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Author(s)	MURAOKA, Daisuke; YAMAMOTO, Hirotoshi; YASUI, Hajime; TERADA, Ryuta
Citation	北海道大學水産學部研究彙報, 49(1), 31-39
Issue Date	1998-03
Doc URL	http://hdl.handle.net/2115/24166
Type	bulletin (article)
File Information	49(1)_P31-39.pdf

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Bull. Fac. Fish. Hokkaido Univ.
49(1), 31-39. 1998.

Formation of Wound Tissue of *Gracilaria chorda* Holmes (Gracilariaceae) in Culture

Daisuke MURAOKA[1], Hirotoshi YAMAMOTO[1], Hajime YASUI[1] and Ryuta TERADA[1]

Abstract

The wound healing of *Gracilaria chorda* is described. At the surface of cut tissues, wounded cells lost their cytoplasm, leaving only the cell walls still attached. However, inside cells (i.e. those next to wounded cells) remained intact, and the nuclei and cytoplasm of some of these cells moved toward the wound within 24 h after the wounding. In these cells, Golgi bodies increased in number and then produced many secretory vesicles. Simultaneously, many multivesicular bodies appeared in these cells and released their contents into the cell wall. Two days after wounding, the first cytokineses occurred in the inside cells, forming new cells along the cut surface. The cell walls between the wounded and new inside cells became thicker and separated the pit plugs connected to wounded cells from the inside cells. The new cells divided further to form wound tissue with up to 3 layers, which eventually developed into cortex-like tissue.

Key Words: Regeneration, Wound healing, Wound tissue, *Gracilaria chorda*, Ultrastructure, Cytokinesis, Cleavage furrow, Pit plug, Cell wall, Multivesicular body

Introduction

Seaweeds are often cut by mechanical stress or grazing, but many species are able to develop wound tissue at cut surfaces (Azanza-Corrales and Dawes, 1989; Hales and Fletcher, 1992) and to regenerate new shoots (Tseng, 1947; Knight and Parke, 1950; Keser et al., 1981; Ang, 1985; Hales and Fletcher, 1992). Since the agar products of *Gracilaria* spp. are in great demand, this genus is cultured all over the world. There are various methods for cultivation, including transplantation, attachment of spores or thalli to ropes, and regeneration of fragments on the sea bottom. The regeneration of plants has been an especially efficient method in various regions, including Burma, India, the Caribbean, Brazil, the USA and Namibia (Critchley, 1993). Bird et al. (1977) cultured *Gracilaria* sp. *in vitro* and observed the formation of wound tissues and the consequent new shoots at cut surfaces within ten days of wounding. Migita et al. (1993) reported luxuriant growth during regeneration of wild *Gracilaria chorda* in the Ariake Sea (Japan). Thus, the regenerative ability of *Gracilaria* plants appears to be very important for propagation and cultivation, but histological and ultrastructural studies have not been conducted on the developmental processes of the wound tissue. This study was conducted using light and electron microscopes to examine the ultrastructures of

[1] *Laboratory of Aquatic Breeding Science, Faculty of Fisheries, Hokkaido University* (北海道大学水産学部育種培養学講座)

cells involved in the formation of wound tissue in *G. chorda* cultured *in vitro*.

Material and Methods

Mature cystocarpic plants of *G. chorda* were collected at Kiire, Kagoshima Pref., Kyushu in May, 1992. Several 1-cm fragments with several cystocarps were cut off and cleaned with sterile sand and seawater (see Yamamoto and Sasaki, 1987). Carpospores released in sterile seawater were transferred into 50-ml glass bottles containing PES medium without vitamins to establish an unialgal culture and were cultured in a growth chamber under conditions of 18°C and 60 μE m^{-2} sec^{-1} in a 14 : 10 L/D photoperiod. Carpospore-derived plants (tetrasporophytes) that grew to 1 cm in length were detached from the bottom of the culture bottles and transferred to 500-ml flasks under the same culture conditions with aeration.

Several 5-mm fragments were cut off from the middle of the lateral branches of the tetrasporophytes raised to 10 cm in length and maintained until they regenerated in glass bottles under the above-mentioned conditions without aeration. Cut surfaces were examined 0, 1, 2, 3, 5, 7, 10 and 24 days after cutting.

All fragments cultured in each period were fixed for 2 h in a mixture of 3% glutaraldehyde, 2% NaCl, 0.1% $CaCl_2$ and 1% caffeine in 0.1 M cacodylate buffer.

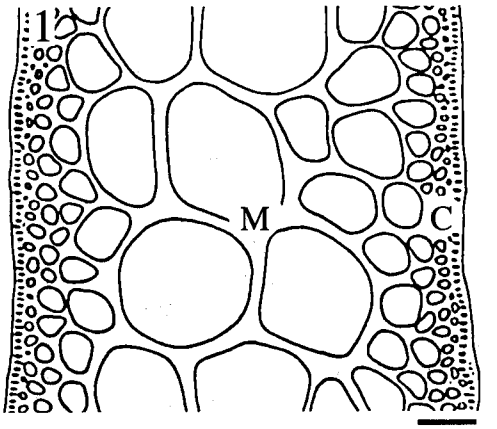


Fig. 1 Diagram of longitudinal section of *Gracilaria chorda*, showing cortex (C) and medulla (M). Bar : 100 μm.

Fig. 2 A longitudinal section 1 day after wounding. Cytoplasm is lost from wounded cell (WC), but cytoplasm in inside cells (IC) remained intact. Bar : 10 μm.

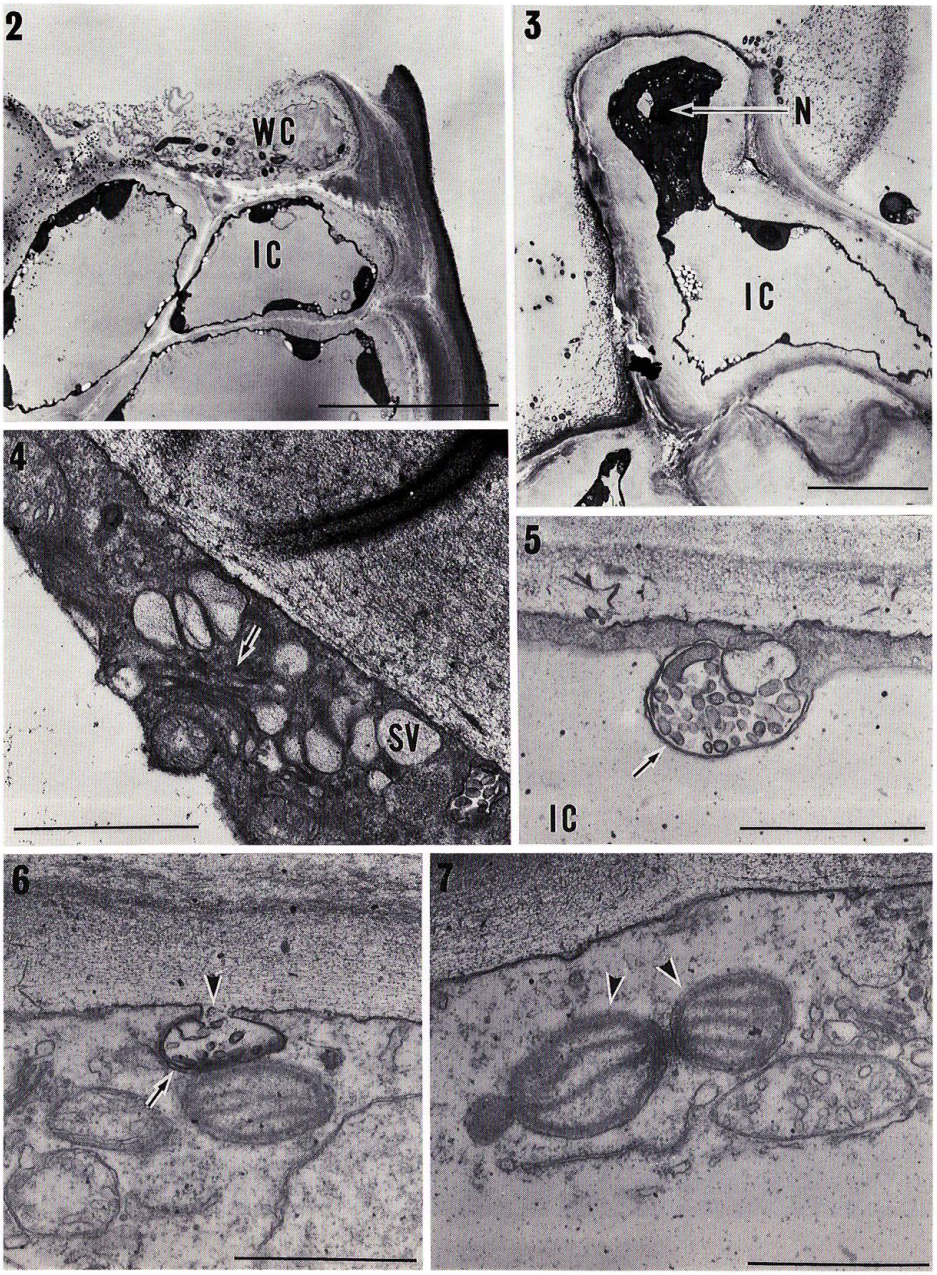
Fig. 3 A longitudinal section of an inside cell (IC) 2 days after wounding. Nucleus (N) and cytoplasm moved toward the wound. Bar : 10 μm.

Fig. 4 Section of an inside cell 2 days after wounding. Golgi body (arrow), which is located near the wound, is producing many secretory vesicles (SV). Bar : 1 μm.

Fig. 5 Section of an inside cell (IC) 2 days after wounding. Multivesicular body (arrow) is located near the wound. Bar : 1 μm.

Fig. 6 Section of an inside cell 2 days after wounding. Multivesicular body (arrow) is releasing its contents into the cell wall (arrowhead). Bar : 1 μm.

Fig. 7 Section of an inside cell 2 days after wounding. Chloroplast has divided into two (arrowheads). Bar : 1 μm.


2
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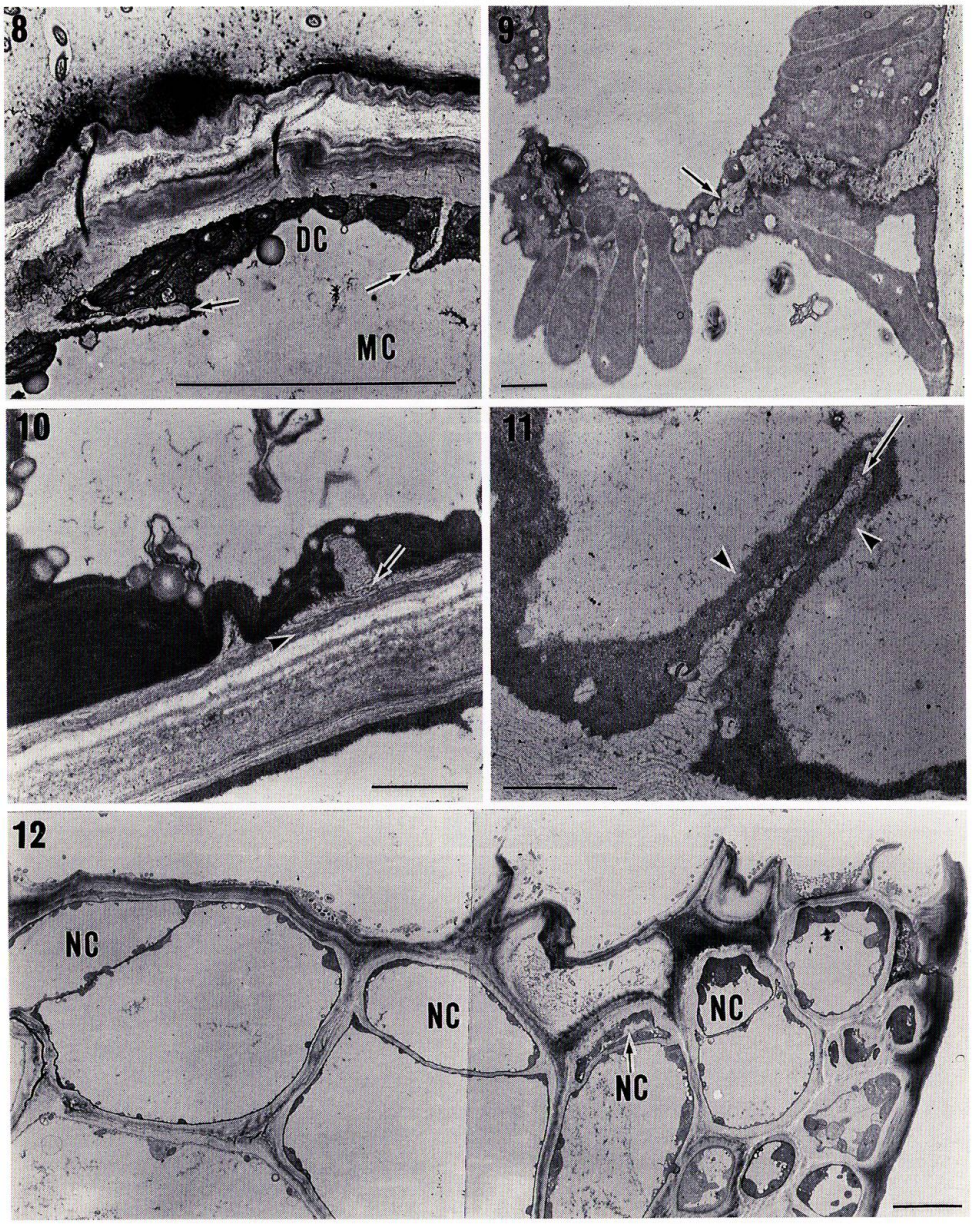
8
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MC
9
10
11
12
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NC
NC
NC

The fragments were rinsed in the same buffer, post-fixed for 3h at 4°C in a 2% osmium tetroxide buffer solution containing 0.05% potassium ferrocyanide, dehydrated through a gradual acetone series and embedded in Spurr's resin (Spurr, 1969). Longitudinal sections were prepared for observation. Thick sections (1 μm) were stained with 1% toluidine blue for light microscopy. Ultrathin sections were placed on formvar-coated one-hole copper slot grids and stained with uranyl acetate and lead citrate for electron microscopy.

Fragments cultured for 0, 1, 2 and 3 days were fixed by ethyl alcohol (3) : acetic acid (1) solution, stained with aceto-iron-haematoxylin-chloral hydrate solution (Wittmann, 1965) and squashed for Nomarski differential interference microscopic observation.

Results

The vegetative structure of *G. chorda* consists of 2 regions: the cortex and the medulla (Fig. 1). The cortex, which is covered with mucilaginous substance, consists of spherical cells (5-10 μm in diam.). These cells are filled with cytoplasm and connect only with their mother cells by primary pit connections (Yamamoto 1973). The medulla is composed of spherical to isodiametric cells up to 1000 μm in diameter. These cells have vacuoles and many secondary pit connections with neighboring cells in addition to the primary pit connections. Cell divisions occur frequently in cortical cells, but rarely in medullary cells.

At cut surfaces, wounded cells (outermost cells) lost their cytoplasm to leave only their cell walls, but "inside cells" (cells directly next to wounded cells) remained intact (Fig. 2).

The nuclei and cytoplasm of some medullary inside cells moved toward wounded cells within 24 h after wounding (Fig. 3).

One or two days after wounding, Golgi bodies of the inside cells increased in number and produced many secretory vesicles (Fig. 4). Simultaneously, many multivesicular bodies with double membranes appeared (Fig. 5) and released their contents into the cell wall (Fig. 6). Many chloroplasts, of which some had divided into two, also appeared (Fig. 7).

Two days after wounding, the first cytokineses occurred in inside cells in the cortex and outer portion of the medulla (Fig. 8). The cleavage furrow first formed centripetally at both sides of the cell, causing the existing cell wall to appear to extend inwards. But the structure of the cleavage furrow was different from that of the existing cell wall (Figs 9, 10). The cleavage furrow forming new cells was

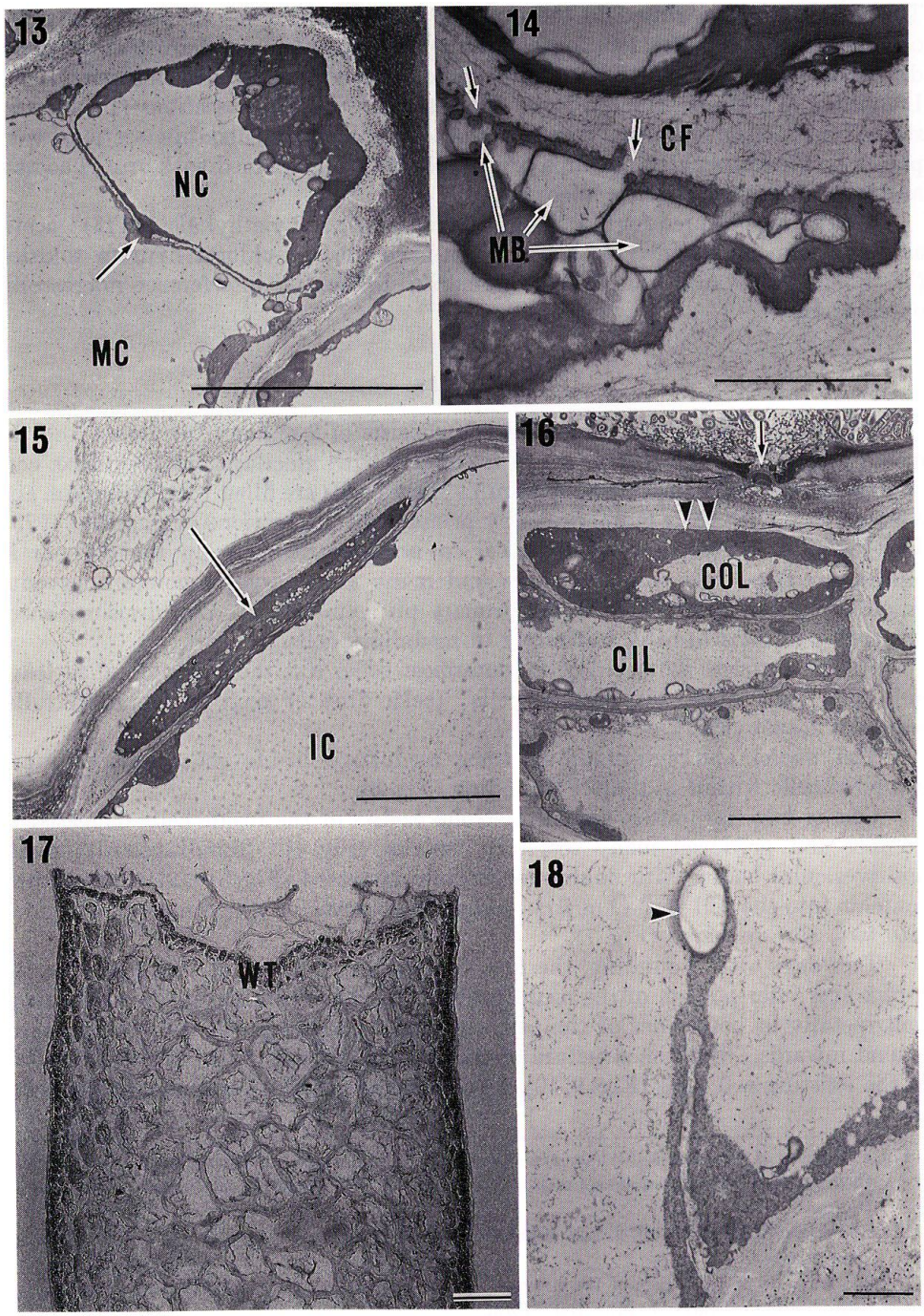
Fig. 8 Section of an inside cell 2 days after wounding. Cytokineses is occurring at the wound side. Cleavage furrows (arrows) are separating a daughter cell (DC) from the mother cell (MC). Bar: 10 μm.

Fig. 9 Section of an inside cell 2 days after wounding, showing developing cleavage furrow (arrow). Bar: 1 μm.

Fig. 10 Close-up of basal part of the cleavage furrow. Structure (arrow) is different from that of original cell wall (arrowhead). Bar: 1 μm.

Fig. 11 Cleavage furrow (arrow) surrounded by cytoplasm (arrowheads). Bar: 1 μm.

Fig. 12 Section of inside cells 2 days after wounding. New cells (NC) are formed along the cut surface. Bar: 10 μm.


13
NC
MC
14
CF
MB
15
IC
16
COL
CIL
17
WT
18

always surrounded by cytoplasm (Fig. 11). Most cleavage furrows were almost parallel to the cut surface. As a result, new cells formed along the cut surface (Fig. 12). It was confirmed by the squash method that each cell had a single nucleus. Each cell was connected by a primary pit connection with its mother cell (Fig. 13). Multivesicular bodies with double membrane structures released their contents into cleavage furrows (Fig. 14).

Three to five days after wounding, cytokineses also occurred in the medullary cells in the central part of the tissue. The new cells were flat, filled with cytoplasm and much smaller than the mother cell (Fig. 15). Five to seven days after wounding, these new cells divided further to form wound tissue with up to 3 layers (Fig. 16). The cells of the inner layers were larger than those of the outermost layer and developed vacuoles. The cells of outermost layer had small chloroplasts containing a few thylakoids. As the cell wall of the new cells became gradually thicker, the pit plugs connecting wounded cells to the new cells separated from the new cells and degenerated. The cells of the outer layer of new cells divided repeatedly to complete the cortex-like wound tissue (Fig. 17). The cleavage furrow sometimes contained floridean starch around its tip (Fig. 18).

Discussion

Hales and Fletcher (1992) reported that cytoplasm was lost from wounded cells in cultured receptacles of *Sargassum muticum*. Wounded cells of *G. chorda* also showed a similar condition. Wound response as a sealing mechanism has been reported in several algae. In *S. muticum*, the plasmodesmata between wounded cells and inside cells, which normally connect medullary cells by their thin sieve plates, collapsed rapidly after wounding (Hales and Fletcher, 1992). In the wound healing of *Eucheuma alvarezii* var. *tambalang* (= *Kappaphycus alvarezii* var. *tambalang*), proteinaceous and phenolic substances concentrate near the pits of cortical and medullary cells that are within 2 cells layers of the wounded cells (Azanza-Corrales and Dawes, 1989). Accordingly *G. chorda* could also have a similar mechanism.

In *S. muticum*, the number of organelles in inside cells increased after wounding (Hales and Fletcher, 1992; Givernaud et al., 1990). In this study, inside-cell organelles, especially Golgi bodies, also increased in number and produced many secretory vesicles, suggesting that metabolic activity increased as a primary reaction

Fig. 13 Close-up of a new cell (NC) of Fig. 12. New cell connects with the mother cell (MC) by primary pit connection (arrow). Bar: 10 μm.

Fig. 14 Multivesicular bodies (MB) discharging their contents (arrows) into the cleavage furrow (CF). Bar: 1 μm.

Fig. 15 Section of an inside cell (IC) 3 days after wounding. A new cell (arrow) produced by medullary cell in the central part of tissue at the cut surface. Bar: 10 μm.

Fig. 16 Section of an inside cell 7 days after wounding. Cell of outer layer (COL) has some small chloroplasts (arrowheads), which have a few thylakoids. Cell of inner layer (CIL) has a developed vacuole. The cell wall of cut surface is thick, and the pit plug (arrow) between the wounded cell and the inside cell was separated from the inside cell by cell wall material. Bar: 10 μm.

Fig. 17 Light micrograph of the longitudinal section of tissue 24 days after wounding. Cortex-like wound tissue (WT) is formed. Bar: 100 μm.

Fig. 18 Developing cleavage furrow has floridean starch at the tip (arrowhead). Bar: 1 μm.

to the wounding of cells.

Two days after wounding in *G. chorda*, the first cytokineses occurred in inside cells. This period was shorter than the three days reported for stipes of *Sargassum filipendula* (Fagerberg and Dawes, 1976), the four days for receptacles of *S. muticum* (Hales and Fletcher, 1992) and the two weeks for thalli of *Fucus* (Fulcher and McCully, 1969). Since almost all *G. chorda* cells are polynucleic, the first cytokineses might occur earlier than in Fucales, which is a mononucleic order.

Cytokinesis began by an inward extension of cleavage furrows from both sides of *G. chorda* cell. On the other hand, in *E. alvarezii* var. *tambalang*, "cellular extensions" (development of new cells) occurred from the pits of cortical and medullary cells of the sub-wounded layer (Azanza-Corrales and Dawes, 1989). The process of producing new cells was completely different. Klepacki et al. (1995) observed in *Agardhiella subulata* that apical cells were divided by the cleavage furrow into two nearly equal parts. In *G. chorda*, the formation of new cells was similar to general cell division, but the new cells were much smaller than the mother cells, especially in the medullary cells. A new cell included only one of several nuclei in the mother cell. The cytokineses resembles the division of a conjunctor cell rather than apical cell division. In this study, medullary inside cells produced new cells capable of dividing, although it has been recognized that the medullary cells of *Gracilaria* plants have no meristematic ability.

Delivopoulos and Tsekos (1986) showed that multivesicular bodies in *G. verrucosa* discharged their contents outside of the carpospores. In *G. chorda*, many multivesicular bodies discharge their contents into the cell walls and cleavage furrows of inside cells. This suggests that multivesicular bodies promote the advance of the cleavage furrow and thicken new cell walls.

Wound tissue composed of new cells was similar in structure to the normal cortex. The wound tissue of *E. alvarezii* var. *tambalang* consists of 5 layers and has features common to the normal cortex (Azanza-Corrales and Dawes, 1989).

Cell walls of the inside cells became thick and cause the pit plugs attached to wounded cells to separate from inside cells, indicating that inside cells seal wounds by forming new cell walls next to wounds. Hales and Fletcher (1992) reported that new cell walls developed around inside cells within 1 day after wounding in *S. muticum*. They noted that the rapid deposition of new cell walls at wound surfaces was essential because the old exposed existing cell walls degenerated within 3 days. Such rapid deposition of new cell walls did not occur in *G. chorda*. This might be because the cell walls of wounded *G. chorda* cells remain attached longer than in other species.

The cell walls of wounded cells remained at cut surfaces until the wound tissue completed forming 24 days after wounding. Azanza-Corrales and Dawes (1989) showed that these cell walls were sloughed off within 12 days of wounding in *E. alvarezii* var. *tambalang*. In *S. muticum*, the old exposed cell walls degenerated within 3 days (Hales and Fletcher, 1992). The attached cell walls might function to protect the inside cells. However, in this study, culture conditions which could remove old tissues, especially aeration, were not used. Accordingly, wound healing should also be studied in the field to determine the relation between the speed of healing and the retention of wounded cell walls.

Acknowledgments

We gratefully acknowledge Dr. T. Motomura, Institute of Algological Research, Hokkaido University, for his technical support.

References

Ang, P.O.Jr. (1985). Regeneration studies of *Sargassum siliquosum* J. Ag. and *S. paniculatum* J. Ag. (Sargassaceae, Phaeophyta). *Bot. Mar.*, **28**, 231-235.

Azanza-Corrales, R. and Dawes, C.J. (1989). Wound healing in cultured *Eucheuma alvarezii* var. *tambalang* Doty. *Bot. Mar.*, **32**, 229-234.

Bird, N., McLachlan, J. and Grund, D. (1977). Studies on *Gracilaria*. 5. *In vitro* life history of *Gracilaria* sp. from the Maritime Provinces. *Can. J. Bot.*, **55**, 1282-1290.

Critchley, A.T. (1993). *Gracilaria* (Gracilariales, Rhodophyta): An economically important agarophyte. pp. 89-112, Ohno, M. and Critchley, A.T. (eds.). *Seaweed cultivation and marine ranching*. JICA, Yokosuka.

Delivopoulos, S.G. and Tsekos, I. (1986). Ultrastructure of carposporogenesis in the red alga *Gracilaria verrucosa* (Gigartinales, Gracilariaceae). *Bot. Mar.*, **29**, 27-35.

Fagerberg, W.R. and Dawes, C.J. (1976). Studies on *Sargassum*. I. A light microscopic examination of the wound regeneration process in mature stipes of *S. filipendula*. *Amer. J. Bot.*, **63**, 110-119.

Fulcher, R.G. and McCully, M. (1969). Histological studies on the genus *Fucus*. IV. Regeneration and adventive embryogeny. *Can. J. Bot.*, **47**, 1643-1649.

Givernaud, T., Cosson, J. and Givernaud-Mouradi, A. (1990). Régénération de la Phéophycée *Sargassum muticum* (Phéophycée, Fucale). *Cryptogamie, Algol.*, **11**, 293-304.

Hales, J.M. and Fletcher, R.L. (1992). Receptacle regeneration in *Sargassum muticum* (Phaeophyta). *Phycologia*, **31**, 591-601.

Keser, N., Vadas, R.L. and Larson, B.R. (1981). Regrowth of *Ascophyllum nodosum* and *Fucus vesiculosus* under various harvesting regimes in Marine, U.S.A.. *Bot. Mar.*, **24**, 29-38.

Klepacki, K.J., Scott, J.L. and Broadwater, S.T. (1995). Ultrastructure of cell division in *Agardhiella subulata* (Gigartinales, Rhodophyta). *Eur. J. Phycol.*, **30**, 159-167.

Knight, M. and Parke, M.W. (1950). A biological study of *Fucus vesiculosus* L. and *F. serratus*. *Journ. Mar. Biol. Assoc.*, **29**, 439-514.

Migita, S., Nakashima, N., Lin, C. and Tamaki, A. (1993). The luxuriant growth of *Gracilaria chorda* (Gracilariaceae, Rhodophyta) off the Kumamoto coasts of the Ariake Sea (in Japanese). *Suisanzoshoku*, **41**, 149-154.

Spurr, A.R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.*, **6**, 31-43.

Tseng, C.K. (1947). Seaweed resources of North America and their utilization. *Economic Botany*, **1**, 69-97.

Wittmann, W. (1965). Aceto-iron-haematoxylin-chloral hydrate for chromosome staining. *Stain Technol.*, **40**, 161-164.

Yamamoto, H. (1973). *Gracilariopsis chorda* (Holm.) Ohmi from Hakodate Bay (in Japanese). *Bull. Jap. Soc. Phycol.*, **21**, 86-91.

Yamamoto, H. and Sasaki, J. (1987). Crossing experiments between populations of so-called *Gracilaria verrucosa* (Huds.) Papenfuss from two localities, Shinori and Kikonai in Hokkaido. *Bull. Fac. Fish. Hokkaido Univ.*, **38**, 335-338.