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A Histological Study of Development of Adventive Embryos in Organ Cultures of *Phoenix dactylifera* L.

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ABSTRACT

Callus cultures from axillary buds of 2-4-year-old offshoots of *Phoenix dactylifera* L. have yielded plantlets adventitiously. Adventive embryos originated from meristematic cytoplasm-rich cells located in the epidermal and adjacent sub-epidermal portions of the periphery of callus on a basal medium supplemented with 2,4-D and isopentenyladenine. The formation of adventive embryos is described. Morphogenetically competent cells proliferate into globular proembryos which differentiate into bipolar structures. Transfer to a medium devoid of hormones enhances embryo development and promotes plantlet growth. Comparative studies were made between zygotic and adventive embryos in various stages of development.

Key words: *Phoenix dactylifera* L., date palm, Palmae, adventive embryogenesis, histological development.

INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is a dioecious, arborescent monocotyledon. Date palms are propagated commercially through offshoots, which arise from axillary buds. Only a limited number of offshoots are produced in the lifetime of a palm. Propagation *via* seed is impractical for two reasons: (1) about half of the progeny will be male and half will be females, and there is no way to determine the sex of the palms until the time of flowering, and (2) fruit from female seedling palms is usually inferior in quality to that of established clonal trees. Plant tissue culture was employed to produce plantlets from economically important date palms. Such procedures should lessen the dependence on offshoot production.

During the last 10 years, numerous attempts have been made to grow arborescent monocotyledons in tissue culture (Ammar and Benbadis, 1977; Corley, Barrett and Jones, 1976; Eeuwens, 1976; Eeuwens and Blake, 1977; Rabechault, Ahee and Guenin, 1970; Reuveni, Adato and Lilien-Kipnis, 1972; Reuveni, 1979; Schroeder, 1970; Smith, 1975; Smith and Thomas, 1973). Some success has been reported in the initiation of adventitious plantlets from embryo callus of the date (Ammar and Benbadis, 1977; Reynolds and Murashige, 1979) and oil palm (Corley *et al.*, 1976; Rabechault *et al.*, 1970). Recently, tissues explanted from lateral buds, shoot tips, stem and rachilla, and excised embryos of date palm have been reported by the first author to initiate plantlets (Tisserat, 1980). In this study, lateral bud cultures grown on a defined medium initiated callus and plantlets. The process of plantlet generation was studied histologically and found to be a type of adventitious embryogenesis.

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MATERIALS AND METHODS

Lateral buds were obtained from two to four-year-old offshoots cut from seedling date palms at Indio, California. Using a hatchet and a knife, offshoot leaves were removed acropetally, revealing the axillary buds. Buds 0·5 mm and greater in length were excised and temporarily stored in an aqueous solution of 150 mg l^{-1} citric acid and 100 mg l^{-1} absorbic acid until surface sterilization could be carried out. All explants were surface-sterilized by soaking in 1 per cent sodium hypochlorite solution (containing one drop of Tween-20[1] emulsifier per 100 ml solution) for 15 min. Bud explants were initially cultured on a basal nutrient medium composed of Murashige and Skoog salts, 170 mg l^{-1} $NaH_2PO_4.H_2O$, 100 mg l^{-1} i-inositol, 0·4 mg l^{-1} thiamine.HCl, 3 per cent sucrose, 3 mg l^{-1} N-(Δ^2-isopentyl)-adenine (2iP), 30 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0·3 per cent Sigma activated charcoal (neutralized), and 0·8 per cent Phytagar. The pH of the medium was adjusted to 5·7 with 0·1 N NaOH or HCl prior to the addition of agar, and autoclaved.

Excised embryos were obtained from mature seeds of 'Deglet Noor' fruits. Seeds were soaked in tap water 48 h prior to surface sterilization. An anvil hand cutter was used to open seeds and the exposed embryos were removed and planted with the aid of a scalpel. Seeds and excised embryos were cultured on medium devoid of hormones. Cultures were maintained at 27 °C under 100 ft-candle Gro Lux lights for 16 h each day in a temperature controlled room.

Tissue cultures and plantlets used in histological observations were fixed in FAA (formalin: glacial acetic acid: 50 per cent ethanol – 5 : 5 : 90 v/v/v), dehydrated in an ethanol series, and embedded in paraplast. Embedded tissue was sectioned 5–7 μm thick and stained with safranin and fast green. Sections of true embryos were stained with aniline blue black and controls were incubated in trypsin and pepsin. Procedures have described previously (DeMason, 1979). All sections were photographed with a No. 29 or No. 22 Kodak Wratten filter. Histological observations were made of the explanted embryos and embryoids at corresponding stages of differentiation. Cell sizes were determined with an ocular micrometer. Callus sections were made from cultures that were grown for 2 weeks on media with and without 2,4-D and 2iP.

OBSERVATIONS AND RESULTS

Development of excised date embryos and seeds in vitro

Explanted white, ovate embryos from mature seeds averaged 2 mm in length. The embryo at this stage shows the rudimentary morphological organization found in the later developmental stages, e.g. epicotyl shoot apex, root pole (radicle and hypocotyl) and cotyledon (Plate 1A). The shoot apex is enclosed by the cotyledon. During normal germination, the distal end of the cotyledon remains within the seed coat and functions as the haustorium which absorbs nutrients from the endosperm during the germination process. An internal cavity which separates the shoot tip region from the rest of the cotyledon occurs within the embryo (Plate 1A, B). Initials of three rudimentary leaves are visible at this stage (Plate 1B). Procambial strands are visible in the embryo and extend from the hypocotyl into the distal portion of the cotyledon (Plate 1A).

The main portion of the cotyledon is composed of cells that are about 40 μm in diameter. These cells contain numerous protein bodies as evident by positive staining with aniline blue black. The shoot apex region is composed of smaller cells; usually averaging around 15 μm in diameter. These cells are characterized as being densely cytoplasmic, and they have thinner cell walls than those found in the cotyledonary portion of the embryo.

[1] Mention of a trademark or proprietary product in this paper does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Within the first 14 days of culture, excised embryos undergo a general greening accompanied by 2–4 cm elongation of the mid-portion of the cotyledon, often referred to as the cotyledonary petiole (Tomlinson, 1960) (Plate 1D). Mature xylem in vascular strands becomes evident at this stage (Plate 1D). During this same time the shoot tip region becomes highly meristematic, forms additional foliar leaves, and the root tip forms a root cap. The haustorial end of the cotyledon is characterized as a round, finger-like projection (Plate 1D, E). It is composed of parenchyma intermixed with several thick-walled cells which contain tannin-like substances.

In the following 2 weeks in culture, the sheath of the cotyledon enlarges considerably, and the foliar leaves also enlarge. Elongation of the primary root and emergence of the plumule from the cotyledonary sheath usually occur after 4–6 weeks in culture. These events may or may not be simultaneous (Plate 2A, B). The first true leaf emerges from the cotyledon through a depression in the cotyledonary sheath called 'the slit-shaped opening' which may be several cm in length. The primary root elongates from the base of the embryo. Subsequent roots are adventitious in origin. Germinating roots grow downwards and the shoot tips grow vertically upwards.

Development of seeds and excised embryos of date palms are similar except for minor deviations in the cotyledonary sheath and haustorium development (Plates 1A, C and 2). The cotyledon emerges from the seed through the centrally-located pore; it grows at a right angle for about 0·5 cm and then exhibits a geotropic response by growing downwards. Expansion of the root and shoot tips occur after the sheath reaches 2–4 cm in length. The cotyledon of the excised embryo usually does not exhibit a geotropic response, but grows on the surface of the agar medium often twisting several times (Plate 2A). Only after emergence of the plumule and elongation of the primary root is there an exhibition of natural geotropism. In excised embryos the haustorium is round and blunt and much reduced in size when compared to the haustorium in the seed (Plate 1E). The seed haustorium initially is bloated, bladder-like in shape and then eventually assumes the shape of the displaced endosperm (cf. Plate 1C, F).

Initiation of adventive embryos on medium containing hormones

Following repeated cultures at 8-week intervals, bud cultures gave rise to a white and nodular callus. Plantlet formation occurred after the bud cultures were transferred to a medium devoid of 2iP and 2,4-D. White nodular callus originated from the leaf margins of bud explants following 2 months in culture. This callus could be sub-cultured (Plate 3A). Adventive embryos were initiated from this callus following 4 months in culture. Sectioned callus consisted of two distinct types of tissues, a loose friable tissue and compact aggregates (Plate 3B). The friable portion of the callus was composed of large single cells and disorganized clumps of highly vacuolated cells ranging in diameter from 20–40 μm. This tissue did not appear to be involved in embryo formation and was generally found surrounding the aggregate clumps.

The compact aggregates serve as the source of embryoids. Aggregate clumps were multicellular structures composed of a variety of cell types which measured from 8–20 μm in diameter. The aggregate clumps were composed of semi-vacuolated cells 20 μm in diameter found in the interior regions and more densely cytoplasmic meristematic cells located in the peripherial layers (Plate 3C, D). Both types of cells had prominent centrally-positioned nuclei commonly containing 2 nucleoli. Early meristematic initials or loci were associated with aggregates located in both the epidermal and subepidermal regions (Plate 3C, D). These loci were meristematic cells that were 8 μm in diameter. Light microscope observations revealed that these meristematic loci had definite fragmentation lines produced by thickened cell walls which separated the smaller, meristematic and cytoplasm rich cells from the surrounding larger, less-meristematic and more

vacuolated cells within the aggregate (Plate 3D). The loci expanded to produce separate aggregate clumps and/or bipolar embryos.

It is suspected that single cells located in both the epidermal and sub-epidermal portions of the aggregate clumps were the precursors to the meristematic loci and embryoids. The meristematic loci were in close association with the callus clump. A sequence of embryogenesis could be traced from a single cell to a bipolar embryoid (Plates 3E, F and 4A, B). Single cells were observed to undergo transverse divisions to yield a 2-celled spherical proembryo (Plate 3E). Further divisions often lead to the formation of a 3- and 4-celled spherical proembryo (Plate 3E) and eventually a spherical embryo of 8 and 16 cells (Plate 3F). A multicellular ovate proembryo developed, often characterized by a thick cell wall enclosing it (Plate 4A). Eventually, this enclosing wall was ruptured by further cell divisions giving way to a multi-celled bipolar structure (Plate 4B). The bipolar structures consisted of a meristematic end and a less meristematic highly vacuolated opposite tip. The opposing ends of the bipolar structure were the precursors of the shoot and root pole region and haustorium at the tip of the cotyledon, respectively. Further divisions of the bipolar structure gave rise to cylindrical embryoids which corresponded to the excised embryo found in the mature seed prior to germination. No suspensor of the embryo was observed. Sometimes a 4-celled linear proembryo occurred. Budding or proliferation of meristematic centres from a single locus origin were commonly observed (Plate 3C).

Development of adventive embryos on medium devoid of hormones

Transfer of this callus, containing meristematic loci and early embryoid stages, to medium devoid of 2,4-D resulted in the formation of plantlets through the enlargement of existing embryoids (Plate 4C, D). Generally, the overall callus appearance became looser in consistency with enlarged embryonic structures being prominent on medium devoid of hormones (Plate 4C). Sectioning of this callus revealed that the haustorium protruded from the callus while the root pole end of the embryo remained embedded in the callus mass (Plate 4D). The root pole continued to be in physical contact with the surrounding callus until the expansion of the plumule and primary root. During the period preceding the emergence of the plumule and primary root, the root pole region was undergoing root and shoot differentiation. Cells of the haustorium exhibited little meristematic activity during this same period. The haustorial region of the embryo was characterized by the same developmental pattern found for the excised zygotic embryo. The haustorium of the embryoids were much reduced in size compared to that found in the seed. Following 2 weeks on medium without 2,4-D, the appearance of embryoids with definite shoot and root tips similar to those found in excised embryos were common (Plate 4D). Later asexual embryo germination stages compared very closely with those found in the germinating excised zygotic embryo (Plates 2 and 4E). The formation of vascular bundles within the asexual plantlet at the 8-week-old stage corresponded to that found in the zygotic seedling.

DISCUSSION

At one time, tissue culture and plantlet initiation in monocotyledons was considered difficult (Johri, 1971). Several investigators have been able to induce plantlets from numerous herbaceous monocotyledons *in vitro* (Constabel, Miller and Gamborg, 1971; Hussey, 1975; Pierik and Steegmans, 1975; Wilmar and Hellendoorn, 1968). The asexual embryogenesis process for *Bromus inermis* Leyes. cell suspensions has been described (Constabel *et al.*, 1971). Asexual plantlets arose from embryoids exhibiting the structures found in normal germination. They observed an intermediate globular

structure that preceded the formation of bipolar structures. This embryogenic stage was unique to the *in vitro* germination process. Less success in the production of plantlets from palms through tissue culture has been reported (Eeuwens, 1976; Eeuwens and Blake, 1977; Reuveni *et al.*, 1972; Schroeder, 1970). However, Rabechault *et al.* (1970) reported the formation of asexual embryos from *Elaeis guineensis* Jacq. (oil palm) using excised embryos. Similarly, Ammar and Benbadis (1977) reported formation of plantlets from date embryo callus. Neither of these reports elaborates on the mode of plantlet formation. Reynolds and Murashige (1979) reported that plantlets from embryo callus arose through an asexual embryogenetic process which paralleled zygotic embryogenesis. They described similar embryogenic structures which have been confirmed in this study.

Few detailed studies of palm embryology have been carried out (Guignard, 1961; Johansen, 1950). Guignard (1961) has described *Chamaerops humilis* L. zygotic embryogenesis in detail. The *C. humilis* L. zygote divides transversely to form a 2-celled proembryo. A subsequent longitudinal division yields a 4-celled spherical proembryo. Further partitions lead to the formation of a multicellular stage composed of four superimposed quadrants. Later divisions of these quadrants yield a spherical mass of two superimposed octants. Eventually, the dividing embryo differentiates epicotyl, cotyledon, and root pole. Davis (1966) considers this type of embryogeny to be an example of the Asterad type of Johansen (1950). Embryo development has also been observed in *Actinophloeus* and *Areca*, both of which conform to the Onagrad type (Davis, 1966). In neither of these two embryo types is a linear embryo formed because the upper cell of the two-celled embryo divides longitudinally. Early date palm embryo development occurs near the micropylar tip of the seed (Lloyd, 1910). Later a spherical embryo migrates to the mid-portion of the seed close to the centrally-located pore where further development continues. The embryo assumes a cylindrical shape with the root end towards the pore opening and the shoot end pointing towards the endosperm (Sachs, 1887; Lloyd, 1910). In date seed germination the embryo's cotyledon is not erect or photosynthetic. The distal portion of the cotyledon serves as the suctorial haustorium or bladder-like absorbing organ, providing endosperm nutrients to the epicotyl region. The cotyledonary sheath emerges through the centrally-located pore of the seed and contains the epicotyl shoot apex at its tip. Germination of palm seeds can be described as two distinct phases: (1) the shoot and root tips are pushed from the seed, still enclosed within the cotyledonary sheath, and (2) the plumule breaks through the sheathing organ, and leaves become externally visible (Carpenter, 1964; Sachs, 1887; Tomlinson, 1961).

All the structures common to zygotic embryogenesis were observed in the asexual phenomenon. McWilliam, Smith and Street (1974) observed that the initial embryogenetic cells could not be distinguished from meristematic cells involved in aggregate proliferation. Similarly, initial precursor and 2-celled proembryoids could not be absolutely identified in the date palm callus. Perhaps there is no difference at this stage between cell division and initial embryogenesis segmentation. However, suspected initial precursor and 2-celled proembryoids corresponding to the zygotic stages were frequently observed. Corresponding spherical 4-, 8- and 16-celled stages were also recognized. Further divisions of these embryonic centers lead to the development of meristematic loci. Early-developing embryoid loci may readily be identified in sections of callus aggregates examined with the light microscope. Definite cleavage walls separated the loci from surrounding vacuolated cells in the aggregates. Further cell divisions resulted in the formation of bipolar embryoids with shoot and root poles and a haustorium. The root pole region of the embryo was always embedded within the callus of origin while the haustorium protruded from the callus onto the agar surface or grew free in the atmosphere. The haustorium of both the excised embryos and embryoids was much reduced in size and nutritional importance compared to the haustorium found in the seed

during *in vitro* germination. Apparently, most of the nutrients necessary for embryo development were absorbed through the root pole region of the asexual and excised zygotic embryos. The development of the haustorium with its peculiar shape in the seed is a function of the endosperm shape and is not inherent to the development of excised embryos. In both the excised embryos and embryoids, the haustorium is essentially a nonfunctional structure. However, the haustorium of excised embryos and embryoids retains its tannin cells. Similarly, Fisher and Tsai (1978) noted that the haustoria of cultured coconut embryos were also much reduced in size and function.

In suspension cultures, carrot proembryos at the globular and heart-shaped stage are released from the physical restraints of the callus and are allowed to develop free-floating (McWilliam *et al.*, 1974). Date palm callus and embryoids in agar nutrient medium develop in close physical contact until the expansion of the embryoid's plumule and root tip. Starch grain development has ascribed various roles in asexual embryogenesis in carrot cultures (Halperin, 1970; McWilliam *et al.*, 1974). Starch grains were conspicuously absent in all stages of date palm zygotic and asexual embryogenesis.

In this study, asexual embryogenesis in date palms was described. The development of asexual embryos from callus closely paralleled excised zygotic embryo germination *in vitro*. This palm species is an especially suitable plant material in which to study asexual embryogenesis in arborescent monocotyledons. To date, several commercial and experimentally-developed date palm cultivars have been successfully tissue cultured. Production of plantlets from callus may offer a potential means to clone superior date genotypes on an accelerated scale. Introduction of new cultivars or increasing the existing date acreage with clonal varieties would no longer be limited to offshoot production.

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EXPLANATION OF PLATES

Abbreviations: AM = apical meristem, C = cotyledon, CA = compact aggregate, E = embryoid, FE = four-celled embryoid, FT = friable tissue, H = haustorium, LP = leaf primordia, PS = procambial strand, RP = root pole, SC = suspected single-celled embryoid initial, TE = two-celled embryoid, X = xylem.

Plate 1

Stages of germination in the date palm. Scale line = 0·5 mm.

A. Longitudinal section through narrow axis of a freshly excised embryo from a 'Deglet Noor' seed.

B. Cross section of a freshly excised embryo from a 'Deglet Noor' seed.

C. Longitudinal section of a developing embryo at the early stage of germination, 2 weeks after culture.

D. Longitudinal section of a developing embryo during the cotyledonary sheath elongation stage, 4 weeks after culture.

E. Longitudinal section through the haustorium of an excised embryo, 6 weeks after culture. Note the the excised embryo's haustorium is a round and finger-like projection.

F. Transverse section through the haustorium of a germinating embryo, 6 weeks after culture. Note the haustorium is bloated and bladder like in shape.

Plate 2

Sequence of germination of 'Deglet Noor' date palm excised embryos and seeds under *in vitro* conditions.

A. Germination of an excised embryo. From left to right: freshly excised embryo from mature seed; stage or early elongation of cotyledon, 2 weeks in culture; later stage of elongation of cotyledon, 4 weeks in culture; emergence of the primary root and the plumule from the cotyledonary sheath, 6 weeks in culture; plantlet with photosynthetic leaves, 8 weeks in culture. × 1.

B. Germination of a date palm seed. From left to right: protrusion of cotyledon through centrally-located pore; 2-weeks-old; elongation of cotyledon and developing primary root, 4-weeks-old; emergence of first leaf through slit-shaped opening of cotyledonary sheath, 6-weeks-old; free living seeding, 10-weeks-old. × 1/2.

PLATE 3

Morphogenesis of date palm callus on nutrient media containing 2,4-D and 2iP.

A. Date palm callus 2 weeks after culture. Note the nodular appearance of the callus which represents proembryonic masses. (× 200)

B. Transverse section through the callus, 2 weeks after culture. Several compact aggregates (CA) are present interdispersed among a matrix of loose friable tissue (FT). Scale line = 50 μm.

C. An example of embryoid budding from a compact aggregate. Scale line = 50 μm.

D. Occurrence of meristematic loci within a callus aggregate. Note the thick walled fragmentation lines demarcating each locus. Scale line = 50 μm.

E. Section through date palm callus. Transverse division of the single cell (SC) embryogenetic progenitor yields a 2-celled embryo (TE). A further division yields a 4-celled spherical tetrad embryo (FE). Scale line = 50 μm.

F. Example of an 8-16-celled embryo. Note thick wall surrounding the proembryonic structure. Scale line = 50 μm.

PLATE 4

Morphogenesis of date palm embryoids.

A. Multicellular spherical proembryonic structure on medium containing 2,4-D and 2iP. Scale line = 50 μm.

B. An example of an early bipolar proembryo on medium containing 2,4-D and 2iP. Scale line = 50 μm.

C. An example of a date palm callus culture on nutrient medium devoid of 2,4-D and 2iP, 2 weeks after culture. Note enlarged embryonic structures. × 200

D. Orientation of embryoids in callus on nutrient medium devoid of 2,4-D and 2iP, 2 weeks after culture. Root pole region is embedded within the callus of origin. Scale line = 1 mm.

E. Sequence of germination of asexual embryos from callus on nutrient medium devoid of 2,4-D and 2iP. From left to right: Cotyledon of asexual embryo protrudes from the callus aggregate, 2 weeks in culture; excised embryoid at early cotyledonary elongation stage; 4 weeks in culture; later cotyledonary elongation stage, 6 weeks in culture; emergence of first leaf and elongation primary root, 8 weeks in culture. × 1

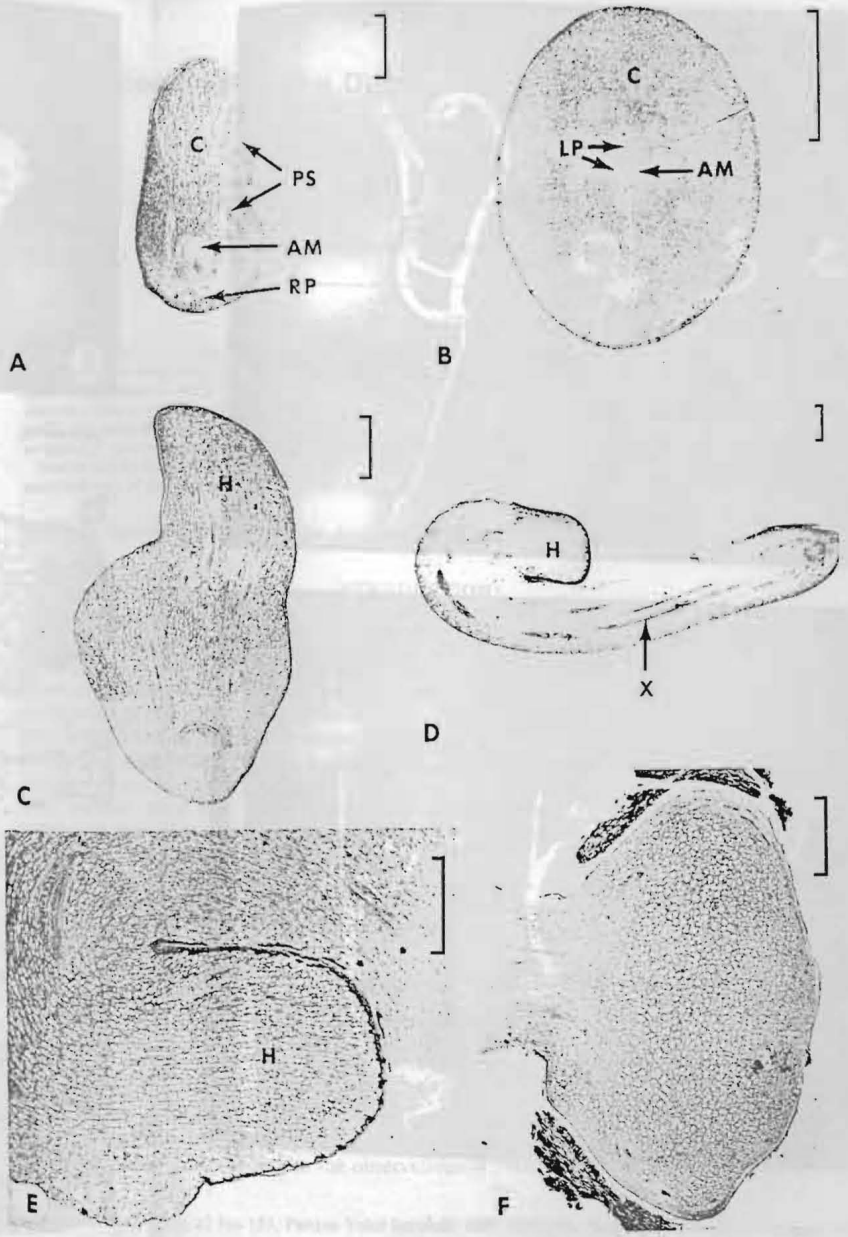
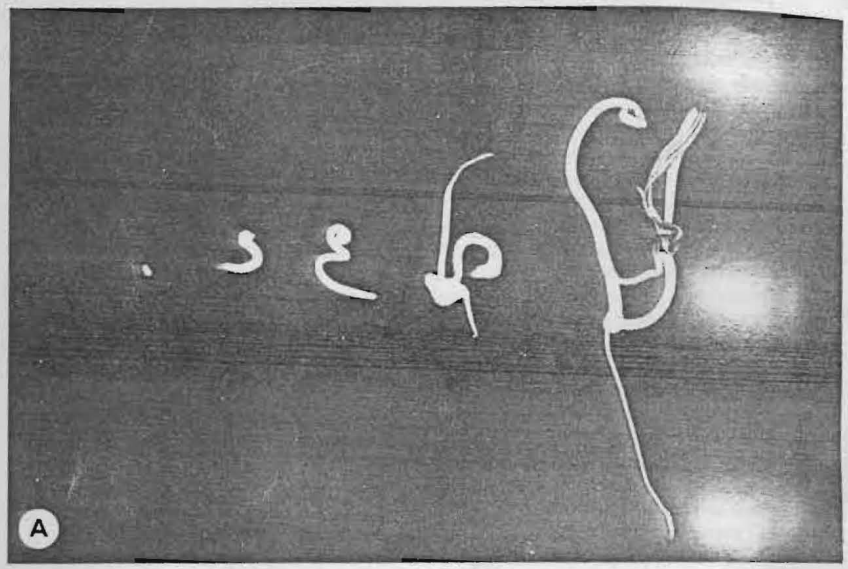


PLATE 1



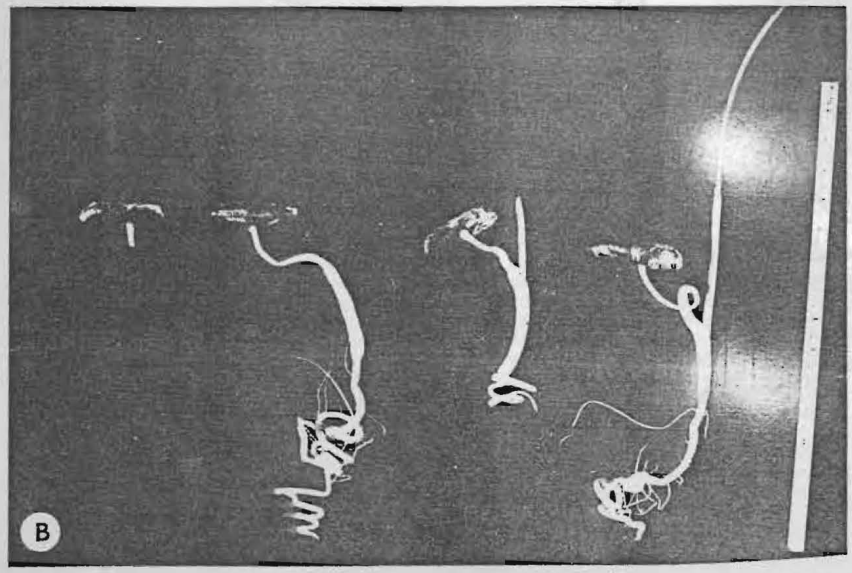
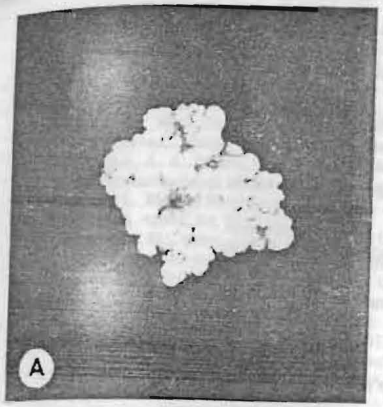
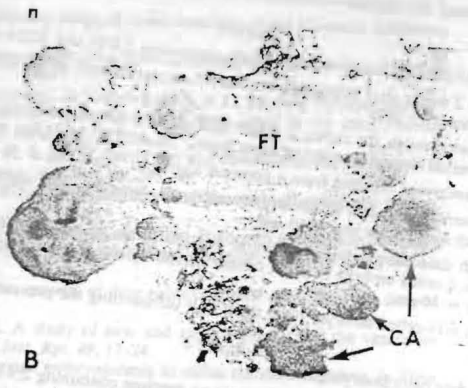
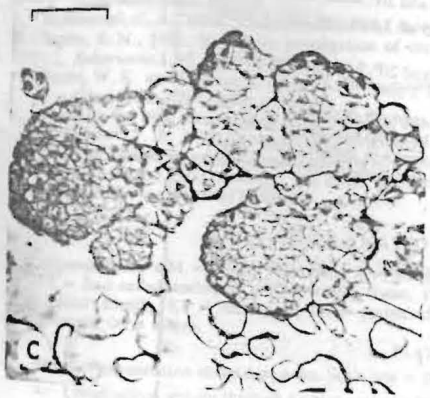
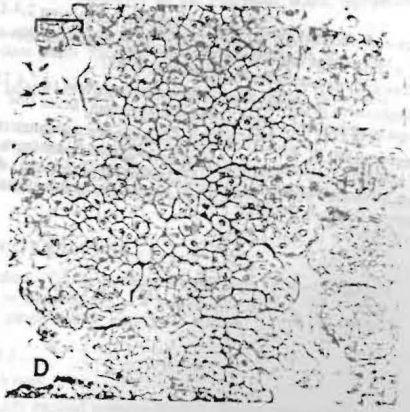


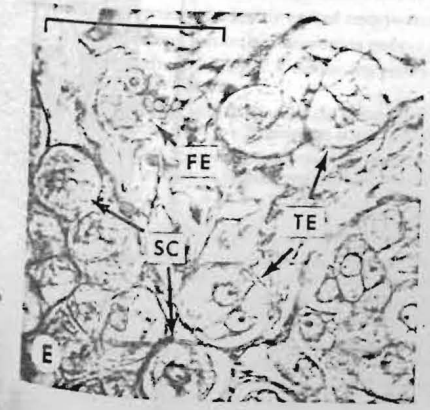
PLATE 2











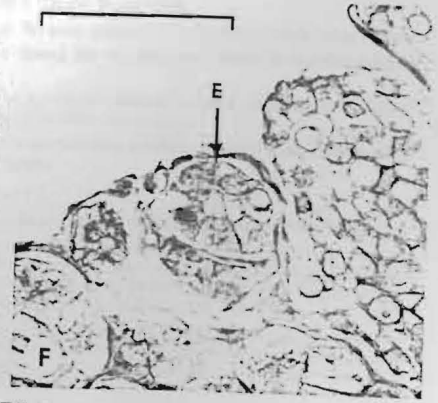


PLATE 3

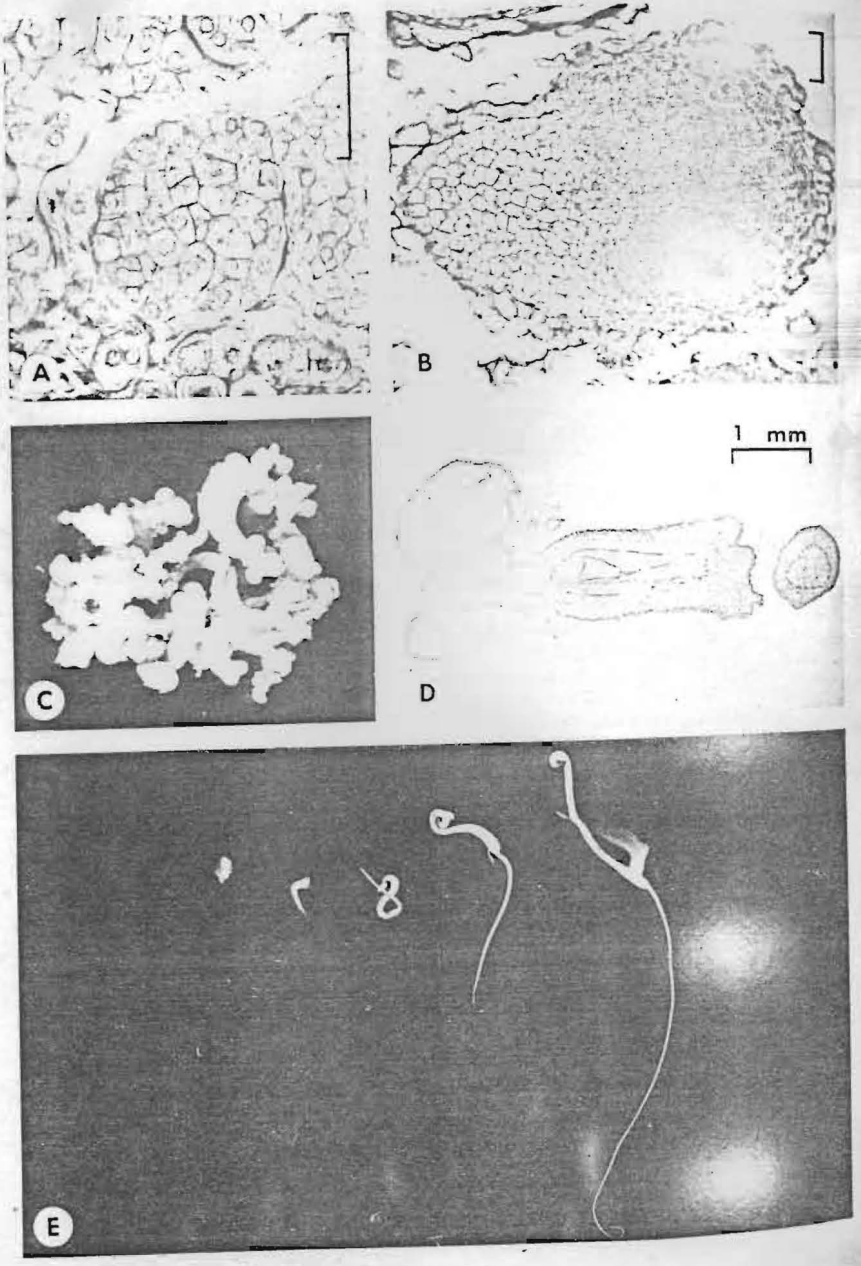


PLATE 4

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Callose Deposition During Megagametogenesis in Two Species of *Oenothera*

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ABSTRACT

Callose deposits are present both in degenerating megaspores of the heteropolar tetrad in *Oenothera hookeri* and in degenerating embryo sacs of the homopolar developing tetrad in *O. biennis*. They are partially continuous with the cell wall and partially enclosed in the degenerating cytoplasm and show electron opaque bands within a less electron opaque material. Vesicles called callose grains are present in the degenerating cytoplasm of the embryo sac in *O. biennis*. These show an electron opaque fibrillar or granular core surrounded by a halo of low electron opacity.

Similarities in fine structure between callose deposits of female gametophytes which follow the degenerating pathway of development, and callose plugs present in pollen tubes during their growth, are discussed.

Key words: *Oenothera*, evening primrose, megagametogenesis, megasporogenesis, callose, ultrastructure.

INTRODUCTION

Megaspore tetrads in species of *Oenothera* may show heteropolar or homopolar development (Noher de Halac and Harte, 1975). Some of the characteristics of cells involved in development of the embryo sacs were investigated earlier in relation to polarity. Recent papers deal with the pattern callose deposition during megasporogenesis (Noher de Halac and Harte, 1977), and with dictyosome and plastid distribution in megaspores mother cells (MMC) and in megaspore tetrads in Onagraceae (Rodkiewicz and Bednara, 1974; Rodkiewicz and Sniezko, 1978; Rodkiewicz, 1978). Some aspects of vacuolation, starch content and nuclear condition in young embryo sacs, after homopolar megasporogenesis in *O. biennis*, have also been investigated (Noher de Halac, 1980*a*). The polarity of nucellus in the ovule in relation to the polarity of the megaspores tetrad has also been examined. (Noher de Halac, 1980*b*).

Despite the previous investigations, several questions about polarity in the ovule remain unanswered. This paper deals with the fine structure of degenerating megaspores and embryo sacs in *Oenothera* species that follow heteropolar and homopolar megasporogenesis, processes in which callose deposits are important. Taking into account the present observations, a new approach to the understanding of callose deposition during female gametophyte development is presented.

MATERIAL AND METHODS

Plants of *Oenothera hookeri* Torr. et Gray and *O. biennis* L. were grown in the experimental garden of the Institute of Developmental Physiology of the University Cologne (W. Germany). Pre-anthesis flowers located above the last open flower were collected during the morning. According to the observations of Noher de Halac and Harte (1977) at this time.

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