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Ultrastructure of cystocarp development in *Gelidium robustum* (Gelidiaceae: Gelidiales: Rhodophyta)

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Abstract The ultrastructure of cystocarp development is described for the red alga *Gelidium robustum* (Gardn.) Hollenb and Abbott. The external cortical cells of the cystocarp remain uninucleate and unmodified, while the internal ones are binucleate, producing large mucilage sacs that discharge their contents into the cystocarpic cavity. Crystalline inclusions occur in both kinds of cells. Nutritive filaments, up to five cells long, are produced. Microtubules are associated with lipid droplets and occur in cells of the third-order filament, which probably function as food suppliers and food-conducting cells for the nutritive filaments. The younger, terminal cells of the nutritive filaments are electron-dense, having a large nucleus and lipid droplets and probably function as nutritive suppliers of the terminal gonimoblast cells. The older cells of the nutritive filaments are electron-light-possessing mucilage sacs, which function as mucilage suppliers of the cystocarpic cavity. Carpospore differentiation proceeds through three developmental stages. The youngest carpospores have an ovoid shape, possessing a large nucleus, proplastids with the peripheral thylakoid and some mucilage sacs, while they are surrounded by the carposporangial mucilage. Starch is polymerized in the perinuclear cytoplasmic area of the later stages of young carpospores. Intermediate-aged carpospores continue their starch polymerization and thylakoid development of plastids. Curved dictyosomes produce vesicles that contribute carpospore wall material. Mature carpospores have a nucleus, fully developed chloroplasts, numerous starch granules and adhesive vesicles, while they are surrounded by a two-layered wall and compressed mucilage, which constitutes a carposporangial wall.

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Introduction

Species of the genera *Gracilaria, Pterocladia* and *Gelidiella* are the most significant source of raw material for the production of agar. But *Gelidium*, in particular, is the main industrial source of agar, which has been manufactured in Japan since 1760 (McLachlan 1985; Akatsuka 1986; Glicksman 1987; Lembi and Waaland 1988). The production of agarose is strongly dependent on the structural organization and on the cohesion of the tissues in which it is biosynthesized (Vignon et al. 1994).

The structure of the female reproductive system in the Gelidiaceae has been described by Kylin (1928), Dixon (1959) and Fan (1961). However, there has been long debate concerning the presence or absence of auxiliary cells, fusions of the fertilized carpogonium and fusions of gonimoblast with nutritive filaments in this family, particularly in *Gelidium* and *Pterocladia,* which are the most diverse genera in the Gelidiaceae. Cystocarp structure is the basic generic distinction between *Gelidium* and *Pterocladia*, but since fertile female thalli of these are infrequent in field collections, vegetative characters have been used (Santelices 1991). According to the same author there are interspecies differences in the cystocarpic structure of *Gelidium* and *Pterocladia*. These include: (1) the manner of carpogonial and nutritive filament production, (2) the direction of gonimoblast divisions resulting in the formation of carposporangia, (3) the shape of immature carpospores and (4) the function of internal cortical filaments, while additional differences in the cystocarp structure might appear in future studies. Moreover, taxonomic elucidation should contribute to a better comprehension of differences in the synthesis and quality among agars produced by various members of the Gelidiales (Santelices 1991).

Gelidium robustum (Gardn.) Hollenb. and Abbott is common, easily identifiable and the largest species along the California coast. Nevertheless, it is sometimes difficult to decide whether a thallus should be assigned in *G. robustum* or should be considered a broad-geniculate *Gelidium purpurascens* Gardn. (Abbott and Hollenberg 1976). Hommersand and Fredericq (1988) presented a thorough light microscopic investigation of cystocarp development in *Gelidium pteridifolium* Norris, Hommersand and Fredericq, 1987, comparing their observations with the data of Kylin (1928), Dixon (1959) and Fan (1961), and provided a revised description of the order Gelidiales. However, the ultrastructure of carposporophyte development has not been examined in the order Gelidiales, and probably an important amount of information could be obtained by studying a representative alga of this diverse order. Due to a lack of cytological data a thorough ultrastructural study of cystocarp development in *G. robustum* was undertaken to provide useful information on the ultrastructure and function of various cystocarpic components of this taxonomically and commercially important genus.

Materials and methods

Thalli of *Gelidium robustum* (Gardn.) Hollenb. and Abbott with cystocarps of various sizes were collected during low tides from rocks of Carpinteria near Santa Barbara, California. The thalli were fixed immediately for 5 h in 5% glutaraldehyde buffered by a mixture of equal amounts of 0.2 M Na-cacodylate buffer and seawater, which was adjusted to pH 7.0 (Kugrens 1974). The fixed thalli were rinsed in decreasing concentrations of seawater and buffer and then finally rinsed in 0.1 M Na-cacodylate buffer. Material was post-fixed for 5 h in 0.1 M Na-cacodylate-buffered 2% osmium tetroxide and then dehydrated with a graded (30%, 50%, 70%, 90%, 100%) ethanol series. Samples were embedded for 2 days in a graded series of mixtures of propylene oxide and Spurr's resin, followed by several changes in pure Spurr's resin and then polymerized at 70°C for 8 h. To obtain thin sections, blocks were cut with a diatome diamond knife on a Reichert-Jung Ultracut E ultramicrotome. Sections were post-stained for 45 min with 1% aqueous uranyl acetate and 15 min with lead citrate and examined with a Zeiss 9S-2 electron microscope.

Results

Mature female thalli of *Gelidium robustum* are up to 40 cm tall. Mature cystocarps protrude equally on both surfaces of a branch, and usually there is one pore on each surface.

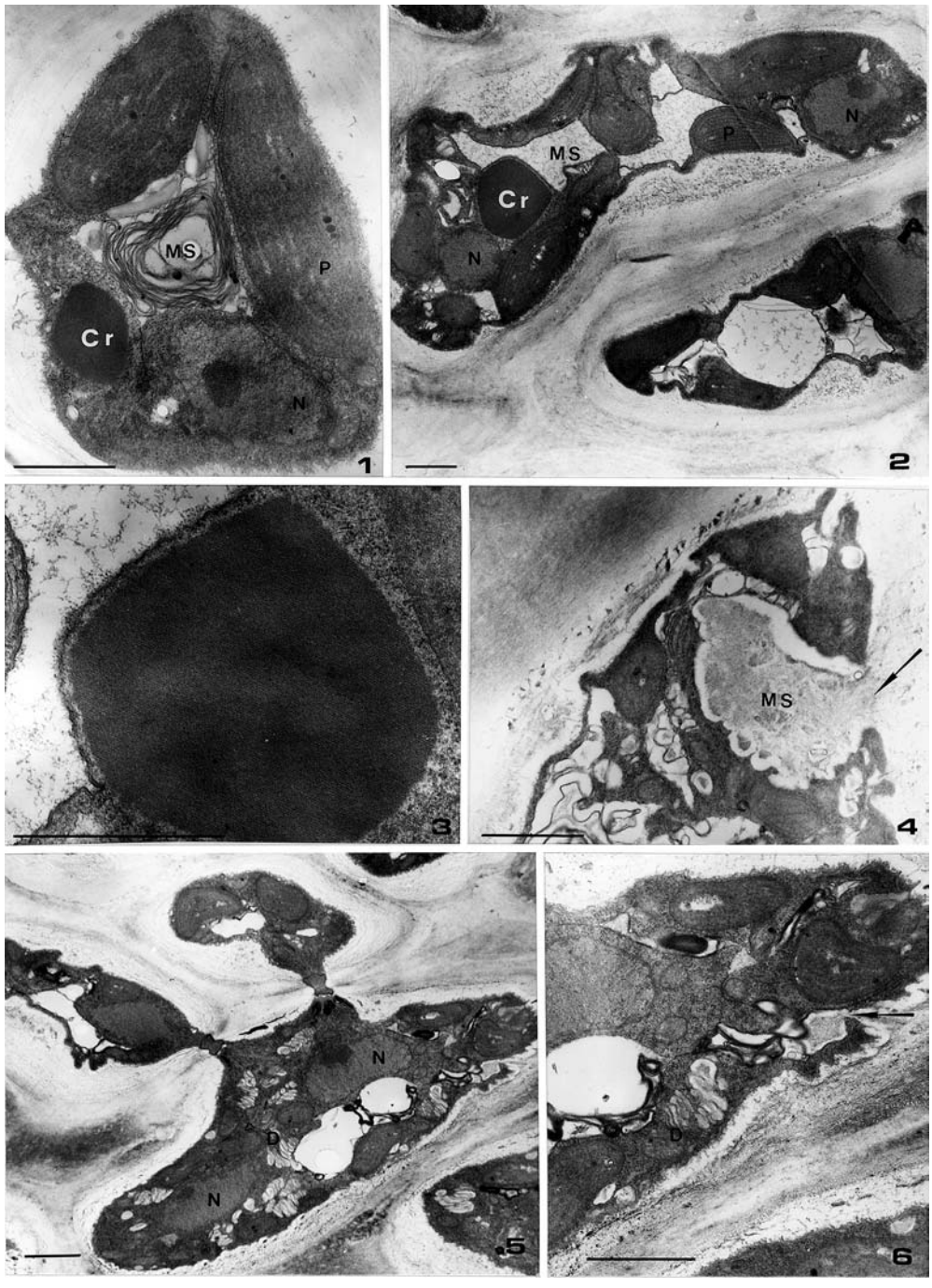
Two types of cortical cells are recognizable. The external cortical cells, which are more distal from the cystocarpic cavity, remain uninucleate and unmodified (Fig. 1). They have fully developed chloroplasts, with discernible phycobilisomes, mucilage sacs and crystalline inclusions (Fig. 1). The internal cortical cells are proximal to the cystocarpic cavity, they become binucleate, and they also have fully developed plastids and large crystalline inclusions (Figs. 2, 3). The crystalline inclusions occur most commonly in the cytoplasm. They lack a bounding membrane but have a periodic substructure of repeated units of 9 nm (Fig. 3). In addition, internal cortical cells possess large mucilage sacs (Fig. 2). The latter are produced by dilation of concentric membrane structures within the expanding areas of the membranes during mucilage formation (Fig. 1). The enlarged mucilage sacs eventually fuse with the plasma membrane and discharge their contents, thus contributing to the huge amount of mucilage filling the cystocarpic cavity (Fig. 4). In addition to the formation of mucilage sacs, very intense dictyosomal activity results in the production of vesicles that also release their contents into the cystocarpic cavity (Figs. 5, 6).

Every cell of a third-order filament in the fertile area has a nutritive filament associated with it. During the early developmental stages the cells of the third-order filament are binucleate, they have very small proplastids, mitochondria and numerous small vacuoles, and they are surrounded by a thick cell wall (Fig. 7). In addition, bundles of microtubules are attached to the surface of lipid droplets in the cytoplasm (Fig. 8). Microtubules also form a system of connections between neighboring lipid droplets (Fig. 8). Cells of the third-order filament in a later stage of development are elongated and multinucleate, with many mucilage sacs and numerous starch granules (Fig. 9).

Nutritive filaments develop from the lateral faces of basal cells of filaments of the third order. Normally from one to three initials are cut off. Each one of these divides (Fig. 10) and produces a filament of up to five cells long (Fig. 11). The distal cells of the nutritive filaments are younger, while the closer ones to the cells of the third-order are the older cells of the nutritive filaments (Fig. 11). The younger cells are electron-dense, with a large nucleus and large lipid droplets (Fig. 11). The older cells of the nutritive filaments have proplastids, remnants of lipid droplets and mucilage sacs (Fig. 12). Mucilage sacs eventually fuse with the plasma membrane and discharge their contents (Fig. 12). Starch granules are absent in all nutritive cells (Figs. 11, 12). The major part of the mature nutritive cells is occupied by large mucilage sacs, which discharge their contents (Fig. 13). Thus, the mature nutritive cells function as mucilage suppliers of the cystocarpic cavity, and not as true nutritive cells.

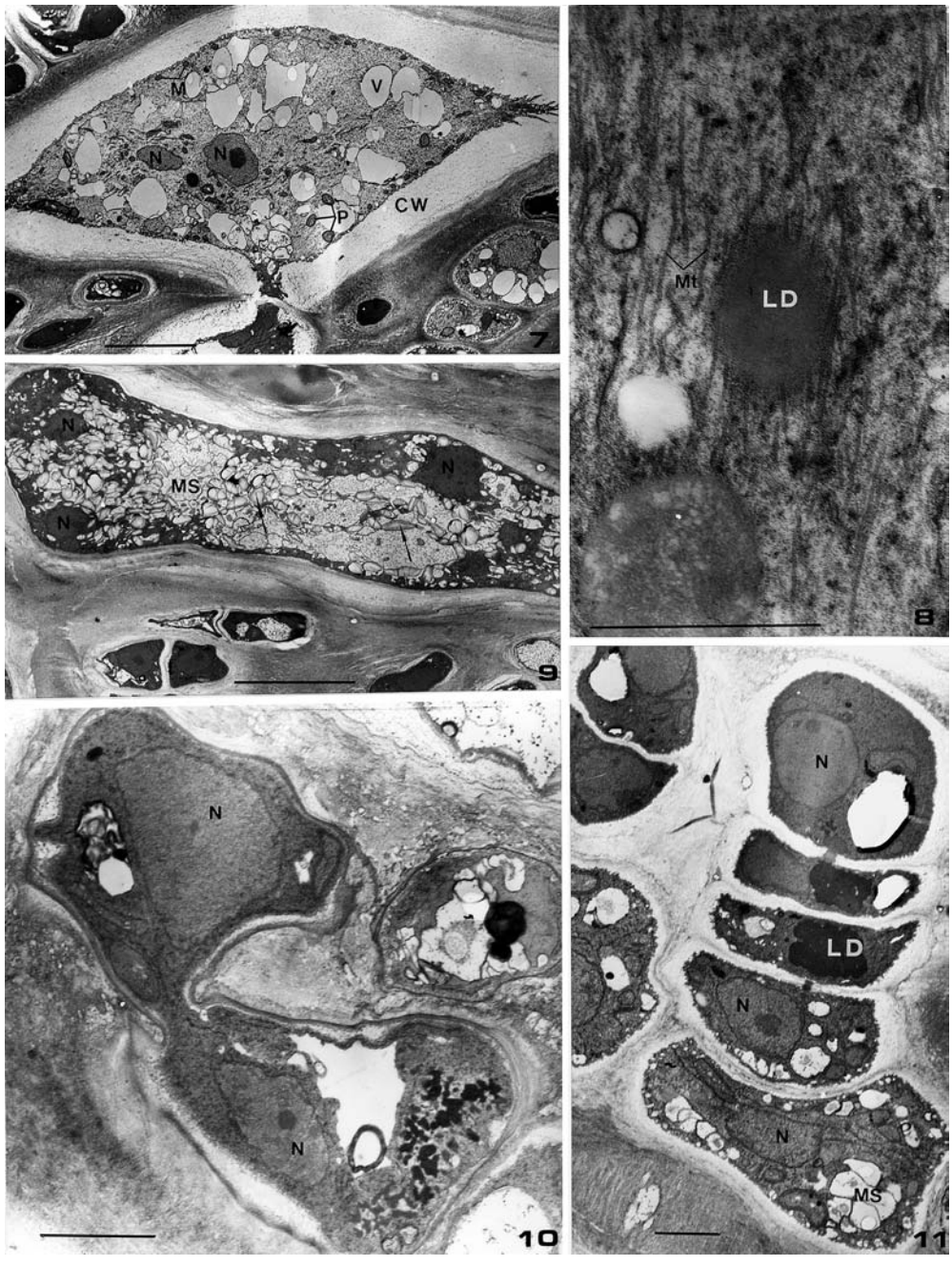
Many carpogonia can be fertilized in a single fertile area. Therefore, a cystocarp is usually a composite structure, containing several individual carposporophytes that develop non-synchronously. Hence, mixtures of carpospores of different developmental stages and of varying sizes coexist in the same area of the cystocarpic cavity. In some cases the first cell cut off from the carpogonium enlarges, distends, becomes multinucleate (Fig. 14) and serves as a secondary site for the production of uninucleate gonimoblast initials. This cell also has small and large mucilage sacs (Fig. 14).

Gonimoblast initials have plastids with developing thylakoids, few starch granules and large mucilage sacs discharging their contents (Fig. 15). Gonimoblast initials



Figs. 1–6 *Gelidium robustum*. Electron micrographs of cortical cells. *Scale bars*: 1 μm (*Cr* crystal; *D* dictyosome; *MS* mucilage sac; *N* nucleus; *P* plastid).
Fig. 1 External cortical with cell plastids, mucilage sacs and crystalline inclusions
Fig. 2 Internal cortical cell with plastids, mucilage sacs and crystalline inclusions
Fig. 3 Higher magnification of the crystal of Fig. 2
Fig. 4 Part of an internal cortical cell with a large mucilage sac discharging its content (*arrow*)
Fig. 5 Binucleate internal cortical cell with increased dictyosome activity
Fig. 6 Higher magnification of part of Fig. 5 showing dictyosome vesicles discharging their contents (*arrow*)

cut off young gonimoblast cells (Fig. 16), which, in turn, produce young carposporangia (Fig. 17). Young gonimoblast cells have proplastids, dictyosomes, active endoplasmic reticulum (ER) and mucilage sacs resulting from the expansion of the concentric membrane structures (Fig. 16). The youngest carpospores have an ovoid shape and contain a large nucleus, proplastids with only the peripheral thylakoid and some mucilage sacs. In addition they are surrounded by the carposporangial mucilage (Fig. 17). As the carpospore differentiates, the number of plastids and mucilage sacs increases considerably (Fig. 18). Mucilage sacs originate from the dilation of concentric membrane structures within the expanding area of the membranes during mucilage formation (Fig. 18). Starch granules are absent during the early stages of carpospore differentiation (Figs. 17, 18). Starch polymerization begins around the nucleus (Figs. 19, 20), and as starch granules enlarge they move away from the perinuclear area, occurring then in various sites of the cytoplasm (Fig. 20). As carpospores mature, they enlarge, and, along with thylakoid development, starch polymerization continues, resulting in the formation of numerous, large starch granules that are scattered throughout the cytoplasm (see Fig. 22). The second stage of



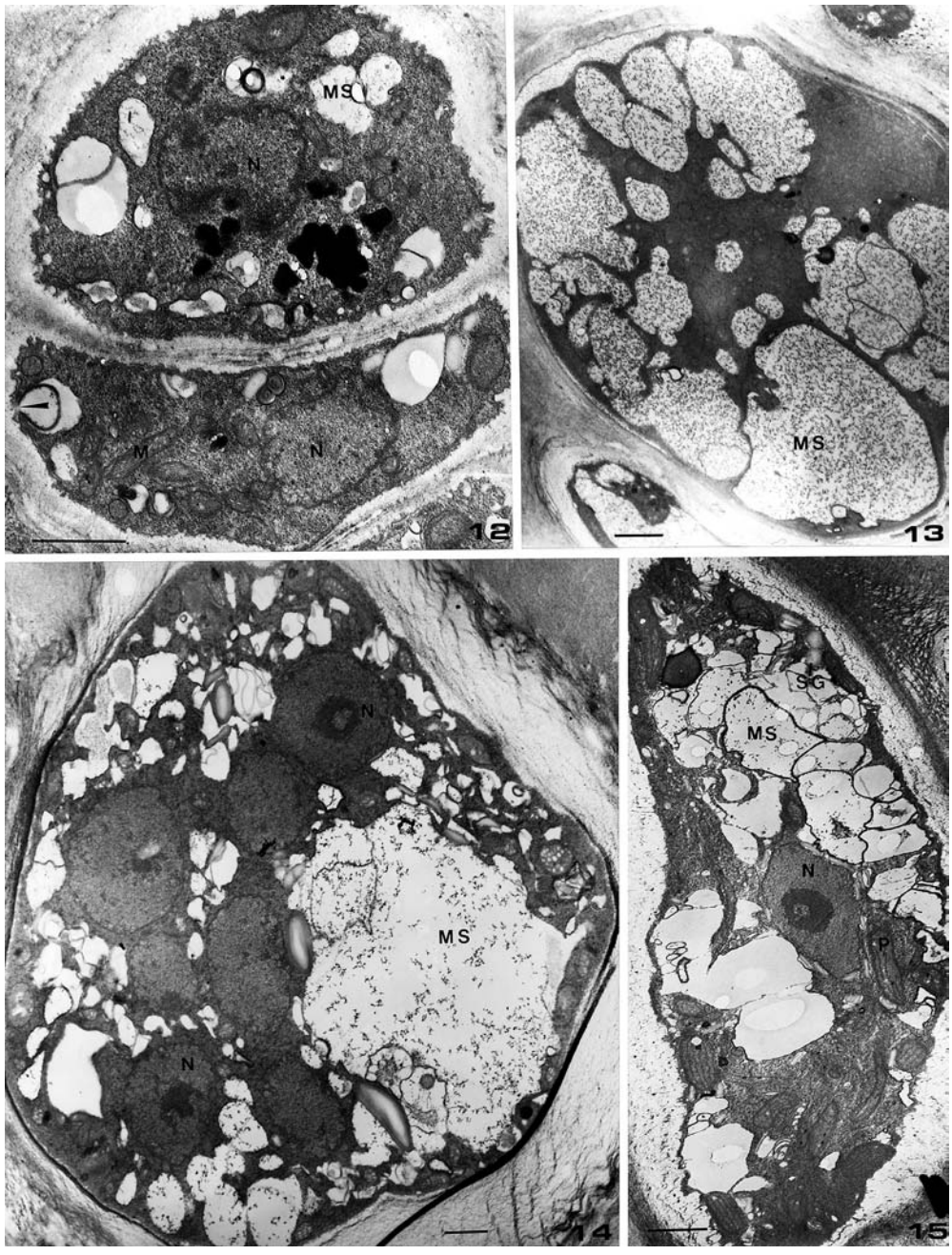
Figs. 7–11 *Gelidium robustum*. Electron micrographs of cells of the third-order filament and of cells of nutritive filaments. *Scale bars*: 1 μm, except Figs. 7 and 9: 5 μm (*CW* cell wall; *LD* lipid droplet; *M* mitochondrion; *MS* mucilage sac; *Mt* microtubule; *N* nucleus; *P* plastid; *V* vacuole)
Fig. 7 Early stage of a binucleate cell of the third-order filament with numerous small plastids, mitochondria and vacuoles
Fig. 8 Microtubules associated with lipid droplets in the cytoplasm
Fig. 9 Later stage of a multinucleate cell of the third-order filament with mucilage sacs and numerous starch granules (*arrows*)
Fig. 10 Dividing, initial cell of the nutritive filament
Fig. 11 A nutritive filament with five cells

carposporogenesis is characterized by the intense activity of dictyosomes. Many curved dictyosomes are scattered mainly in the peripheral cytoplasm, and these produce numerous vesicles with fibular material (Figs. 21, 22). Subsequently, these vesicles deposit additional wall material, creating a two-layered carpospore wall surrounded by carposporangial mucilage (Fig. 23).

The final carpospore stage is characterized by the presence of dark-staining vesicles (Figs. 24, 25), which appear similar to the ''adhesive'' vesicles described by Pueschel (1979). These also are derived from dictyosomes, but they are never released into the existing wall, while the carpospores remain in the cystocarpic cavity. The mature carpospore has a spherical shape, it has a centrally located nucleus, chloroplasts, floridean starch, adhesive vesicles, and is surrounded by a two-layered wall with compressed mucilage that constitutes a carposporangial wall (Figs. 23, 24).

Discussion

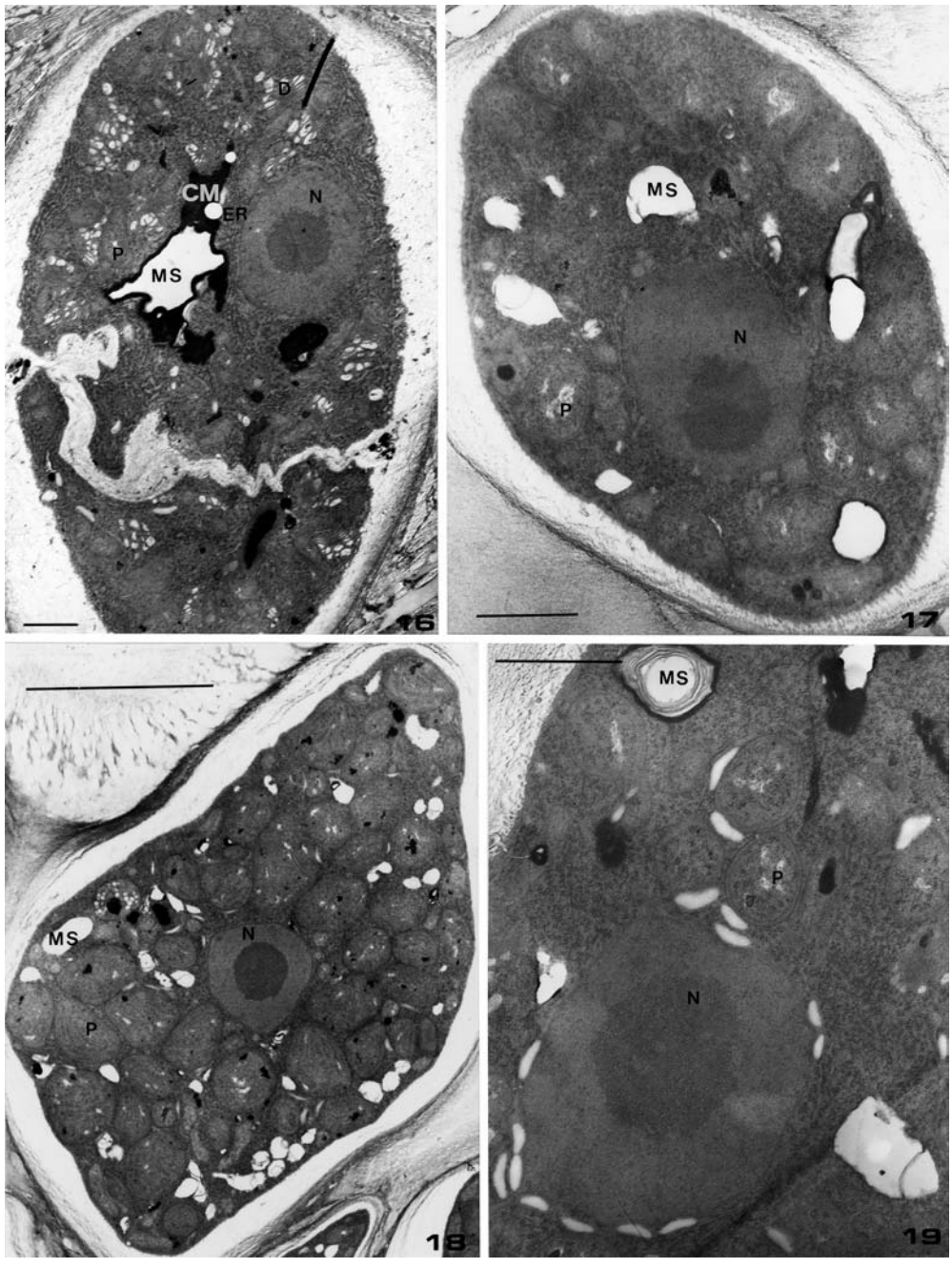
This report is the first ultrastructural study of cystocarp development in a member of the Gelidiales. In the vicinity of the several carposporophytes many chains of nutritive cells are produced by the vegetative tissue, and these comprise the nutritive filaments (Bold and Wynne 1985). It has been suggested that the nutritive filaments



Figs. 12–15 *Gelidium robustum*. Electron micrographs depicting old cells of the nutritive filament and gonimoblast initials. *Scale bars*: 1 μm (*M* mitochondrion; *MS* mucilage sac; *N* nucleus; *P* plastid; *SG* starch granule)
Fig. 12 Older cells of the nutritive filament with mucilage sacs and remnants of lipid droplets. Note a mucilage sac discharging its content (*arrowhead*)
Fig. 13 Mature nutritive cell with large mucilage sacs prior to discharging their contents
Fig. 14 The first cell cut off from the carpogonium
Fig. 15 Gonimoblast initial having plastids and mucilage sacs

are formed and specially adapted for a nutritive function prior to fertilization (Hommersand and Fredericq 1988). According to the same authors the diffusely developing carposporophytes possibly receive some nourishment from these nutritive filaments, and for this reason the terminal cells of the gonimoblast filaments fuse with the terminal cells of the nutritive filaments (Hommersand and Fredericq 1988). Thus, the terminal nutritive cells act as nutritive suppliers. This is suggested by the presence of large lipid droplets in the terminal nutritive cells of *Gelidium*. Hommersand and Fredericq (1988) postulated that ultimately the cells of the nutritive filaments become vacuolated as their contents are consumed. However, it is evident in the present study that the basal cells of the nutritive filaments, which are the older and the more mature ones, have large mucilage sacs and function as mucilage suppliers for the cystocarpic cavity. These may appear as clear vacuoles when observed with light microscopy.

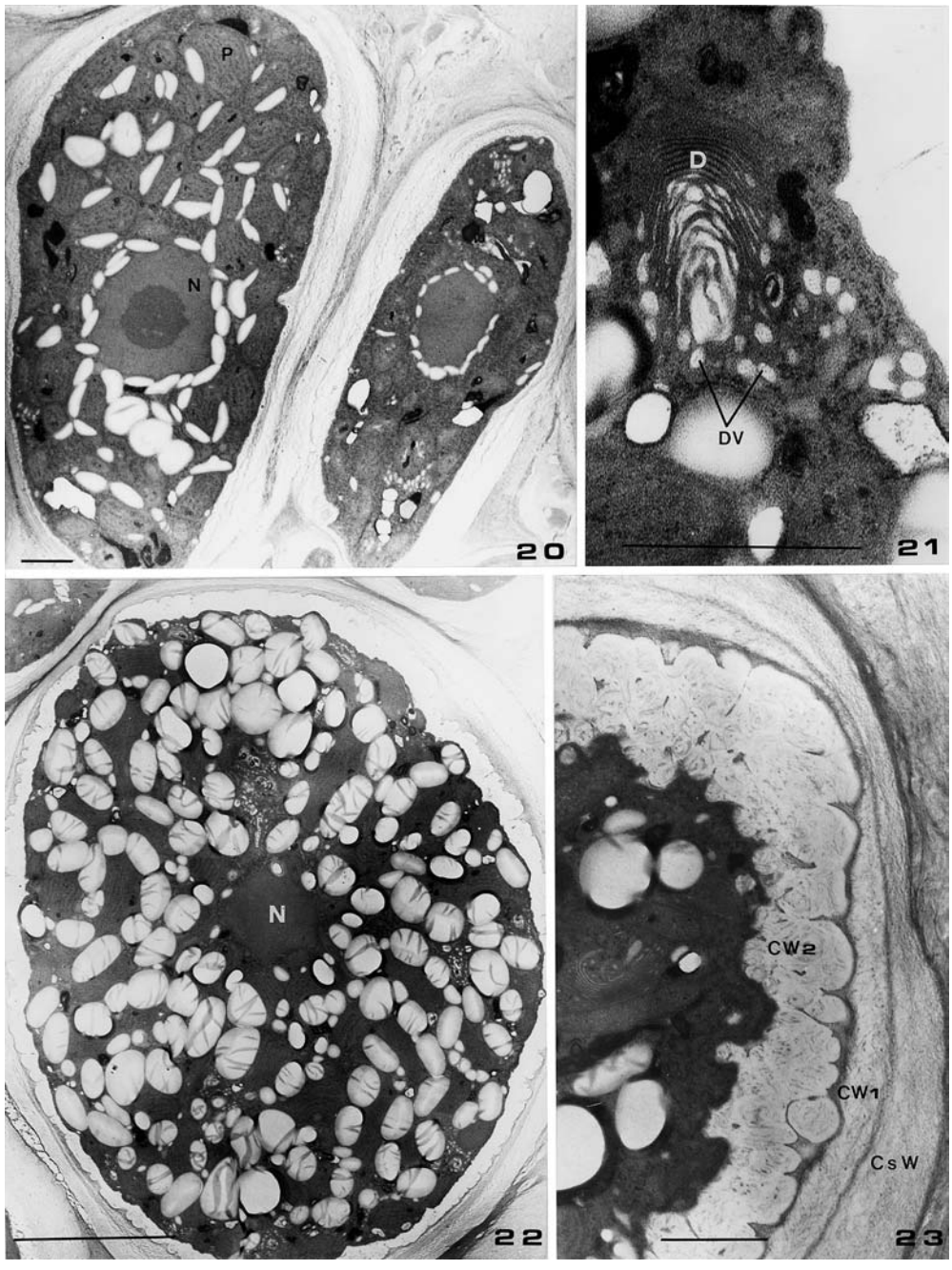
The third-order filament cells, from which nutritive filaments develop, are multinucleate cells. In these cells bundles of microtubules are attached to the surface of cytoplasmic lipid droplets lacking a bounding membrane. Cytoplasmic lipid droplets lacking a bounding membrane appear to be ubiquitous, and are particularly conspicuous in storage cells of bryophytes (Doyle 1962; Valanne et al. 1976; Ligrone and Lopes 1989; Duckett and Ligrone 1992; Duckett and Renzaglia 1993).



Figs. 16–19 *Gelidium robustum*. Electron micrographs of young gonimoblast cells and young carpospores. *Scale bars*: 1 μm, except Fig. 18: 5 μm (*CM* concentric membranes; *ER* endoplasmic reticulum; *MS* mucilage sac; *N* nucleus; *P* plastid)
Fig. 16 Young gonimoblast cell with proplastids, dictysomes, active ER, concentric membrane structures and a mucilage sac
Fig. 17 Very young carpospore with proplastids and some mucilage sacs
Fig. 18 Differentiating young carpospore with increased number of plastids
Fig. 19 Young carpospore with some perinuclear starch grain formation. Mucilage is forming within expanding areas of the concentric membranes, resulting in the formation of mucilage sacs

Association of microtubules with lipid droplets has been reported in food-conducting cells in bryoid mosses (Bryophyta) (Lignore and Duckett 1994). There is also an increase in the number of microtubules in oil-body cells of *Marchanthia* (Galatis and Apostolakos 1976). In addition, microtubules have been observed to be closely connected with lipid droplets participating in their rotary motion in the cytoplasm (Kwiatksowska 1972, 1973). This is the first report of microtubule association with lipid droplets in red algal cells. The presence of microtubules with lipid droplets in young cells of the third-order filament of *Gelidium* reinforces our suggestion that these cells function as food suppliers and food-conducting cells to the nearby cells of the nutritive filament. Cells of the third-order filaments have, at maturity, mucilage sacs and numerous starch granules, many of which occur in the mucilage sacs. Therefore, mature cells of the third-order filaments function as mucilage suppliers for the cystocarpic cavity.

The cystocarpic cavity enlarges gradually, beginning with the earliest stages of gonimoblast formation. It is evident that mucilaginous material is secreted into this shape, filling it and expanding it as it has been assumed in *Gelidium pteridifolium* (Hommersand and Fredericq 1988). The numerous dictyosome vesicles and large mucilage sacs of the inner cortical cells supply the cystocarpic cavity with large quantities of mucilage. Therefore, inner cortical cells also function as mucilage

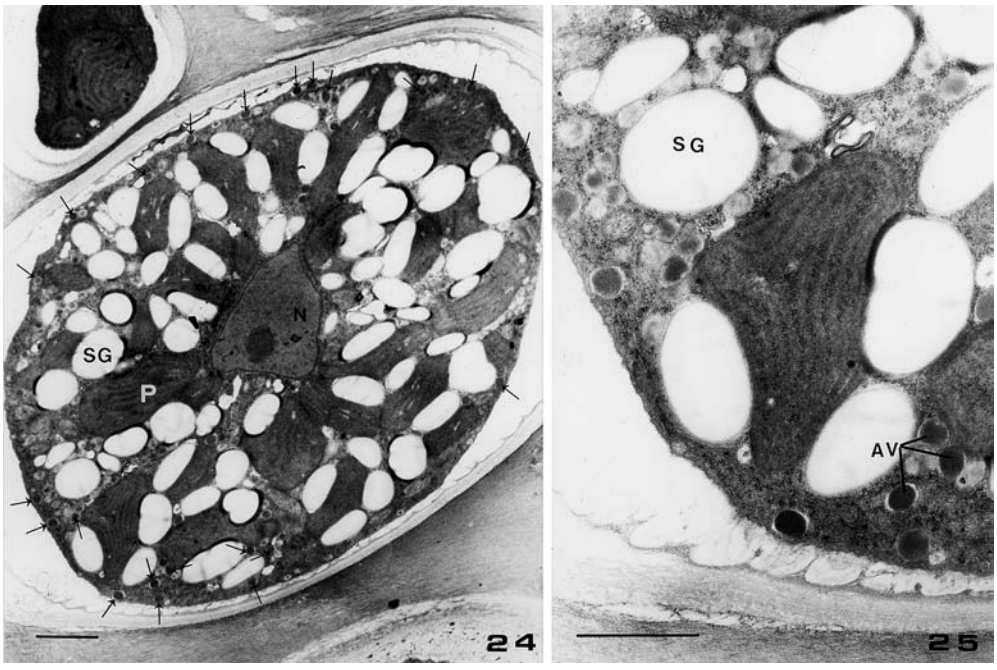


Figs. 20–23 *Gelidium robustum*. Electron micrographs of second and final stage of carposporogenesis. *Scale bars*: 1 μm, except Fig. 22: 5 μm (*CsW* compressed mucilage of carposporangial wall; CW_1 first carpospore wall layer; CW_2 second carpospore wall layer; *D* dictyosome; *DV* dictyosome vesicle; *N* nucleus; *P* plastid)
Fig. 20 Differentiating carpospores with starch grain formation, and granules scattered throughout the cytoplasm
Fig. 21 Portion of a second-stage carpospore having curved dictyosomes producing vesicles with fibular material
Fig. 22 Low magnification of a medium-aged carpospore with curved dictyosomes and numerous, large starch grains scattered throughout the cytoplasm
Fig. 23 Portion of a medium-aged carpospore showing the two-layered carpospore wall and compressed mucilage that constitutes a carposporangial wall

suppliers for the cystocarpic cavity. Thus, part of this tremendous amount of mucilage surrounding the carposporophyte derives from cortical (pericarp) cells, as has been described in the cystocarp of the well-known agarophyte *Gracilaria verrucosa* (Hudson) Papenfuss (Delivopoulos and Tsekos 1983). In that case, the massive production of mucilage by the pericarp cells results in their degeneration. This accounts for the fact that the pericarp of mature cystocarps of *Gracilaria* is considerably decreased in comparison with that of immature ones, as in *Gelidium robustum*. In *Levringiella gardneri* (Setch.) Kylin, however, mucilage is formed by a special group of secretory cells adjacent to the pericarp (Kugrens and West 1973).

Crystalline, proteinaceous inclusions have previously been reported in red algal cells of many species, and their function is generally regarded to be the storage of protein or nitrogen for later utilization (Pueschel 1992). Crystals in the Gelidiales have only previously been observed in *Pterocladia caloglossoides* (Howe) Dawson and have a repeated unit of 10 nm, while in *Gelidium* the respective unit is 9 nm. The presence of crystals in the cortical cells of *G. robustum*, which are near the developing carpospores, tends to support the idea of Wetherbee et al. (1984) that crystals might be mobilized and utilized during the developmental stages of sporogenesis.

Young carpospores in *G. robustum* are ovate, while the mature ones are spherical and borne solitarily.



Figs. 24–25 *Gelidium robustum*. Electron micrographs of mature carpospores. *Scale bars*: 1 μm (*AV* adhesive vesicle; *N* nucleus; *P* plastid; *SG* starch granule)
Fig. 24 Low-magnification view of a mature carpospore. Note the distribution of darker staining adhesive vesicles (*arrows*)
Fig. 25 Higher magnification of a portion of Fig. 24, showing the structure of the adhesive vesicles

On the other hand, carposporangia in *G. pteridifolium* are clavate to obovate and borne either in chains on a basal placenta or singly, and either solitarily or in terminal clusters on paired placenta alongside the central partition (Hommersand and Fredericq 1988).

Cytological differentiation of carpospores in *Gelidium* is reported here for the first time in a member of Gelidiales. There are similarities and differences with the respective process described in other species. When starch polymerization begins, a ring of developing starch grains just external to the nuclear envelope is observed. A similar association of starch granules with the nuclear envelope was also observed in *Pterocladia capilacea* (Gmel.) Born. et Thur. (Tripodi 1971), *Polysiphonia novae-angliae* Taylor (Wetherbee and Wynne 1973) and *Faucheocolax attenuata* Setch. (Delivopoulos and Kugrens 1984). The observations imply a possible role for the nuclear envelope in starch polymerization. The metabolic pathway of this process could be similar to that described by Pueschel (1979) for the relative role of ER in *Palmaria palmata* (L.) O. Kuntze.

Fibrous vacuoles are a common feature of carpospore differentiation in various red algal species (for references see Pueschel 1990). However, fibrous vacuoles are absent from maturing carpospores of *Gelidium*, which also was the case in *Gloiosiphonia verticillaris* Farl. (Delivopoulos and Kugrens 1985), *Plocamiocolax pulvinata* Setch. (Kugrens and Delivopoulos 1986) and *Alsidium corallinum* C. Agardh (Delivopoulos and Diannelidis 1991). It is remarkable that mature carpospores of species lacking fibrous vacuoles possess large amounts of starch, while carpospores from species with fibrous vacuoles possess considerably fewer starch granules. The fibrous vacuoles, after cytochemical staining, showed a polysaccharide component (Tripodi and De Masi 1975). Therefore, the numerous starch granules could potentially represent a large reservoir of nutrients (energy) for the developing carpospores of some species, replacing the lacking fibrous vacuoles.

Instead of the commonly observed cored vesicles in mature carpospores of red algae, there are condensed, cored vesicles in those of *Gelidium*. These are dictyosome-derived vesicles that undergo modifications to gradually form dark-staining granular vesicles, originally called adhesive vesicles by Pueschel (1976), as shown in *Faucheocolax* (Delivopoulos and Kugrens 1984) and *Plocamiocolax* (Kugrens and Delivopoulos 1986).

The mucilage sac is the main structure involved in the production of mucilage. Mucilage sacs originate from the concentric membrane structures. They have been described in many other species (for references see Pueschel 1990), but their presence in *Gelidium* is the most notable case observed to date. The extensive mucilage produced during carposporophyte development and surrounding the carposporophyte in various red algal species has been suggested to play an adhesive, antibiotic and/or an-desiccation role (Kugrens and West 1973; Kugrens and Arif 1981), or, alternatively, to have a nutritive function for the developing carposporophyte (Delivopoulos and Kugrens 1984). It has also been suggested that cell walls and the intercellular matrix contain agar and other polymers characteristic of agarophytes (Hommersand and Fredericq 1988). This suggestion reinforces the idea that the extensive mucilage surrounding carposporophytes of various red algae could have a nutritive function (Delivopoulos and Kugrens 1984). It seems, therefore, that this tremendous amount of mucilage in the agarophytes *Gracilaria* and *Gelidium* is correlated with the production of agar.

From the structural point of view the carpospore wall in *Gelidium* most resembles that in *Plocamiocolax* (Kugrens and Delivopoulos 1986). A two-layered wall surrounds each mature carpospore in both cases. The carposporangial mucilage that is outside the two carpospore wall layers would constitute the carposporangial wall. However, it is not called carposporangial wall, because it appears as a distinct layer only upon compression of diffuse mucilage fibrils during carpospore formation.

Additional studies of the cystocarpic composition and ultrastructure are warranted in order for the correlation between the cystocarpic structure and other morphological characters in *Gelidium* species to be clarified.

The experiments performed during this study comply with the current laws of Greece.

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