4 Cytological and cytochemical aspects in selected carrageenophytes (Gigartinales, Rhodophyta)

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Introduction

Red algae (Rhodophyta) are a widespread group of uni- to multicellular aquatic photoautotrophic plants. They exhibit a broad range of morphologies, simple anatomy and display a wide array of life cycles. About 98% of the species are marine, 2% freshwater and a few rare terrestrial/sub-aerial representatives (Gurgel and Lopez 2007).

Red algae are true plants in the phylogenetic sense since they share, with the green lineage (green algae and higher plants), a single common ancestor (Adl et al. 2005). However, the phylum Rhodophyta is easily distinguished from other groups of eukaryotic algae due to a number of features listed below (Woelkerling 1990; Grossman et al. 1993; Gurgel and Lopez 2007):

(i) Total absence of centrioles and any flagellate phase.
(ii) Presence of chlorophylls *a* and *d*, and accessory pigments (light-harvest) called phycobilins (phycoerythrin and phycocyanin).
(iii) Plastids with unstacked thylakoids, and no external endoplasmic reticulum.
(iv) Absence of parenchyma and presence of pit-connections between cells (i.e. incomplete cytokinesis).
(v) Floridean starch as storage product.

Traditionally, red algae can be morphologically separated in three major groups: (1) a unicellular group with reproduction by binary cell division only, (2) a multicellular group where a carpogonial branch is absent or incipient (Bangiophyceae *sensu lato*) and (3) a multicellular group with well developed carpogonial branches (Florideophyceae).

Gigartinales

This family presents, like other Florideophyceae, development of a specialized female filament called carpogonial branch. The female gamete (carpogonium) is easily recognizable by the presence of the trichogyne, an elongated extension responsible for receiving the male gametes (spermatium). Germination *in situ* of the zygote and consequent formation of a group of spores (carpospores) or a parasite generation of female gametophyte, which produce carpospores (carposporophyte) inside the cystocarp.

The cystocarp is composed of the carposporophyte plus all protective sterile haploid tissue of the female gametophyte encircling and interacting with it (pericarp). Carpospores develop into a second free-living phase called tetrasporophyte, which can be

morphologically similar (isomorphic alternation of generations) or different (heteromorphic alternation of phases) from the gametophytes. Tetrasporophyte plants produce tetrasporangia by meiosis, which release tetraspores. This pattern of meiotic cell division in the tetrasporangium is stable in red algae and can be one of three types: cruciate, tetrahedral and zonate. When released, each tetraspore will give rise to either a male or a female haploid gametophyte (Gurgel and Lopez 2007).

In general Gigartinales present triphasic isomorphic or heteromorphic, diplo-haplotic (haploid gametophyte, diploid carposporophyte and diploid tetrasporophyte) or diphasic diplo-haplotic lifecycles (Maggs 1990; Brown et al. 2004; Thornber 2006).

A large number of genera of high economic interest (carrageenophytes), are members of this order and most of them are phylogenetically related (Freshwater et al. 1994; Fredericq et al. 1996).

Algal cell walls are composed of cellulose fibrils (rarely xylan fibrils) and a matrix of hydrocolloids. Cell wall hydrocolloid matrixes in red algae are formed by sulphated polysaccharides classified in two main groups: agar and carrageenan.

Red algal hydrocolloids (phycocolloids) are polysaccharides with gel-forming capabilities. They can be classified in two classes, each bearing a basic sugar skeleton consisting of 1,3-linked ß-D-galactopyranose plus either 1,4-linked 3,6-anhydro-α-L-galactopyranose (i.e. agars) or 1,4-linked 3,6-anhydro-α-D-galactopyranose units (i.e. carrageenans). However, several other sugar residues are present making all natural phycocolloid products (whether agars or carrageenans) a complex mixture of neutral and charged polysaccharides. Phycocolloid gel quality is measured by its rheological properties such as gel strength, density, gelling and melting points. These properties are in turn influenced by the overall chemical composition of the gels, i.e. the ratio among different polysaccharides and modified sugar residues found in it. Some groups of red algae exhibit higher concentrations of one particular class and thus are known as either agarophytes (agar-producers, e.g. Gracilariales, Gelidiales and Ceramiales) or carrageenophytes (carrageenan-producers, e.g. most families currently in the Gigartinales such as Caulacanthaceae, Cystocloniaceae, Dumontiaceae, Furcellariaceae, Gigartinaceae, Hypneaceae, Kallymeniaceae, Polyideaceae, Rhizophyllidaceae, Solieriaceae, Sphaerococcaceae, Tichocarpaceae, to name a few) (Pereira et al. 2003; Gurgel and Lopez 2007).

Cytological and spectroscopic analysis techniques

Vibrational spectroscopy

Samples of ground, dried algal material were analyzed by FTIR-ATR and FT-Raman for determination of natural phycocolloids composition, according to the method described by Pereira (2006) and Pereira et al. (2009). The FTIR spectra of ground dried seaweed, native and alkali-modified carrageenan, were recorded on an IFS 55 spectrometer, using a Golden Gate single reflection diamond ATR system, with no need for sample preparation. All spectra reported in this paper are the average of two counts, with 128 scans each and a resolution of 2 cm^{-1}. The room temperature FT-Raman spectra were recorded on a RFS-100 Bruker FT-spectrometer using a Nd:YAG laser with excitation wavelength of 1064 nm. Each spectrum was the average of two repeated measurements, with 150 scans at a resolution of 2 cm^{-1}.

NMR spectroscopy

^{1}H-NMR spectra were taken on a Bruker AMX600 spectrometer operating at 500.13 MHz at 65°C. Typically 64 scans were taken with an interpulse delay of 5 s (T_1 values for the resonance of the anomeric protons of κ- and ι-carrageenan are shorter than 1.5 s). Sample preparation for the ^{1}H-NMR experiments involved dissolving the carrageenan sample (5 mg mL^{-1}) at 80°C in D_2O containing 1 mM TSP (3-(trimethylsilyl) propionic-*2,2,3,3-d*$_4$ acid sodium salt) and 20 mM Na_2HPO_4, followed by sonication three times for 1 h each in a sonicator bath (Branson 2510). Chemical shifts (δ) are referred to internal TSP standard (δ = −0.017 ppm) relative to the IUPAC recommended standard DSS for ^{1}H according to van de Velde et al. (2004). Assignments of the ^{1}H-NMR spectra were based on the chemical shift data summarized by van de Velde et al. (2002, 2004) and Pereira and van de Velde (2011).

Cytological localization of polymers

Two methods were applied for the localization of the cellulose (β-glucan): a – observation of fresh sections in fluorescence microscopy (ultraviolet light, 345–365 nm, Nikon Diaphot microscope equipped with UV 2A filter), after staining with Calcofluor-white (fluorescent brightener 28 of Sigma) at 0.04% (Gretz et al. 1997); b – observation of identical sections in polarization microscope (Nikon Optiphot), with cross polarization filters (birefringence studies) looking for crystalline or para-crystalline structures (Gretz et al. 1997).

For the identification and localization of sulphated polysaccharides (carrageenans), two techniques were also used: a – staining of the sections with toluidine blue (0.05% in 0.1 M acetate buffer, pH 4.4) using light microscopy, for the detection of acid polysaccharides metachromasia (Gretz et al. 1997); b – energy dispersive X-ray analysis (EDX), to detect the variation of sulphur concentration (Russ 1974; McCandless et al. 1999), conducted on a Hitachi H900 electron microscope, associated with an X-ray spectrometer and respective detector.

Other cytochemical stains used were: e.g. Thiéry test, for the identification and localization of floridean starch in electron microscopy; Periodic acid Schiff (PAS), for the detection of polysaccharides (floridean starch); and Black Sudan B, for the detection of lipids using light microscopy (MacManus 1948; Thiéry 1967; Bronner 1975).

Morphological, anatomical and cytological aspects

Ahnfeltiopsis devoniensis and *Gymnogongrus crenulatus* (Phyllophoraceae)

G. crenulatus thallus (see Figure 4.1A–B) consists of cartilaginous flattened dark red fronds a rising from a short cylindrical stipe, attached to substrate by a small disc (holdfast) with 10 mm in diameter. Fronds repeatedly dichotomous with rounded apices sometimes bleached (Ardré 1977; Gayral and Cosson 1986; Cabioch et al. 1995).

This alga has a diphasic lifecycle without carposporophyte. The monoecious gametophytes of *G. crenulatus* form a post-fertilization structure (see Figure 4.1C–D) variously termed a tetrasporoblast (Schotter 1968; Ardré 1977) or carpotetrasporophyte (Dixon and Irvine 1977). These develop from auxiliary cells and form wart-like excrescences from the gametophyte that bear tetrasporangial nemathecia. Meiosis apparently occurs during tetrasporogenesis (McCandless and Vollmer 1984).

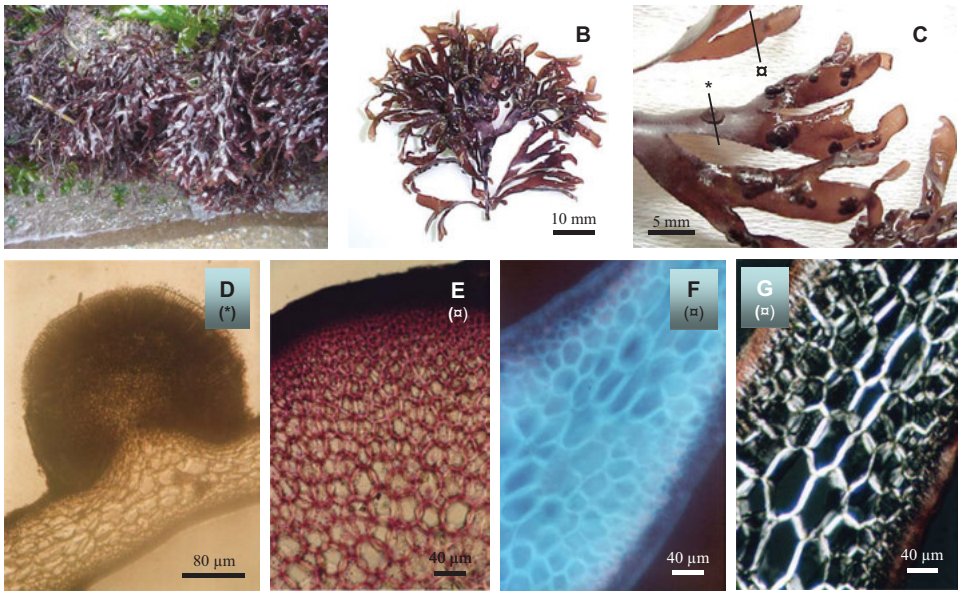

Figure 4.1 *Gymnogongrus crenulatus*: tetrasporoblastic thalli (A–D); branches with tetrasporoblasts (C); tetrasporoblast cross section according to the orientation shown in Figure C (D); thalli cross section according the orientation shown in Figure C (E–G), stained with toluidine blue (E), calcofluor-white (F) and observed with polarizing microscopy (G).

A. devoniensis is difficult to distinguish from *G. crenulatus*, however this species has generally smaller dimensions, with a maximum length of 10 cm (see Figure 4.2A–B). It forms a medium-sized flattened frond with regular dichotomous branching. The branches have parallel sides and the reproductive structures (cystocarps) are internal (Cabioch et al. 1995).

A. devoniensis (formerly *Gymnogongrus devoniensis*) was separated from the *Gymnogongrus* genus because it has a heteromorphic triphasic lifecycle, in which carpospores give rise to an encrusting tetrasporophyte (Maggs et al. 1992; Cabioch et al. 1995).

In Portugal, *A. devoniensis* grew abundantly on damp shaded rocks and in rock-pools at and below water of neap tides, on moderately wave-sheltered shores. Plants were brownish-red, and many were bleached to a pale colour. Small tufts of fronds were attached by holdfasts about 3 mm wide. According to Maggs et al. (1992), approximately 50% of the thalli were male. Males were softer than the succulent female plants, and small, pale, slightly raised spermatangial sori were visible a few millimetres from the apices.

Chondracanthus teedei and *C. teedei* var. *lusitanicus* (Gigartinaceae)

Chondracanthus teedei (Roth) Kützing (formerly *Gigartina teedei*) present flattened main axes, regularly pinnately branched (Guiry; Zinoun et al. 1993). The fronds, cartilaginous-membranous, have a purplish-violet colour that darkens by desiccation, becoming greenish-yellow through decay (see Figure 4.3A–I) (Rodrigues 1957; Gayral 1982). This

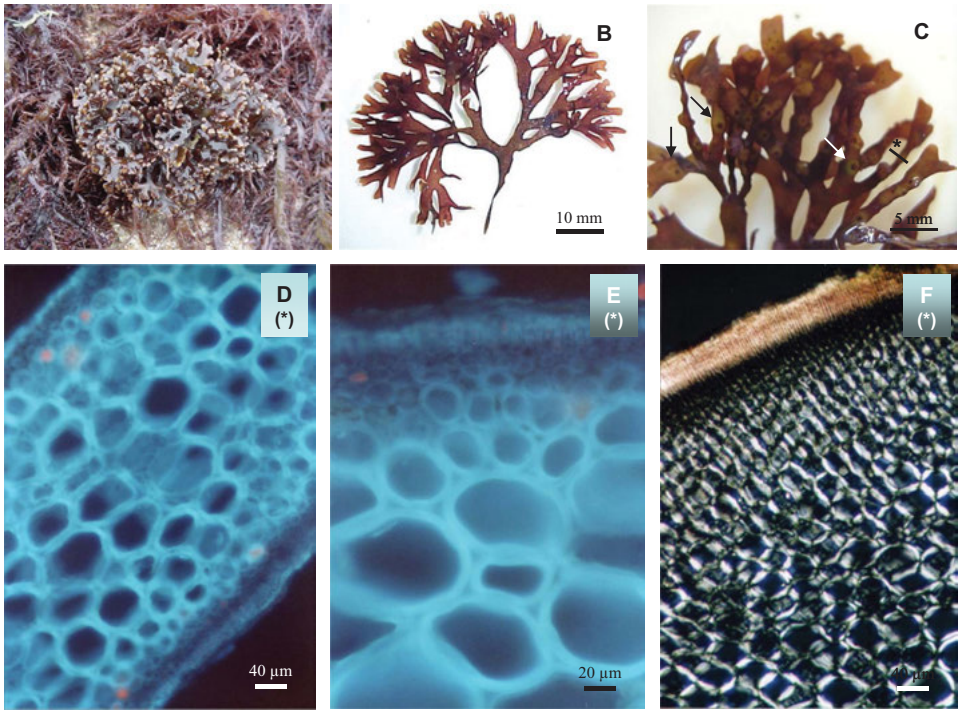

Figure 4.2 *Ahnfeltiopsis devoniensis*: fructified female gametophyte (A–C); branches with cystocarps – arrows (C); thalli cross section according to orientation shown in Figure C (D–F); light microscopical cytochemical aspects showing a cross section after calcofluor-white staining (D–E) and polarized microscopy (F).

species is abundant throughout the year in the lower horizon of the intertidal zone and is often confused with *Gelidium* sp. (Zinoun et al. 1993).

However, the specimens collected in Buarcos bay (Portugal) have very obvious differences compared to specimens taken from Brittany (France), Barcelona (Spain) and the Mediterranean Sea. Thus, the principal axes of the fronds and their ramifications are wider (reaching 1 cm in the older portions) and, therefore, the plants look more robust, often reaching 20 cm in length; it's branches are more dense and lush and the pinnules not only develop on the margins of the branches, but also on their surfaces; the cystocarps are spherical and sessile, being present in large numbers on the pinnules, margins of the branches and thalli surface (see Figure 4.3J–M and Figure 4.7A–C) (Rodrigues 1958; Pereira 2004). Due to these characteristics, the specimens collected in central and northern Portugal belong to the taxon *Chondracanthus teedei* var. *lusitanicus* (Rodrigues 1958; Bárbara and Cremades 1996). This alga has an isomorphic triphasic life cycle (Guiry 1984; Braga 1985, 1990) and appears on the rocks and intertidal pools, generally in shallow water (Gayral 1982).

Tetrasporophytes (Figure 4.4) exhibit tetrasporangial sori with the appearance of dark red spots, prominent in the thallus, on the main axis and lateral branches (Figure 4.4C–D). Sori, with cruciately divided tetrasporangia (Figure 4.4E–G), are released by rupturing

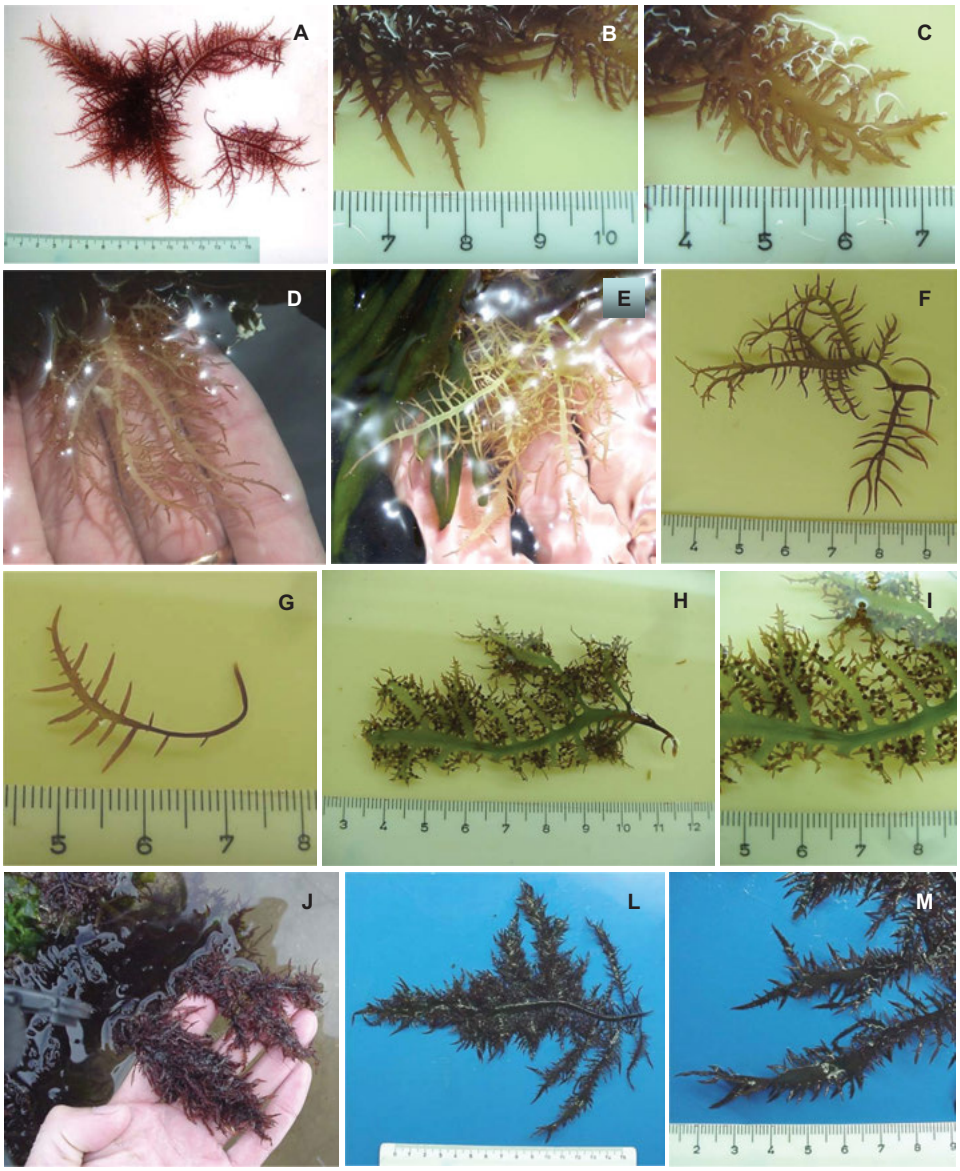

Figure 4.3 *Chondracanthus teedei*: non-fructified thalli collected in Roscoff, France (A–C); tetrasporic thallus (D, F), non-fructified (E, G) and female gametophyte (H–I), collected in Baleal, Portugal; non-fructified thallus of *C. teedei* var. *lusitanicus* collected in Buarcos (J, L), detailed view (M).

of the cortex and old sori, after tetraspore liberation, are filled with medullary filaments (Gury 1994; Pereira 2004).

Examination of thallus transverse sections shows a multiaxial structure (Figure 4.4G–L), with two types of filaments: with unlimited or limited growth. Filaments of unlimited

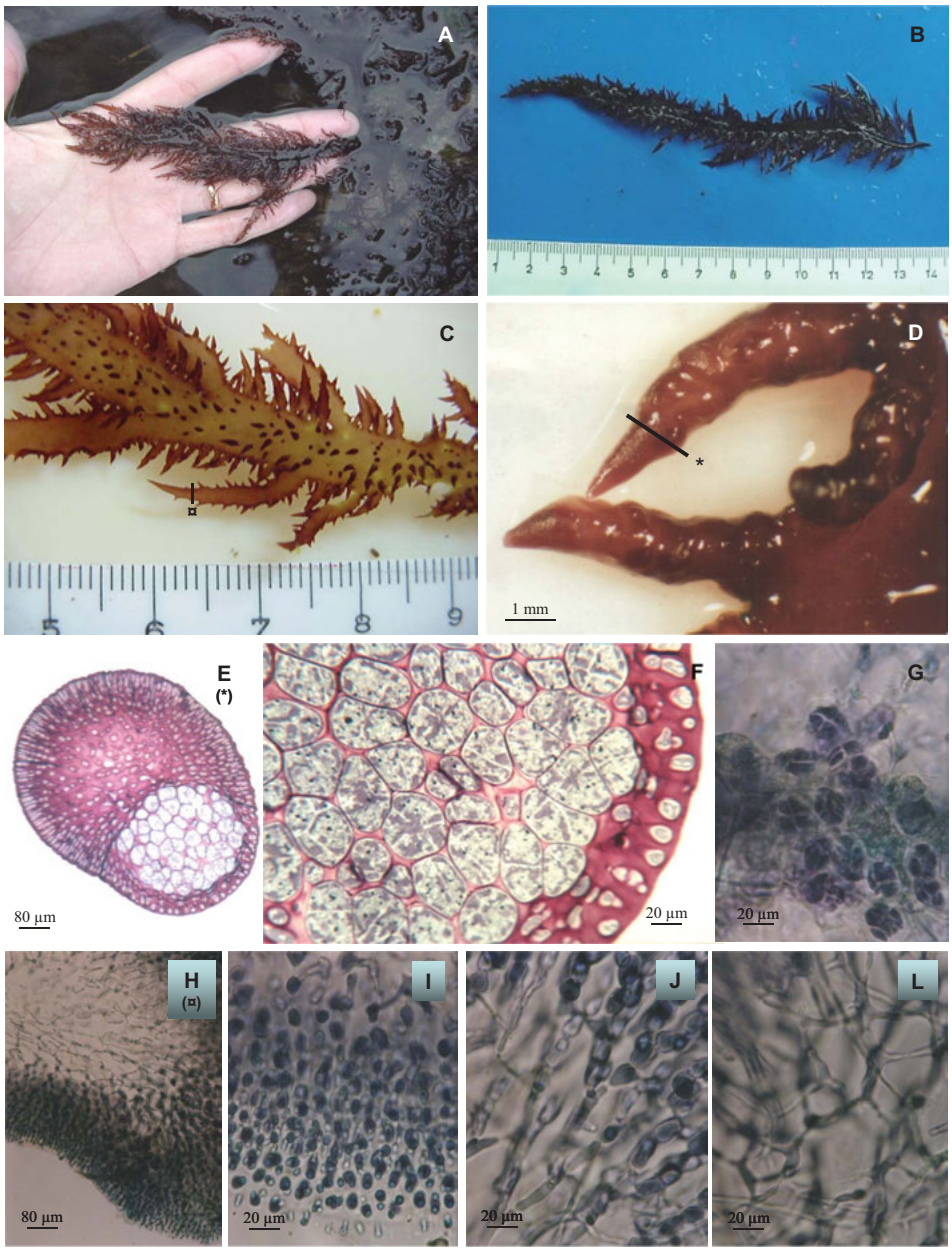

Figure 4.4 *Chondracanthus teedei* var. *lusitanicus*: fructified tetrasporophyte, with tetrasporangial sori, collected in Buarcos bay, Portugal (A–D); detail of branches with tetrasporangial sori (C); branch cross section at the level of tetrasporangial sori, according to the orientation shown in Figure D (E); detail of tetrasporangia and tetraspores (F–G); thalli cross section according to the orientation shown in Figure C (H); details of cortex (I), transition zone (J) and medulla (L). Staining with Toluidine blue (E–F) and lactophenol blue (G–L).

growth, present in the medullary zone (Figure 4.4H, L), show hyaline elongated cells, with apparent star shapes (Figure 4.4L). The limited growth filaments form the cortical zone (Figure 4.4H, I), whose hyaline irregular cells shape, located on the inside, become elliptical and rounded towards the edges. The cortical zone (at the thallus middle region) shows 8 to 9 (± 2) cell layers. It is possible to define a transition zone between the cortical and medullary zone (Figure 4.4J).

In Figure 4.4E and F (semi-thin sections at the level of tetrasporangial sorus) the areas most intensely stained by toluidine blue were located in the intercellular spaces, especially in the cortical zone.

The ultrastructure of tetrasporogenesis (Figure 4.5) in *C. teedei* var. *lusitanicus* can be summarized as follows: the nucleus with a well developed nucleolus, contains

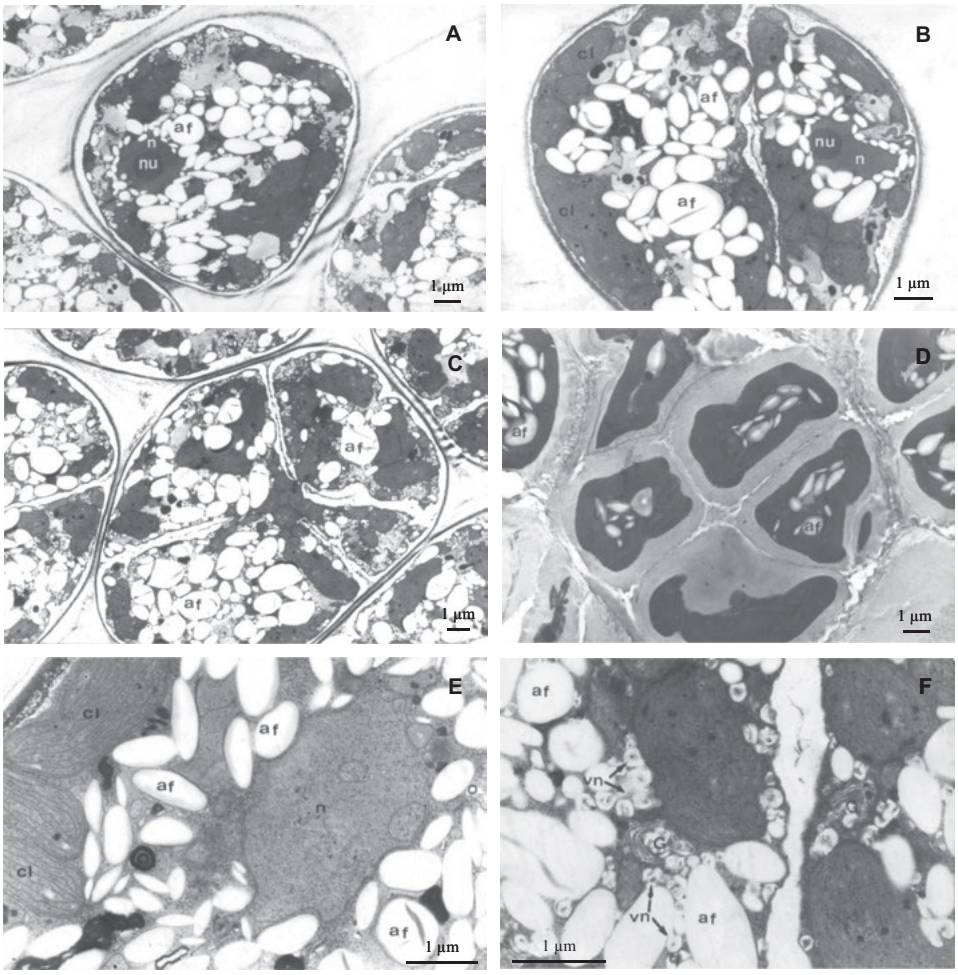

Figure 4.5 *Chondracanthus teedei* var. *lusitanicus* (ultrastructure of tetrasporogenisis): successive tetraspore stages of division (A–D); partial views of tetrasporangial maturation stages showing ultrastructural details of the nucleus and chloroplasts (E), Golgi and cored vesicles (F). **af** – floridean starch, **cl** – chloroplast, **G** – Golgi, **n** – nucleus, **nu** – nucleolus, **vn** – cored vesicles.

little condensed chromatin (Figure 4.5A–B); chloroplasts, in addition to their typical non-association with internal and peripheral thylakoids, show several areas of DNA and electron-dense inclusions (Figure 4.5B, E); in addition to floridean starch grains, which represent the most abundant cellular component, a large part of the remaining cytoplasm is occupied by very active dictyosomes producing numerous vesicles with a dense core (cored vesicles) (Figure 4.5F). This system and starch grains react positively the Thiéry test (Figure 4.6A–C). As in tetrasporogenesis in *Chondria tenuissima* (Tsekos et al. 1985), the ultrastructure of *C. teedei* var. *lusitanicus* during tetrasporogenesis shows straight, large dictyosomes which produce cored vesicles, an abundance of starch grains and form fully developed chloroplasts. The cored vesicles contain Thiéry-positive material and contribute to the formation of vacuoles (Figure 4.6C) with fibrous content (fibrillar vacuoles) which are dominant in tetraspores before liberation.

Female gametophytes (Figure 4.7) present prominent spherical cystocarps (Figure 4.7C), producing carpospores (Figure 4.7D–F). The thallus transverse section shows a multiaxial structure (Figure 4.7G–H), similar to that of tetrasporophyte one. In general, carposporangial ultrastructure (female gametophyte) is not significantly different from that observed during tetrasporogenesis (tetrasporophyte), before cell division (Figure 4.8) and similar to that described in *C. teedei* by Tsekos (1984).

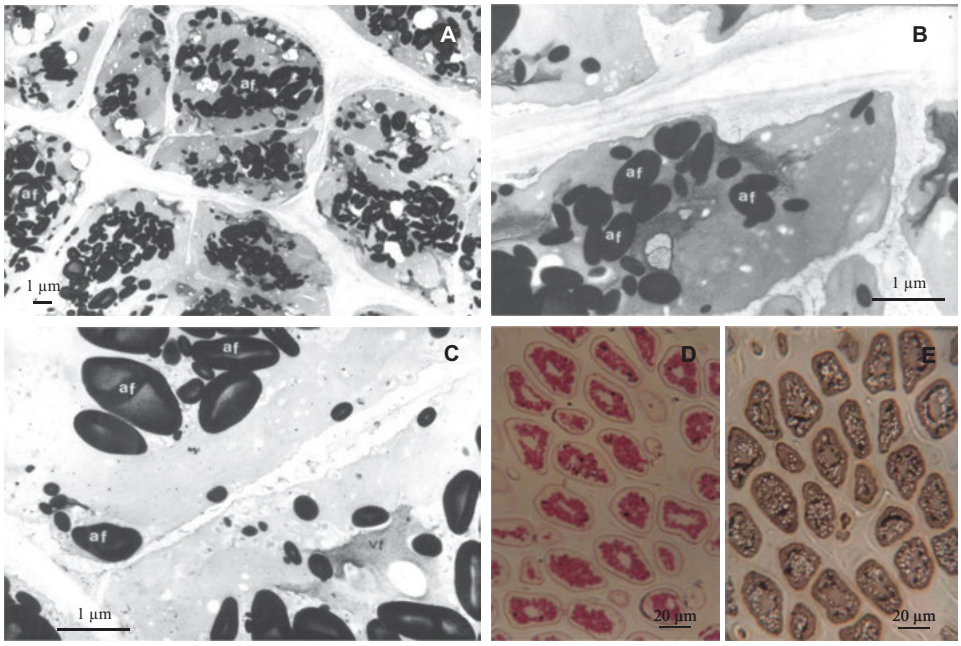

Figure 4.6 *Chondracanthus teedei* var. *lusitanicus*: light- (D–E) and electron microscopical aspects (A–C) of the tetrasporophyte; Thiéry test (A–C); PAS (D) and Black Sudan B staining (E). Note the positive reaction to Thiéry test not only with the floridean starch, but also with the fibrillar vacuoles and portions of the cell wall. **af** – floridean starch grains, **vf** – fibrillar vacuoles.

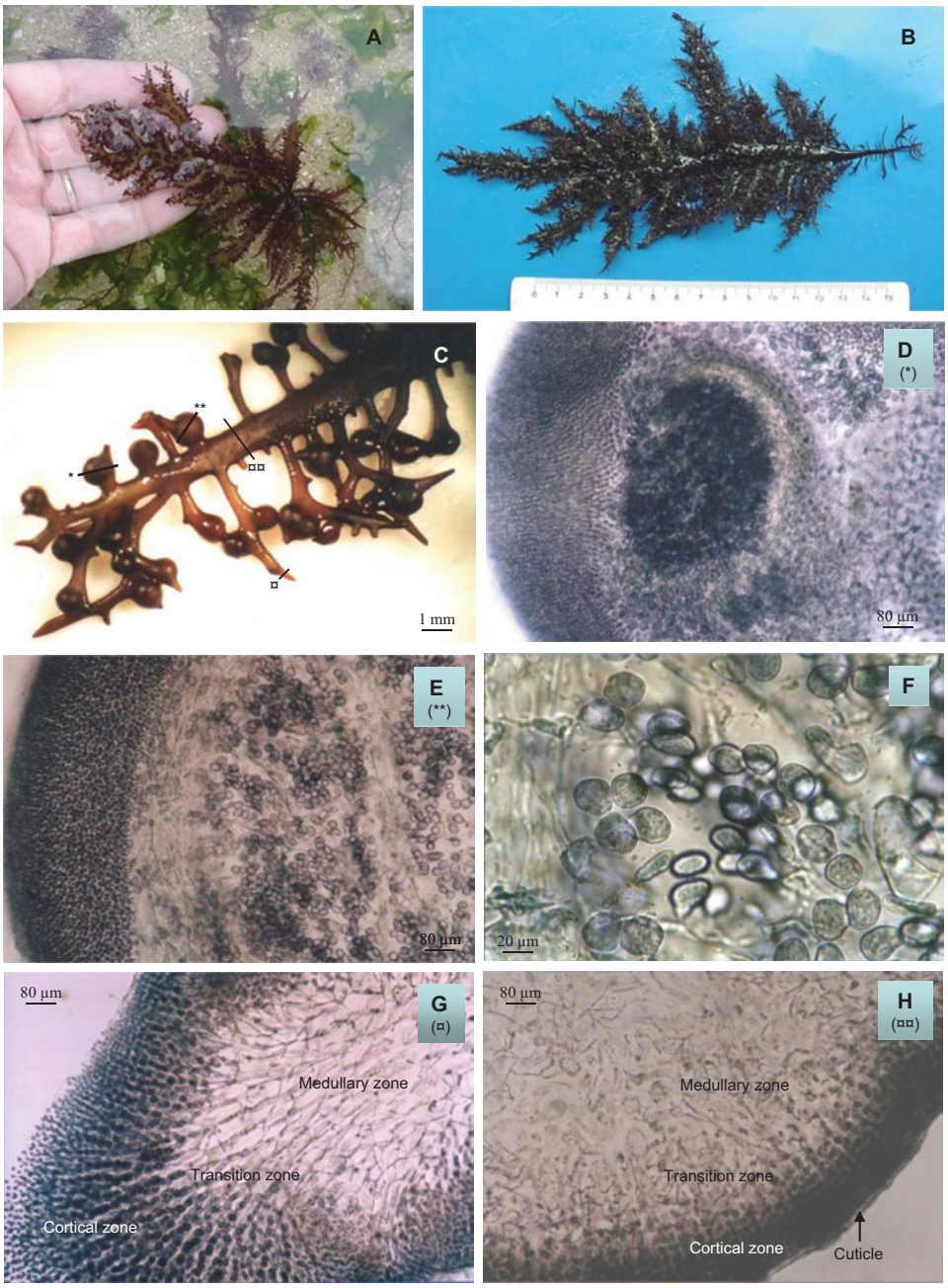

Figure 4.7 *Chondracanthus teedei* var. *lusitanicus*: fructified female gametophyte, with cystocarps, collected in Buarcos, Portugal (A–B); detail of a branch with cystocarps (C); cystocarp cross section according to the orientation shown in Figure C (D–E); carpospores within cystocarps (F); thallus cross section according to the orientation indicated in Figure C (G, H); staining with lactophenol blue (D, H).

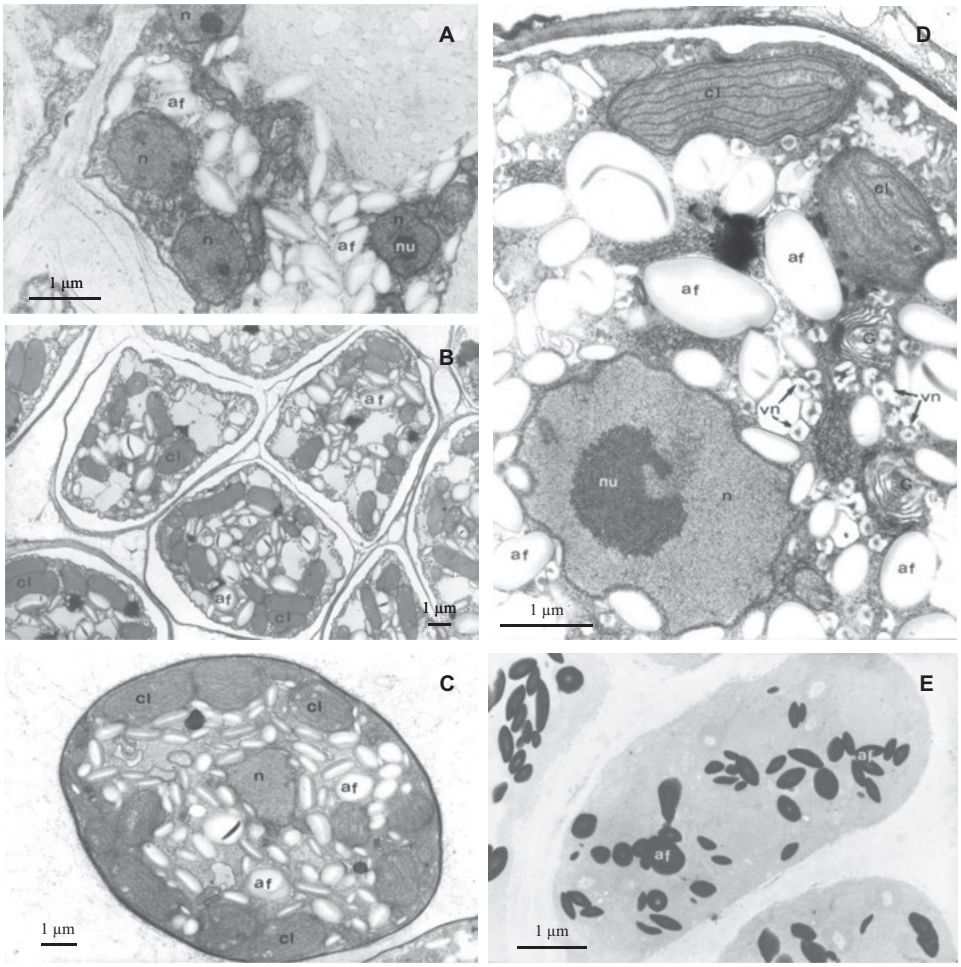

Figure 4.8 *Chondracanthus teedei* var. *lusitanicus*: ultrastructure of the female gametophyte showing partially a multinucleate cell (A) and some aspects of the differentiation of carpospores (B–C); ultrastructural details of the carposporangium, in particular with respect to the nucleus, chloroplasts, Golgi and cored vesicles (E); note the abundance of floridean starch grains giving a positive reaction with the Thiéry test (E). **af** – floridean starch grains, **cl** – chloroplast, **G** – Golgi, **n** – nucleus, **nu** – nucleolus, **vn** – cored vesicles.

Gigartina pistillata (Gigartinaceae)

G. pistillata is the type species of the genus *Gigartina* (Kim 1976; Hommersand et al. 1993). The thallus consists of a group of erect fronds, up to 20 cm tall, dark-red or reddish-brown, cartilaginous, elastic, dichotomously branched (see Figure 4.9A–D and Figure 4.10A–D), attached to the substrate with a small disk (10 mm in diameter). Branching is repeatedly dichotomous (up to 4 times), a few secondary lateral branches sometimes appear in old plants and after damage; branches usually in one plane, but with

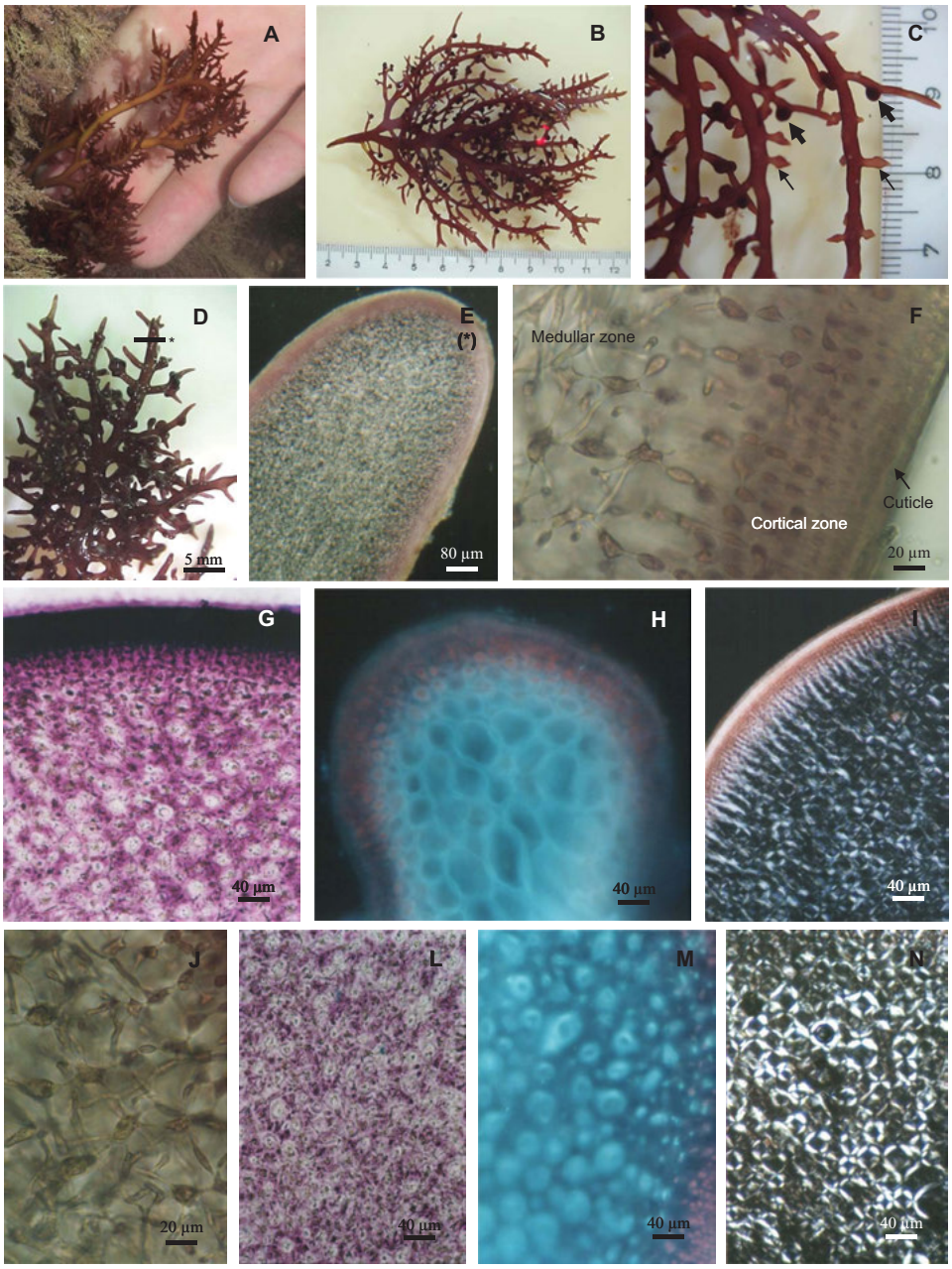

Figure 4.9 *Gigatina pistillata*: fructified female gametophyte (A, N); branches with cystocarps (C–D); thalli cross section according to the orientation shown in Figure C (E–F); Light microscopical cytochemical aspects after staining with toluidine blue (G) and calcofluor-white (H), and observation with a polarizing microscope (I); cytochemical aspects of the medullar zone (J) after staining with toluidine blue (L) and calcofluor-white (M) and observation with a polarizing microscope (N).

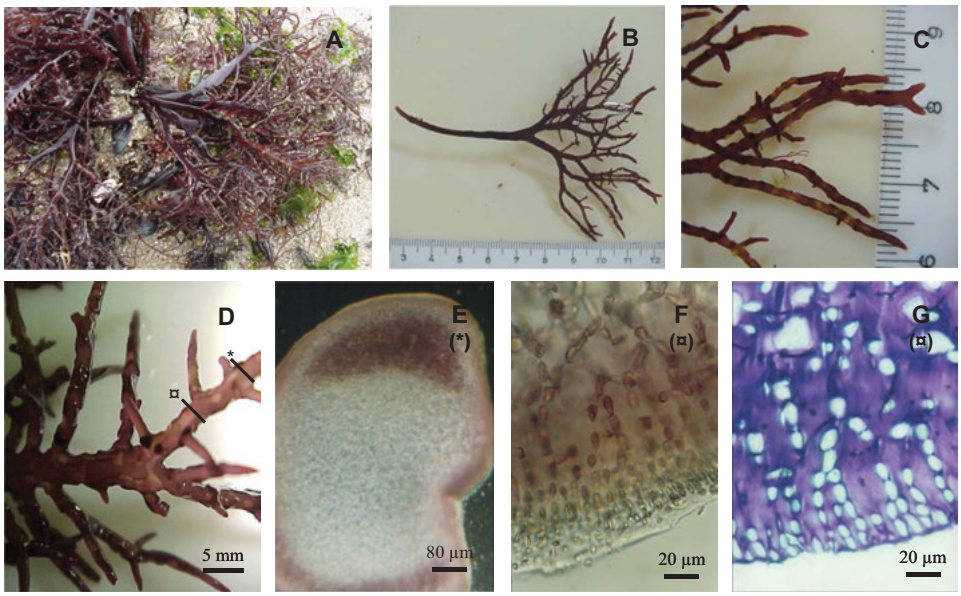

Figure 4.10 *Gigartina pistillata*: fructified tetrasporophyte (A–G); branch with tetrasporangial sori (C–D); branch cross section at the level of the tetrasporangial sori according to the orientation shown in Figure D (E); thalli cross section according to the orientation shown in Figure D (F–G). Staining with toluidine blue (G).

twisted axes. The female gametophytes exhibit a dichotomous branching more profuse than tetrasporophytes (Dixon and Irvine 1995; Pereira 2004).

G. pistillata has an isomorphic triphasic lifecycle (Hommersand et al. 1992); however, heterosporous thalli (with cystocarps and tetrasporangia in the same thallus) were identified in populations of South Africa (Isaac and Simons 1954) and Portugal (Pereira et al. 2002).

The thallus of this alga exhibits a multiaxial structure, where it is possible to recognize a central area with large stellate elements (Figure 4.9F) and a cortical zone of dense small cells, heavily pigmented, forming branched chains (Figure 4.9F and Figure 4.10F).

The female gametophyte has cystocarps which initially do not protrude from the thallus surface (Figure 4.9C – narrow arrows) becoming evident in the final stage of their development (Figure 4.9C – large arrows). These cystocarps are usually sessile (Figure 4.9B–D), but when present in a terminal position, appear to be pedunculated.

The tetrasporophytes present sori, with cruciately divided tetrasporangia, embedded in the surface of the thallus (Figure 4.10D, E) in marginal areas, mainly located in branching zones. Sporangia (tetraspores) in each tetrasporangial sorus are evidently released as a unit at maturity, leaving an exposed surface which is repaired by means of proliferation of subcortical cells (Hommersand et al. 1992; Pereira 2004).

In both phases (gametophytes and tetrasporophytes), the medullary region of the frond is composed of star-shaped cells, very similar to those of the inner cortical cells (Figure 4.9F), the demarcation between medulla and cortex is not clear.

Ultrastrucural features during carposporogenesis (Figure 4.11A), in addition to typical features of the Rhodophyta (chloroplasts with peripheral thylakoid and non-associated internal thylakoids, extraplastidial floridean starch grains, etc.), show well developed Golgi apparati which are very active with an abundance of dictyosomes and cored vesicles. As already mentioned, this would appear to play a key role in the genesis of fibrillar vacuoles and subsequent cell wall edification (Figure 4.11) (Pereira 2004).

Apart from its cruciately divided tetrasporangia (Figure 4.11B – arrows), the ultrastructural characteristics of the tetraspore mother-cells (Figure 4.11B) appear to be identical to those observed during carposporogenesis. During the final stage of tetraspore differentiation, the number and size of floridean starch grains are substantially reduced, indicating that this reserve polysaccharide is probably used in the synthesis of the structural components of the cell wall (Zinuon 1993). The walls of these cells result from the contribution of dictyosome-derived vesicles and also from mucilage sacs originating from cytoplasmic concentric membranes (Delivopoulos and Diannelidis 1990a, b) or from the endoplasmic reticulum (Delivopoulos and Tsekos 1986; Tsekos and Schnepf 1991; Tsekos 1996).

Overall, the reproductive cells (carpospores and tetraspores), while they are still contained in their reproductive structures (cystocarps and tetrasporagial sori, respectively), are embedded in a confluent mucilage (see Figures 4.12 and 4.13) and have characteristics that, in general, are consistent with previous descriptions (see Tsekos 1981, 1983; Tsekos and Schnepf 1983; Delivopoulos and Tsekos 1986; Delivopoulos and Diannelidis 1990a, b).

Spectroscopic analysis

In relation to the phycocolloid nature, spectroscopic analysis showed that the selected carrageenophytes (see Table 4.1) have a similar variation as seen in other species of the Gigartinales family (Chopin et al. 1999; Pereira 2004). The gametophyte and non-fructified stages of *C. teedei* var. *lusitanicus, G. pistillata, A. devoniensis* and *G. crenulatus* produce carrageenans of the kappa family (hybrid kappa/iota/mu/nu-carrageenan). The tetrasporophytes produce carrageenans of the lambda family (hybrid xi/theta or xi/lambda-carrageenan). The alkaline-extracted carrageenan from female gametophytes showed lower sulphate content and a decrease in galactose to the benefit of 3,6-anhydrogalactose. This corresponds to the conversion of the 4-linked galactose-6-sulfate in native samples to anhydro-galactose in the alkaline modified samples. Thus the biological precursor's mu- and nu-carrageenan were converted into kappa and iota carrageenan, respectively (Pereira and Mesquita 2004).

Cytochemical aspects

Transverse sections of *G. pistillata* (Figure 4.9G, L and Figure 4.10G) and *G. crenulatus* (Figure 4.1E) show a strong reaction with toluidine blue in the cortical zone, and weaker staining of the inner areas (medullary zone). Also noteworthy, in *G. pistillata*, the intercellular material between the tetrasporangia (Figure 4.12A–B) had a stronger reaction with toluidine blue than did the intercellular material present between carpospores (Figure 4.13A–B).

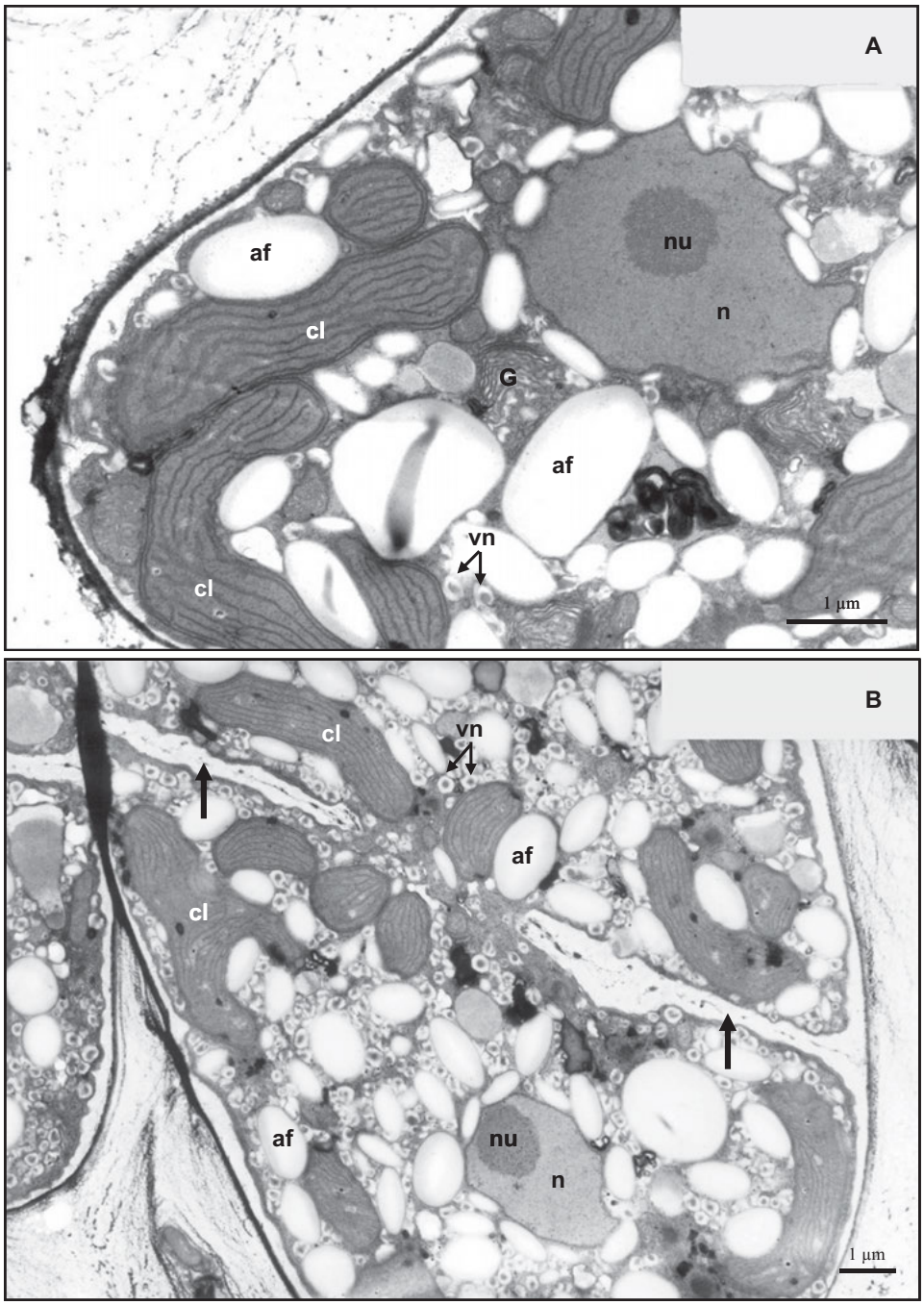

Figure 4.11 *Gigatina pistillata*: A – ultrastructure of developing carpospores (carposporogenesis); B – ultrastructure of developing tetraspores (tetrasporogenesis). **af** – floridean starch, **cl** – chloroplasts, **G** – Golgi, **n** – nucleus, **nu** – nucleulus, **vn** – cored vesicles.

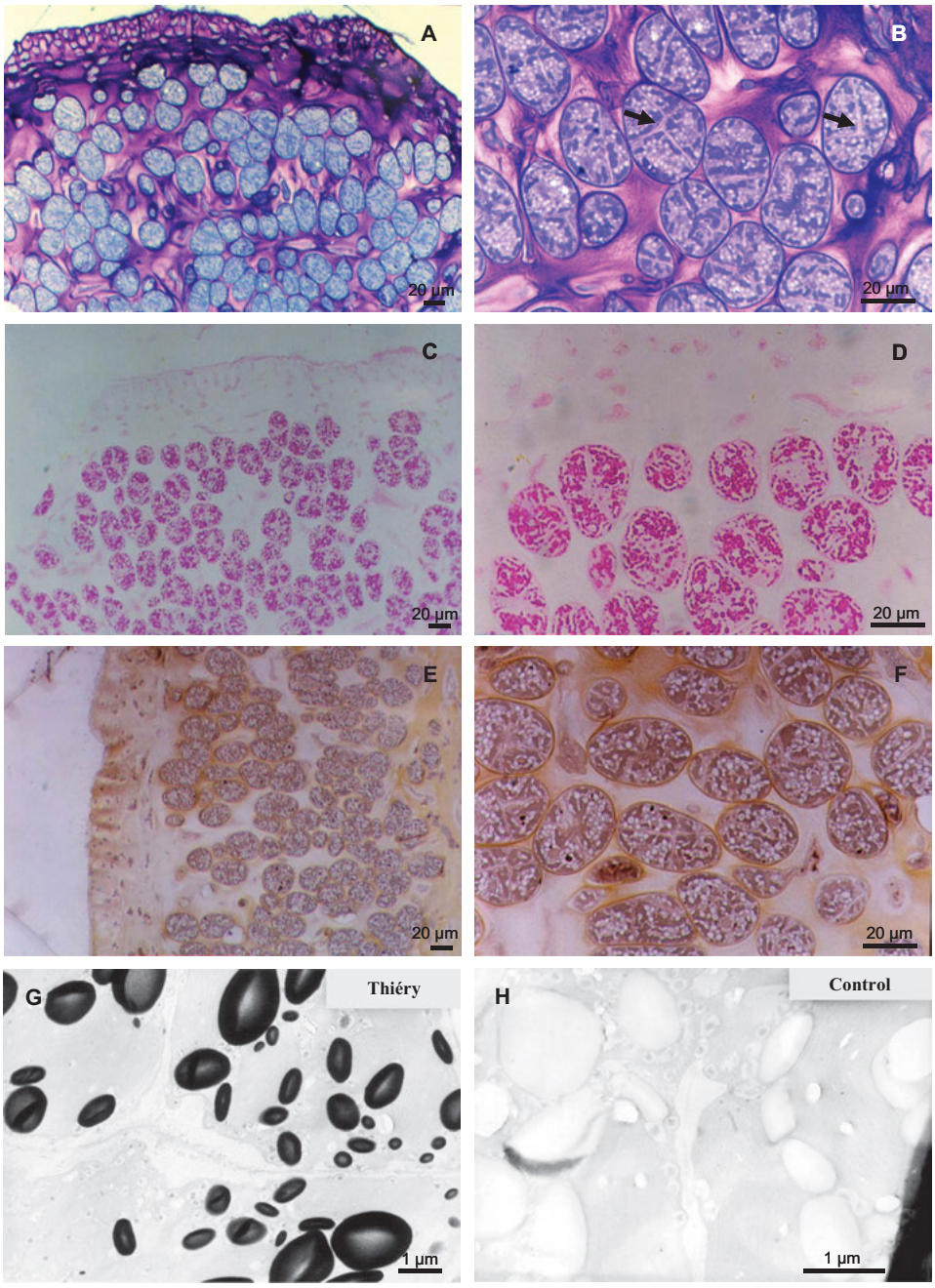

Figure 4.12 *Gigartina pistillata*: tetrasporogenesis (A–H); light- (A, F) and electron microscopical (G–H) cytochemical aspects; toluidine blue staining (A–B), PAS reaction (C–D), Black Sudan B staining (E–F) and Thiéry test (G).

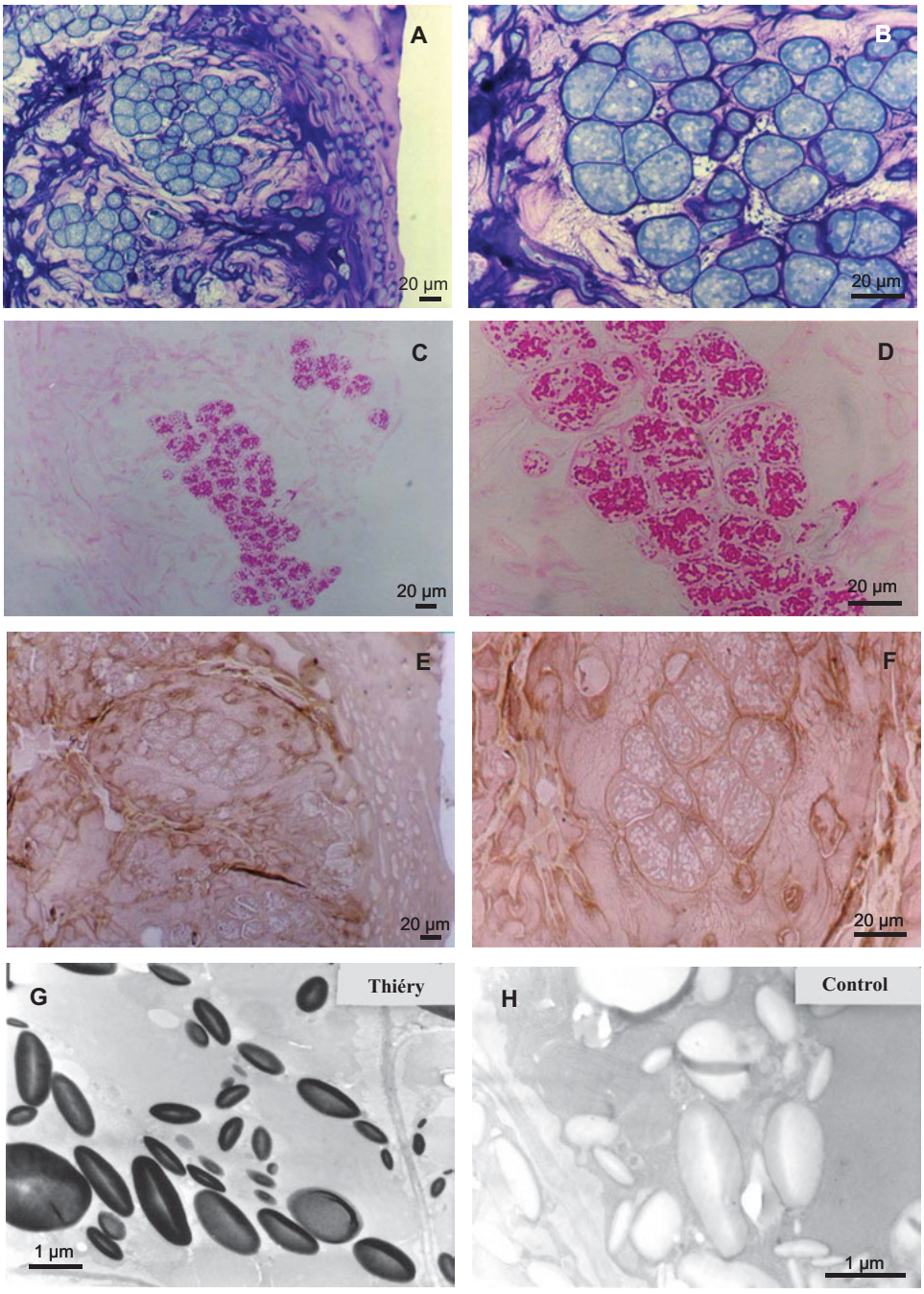

Figure 4.13 *Gigartina pistillata*: carposporogenesis (A–H); light- (A, F) and electron microscopical (G–H) cytochemical aspects; toluidine blue staining (A–B), PAS reaction (C–D), Black Sudan B staining (E–F) and Thiéry test (G).

Table 4.1 Carrageenan composition determined by vibrational spectroscopy (FTIR-ATR and FT-Raman) and ^{1}H-NMR (adapted from Pereira and Mesquita 2007; Pereira and van de Velde 2011).

Species	Lifecycle phase	Date of harvest	Carrageenan		
			Yield[1]	Alkali extracted[2] (%mol)	Native[3]
Gigartina pistillata	Female gametophyte	Mar. 2002	49.8 %	48.7 κ, 44.5 ι	κ – ι (μ/ν)
Chondracanthus teedei var. *lusitanicus*	Non-fructified	Jun. 2001	35.0 %	55.8 κ, 44.2 ι	κ – ι (μ/ν)
C. teedei var. *lusitanicus*	Female gametophyte	Jun. 2001	43.6 %	58.1 κ, 41.9 ι	κ – ι (μ/ν)
Ahnfeltiopsis devoniensis	Gametophyte	Jul. 2001	13.6 %	16.7 κ, 81.1 ι, 2.2 ν	ι – κ (ν)
A. devoniensis	Non-fructified	Aug. 2001	11.5 %	19.8 κ, 80.2 ι	ι – κ (ν)
Gymnogongrus crenulatus	Tetrasporoblastic	Apr. 2002	9.7 %	64.1 κ, 30.8 ι	κ – ι (μ)
A. devoniensis	Gametophyte	Dec. 2001	11.5 %	22.3–34.7 κ, 65.3–77.7 ι	ι – κ (ν)
G. crenulatus	Tetrasporoblastic	Nov. 2001	11.0 %	60.0 κ, 28.9 ι	κ – ι (μ)
G. pistillata	Tetrasporophyte	Apr. 2002	55.6 %	ξ, λ	ξ – λ
C. teedei var. *lusitanicus*	Tetrasporophyte	Jun. 2001	36.6 %	67.0 ξ, 37.0 θ	ξ – θ

[1]expressed in percentage of dry weight; [2]composition determined by ^{1}H-NMR; [3]composition determined by FTIR-ATR and FT-Raman analysis of ground seaweed samples; the carrageenans are identified according to the Greek lettering system (Chopin et al. 1999); the letters between parenthesis () correspond to the biological precursors of the carrageenans, present in native samples.

The cytoplasmic reserve inclusions (floridean starch grains) show a positive PAS reaction (see Figures 4.6D and 4.12, 4.13C, D); these inclusions also react positively with the Thiéry test (Figures 4.6A–B, 4.8E, 4.12G and 4.13G). It is noted that the cytoplasmic reserve inclusions (floridean starch grains), which react positively to PAS and the Thiéry test, do not stain with toluidine blue or with Sudan black B (Figures 4.12E–F and 4.13E–F).

Cellular localization and characterization of cellulose and carrageenan polymers

As red algae inhabit aquatic environments quite different to those typical of land plants, it is not perhaps unexpected to discover that the composition and organization of their

intercellular matrix are distinct from those commonly found in the latter (Gretz et al. 1997). The extracellular matrix of red algae is composed of cellulose microfibril networks associated with amorphous polymers containing sulphated galactans, mucilage and cellulose. In contrast to the relatively rigid walls in other algae, the majority of red algae are flexible with a soft consistency due to the presence of large amounts of amorphous material and less fibrillar components (cellulose) (Gretz and Vollmer 1989; Gretz et al. 1997; Graham and Wilcox 2000).

Inner zones of *G. pistillata* react with Calcofluor-white more intensely than peripheral areas (Figure 4.9H, M), due to the presence of β-glucan in the walls of the medullary cells. Note also the very strong reaction in medullary cells of *G. crenulatus* (Figure 4.1F), and in particular of *A. devoniensis* (Figure 4.2D–E). These areas come birefringent when observed with a polarizing optical microscope, confirming the presence of a structure with crystalline organization (Figures 4.1G and 4.2F).

The intercellular matrix of carrageenophytes is mainly composed of hydrophilic sulphated oligogalactans, with D-galactose and anhydro-D-galactose, in contrast to the less sulphated agars, where anhydro-L-galactose is predominant (Knutsen et al. 1994; Pereira and Mesquita 2007). The function and physiological significance of the matrix are related to the mechanical adaptation, hydration and osmotic regulation of carrageenophytes in their marine habitat (Kloareg and Quatrano 1988; Carpita and Gibeaut 1993; Whitney et al. 1999).

The distribution of sulphated polysaccharides in the thalli of *C. teedei* var. *lusitanicus* (Figure 4.4E–F), *G. pistillata* (Figure 4.9G, L), and *Gymnogongrus crenulatus* (Figure 4.1E) shows a gradient similar to that observed in several other carrageenophytes, particularly in *C. crispus* (Gordon and Mccandless 1973; Gordon-Mills and Mccandless 1975; Gordon-Mills et al. 1978) and agarophytes such as *Pterocladiella capillacea* (Rascio et al. 1991); there is a higher concentration of sulphated polysaccharides in the outer part of the thalli, especially in the cortical zone (see Figures 4.1E and 4.9G) compared to the innermost part (medullary zone).

EDX microanalysis

The data obtained by EDX analysis of tissues from *G. pistillata* (female gametophyte), in the form of % (atomic percentage) of sulphur, are interpreted as corresponding to the presence of carrageenan (McCandless et al. 1977). Within cortical cells (cytoplasm, plastids and floridean starch grains) sulphur levels are below detection, but significant quantities are present in cell walls and in the intercellular matrix. The amounts of sulphur detected shows a concentration gradient with the inner part of the cell wall containing far less compared to the intercellular matrix (see Figure 4.14): inner cell wall (3.98%); outside the cell wall (7.19%); transition zone between the wall and the matrix (10.39%); intercellular matrix (11.43%).

The results of the analysis by EDX show that the highest concentrations of sulphur and, therefore, carrageenan are located in the cell walls and intercellular matrix and a

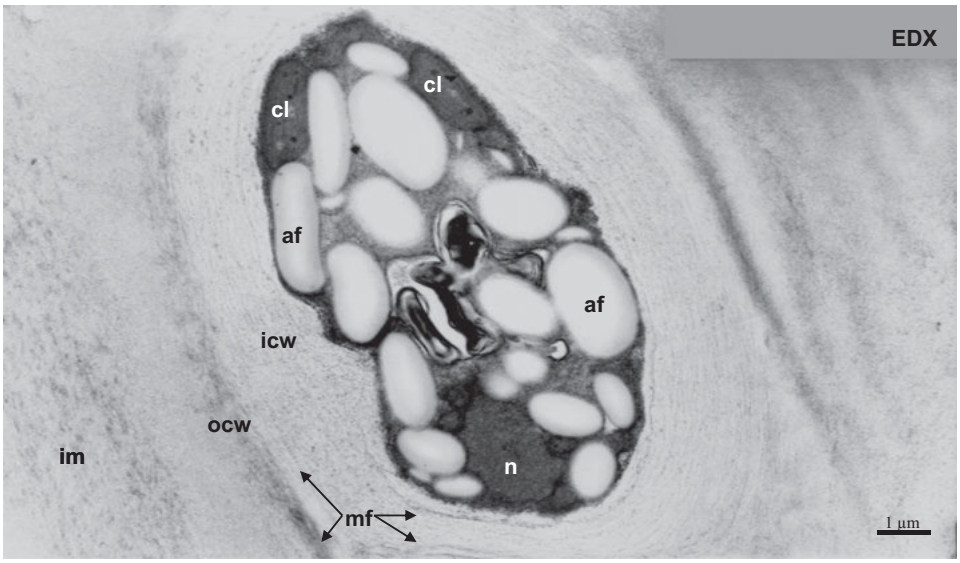

Figure 4.14 *Gigartina pistillata*: cortical cell and their cell wall ultrastructure – localization of sulphated polysaccharides (carrageenan) (EDX analysis for sulphur); the microfibrills (**mf**) are arranged concentrically in a less dense way on the inside zone (**icw**) and denser in the outer zone (**ocw**); the intercellular matrix (**im**) has a granular appearance. **af** – floridean starch grains, **cl** – chloroplasts, **n** – nucleus.

gradient of increasing carrageenan innermost region of the cell wall towards the intercellular matrix, as observed in *C. crispus* (McCandless et al. 1977).

Conclusions

The combination of FTIR and Raman spectroscopy allows identification of the natural composition of phycocolloids present in seaweeds (Pereira 2004; Pereira 2006). Since the vibrational spectrometers (FTIR and FT-Raman) are now standard equipment in many laboratories, the techniques used (Pereira et al. 2003; Pereira and Mesquita 2004; Pereira et al. 2009) are useful for studying carrageenophyte populations, substituting traditional tests of iridescence and resorcinol (Braun et al. 2004). These techniques are also useful for the development and implementation of strategies for sustainable seaweed harvest, the evaluation of the natural seaweed composition and for control of the quality of the different batches of algal material harvested and/or cultivated (Pereira et al. 2003; Pereira et al. 2009).

However, ^{1}H-NMR spectroscopy is necessary for the quantitative analysis of the different repeating units of the hybrid carrageenans, extracted from the studied algae (Pereira 2004). ^{1}H-NMR allows for the identification and quantification of the different carrageenan types based on the intensity and the chemical shift of the resonances of the anomeric protons (van de Velde et al. 2002; Pereira and van de Velde 2011).

Cytological localization of intercellular matrix polymers *in situ* provides valuable information complementary to chemical and physical characterization of extracted

components. Thus, based on this cytochemical study, the following conclusions might be drawn: in the carrageenophytes studied here (*C. teedei* var. *lusitanicus, G. pistillata, G. crenulatus* and *A. devoniensis*), the two main components of the cell walls and intercellular matrix are cellulose and carrageenans (polymers of sulphated galactans). At the thallus level, concentrations of theese sulphated polysaccharides decrease from the cortex to the medulla, while the cellulose gradient is inversed. At the cellular level, cellulose is the main fibrillar component of the cell walls. This has also been observed in carrageenophytes with a high commercial value (*Chondrus crispus* and *Kappaphycus alvarezzi*). Moreover, the cristallinity of cellulose changes with the cell size (Gordon and McCandless 1973; Gretz et al. 1997).

The EDX analysis for sulphur showed (like in *C. crispus*) an increasing gradient of carrageenans from the innermost region of the cell wall to the intercellular matrix (Gretz et al. 1997). This is in agreement with the direct analyses of carrageenan concentrations.

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