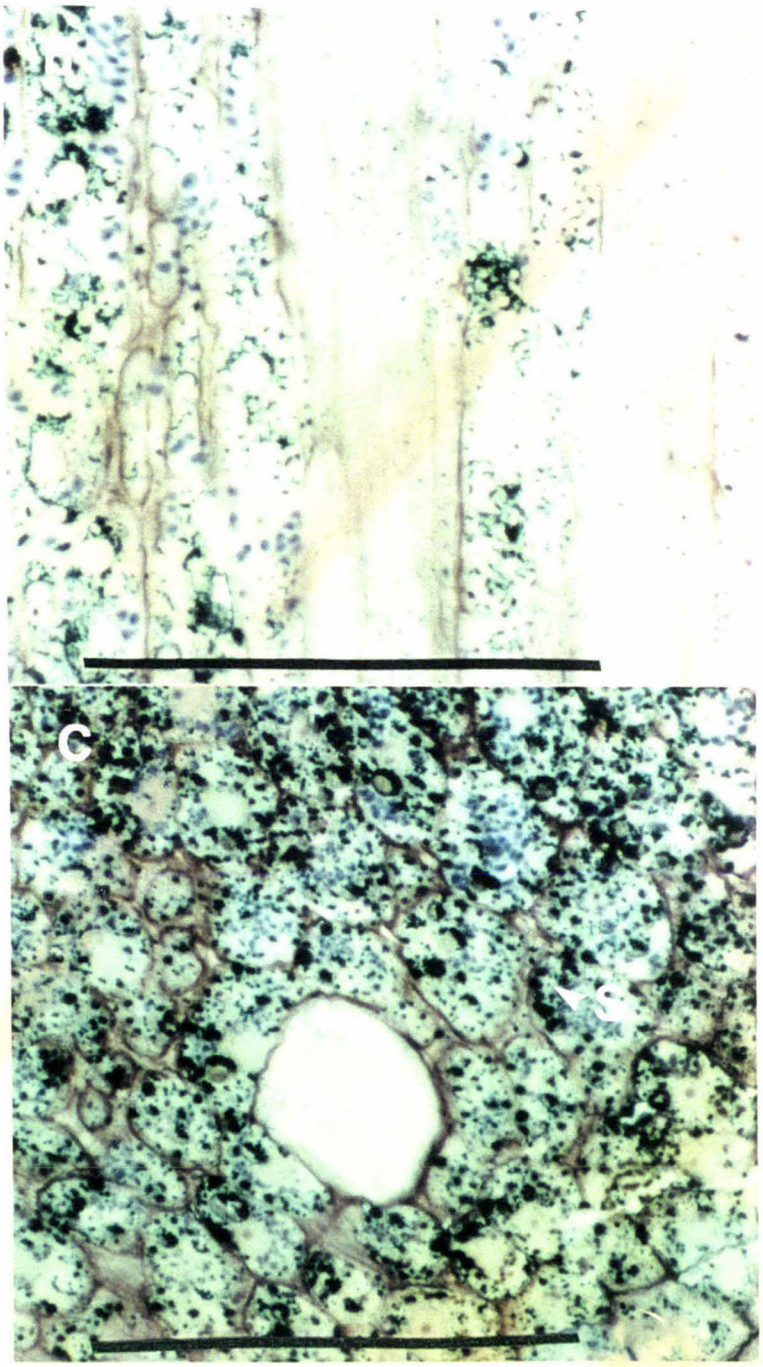

Plate 25

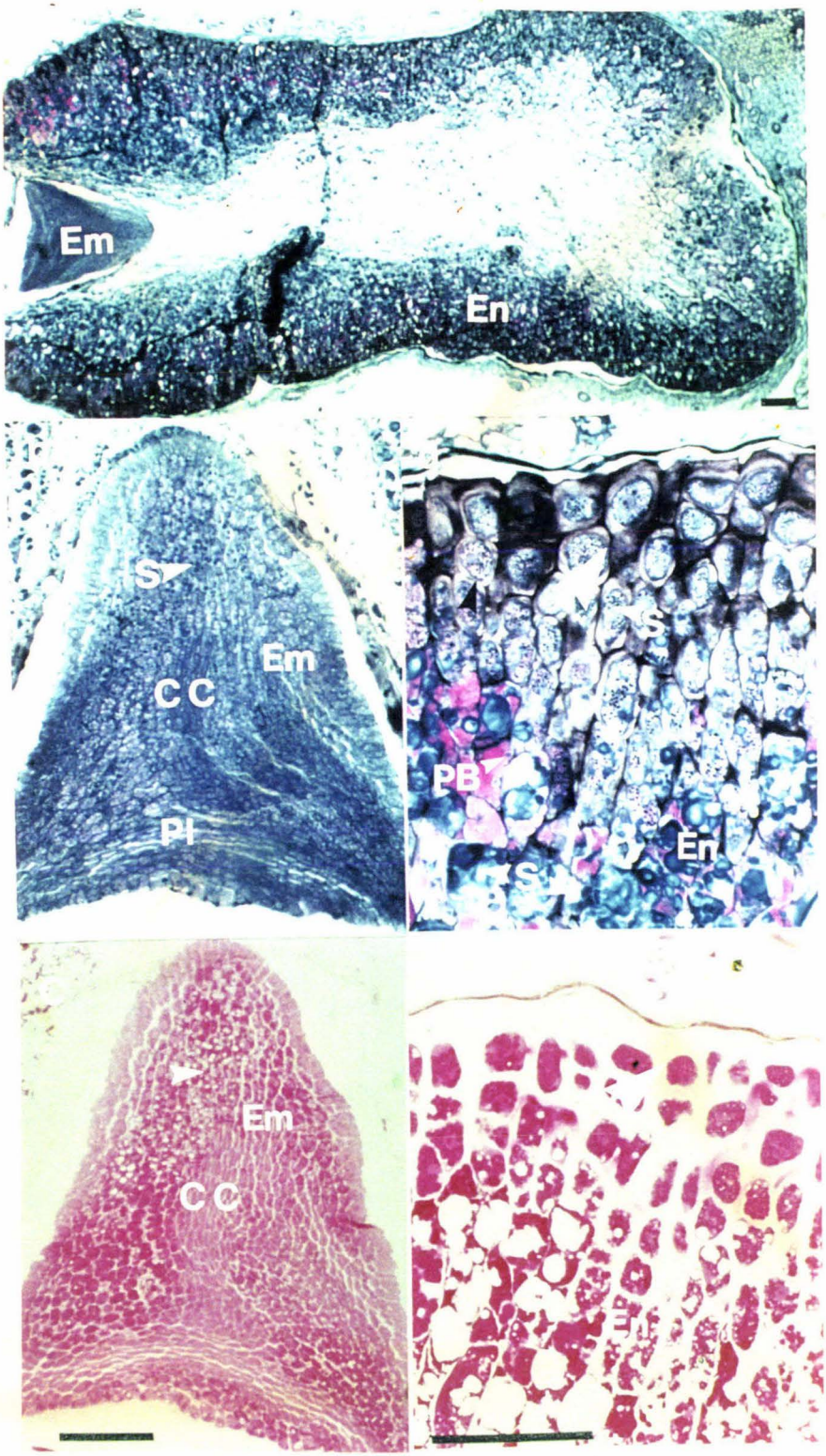

Plate 26

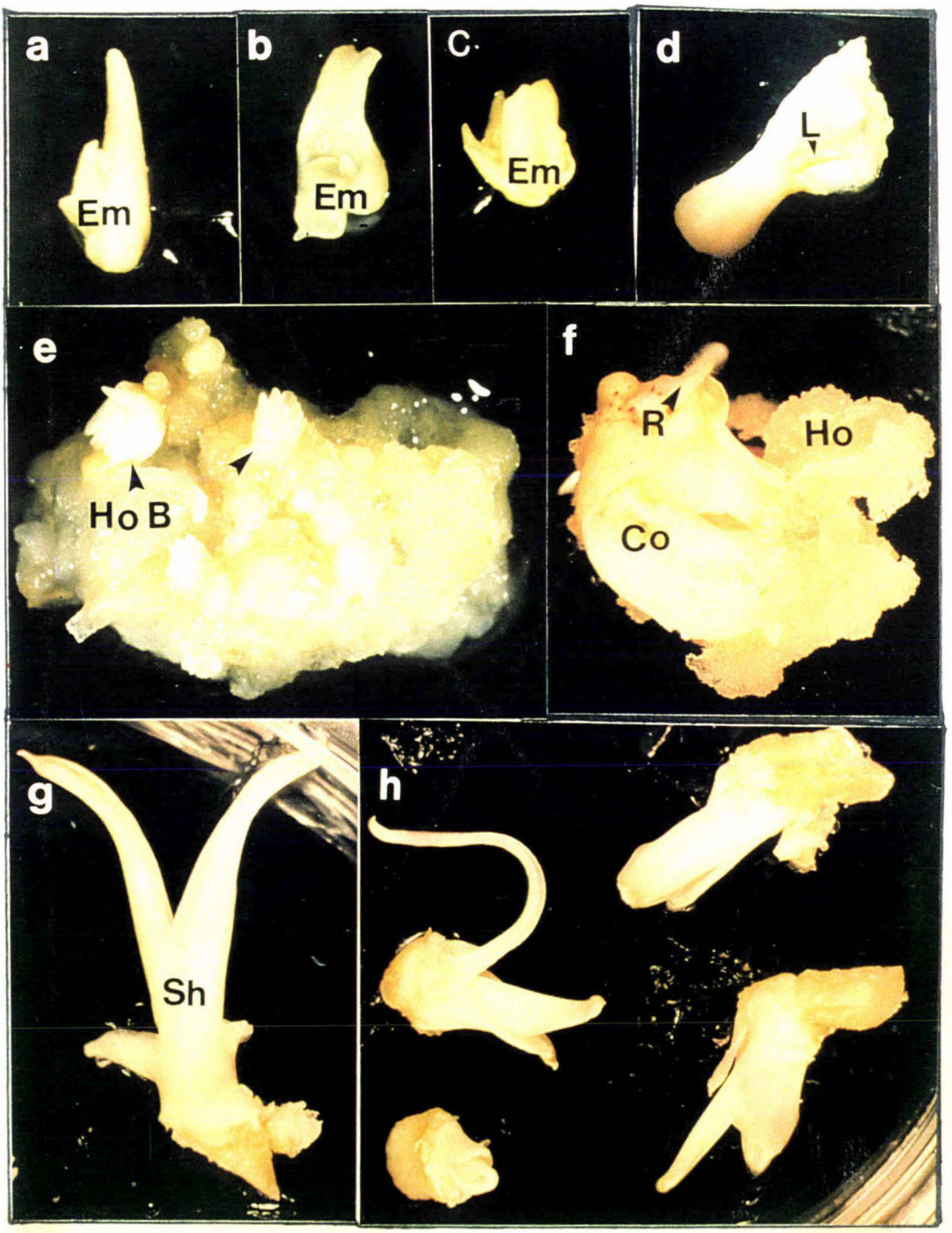

Plate 27

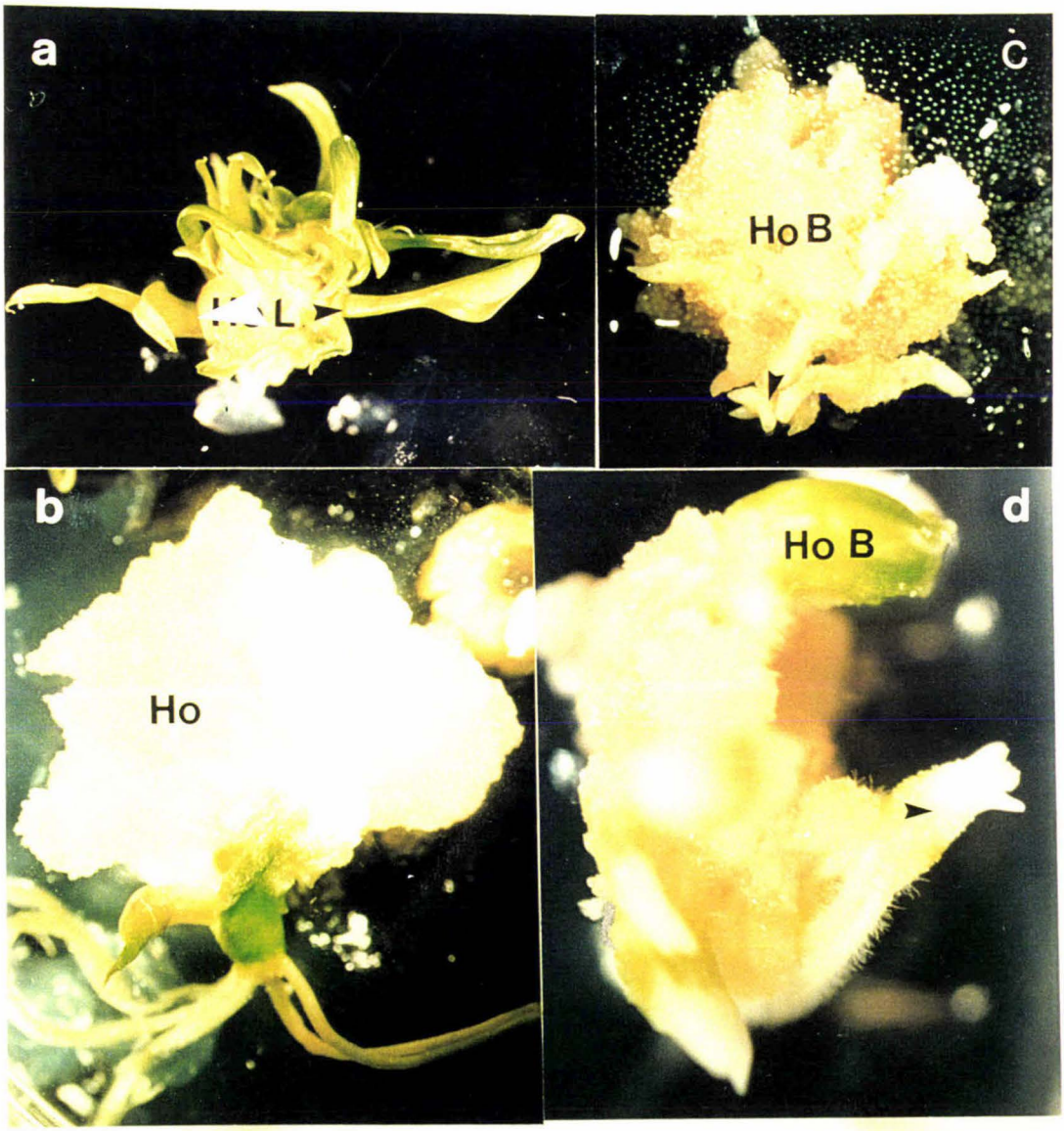

Plate 28