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Ultrastructure of some species of *Halimeda* (Bryopsidales, Chlorophyta) from Brazil

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Abstract – Here we report for the first time the ultrastructure of *Halimeda discoidea*, *H. incrassata*, *H. opuntia*, *H. simulans* and *H. tuna*, collected on the northeastern coast of Brazil. The genus is heteroplastidic, showing chloroplasts and amyloplasts in different stages of development. Large chloroplasts are more abundant in the cortical utricles, close to the outer cell walls, whereas small chloroplasts, amyloplasts, nuclei and mitochondria are more abundant in the medullary siphons. Chloroplasts are similar to those of terrestrial plants, with a system of thylakoids parallel to the chloroplast's long axis and a large variation in the number of thylakoids per granum. A characteristic system of concentric lamellae is formed in one pole of the plastids. Chloroplast aging is characterized by an increase in number and size of the plastoglobules. In addition to the usual organelles, the cytoplasm has an unusual set of spherical bodies of different texture and electron-densities. Another kind of spherical bodies, with a fibrillar structure, is found inside the vacuoles. The function and composition of these bodies have not yet been ascertained.

Brazil / Bryopsidales / coenocytic / *Halimeda* / ultrastructure

Résumé – L'ultrastructure d'*Halimeda discoidea*, d'*H. incrassata*, d'*H. opuntia*, d'*H. simulans* et d'*H. tuna,* récoltés sur les côtes nord-est du Brésil est étudiée pour la première fois. Le genre est hétéroplastidié, avec des chloroplastes et des amyloplastes à différents stades de développement. Les grands chloroplastes sont plus abondants dans les utricules corticaux, liés aux parois cellulaires externes, tandis que les petits chloroplastes, les amyloplastes, les noyaux et les mitochondries sont plus abondants dans les siphons médullaires. Les chloroplastes sont semblables à ceux des plantes terrestres, avec un système de thylacoïdes parallèle à l'axe long des chloroplastes, et une grande variation dans le nombre de thylacoïdes par granum. Un système caractéristique de lamelles concentriques est formé dans un pôle des plastids. Le chloroplaste âgé est caractérisé par un accroissement en nombre et en taille des plastoglobules. En plus des habituels organelles, le cytoplasme a un lot particulier de corps sphériques de texture différente et dense aux électrons. Une autre sorte de corps sphériques, avec une structure fibrillaire, est présente à l'intérieur des vacuoles. La fonction et la composition de ces corps demeurent incertaines.

Brésil / Bryopsidales / coenocytique / *Halimeda* / ultrastructure

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INTRODUCTION

Members of the genus *Halimeda* Lamouroux are calcified green algae characterized by a macroscopic thallus that is composed of flattened to discoid segments connected by non-calcified flexible joints. Segments consist of interwoven coenocytic siphons with expanded portions, the utricles; terminal utricles are coalescent at the thallus surface forming a distinctive cortex. The genus has a pantropical distribution and is comprised of 33 species recognized by a combination of characters that include segment morphology, thallus branching pattern, holdfast development, and anatomical details including fusion of nodal siphons and diameter of peripheral utricles (Hillis-Colinvaux, 1980; Hillis *et al.*, 1998, Kooistra *et al.* 2002). In a recent molecular systematics treatment Kooistra *et al.* (2002) were able to recognize five well-supported lineages and suggested a recent diversification of the genus.

The genus may have a key ecological role in some tropical regions owing to its large biomass and ability to provide shelter and substrate for a large variety of algae and invertebrates. Mechanisms of defense against herbivory on *Halimeda* were studied by Hay *et al.* (1988), Hay *et al.* (1994) and Hay (1997).

Although species of *Halimeda* in Brazil have been mentioned in general floristic papers (e.g. Taylor, 1960; Joly *et al.*, 1968; Oliveira Filho, 1977; Pereira & Accioly, 1998), little is known about them. Information on the ultrastructure of *Halimeda* spp is scarce and deals mostly with calcification (Lowenstam, 1955; McConnell & Colinvaux, 1967; Wilbur *et al.*, 1969; Perkins *et al.*, 1972; Palandri, 1972; Böhm, 1972; Böhm, 1973; Böhm & Goreau, 1973; Borowitzka & Larkum, 1976abc; Borowitzka & Larkum, 1977; Borowitzka *et al.*, 1974). Borowitzka & Larkum (1974) studied chloroplast development of *Halimeda* and Colombo & Orsenigo (1977) compared the ultrastructure of specimens of *Halimeda tuna* from different depths and found differences equivalent to what is known for sun and shade adapted land plants. Here we present some basic information on the ultrastructure of five species of *Halimeda* collected in Brazil. The ultrastructure of the studied species is compared with the ultrastructure of *H. cuneata* Hering newly referred to Brazil (Bandeira-Pedrosa, E. *et al.,* submitted to Phycologia).

MATERIALS AND METHODS

Specimens of *Halimeda incrassata* (Ellis) Lamouroux, *H. opuntia* (Linnaeus) Lamouroux and *H. tuna* (Ellis *et* Solander) Lamouroux were collected during low water spring tide in the State of Pernambuco (8°43'S/35° 06'W) in May 2000. *Halimeda discoidea* and *H. simulans* were collected in the State of Bahia (12°34'S/37°59'W, and 13°00'S/38°36'W) in June 2000.

For TEM observations, fragments of mature segments were fixed in 2.5% glutaraldehyde in phosphate buffer 0.1M, pH 7.0, in the field, in the morning (cf. Drew & Abel, 1990) and transported to the laboratory in an icebox. Fragments were decalcified overnight in EDTA 5% in a phosphate buffer 0.1M, pH 7.0, and transferred to 2% osmium tetroxide in the same buffer. The material was dehydrated in an acetone series and embedded in Spurr resin (Spurr, 1969). Sections were made with a Sorvall Porter-Blum microtome and stained with uranyl acetate and lead citrate (Reynolds, 1963). Observations and photographs were taken with a Zeiss EM900 electron microscope.

RESULTS

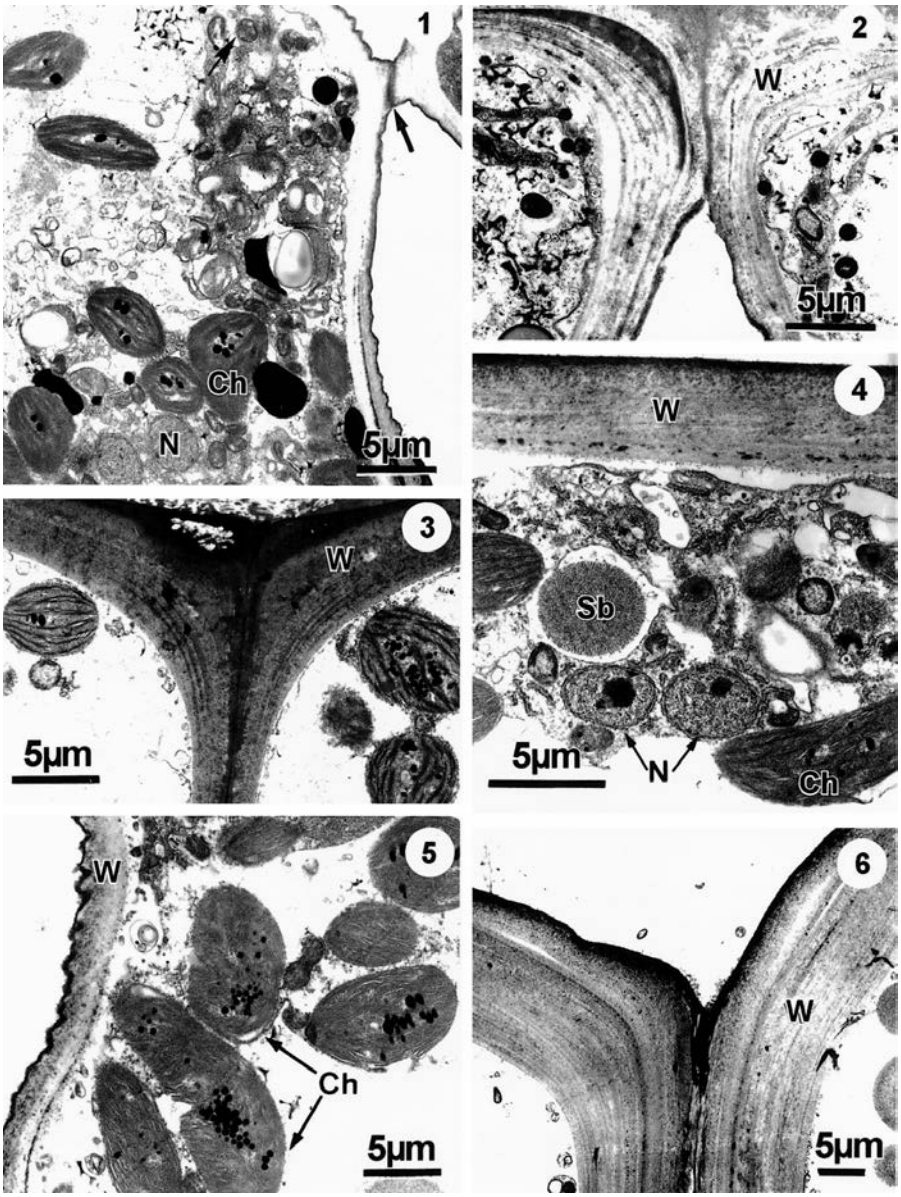
Siphons of all studied species are covered with an electron dense layer similar to a thin "cuticle" on top of the cell wall. This cuticle seems to cement the external utricles together (Figs 1-3). On medullary siphons this cuticular layer may be smooth, as in *H. discoidea* (Fig. 4), or rippled, as in *H. incrassata* (Fig. 5). Two regions can be observed in the cell wall: a granular external one and an internal region consisting of concentric microfibrils, both embedded in an amorphous matrix (Figs 2-4). The siphon wall is thicker on the utricles (*ca* 20 μm) than on the medullary siphons (*ca* 7 μm) (Figs 4, 5). On the siphons that form the joints, the cell wall is as thick as in the external utricles, but without concentric layers of microfibrils and with less variation in electron density (Fig. 6).

As the central vacuole increases below the utricular region, the cytoplasm becomes gradually compressed appearing as a thin parietal layer in the medullary siphons, with most of the cell volume being occupied by the central vacuole (Fig. 7). The most conspicuous organelles are the various plastids, proplastids, chloroplasts and amyloplasts. The proplastids are about 5 μm in diameter; they are surrounded by two external membranes and have a poor system of thylakoids within a finely granular stroma. The presence of 3-4 concentric thylakoids at one pole is characteristic of proplastids (Fig. 8). Proplastids are more frequent in the medullary siphons, whereas chloroplasts are more common in utricles, where they account for most of the cell content. Chloroplasts vary from ellipsoid to fusiform and are 5-13 μm long (Figs 9, 10, 12, 13) ; they have the typical two-membrane envelope and a variable number of thylakoids disposed parallel to the long axis of the chloroplast; in some points, stacks of 2-14 thylakoids may be seen (Figs 10, 11). Osmiophilic drops (plastoglobuli) up to 900 nm in diameter are common among the thylakoids, forming aggregates in the senescent chloroplasts (Fig. 14). The plastidial stroma is uniformly granular and has a moderate electron-density, except for some electron-transparent spots where a fibrillar material, probably the plastidial genome, is present (Fig. 15). Some chloroplasts viewed in longitudinal sections may present the same polar system of concentric lamellae that we described for the proplastids in one, or rarely both, poles (Figs 12 and 13).

Although we have seen small starch grains among the thylakoids of mature chloroplasts (Figs 7 and 15), we believe that amyloplasts develop only from proplastids. Young amylopasts deposit a central starch grain (Figs 16 and 17). As the amyloplast matures the stroma gradually disappears and the growing starch granule eventually fills the structure completely (Figs 17 and 18). Mature amyloplasts are elliptic, 4-9 μm on the larger diameter, surrounded by two membranes and do not have thylakoids, but have the concentric system of lamellae on one pole (Fig. 16). Amyloplasts of varying stages of maturation are especially common in the medullary region (Figs 16-19).

In the medullary siphons the cytoplasm is restricted to a thin layer close to the cell wall, with several nuclei, 4-7 μm in diameter, and can be seen isolated or in groups, each with a distinctive nucleolus (Fig. 20).

Mitochondria, about 1.5 μm in diameter, are abundant and scattered through the cytoplasm, whereas the rough endoplasmic reticulum, dictyosomes and ribosomes are always present but not particularly abundant (Fig. 20). Inclusions such as osmiophilic granules and electron-dense spherical structures are also present in the cytoplasm of the medullary siphons. The presence of a bounding membrane in those structures is difficult to ascertain due to the structures' high electron-density, which suggests a lipidic composition (Fig. 21). Another com-



Figs 1-6. Electron micrographs of *Halimeda* species Fig. 1. *Halimeda opuntia*. Longitudinal section showing two utricles partially attached by the cuticular layer (arrow). Note chloroplasts, nuclei and mitochondria. Fig. 2. *Halimeda opuntia*. Cell wall formed by layers of microfibrils. Note partial adhesion of the utricles. Fig. 3. *Halimeda tuna*. Utricle cell wall. Note extensive adhesion of cell walls of neighbour utricles. Fig. 4. *Halimeda discoidea*. Detail of medullary siphon, showing a parietal disposition of the cytoplasm and organelles. Note smooth cuticle in contrast with the granular aspect of the cell wall. Fig. 5. *Halimeda incrassata*. Detail of medullary siphon with a crenulate cuticle. Note senescent chloroplasts. Fig. 6. *Halimeda simulans*. Microfibrillar structure of the cell wall of a medullary siphon.
(Abbreviations: W, cell wall; Ch, chloroplasts; N, nuclei; Sb, spherical bodies).

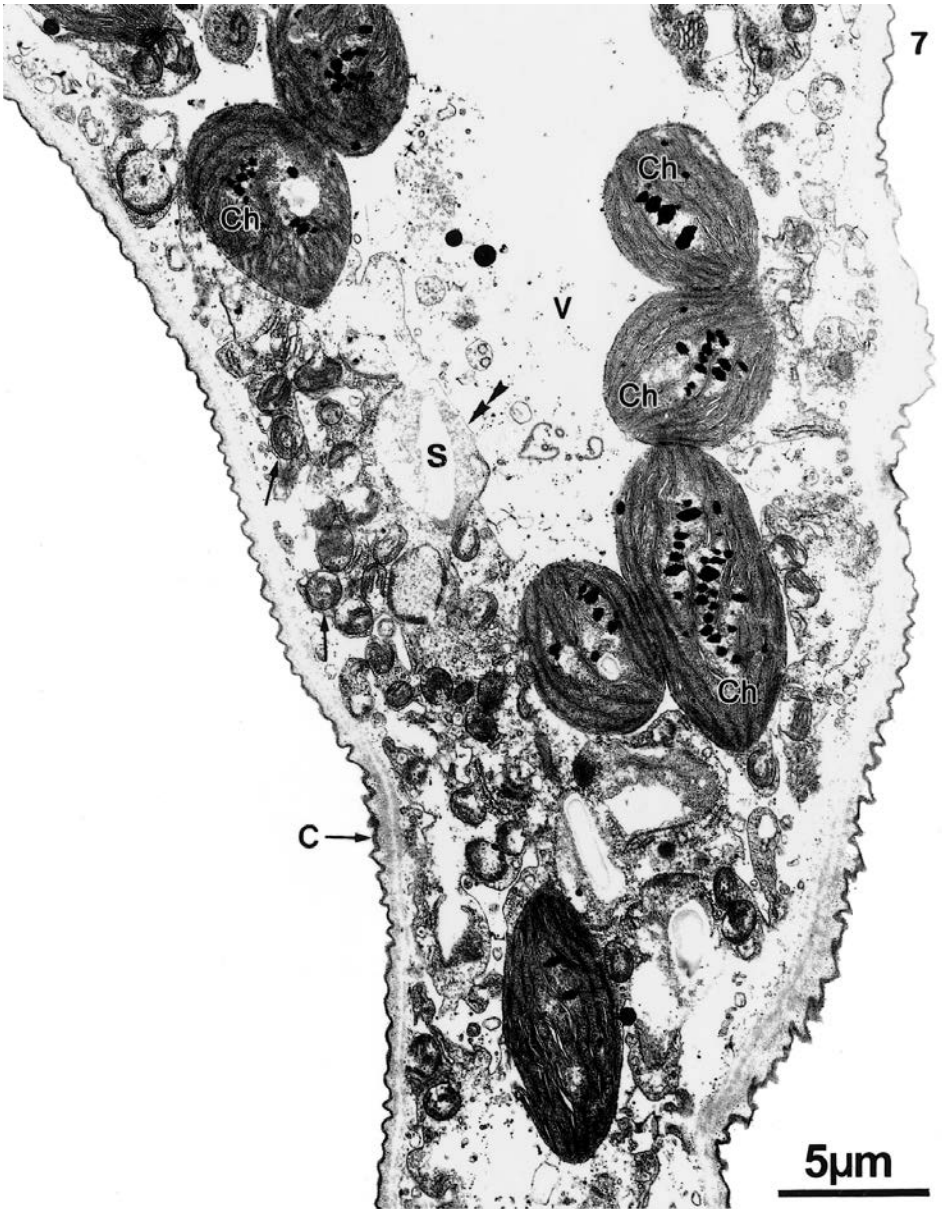
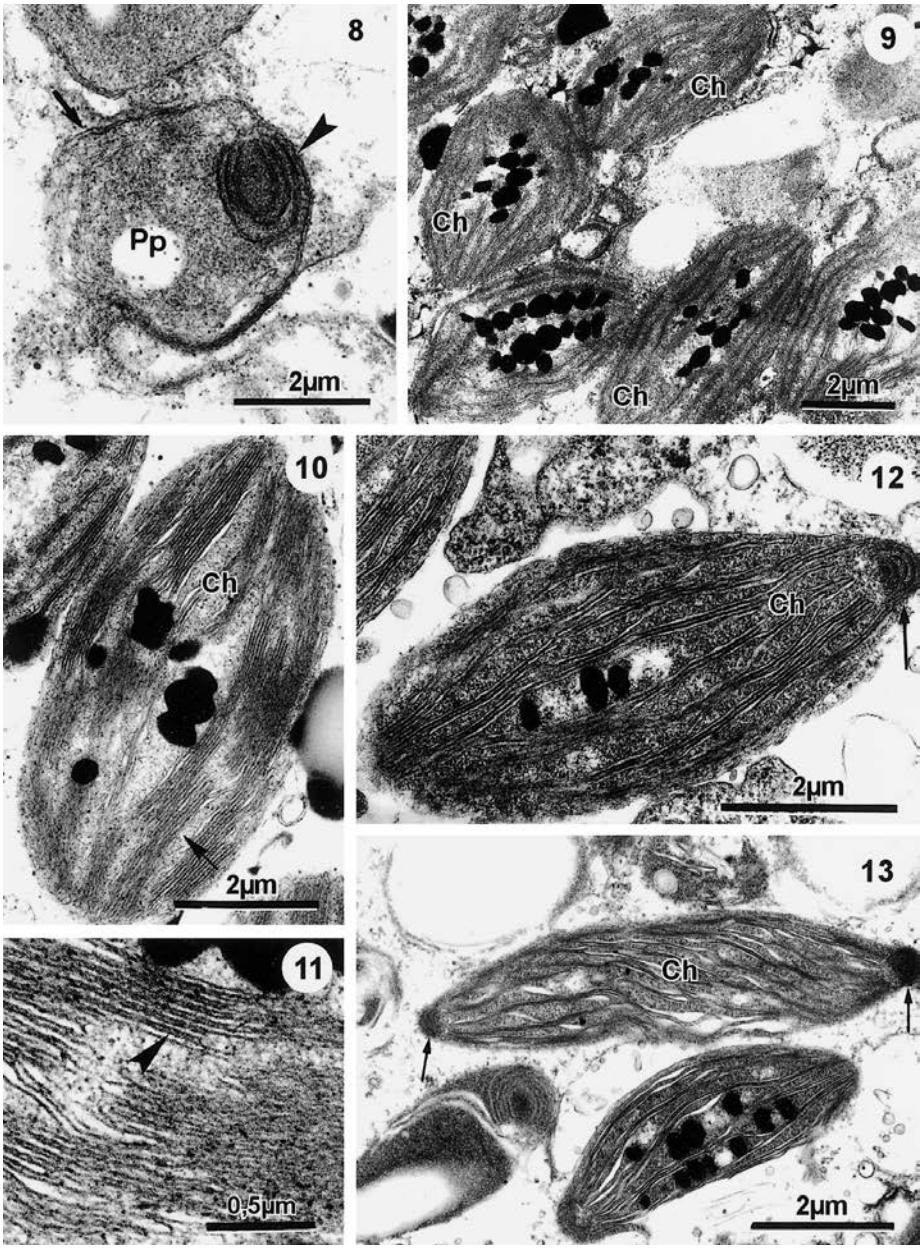
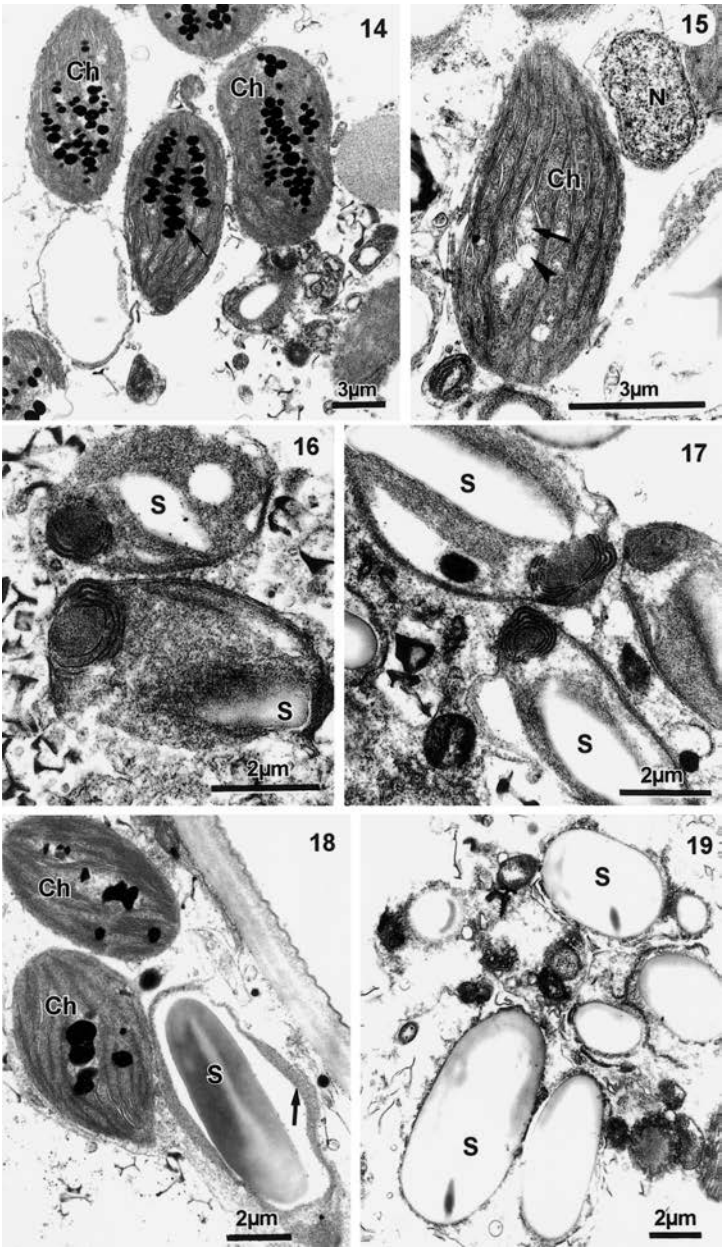


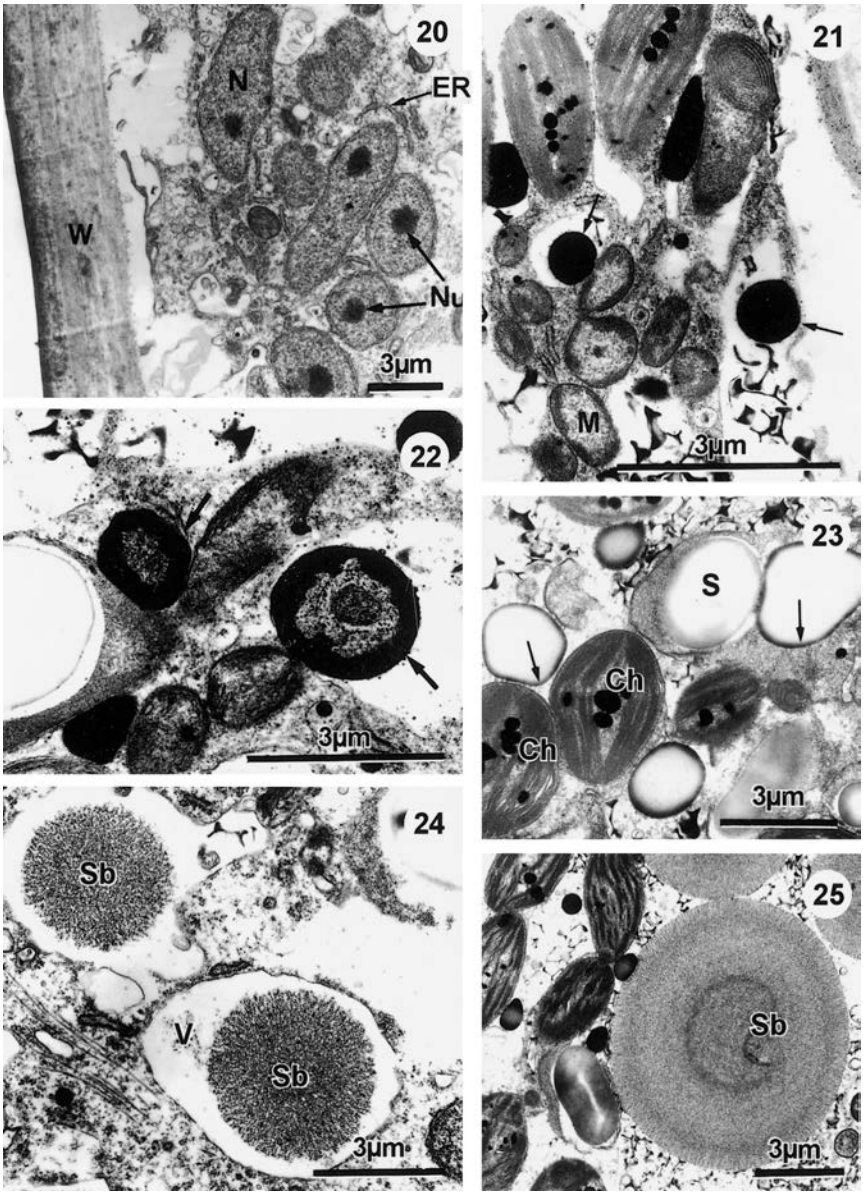
Fig. 7. *Halimeda tuna*. Longitudinal section of a siphon just below the utricular region showing the large vacuolization of the utricle and many organelles. Arrows point to mitochondria and arrow head points to the tonoplast. Note crenulate cuticle. (Abbreviations: C, cuticle; Ch, chloroplasts; S, starch; V, vacuole).



Figs 8-13. Electron micrographs of *Halimeda* species. Fig. 8. *Halimeda opuntia*. Proplastid with double membrane (arrow) and concentrically distributed lamellae (arrow head). Fig. 9. *Halimeda opuntia*. Group of chloroplasts with osmiophilic drops among thylacoids. Fig. 10. *Halimeda tuna*. Detail of a chloroplast. Fig. 11. *Halimeda tuna*. Detail of a chloroplast showing grana-like arrangement of the thylacoids (arrow head). Fig. 12. *Halimeda discoidea*. Detail of a chloroplast showing arrangement of the thyllakoids, not forming grana, and concentric lamellae at one of the poles (arrow). Fig. 13. *Halimeda tuna*. Detail of a chloroplast showing absence of grana and TOBs. (Abbreviations: Ch, chloroplasts; Pp, proplastid; TOB, thylakoid organizer body).



Figs 14-19. Electron micrographs of *Halimeda* species. Fig.14. *Halimeda incrassata*. Senescent chloroplasts in a medullary siphon with abundant osmiophilic granules (arrow). Fig. 15. *Halimeda discoidea*. Chloroplast with small starch granules (arrow head) and DNA-like fibrils (arrow). Fig. 16. *Halimeda opuntia*. Amyloplast in a medullary siphon and concentric lamellae at one pole (arrow). Fig. 17. *Halimeda opuntia*. Amyloplasts (arrow) with starch granules. Fig. 18. *Halimeda tuna*. Amyloplasts with a starch granule surrounded by a thin layer of stroma (arrow). Fig. 19. *Halimeda simulans*. Medullary siphon with starch filling completely the amyloplasts. (Abbreviations: Ch, chloroplasts; S, starch; N, nuclei).



Figs 20-25. Electron micrographs of *Halimeda* species. Fig. 20. *Halimeda discoidea*. Detail of a medullary siphon with several nuclei with prominent nucleoli and rough endoplasmatic reticule. Fig. 21. *Halimeda opuntia*. Osmiophilic granules of unknown origin in the cytoplasm of medullary siphons (arrow). Fig. 22. *Halimeda opuntia*. Thick bordered osmiophilic granules with fine granular core in a medullary siphon (arrow). Fig. 23. *Halimeda opuntia*. Electron translucent granules with a thin electron dense layer (arrow) among amyloplasts and chloroplasts are probably starch granules. Fig. 24. *Halimeda discoidea*. Striated electron dense bodies developing inside vacuoles. Fig. *25. Halimeda tuna*. Spherical electron dense body with concentric layers surrounded by a striated border. (Abbreviations: Ch, chloroplasts; S, starch; N, nuclei; ER, rough endoplasmatic reticule; V, vacuole; Sb, spherical bodies).

mon kind of vesicles has a granular center and is surrounded by a thick electron-dense halo (Fig. 22) ; some other vesicles enveloped by a membrane and with an electron-dense halo are probably starch granules (Fig. 23). Second to the chloroplasts, the most numerous structures in the medullary siphons are the electron-dense spherical bodies in vacuoles. These structures are not surrounded by membranes and are composed of an agglomeration of small osmiophilic granules scattered or in radial rows (Fig. 24). When fully developed, these bodies measure up to 15 μm in diameter and exhibit concentric granular layers with distinct electron-densities formed by microfibrils (Fig. 25). In this last figure the spherical bodies appear to be in the cytoplasm, but this may be so because the vacuolar membrane was disrupted in the preparation.

DISCUSSION

The ultrastructure of several taxa of Chlorophyta has been described by Dawes (1966), Hori (1974), Borowitzka & Larkum (1974), Borowitzka (1976), Calvert *et al.* (1976) and Roth & Friedmann, (1987), among others.

The general ultrastructure of the coenocytic siphons agrees with observations in the literature for members of the Bryopsidales, which now includes the former Caulerpales (Dawes & Rhamstine, 1967; Sabnis, 1969; Wilbur *et al.*, 1969; Palandri, 1972; Colombo & Orsenigo, 1977).

A type of cuticular layer is present on the siphon external wall, except when there is a fusion of neighbor utricles. When this occurs the cuticle gets detached and the space filled with granular material. External to the cuticle there is a pilose layer that corresponds to the site of carbonate deposition (Borowitzka & Larkum, 1977).

The cell wall is composed of xylans with β-1-3 links, corresponding to the Group C of Dawes (1966) and characterized by having axially oriented microfibrilles. In our material the microfibrillar structure may be replaced by a granular deposition, apparent in some cross sections. On the siphons of the joints, between two contiguous segments, discrete layers of concentric, electron-dense microfibrils form the cell wall, which may be associated with calcification (Borowitzka & Larkum, 1977). According to Hay *et al.* (1988), 48 h old segments are already well calcified and protected from grazing, being new tissue produced at night when herbivorous reef fishes are inactive.

The cortical utricles have a large number of chloroplasts whose orientation varies pending on light quantity (Drew & Abel, 1990), but are always close to the external cell wall.

It is well known that chloroplast structure differs among the phyla of the algae, (e. g., Calvert *et al*., 1976; van den Hoek *et al*., 1995). However, some basic features, such as enveloping membranes, presence of plastoglobuli and longitudinally oriented thylakoids, occur in all groups. Variations also occur in the number and arrangement of the thylakoids, presence or absence of pyrenoids and kind of storage compounds, and these are considered to have phylogenetic importance.

Members of the group show ultrastructural similarity among themselves and with the land plants. The presence of two kinds of plastids (heteroplasty), namely chloroplasts and amyloplasts, in the green algae was recognized by Feldmann (1946).

Within the Bryopsidales, the genus *Caulerpa* Lamouroux is well studied. In this genus three kinds of chloroplasts have been described: i) the "prolifera-type", ii) the "microphysa-type", and iii) the "paspaloides-type" (Calvert *et al*., 1976). The species of *Halimeda* that we studied have a prolifera-type of chloroplast. This is also the most common type in the Bryopsidales and is considered the most advanced. This type of chloroplasts organization is characterized by the presence of numerous small chloroplasts with 8-12 thylakoids uniformly distributed in the stroma, although eventually stacks of 6-10 thylakoids can be recognized (Calvert *et al*., 1976).

In contrast to the amyloplasts, which occupy a more internal position, the chloroplasts are always close to the external cell wall. This suggests that the photosynthate produced in the chloroplasts is transported to and polymerized in the amyloplasts. The disposition of the thylakoids in grana-like arrangements is not always present, as has been observed for other Bryopsidales (e. g., Dawes & Rhamstine, 1967).

The concentric lamellae at the poles of proplastids and some chloroplasts and amyloplasts have been designated as "circular bodies" (Dawes & Rhamstine, 1967), "lamellar concentric body" (Hori, 1974), "thylakoid organizing bodies", TOB, (Borowitzka & Larkum, 1974) and "concentric lamellar body" (Calvert *et al.*, 1976). This system has a close relationship to the inner membrane of the chloroplasts envelope, suggesting its participation on plastid ontogeny, as proposed by Borowitzka (1976). However, Dawes & Rhamstine (1967) suggested that the circular bodies participate in the production of starch and indicate an intermediate stage in the development of amyloplasts.

According to Borowitzka & Larkum (1974), TOBs are not the same as the prolamellar body of the flowering plants. TOBs disappear in the earlier stages of plastid differentiation, whereas TOBs in *Halimeda* remain throughout the maturation process. These authors consider that in *Halimeda* chloroplasts differentiate from proplastids that already include starch, and later on differentiate as mature chloroplasts losing the starch, or amyloplasts, losing the thylakoids. In our material TOBs were always present in one pole of proplastids. They were also seen in one or rarely both poles of some mature chloroplasts. The distance between the concentric lamellae of the TOBs (200 nm) are about the same as the distance between thylakoids. Intermediate phases between proplastids and chloroplasts, or amyloplasts, were common. Although we saw chloroplasts with starch granules, these are small and rare, suggesting a specialization of chloroplasts and amyloplasts in their respective functions of production of photosynthate and starch storage. This evolutionary trend had already been pointed out by Calvert *et al.* (1976).

The cytoplasm of the medullary siphons presents numerous bodies of variable electron-densities, and probably different composition, whose function is unknown. Some are similar to starch granules, with low electron-density, but differ from them in having a thin electron-dense layer and lacking a bounding membrane. The other bodies with strong electron-density may represent an accumulation of lipids and are similar to the plastoglobuli seen within the chloroplasts. The most peculiar structures in the genus *Halimeda* are the spherical bodies found within the vacuoles in various stages of "maturation". These structures have been seen by other authors (e.g., Wilbur *et al.*, 1969; Sabnis, 1969; Palandri, 1972; Colombo & Orsenigo, 1977; Borowitzka & Larkum, 1977), but thus far there are no explanations for their content or function. Menzel (1987) found similar bodies in *Chlorodesmis fastigiata* and presents evidence suggesting that these bodies may have an important role in wound plug formation.

When we initiated this project we hoped to find some additional characters of taxonomic value to distinguish the studied species. However, with the exception of the lenticular thickenings peculiar to the outer cell wall of *H. cuneata* (Bandeira-Pedrosa, E. *et al*., submitted for publication in Phycologia) we saw a great ultrastructural uniformity, even though we included species of three taxonomic sections of the genus: *Rhipsalis* J. Agardh *ex* De Toni, *Opuntia* J. Agardh *ex* De Toni and *Halimeda* J. Agardh *ex* De Toni (*sensu* Hillis-Colinvaux, 1980), and three of the five lineages of Kooistra *et al.* (2002). Besides that wall feature we found only variations in the size of the chloroplasts, (from 5,0-12,5 μm in their largest diameter) and the number of thylakoids in the grana, from none to 14. These are not of practical value. The situation is different from what was found by Calvert *et al*. (1976) for species of *Caulerpa*, which exhibited different patterns of chloroplast structure.

Abbreviations: W, cell wall; C, cuticle; Ch, chloroplasts; Pp, proplastid; TOB, thylakoid organizer body; A, amyloplasts; S, starch; N, nuclei; Nu, nucleoli; ER, rough endoplasmatic reticule; V, vacuole; Sb, spherical bodies; TEM, transmission electron microscopy.

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