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Fertilization and rejection of spermatozoids by egg cells in artificially pollinated ovules of *Encephalartos* (Zamiaceae)

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Abstract The structure and behaviour of free female, male and proembryonal nuclei of *Encephalartos villosus* Lem. were studied during a light-microscopical investigation of serially sectioned archegonia in successfully pollinated ovules. Before spermatozoids were released from the pollen tubes into the archegonial chamber, the ventral canal nucleus had disintegrated in the neck region of the egg cell among minute, amoeboid bodies with PAS-positive granules. In archegonia containing multiple spermatozoids, the egg nucleus was unobtrusive and syngamy followed by proembryo formation regularly resulted. The egg cell usually reacted violently in archegonia penetrated by a single spermatozoid. These reactions were regarded as rejection phenomena and considered as indicators that the egg cell can differentiate between compatible and incompatible male gametes.

Key words Cycads · *Encephalartos* · Female choice · Fertilization · Prezygotic recognition

Introduction

All over the world, and despite national and international conservation strategies, wild populations of cycads are becoming extinct because of drastic human exploitation practices (Johnson and Wilson 1990). In southern Africa, treated here as the "flora of southern Africa" region as traditionally defined, only six of the 37 species of *Encephalartos* Lehm., one of 11 extant cycad genera, are regarded as safe or at low risk (Osborne 1995). Conservation of the species may well depend on the successful execution of ex situ cultivation programmes. However, trials with artificial pollination of female plants have revealed that reproductive processes in the genus are poorly understood and there is dire need of detailed data on post-pollination events.

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The cycads are an ancient, isolated group of primitive gymnosperms, not closely related to conifers but with many resemblances to some of the extinct (chiefly late Palaeozoic) pteridosperms (Johnson and Wilson 1990). The seed ferns are considered a likely ancestral source of cycads as well as flowering plants (Dilcher 1979; Mamay 1976; Pettitt 1982). Claims that gymnosperms, unlike angiosperms, generally lack prezygotic mechanisms of mate selection were based on studies conducted on conifers (Willson and Burley 1983 and references cited therein) and may not be valid for cycads.

The many, but often fragmentary, reports in literature dealing with life-historical aspects of *Encephalartos* reveal no data on sexual fusion or on phenomena suggesting the presence of prezygotic mate choice in this totally African genus of the cycads. No information pertaining to possible prezygotic detection abilities of other cycad genera can be derived from studies, conducted during the late 1800s and early 1900s on material fixed in coagulating fixatives and sectioned in paraffin wax, describing fertilization in cycads. Data in these reports do reveal, however, that the reproductive biology of *Encephalartos* conforms to the general cycad pattern.

Representatives of the genus are long-living, strictly dioecious plants bearing single or small numbers of massive cones (up to a mass of 45 kg, the largest known in either living or extinct plants) infrequently after an extended juvenile period. Meiosis occurs in the sporangia while the cones are still minute and completely concealed by bud scales covering the stem apices (De Sloover 1961; Sedgwick 1924). In the megasporangium, a single megaspore mother cell develops, deeply imbedded in a massive nucellus which is partly enclosed by the young integument. The chalazal megaspore of a T-shaped tetrad develops into the megagametophyte (De Sloover 1961). Free nuclear divisions occur in the latter structure which enlarges enormously so that a central cavity, lined with a thin layer of cytoplasm and imbedded free nuclei, is formed within the megaspore membrane. Cell wall formation occurs centripetally (De Sloover 1961, 1964; Sedgwick 1924). The enlarging mega-

gametophyte displaces the nucellar tissue almost completely and, eventually, forms an ovoid body (ca. 2 cm×1.5 cm in *E. villosus*) of megagametophytic tissue, usually referred to as endosperm. In the mature ovule the endosperm constitutes the major part of the ovular region which lies inside the sclerotesta. In the extreme apical region, directly under the micropyle, a small nucellus cap remains (De Sloover 1964; Steyn and Strydom 1993). When cell wall formation in the megagametophyte is completed, archegonia initiate from epidermal, megagametophyte cells opposite the nucellar cap. In this region an indentation, which ultimately forms the archegonial chamber, develops in the megagametophytic tissue. During their development the egg-containing archegonia become deeply sunken into the tissue under the indentation and only their inflated neck cells protrude into the archegonial chamber (Steyn and Strydom 1993).

At the beginning of the long pollination process, three-celled pollen grains, each containing a prothallial cell, tube cell and a generative cell, are dispersed by wind and insects to enter receptive ovules. Prior to pollination, a small cavity, the pollination chamber, develops in the beak-like extension of the nucellus into the micropyle (De Sloover 1964; Steyn and Strydom 1993). When germination occurs within the pollination chamber, each pollen grain produces a haustorial, microgametophytic tube which initially invades the subepidermal cell layers of the nucellus (Steyn, unpublished findings). The basal part of the tube, still partly incased in the exine of the pollen grain, eventually starts growing towards the megagametophyte. The generative cell divides to form a sterile cell (stalk cell of older literature) and a spermatogenous cell (body cell of older literature). As the pollen chamber with developing pollen tubes extends downwards, the nucellus cells separate along a splitting layer so that the tubes enter the archegonial chamber. At this stage the swollen base of each tube contains a small tube nucleus, two amoeboid, immature spermatozoids (derived by division of the spermatogenous cell), a sterile cell and a prothallial cell (Figs. 5, 6, Steyn 1993). Before the tubes spill their contents into the archegonial chamber, the male gametes mature (Fig. 1, Steyn 1993). At maturity, a living spermatozoid is an ovoid (ca. 270 μm×230 μm) cell, resembling a spinning top (Fig. 2, Steyn 1993). Motility is provided by numerous cilia occurring in a spiral band consisting of at least four gyres encircling the anterior half of the cell (Figs. 1, 3, 4, Steyn 1993).

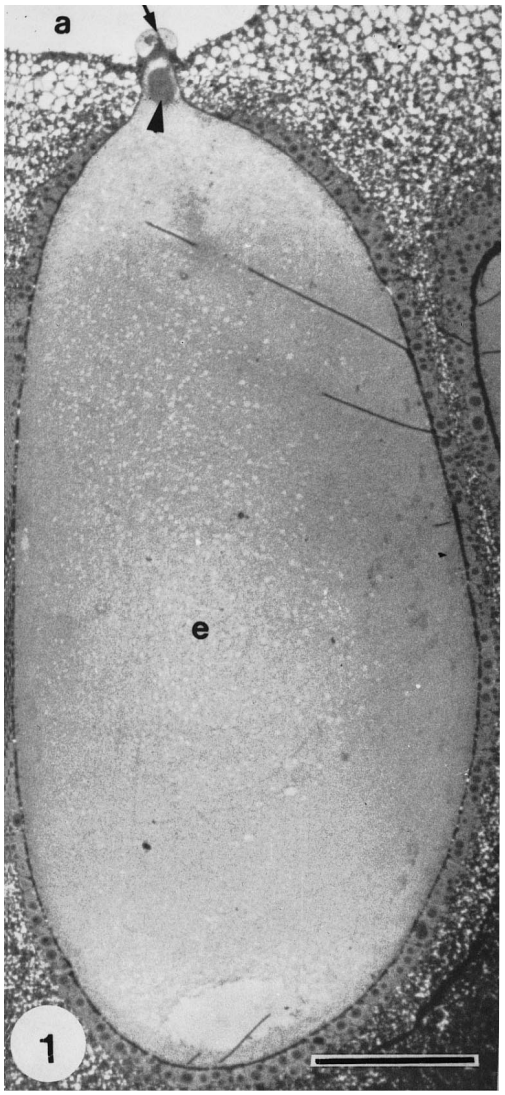
During a light-microscopical study of artificial pollination in *E. villosus* Lem., we observed the penetration of pollen tubes into the archegonial chambers of the ovules. This paper reports on a light-microscopical study of archegonial structure and the behaviour of free nuclei inside the archegonia before and after spermatozoids have entered the egg cells. Our results present clear evidence that prezygotic choice exits in cycads and indicate that the recognition site is located within the egg cell. Such a recognition system operating between male and female gametes has not yet been proposed for cycads.

Material and methods

Material for this study was collected from four coning female plants of *E. villosus*. The plants came from diverse sources and were relocated in the Pretoria National Botanical Garden where they grew widely spaced under diverse micro-environmental conditions. Fresh pollen from conspecific male plants in the same garden was manually syringed into the strobili when the megasporophylls separated during the first 2 weeks of April 1994. Throughout the processing period, material collected from each of the four cones was handled separately in labelled containers. Regular collection and dissection of ovules revealed the stage when the first pollen tubes started protruding through the nucellus into the archegonial chambers. The ovules were halved and the micropylar portions of megagametophytes, with attached nucellus caps, were carefully removed from the sclerotestal coverings. At this stage, the number of archegonia per megagametophyte and the number of protruding pollen tubes per corresponding nucellus cap and, thus, per archegonial chamber, were noted. Megagametophytes were further processed if the archegonial chambers showed the following easily detectable signs that pollination had been successful: (1) emerging or well-formed pollen tubes; (2) collapsed pollen tubes in wet archegonial chambers and (3) dry remains of pollen tubes. Ovules containing fewer pollen tubes than the number of archegonia present were discarded. Since each pollen tube contained two spermatozoids, this precaution ensured the sectioning of megagametophytes that held a surplus of male gametes for penetrating the archegonia. Additional ovules were collected weekly for 4 weeks after the first collapsed pollen tubes, indicative of spermatozoid release into the archegonial chamber, were found.

The micropylar halves of megagametophytes were immersed in fixative and trimmed to cores (ca. 1 cm^2) which contained the intact archegonia. Attempts to obtain smaller cores by excessive trimming of the surrounding tissue or by separating the two to five closely associated archegonia resulted in the destruction of the massive, turgid egg cells with their thickened, but unlignified cell walls. The cores were fixed for 16–24 h in a phosphate-buffered solution (pH 7.4) of 5% formaldehyde that contained 0.5% caffeine. Processing for semi-thin (2 μm) sectioning in glycol methacrylate (GMA) was performed according to conventional procedures. Some 93 cores and two to four archegonia per core were sectioned longitudinally. Ultimately, 287 complete sets of serially sectioned archegonia were obtained. Sections were pre-stained in 0.05% (w/v) toluidine blue in a phosphate buffer at pH 6.8 (O'Brien et al. 1964) to facilitate the selection of appropriate sections for permanent staining with periodic acid-Schiff's reagent (PAS), Haidenhain's haematoxylin and/or toluidine blue according

Figs. 1–6 Structure of archegonium before spermatozoids were released into the archegonial chamber. **Fig. 1** Archegonium in median longitudinal section illustrating the size of the egg cell (*e*), position of the egg nucleus (*arrowhead*) and the two neck cells (*arrow*) protruding into the archegonial chamber (*a*). *Bar* 500 μm. **Fig. 2** Neck region of egg cell containing the egg nucleus (*n*), fragmented ventral canal nucleus (*arrowhead*) and bodies (*small arrows*) containing PAS-positive granules. Note subsidiary neck cells (*large arrows*). *Bar* 100 μm. **Fig. 3** Neck region of egg cell with vestiges of neck canal cell (*arrowhead*) surrounded by bodies (*small arrows*) containing PAS-positive granules. *Bar* 25 μm. **Fig. 4** Cell wall and adjacent tissues of egg cell illustrated in Fig. 1. Note delicate cell walls (*arrowheads*) of jacket cells (*j*), thickness of egg cell wall (*white arrows*) and bodies (*small arrows*) containing PAS-positive granules in peripheral cytoplasm of egg cell (*e*). *Bar* 25 μm. **Fig. 5** Neck region of egg cell before division of neck cells, illustrating egg nucleus (*n*) with two nucleoli (*white arrows*), cytoplasm (*c*) surging up into the neck region and starch grains (*black arrows*) in archegonial chamber (*a*). *Bar* 100 μm. **Fig. 6** Median, apical region of archegonium showing recently devided neck cell (*arrowheads*), starch grains (*arrows*) and egg nucleus (*n*) descending into central cytoplasm of egg cell. *Bar* 100 μm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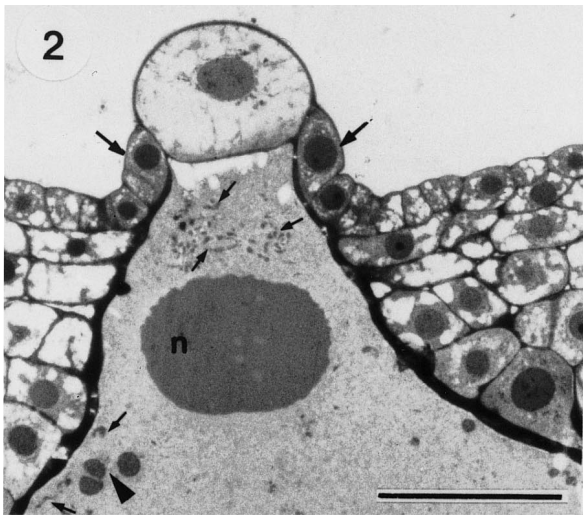
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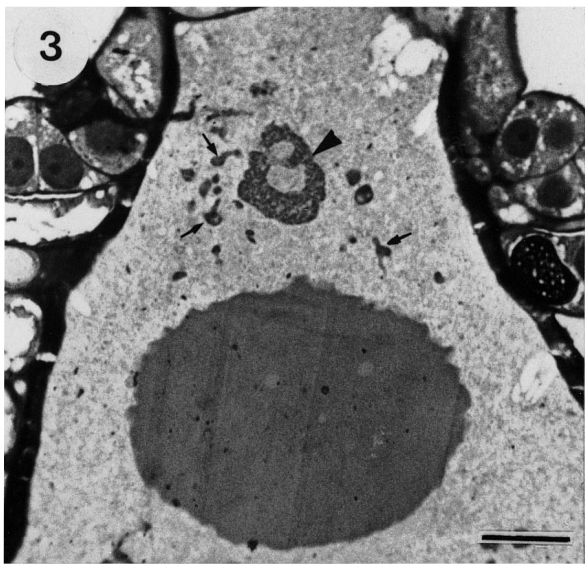
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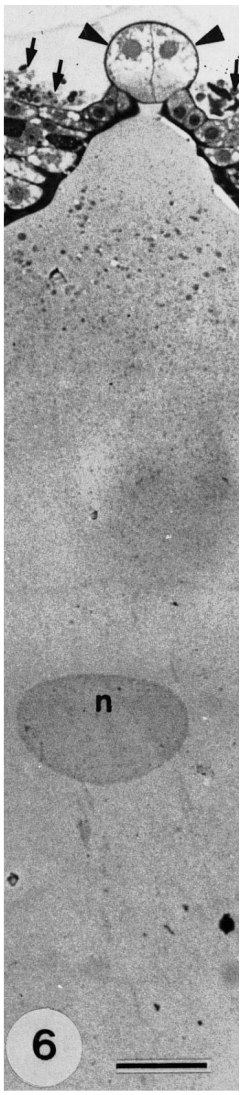
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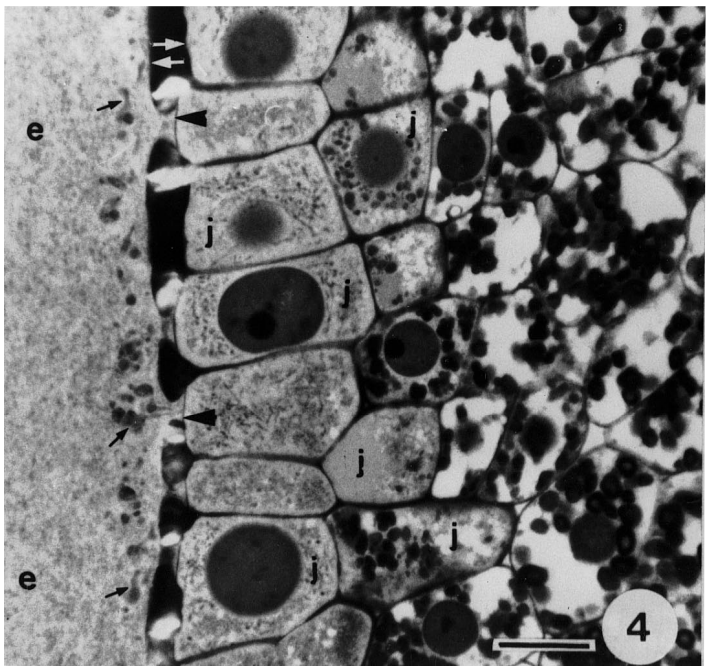
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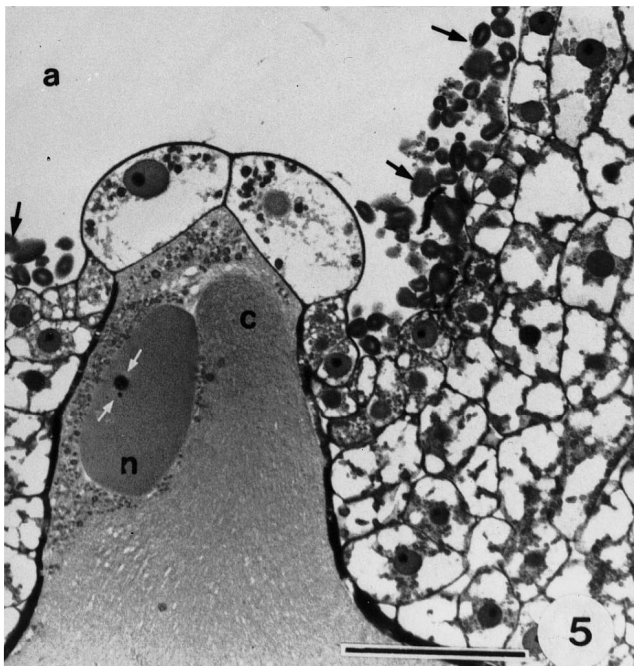
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to the methods described by O'Brien and McCully (1981). PAS staining revealed total carbohydrate contents of the tissue (Jensen 1962). Unstained sections were used for additional histochemical tests: (1) detection of DNA (Jensen 1962) by using the Feulgen reaction as described by O'Brien and McCully (1981); (2) staining for total proteins in 0.25% (w/v) Coomassie brilliant blue in 7% acetic acid (Fisher 1968) or in a 1% (w/v) aqueous solution of acid fuchsin (O'Brien and McCully 1981).

Results

Structure of archegonia after pollen tube entrance into the archegonial chamber

Ovules that during dissection showed the presence of intact, well-developed pollen tubes in the archegonial chamber held two to five closely associated, ovoid archegonia. Each archegonium comprised an enormous (ca. 3 300–3 500 μm×2 000 μm), turgid, inner cell with a thickened but unlignified cell wall and two neck cells. Above the small attenuate apex (hereafter referred to as the neck region) of the inner cell the dilated, thin-walled neck cells protruded into the archegonial chamber (Figs. 1, 2). The protrusion of the neck cells was partly effected by one or two tiers of adjacent epidermal cells acting as subsidiary neck cells and raising the bulbous cells above the flattened epidermal surface of the megagametophytic tissue (Fig. 2). The small neck region of the inner cell contained a prominent nucleus with an undulating nuclear membrane. In the proximity of the nucleus, small fragments of an organelle which stained like the large nucleus and the nuclei of the neck cells (Fig. 2) invariably occurred. The nuclear fragments were found separately or in small groups in the peripheral cytoplasm of the neck region, above or slightly to the side of the large nucleus. In many archegonia, vacuolate and disintegrating nuclear fragments were surrounded by minute, irregularly shaped bodies containing PAS-positive (starch?) granules (Fig. 3). The amoeboid bodies also occurred in the wider part of the egg cell.

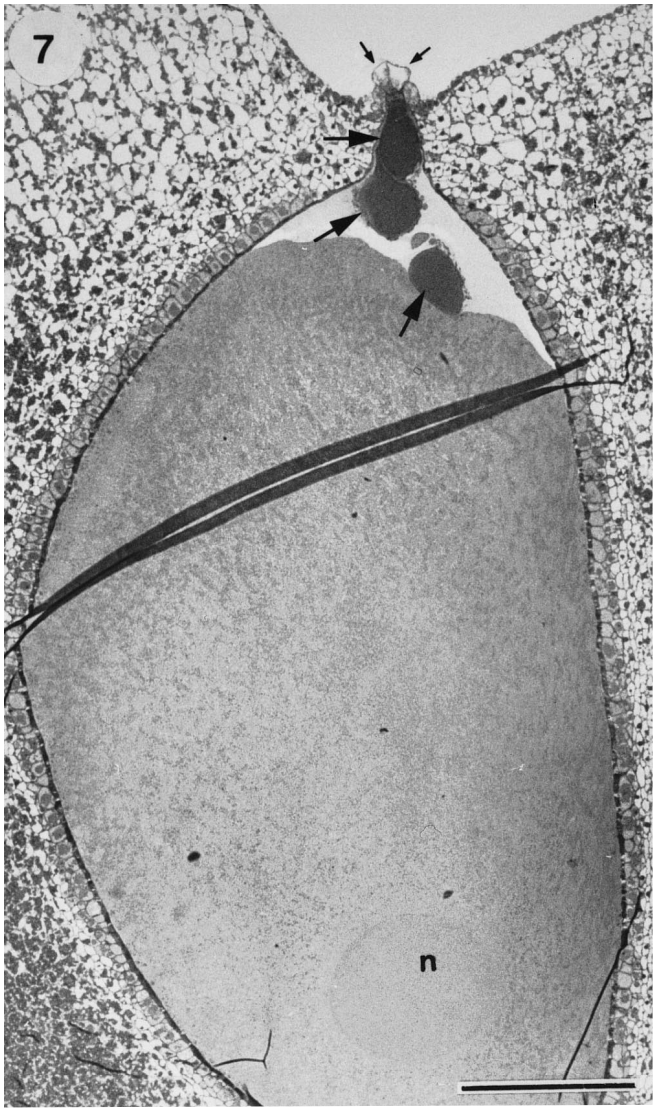
The position, size and structure of the fragmented nucleus corresponded with descriptions in cycad literature of the ventral canal nucleus, sister to the egg nucleus (Brough and Taylor 1940; Chamberlain 1906). In cycads, the ventral canal nucleus characteristically disappears at an early stage (Bryan and Evans 1957 and references cited therein). The enormous inner cell and prominent nucleus therefore represented the egg cell with enlarging egg nucleus.

The thick-walled egg cell was invested by one to four tiers of jacket cells (Fig. 4), except for a small area immediately below the neck cells where no jacket cells occurred between the egg cell and the nutritive tissue of the megagametophyte. The walls of adjacent jacket cells were slightly and unevenly thickened, but cell walls bordering on the thickened egg cell wall were extremely delicate so that the pit-closing membranes were displaced towards the side of the jacket cells (Fig. 4). The minute, amoeboid bodies that surrounded the disintegrating ventral canal nucleus occurred in large numbers in the peripheral cytoplasm of the egg cell. In this region the bodies contained PAS-positive granules and were often closely associated with the pit membranes (Fig. 4). In the interior granular cytoplasm the bodies were devoid of granules. In structure, size (up to 1.5 μm) and staining abilities the bodies corresponded with descriptions of proplastids (Kirk 1978).

The egg nucleoplasm stained homogeneously in toluidine blue, Coomassie brilliant blue and haematoxylin, indicating the presence of protein (Fisher 1968; Jacobsen et al. 1971; Jensen 1962). Two nucleoli of extremely unequal size occurred in the homogeneous nucleoplasm (Fig. 5). They reacted easily and intensely with the aforementioned protein stains, but negatively with PAS and acid fuchsin, indicating the absence of total carbohydrates (Jensen 1962) and aromatic amino acids (Ling-Lee et al. 1977), respectively. The nucleus showed no affinity for Feulgen; the presence and position of the chromatin was therefore not established, possibly because of the non-coagulating properties of the fixative (O'Brien and McCully 1981). All cell walls of the gametophyte, including the prominently thickened egg cell wall, showed a distinct affinity for PAS and toluidine blue, indicating the presence of complex polysaccharides and the total absence of lignin (O'Brien et al. 1964). Staining with haematoxylin, Coomassie brilliant blue, acid fuchsin and Feulgen gave negative results.

In some ovules collected 1 day before the pollen tubes started to burst, the two neck cells had divided longitudinally (Fig. 6) so that in successive sections a tier of four cells could be seen. Numerous starch grains occurred in the areas of the archegonial chamber that contained the protruding neck cells before (Fig. 5) and after (Fig. 6) their division. The neck cells contained small starch grains. In archegonia with four neck cells, the apices of the egg cells were devoid of nuclei, but enlarging, faintly staining egg nuclei were found lower down in the wide, central region of the egg cells (Fig. 6). An upward surge of cytoplasm might possibly have forced the nucleus downwards and out of the neck region (Fig. 5).

Figs. 7–12 Micrographs depicting the course of syngamy in archegonia containing multiple spermatozoids. **Fig. 7** Egg cell containing three spermatozoids (*large arrows*) and tangentially sectioned egg nucleus (*n*). Note low affinity of nucleus for histochemical stains (PAS, haematoxylin and toluidine blue) and thin walls of deflated neck cells (*small arrows*). *Bar* 500 μm. **Fig. 8** Part of egg cell with three unsuccessful spermatozoids (*arrows*), liberated male nucleus (*arrowhead*) of fourth spermatozoid and egg nucleus (*n*) with two small nucleoli (*small arrows*). *Bar* 500 μm. **Fig. 9** Part of egg cytoplasm containing the cytoplasmic sheath (*small white arrows*) and cilia of the liberated male nucleus (*arrowheads*). Note bodies (*large white arrows)* with PAS-positive granules. *Bar* 100 μm. **Fig. 10** Apical region of proembryo containing the first two proembryonal nuclei. Note the spiral (*small white arrows*) in the dense proembryonal cytoplasm (*p*) and the position of the first two proembryonal nuclei (*black arrows* at *11, 12*). *Bar* 500 μm. **Figs. 11, 12** Micrographs depicting nuclei (*large white arrows*) at positions indicated in Fig. 10. Note amoeboid bodies (*small white arrows*) containing PAS-positive granules. *Bars* 25 μm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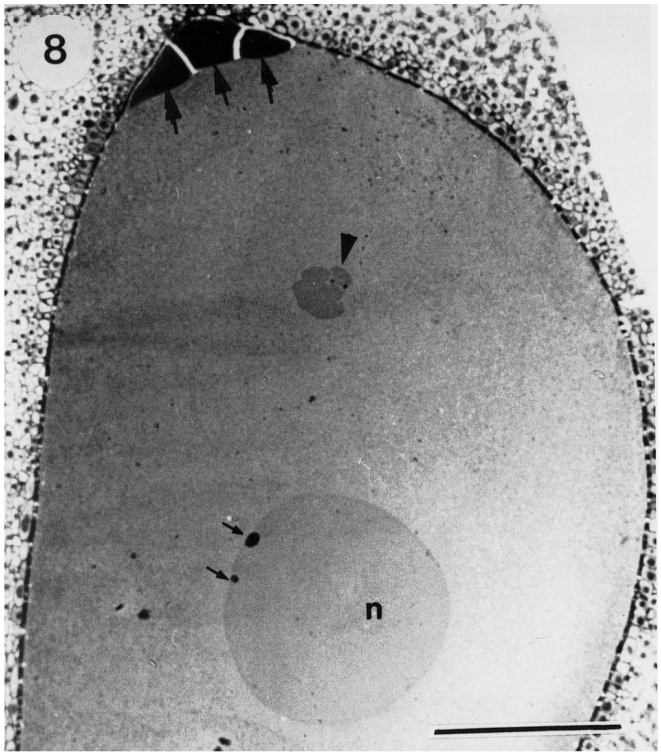
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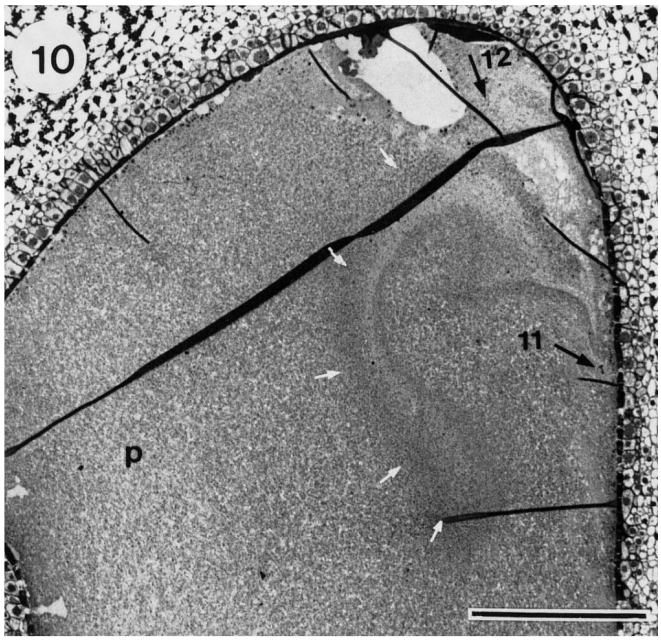
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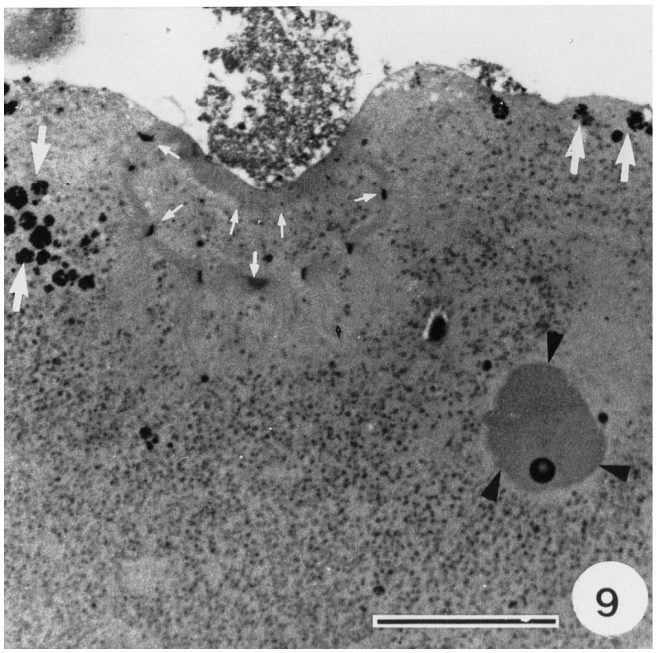
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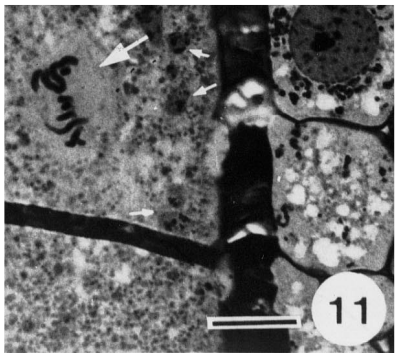
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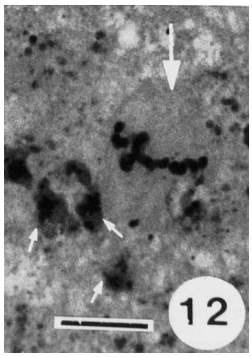
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Structure of nuclei in the archegonium after spermatozoid release into the archegonial chamber

In ovules with wet archegonial chambers and collapsed pollen tubes, all archegonia had been penetrated by either one or several male gametes. The neck cells were deflated and the egg nucleus had moved approximately halfway or lower down into the archegonium (Figs. 7, 8). The structure and conduct of the egg nucleus and the spermatozoid without its cytoplasmic sheath (hereafter referred to as the liberated male nucleus) seemed to depend on the number of spermatozoids that had succeeded in entering the archegonium.

Structure and conduct of nuclei in archegonia with multiple spermatozoids

In all ovules collected from three of the four cones, one or more archegonia had been penetrated by multiple spermatozoids (Figs. 7, 8). After PAS and haematoxylin staining, sections of such archegonia had to be stained repeatedly in toluidine blue to reveal the position of the spherical (approximately 500 μm diameter) egg nucleus. The nuclear membrane appeared smooth, the nucleoplasm was homogeneous and the nucleoli were usually not visible. In only two of the numerous serially sectioned archegonia could nucleoli be seen in the egg nuclei (Fig. 8).

The number of male gametes that entered the archegonia ranged between three and seven. The egg cell cytoplasm seemed to have contracted to form a vacuole in the neck region. The spermatozoids were received in this space (Fig. 7). Initially, only one, and seemingly the first that entered, made contact with the membrane of the egg cytoplasm (Fig. 7). After this male gamete had lost its cytoplasmic sheath with cilia (Fig. 9) and the liberated male nucleus entered the cytoplasm (Figs. 8, 9), additional spermatozoids came into contact with the egg cytoplasm (Fig 8). These spermatozoids remained within their ciliated sheaths. The liberated male nucleus was much smaller than the egg nucleus, irregular in shape and variable in size.

The union of the egg and male nuclei was not observed. Several archegonia contained no egg nucleus or liberated male nucleus, although the cytoplasmic sheath of the latter nucleus was found in the vicinity of the unsuccessful spermatozoids. In such archegonia a conspicuous spiral could be seen in the cytoplasm which had become dense and finely granular (Fig. 10). The first two nuclei of the proembryo were found in close proximity to the spirals (Figs. 10–12) and were minute organelles in the vast interior of the proembryo. The arrangement of the darkly staining chromosomes indicated that the nuclei had already entered into the next division. Amoeboid bodies containing numerous PAS-positive granules were closely associated with the dividing nuclei.

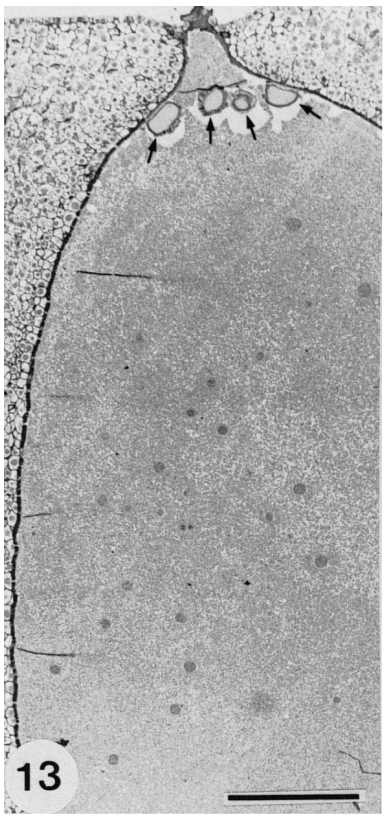
In ovules containing dried up pollen tubes, more advanced proembryonal stages were found. The nuclei had increased in number and were scattered throughout the cytoplasm (Fig. 13). The cytoplasmic sheath of the spermatozoid that had fused with the egg cell could still be seen in these proembryos. Sections of megagametophytes processed in the ensuing weeks showed proembryonal nuclei gathering at the base of the proembryo (Fig. 14). The free nuclei were multi-lobed, amoeboid organelles surrounded by bodies containing PAS-positive granules (Fig. 15). Subsequently, cell walls were laid down between adjacent nuclei and their amoeboid nature seemed to disappear (not shown).

Structure and conduct of nuclei in archegonia with single spermatozoids

In ovules collected from one specific cone, all archegonia had been penetrated by a single spermatozoid. Such archegonia also occurred among those that showed multiple male gamete penetration and were representative of the other three cones. In ovules with wet archegonial chambers, the liberated male nucleus lay in the neck region of the archegonium, just below the neck cells (Figs. 16, 17). Alternatively, the nucleus occurred in the peripheral egg cell cytoplasm slightly distant from the neck cells (Fig. 18). The cell walls of the neck cells were heavily thickened and the contents of the cells stained intensely. The cytoplasmic sheath and cilia of the spermatozoid had been stripped off, but did not remain intact in the egg cytoplasm. Fragments of male cytoplasm and cilia were dispersed over a large area of the egg cell. The fragments were pressed tightly against the egg cell wall outside the egg cytoplasm and tufts of cilia were also seen as intensely staining, undulating structures inside the egg cytoplasm (Fig. 17). The liberated male nucleus and remains of cilia were always associated with numerous, amoeboid bodies containing PAS-positive granules. In sections, the size and shape of the many-lobed, amoeboid male nucleus were extremely variable. The homo-

Figs. 13–15 Later stages in proembryo development. **Fig. 13** Part of proembryo showing remains of four unsuccessful spermatozoids (*small arrows*) and proembryonal nuclei dispersed in cytoplasm. *Bar* 500 μm. **Fig. 14** Part of proembryo showing free nuclei accumulating at the chalazal side. *Bar* 500 μm. **Fig. 15** Free nuclei of Fig. 14 at higher magnification to illustrate their amoeboid shape and association with bodies (*small arrows*) containing PAS-positive granules. *Bar* 100 μm ▶

Figs. 16–18 Micrographs illustrating the neck cells and a part of the egg cell in archegonia containing a single spermatozoid. **Fig. 16** Neck region of egg cell surmounted by two thick-walled, darkly staining neck cells. Note the liberated, multi-lobed male nucleus (*m*) with several vacuolate, distinctive nucleoli (*small white arrows*) and the amyloplasts (*small black arrows*) in the egg cytoplasm. Compare with Fig. 21 illustrating the nucleoli of the egg nucleus in the same egg cell at same magnification. *Bar* 50 μm. **Fig. 17** Part of egg cell with male nucleus (*black arrows*) in the neck region and tufts of cilia (*small white arrows*) dispersed in the egg cytoplasm. *Bar* 100 μm. **Fig. 18** Part of egg cell with male nucleus (*black arrow*) moving out of the neck region towards the egg nucleus (*n*). Section stained in PAS and toluidine blue *Bar* 500 μm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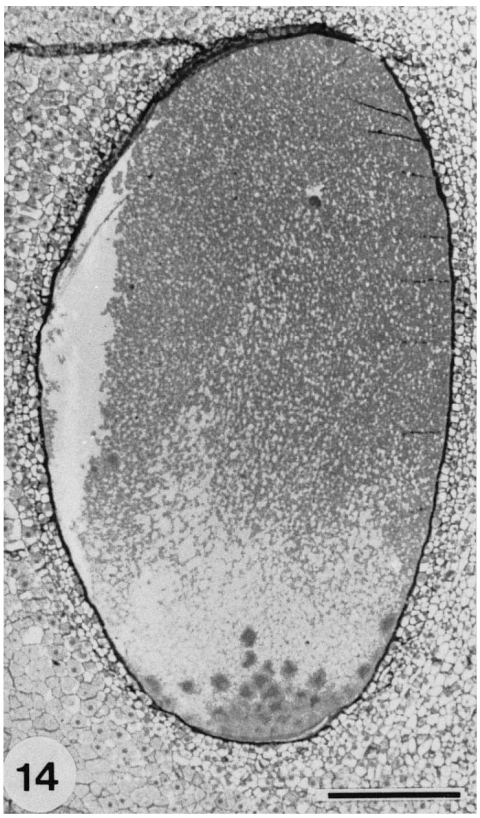
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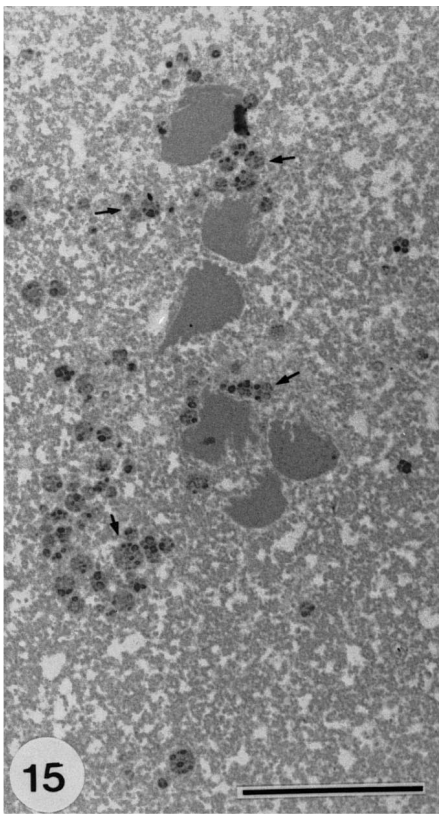
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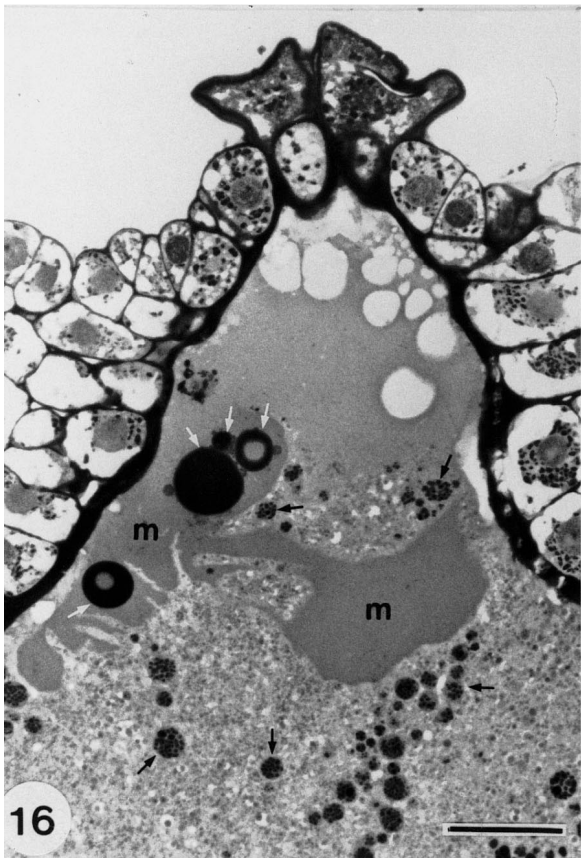
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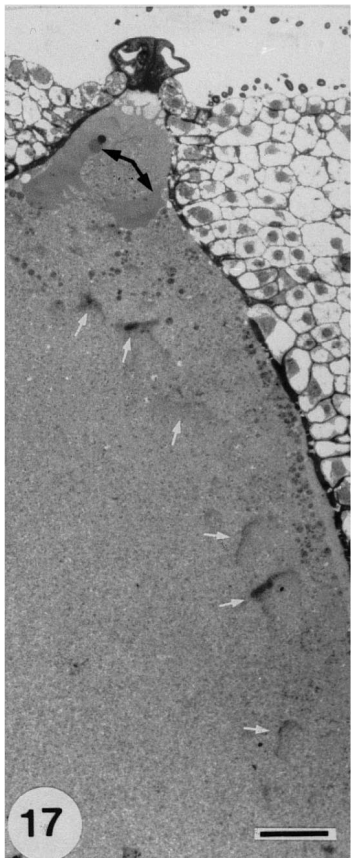
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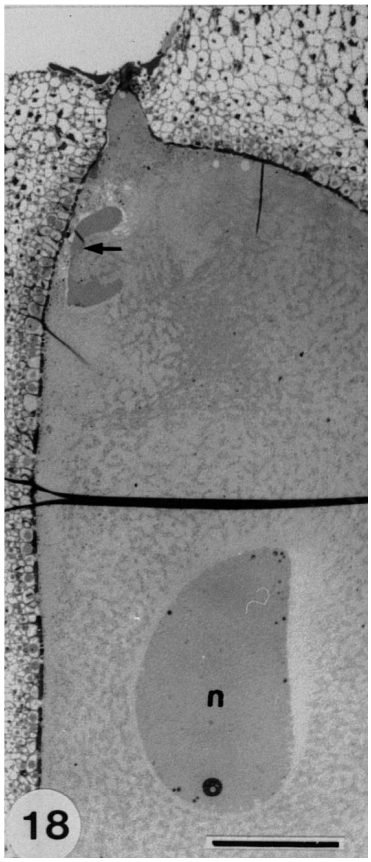
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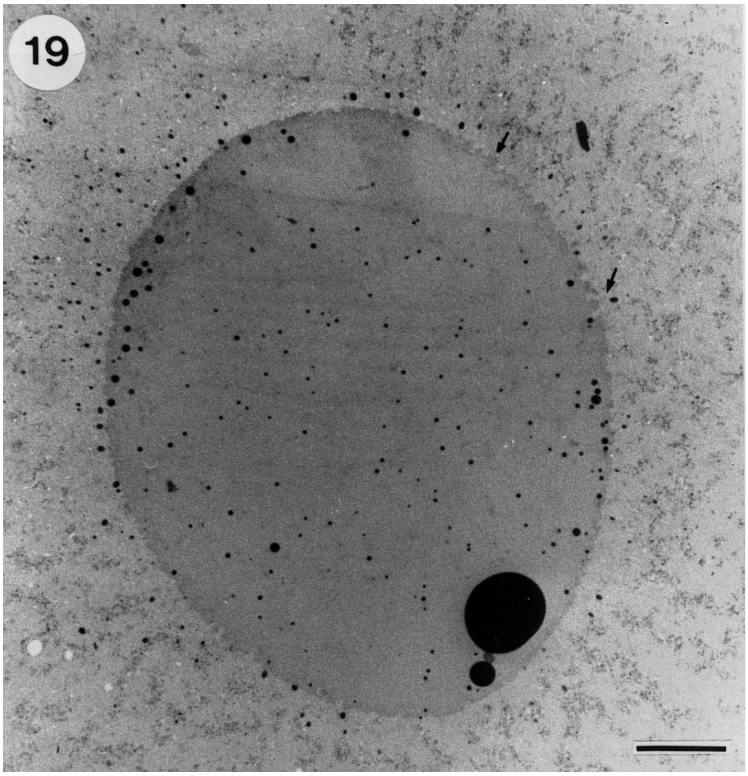
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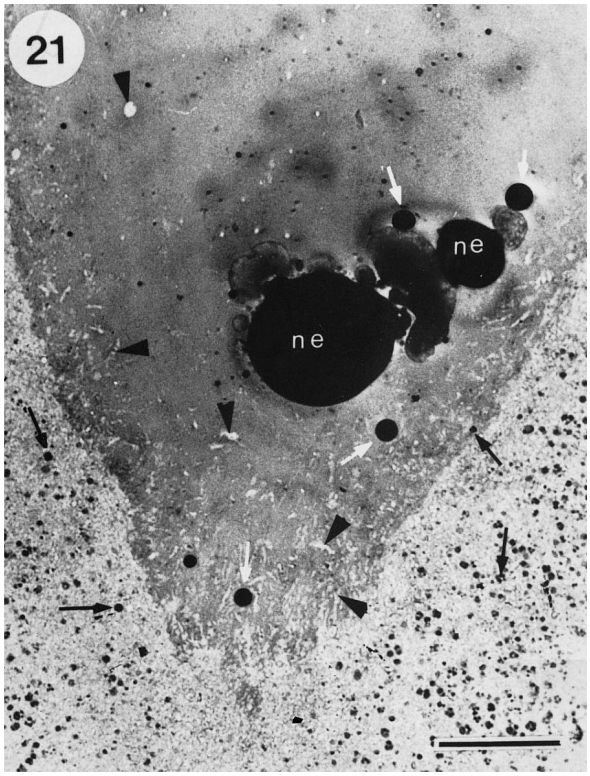
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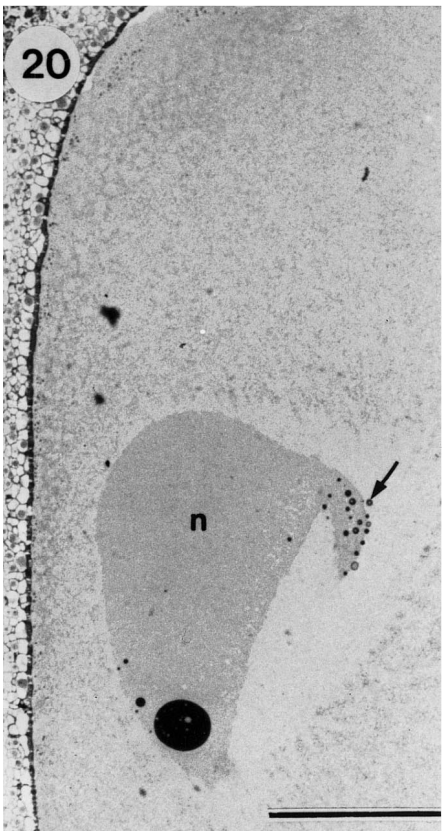
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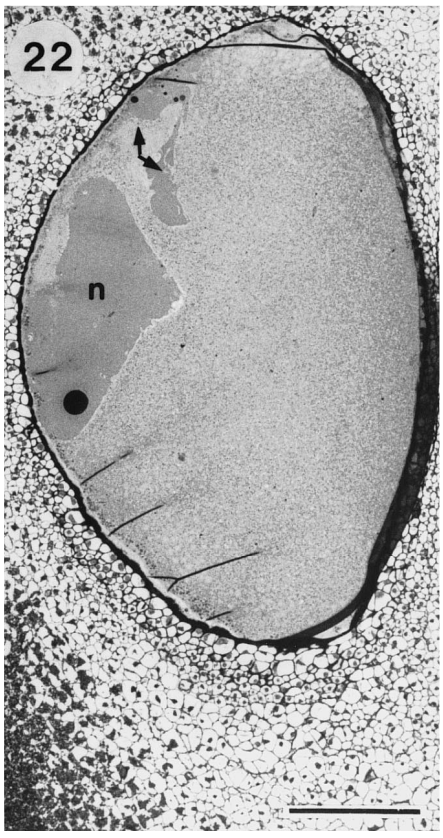
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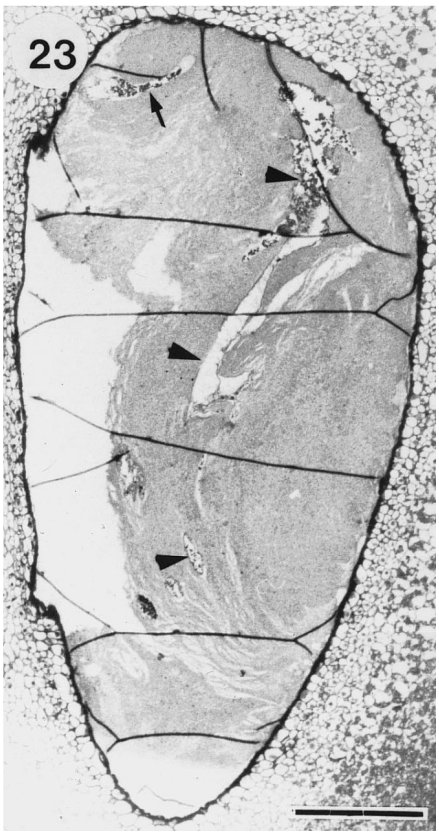
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geneous nucleoplasm stained easily and included many prominent nucleoli (Fig. 16) which varied in size and reacted strongly with haematoxylin, toluidine blue and Coomassie brilliant blue, but negatively with PAS and Feulgen.

The egg nucleus stained easily and distinctly (Figs. 19, 20) in either haematoxylin or toluidine blue and seemed to react strongly in archegonia that were fixed while the archegonial chambers were wet. Initially, the egg nucleus remained spherical, but the nuclear membrane became irregular in outline as small ridges started to project into the cytoplasm. Small intensely staining globules were seen in the depressions between the ridges and dispersed in the cytoplasm surrounding the nucleus (Fig. 19). The two nucleoli were prominent, often vacuolate, and showed histochemical reactions like those of the nucleoli of the spermatozoid. Subsequently, the egg nucleus increased considerably in length and became very irregular in shape (Figs. 18–22). A prominent hook or long tail often developed. The nuclear membrane became very irregular so that extensive labyrinths were formed in the cytoplasm and channels developed in the nucleoplasm. Numerous small nucleoli seemed to originate in close proximity to the original nucleoli (Fig. 21). This reaction was seen in egg cells while tufts of cilia were being dispersed in the cytoplasm (Fig. 17) and the male gamete lay directly inside the neck cells (Fig. 16). The extra nucleoli became scattered in the labyrinths and were found in large numbers in the hook-like or tail-like appendages of the egg nucleus (Fig. 20).

Syngamy seemed to occur very rarely in archegonia with single male gametes. In ovules with wet archegonial chambers, only two proembryos were found which occurred in the same megagametophyte. In these two proembryos the cytoplasmic sheath of the male gamete was still visible in the neck region. In ovules with dried up pollen tubes, no advanced proembryonal stages were seen. The large, irregularly shaped egg nuclei with prominent nucleoli almost invariably lay in an off-centre position. A large part of each nucleus was tightly appressed to the egg cell wall. The approaching male nucleus occurred on the same side as the egg cell. While a part of the enlarged male nucleus remained against the egg cell wall, a multi-lobed tail was being projected towards the egg nucleus (Fig. 22). However, sexual fusion did not seem to occur. In subsequent stages the cytoplasm of archegonia containing irregularly shaped, multi-nucleolated egg nuclei and multi-lobed, liberated male nuclei showed signs of deterioration, while the egg nucleus and liberated male nucleus were still apart and disintegrating (Fig. 23). The nucleoplasm contained dark-staining, fibrous strands and coarse granules, suggesting the denaturation and precipitation of nucleoproteins (O'Brien and McCully 1981). In megagametophytes that were representative of three of the cones investigated, aborting archegonia occurred among those that showed multiple sperm penetration and advanced proembryonal stages (Figs. 13–15). In megagametophytes representing the fourth cone, all archegonia subsequently aborted and no proembryos were found.

◀ **Figs. 19–23** Micrographs depicting behaviour of the egg nucleus in archegonia containing a single spermatozoid. **Fig. 19** Egg nucleus with two prominent nucleoli, uneven nuclear membrane (*small arrows*), and small dark granules moving from the nucleus into the surrounding cytoplasm. Section stained in PAS and haematoxylin. *Bar* 100 μm. **Fig. 20** Enlarged, irregularly shaped egg nucleus (*n*) in peripheral cytoplasm. Note prominent, vacuolate nucleolus and numerous, small nucleoli in hook-like appendage (*arrow*). Section stained in PAS and haematoxylin. *Bar* 500 μm. **Fig. 21** Part of egg nucleus containing the two original nucleoli (*ne*). Note the formation of extra nucleoli (*white arrows*), channels (*arrowheads*) extending into the nucleoplasm and small granules (*black arrows*) in the cytoplasm. Compare with Fig. 16 illustrating the neck region of the same egg cell at same magnification. *Bar* 50 μm. **Fig. 22** Tangential section of egg cell illustrating the peripheral position and irregular shape of the egg nucleus (*n*) and male nucleus (*arrows*). *Bar* 500 μm. **Fig. 23** Egg cell with disintegrating male nucleus (*black arrow*) and egg nucleus (*arrowheads*) in degenerating cytoplasm. *Bar* 500 μm

Discussion

To our knowledge, this study provides the first evidence of a positive relationship between the presence of pollen tubes in the nucellus and the disintegration of the ventral canal nucleus. The degeneration of this nucleus before fertilization is regarded as characteristic for cycads (Bryan and Evans 1957 and references cited therein) and many conifers (Bell 1994). When pollen tubes appeared in the archegonial chambers of *E. villosus*, the ventral canal nuclei had entered into a stage that, in animal cells, is indicative of normal (programmed) cell death (Raff 1992). Before the egg nucleus of *E. villosus* left the neck region of the archegonium, the fragments of the ventral canal nucleus were disappearing among small, amoeboid bodies containing PAS-positive granules.

Claims have been made that the ventral canal nucleus sometimes reacts aberrantly in *Zamia*, *Encephalartos* and *Ceratozamia* (Bryan and Evans 1957; Chamberlain 1912; Sedgwick 1924). In a very cursory study of embryo development in *E. altensteinii* Lehm., *E. friderici-guilielmi* Lehm. and *E. villosus*, Sedgwick (1924) could find no sperm sheaths or ciliated bands in the egg cells and assumed that the ovules were unpollinated. A nucleus seen to approach the egg nucleus was regarded as the enlarged ventral canal nucleus. It was concluded that embryos found in the material had resulted from the fusion of egg nuclei and ventral canal nuclei. Chamberlain (1912) had previously suggested that a union between the aforementioned nuclei could have effected occasional embryo formation in unpollinated ovules of *Encephalartos* and *Ceratozamia*. Norstog (1977) could, however, find no embryos in unpollinated ovules of *E. ferox*.

In detailed studies conducted on megagametophytes fixed in coagulating fixatives, Bryan and Evans (1956, 1957) reported the maturation of the egg nucleus and the presence of persistent and enlarged ventral canal nuclei in ovules of *Zamia umbrosa*. The behaviour of mature egg nuclei in these ovules corresponded to the behaviour

of the egg nucleus of *E. villosus* (Fig. 19 of the present investigation) in archegonia with single spermatozoids that could not effect fertilization and proembryo formation. The escape of globules from depressions in the nuclear membrane was depicted and described (Figs. 14–20 in Bryan and Evans 1956), but the origin and function of the globules were unknown. It was reported that, at this stage, the process of fertilization had begun, but would be described in a subsequent communication. However, the envisaged account of fertilization did not, to our knowledge, appear in the literature. In a subsequent study conducted on the same material, Bryan and Evans (1957) described nuclei, regarded as enlarged ventral canal nuclei, occurring with egg nuclei which were behaving similarly to the description above. The ventral canal nuclei remained in the peripheral cytoplasm, became multi-lobed and were associated with dark-staining masses in the cytoplasm (Figs. 17–21, 27 in Bryan and Evans 1957). The dark-staining substances could not be identified. The conduct of the persistent ventral canal nuclei in *Z. umbrosa* (Bryan and Evans 1957) conforms to that of the liberated male nuclei of incompatible spermatozoids in archegonia of *E. villosus*. We suggest that the unidentified, dark-staining masses that were associated with the nuclei in archegonia of *Z. umbrosa* were, in fact, the remains of the cytoplasmic sheaths of male gametes and that the persistent nuclei did not represent ventral canal nuclei, but were the liberated nuclei of incompatible spermatozoids.

Our study revealed a remarkable and microscopically discernable contrast in the behaviour of the egg cell towards spermatozoids that could effect fertilization and proembryo formation (hereafter referred to as compatible spermatozoids/male gametes) and incompatible spermatozoids. These behavioural patterns clearly show that the egg cell of *Encephalartos* can distinguish between male gametes and that members of the genus have prezygotic detection abilities.

Compatible spermatozoids were immediately accepted by the egg cell and their entrance caused no conspicuous reaction in the archegonium. The neck cells remained open to allow entry of additional male gametes. The cytoplasmic sheath of the compatible spermatozoid remained intact in the egg cytoplasm for an appreciable period. The liberated male nucleus and egg nucleus did not enlarge and the nuclear membranes were smooth, without labyrinths. The nucleoplasm of both nuclei remained homogeneous and showed little affinity for histochemical stains, while the nucleoli were inconspicuous or not visible. Syngamy, which seemingly occurred rapidly and did not result in a resting zygote, was followed by proembryo formation.

Pollen tubes containing incompatible spermatozoids were not screened out in the nucellus or in the archegonial chamber. The entrance of such a spermatozoid into the egg cell evoked easily discernable reactions in the archegonium. We suggest that, although the spermatozoid had been allowed into the archegonium, it was immediately rejected by the egg cell and that the entry of additional spermatozoids was excluded by the closure of the neck cells. Our results also suggest that the cilia of the male gamete might have been involved in evoking the following responses, considered as rejection phenomena. (1) The cell walls of the neck cells thickened and the cell contents stained intensely, indicating that the cells had died and were no longer functioning. (2) The nucleoli of the egg nucleus developed a high affinity for protein stains and showed a marked increase in size and vacuolation. Simultaneously, the nuclear membrane developed ridges. Numerous globules that were formed in the depressions between the ridges started to spread outwards through the cytoplasm. (3) The nucleus of the incompatible male gamete was not accepted into the interior cytoplasm, but kept in a peripheral position, while its cilia and cytoplasm were distributed over a large area and destroyed. During this process, the vestiges of the cytoplasmic sheath caused an intensely staining reaction in the cytoplasm and were associated with amoeboid bodies containing PAS-positive granules. (4) The egg nucleus and the liberated male nucleus increased in size and developed elaborate lobes or labyrinths, whereby the contact areas of the nuclear membranes with the egg cytoplasm were enhanced. (5) The nucleoli of the egg nucleus as well as the male nucleus formed extra nucleoli which were scattered into the many lobes of the male nucleus and the labyrinths of the egg nucleus. (6) The egg nucleus showed an increased affinity for histochemical stains with a better differentiation of the boundary between the nuclear membrane and the cytoplasm of the egg cell, suggesting an intensification of chemical reactions between the egg nucleoli and the cytoplasm. (7) In archegonia with incompatible male gametes, there were no syngamy and proembryo formation; the egg and male nuclei ultimately died and the egg cell cytoplasm degenerated.

It cannot be assumed that the incompatible and compatible spermatozoids that entered the archegonia were solely representative of conspecific pollen sources. The cones were not covered during the receptive stage and open pollination could have resulted in penetration of interspecific pollen grains. It is well known that interspecific hybridization can occur in *Encephalartos*, although *E. villosus* does not readily form hybrids (unpublished data). The incompatible spermatozoids could therefore have come from an interspecific pollen source. This will have to be determined in future studies, during artificial pollination experiments conducted under carefully controlled conditions. However, the abortion phenomenon is not indicative of hybrid failure. Abortion occurred before nuclear fusion and not because of improper pairing or segregation of chromosome sets. Furthermore, the total absence of proembryonal stages in successfully pollinated ovules of one specific cone does suggest the possible presence of a common self-incompatible gene in the female plant.

It has previously been suggested that cycads have abilities to discriminate between male gametes prezygotically (Pettitt 1977). Proteins and glycoproteins regarded

as lectin had been extracted from cycad megagametophytes and were thought to act as a screening device in the archegonial chamber to control intraspecific mating in cycads (Pettitt 1977). To our knowledge, this hypothesis has attracted no further attention in cycad literature. Consequently, no attempts have been made to explore avenues for testing, by experimental means, the effectiveness of an intraspecific screening mechanism operating in the archegonial chamber. It seems possible that our results may suggest such an avenue for embryologists, horticulturists and molecular biologists concerned with cycad conservation.

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