ASPECTS OF ZYGOTIC AND SOMATIC EMBRYOGENESIS IN *ANTHURIUMS*

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By

Tracie K. Matsumoto

Thesis Committee:

Adelheid Kuehnle, Chairperson
Richard Criley
William Sakai

We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Horticulture

THESIS COMMITTEE

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Chairperson

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CHAPTER 1

LITERATURE REVIEW AND QUESTIONS TO BE ANSWERED

ARACEAE

Araceae consists of 106 genera and approximately 3,500 species. Uncertainty of this species count is largely attributed to insufficient information in the two largest genera, *Anthurium* and *Philodendron*, consisting of about 1,000 and 700 species, respectively (Croat 1992). Members of the Araceae are found on every continent with the exception of Antarctica. Predominantly tropical, there are only ten genera found in the northern temperate zone (Grayum 1990).

The most prominent characteristic of the Araceae is the presence of a large spathe, often conspicuous and petaloid, either subtending and/or sometimes enveloping a spadix of numerous small flowers (Chant 1993). The flowers are unisexual or bisexual. If unisexual flowers occur, the male flowers are located proximally and female flowers distally on the spadix. All flowers, bisexual or unisexual, are protogynous (Dahlgren et al. 1985).

Other characteristics of the Araceae, as described by Grayum (1991), include a bractless spicate inflorescence, extrose anther dehiscence, unipistillate gynoecium, a berry like fruit, amoeboid anther tapetum, helobial endosperm development, and presence of tannins. Also noted are bifacial leaves, presence of raphides, lack of ethereal oil cells, hypogynous, actinomorphic flowers, basifixed anthers, successive pollen mother-cell cytokinesis, absence of perisperm, absence of silica, and absence of tricin.

A family of monocots, Araceae is grouped under the superorder Ariflorae and the order Arales. Three recent classifications of Araceae are compared by Croat (1990) to Engler's original 1905. Included in this comparison are classifications by Hotta (1970), Bogner and Nicolson (1991) and Grayum (1990). In Grayum's (1990) classification the

genus *Anthurium* is kept in the subfamily Pothoideae as first described by Engler(1905). In Bogner and Nicolson's (1991) classification, *Anthurium* is moved to the subfamily Lasioideae. Hotta's (1970) classification is limited to Araceae in Japan and adjacent areas and does not include *Anthurium*. Relationships of the Araceae to other monocotyledonous groups such as the Liliiflorae, Areciflorae and the Typhales have been suggested and are summarized in Dahlgren and Clifford (1982).

Characteristics of subfamily Pothoideae and Lasioideae described by Engler in Das Pflanzenreich (1905) and translated by Nicolson (1982) are given below.

> Subfamily Pothoideae
> Plants terrestrial. Stems, petioles, and roots without lacticiferous cells ('vessels') and trichosclereids; very rarely trichosclereids occurring (in leaves of *Pothos rumphii* var. *giganteus*). Leaves in two ranks or spiralled. Secondary and tertiary lateral veins reticulate, rarely subparallel (Acoreae). Flowers usually bisexual and with perigonium [tepals], rarely unisexual and monoecious.
>
> Subfamily Lasiodeae
> Plants terrestrial or paludose. Fibrovascular bundles with superposed simple lactiferous cells ('vessels'). Shrubs or tuberous plants, not uncommonly aculeate or verrucose. Flowers bisexual, or unisexual and monoecious. Ovules anatropous. Seeds usually without endosperm. Leaves sagittate, very often multiparted, with reticulate veins.

Bogner and Nicolson's description of the two subfamilies is available in a dichotomous key (not shown here) (Bogner and Nicolson 1991).

A most interesting relationship suggested is that between Araceae and Piperaceae, noted as early as 1905 in Campbell's embryological study of *Anthurium violaceum* Schott var. *leucocarpum* (*A. scandens)* (Campbell 1905) (Sheffer et al. 1980). The relationship between the Arales and Piperales, as described by Dahlgren et al. (1985), is thought to be due to convergent evolution. Both orders consist of mainly rain-forest herb and vines. The leaves are petiolate and often cordate with randomly oriented stomata. The inflorescence is in the form of spikes or spadices and consists of strongly reduced flowers

with a sessile stigma and baccate fruit (Dahlgren et al. 1985). Botanical similarities with dicots are augmented by physiological characteristics. Members of the Arales are susceptible to tumor formation by infection with *Agrobacterium tumefaciens,* a typical dicot pathogen (DeCleene 1982, Kuehnle and Sugii 1991).

ANTHURIUM

Within the family Araceae, *Anthurium* is the largest, most morphologically diverse and taxonomically complex genus in the American tropics. Native to Central and South America, members of *Anthurium* are found at elevations ranging from sea level to 3000 m, most commonly in cloud forests at 1500 m (Croat 1986).

Anthurium, a perennial monocot, is an herbaceous terrestrial or epiphytic plant. It has a multitude of unobtrusive true flowers supported by a fleshy axil, collectively known as the spadix. The protogynous nature of the bisexual flowers favors cross-pollination. The commercial anthurium flower is a combination of the spadix and a colorful modified leaf, termed spathe. Attractive foliage of some species makes anthurium also suitable for leaf harvest and cultivation as a potted plant.

Anthurium is divided into sections based originally on Engler's classification of the genus (Engler 1905). Both morphological and cytological methods are used to divide members of this diverse group into sections (Sheffer and Croat 1983). The 19 sections of *Anthurium* are: *Belolonchium* with *andicola* and *verapazense* alliances, *Calomystrium, Cardiolonchium, Chamaerepium, Dactylophyllium, Digitinervium, Episeiostenium, Gymnopodium, Leptanthurium, Oxycarpium, Pachyneurium, Polyneurium, Polyphyllium, Porphyrochitonium, Schizoplacium, Semaeophyllium, Tetraspermium, Urospadix,* and *Xialophyllium* with *microspadix* and *caucanum* alliances (Croat 1991). A preliminary key to the natural groups of anthurium (Croat 1983) and important characteristics of major sections are listed in Croat (1986). *Anthurium scandens*, the species used in Campbell's

(1905) embryology study, is included in sections *Tetraspermium*. *A. andraeanum* and *A. scherzerianum*, the two most economically important species, are included in section *Pachyneurium* and *Calomystrium*, respectively (Sheffer and Croat 1983).

Commercial production has focused on plants derived from two major species: *Anthurium andraeanum* Lind ex. Andre. and *A. scherzerianum* Schott. A majority of the plants used in the cut flower industry are thought to be hybrids of *A. andraeanum* Lind ex. Andre, and other species (Madison 1980), and will further be referred to as *A. andraeanum* Hort. Main production areas are Hawaii and the Netherlands, with additional production in other tropical and subtropical regions. The 1991 combined Dutch auctions ranked anthurium 14th of all cut-flower sales, with over 20 million stems sold for an approximate wholesale value of $21.5 million (International Floriculture Quarterly Report 1992). Estimates for anthurium sales in 1992 are approximately 33 million stems based on reports for the first and second quarter of 1992 (International Floriculture Quarterly Report 1993). In Hawaii, anthurium is one of the top cut flowers with a 1993 "farmgate" value of $7.5 million for 10.5 million stems sold (Hawaii Agricultural Statistics Service 1994). *A. scherzerianum* is sold as a flowering potted plant, with main production areas located in Europe. Recently, there has been an increase in the production of anthurium hybrids as potted plants in the USA.

Only a few morphological and anatomical studies have been conducted on the inflorescence and flowers of *Anthurium andraeanum*. Kamemoto (1962) and Watson and Shirakawa (1967) related the morphology of the anthurium flower to water loss and ultimately shortened shelf life. Dai and Paull (1990) correlated subtending leaf development with flower growth. A study by Szendel and Weryszo (1973) provided a brief description of the flowers of *A. andraeanum*. Anatomy of the flower structures was studied by Higaki et al. (1984) using light microscopy and scanning electron photographs were used to observe flower structure.

ZYGOTIC EMBRYOGENESIS

Zygotic embryogenesis is the period of development following double fertilization in which the zygote undergoes a series of complex morphological and cellular changes to become a fully mature embryo. Mature embryos consist of shoot and root apices and cotyledons, which often contain high levels of storage macromolecules especially proteins and lipids (West and Harada 1993). In contrast, embryology includes all events connected with microsporogenesis, megasporogenesis, development of the male and female gametophytes, fertilization, endosperm and embryo formation (Ragavahan 1976). Several methods are used to study zygotic embryogenesis. These include microscopy, histochemistry, cytochemistry, autoradiography, tissue culture, and molecular techniques.

Microscopy

Microscopy has provided the most information on embryogenesis in angiosperms. To follow the entire sequence of embryogenesis, ovules of different ages are fixed over a span of time (Raghavan 1986). Specimens are fixed, sectioned and stained. Fixation is possible with a combination of formalin, glacial acetic acid and ethanol. However, mitochondria and fine transvacuolar strands of cytoplasm are not usually visible. To remedy this, non-coagulant fixatives are recommended (ie. osmium tetroxide, acrolein, glutaraldehyde, formaldehyde) in conjuction with embedding in a glycol methacrolate polymer (Feder and O'Brien 1968). Dehydration, which follows fixation, consists of treating tissues with a series of solutions containing progressively increasing concentrations of the dehydrating agent, usually alcohol, and decreasing concentration of water. Embedding is possible in paraffin wax, glycol methacralate resins or epoxy plastic. Sections of approximately 10 μm may be cut with specimens embedded in paraffin (Berlyn and Miksche 1976) and 0.5-4 μm with glycol methacrolate polymers (Feder and O'Brien

1968). Specific staining of sections has been reviewed by Johansen (1940), Feder and O'Brien (1968), and Berlyn and Miksche (1976).

Histochemistry, Cytochemistry and Autoradiography

Histochemistry, cytochemistry and autoradiography are methods used to localize biochemical events at the cellular level. Histochemistry is useful when heterogeneity of the tissue discourages conventional biochemical techniques. To prevent alteration of the chemical structure by fixatives, specimens are usually rapidly cooled in liquid nitrogen and sectioned on a special low temperature microtome, the cryostat. Sections are then treated with a reagent specific for the chemical substance. Under controlled conditions, histochemical methods may be used to quantify specific substances such as the Feulgen reaction for deoxyribonucleic acid (DNA). Autoradiography enables the detection of chemical substances with the use of a radioactive isotope in methods similar to those used in photography. This method is often used to follow the synthesis of nucleic acid or protein. The material is incubated in a medium containing radioactive precursors of DNA or RNA, H^3-thymidine and H^3-uridine, respectively. Specimens are then fixed and sectioned as in regular microscopy preparation. Slides are covered with a sensitive photographic emulsion and exposed in the dark. Once processed, autoradiographs locate the sites of synthesis for specific macromolecules (Raghavan 1986).

Tissue Culture

Tissue culture involves the aseptic culture of plant organs, tissues, cells and protoplasts. Embryo culture is commonly used as an application of embryo rescue in unviable crosses. Non-viable crosses may be the result of deleterious genes, wide hybridization and embryo-endosperm incompatibilities (Raghavan 1986). Precocious germination of embryos in culture help elucidate the requirements and control of embryo

development. A model to study the process of embryogenesis in plants using somatic cells, somatic embryogenesis, is also used for propagation. Further information on somatic embryogenesis will be covered later in this review.

Molecular Techniques

Molecular methods such as molecular hybridization, in situ hybridization and cDNA cloning are used for the isolation and characterization of genes involved in embryogenesis (Raghavan 1986). Genes have been found to regulate the development and maturation of the plant embryo. Zygotic and somatic embryogenesis systems have also been compared. Gene expression during embryogenesis is reviewed by Thomas (1993).

Morphological Description of Zygotic Embryogenesis

Double fertilization preceeds embryogenesis. After the pollen tube penetrates the ovule through the micropyle, the generative sperm cell from the pollen tube fuses with the egg cell to form the embryo. The second sperm nucleus combines with the two polar nuclei in the central cell to form the triploid endosperm. The endosperm provides nutrients to the developing embryo and will persist in the mature seed or will be absorbed during seed development. The integument or integuments of the ovule becomes the seed coat and serves to protect the enclosed embryo and endosperm (West and Harada 1993).

Embryogenesis can be divided into three phases: morphogenesis, embryo maturation and embryo dessication. Morphogenesis includes formation of embryonic tissue and organ systems, with the specification of shoot and root apices. Embryo maturation is characterized by the accumulation of storage reserves. During the final phases, the embryo enters a period of developmental rest (West and Harada 1993).

During the period following the union of the sperm and egg cells, the zygote enters a quiescent phase which lasts a period of a few days to a few months. During early phases

of development, dicotyledonous and monocotyledenous embryo development is indistinguishable. Initial asymmetrical division of the zygote results in a large basal cell and small apical cell, located at the chalaza and micropyle respectively. Based on the plane of further divisions, five different types of embryo ontogeny have been described (Raghavan 1986). In the Crucifer type of division, the terminal cell divides longitudinally and the basal part plays only a minor role in the subsequent development of the embryo. Likewise, in the Asterad type, the terminal cell also divides longitudinally; however, both the basal and terminal cells contribute to the development of the embryo. In the other types of development, the terminal cell divides transversely. In the Solonad type, the basal cell usually forms a suspensor of two or more cells. In the Caryophyllad type, the basal cell undergoes no further division and the suspensor, if present, is derived from the terminal cell. When both the basal cell and terminal cell contributes to the development of the embryo, it is classified as the Chenopodial type. Further descriptions of the types of division are covered in Maheshwari (1950).

Embryonic polarity is well-established before the egg cell is fertilized. The nucleus and cytoplasm of many plants are found in the chalazal pole while a large vacuole is in the micropylar pole (West and Harada 1993). Following asymmetric division, the chalazal pole is the main area of growth while the micropyle end produces the suspensor. The suspensor is actively involved in the absorbtion of nutrients, movement of the embryo into the endosperm and production of proteinaceous substances and growth regulators for the development of the embryo (Yeung and Meinke 1993). The proembryo then divides and differentiates into a nearly spherical structure.

Cells within the embryo begin to differentiate into various tissue systems. The protoderm (future epidermis) is formed by periclinal divisions in the outermost cells. Highly-vacuolated, less-dense ground meristem gives rise to the ground tissue. Within the ground meristem is the less vacuolated and denser procambium. The primary meristems

(protoderm, ground meristem and procambium) are continuous between the axis of the embryo and cotyledon (Wardlaw 1955).

Development of the cotyledon may begin before or after the initiation of the primary meristem. Monocots become cylindrical in shape (Raven et al. 1987). It has been suggested that the monocot cotyledon maybe derived from the fusion of the two separate cotyledons or one cotyledon may be suppressed or only one cotyledon is truly formed initially lateral but now occupying a distal area (Wardlaw 1955).

Embryogenesis in Araceae

Aroid embryology (embryogenesis) has been impeded by the unavailability of seeds due to vegetative propagation, embryo cell deterioration during seed storage (Nyman et al. 1986), difficulties in fixation and embedding (Johansen 1940; Eyde 1967; Mayo 1989), and juvenility of cultivated species. Currently, the embryology is known for only nine aroid genera. Onagrad embryogeny is predominant among the genera with unisexual flowers. However, in two genera, *Synandrospadix* and *Pistia*, asterad embryogeny is established. In hermaphroditic genera the caryophyllad and/or the solanad embryogeny appear to be dominant (Grayum 1991).

Embryogenesis in *Anthurium*

In *Anthurium violaceum* (*A. scandens*) embryogeny appears to be of the solanad and caryophyllad type (Grayum 1991). Once early phases of division are complete, the embryo assumes a nearly globular form. The rudimentary suspensor is apparently of little or no importance for nutrient absorption and remains reduced, similar to other Araceae members. As the embryo enlarges, it becomes conical in shape with the basal portion becoming somewhat flattened. At this stage there is no apparent differentiation of the organs of the young plant.

The shoot apex later develops on one side of the embryo and is indicated with a depression, similar to other monocots. At the same time that the shoot apex develops, the root region is apparent and has a clearly defined line marking the boundary of the root cap. While the root and shoot central cylinder are evident, their extent are not clear in the young embryo. The embryo then rapidly grows to approximately 2 mm in length and is fully mature before the berry is ripe. In the mature embryo, the young vascular bundles are evident but no tracheary tissue has developed. Each primary organ (except the stem) has axial bundles which unite with the other organ bundles. Tissues within the roots are similar to those found in other monocots. The root cap is clearly separated from the tissues beneath it and has its own cells independent of those of the other primary tissues. Calcium oxalate cells are present in the embryo in compact bundles (Campbell 1905).

SOMATIC EMBRYOGENESIS

Asexual embryogenesis in plants was first reported by Leeuwenhoek in 1719 as he described the presence of multiple embryos in orange seeds. Since then, asexual embryogenesis has been noted to occur *in vivo* from tissues such as the nucellus, inner integument, synergids, endosperm, zygote, zygotic embryo and suspensor (Tisserat 1979).

A review of embryogenesis by Vasil and Vasil (1972) gives a historical overview on somatic embryogenesis. In 1838-1839 the concept of cellular totipotency was proposed in the Cell Theory of Schleiden and Schwann. However, no experimental evidence was given in support of these theories until 1878 when Vochting in 1878 and 1884, was able to dissect plants to smaller and smaller fragments while keeping them viable. In 1902, Haberlandt proposed the use of embryo sac fluids for inducing cell divisions in vegetative cells and the possibility of successfully cultivating artificial embryos from vegetative cells using isolated plant cells in nutrient solution (Vasil and Vasil 1972). However, it was not until 1958 that two groups working independently, Steward et al. (1958) and

Reinert(1958), were able to obtain somatic embryos from the somatic tissues of carrot (*Daucus carota*) cultured *in vitro* (reviewed by Tisserat 1979). Presently, many plants are known to produce asexual embryos *in vivo* and *in vitro* and are cited in the reviews by Tisserat (1979) and Williams and Maheswaren (1986).

The origin of the somatic embryo is believed to be through Pre-Embryonic Determined Cells (PEDC) or Induced-Embryonic Determined Cells (IEDC). The process of PEDC cells dividing directly into an embryo is known as direct embryogenesis. IEDC cells, which require redifferentiation of the cells in the embryogenic pattern by an auxin or auxin/cytokinin treatment, go through the process known as indirect embryogenesis (Sharp et al. 1980). Homologies have also been noted between the direct and indirect somatic embryogenesis and from single cell, and also between multiple cell initiation of somatic embryos (Williams and Maheswaran 1986).

Factors Influencing Somatic Embryogenesis

Factors affecting somatic embryogenesis include genetic, physiological and environmental conditions. The ability of a plant to form somatic embryos *in vitro* is heritable in some crops including potato (*Solanum tuberosum*). In alfalfa and cucumber, dominant genes have been identified as being related to high frequency regeneration. Similarly, in red clover and wheat a recessive gene or genes on certain chromosomes in the cytoplasm have been associated with regeneration capacity (Parrott et al. 1991).

The explant source and the ontogenic stage also are important in the somatic embryogenesis response of the explant *in vitro*. In general, younger or vigorously growing tissues from healthy plants are the ideal explant source (Ritchie and Hodges 1993). As shown in the summary of plants known to undergo somatic embryogenesis, most successful embryogenesis systems use explants derived from developmentally

younger plant material such as hypocotyls, immature embryos and seedling segments (Tisserat et al. 1979).

In general, modifications of the medium and culture environment are highly dependent on the plant in question. The roles of nitrogen, mineral salts, defined and undefined organics and growth regulators have all been extensively reviewed by Sharp (1980), Tisserat et al. (1979) and Vasil and Vasil (1972).

Applications of Somatic Embryogenesis

Applications of somatic embryogenesis include the process of rapid clonal propagation and its use in gene transfer systems (Parrott et al. 1991). In plant tissue culture there are three basic forms of clonal multiplication: 1) enhanced axillary branching; 2) production of adventitious shoots and roots from callus culture; and 3) somatic cell embryogenesis. It is hypothesized that expression of many more developmental genes are required for the ontogeny of somatic embryos. To be sucessful, somatic embryos must be less tolerant to mutations and epigenetic changes when compared to organogenic cultures (Parrott 1991).

The use of somatic embryos in synthetic seed technology is still in the intermediate stages of development. A synthetic seed is defined as a somatic embryo inside a coating. This coating serves as the synthetic endosperm of the seed and provides nutrients, carbon sources and growth regulators for the somatic embryo (Redenbaugh 1993). Potential applications for this system include long term storage similar to those of the zygotic seed and rapid propagation for clonal material. Techniques for obtaining somatic embryos, encapsulating material and methods and systems in crops are reviewed by Redenbaugh (1993).

PROPAGATION OF ANTHURIUM

Seeds

Propagation of anthuriums with seeds can result in an extremely heterogeneous population varying in flower color, size, form and texture. Since flowers of anthurium are protogynous, the stigma is receptive prior to anthesis. Crosses require two spadices, one receptive and one shedding pollen. Receptive stigmas are evident by a clear liquid exudate. Anthesis is evident by a powdery appearance of the spadix. Cross pollination is achieved by grasping the spadix to obtain pollen, then rubbing the stigma of the other receptive spadix. Once fertilization occurs, the spadix will appear swollen and disfigured (Kamemoto and Nakasone 1955).

To reach maturity, six months are generally required for seeds of *A. andraeanum* and ten to twelve months for seeds of *A. scherzerianum*. Fresh seeds are usually required for successful germination. Seeds of *A. scherzerianum* hybrids may be stored for 20 weeks by treating the undamaged berries with a fungicide and storing at 11 ^{o}C and 700 hPa (Bachthaler 1993). After removal of the seed by gentle squeezing of the berry, the seeds are planted on a media such as finely ground tree fern (Kamemoto and Nakasone 1955) or Cornell epiphytic-African violet mix (Croat 1979). The time required for all progeny to flower is approximately two years (Kamemoto and Nakasone 1955).

Division

Division relies on lateral shoots arising from the basal stem portion of the anthurium plant. Some cultivars produce lateral shoots easily while others produce very few. Plant growth regulators have been used to increase lateral shoot development. Benzyladenine (BA) at 1000 mg/L applied as a foliar spray to intact anthurium plants increased lateral shoot development of 'Ozaki' from no lateral shoot formation to 3.6 shoots per plant (Higaki and Rasmussen 1979). These results were confirmed and later refined by Imamura

and Higaki (1988) using the cultivar 'Mauna Kea'. Through removal of the apical portion of a juvenile plant followed by a 500 ppm GA_3 spray, shoot production of 'Mauna Kea' was increased from 3.3 shoots to an average of 5.8 shoots per plant.

Cuttings

Tip cuttings, consisting of the uppermost stem with two to three leaves, are removed from the plants and rooted in a well-aerated medium. Roots develop within two to three weeks with the first flower produced in approximately six months. Removal of the tip cutting stimulates development of lateral shoots from the mother plant. Basal cuttings utilize one or two leafless nodal cuttings taken from the bottom portion of the anthurium plant. These cuttings are placed horizontally on a well-aerated medium with plants arising from each node. Although more plant material may be generated through this method, plants take longer to develop and often take two to three years to reach full production.

TISSUE CULTURE OF ANTHURIUM

Establishment of Cultures

Seeds

Seeds are disinfested by first soaking the berry in 3% sodium hypochlorite for 15 minutes then excising the seeds and soaking in a 1% sodium hypochlorite solution for 20 minutes. Both sodium hypochlorite soaks are followed by three sterile water rinses for 30 minutes. The seed coat is then removed and the explant, consisting of the embryo and endosperm, is cultured onto the appropriate medium (Pierik 1974). Successful disinfestation has also been achieved by one soak of the isolated seed in 1% calcium hypochlorite, 10% sodium hypochlorite or LD, Laboratory Disinfestant (Alcide Corporation) at 1 part activator : 1 part base : 10 parts water (Rosario and Lapitan 1980; Zens and Zimmer 1988; Tanabe et al. 1989). Presence of a gelatinous or sticky substance

surrounding the seed often hinders the handling of the anthurium seeds with standard tissue culture tools. Removal of this substance is possible with a soak in 13% sodium carbamate solution (Maurer and Brandes 1979).

Leaf, Spathe and Spadix

Lamina, petiole, pedicel and spathe sections are generally disinfested by an initial dip in 70-95% alcohol, followed by a 10 to 30 minute soak in 1.5% to 3% sodium hypochlorite. An alternative method using a 5 minute soak in 0.1% mercuric chloride solution with 0.25 ml/L Tween 20 has also been used in place of sodium hypochlorite (Eapen and Rao 1985). Similar methods are used for explants derived from spadix tissue, in which the spathe surrounding the young spadix is disinfested, followed by the disinfestation of the spadix proper (Geier 1982). In sub-tropical and tropical areas, high humidity and warm temperature are conducive for microbial growth leading to higher contamination from field grown material. In Jamaica, a 70% alcohol dip for 45 seconds and 1.25% sodium hypochlorite soak for 15 minutes, resulted in 70% contamination. Reduction of the contamination to 10% was achieved by a pre-sterilization soak in Benlate (Dupont), a fungicide, for 15 minutes (Lightbourn and Devi Prasad 1990). Effective disinfestation has been obtained using an initial 10 minute soak in 0.14% Physan 20 (Maril Products Inc.) followed by two consecutive soaks of 30 minutes in 0.53% and 0.27% sodium hypochloride solutions with Tween 20 at one drop per 100ml (Kuehnle and Sugii 1991). Contamination percentages as low as 5% have been achieved. Cultures are rinsed in sterile water for the complete removal of the disinfesting solution. Geier (1990) recommends three consecutive rinses of 10, 30 and 60 minute durations.

Axillary Buds

Contamination is also a major problem in dealing with axillary buds. Initial reports record a contamination rate of 33% using two soaks for 20 and 45 minutes in 0.53% and 0.27% sodium hypochloride solution with Tween 20 and removal of bud scales with the aid of a dissecting microscope (Kunisaki 1980). Reduction of the soak period in the disinfesting solution and elimination of the dissecting microscope is possible through the use of LD (Laboratory Disinfectant) and Exspor (Alcide Corporation). Leaves, roots and dead plant parts are removed from the stem. The stem with axillary buds is washed under running water and soaked in dilute LD at a rate of 1 part base : 1 part activator: 10 parts water or Exspor at a rate of 1 part base: 1 part activator : 4 parts water for 5 minutes and air dried for 2 to 3 days. Buds are removed from the stem and cut into 1 cm base segments. One bud scale was removed prior to a 30 minute soak in LD or Exspor; addition of 35% isopropyl alcohol to the disinfesting solution successfully decreased contamination percentage from 29 to 10%. Two to three bud scales are removed and the base trimmed. Explants are soaked in the disinfestation solution for 5 minutes, transferred to sterile water and then to the culture medium (Tanabe and Matsumoto 1992).

Successfully disinfested explant material show minimal discoloration. Seeds or cultured embryos germinate within 4 weeks, with proliferation of callus usually occurring within 12 to 16 weeks (Rosario and Lapitan 1981). Lamina sections show signs of proliferation as early as 2 to 4 weeks (Finnie and van Staden 1986) through 12 to 16 weeks (Lightbourn and Devi Prasad 1990). Emergence of the first leaf from an axillary bud may take from 13 weeks to one year, depending on the particular cultivar.

Multiplication and Plant Regeneration

Callus

Callus culture is customarily the method used in micropropagation in the Netherlands and numerous reports have been published utilizing this method. Organogenic callus and plant regeneration have been successfully achieved using seeds, embryos and explant material of leaf lamina, petiole, spadix, spathe, and etiolated shoots (Geier 1990; Lightbourn and Devi Prasad 1990; Kuehnle and Sugii 1991; Liu and Xu 1992). Callus culture usually consists of callus induction, callus multiplication, shoot and root regeneration. In general, callus induction and multiplication are favored by addition of an auxin, usually 2,4-D and cytokinin, usually BA, to the solid or liquid medium under dark conditions. Shoot proliferation is stimulated with the removal of auxin from the medium, reduction of ammoniacal nitrogen, and increased light. Cytokinins such as 2iP (N^6 -isopentenyladenine) or kinetin (N^6 -furfuryladenine) may be required for shoot formation in some cases. Root formation usually occurs readily with shoot formation in a liquid medium devoid of plant growth regulators.

Enhanced Axillary Branching

Enhanced axillary branching without an intervening callus was described by Kunisaki (1980). In this system, lower rates of growth regulators are used for a slower and more organized shoot formation. This reduces the possibility of abnormal plants recovery due to somaclonal variation but results in slower regeneration of plants. According to Kunisaki (1980) and Kunisaki's later adaptation (personal communications), 5 to 10 lateral buds are obtained from the stem of an anthurium plant, surface sterilized and trimmed to 2 mm at the base Axillary buds within the leaf sheaths of the lateral bud are also removed and cultured.

Shoot formation is encouraged in a liquid modified Murashige and Skoog (MS) medium consisting of MS salts at 3/8 strength, 15% coconut water and 2% sucrose. After the shoots develop in 12 to 18 months top cuttings, consisting of the apex and top two or more leaves are cultured on filter paper bridges. Basal portions of the stem are placed in the modified liquid medium supplemented with 0.2 mg/L BA. Explants are kept on BA-supplemented medium for a maximum of 2 months for multiple shoot formation. Top-cuttings taken from the multiple shoots are placed on medium lacking BA and solidified with 0.18% Gelrite for shoot growth and root formation. The basal explants are subcultured again to medium lacking BA for additional shoot formation. Once shoots form, top cuttings are taken again and the remaining base discarded.

Somatic Embryogenesis

The first report of somatic embryogenesis utilized the spadix of *Anthurium scherzerianum* (Geier 1982). Somatic embryogenesis in spadix callus was induced by lowering the NH_4NO_3 to 1.25 mM in a Nitsch medium with 4.44 µM BA and 0.45 µM 2,4-D. Somatic embryos were confirmed by histological comparison with those of zygotic embryos. Similar storage products of starch and protein were observed along with calcium oxalate deposits. However, no plants were recovered through this system (Geier 1990).

Somatic embryogenesis and subsequent plant regeneration have been achieved in *A. andraeanum* hybrids. Whole lamina explants were harvested from *in vitro* grown plants and plated on a modified half-strength Murashige and Skoog medium with 2% sucrose, 1% glucose supplemented with 1.0 to 4.0 mg/L 2,4-D and 0.33 to 1.0 mg/L of kinetin. Induction of embryos and proliferation of secondary embryos occurred under complete darkness. Conversion and maturation occurred on the same basal medium plus 2% sucrose, 0.2 mg/L BA and 0.18% Gelrite under a 16 hour photoperiod (Kuehnle et al. 1992).

Petioles from *in vitro* grown plants of an *A. andraeanum* and *A. antioquiense* hybrid, plated on a modified MS medium with 2 μM BAP (6-benzylaminopurine), 2 μM zeatin and 1 μM 2iP also appeared to produce somatic embryos. Cultures are kept in the dark for 2 to 3 months at 28°C and are maintained on the same medium which may be either solid or liquid. Shoots are regenerated on a medium containing 0.5 μM IBA (indolebutyric acid) (J. Novak, personal communication).

Factors Affecting Multiplication

Genotype

First alluded to by Pierik (1974), genotype plays an important role in the multiplication and regeneration of anthuriums. Cultivar variation extends to enhanced axillary branching, in which the time of first leaf emergence may range from 13 weeks for 'Hawaiian Butterfly' to 38 weeks for 'Fuji Pink' (Tanabe et al. 1991). Genotype influence has been cited for callus formation (Geier 1990; Lightbourn and Devi Prasad 1990; Kuehnle and Sugii 1991; Liu and Xu 1992) and for somatic embryogenesis (Kuehnle et al. 1992).

As reviewed by Geier(1990), a particular genotype will usually proliferate at the same rate, on different media. Different genotypes may depend on the cytokinin component in the medium with certain genotypes reacting differently to a specific cytokinin. In addition, relationships between genotype and specific explant source are thought to exist (Geier 1990). This was confirmed by Kuehnle and Sugii (1991), in which regeneration was strongly affected by genotype and callus organization prior to regeneration.

Explant Source

Selection of the explant is often dependent upon the material available and the objective of study. For rapid clonal propagation, the use of seeds is highly discouraged due to the high variability in offspring. However, seeds may be used as a breeding tool for *in vitro* selection for temperature tolerance or high *in vitro* regeneration (Geier 1990). Seeds have also been used as the initial explant material before selection is made by the breeder. Multiplying plants produced by seeds enables the breeder to evaluate clones of one seed *in vivo* while retaining other plants of the same clone *in vitro*. Once the selection of promising cultivars is made, the material *in vitro* is available for multiplication (Tanabe et al. 1989).

For clonal propagation, use of axillary buds is a viable possibility, however few explants may be obtained and the recovery from the removal of a large stem section may be lengthy. Removal of leaf, petiole, spathe and spadix will not cause substantial injury to the plant with the exception of decreased flower yield. In general, it is suggested that young, soft leaves about half to two-thirds of the final length are most useful for *A. scherzerianum*. If leaves are allowed to lignify, callus formation will not occur (Geier 1990). Others suggest the use of very soft tissue of newly unfolded leaves. Explants should include a major vein with vascular tissue for better proliferation (Finnie and van Staden 1986). If the explants are taken from different sections of the leaf, this influences hinders callus formation in *A. andraeanum* (Geier 1990).

Commercial Value

Commercial micropropagation in 15 West European countries and Israel was conducted in an analysis by Pierik (1991). In Western Europe, a total of 248 commercial tissue culture laboratories produced 212.5 million plants in 1988. A survey for the most frequently propagated species or genera in 14 West European countries in 1988 revealed

Anthurium was ranked 14th, with three countries producing more than 100,000 anthurium plants. In the Netherlands, which accounts of 29% of the West European production, *A. andraeanum* ranks third, after Gerbera and Aster, in the quantity of plants micropropagated for the cut-flower industry. *A. scherzerianum* ranks third, after *Nephrolepis* and *Spathiphyllum*, in the quantity of plants produced *in vitro* for pot plant market (Pierik 1991). Trends for the production of micropropagated plants from 1988 to 1990 have been compiled by Pierik.

Anthurium micropropagation is developing gradually in the United States. The demand for anthurium plants is increasing, to replace plants lost to disease and to acquire new releases of cut flower and pot plants cultivars. A survey of plant tissue culture laboratories in the United States was conducted for an estimate of micropropagated anthurium plants. Fifty-two percent of the plant tissue culture laboratories surveyed responded to the survey; sixty-four percent of the responding laboratories are currently propagating anthuriums *in vitro*. Anthuriums for cut flower, potted flowering and foliage plants are included in the estimate of 2.5 to 3 million plants produced in 1993. All plant tissue culture labs surveyed use enhanced axillary branching as a part of their propagation system; enhanced axillary branching is used exclusively by 36% of these labs. Callus culture and somatic embryogenesis is used by 43% of the labs. This is a strong deviation from the Netherlands where production concentrates on callus culture as a main proliferation method. Plants sold in the United States include unrooted microcuttings, *in vitro* rooted microcuttings and acclimatized plantlets. The vast majority of the plants are sold as Stage 3 rooted microcuttings *in vitro* and Stage 4 acclimatized plants.

QUESTIONS TO BE ANSWERED

Improvement of Microtechnique Procedures for *Anthurium*

Is it possible to improve microtechnique procedures for *Anthurium*? Histological studies of the floral structures and embryogenesis have been completed in other members of Araceae. However, these studies are often hindered by the presence of mucilage in the locules (Mayo 1989; Edye 1967; Grayum 1991). The progressive thickening of the carpel walls is also cited as a problem in fixation and infiltration of the spadix (Johansen 1940). Since new fixation methods and embedding materials are currently available, a method to improve light microscopic observations of floral structures of Araceae may be possible.

Anthurium Zygotic Embryogenesis

What are the morphological and anatomical developmental stages of an *Anthurium* zygotic embryo? *Anthurium* is the largest genus in the family Araceae, yet little work has been done in studying the complete embryology of this group. Most work completed in this family has been done half a century ago using the crude techniques available during that period (Grayum 1991). *Anthurium* embryology dates back to 1905 from the work done by Campbell; however, no current study has since been completed. In addition to aiding the systematic taxonomy of the Araceae, embryological data is useful in providing a framework for the study of meristematic and organizational structure within the organism (Wardlaw 1955).

Somatic Embryogenesis in Greenhouse-Grown *Anthurium*

Is it possible to obtain somatic embryos directly from *in vivo* grown plant material? Somatic embryogenesis is the formation of the embryo structure from somatic cells closely resembling their zygotic counterparts in gene regulation and structure (Parrott 1991). Therefore, somatic embryogenesis should be able to provide a means of anthurium clonal

propagation. Procedures for obtaining somatic embryo in anthurium have been previously described for leaf blades grown *in vitro* (Kuehnle et al. 1992). The production of somatic embryos from *in vivo* conditions has been limited to spadix tissue (Geier 1982).

Histology of the Somatic Embryo

From which tissue layers does the *Anthurium* somatic embryos arise? What type of cells characterize the somatic embryo in *Anthurium*? Histological techniques have been used to characterize the cells forming callus and somatic embryos (El Maataouli et al. 1990), elucidate their origin (Halperin 1970), follow somatic embryo development (Schwendiman et al. 1988) and determine optimal timing for subculture (Michaux-Ferriere and Carron 1989).

One application of somatic embryogenesis is rapid clonal propagation and regeneration of transformed plants (Parrot et al. 1991). Histological observations of explants co-cultivated with *Agrobacterium tumefaciens* are useful in determining vascular connections between adventitious shoots and the original explant. If a connection does occur, the use of antibiotic selection on the untransformed explant may be detrimental to the transformed shoot. Determination of the number of cells forming the meristem is important in detection of a chimeral shoot (Colby et al. 1991). The layer from the plants' origin is also important in the regeneration of transgenic plants. In the study of Agrobacterium-mediated transformation on grape leaf explant, the layer of transfomation was found to be different from the layer of regeneration (Colby et al. 1991b). Histological studies of anthurium somatic embryos could yield similarly useful information.

Aim and Scope of Work

This thesis holds that procedural and technical improvements will yield basic and applied knowledge pertinant to zygotic and somatic embryogenesis in *Anthurium*. This

work presents 1) a histological method for the observation of embryo development in anthurium, 2) a complete description of zygotic embryogenesis in anthurium, 3) methodology for somatic embryogenesis in greenhouse-grown anthurium leaves, and 4) the layer of origin of the anthurium somatic embryo.

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CHAPTER 2

MICROTECHNIQUE IMPROVEMENT FOR *ANTHURIUM*

INTRODUCTION

Araceae is a large family of monocots that consists of 106 genera and approximately 3,500 species. In a recent comparison of the four current taxonomic treatments of Araceae, disparities are noted at all levels Croat (1990). These disparities may be attributed to inadequate information for certain features revelant to taxonomic groupings. Classification of Araceae is based on surveys of floral and vascular stem patterns, leaf blade nervature, embryology, seedling morphology, patterns of endothecial wall thickenings, ovule and stamen vasculature, structure of ovular and placental tricomes, occurrance of sclerotic hypodermis and resin canals in roots, presence of anastomosing laticifers and latex particles, plant chemistry, cytology, and molecular systematics (Croat 1990).

One area in which information is lacking is Araceae embryology. Most embryological studies were done in the 1900's using crude techniques and facilities present at that time (reviewed by Grayum 1991). Of the studies that have been completed, little information exists on the full spectrum of embryo development of *Anthurium*. *Anthurium* is the largest genus in the family in Araceae, with approximately 1,000 species (Croat 1992). Similar to other aroids, *Anthurium* produces a multitude of unobtrusive true flowers supported by a fleshy axil, collectively known as the spadix.

One major hinderance in *Anthurium* embryology appears to be the presence of mucilage in the locules. In the study of *Anthurium* embryology in Campbell (1905), it is noted that aqueous fixatives should be avoided due to the presence of this mucilage. Other anatomical studies of Araceae floral structure mention mucilage as problematic. Difficulties were encountered in sections of *Philodendron* ovaries, where mucilage hindered the study of gynoecial vasculature (Mayo 1989). Eyde et al. (1967) observed mucilage as a constant

feature in the araceous ovary. The source of the mucilage was hypothesized to be from the breakdown of glandular stigmatic cells and transmitting tissue of the stylar canal and placental region. Eyde et al. (1967) also observed that several layers of cells lining the locule of *Lasia* were involved in mucilage production.

The function of locular mucilage is not known, however speculation of its importance have been made and are reviewed by Eyde et al. (1967) and French (1987). Hypothesized functions include: support of the germinating pollen, nourishment of the insect pollinators, and protection and germination of the seeds (Eyde et al. 1967). Campbell (1905) suggested that the mucilage facilitates the attachment of the seed to the medium where they germinate.

Procedures for removal of mucilage from seeds have been developed for *Anthurium scherzerianum* hybrids (Maurer and Brandes 1979). A soak in a 13% crystalline sodium carbonate solution for 2.5 hours at 20 ^{o}C was found to be effective in the removal of mucilage while retaining seed viability.

Mucilages in plants usually consist of polysaccharides and proteins (Smith and Montgomery 1959). For histological studies, formaldehyde is not effective in preserving soluble polysaccharides (Hayat 1989). Tannic acid is noted to form insoluble precipitates with albumin, starch, gelatin and most alkaloidal and metallic salts. As a supplemental fixative, tannic acid also acts to improve fixation with glutaraldehyde (reviewed by Hayat 1989). Cetylpyridinium chloride (CPC) at 0.5% and 1% combined with an aldehyde fixative also has been noted to precipitate and immobilize polysaccharides in animals and seaweed (McCully 1970; O'Brien and McCully 1981).

In addition to mucilage in the locule, hardened carpel walls have been noted as a problem in anatomical studies of floral structures in Araceae. As the seed develops, the carpel wall (which becomes the berry) hardens and prevents penetration of the embedding

medium into the locule (Johansen 1940). In addition, we have observed that as the seed coat develops, it also hinders fixation and infiltration (Fig. 3.4).

New methods to aid infiltration and embedding are now available. In this paper we examine various methods to: 1) remove or fix mucilage, 2) overcome hardening of the carpel wall during development, and 3) overcome hardening of the seed coat. A complete procedure for fixation of *Anthurium* spadices is described.

MATERIALS AND METHODS

Basic Procedure

Cross sections of pollinated 'Kalapana' spadices of various ages were placed in a fixative overnight. Samples were dehydrated through a standard tertiary-butyl alcohol series, infiltrated and embedded in paraffin. Paraffin blocks were sectioned on a rotary microtome at a thickness of 16 µm. Sections were spread with 4% formalin and mounted on a slide with Haupt's solution or poly-L-lysine. Sections were stained with saffranin and counterstained with fast green (Berlyn and Miksche 1976) or toluidine blue O (Sakai 1973). Paraffin was removed with xylene.

Methods to Remove or Fix Mucilage

Both physical and chemical methods were used to remove or fix mucilage within the anthurium locule. Physical methods included the use of spadix cross sections cut into various widths for a thinner specimen. Pistils were excised from the rest of the spadix. Klerzyme and tomato pulp, compounds effective in removal of mucilage from papayas and cactus fruits, were used prior to fixation in FAA (F. Zee; K. Porter, personnel communication). A piece of spadix approximately 0.5 cm was soaked in boiling water for 5 minutes. This treatment with boiling water was also used to overcome a hardened seed coat.

Chemical methods to remove or fix mucilage in the locule included the use of tannic acid. The fixative contained 1% glutaraldehyde, 0.5 mg/ml saponin, and 2 mg/ml tannic acid in a 100 mM sodium phosphate, 50 mM potassium chloride (KCl), and 5 mM magnesium chloride (MgCl) buffer, at pH 7.0 (Hayat 1986).

Three treatments of 13% sodium carbonate were tested to determine the best procedure in removing mucilage and retain correct anatomical structure. Spadices were soaked in the sodium carbonate solution: 1) prior to fixation in FAA, 2) following sectioning and prior to mounting on slides, or 3) following sectioning and after mounting.

CPC (Cetylpyridinium chloride) was added at a concentration of 0.5% and 1.0% to 6% glutaraldehyde and modified Karnovsky (2% glutaraldehyde and 2.5% paraformaldehyde) in 0.05M sodium cacodylate buffer at pH 6.8 (Karnovsky 1965). CPC was also added to the FAA (47.5% ethyl alcohol, 5% glacial acetic acid and 3.8% formalin) at the same concentrations, however, pH was not adjusted. Spadices were cut transversely so one row of intact flowers was included in each section.

Methods to Overcome a Hardened Carpel Wall and Seed Coat

After pollination, spadices of 'Kalapana' self-pollinated plants were harvested at intervals of two weeks. To aid the infiltration process, vacuum was used during fixation and infiltration, punctures in the carpel wall were made with a needle, and the formulation of paraffin was changed from Paraplast to Paraplast X-tra. In order to provide additional support for the tissue, a harder embedding medium was used. Historesin, a glycol methacrylate from Reichert-Jung (Leica Inc. Deerfield, Illinois), may be cut thinner than paraffin blocks while producing serial sections like paraffin embedded material (Yeung and Law 1987).

Treatments employed to soften the seed coat without removal of the ovule from the pistil included soaking the spadix piece in boiling water or in concentrated sulfuric acid for 5 minutes.

RESULTS AND DISCUSSION

Methods to Overcome Mucilage

Mucilage was present in the ovular locule at all stages of development. The mucilage expands in the locule upon rehydration in the mounting solution. This mucilage covers the ovule and expands past the carpel wall, hindering observations of structures in and around the locule. Fig. 2.1 shows the botanical flower of *Anthurium*, for orientation and comparison to the cross sections of *Anthurium* spadix.

Decreasing the specimen size or increasing the infiltration period did not decrease the presence of the mucilage in the locule. The mucilage remained in the locule area or obstructed the view of ovules. Similarly, pretreating the spadix with boiling water and sodium carbonate did not result in adequate sections. Treatment of the mounted sections with the sodium carbonate solution also did not clear up the mucilage. This may be due to the adherence of the mucilage to the slide during mounting. Addition of tannic acid to the fixative also did not contain or remove mucilage in the locule.

The only treatments that were able to fix or remove mucilage in the locule were fixatives containing CPC at 1.0%. All fixatives with 0.5% CPC gave inconsistent control of the mucilage. Ranking of the treatments for control of mucilage revealed 6% glutaraldehyde plus 1% CPC (Figs. 2.2 G and H) as the best treatment followed by modified Karnovsky plus 1% CPC (Figs. 2.2 E and F) and FAA plus 1% CPC (Figs. 2.2 C and D).

Six percent glutaraldehyde and 1.0% CPC seemed to remove the mucilage from the locule. Coagulation of mucilage was evident during the dehydration of the treated spadix.

This resulted in sections free of the densely stained mucilage and enabled clear observations of the ovule (Figs. 2.2 G and H). Fixation with modified Karnovsky limited the mucilage to within the locule area. This is evident in the comparison of the spadix treated with 1.0% CPC in modified Karnovsky (Figs. 2.2 E and F) with the FAA control (Figs. 2.2 A and B). One percent CPC in FAA resulted in inconsistent preservation of the mucilage. The preserved mucilage is contained within the locules of the spadix and unpreserved mucilage are present in areas outside the locule (Fig. 2.2 A).

Since 6% glutaraldehyde with 1% CPC was effective in the preservation of mucilage in the locule, this fixative was used on selfed 'Kalapana' spadices 10 and 16 weeks after pollination. Procedures were the same as the basic procedures except spadices were cut at 10 μm instead of 16 μm. Sections from the spadices showed good preservation of the tissue and cytoplasm (Figs. 2.3 B and C). Ovules were seen easily without the hindrance of mucilage. These may be compared to the FAA-fixed spadix where the ovule is covered by the dark stained mucilage (Fig. 2.3 A). In the 10 week old ovule, the early cell divisions of the endosperm can be observed. Storage products are evident in the ovule at 16 weeks following pollination. The well developed endosperm and embryo may be seen, as well as the shoot apex (Fig. 2.3C).

Figure 2.1. Botanical flower of anthurium. For orientation and comparison to spadix cross sections. t = tepals, s = stamen, p = pistil

Figure 2.2. Photomicrographs of anthurium spadices processed with different fixative solutions. A = Cross section of an anthurium spadix fixed with FAA. B = close up of a locule in a spadix fixed with FAA. Note the mucilage (m) covering the entire locule area. C = Cross section of an anthurium spadix fixed in FAA plus 1% cetylpyridium chloride (CPC). D = Close up of the locule of the anthurium fixed in FAA plus 1% cetylpyridium chloride. Mucilage is still covering the area around the ovule. t= tepals, s = stigma, p = pistil, r = rachis, m = mucilage, l = locule, o = ovule, cp = carpel wall, f = filament

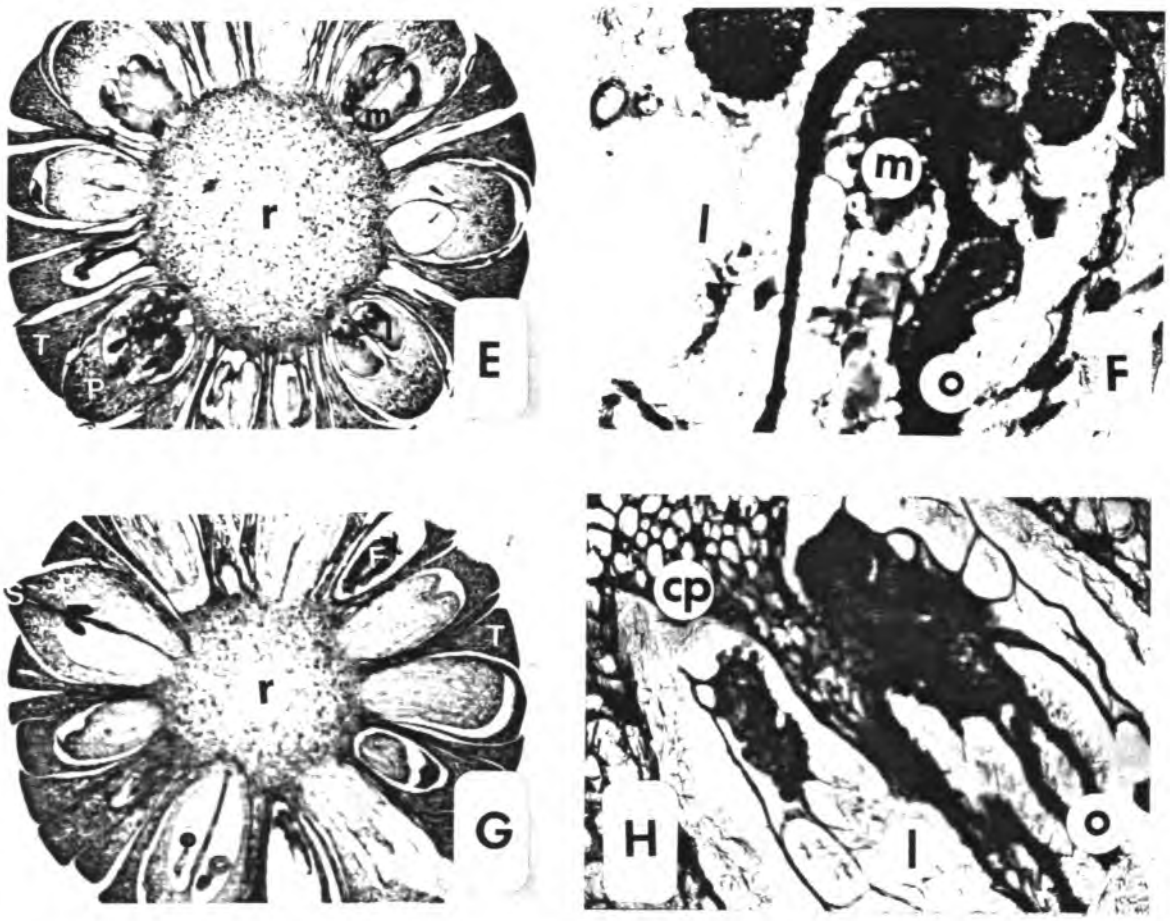

Figure 2.2 (continued). Photomicrographs of anthurium spadices processed with different fixative solutions. E = Cross section of anthurium spadices fixed in 1/2 strength Karnovsky plus 1% CPC in 0.5 M sodium cacodylate buffer. F = Close up of a locule of anthurium fixed in 1/2 strength Karnovsky plus 1% CPC in 0.5 M sodium cacodylate buffer. Mucilage present in the locule is slightly fixed, however, mucilage is external to the locule and obscures the ovule. G = Cross section of anthurium spadices fixed in 6% glutaraldehyde plus 1% CPC in 0.5 M sodium cacodylate buffer at pH 6.8. H = Close-up of the locule of anthurium fixed in 6% glutaraldehyde plus 1% CPC in 0.5 M sodium cacodylate buffer at pH 6.8. Very little mucilage is evident in the locule. The view of the ovule is unobstructed. t= tepals, s = stigma, p = pistil, r = rachis, m = mucilage, l = locule, o = ovule, cp = carpel wall, f = filament

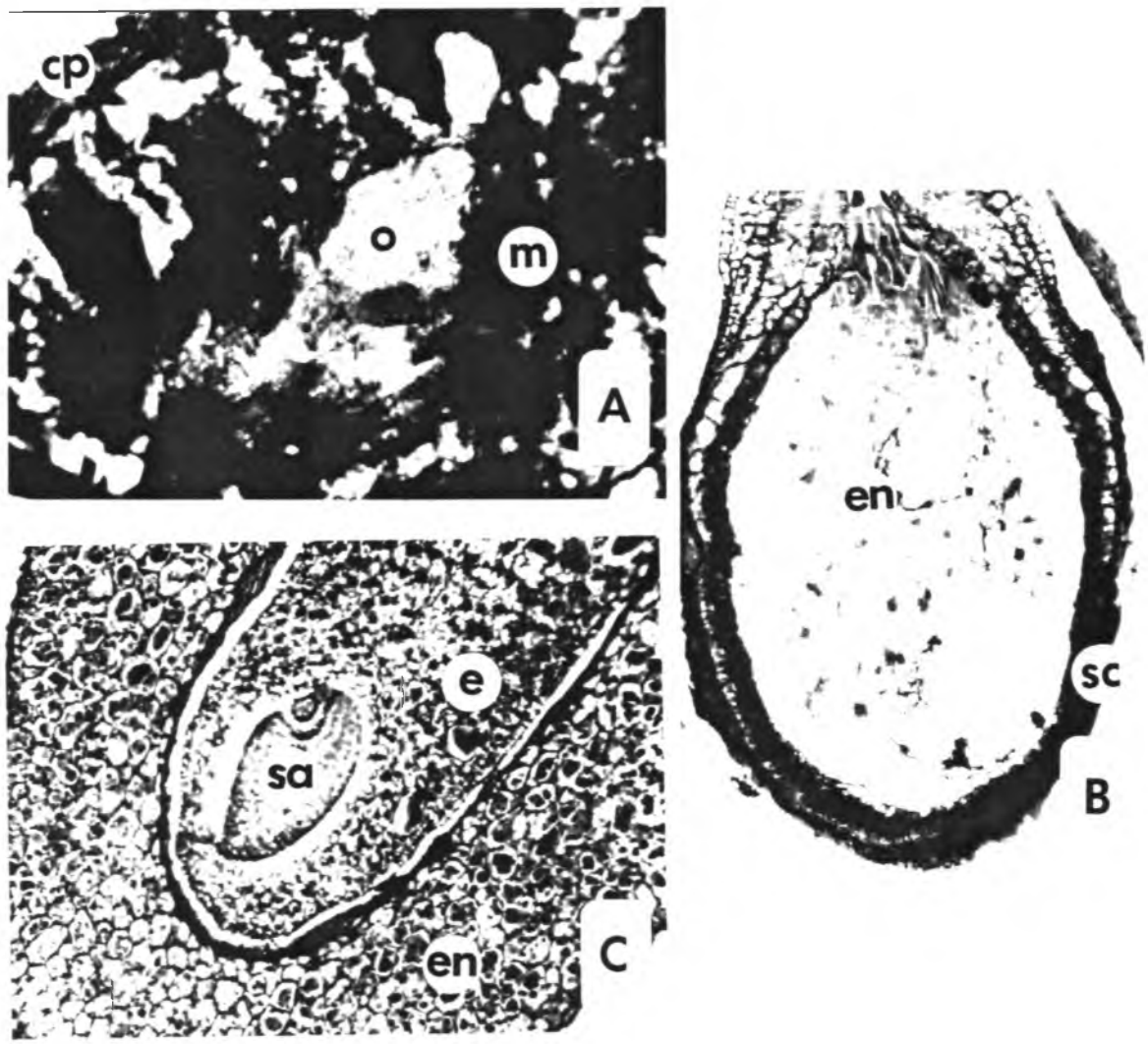

Figure 2.3. Anthurium spadices fixed in FAA and 6% glutaraldehyde plus 1% CPC in 0.5 M sodium cacodylate buffer at pH 6.8. A = Cross section of an anthurium spadix 12 weeks after pollination fixed in FAA (control). View of the ovule is hindered by the presence of the darkly staining mucilage (m). B = Cross section of an anthurium spadix 10 weeks after pollination, fixed in 6% glutaraldehyde + 1% CPC in 0.5 M sodium cacodylate buffer at pH 6.8. The view of the ovule is unobstructed by the mucilage and the formation of the endosperm (en) is evident. C = Cross section of an anthurium spadix 16 weeks after pollination, fixed in 6% glutaraldehyde plus 1% CPC in 0.5 M sodium cacodylate buffer at pH 6.8. The view of the ovule is unobstructed by mucilage, and the endosperm (en), embryo (e), and shoot apex (sa) may be observed. cp = carpel wall, m = mucilage, o = ovule, sc = seed coat, en = endosperm, e = embryo, sa = shoot apex.

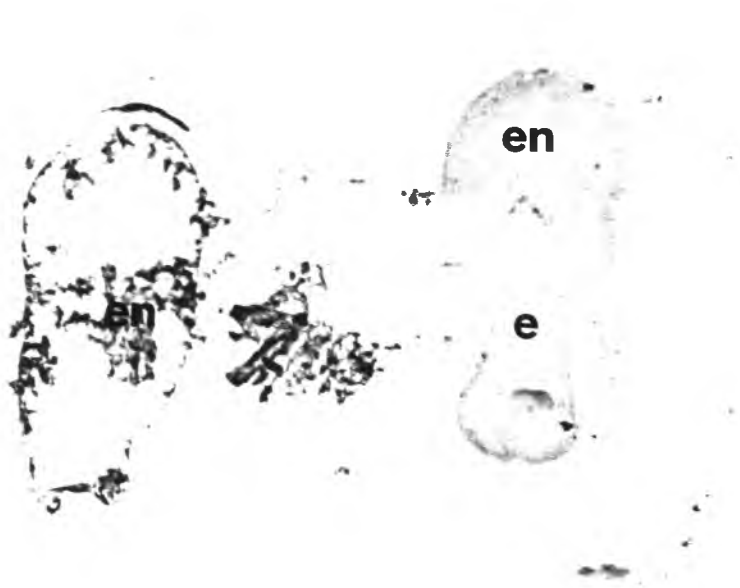

Figure 2.4. Anthurium seeds of the same age fixed with and without seed coat. Seeds were simultaneously fixed and infiltrated, one with the seed coat removed (right) and the other with the seed coat intact (left). Seeds were fixed in 1/2 strength modified Karnovsky, embedded in Historesin and stained with toluidine blue O. sc = seed coat, e = embryo, and en = endosperm.

Methods to Overcome Hardened Carpel Wall and Seed Coat

Johansen, in 1940, cited the hardening of the carpel wall as a problem in the study of Araceae floral development (Johansen 1940). Personal observations has shown this to be true. Holes were evident within the locule area of spadices 12 weeks after pollination. These holes often caused the paraplast ribbon to tear during sectioning. In addition to holes in the locule, the ovules of older spadices, approximately 22 weeks old, shattered during sectioning (Fig. 2.4). This is thought to be due to hardening of the sample by the FAA fixation (Berlyn and Miksche 1976).

Independently, none of the tested treatments were effective for complete infiltration of the area within the carpel wall. However, a treatment of 6% glutaraldehyde with 1.0% CPC was somewhat effective in reducing the size and occurence of holes in the sample. This may be due to the preservation of mucilage within the locule. Preservation of the mucilage would maintain a uniform specimen, free of any air space. Paraplast is then able to infiltrate the fixed material and fill the locule.

Methods to scarify the seed coat *in situ* were not effective for proper fixation and infiltration. The acid treatment resulted in blackening of areas on the seed that were not protected by the seed coat. Blackening color probably indicates cell damage. This is undesirable since cellular structure and anatomy is changed.

The seed coat is not a major problem until late in the development of the embryo, at approximately 22 to 24 weeks. This problem may be unique to *Anthurium*; the hardened seed coat was not identified as a hinderance in a histological survey of other aroid seeds (Kulkarni et al. 1990). Removal of the embryo from the seed is possible at this time. The embryo is readily seen at this stage of development without the use of a dissecting microscope. If sections of the embryo and endosperm are desired, only the seed coat may be removed in place of excision of the embryo. Some information may be lost when

removing the ovule from the spadix with regard to the remaining floral structure. However, the majority of the growth in the spadix should occur within the ovule region. To remedy this, separate observations of the spadix and the ovule may be made.

CONCLUSION

Three major obstacles must be overcome in the anatomical study of *Anthurium* embryology. These are the presence of mucilage, hardened carpel wall and seed coat in the developing fruit and seed. During early stages of development, up to 16 weeks following pollination, the best procedure found for fixation of anthurium spadices is the use of 1.0% CPC in a 6% glutaraldehyde 0.05 M sodium cacodylate buffer pH 6.8. This enables removal of mucilage from the observed sections and aids the infiltration of Paraplast into the locule. Cytoplasm features are well preserved with the 1.0% CPC in a 6% glutaraldehyde 0.05 M sodium cacodylate buffer pH 6.8 and may be useful in histochemical studies of the embryo ontogeny. For later stages of development it is necessary to remove the seed from the spadix and mechanically remove the seed coat prior to fixation. The use of these methods should facilitate the study of *Anthurium* embryology.

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CHAPTER 3

MORPHOLOGY AND ANATOMY OF *ANTHURIUM* ZYGOTIC EMBRYOGENESIS

INTRODUCTION

Anthurium is the largest genus in the family Araceae and is made up of approximately 1,000 members (Croat 1992). Two recent taxonomic treatments of Araceae, the genus *Anthurium* is placed in two different subfamilies. Grayum (1991) retains *Anthurium* in the subfamily Pothoideae as described by Engler (1905). Bogner and Nicolson (1991) places *Anthurium* in the subfamily Lasiodeae. Such inconsistencies in Araceae taxonomy are due to insufficient information on members of this diverse group (Croat 1990). The area of embryology, which includes gametogenesis and embryogenesis, in particular is poorly studied (reviewed by Grayum 1991). In *Anthurium*, the most recent embryological study only briefly covered embryogenesis and did not provide a time frame for embryo development (Campbell 1905).

Embryogenesis is the study of embryo development from the single-celled zygote to the matured embryo capable of germination. Higher plant embryogenesis has been studied through the use of microscopy, histochemistry, cytochemistry, autoradiography, tissue culture, biochemical techniques, and recently molecular techniques (Raghavan 1986). Embryogenesis of many plants has been reviewed in the past (Johansen 1950; Maheshwari 1950; Wardlaw 1955; Raghavan 1976; Raghavan 1986). Information on monocot embryogenesis is deficient. Embryos of the Graminae are often used as a model of the monocotyledonous embryo; however, the mature structure and the ontogeny of the Graminae embryos have few features in common with other monocot embryos (Raghavan 1986).

Investigations of Araceae floral structures have been hindered by the presence of mucilage in the locule of the flower (Campbell 1905; Eyde et al. 1967; Mayo 1989) and hardening of the carpel wall (Johansen 1940). These problems have made the study of Araceae embryology difficult. A new method is able to remove mucilage from the pistil locule (Chapter 2). However, prior to this discovery, removal of the ovule from the pistil was the best method to avoid the problem with mucilage.

Horticulturally, *Anthurium* is an important genus. Anthuriums are used as cut flowers and foliage, potted plants, and landscape plants. Commercially, anthuriums are usually asexually propagated by cuttings, divisions or tissue culture. Species of *Anthurium* are rarely used for commercial production. Instead, hybridization of species has been used to increase marketability of the plants. Within certain groups of *Anthurium,* seeds cannot be obtained due to incompatible crosses (Sheffer and Kamemoto 1976). If crosses are viable, seed formation of *Anthurium* is fairly long, 6 month in *A. andraeanum* Hort. and 10 to 12 months in *A. scherzerianum.* Ovule or embryo culture has been commonly used to rescue unviable crosses and prematurely harvested fruit (Raghavan 1986).

The aim of this study is to 1) provide basic knowledge on anthurium (monocot) embryogenesis, and 2) correlate anatomical features with embryo germination.

MATERIALS AND METHODS

Pollination and Harvest

Spadices of anthurium cultivar 'Kalapana' (formerly UH1016) were sib pollinated during January to August 1994. The pedigree for 'Kalapana' is given in Fig. 3.1 (Kamemoto et al., in press). Pollination was accomplished by grasping a spadix at anthesis between the thumb and first two fingers and rubbing the pollen on a spadix with receptive stigma. Flowers were bagged for one to two days after pollination. Plants were

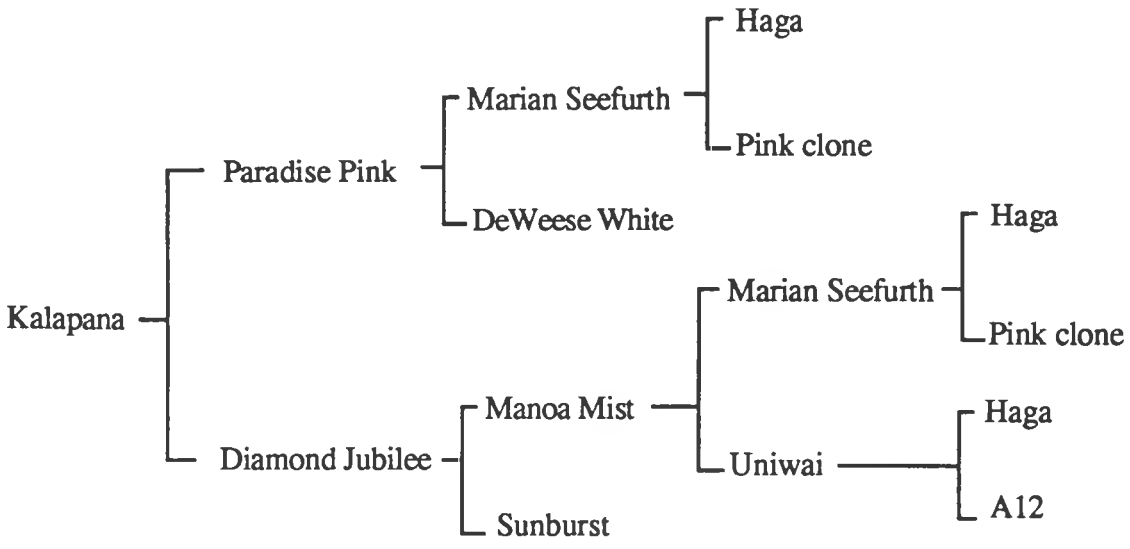

Figure 3.1. Pedigree of anthurium cultivar 'Kalapana' (formerly UH1016)

maintained at the Magoon greenhouse facilities in the shade house under 80% shade approximately 1500 foot candles.

Microtechnique

Flowers were harvested every two weeks over a 24 week period. Flowers were photographed and spadices were removed. Pistils or ovules were removed from the botanical flower and fixed in 1/2 strength modified Karnovsky, 2% glutaraldehyde and 2.5% paraformaldehyde in a 0.5 M sodium cacodylate (Na cacodylate) buffer, pH 7.0 (Kanovsky 1965). Following fixation, specimens were washed three times with the 0.5 M Na Cacodylate buffer. Specimens were dehydrated with a standard ethyl alcohol (EtOH) series from 10% EtOH to 100% EtOH, with a 10% increase every 15 minutes. Absolute alcohol change was repeated twice, then 3 ml of fresh alcohol were added to the specimens. One ml of Historesin infiltration solution (50 ml base + 1 packet activator) was added to the alcohol every other day. The infiltration solution was replaced every two days until the specimens appeared translucent. Specimens were embedded in Historesin embedding medium in inverted Beem capsules and placed under vacuum for polymerization. Once the medium hardened, the Beem capsule was removed and the block face was trimmed around the specimen.

Blocks containing the specimen were placed in an oven at 60 °C for at least 2 days before sections were made. This ensured a harder block through the removal of water and decreased the amount of chatter marks on sections. Twenty-five mm glass knives, 8 mm in width, were cut with an LKB Knifemaker type 7801B. Trimmed blocks were sectioned with a Sorval Porter-Blum MT2-B Ultra-Microtome to 4.8 µm. Sections were floated on distilled water and heated on a slide warmer at 40 °C. Slides were stained with PAS for carbohydrates and counterstained with aniline-blue-black for proteins (Feder and O'Brien

1968). Sections were observed with a Zeiss compound microscope and photographed with a Zeiss M35W camera.

Ovule / Embryo Culture

Extra flowers not used in the microtechnique experiment were used in this study. Flowers were removed from the spadix. Under a dissecting microscope, the 4 tepals and 4 stamens were removed. The isolated pistil was placed in 10% Clorox plus 1 drop / 100 mL Tween 20 on a gyratory shaker. After 30 minutes, the 10% Clorox solution was decanted off and replaced by a 5% Clorox plus 1 drop / 100 mL Tween 20 solution on a gyratory shaker for 30 minutes. The pistils were moved into sterile conditions under the laminar flow hood and rinsed three times with distilled sterile water. Under a dissecting microscope the ovules were removed from the pistil and plated onto the medium. In some cases, the embryo was excised from the ovule and plated on the same medium. Ten explants were plated per medium.

The medium consisted of 1/2 MS macronutrients, full micronutrients, 2% sucrose, 100 mg/l myo-inositol, 100 $mg \cdot l^{-1}$ myo-inositol, 25 mg l^{-1} NaFeEDTA, MS vitamins modified to include 0.4 $mg \cdot l^{-1}$ thiamine • HCl, 2% sucrose and 15% v/v coconut water. The pH was adjusted to 5.7-5.8 and 0.25 % Gelrite was added. The medium was autoclaved at 121 °C and 15 psi for 15 minutes. Following sterilization, the medium was dispensed into 100 mm petri plates.

Each plate of embryos or ovules was placed in the dark or under light at 23 °C. Cultures were monitored for contamination and seed germination.

RESULTS AND DISCUSSION

Microtechnique

Embryo development

Week 4

Four weeks after pollination none of the flowers on the spadix were receptive and the surface of the stigma was dry. Most flowers had finished anthesis (Fig. 3.2). Remnants of the filament are seen as four brown spots on each side of the stigma. The zygote, located at the micropyle end of the ovule is indicated by a stained nucleus with two prominent nucleoli (Fig. 3.3A). The nucellus is evident as a single line of cells between the zygote and micropyle. This is similar to the observation made by Campbell in which the nucellus is present as a "cap" (Campbell 1905). The inner integument of the ovule, which eventually gives rise to the inner seed coat, or tegmen, is two layers thick. The outer integument which becomes the outer seed coat, or testa, is three layers thick. Yellow deposits are present in both integuments. These are probably tannins, often present in the seed coat. Tannins are thought to protect the plant against dehydration, rotting, and damage by animals (Fahn 1982). The cell closest to the nucellus and adjacent to the zygote may be the remaining synergid (Fig. 3.3A).

The endosperm is present as a faint webbed structure in the center of the ovule. As described by Campbell (1905), endosperm formation in *Anthurium* does not form free-endosperm-nuclei. Each nuclear division is accompanied by the formation of a wall between each daughter nuclei (Campbell 1905). A mitotic nucleus of the endosperm, with a thin wall between the two nucleoli, in one section of the same ovule (Fig. 3.3B).

A section of a different ovule shows the embryo at a slightly more advanced stage (Fig. 3.3C). Here the zygote has undergone one or more periclinal divisions to become a multicelled embryo. Again, thin webbed structures indicate an early developmental stage of the endosperm. A prominent nucleus is also oberved in the middle of the ovule.

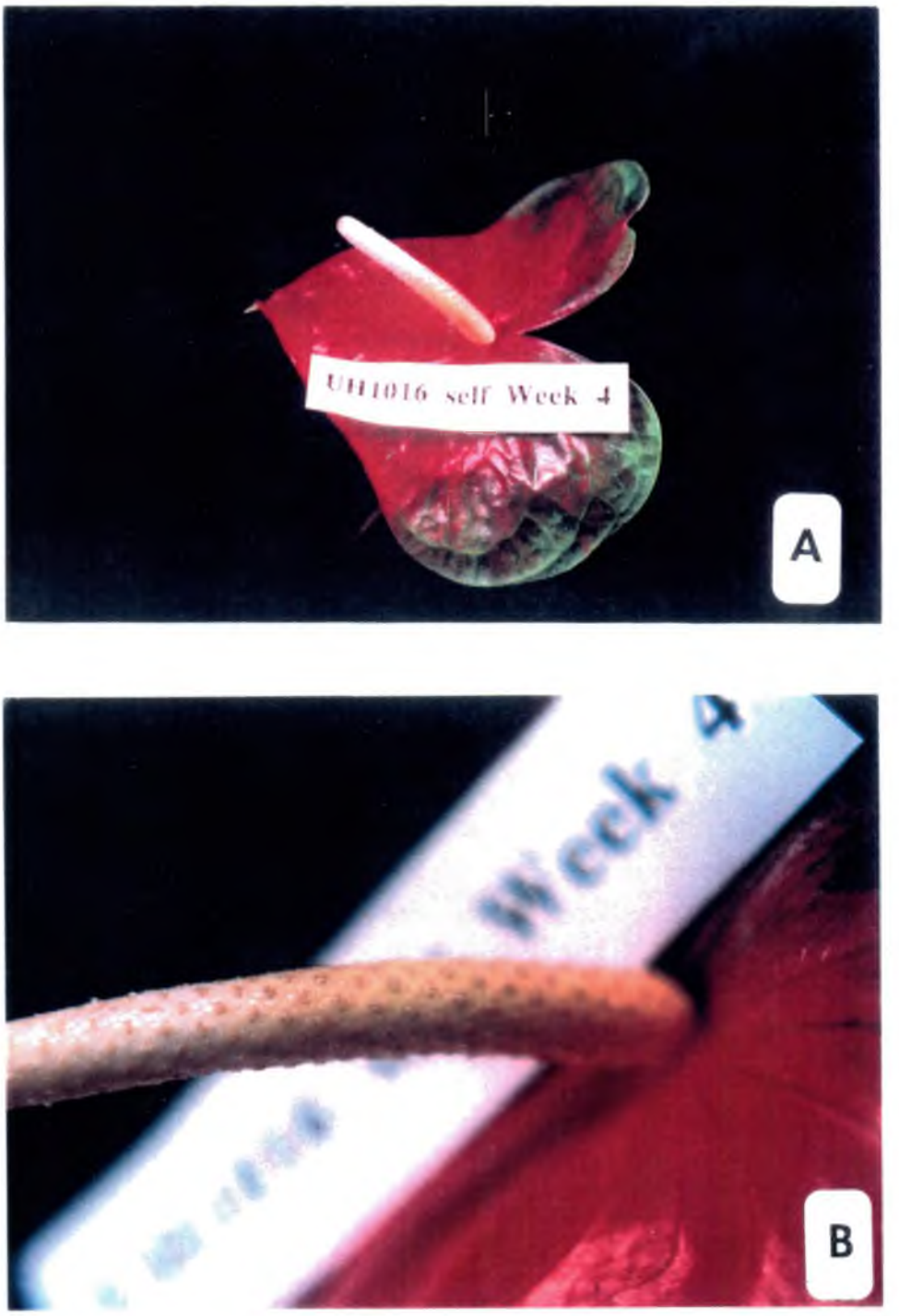

Figure 3.2. Spadix of 'Kalapana' four weeks after pollination. A = overview of anthurium flower. B = Close up view of the spadix.

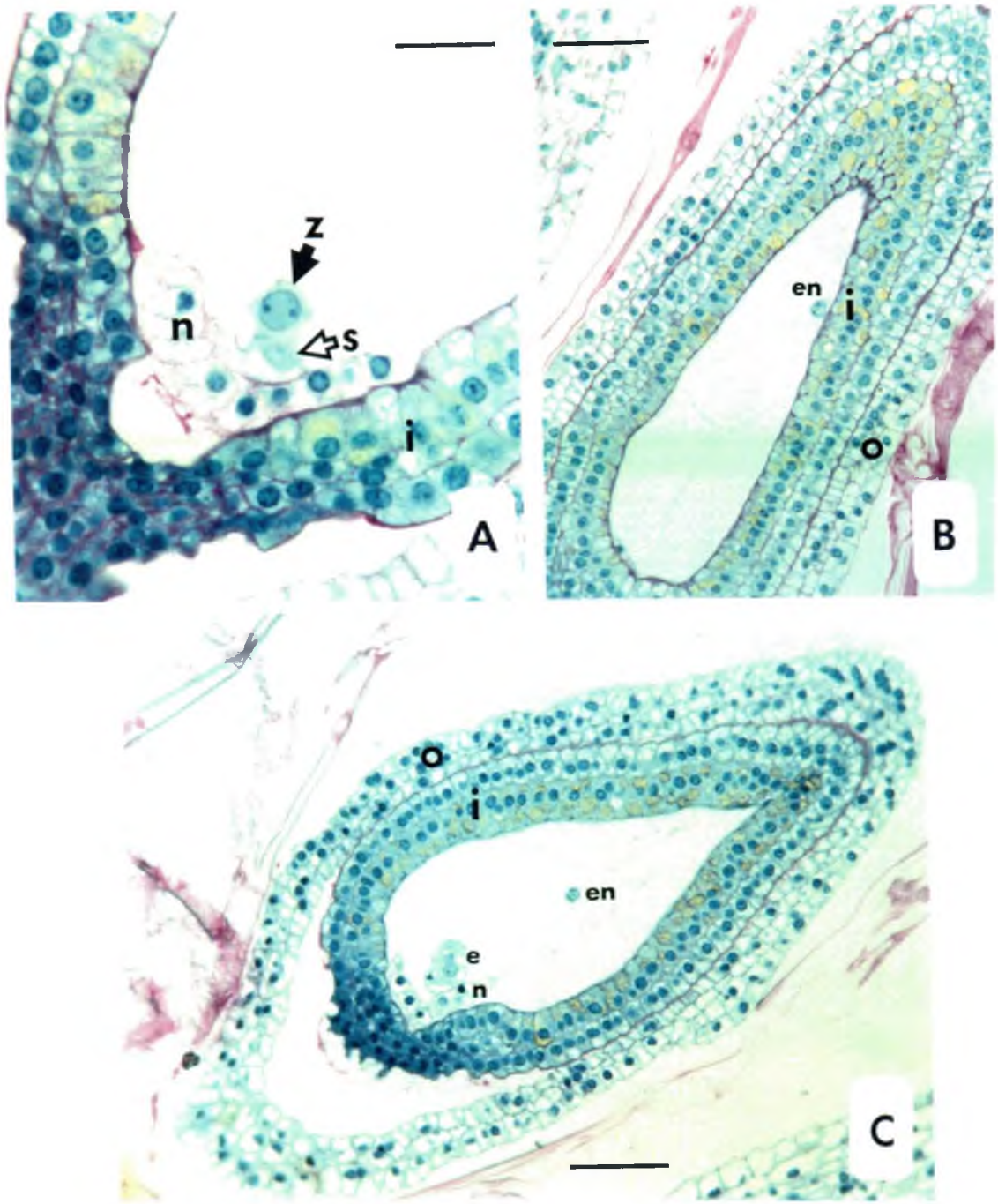

Figure 3.3. Light micrographs of ovules 4 weeks after pollination. A and B = Zygote (A) and an endosperm nucleus, bar = 25µm, (B) within one ovule. Bar = 100 µm. C = Advanced embryo and endosperm nucleus in a second ovule. Bar = 100 µm. Sections were stained with PAS (carbohydrates stain pink) and aniline blue black (proteins stain blue). n = nucellus, o = outer integument, i = inner integument, z = zygote, s = synergid, e = embryo and en = endosperm.

Week 6

Morphological observations of the spadix six weeks after pollination shows swelling of the pistil and a slight protrusion of the stigma area (Fig. 3.4A). Histological sections of the ovule show the embryo at a multicellular state (Figs. 3.4B and 3.4C). Sometransverse divisions of the two celled embryo seemed to have taken place. The walls of the endosperm are more noticeable, as are the yellow deposits in the inner integument. At this point in development most of the nucellus appears to be degraded.

Week 8

Morphologically the pistil continues to swell and protrude out of the spadix surface (Fig. 3.5A). The embryo continues to divide, mainly at the terminal end; however, the basal end seems to be unchanged (Fig. 3.5B). Divisions are both transverse and vertical. The endosperm has also divided extensively and many nuclei may be seen (Fig. 3.5C). At the chalazal end of the ovule, the endosperm is more developed. Carbohydrate is present in the intracellular spaces and protein is stored in the cells (Fig. 3.5D). This food reserve appears to be imported from the mother plant by the funiculus to the developing endosperm and embryo.

Week 10

The pistil continues to swell and slight disfigurement of the spadix is observed (Fig. 3.6A). At this stage, the basal cells are developed into a suspensor-like structure and are curved up toward the embryo (Fig. 3.6B). A multicellular suspensor derived from basal cells is typical of solonad embryogeny (Ragavan 1986). This conforms to previous descriptions of Araceae and *Anthurium* embryogeny (Johansen 1950; Grayum 1991; Campbell 1905). Interestingly, Campbell did not observe a suspensor in his study on *A.*

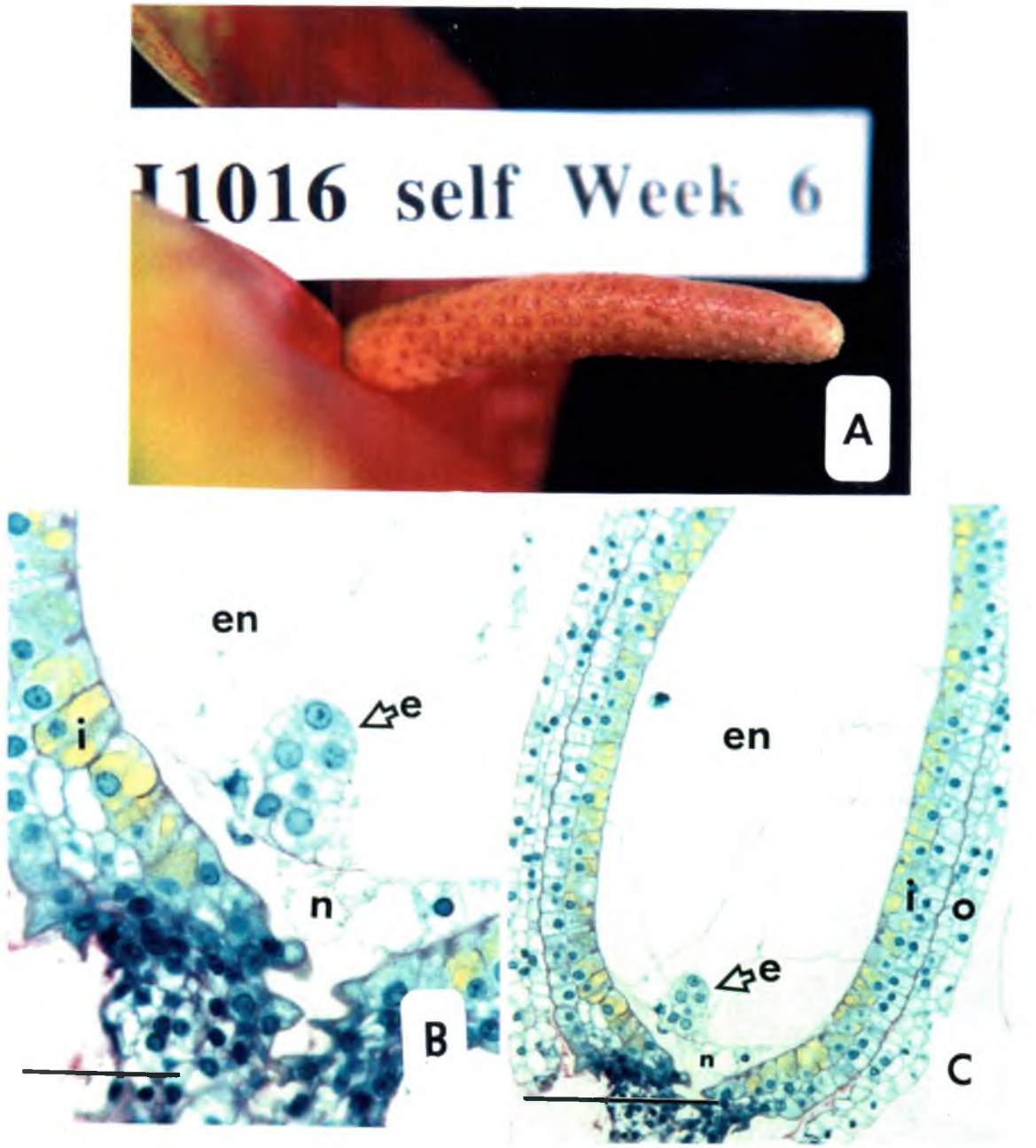

Figure 3.4. 'Kalapana' 6 weeks after pollination. A = Spadix of 'Kalapana'. B = Multicellular embryo. Embryo has undergone transverse and vertical divisions from a previous two celled state. Bar = 25 µm. C = Overview of ovule with embryo and endosperm Bar = 100 µm. Sections were stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). n = nucellus, o = outer integument, i = inner integument, e = embryo and en = endosperm.

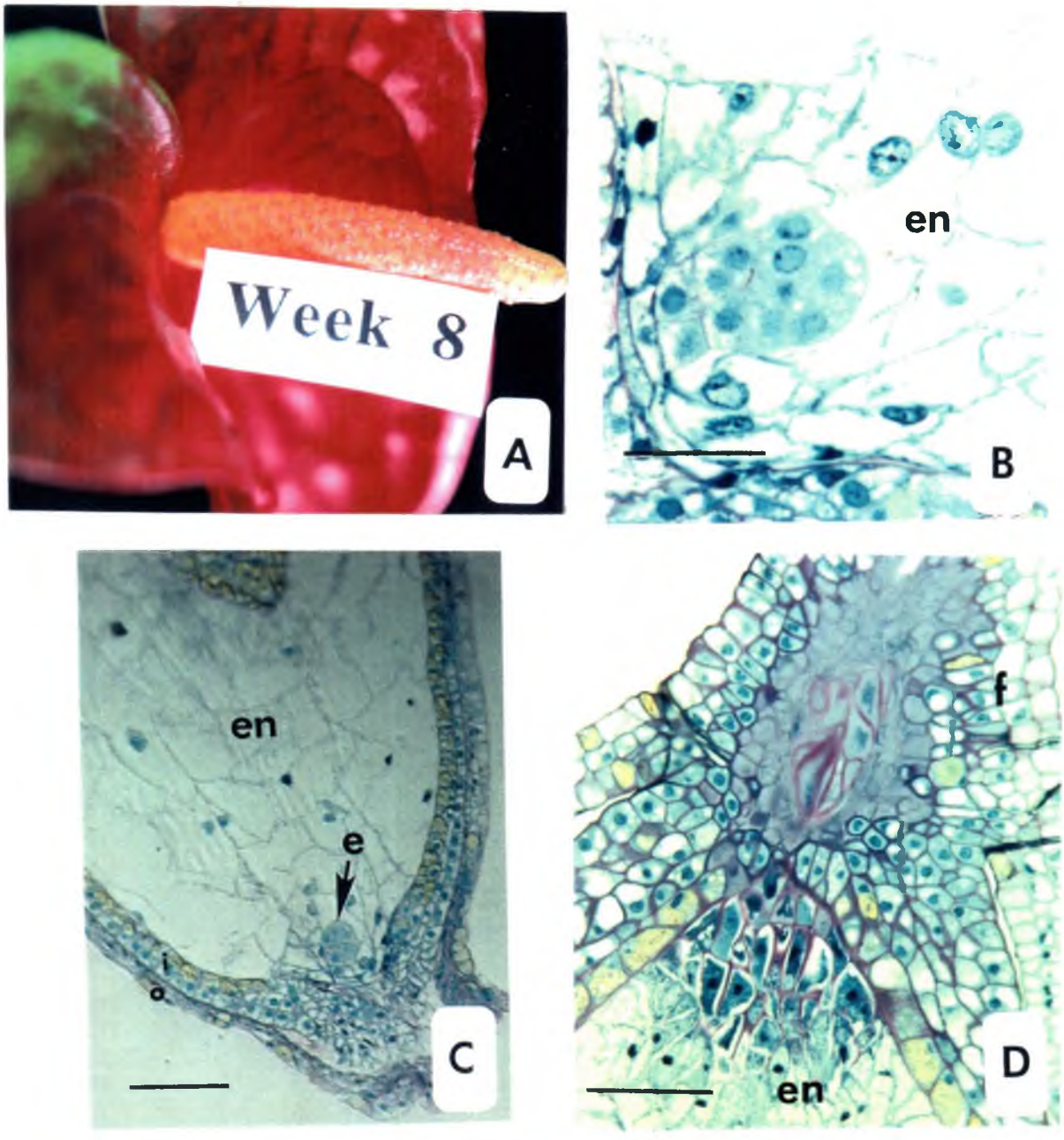

Figure 3.5. 'Kalapana' 8 weeks after pollination. A = Spadix of 'Kalapana'. B = Multicellular embryo. Embryo has undergone transverse and vertical divisions at the terminal end. Bar = 25 µm. C = Overview of ovule with embryo and endosperm. More endosperm nuclei are present. Bar = 100 µm. D = Import of food reserves through the funiculus of the ovule. Bar = 100 µm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). o = outer integument, i = inner integument, e = embryo,en = endosperm, f = funiculus and fd = food reserves.

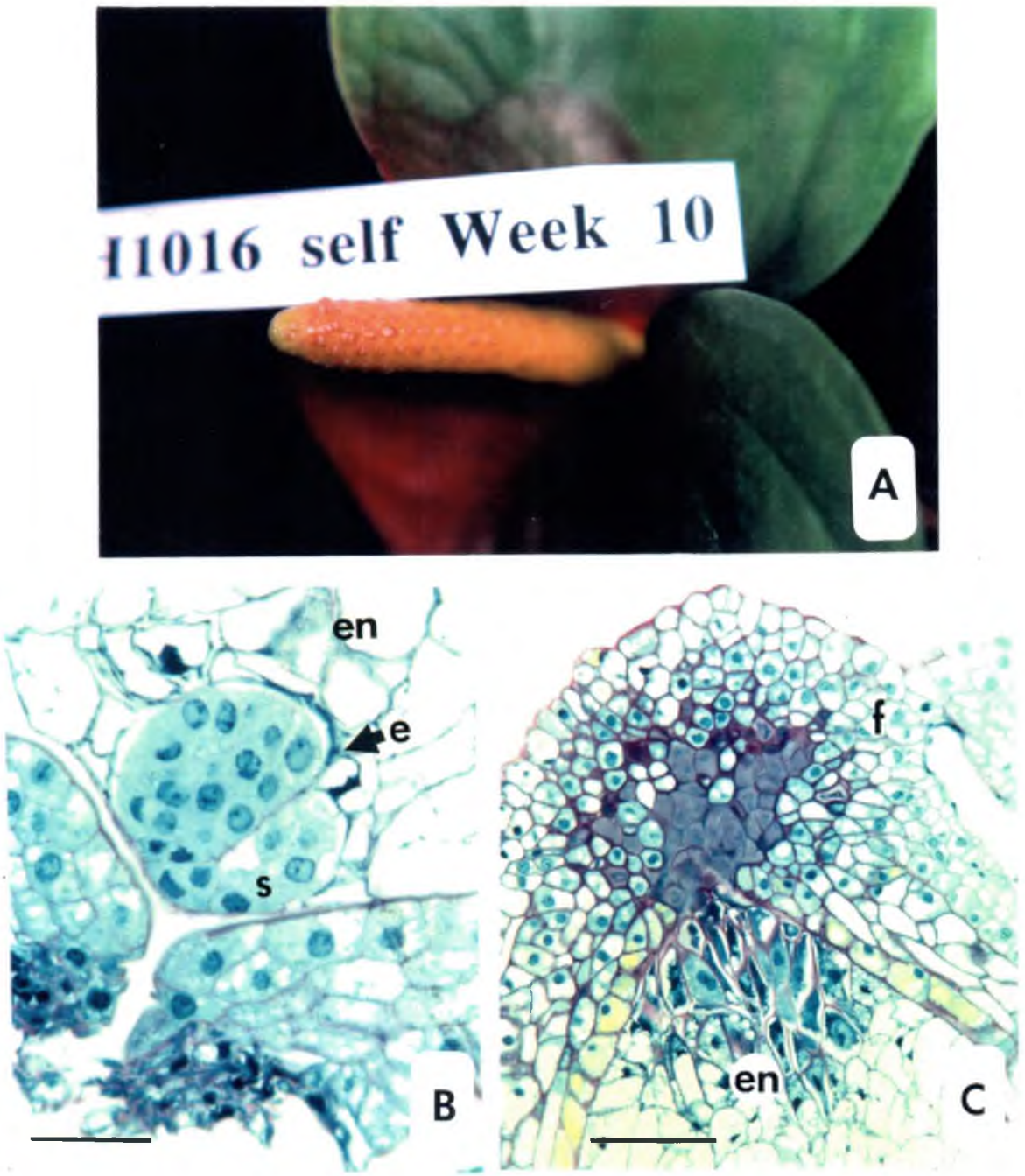

Figure 3.6. 'Kalapana' 10 weeks after pollination. A = Spadix of 'Kalapana'. B = Multicellular embryo. Suspensor is evident at basal end of embryo and is curving upward toward terminal end. Bar = 25 μm. C = Import of food reserves from the funiculus of the ovule. Bar = 100 μm. Sections stained with PAS (carbohydrates stain pink) reagent and aniline blue black (proteins stain blue). en = endosperm, e = embryo, s = suspensor, f = funiculus, and fd = food reserves.

scandens. Import of the nutrients to the endosperm is still evident at the chalazal end of the ovule (Fig. 3.6C).

Week 12 / Week 14

Twelve weeks after pollination the spadix is disfigured due to the swelling of the pistils and probable compression of the lower portion of the tepals (Fig. 3.7A). Sections of ovules taken at week 12 show a globular structure made up of highly vacuolated cells (Fig. 3.7B). However, sections taken from this date show deformed cells indicating improper fixation or infiltration. Therefore, multiple samples were sectioned at week 14 to seek a developmental stage similar to that seen in week 12 (Fig. 3.7C). A distinct suspensor is observed and the embryo has divided rapidly to form a multiple cell structure. The presence of highly vacuolated cells in the embryo is a contrast to the cytoplasmically dense cells seen earlier in development. The pink area surrounding the embryo is probably soluble carbohydrates utilized by the embryo.

A different sample sectioned from week 14 shows a larger multicellular embryo (Fig. 3.8A). In this section a distinct suspensor is still detected. The embryo proper is predominantly vacuolate cells. A region of densely staining cells is shown and is the probable origin of the shoot apical meristem. At this stage the endosperm is made up of cell with densely stained cytoplasm the development of calcium oxalate crystals is observed in the outer integument (precursor of the seed coat) (Fig. 3.8B).

Embryo maturation

Week 16

The spadix 16 weeks from pollination is disfigured (Fig. 3.9A). Tepals and pistils are slightly green in color. The embryo at this time is well developed, about 2 mm long, (Fig. 3.9b) with a shoot apical meristem (Fig. 3.10A), root meristem (Fig. 3.10B) and

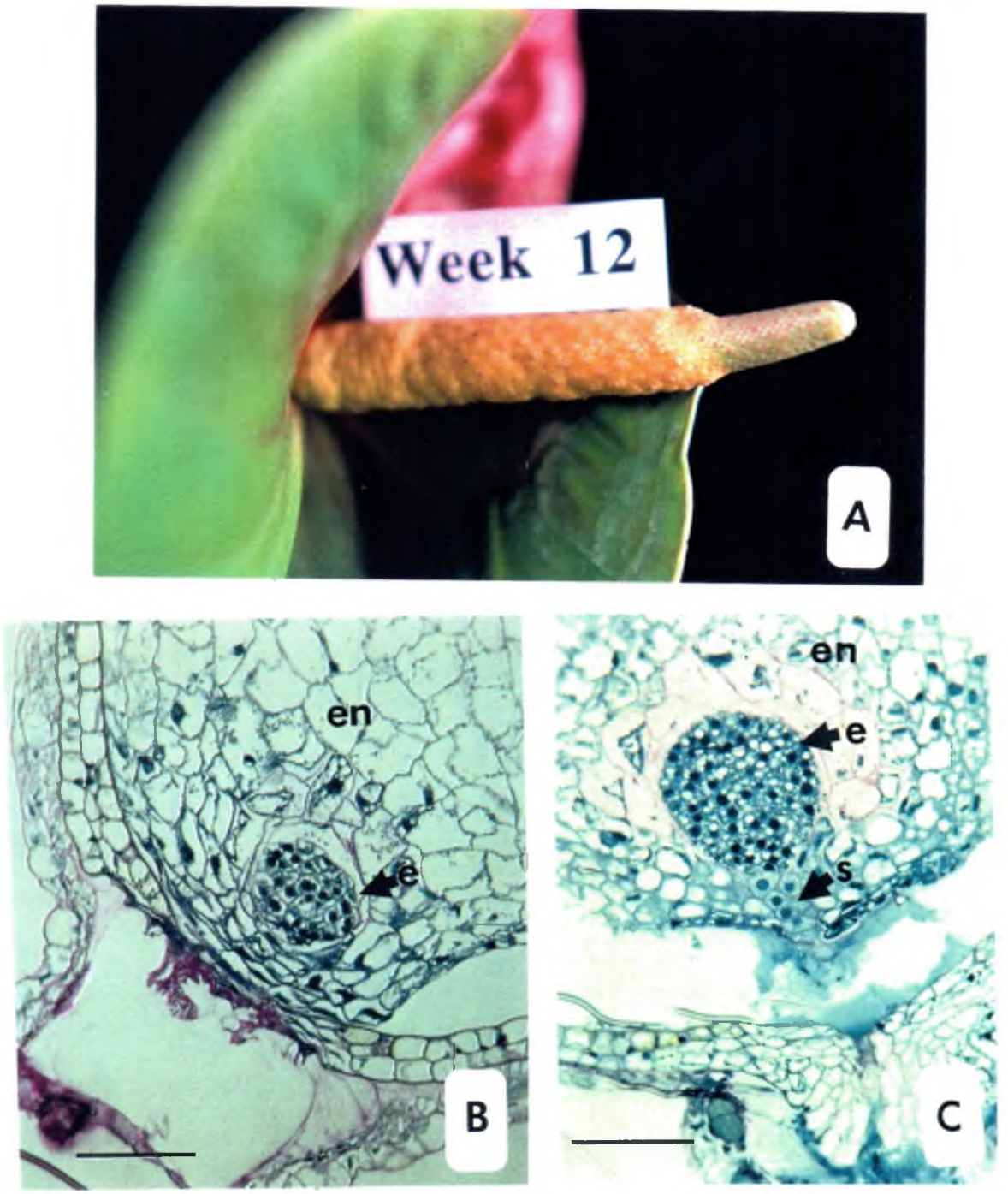

Figure 3.7. 'Kalapana' 12 weeks after pollination. A = Spadix of 'Kalapana'. B = Multicellular embryo. Specimen was not properly fixed and infiltrated. Bar = 100 µm. C = Earliest developmental stage of embryo 14 weeks after pollination. Bar = 100 µm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). e = embryo, en = endosperm, and s = suspensor.

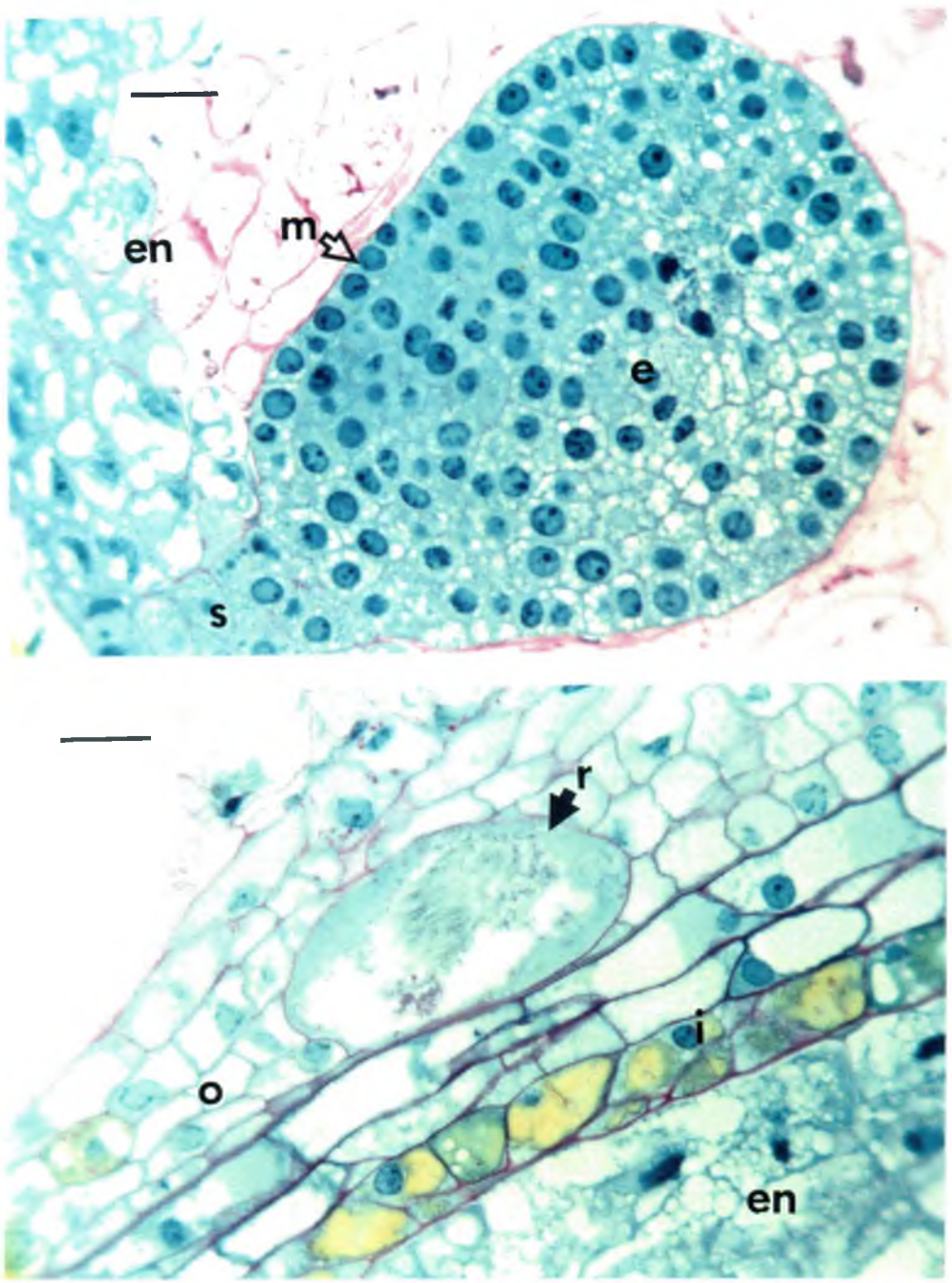

Figure 3.8. 'Kalapana' 14 weeks after pollination. A = Embryo at 14 weeks after pollination. The darkly stained cells indicate formation of the shoot apex. The pink color surrounding the embryo is probably carbohydrate breakdown and utilization. Bar = 25 µm. B = Calcium oxalate crystal (raphide) deposit in seed coat. Bar = 25 µm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). o = outer integument, i = inner integument, e = embryo, en = endosperm, s = suspensor, m = meristematic cells, and r = raphides.

Figure 3.9. 'Kalapana' 16 weeks after pollination. A = Spadix of 'Kalapana'. B = Embryo under dissecting scope.

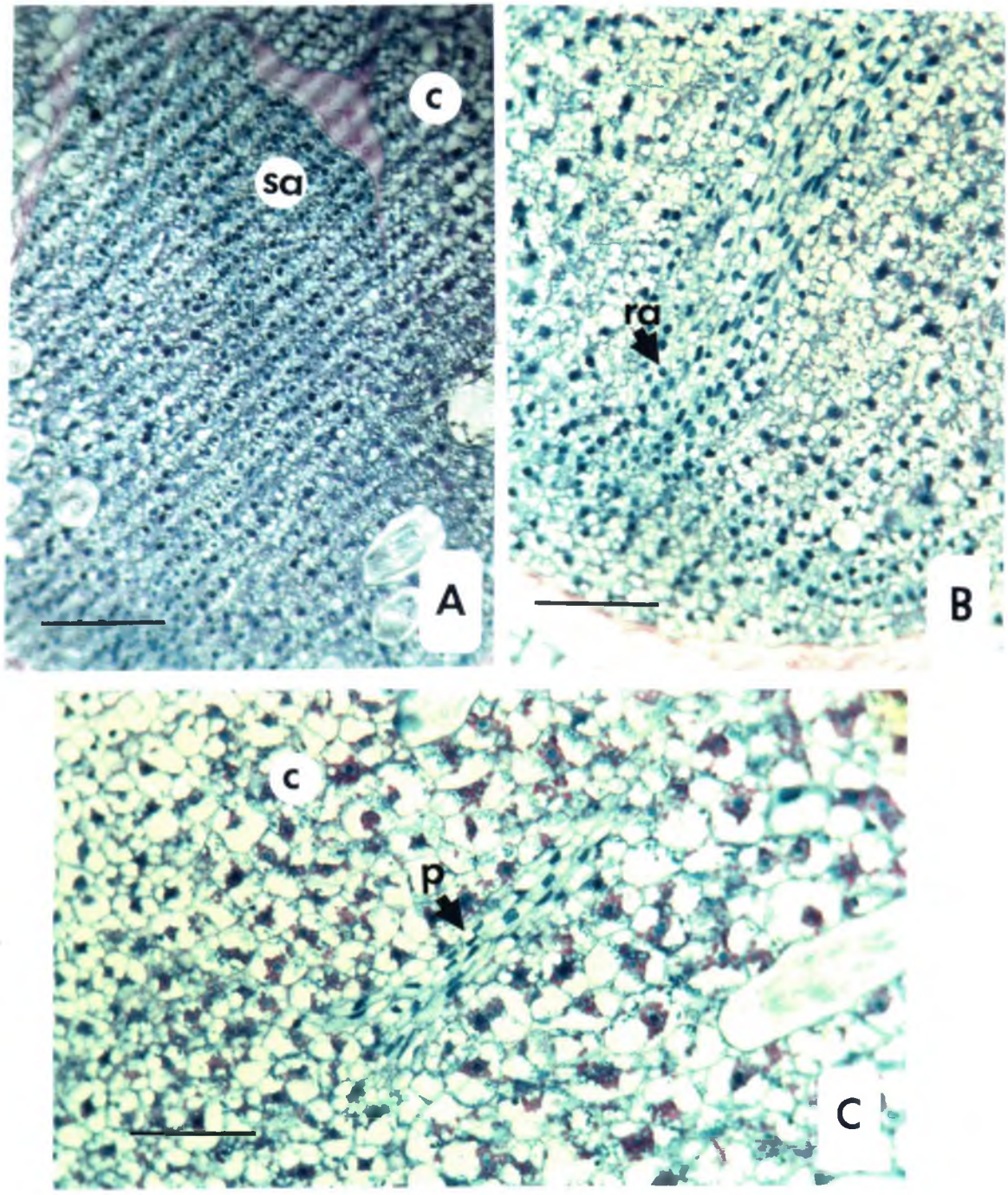

Figure 3.10. 'Kalapana' embryo 16 weeks after pollination. A = Shoot apex within embryo structure. Bar = 125 µm. B = Root apex within embryo. Bar = 100 µm. C = Procambium within the cotyledon of the embryo. Bar = 100 µm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). c = cotyledon, p = procambium, sa = shoot apex, and ra = root apex.

procambium (Fig. 3.10C). Storage products present in the embryo include calcium oxalate crystals (Fig. 3.11A), proteins and starch in the endosperm and cotyledon (Fig. 3.11B).The cotyledon is also well developed and envelops the shoot meristem within it. This encircling of the shoot meristems is shown in Figs. 3.11B and 11C. The cotyledonary node is shown on Fig. 3.11C where the cotyledon is present and wraps around the shoot meristem twice. This is the area that Campbell refers to as the "sheath-like base of the cotyledon" (Campbell 1905). Fig. 3.11C shows the top portion of this enveloping layer of cotyledon.

Week 18

At 18 weeks following pollination, growth is manifested as formation of new leaves with storage of food reserves. The suspensor is still evident at the base of the embryo between the embryo and seed coat (Fig. 3.12A). In addition to the suspensor, the cotyledon appears involved in nutrient absorption (Fig. 3.12B). The light pink region around the cotyledon is a probable indication of storage product breakdown; protein and starch reserves in the adjacent endosperm cells are depleted. The procambium of the cotyledon is also evident, extending down through the cotyledon to the shoot meristem (Fig. 3.12C).

Week 20

At week 20 the spadix is swollen and disfigured and the pistils are green (Fig. 3.13A). The seed continues to store products in the cotyledon of the embryo and endosperm (Fig. 3.13B). This is may be the result of mobilization of the nutrients from the mother plant to the developing sink. The cells of the cotyledon are rich in starch, as indicated by the pink stain, and the endosperm predominantly contains protein, as indicated

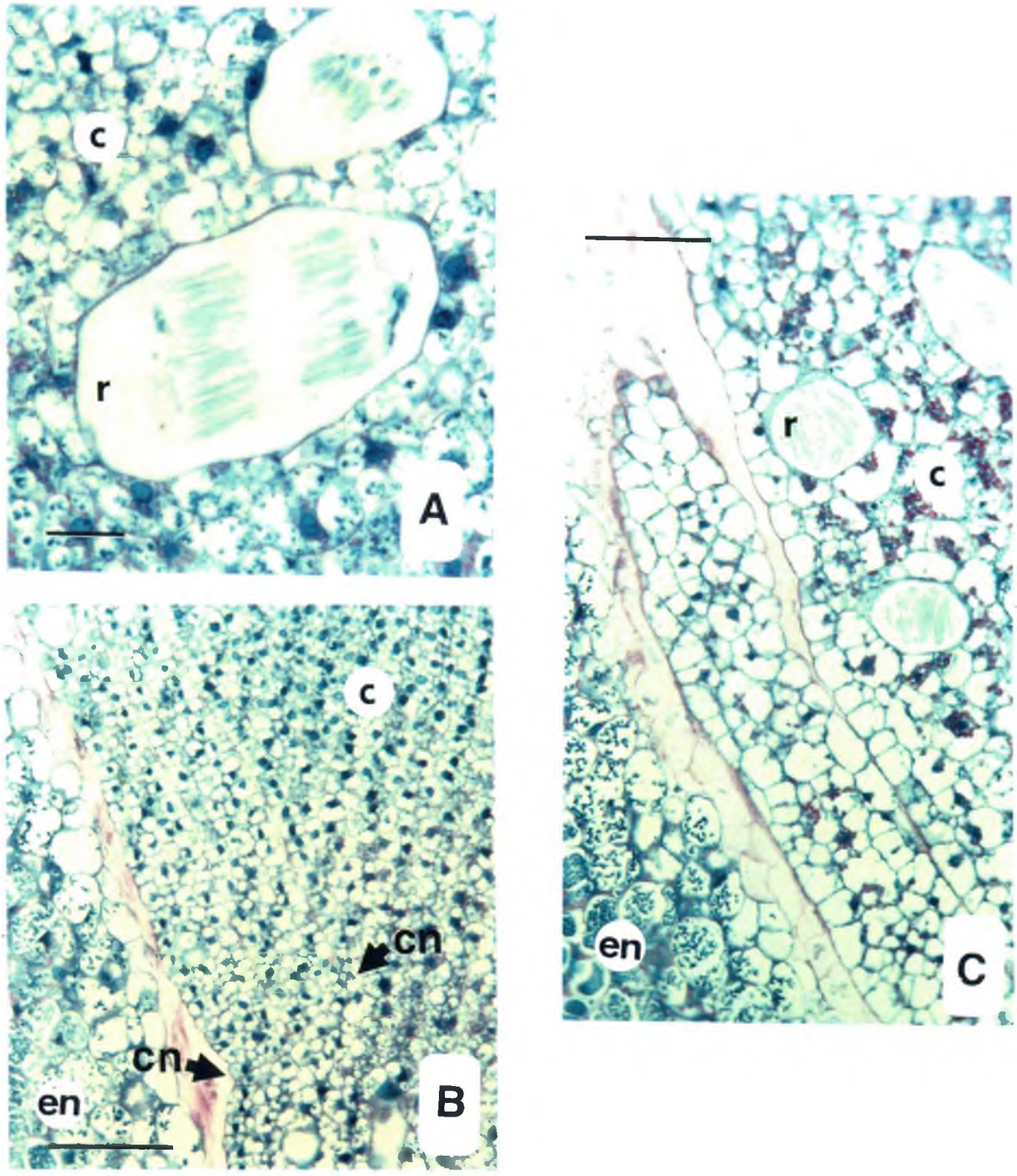

Figure 3.11. 'Kalapana' 18 weeks after pollination. A = Calcium oxalate crystal (raphides) deposits in the embryo. Bar = 25 µm. B = Cotyledonary node of the embryo. Point at which the cotyledon wraps around itself and envelopes the shoot apex. Bar = 100 µm. C = Top portion of the enveloping cotyledon. Bar = 100 µm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). c = cotyledon, cn = cotyledonary node, en = endosperm, and r = raphides.

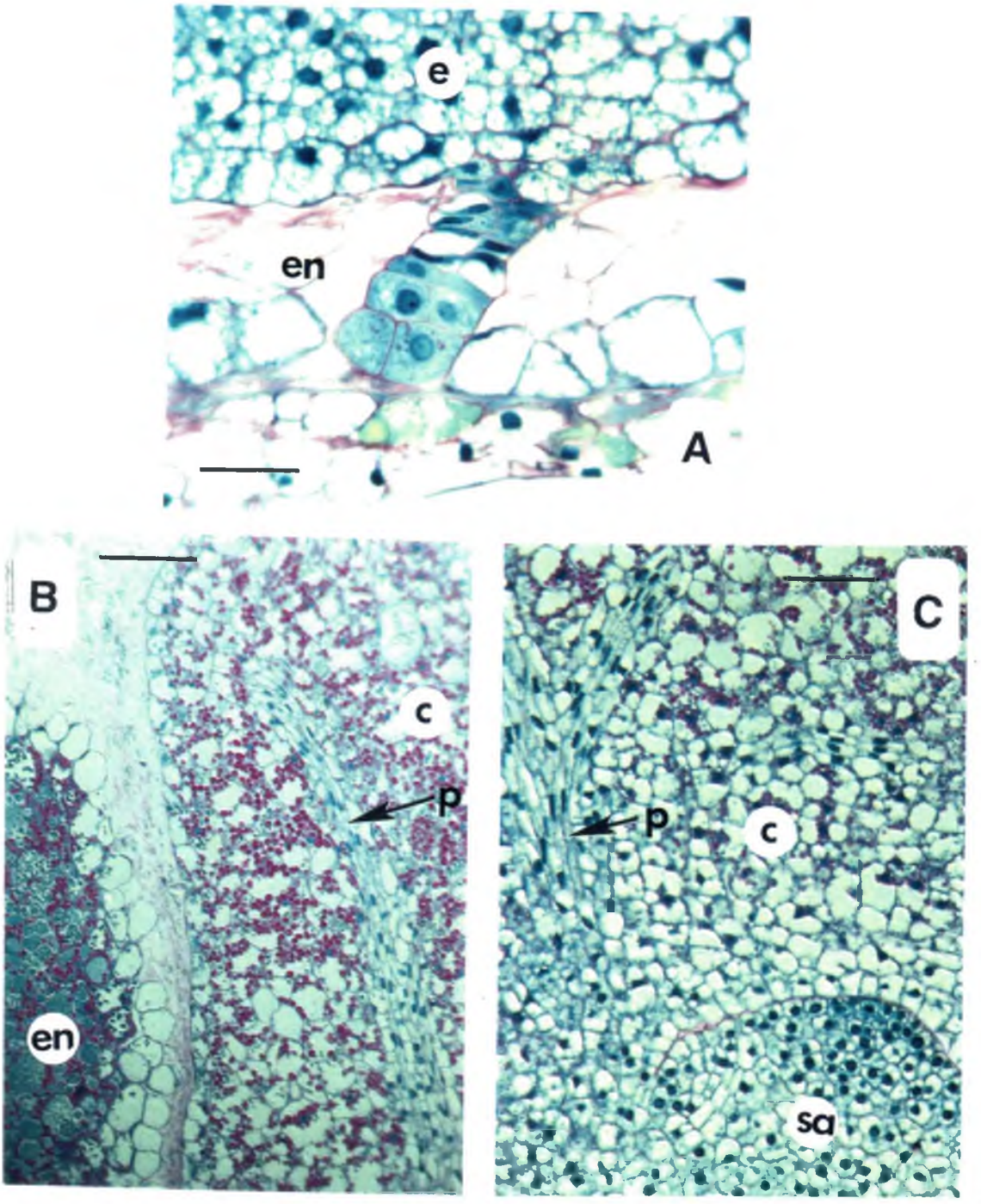

Figure 3.12. 'Kalapana' 18 weeks after pollination. A = Suspensor of embryo. Bar = 125 µm. B = Cotyledon with procambium and endosperm. Bar = 125 µm. C = Procambium from the cotyledon connecting to the shoot apex region of the cotyledon. Bar = 125 µm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). s = suspensor, c = cotyledon, e = embryo, en = endosperm, p = procambium, and sa = shoot apex.

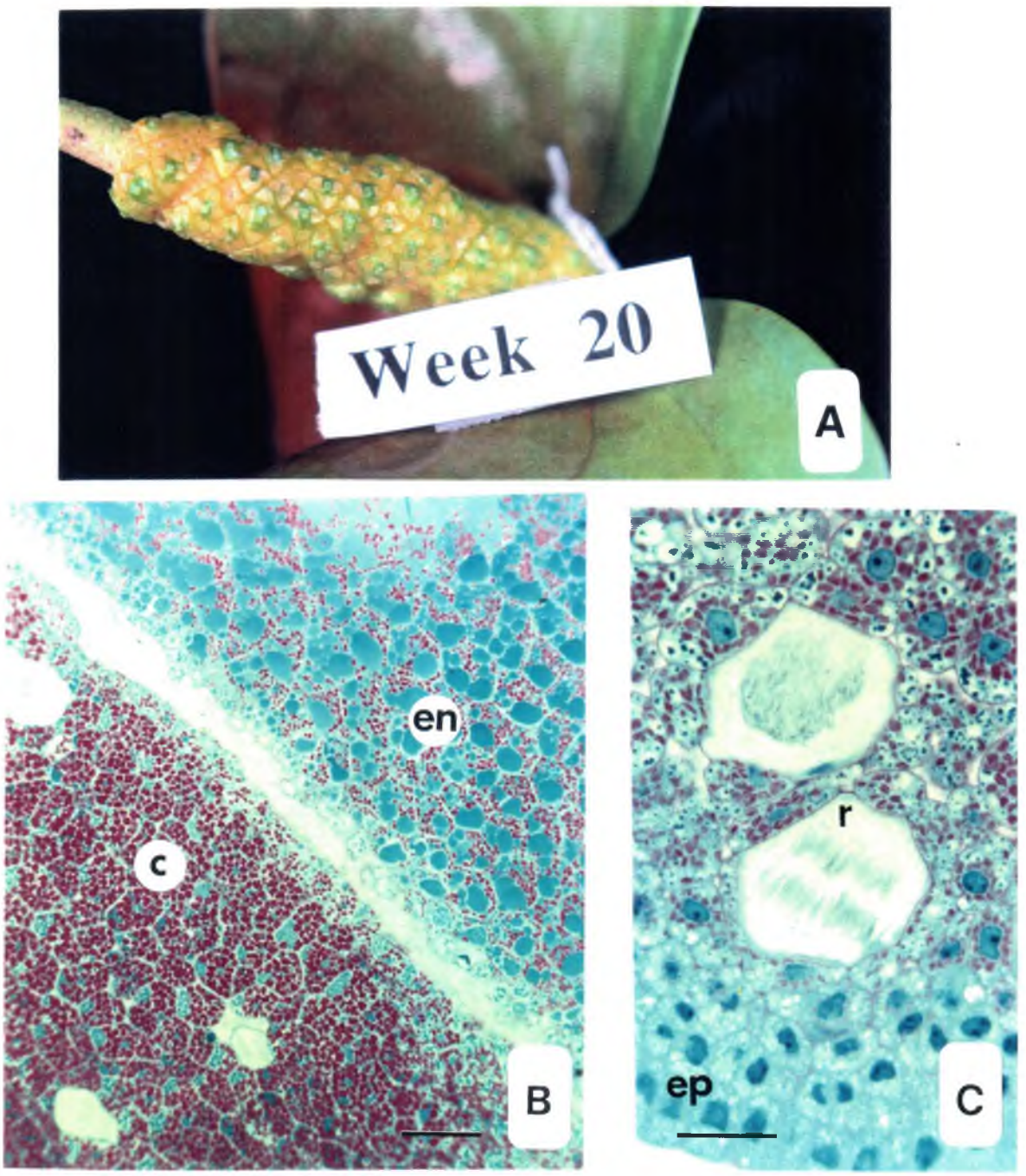

Figure 3.13. 'Kalapana' 20 weeks after pollination. A = Spadix of 'Kalapana'. B = Storage products of the cotyledon and endosperm. Bar = 125 µm. C = Calcium oxalate crystal deposits (raphides) and epidermis of the embryo. Bar = 25 µm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). c = cotyledon, en = endosperm, ep = epidermis, and r = raphides.

by the blue stain. Calcium oxalate crystals are present in the cotyledon as well as at the base of the embryo (Fig. 3.13c).

Common to members of Araceae such as *Colocasia, Alocasia* and *Xanthosoma*, these calcium oxylate crystals are in the form of raphides (Sakai and Hanson 1974). Raphides in the anthurium embryo appear as two sets of parallel crystal formation in one cell and a disorganized bundle in the adjacent cell.

The root and shoot meristems are well formed. Within the shoot meristem region the leaf primordium is present next to the apical meristem (Fig. 3.14A). In an earlier section the procambium connection between the shoot, root and cotyledon was evident (Fig. 3.14B). A close up of the root shows the formation of a procambium within the root (Fig. 3.14C). The suspensor is still present and visible at the base of the embryo. A close up view of the suspensor is given in Fig. 3.14D.

Week 22

At week 22, some of the pistils on the spadix are yellow in color and could be considered a ripe berry (Fig. 3.15A). The embryo continues to store proteins and starch in the endosperm and cotyledon. The leaf primordium is more developed and is located in the area in which the cotyledon wraps around itself (Fig. 3.15B). The shoot and root vascular connection is evident in Fig. 3.15C. A suspensor, which was present in all previously viewed specimens, could not be detected in samples taken from week 22. The suspensor may have broken down after the embryo reached maturity or it may have been crushed against the seed coat after the embryo reached the final height.

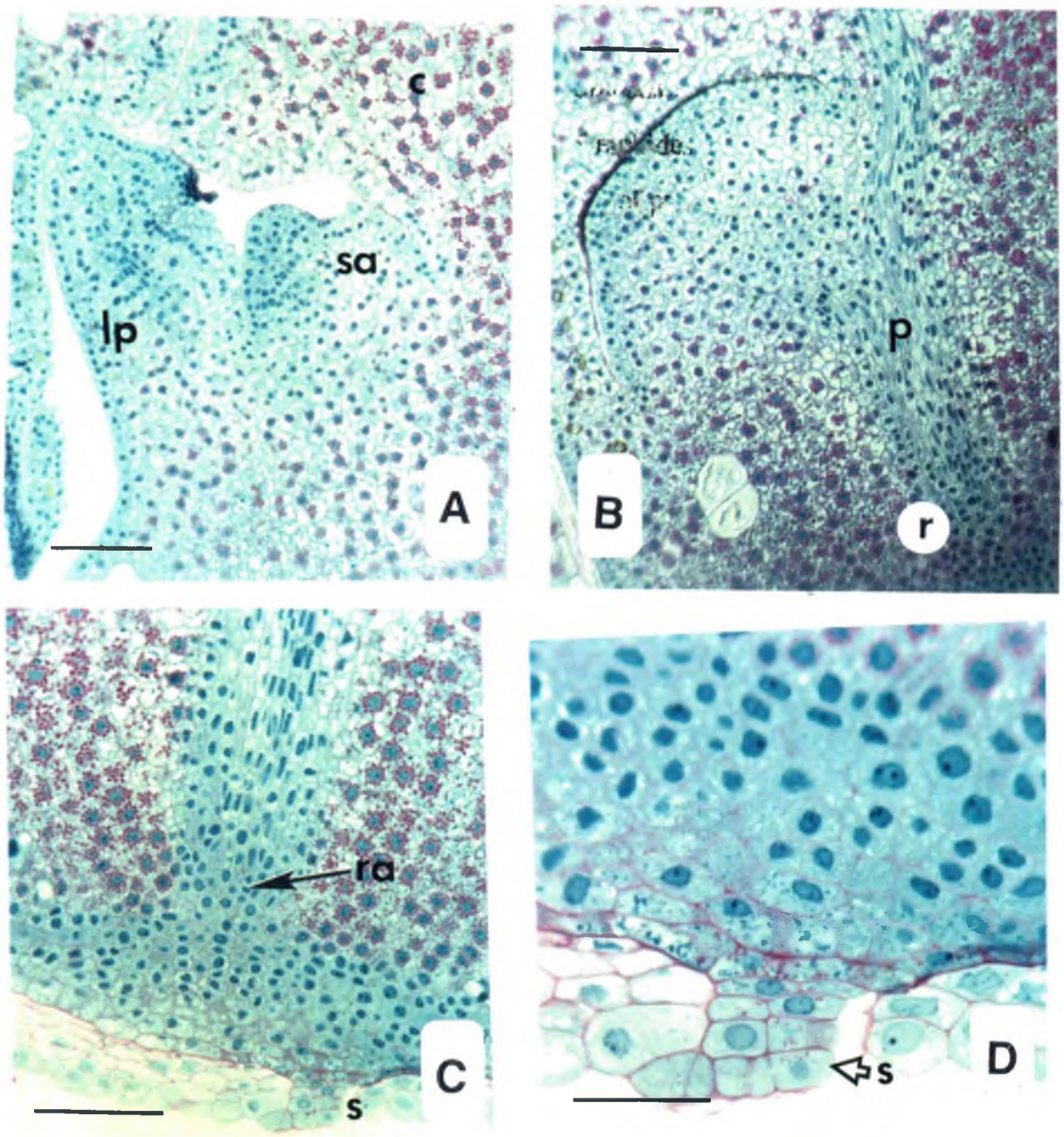

Figure 3.14. 'Kalapana' 20 weeks after pollination. A = Shoot apex and leaf primordium in embryo. Bar = 125 µm. B = Procambium from cotyledon connecting to shoot apex region. Bar = 125 µm. C = Root apex of the cotyledon. Bar = 100 µm. D = Close up of embryo suspensor. Bar = 25 µm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). sa = shoot apex, lp = leaf primordium, c = cotyledon, p = procambium, s = suspensor, and ra = root apex.

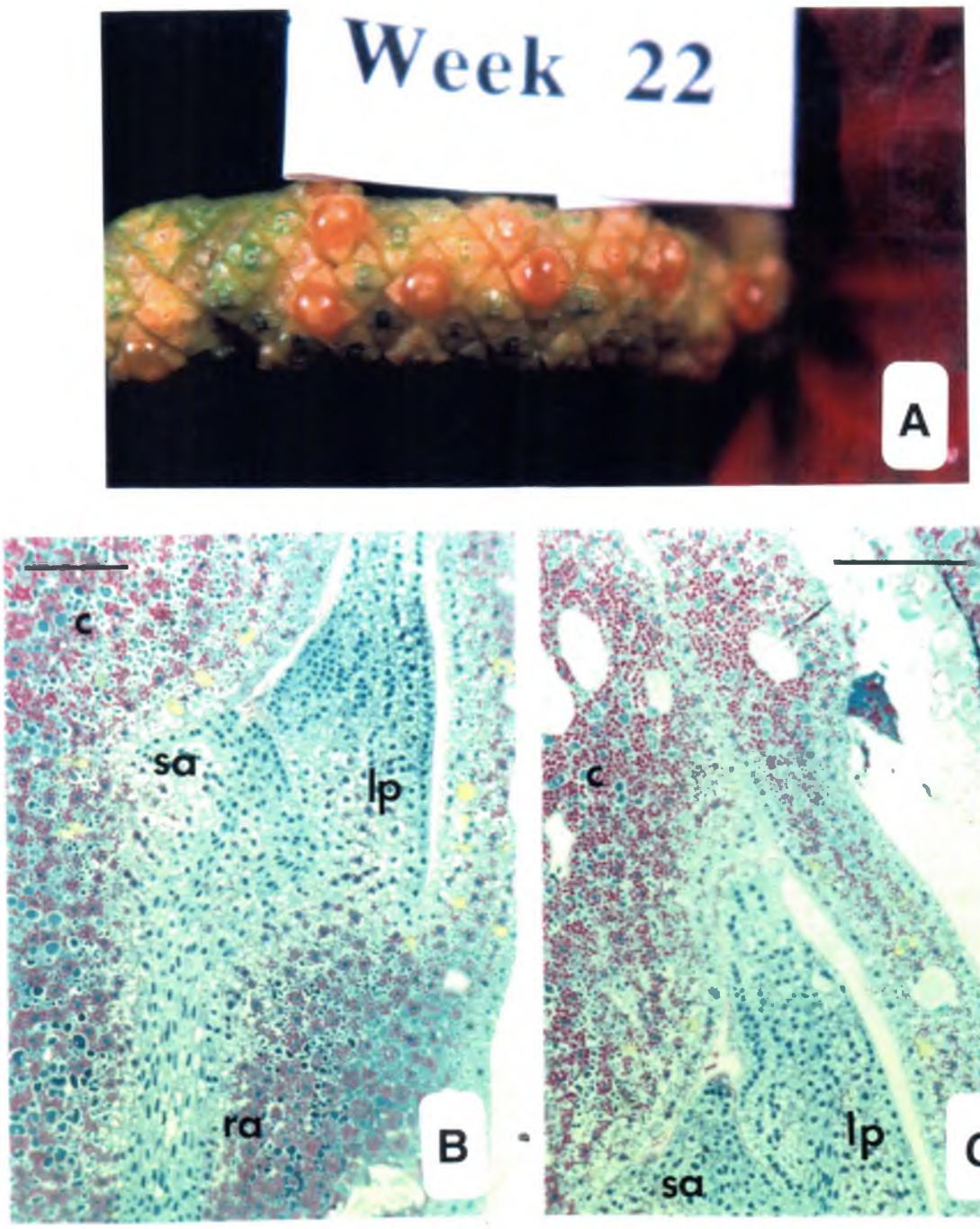

Figure 3.15. 'Kalapana' 22 weeks after pollination. A = Spadix of 'Kalapana'. B = Shoot apex and cotyledon enveloped by cotyledon. Bar = 125 μm. C = Shoot apex and leaf primordium connected to root apex by procambium. Bar = 100 μm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). sa = shoot apex, lp = leaf primordium, c = cotyledon, and ra = root apex.

Week 24

At week 24, all berries are yellow and considered ripe (Fig. 3.16A). The embryo is fully matured and is approximately 4 mm in length (Fig. 3.16B). The leaf primordium is larger (Fig. 3.17A) and the xylem tracheids are present in the vascular system (Fig. 3.17C). Storage products in the cotyledon and endosperm are shown in Fig. 3.17B. The cotyledon contains starch with some protein bodies while the endosperm contains a mixture of starch and proteins in seemingly equal amounts. The cells of the endosperm and cotyledon are less dense and it appears some of the storage products are being utilized.

Ovule and Embryo Culture

Three of 22 (14%) ovules, aged 8 weeks post-pollination, formed callus after approximately 10 weeks when cultured under light. In the dark, one of 23 (4%) of the ovules had germinated indicated by an emerged radicle. The origin of the callus from the ovules cultured in the light was not determined. In one of the ovules it appeared that the callus arose from the outermost layer of the ovule; however in another ovule the callus appeared to emerge from within the ovule, possibly from the embryo.

Ovules aged 12 weeks, but not 10 weeks, germinated under dark and light conditions. Two of 20 (10%) ovules in the dark and 2 of 21 (9.5%) ovules germinated under light conditions. Ovules grown under light formed callus while ovules grown in the dark germinated normally.

Excised embryos cultured at week 14 had a recognizable cotyledon, however, many of the embryos were not green, indicating chlorophyll had not developed. Older embryos were cultured and isolated because less developed embryos could not be easily isolated. Under dark conditions, 12 of 27 (44%) ovules and 20 of 20 (100%) of the excised embryos germinated. Under light conditions, 5 of 9 (56%) of the ovules and 9 of 11

Figure 3.16. 'Kalapana' 24 weeks after pollination. A = Spadix of 'Kalapana'. B = Fully matured embryo approximately 4 mm in length.

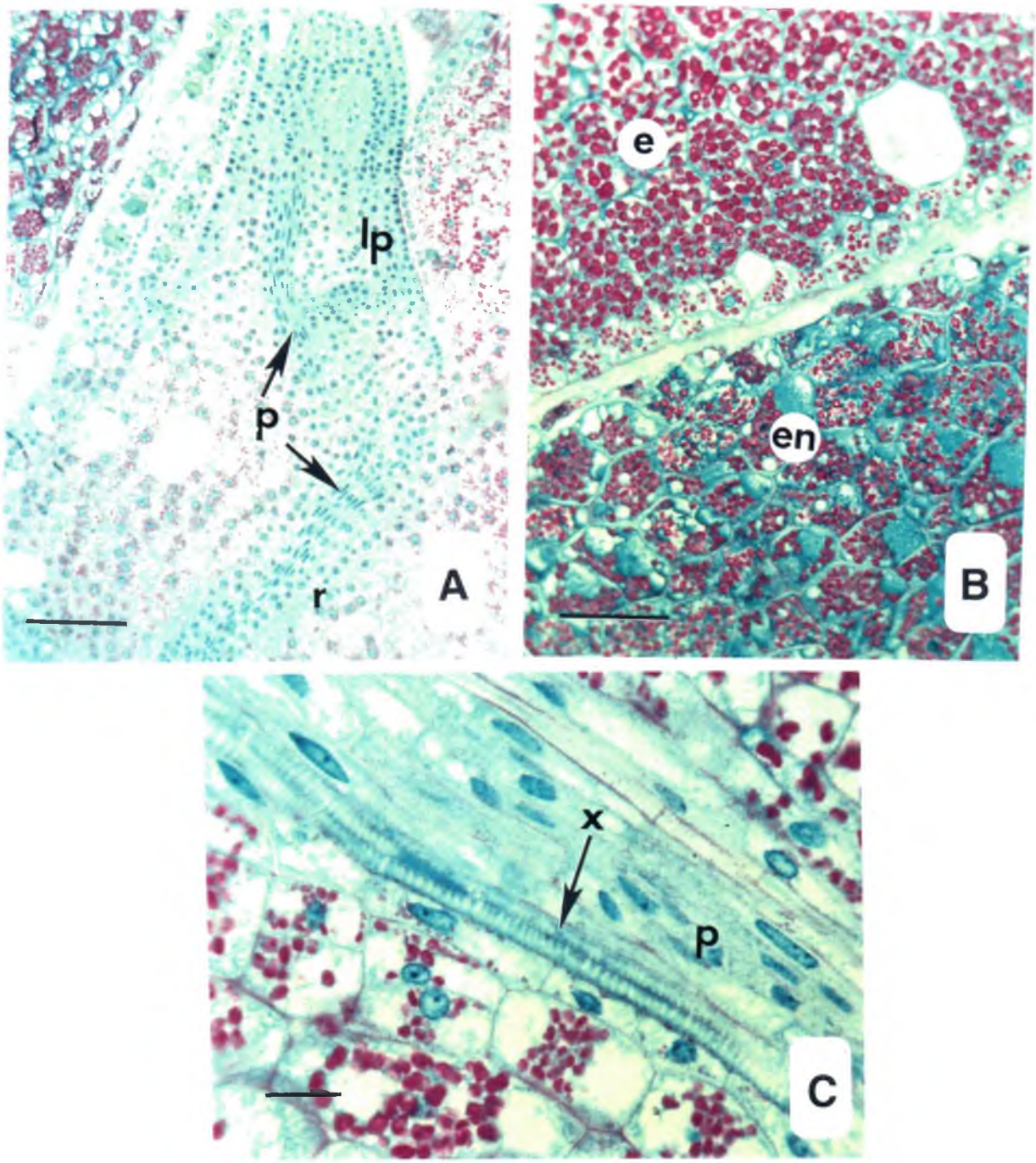

Figure 3.17. 'Kalapana' 24 weeks after pollination. A = Leaf primordium connected to root by procambium. Bar = 125 µm. B = Storage products in cotyledon and endosperm. Bar = 100 µm. C = Tracheary xylem in the cotyledon. Bar = 25 µm. Sections stained with PAS reagent (carbohydrates stain pink) and aniline blue black (proteins stain blue). e = embryo, en = endosperm, x = xylem, p = procambium, lp = leaf primordium and ra = root apex.

(82%) embryos germinated. The zygotic embryos cultured in the dark formed either callus, multiple shoots or what appeared to be somatic embryos (Fig. 3.18). Other cultures of ovules grown in the dark and embryos and ovules grown under light showed normal growth, with one shoot formed per explant.

Among 16-week-old ovules grown under dark conditions 13 of 20 (65%) germinated in the dark and 15 of 20 (75%) germinated under light. All ovules grown in the dark germinated normally except for two ovules which formed callus at the micropyle end of the ovule. Most of the ovules germinated under light were normal except for two which formed two shoots per ovule. These extra shoots appeared to have formed without an intervening callus phase.

All ovules or excised embryos, 20 to 24-weeks-old, grown under light or dark conditions had virtually 100% germination. Zygotic embryos cultured in the dark formed somatic embryos or callus. Ovules cultured in the dark and embryo and ovules cultured under light, usually germinated normally, rarely formed callus, but did occasionally produce multiple shoots.

CONCLUSION

Events of embryogenesis in *A. andraeanum* Hort. cultivar 'Kalapana' are given in Table 3.1. Similar to the observations of Campbell (1905), the initiation of cell division of the anthurium embryo occurs later than division of the endosperm. A single cell zygote is present 4 weeks after pollination. At this time the endosperm has undergone several divisions. The presence of the nucellus is detected until 6 weeks after pollination. Upon breakdown of the nucellus, storage products, of protein and starch, are formed in the endosperm at the chalazal end of the ovule. Apparently these storage products are imported from the mother plant to the endosperm and embryo through the funiculus of the ovule.

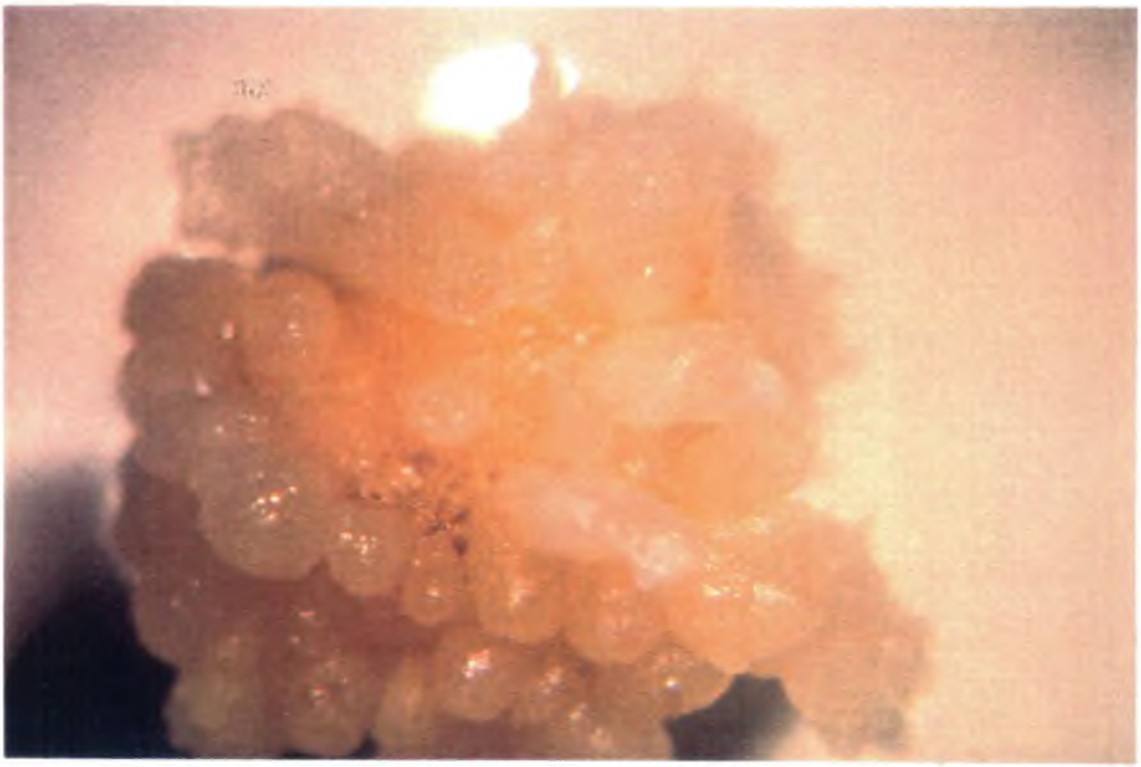

Figure 3.18. Somatic embryo-like structures from a zygotic embryo placed into culture 14 weeks after pollination.

As described earlier, anthurium embryogeny is of the solonad type (Campbell 1905). The suspensor, not detected by Campbell (1905), is evident in this study at all stages of embryo formation until week 22. Whether the absence of the suspensor in Campbell (1905) is due to the use of a different species or difference in microtechnique used should be further investigated. By 14 weeks post-pollination, the embryo was at a multicellular state and the shoot apex was defined by densely staining mitotic cells. The definition of the shoot apex region may aid in the growth of the embryo. At this stage most embryos and ovules are capable of germination *in vitro*.

At 16 weeks, the embryo is completely formed with a cotyledon, shoot apex and root apex are present. Storage products are evident in the cotyledon and endosperm and the embryo is green which indicates the presence of chlorophyll and implies the embryo is capable of photosynthesis. At this age, the majority of the embryos and ovules are capable of germination *in vitro*.

Starting at 16 weeks post-pollination, the emphasis of seed development is accumulation of storage products in both the cotyledon and endosperm. Storage in the cotyledon is predominantly in the form of starch and storage in the endosperm consists of both starch and protein. After week 16, the embryo may be considered fully mature and capable of germination. All embryos and ovules are capable of germination *in vitro* after week 20.

The use of a horticultural cultivar may be an area of concern when evaluating the information presented in this study. Campbell (1905) used *A. violaceum*, var. *leucocarpum*, hort., as his study material. As shown in the pedigree of 'Kalapana' (Fig. 3.1), parental plants are the *A. andraeanum* Hort. type. While an acceptable taxonomic assignment cannot be determined by information generated in this study alone, this investigation has set up a time frame for embryogenesis and has provided a general overview with emphasis on key structures in embryogenesis. A combination of this study

which gives the time frame for embryogenesis and the procedure for the fixation of mucilage (Chapter 2) should provide the basis for further embryological work for taxonomic purposes.

Horticulturally, this study provides a procedure for the germination of ovules and embryos cultured *in vitro*. This may be used as a embryo rescue method for important crosses with embryo and endosperm incompatibility. Additionally, *A. scherzerianum* seeds are often used as explant material for propagation. This paper also documents zygotic embryos as starting explant material for generating embryoid-like structures.

Table 3.1. Time frame for embryogenesis in *Anthurium andraeanum* Hort. cultivar 'Kalapana'

Weeks following pollination	Status of Zygote/Embryo	Endosperm
4	single cell to a few cells	several cells large cells present with thin cell walls
6	few cells	several cells large cells present with thin cell walls
8	terminal cells cytoplasmically dense	many cells evident, cells at chalazal end storing proteins
10	terminal cells forming embryo proper and basal cells form the suspensor	many cells evident, cells at chalazal end storing proteins
12/14	embryo globular, cells more vacuolated and densely stained mitotic area present and thought to give rise to shoot and root apices	endosperm storing some protein but still highly vacuolated
16	embryo has root and shoot apices and well developed cotyledon with procambium, raphides present in embryo	cells storing some carbohydrates and proteins
18	storage of starch in cells of the cotyledon	cells dense with proteins and starch
20	well developed leaf primordium present	cells continue to store proteins and starch
22	suspensor no longer evident	cells continue to store proteins and starch
24	xylem tracheids present in procambium in cotyledon	utilization of storage material by embryo

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CHAPTER 4

EVALUATION OF FACTORS FOR INDUCTION OF SOMATIC EMBRYOGENESIS FROM LAMINA DERIVED GREENHOUSE-GROWN *ANTHURIUM*

INTRODUCTION

Cut anthuriums are one of Hawaii's most valuable cut flower crops. The value of cut anthuriums reached a peak of $9.9 million in 1986 with 28 million dozens sold. Hawaii's diversified agriculture in 1993, in value worth an estimated "farmgate" value of $ 7.5 million with 10.5 million stems sold (Hawaii Agriculture Statistics Service 1994). The decline in anthurium production since 1986 may be largely attributed to bacterial blight caused by *Xanthamonas campestris* pv. *diffenbachiae* (*Xcd*).

The bacterial blight was first observed on Kauai in 1971 on the cultivar 'Kansako Red' and spread to Oahu and Hawaii in 1980 (Nishijima and Fujiyama 1985). Transmission of the pathogen to the plant is possible through hydathodes, stomata, open wounds, cultivation practices, and airborne inoculum. Favored by warm and wet conditions, the pathogen may spread rapidly in anthurium fields. Foliar symptoms of the disease have been described as chlorotic water-soaked spots on the underside of anthurium leaves. Systemic infection results from the invasion of the pathogen into the vascular system (Nishijima and Fujiyama 1985). Current recommendations to control the bacterial blight include use of clean plant material free of the pathogen, strict sanitation and removal of infected plants, proper use of antibiotics, chemical treatment of medium or a fallow period prior to replant, maintenance of clean replant area isolated from other infected plants, use isolated areas for propagation stock, and the use of resistant varieties and maintenance of a healthy plant (Nishijima 1989).

Plant tissue culture serves as an excellent tool in the production of disease-free plants, rapid clonal propagation and cultivar improvement. Plants derived from plant tissue culture are thought to be free of microorganisms because of the small size of the initial explant. The medium for explant growth may also be conducive for microorganism growth and, thus, detection. However, plants produced through tissue culture are not always free from all microorganisms and it is necessary to screen for a specific pathogen. It has been shown that *Xcd* may survive for several months on MS anthurium medium, and *in vitro* grown anthurium plants infected *in vitro* with *Xcd* may not display any visible disease symptoms (Tanabe et al. 1990). Other studies have shown that callus and subsequent plant formation may be obtained from explants taken from with *Xcd* infected source (D. Norman personnal communication). However, the plants obtained from the callus test negative for the presence of the bacteria. Efforts are now underway to devise a triple-index system for the elimination of *Xcd* in micropropagated anthuriums (Tanabe et al. 1992).

Bud culture with enhanced axillary branching is the preferred method of anthurium micropropagation in Hawaii. Enhanced axillary branching produces slow, orderly growth of the plants *in vitro*. It is presumed that plants propagated by this method will be true to type with limited somaclonal variation. This method is slow with certain cultivars, and may range from 13 to 35 weeks or more to produce the first leaf from bud. In contrast, leaf callus cultures are able to produce initial plants more quickly. However, somaclonal variation may be increased, due to the rapid division of the callus cells.

Somatic embryogenesis is a promising method for the rapid propagation of anthuriums. Somatic embryos may be obtained via direct embryogenesis, from an explant, or via an indirect embryogenesis system, with callus formed prior to plant regeneration. One of the biggest advantages of somatic embryogenesis is in reduction of labor through the use of synthetic seeds (Parrott et al. 1991). Through large-scale batch culture, cell

suspensions are induced to form somatic embryos, which are then harvested and encapsulated. Thus, manipulation of the explant during subculture is minimal and the need for acclimatization of the microcutting is eliminated.

As an aid to breeding, plant tissue culture is used in genetic engineering for the production of transgenic plants. Most genetic engineering systems require the use of an tissue cultures, one exception is seed transformation in *Arabidopsis* (Ritchie and Hodges 1993). Somatic embryogenesis has excellent potential for a regeneration system because it produces discrete propagules that may be tested for the presence of the selectable marker. The origin of the somatic embryo may be from single cells (Williams and Maheswaran 1986); this would ensure selection of non-chimeric plants. However, if somatic embryos are derived from multiple cells, production of secondary embryos in conjunction with a selectable marker can also ensure non-chimeric development (Parrott et al. 1991). A gene transfer system via *Agrobacterium tumefaciens* is available for anthurium and has been used for the genetic engineering of bacterial blight resistance into anthuriums (Chen 1993). The major advantage of genetic engineering anthuriums is the incorporation of a specific gene into an established cultivar. The characters of the plant may be retained with only the specific modification made. This is of considerable advantage since the conventional breeding of an anthurium cultivar may take up to ten years and market acceptance of the new cultivar cannot be guaranteed.

Therefore, somatic embryogenesis of anthurium should be considered as a rapid propagation tool for the production of *Xcd*-free or genetically engineered plants. Currently, plant production via somatic embryogenesis is limited to explants taken from *in vitro* material (Kuehnle et al. 1992). A somatic embryogenesis procedure from mature field grown anthuriums would be highly desirable for the rapid clonal propagation of anthuriums. Unfortunately, the interchange of *in vivo* and *in vitro* tissues is not always possible without making other culture modifications.

There are many differences between *in vivo*- and *in vitro*- grown plant material. According to Pierik (1987), plants grown *in vitro* have a poorly developed cuticle and are not very photosynthetically active. Anatomically, *in vitro* grown leaves have smaller and fewer palisade cells and larger mesophyll air spaces. Stomata function is poor in the leaves grown *in vitro* (Pierik 1987). Comparisons of hydathodes in micropropagated plantlets and greenhouse-grown rose plants revealed fewer water pores per unit area, in the former (< 20 compared with 35). Water pores and stomata of the *in vitro* grown plantlets were open while those of the greenhouse grown plants had smaller apertures and were completely closed (Donnelly and Skelton 1989). Abscisic acid, which is known to play a role in stomata function, is also thought to be involved with the maturation of zygotic and somatic embryos.

Other factors affect competence of tissues to undergo somatic embryogenesis. Low light intensity and high relative humidity accounted for the difference in the leaf structure of *in vitro*-grown rose plants versus those grown under greenhouse conditions. An increase in light intensity from 25 $\mu mols \cdot s^{-1} \cdot m^{-2}$ to 80 $\mu mols \cdot s^{-1} \cdot m^{-2}$ and a decrease in relative humidity from 100% to 75% resulted in modification of the epicuticular wax, stomata, and epidermal cells of *in vitro* leaves similar to those of greenhouse grown rose plants (Capellades et al. 1990). Similar results have been demonstrated in anthurium (Tanabe et al. 1992). Plants grown in a culture vessel covered with Parafilm have increased numbers of non-functional stomates with larger stomata openings, compared to those that are not subjected to Parafilm enclosure. Lastly, it should be noted that rejuvenation often occurs in plants cultured *in vitro*. These rejuvenated plants usually have a greater capacity to regenerate in comparison to adult plants (Pierik 1987).

This chapter investigates methods for obtaining somatic embryos from greenhouse-grown anthurium leaves from a mature plant source. The effects on somatic

embryogenesis by wounding, juvenility, modification of nitrogen source, disinfestation, and ABA were investigated.

MATERIALS AND METHODS

Basic Procedure

The procedure for the experiments are given below. Specific modifications to the basic procedure are indicated for each experiment.

Disinfestation

The disinfestation procedure of Kuehnle and Sugii (1991) was used. Anthurium leaves were harvested, washed with detergent under running water, and soaked in a 0.14% Physan solution for 10 minutes. Leaves were then placed on a gyratory shaker in 10% Clorox plus 1 drop per 100 ml of Tween 20 for 30 minutes and 5% Clorox plus 1 drop per 100 ml of Tween 20 for 30 minutes. Leaves were rinsed three times in sterile water and dissected into rectangular explants approximately 1 cm x 1.5 cm. A major vein was present in each of explant and a total of ten explants were plated with edges embedded in the medium per Petri plate (Kuehnle and Sugii 1991).

Somatic Embryogenesis Medium

The somatic embryogenesis media recommended by Kuehnle et al. (1992) were used. The basal medium consisted of 1/2 MS macronutrients, full MS micronutrients, 25 mg/l NaFeEDTA, modified MS vitamins with 0.4 mg/l thiamine•HCl, 2% sucrose, 1% glucose, 100 mg/l myo-inositol and 0.18% Gelrite (Kuehnle et al. 1992). Growth regulators 2,4-D and kinetin were added at the concentrations given in Table 1 and each medium was designated by assigned letters (Kuehnle et al. 1992). The pH of the medium

was adjusted to 5.7-5.8 prior to autoclaving and approximately 20 ml of medium was dispensed per 100 x 15 mm petri plate.

Table 4.1: Growth regulator components in somatic embryogenesis media (Kuehnle et al. 1992)

Medium	2,4-D (mg/l)	Kinetin (mg/l)
B	2.0	0.33
D	1.0	0.5
E	2.0	0.5
F	3.0	0.5
G	4.0	0.5
O	1.5	0.5
S*	1.5	0.5

* sugars are doubled to 4% sucrose, 2% glucose

Explant Source

Phase of Explant Material

Leaves were harvested from a community pot of seedlings from a cross of UH931 x UH1016. Following disinfestation, petioles were removed from the lamina and the lamina was trimmed into a square-shaped explant. All seven somatic embryogenesis media were used (Table 4.1). Younger leaves (leaves that have just unfurled) were harvested and plated on medium O and S. Two replications were used for each medium. Ten leaf sections were placed on each Petri plate, with 2 plates per medium. Cultures were kept in complete darkness and monitored for contamination and growth.

Sectioned versus Intact Lamina

Lamina Grown *in vitro*

In vitro lamina were included in this study as a positive control. Leaves were harvested from UH1060 plantlets grown *in vitro* on a medium containing 1/2 MS macronutrients and micronutrients, vitamins, 3% sucrose, 0.2 mg/l benzyladenine and 2.5% Gelrite designated H-1. Leaves were disinfested to determine if the sodium hypochlorite soak was detrimental to somatic embryogenesis production. The petiole was removed and remaining lamina were separated into two groups. The first set of lamina was placed onto somatic embryogenesis media B through G. Ten intact lamina were used per medium. The second set of lamina was sectioned into square-shaped explants 1 cm by 1 cm. Each explant contained the area of petiole attachment. Ten lamina sections were placed on somatic embryogenesis media B through G. Cultures were kept in complete darkness and monitored for contamination and growth.

Lamina Grown Under Greenhouse Conditions

Leaves were harvested from five-month-old UH1060 greenhouse-acclimatized plants and fully mature greenhouse material grown in 1 gal. pots. All harvested leaves were disinfested, petioles of all leaves were removed, and lamina were used as the explant. Lamina from the UH10160 *in vitro* source were kept intact to serve as a control. Lamina from the five-month-old acclimatized plants were divided into two groups, one group was kept intact and the other sectioned to 1 cm^2 pieces. All lamina derived from the mature plant were sectioned. Intact leaves of the mature plant were too large and could not be cultured in the Petri plates. Media B through G were used for the lamina derived from the *in vitro* source and from mature plants. Ten explants were used per plate and two plates were used per medium. Lamina from the five-month-old acclimatized plants were plated on media B through F. Ten explants per plate and one plate per medium were used for

sectioned and intact lamina. Plates were kept in complete darkness and monitored for contamination and growth.

Modification of Nitrogen Salts in Medium

Total Nitrogen

Modification to the amount of total nitrogen (19.1mM) was made to medium F (Kuehnke et al, 1992). Nitrogen levels were used at full-strength (39.4 mM), half-strength (19.7 mM), one-eighth-strength (4.93 mM) and one-thirty-two-strength (1.23 mM). KCl was added to the media to compensate for potassium lost with the elimination of KNO_3.

Explants were obtained from greenhouse-grown fully mature plants of 'Rudolph', 'Kalapana' and UH1060. Following disinfestation, petioles were removed from the leaves and lamina were sectioned into 1.5 cm^2 square-shaped explants. Each section contained a major vein. Ten explants were placed in each petri plate and two plates were used per medium.

Nitrate to Ammonium Ratio

Nitrate to ammonium molar ratio in somatic embryogenesis medium F was modified to a 1:1 ratio and 1:0 (nitrate only medium). The unmodified 1:2 ratio was used as the control. A total nitrogen level of 19.7 mM was kept constant for all treatments.

Explants were obtained from greenhouse-grown fully mature plants of 'Rudolph', 'Kalapana' and UH1060. Following disinfestation, petioles were removed from the leaves, and lamina were sectioned into 1 cm square-shaped explants. Each section contained a major vein. Ten explants were placed in each petri plate and two plates were used per medium.

Supplemental ABA

Somatic embryogenesis medium B was used as the basal medium. Abscisic acid (ABA) was added to the medium to obtain concentrations of 0, 0.01, 0.1, 1.0, and 10 μM ABA.

Explant material was acquired from *in vitro* grown 'Midori' plants and greenhouse-grown, adult 'Rudolph' plants. Following disinfestion of the greenhouse material, petioles were removed from the leaves and lamina were sectioned into square explants. The *in vitro*-grown 'Midori' served as a control and was not disinfested. Each section contained a major vein. Ten explants were placed in each petri plate and six plates were used per medium. Ten explants of *in vitro* grown intact lamina were used per medium, with no replication.

RESULTS

Somatic-embryo like structures from *in vitro*-grown anthurium lamina derived from the methods of Kuehnle et al. (1992) were evaluated histologically (Chapter 5). These structures were confirmed to be somatic embryos. Morphologically similar structures of to the *in vitro* lamina somatic embryos derived from greenhouse-grown lamina explants are thought to be somatic embryos.

Explant Source

Phase of Explant Material

One explant (5%) produced a somatic embryo on each medium B and D. Lamina harvested at a younger developmental age and placed on medium O produced somatic embryos on two of the explants. The somatic embryos appeared at the cut surface and were globular in shape and whitish-yellow in color. Explants on medium E and younger lamina explants on medium S produced prolific root growth. All activity occured on the cut

surface of the explant. The remaining explants did not show signs of growth or multiplication.

Sectioned versus Intact Lamina

Lamina Grown *in vitro*

Observations of the UH1060 explants after 10 weeks of culture resulted in the figures shown in Table 4.2. What appeared to be somatic embryos were present on the sectioned explants on medium F after 4 weeks in culture. After 6 weeks in culture somatic embryos were formed on media B, F and G on sectioned and intact lamina. Only intact explants formed somatic embryos on medium E. Neither intact or sectioned explants formed somatic embryos on medium D.

Table 4.2 : Somatic embryo production on lamina sections and intact lamina from *in vitro* plants ten weeks after culture

Somatic Embryogenesis Medium (2,4-D : Kinetin) (μM)	Number of Somatic Embryos		Number of Explants* forming Somatic Embryos	
	Intact	Section	Intact	Section
B (5.9 : 1.0)	14	4	7	2
D (2.0 : 1.0)	0	0	0	0
E (3.9 : 1.0)	9	0	3	0
F (5.9 : 1.0)	8	41	3	4
G (7.8 : 1.0)	12	27	6	7

* n = 20

Lamina Grown Under Greenhouse Conditions

At six weeks, control UH1060 lamina harvested from *in vitro* -grown plants did not produce somatic embryos. Globular cell protrusions were noted at the cut leaf base. Similar results were obtained from the lamina sections harvested from the five-month-old acclimatized plants seven weeks after initial culture. Round, translucent cell protrusions. possibly callus or cell expansions,. were present at the cut surface, but no somatic embryos. Intact lamina from the five-month-old acclimatized plants remained green and did not show any sign of proliferation at this stage. Lamina sections from the fully matured greenhouse grown plants were chlorotic and did not show any signs of cellular division or somatic embryos.

Final observations at week 17 revealed no activity in sections obtained from the mature plant material. At this time, most sections were necrotic and considered dead. Round cell protrusions were evident on the cut surface of the lamina sections, from the acclimatized plants, on medium B and D. One possible somatic embryo was formed on the acclimatized lamina section placed on medium F. Intact lamina from the acclimatized plants produced also produced one somatic embryo on each of the 10 leaf sections on medium F. An average of 40% of the control lamina formed somatic embryos on all media tested.

Modification of Salts in Medium

Total Nitrogen

Following ten weeks of culture, explants from 'Kalapana' remained green but showed no signs of callus or somatic embryos. Hard, yellow organogenic callus was present on explants of 'Rudolph' explants grown on medium with 1/2-strength nitrogen. Morphologically similar callus was present on UH1060 explants placed on 1/32-strength medium. On 1/8-nitrogen medium, explants of UH1060 produced the round celled, translucent protrutions.

By week 13, all 'Kalapana' explants were necrotic. Callus on the 1/8-strength nitrogen medium was also brown. Remaining callus was then transferred to H-1 medium. No differentiation was achieved and callus turned necrotic approximately 2 weeks after subculture.

Nitrate to Ammonium Ratio

Ten weeks after explants were placed into culture, the explant remained green but no activity was detected on 'Kalapana' explants. Hard, yellow organogenic callus was present on explants on 'Rudolph' explants grown on medium with the standard 1:2 nitrate to ammonium molar ratio. Minimal cellular activity was present on the cut edges of the explants grown on medium containing nitrogen at a 1:1 nitrate to ammonium ratio. The cellular activity was brown in color with a mushy, fuzzy like texture, similar in appearance to fungal hyphae. The same type of callus, as obtained on medium with a 1:1 nitrate to ammonium ratio, was present and more prolific on UH1060 explants grown on nitrate only medium.

Modifications of Plant Growth Regulators

Effect of ABA on Somatic Embryo Production

No somatic embryos were obtained from greenhouse-grown explant material during the 19 weeks of culture. Explants became necrotic as early as two weeks after the initial culture with the majority of the explants dead at week 19. Explants taken from *in vitro* source did produce somatic embryos on all medium except for medium containing 10 µM ABA. Percentage of lamina forming embryos were 50% for medium containing no ABA, 30% for 0.01 µM ABA, 20% for 0.1 µM ABA, 1% for 1.0 µM ABA, and no somatic embryos for medium containing 10.0 µM ABA. All *in vitro* derived explants remained green after 26 weeks in culture.

DISCUSSION AND CONCLUSION

To date, an efficient method for the production of somatic embryos from explants of mature lamina of anthurium plants has not been achieved for the genotypes and media tested. There may be several possible explanations for the observed results.

In general, propagation is more difficult of plants in the mature or reproductive phase of development than in the juvenile phase. Plants placed into culture are thought to be rejuvenated. It has been shown that repetitive grafting of explants *in vitro* onto seedlings will effectively rejuvenate avocado plants and enable them to produce roots (Pliego- Alfaro and Murashige 1987). Plants in the juvenile phase are easily manipulated in culture and provide exceptional explants for producing somatic embryos (Tisserat et al. 1979).

Zygotic embryos excised from anthurium will readily produce somatic embryo-like structures when placed in the dark on a medium containing no plant growth regulators other than 15% v/v coconut water (see Chapter 3). Conversely, explants taken from the leaves of mature greenhouse grown plants will not produce somatic embryos, even on medium containing additional growth regulators. In the middle of these two extremes are the plants grown *in vitro*. The *in vitro* plants from the mature plants are rejuvenated in culture. *In vitro* plants are capable of forming somatic embryos given the right combination of auxin and cytokinin with additional carbohydrate sources of sucrose and glucose. Once taken out of culture, the newly acclimatized plants are able to form somatic embryos although the quantity of somatic embryos produced is dramatically decreased. Likewise, plants taken from seedlings, which are also considered juvenile tissue, are able to form somatic embryos at reduced rates similar to those of acclimatized plants. Methods to obtain callus from mature anthurium tissues have been demonstrated in numerous studies (Geier 1990).

Therefore, it was reasoned that besides factors other than juvenility must influence the production of somatic embryos from mature tissue.

Wounding of the tissue by excision of the explant from the lamina was considered to be a possible factor affecting somatic embryogenesis. Kuehnle et al. (1992) indicated that sectioned lamina taken from an *in vitro* source did not form somatic embryos. However, comparison of sectioned and intact lamina of UH1060 has shown that the production of somatic embryos was not significantly reduced by wounding (Table 4.2). It is also unrealistic to culture greenhouse-grown lamina without wounding the tissue.

A second factor, the use of sodium hypochlorite, also did not have an effect on somatic embryogenesis on UH1060 explants. Intact lamina of *in vitro* grown UH1060 were disinfested for use as a comparision for the intact lamina taken from the five-month old acclimatized plants. Somatic embryogenesis on UH1060 was not hindered by disinfestation. There may have been a slight delay in somatic embryo production. An average of 6 weeks are required for the first somatic embryo to form on the intact UH1060 leaves used in the *in vitro* sectioned vs intact leaf experiments. Somatic embryos were not obtained from the disinfested intact lamina until approximately week 13. Total number of lamina forming somatic embryos and the amount of somatic embryos formed was not affected by disinfestation. However, since explants were harvested at different times, other factors may have effected somatic embryo formation.

The amount and form of nitrogen present in the medium are important considerations in somatic embryogenesis (Kamada and Harada 1982). Reduced nitrogen levels has shown to be beneficial in the regeneration of anthuriums in culture. Somatic embryogenesis in spadix callus was induced by lowering the NH_4NO_3 to 1.25 mM in a Nitsch medium with 4.44 μM BA and 0.45 μM 2,4-D (Geier 1990). In work conducted by Pierik and reviewed by Geier (1990) on *A. andraeanum* plant regeneration, shoots could be induced from organogenic callus by lowering the NH_4NO_3 concentration from

10.3 mM to 2.5 mM. This effect was found to be caused by the ammonium ion, not the nitrate ion. Likewise, plant regeneration in *A. scherzerianum* was increased by lowering of nitrogen to 2.5 mM (Geier 1986). In this present study, an increase in callus production was observed on medium containing 1/2 (19.7 mM), 1/8 (4.93 mM) and 1/32 (1.23 mM) strength total nitrogen. However, no somatic embryos were formed on any of the media tested.

The form of nitrogen added to the culture medium significantly influences somatic embryogenesis. In a study conducted by Halperin and Wetherall (1965) on phloem-derived wild root callus, pro-embryos would not form callus on a medium with nitrate as the sole nitrogen source. Addition of at least 5 mmoles/l of ammonium were need to supplement the 60 mmoles/l of nitrate for embryogenesis to occur (Halperin and Wetherell 1965). Tazawa and Reinert (1969) reported intracellular ammonium present in carrot root cultures grown in a 60 mM nitrate only nitrogen medium. From this study they speculated that the reducing enzymes produced the ammonium ions required for the embryogenesis to occur (Tazawa and Reinert 1969).

Wetherall and Dougall (1976) sieved and washed wild carrot cultures to remove the initiation medium before subculture. They found that nitrate alone did not promote somatic embryogenesis. Nitrate concentrations from 5 to 95 mM KNO_3 supported weak growth of the cultures with little embryogenesis. A supplement as low as 0.1 mM NH_4Cl added to the medium allowed more frequent embryogenesis to occur, with a optimal effect seen at 10 mM. Reduced forms of nitrogen such as glutamine and alanine could partially replace the ammonium as the nitrogen source (Wetherell and Dougall 1976).

Studies on nitrogen metabolism during somatic embryogenesis also revealed that various amino acids added as a supplement to nitrate were effective in the production of somatic embryos in the absence of 2,4-D (Kamada and Hamada 1982). Equimolar concentrations of proline in combination with serine or threonine promoted somatic

embryogenesis of *Dactylis glomerata* (orchardgrass) (Trigiano et al. 1992). Ammonium ions supplemented at 6 to 25 mM also promoted somatic embryogenesis. Protein hydrolysates, such as casein hydrolysate, were effective for evaluation of the need for amino acids or amides very quickly (Huang and Murashige 1976).

Anthuriums are thought to be root convertors of nitrogen, nitrate is changed to ammonia and ammonia forms of amino acids and amines in the root (Sakai et al. 1992). This was determined by evaluation of the guttation fluid of anthurium plants given different levels of nitrogen and different ratios of nitrate and ammonium fertilizers. It is possible the lamina explants could not convert the nitrate-nitrogen to a utilizable form of nitrogen, such as ammonium and its derivatives.

The ratio of nitrate to ammonium at 5:1 was more effective than a 1:1 ratio in the formation of callus and adventitious shoots of *A. scherzerianum* (Zens and Zimmer 1986). This ratio was tested at total nitrogen levels of 14.89 mM and 29.95 mM.

The experiment conducted here on the nitrate to ammonium ratio in anthurium showed no viable callus or somatic embryos on media containing a nitrate : ammonium ratio of 1:1 or less. At the standard ratio of 1:2 nitrate to ammonium ratio, callus was formed. It is recommended that a ratio with a higher ammonium ion component or amino acid supplement should be investigated to see if somatic embryogenesis will be promoted. Casein hydrolysate should be tested for anthurium as a source of reduced nitrogen.

The experiment conducted on the influence of ABA on somatic embryo production on mature anthurium explants showed no effect of ABA compared to the control. The use of *in vitro* leaves on medium without ABA demonstrates that somatic embryogenesis could be achieved by certain tissues on the tested medium. An interesting trend of decreased embryogenesis with increased ABA concentration was observed in the experiment. However, more explants would be needed to evaluate this observation statistically. Prevention of somatic embryo production with increased ABA concentrations may be

attributed to inhibition of mitosis. Levi et al. (1993) demonstrated that, in the presence of exogenous ABA, only a few meristematic root tip nuclei of culture peas embryos progressed through the cell cycle during germination. This effect was also dependent on the presence of potassium ions. In the absence of KNO_3 and presence of ABA the inhibitory action of ABA increased (Levi et al. 1993).

In conclusion, the factors of wounding (sectioning) and surface sterilization do not hinder somatic embryogenesis in anthurium lamina explants. Supplemental ABA may be eliminated as a factor that promotes anthurium somatic embryogenesis. It is recommended that modification of the nitrogen source with special consideration of ammonium and amino acids may be beneficial and should continue to be investigated. It is also recommended that an empirical study of various growth regulators and growth regulator combinations be undertaken in future experimentation.

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CHAPTER 5

HISTOLOGY AND ORIGIN OF *ANTHURIUM* SOMATIC EMBRYOS

INTRODUCTION

Anthuriums are an important florist crop in Hawaii, the Netherlands and subtropical and tropical countries. As a cut flower, the 1991 combined Dutch auctions ranked anthurium 14th of all cut-flower sales, with over 20 million stems sold for approximate wholesale value of $21.5 million (International Floriculture Quarterly Report 1992). In Hawaii, anthurium is one of the top cut flowers with a 1992 "farmgate" value of 64 million for 9.9 million stems sold (Hawaii Agricultural Statistics Service 1993).

Pierik (1991) conducted an analysis of micropropagation commodities in 15 West European countries and Israel. In Western Europe, a total of 248 commercial tissue culture laboratories produced 212.5 million plants in 1988. A survey for the most frequently propagated species or genera in 14 West European countries in 1988 revealed *Anthurium* was ranked 14th in volume, with three countries producing more than 100,000 anthurium plants each. In the Netherlands, which accounts for 29% of the West European production, *A. andraeanum* ranks third, after *Gerbera* and *Aster*, in the quantity of plants micropropagated for the cut-flower industry. *A. scherzerianum* ranks third, after *Nephrolepis* and *Spathiphyllum*, in the quantity of plants produced in vitro for the flowering potted plant market (Pierik 1991).

Anthurium micropropagation is gradually developing in the United States (Matsumoto and Kuehnle, in press). The demand for anthurium plants is increasing, to replace plants lost to disease and to acquire new releases of cut flower and pot plants cultivars. A survey of plant tissue culture laboratories in the United States was conducted for an estimate of micropropagated anthurium plants. Anthuriums for cut flower, potted

flowering and foliage plants are included in the estimate of 2.5 million plants produced in 1993.

Although not a common practice, somatic embryogenesis is one form of micropropagation available for anthurium (Matsumoto and Kuehnle, in press). Somatic embryogenesis in anthurium was first observed by Geier in *Anthurium scherzerianum* spadix cultures (Geier 1982). Somatic embryos have also been derived from in vitro grown leaves of *A. andraeanum* Hort. cultivars and *A. andraeanum* hybrids (Kuehnle et al. 1992). Both systems of *A. scherzerianum* and *A. andraeanum* resulted in somatic embryos that germinated into bipolar structures or multiple clumps.

Somaclonal variation in plants regenerated from somatic embryos in anthuriums cannot be determined at this time. Geier (1982) tested two hundred plants derived by spadix culture. This resulted in plants free of morphological deviation or change in somatic chromosome numbers; however, the study does not differentiate between plants derived from organogenesis or somatic embryogenesis.

Histological studies of somatic embryos are important in the confirmation of the somatic embryo structure. Determination of the layer of origin is also important, especially in gene transfer systems. Colby et al. (1991) has shown that the layer of tranformation is different from the layer of plant origin in *Vitis vinifera* infected with *Agrobacterium tumefaciens*. Thus, transformed cells do not regenerate plants. *Anthurium* is amenable to transformation by *A. tumefaciens* (Kuehnle and Sugii 1991; Chen 1993).

Unicellular versus multicellular origin of somatic embryos is difficult to determine since the development of the somatic embryo is permanently halted once the specimen is fixed. However, many authors have been able to speculate about the cellular origin by observations of different developmental stages in somatic embryo production (McWilliam et al. 1974; Gray and Conger 1985; Conger et al. 1987; Trigiano et al. 1989; Eapen et al. 1989; Kim and Janick 1989; Michaux-Ferriere et al. 1992; Wilson et al. 1994). Unicellular

origin of the somatic embryo is desirable in the production of non-chimeric genetically engineered plants, and is the criterium for a somatic embryo as defined by Haccius (1978). A system has been developed for genetically engineering anthuriums for bacterial disease resistance (Chen 1993) using *Agrobacterium tumefaciens.*

In this study, the origin of the somatic embryo will be determined and the cellular source the anthurium somatic embryo will be discussed.

MATERIALS AND METHODS

Somatic Embryogenesis

In vitro lamina of *A. andraeanum* Hort. cultivars 'Anuenue' and 'Toyama Peach' were placed on somatic embryogenesis media B and D (Kuehnle et al. 1992). The basal components of the somatic embryogenesis medium contained 1/2 MS macro-nutrients, full strength MS micro-nutrients, 100 $mg \cdot l^{-1}$ myo-inositol, 25 $mg\ l^{-1}$ NaFeEDTA, MS vitamins modified to include 0.4 $mg \cdot l^{-1}$ thiamine • HCl, 2% sucrose, 1% glucose and 0.18% Gelrite. Medium B contained 2.0 $mg \cdot l^{-1}$ 2,4-D and 0.33 $mg \cdot l^{-1}$ kinetin and medium D contained 1.0 $mg \cdot l^{-1}$ 2,4-D and 0.33 $mg \cdot l^{-1}$ kinetin. Cultures were kept in complete darkness at 23°C.

Microtechnique

Lamina producing somatic embryos were harvested from the medium approximately 10 weeks after culture initiation. Various developmental stages were present so lamina were divided into 4 categories by morphology : Group I - initial swelling at surface and glass cell protrusions, Group II - globular protrusion on cut surface, and Group III - multiple somatic embryos present with secondary embryos formed. Somatic embryos and a piece of the explant were excised and placed in a modified Karnovsky fixative containing 2% glutaraldehyde and 2.5% paraformaldehyde in a 0.5 M sodium

cacodylate buffer pH 7.0 (Karnovsky 1965). Specimens were placed under vacuum and fixed overnight at 5°C. Specimens were rinsed three times with 0.5 M sodium cacodylate buffer pH 7.0 and dehydrated through an ethyl alcohol series. A ten percent increase in alcohol was used every fifteen minutes. To infiltrate the specimens, 3 ml of alcohol were added to the specimen, and 1 ml of Historesin infiltration solution was added every other day. After one week, the Historesin-alcohol mixture was replaced by 100% Historesin infiltration solution. The solution was changed every other day until the tissue looked translucent. Specimens were embedded in Historesin embedding medium in inverted Beem capsules and placed under vacuum. Once the medium hardened, the Beem capsule was removed and the block face was trimmed around the specimen.

Twenty-five mm glass knives, 8 mm in width, were cut with an LKB Knifemaker type 7801B. Trimmed blocks were sectioned with a Sorval Porter-Blum MT2-B Ultra-Microtome to 4.8 μm. Sections were floated on distilled water and heated on a slide warmer at 40 °C. Slides were stained with PAS for carbohydrates and counterstained with aniline-blue-black for proteins (Feder and O'Brien 1968). A total of 8 different specimens from 'Anuenue' and 'Toyama Peach' lamina were evaluated. Sections were examined with a Zeiss compound microscope and photographed with a Zeiss M35W camera.

RESULTS AND DISCUSSION

A cross section of a lamina explant is shown in Figure 5.1. Evident in the light micrograph are the two layers of isodiametric cells which make up the epidermis. Between these two layers of epidermal cells are the spongy parenchyma mesophyll cells. Anatomy of the lamina grown *in vitro* conditions are different from lamina grown *in vivo*. Lamina cross sections of the in vitro leaves done in this study were compared to the photos of cross sections of lamina of greenhouse grown leaves in a study by Higaki (1976). A well developed palisade parenchyma cell layer is not evident in the former but present in the

latter. The epidermal cells are larger than those of the in vivo grown leaves and do not have a well defined cuticle.

Within the epidermal layer of the lamina explant, darkly stained cells with starch reserves, darkly stained nuclei and few or no vacuoles were observed. Since these are characteristics of meristematic cells, these structures are thought to be promeristemoids which will give rise to the somatic embryos. Structures such as those seen in Figure 5.2A may be derived from a periclinal division followed by several anticlinal divisions. A more developed structure of this type of somatic embryo development may be seen in Figure 5.2B.

This somatic embryo appears to have a suspensor-like organ embedded within the mesophyll layer of the lamina explant. The layer of cells above the somatic embryo is the lamina epidermis. Unicellular origin as indicated by Haccius suggests the somatic embryo may rise directly from the somatic cell or from a proembryonal cell complex (Haccius 1978). A densely staining area of mitotic cells was observed in the mesophyll layer of the lamina explant in an area adjacent to the somatic embryo shown in Figure 5.2B. These cells are assumed to comprise the proembryonic cell complex (Figure 5.3). Within this region, a line of meristematic cells is evident. This may be polarity establishment in the complex or the formation of somatic embryos in the complex.

A somatic embryo at a later developmental stage than the somatic embryo in Figure 5.2 is shown in Figure 5.4. In this structure, a layer of meristematic cells is evident around the circumference of the somatic embryo. From this layer the start of an epidermis is apparent. This is evident by the presence of a cuboidal shaped layer at the outermost edge of the meristematic region. The meristematic region also separates the somatic embryo from the rest of the explant source with no vascular connection evident between them. Storage products of the somatic embryo are similar to those seen in the zygotic embryo (Chapter 3) with the storage of starch predominant over the storage of protein. The storage

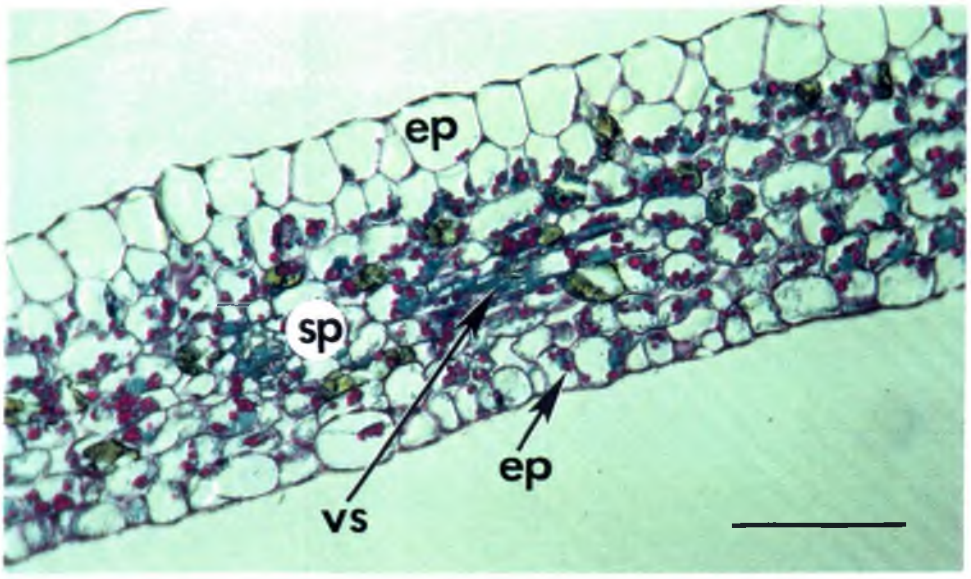

Figure 5.1. Cross section perpendicular to the midrib of a lamina taken from *in vitro* conditions. Sections were stained with PAS (carbohydrates stain pink) and aniline blue black (proteins stain blue). ep = epidermis, vs = vascular system, sp = spongy parenchyma, and se = somatic embryo. Bar = 100 μm.

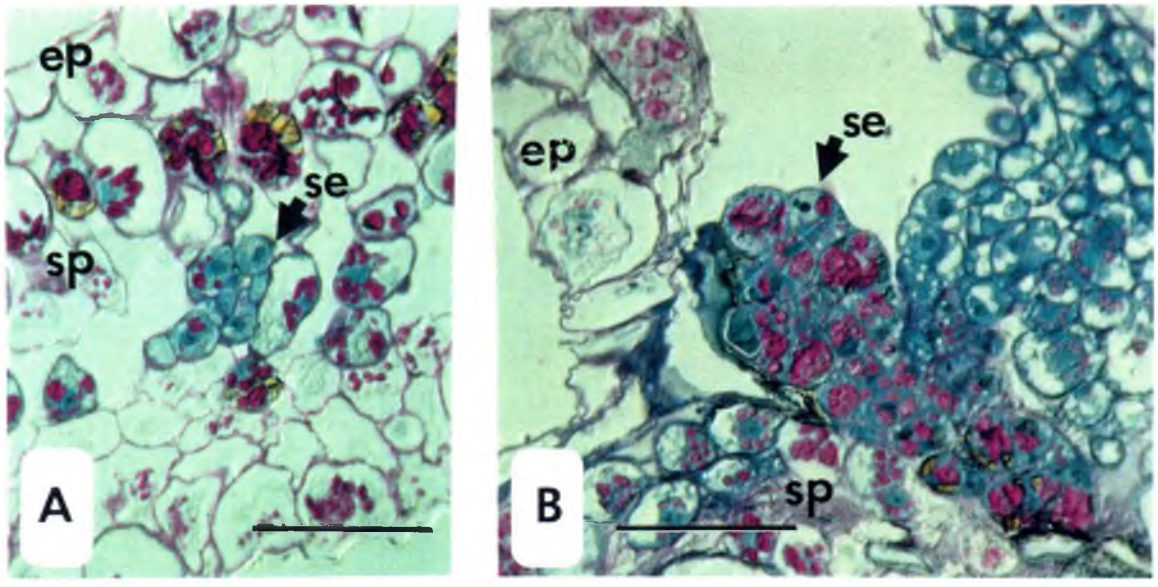

Figure 5.2. Cross section of an in vitro grown lamina containing somatic embryos 10 weeks after culture. A = Early stage of somatic embryo. B = Later stage of somatic embryo. Sections were stained with PAS (carbohydrates stain pink) and aniline blue black (proteins stain blue). ep = epidermis, vs = vacular system, and sp = spongy parenchyma. Bar = 100 μm.

of starch and protein and the presence of calcium oxalate crystals were also observed by Geier (1990) in somatic embryos from *A. scherzerianum*. Surrounding the somatic embryo are large isometric shaped cells with lightly stained cytoplasm and prominent nuclei. Their function or importance, if any, to the somatic embryo is unknown. It may be speculated that these cells act as "suspensor-like" structures which aid the somatic embryo in nutrient absorbtion. The lighly stained pink area around the cells of the somatic embryo may be carbohydrates broken down for utilization by the cells of the somatic embryo (Fig 5.4).

In a fully mature somatic embryo, a root and shoot meristem is present, as well as a procambium and epidermis. The root meristem of a mature somatic embryo may be seen in Figure 5.5. A procambium that has differentiated to xylem in some areas are also evident and is shown to connect the shoot and root meristem areas (Figure 5.6). Also evident in the structure are the presence of calcium oxalate crystals. These are similar to the crystals formed in the zygotic embryo. Along with the epidermis, the calcium oxalate crystals were used by Geier (1982) to confirm the presence of somatic embryos in *A. scherzerianum* spadix cultures.

CONCLUSION

This study confirms the formation of somatic embryos in *A. andraeanum* Hort. using a protocol established by Kuehnle et al. (1992). An epidermis, calcium oxalate, starch and protein storage were found in both somatic and zygotic embryos of *A. scherzerianum* (Geier 1982). In addition to these observations we have presented evidence of the shoot and root meristems of the somatic embryos as well as a connecting procambium. The somatic embryos are not connected by vascular tissue to the explant and are individual units.

The origin of the somatic embryo is within the mesophyll layer of the lamina. For absolute confirmation of the origin of the somatic embryo, a nondestructive methodology for the continuous study of the embryo is required. However, the observations presented in this study suggest that the somatic embryo is of unicellular origin either by direct formation or through a proembryonic cell complex. This scenario may account for observations of direct bipolar germination in some somatic embryos or formation of multicellular clumps in other somatic embryos within the same culture (Geier 1982; Kuehnle et al. 1992).

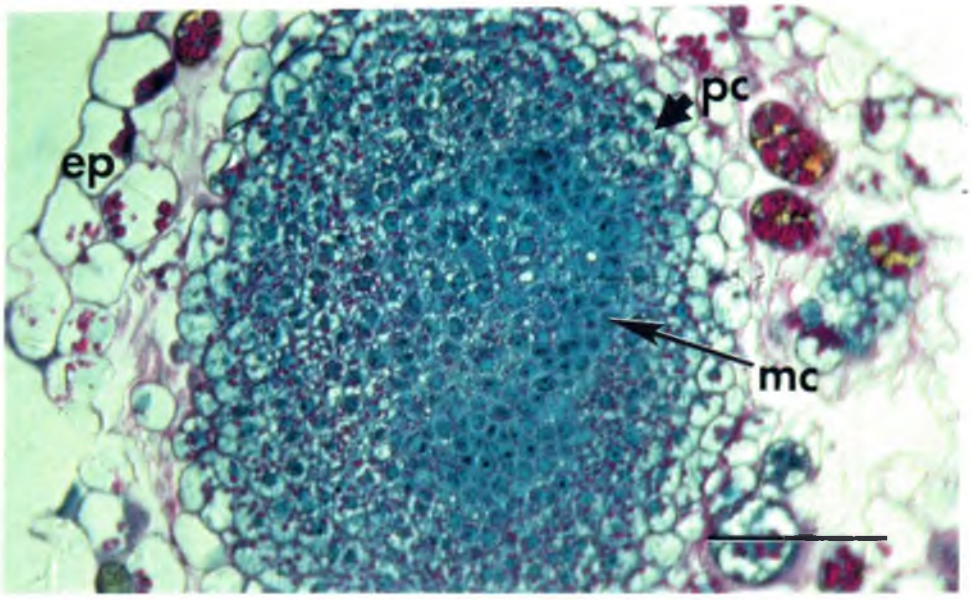

Figure 5.3. Cross section of a proembryonic cell complex. Sections were stained with PAS (carbohydrates stain pink) and aniline blue black (proteins stain blue). ep = epidermis, pc = proembryoic complex, and mc = meristematic cells. Bar = 100 µm.

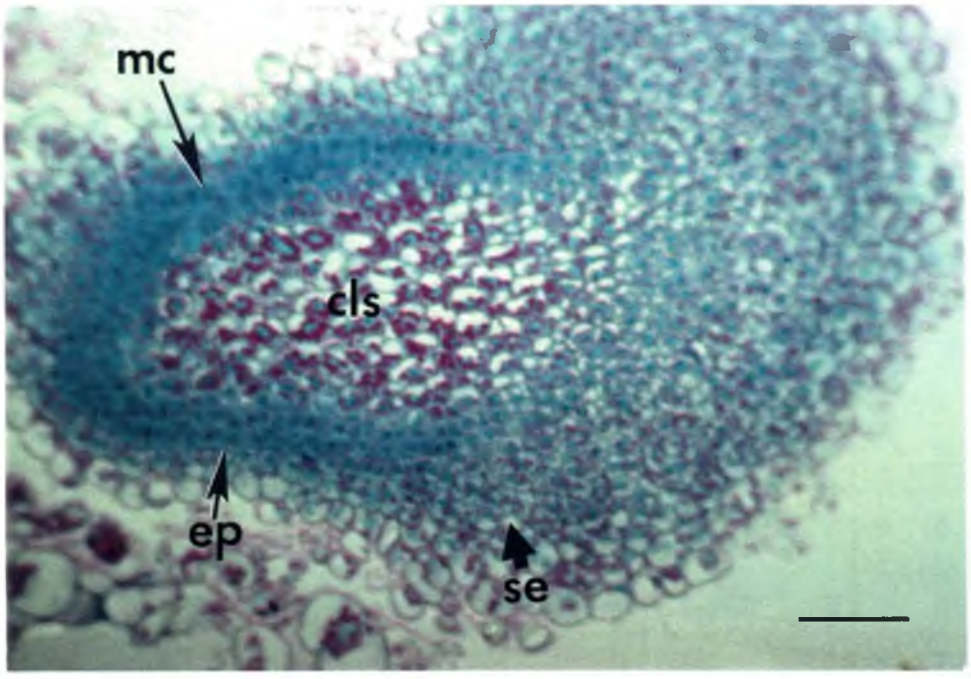

Figure 5.4. Cross section of a somatic embryo. Sections were stained with PAS (carbohydrates stain pink) and aniline blue black (proteins stain blue). ep = epidermis, mc = meristematic cells, se = somatic embryo and cls = "cotyledon-like structure". Bar = 100 µm.

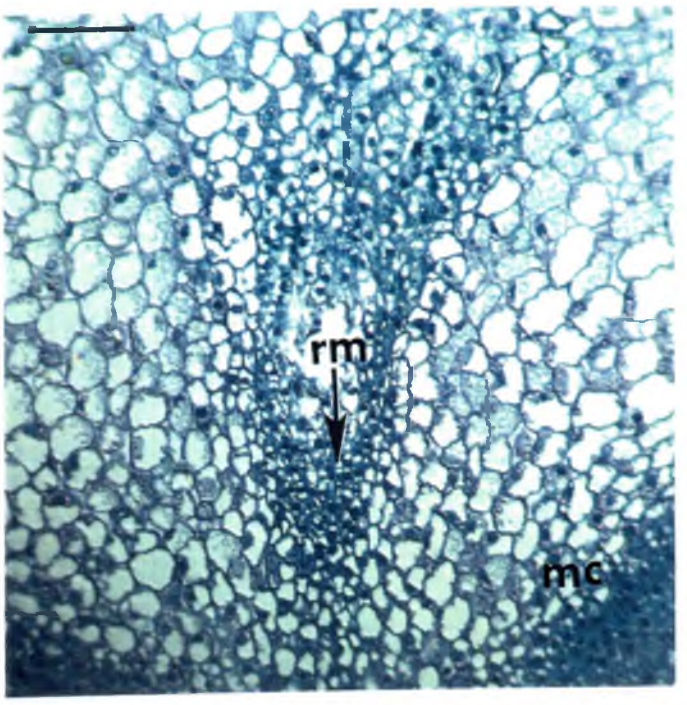

Figure 5.5. Root meristem of a mature somatic embryo. Sections were stained with PAS (carbohydrates stain pink) and aniline blue black (proteins stain blue). rm = root meristem and mc = meristematic cells. Bar = 125 µm.

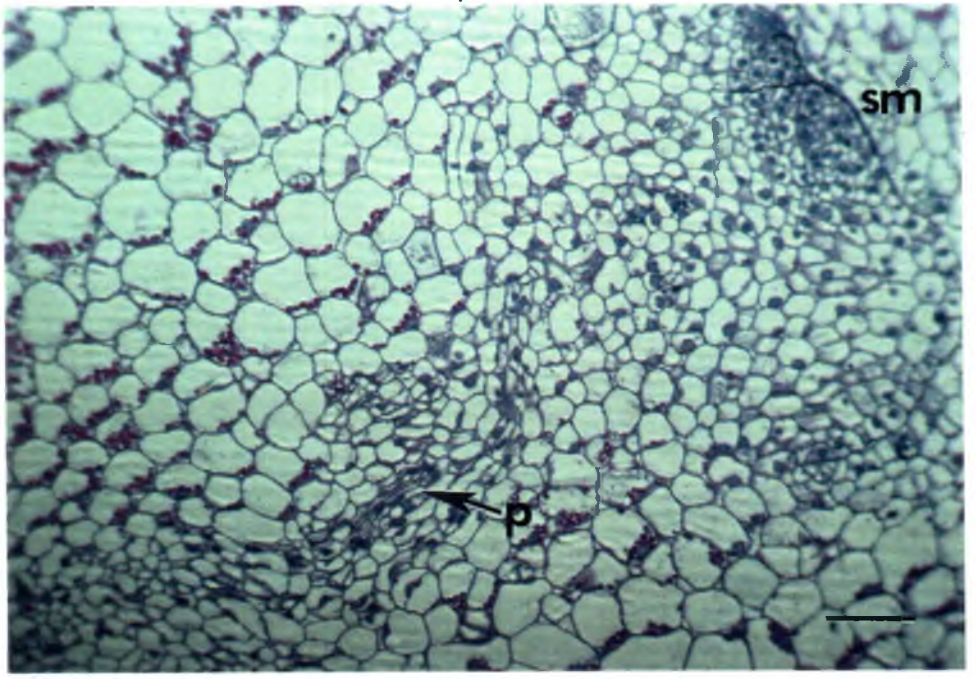

Figure 5.6. Cross section of a somatic embryo with shoot meristem and procambium. Sections were stained with PAS (carbohydrates stain pink) and aniline blue black (proteins stain blue). sm = shoot meristem and p = procambium. Bar = 125 µm.

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