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ORIGINAL ARTICLE

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Induction of phlorotannins in the brown macroalga *Ecklonia radiata* (Laminariales, Phaeophyta) in response to simulated herbivory—the first microscopic study

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Abstract We investigated, for the first time in a microscopic study, the accumulation of phlorotannins as a possible inducible chemical defence against herbivory. *Ecklonia radiata* (C. Agardh) was mechanically wounded with a cork borer, simulating grazer action, and at intervals of 0, 1, 2, 3, 5, 7 and 9 days after wounding the distribution of phlorotannins and other structural changes were examined by light, fluorescence and electron microscopy. In brown algal cells, phlorotannins (polyphenolic compounds) occur in vesicles known as physodes. In *E. radiata*, most of the physodes were found in the outer epidermal cell layer, but some were present in the cortical cells and in the innermost medullary cells (sieve elements and hyphal cells). The wound-healing process could be divided into three stages: (i) 'closing' of the medulla by the formation of new medullary cells, (ii) accumulation of phlorotannins (physodes) at the wound area (first in the medullary cells and then in the cortical cells) and in the medullary tissue further away from the wound, and (iii) formation of a new epidermis. The accumulation of phlorotannins started on day 1 and was evident from day 3 on. Our results show structural wound-healing and support wound-sealing functions for phlorotannins and the view that phlorotannins might be considered as inducible anti-herbivory agent in *E. radiata*. Our results strongly demonstrate the importance of detailed microscopic studies, in addition to chemical analysis, for revealing the localised nature of the brown algal response to wounding.

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Introduction

Phlorotannins are polymers of phloroglucinol, a class of polyphenolic compounds, restricted to the brown algae (Phaeophyceae) and making up 1–20% of the dry weight (Ragan and Glombitza 1986; Schoenwaelder 2002). They are secondary metabolites that occur in a wide range of molecular sizes, have an astringent taste, bind to metal ions and precipitate proteins (Ragan and Glombitza 1986). The occurrence and role of phlorotannins in brown algae were recently reviewed by Schoenwaelder (2002). They are located within the cell in the cytoplasm in vesicles called physodes, and are also a constituent of the cell wall. Several functions have been proposed, including a strengthening role in cell walls, a role in adhesion and a role in protection against excess irradiance, in particular ultraviolet radiation. Phlorotannins are commonly considered to be herbivore deterrents, digestive inhibitors and antibacterial agents even though the experimental evidence remains inconclusive (Targett and Arnold 1998; Amsler 2001). Polyphenolics are also found in all vascular terrestrial plants and have a broadly similar functional role (Hagerman and Bulter 1991). Even in terrestrial plants, where since 1995 more than 200 studies have quantified phenolics to investigate plant anti-herbivore defence, many studies have failed to confirm a close correlation between content of phenolics and effects on the investigated herbivores (Heil et al. 2002).

There are two chemical defence theories. The defence can be 'constitutive', i.e. there is a generally high concentration of phlorotannins that prevents the alga being eaten by herbivores, or 'inducible', when the herbivore attack stimulates an increase in phlorotannins that

prevents further herbivory. The role of phlorotannins as a constitutive defence against herbivory is well documented in several algal species (Targett and Arnold 1998), but the function of phlorotannins as an inducible defence has been investigated in very few studies. Accumulation of phlorotannins has been shown in response to grazing by snails (Pavia and Brock 2000; Pavia and Toth 2000) and in response to simulated herbivory (mechanical wounding; Van Alstyne 1988; Yates and Peckol 1993; Peckol et al. 1996; Hammerstrom et al. 1998). In contrast, *Laminaria hyperborea* showed no phlorotannin induction in response to natural herbivory (Toth and Pavia 2002). No induction in response to simulated herbivory was reported for *Ecklonia radiata* and *Sargassum vestitum* (Steinberg 1994), *Lessonia nigrescens* (Martínez 1996), *Ascophyllum nodosum* (Pavia et al. 1997; Pavia and Toth 2000), *Hedophyllum sessile* (Hammerstrom et al. 1998), and *Laminaria hyperborea* (Toth and Pavia 2002).

We have observed an accumulation of phlorotannins around small holes, probably caused by grazers, in blades of *Ecklonia radiata* found in the field in April. In the present study, we simulated this kind of wounding by making a hole with a cork borer in blades of *E. radiata* and investigated the healing process over 9 days in the laboratory. Instead of measuring changes in phlorotannin concentrations, as in all the previously cited induction studies, we investigated for the first time the phlorotannin accumulation together with structural changes visible in light, fluorescence and transmission-electron microscopes. We showed an accumulation of phlorotannins in *E. radiata* as a short-term and primarily localised response to wounding.

Materials and methods

Algal material and experimental design

Three individuals of *Ecklonia radiata* (C. Agardh) were collected in April at Sorrento, Victoria, Australia (144°40′E 38°14′S). Several blades from each individual were cut into 5-cm segments. 16 segments from each individual were placed in a culture dish containing filtered sea water (2-μm filter; Millipore) and cultured at 16°C, under a constant photon fluence rate of 130 μmol photons m^{-2} s^{-1} using wide-spectrum fluorescent tubes (Gro-Lux WS; Sylvania, Danvers, MA, USA) and a 16-h-light/8-h-dark cycle. To ensure sufficient water movement, the culture dishes were shaken slowly on an orbital shaker.

To simulate wounding by herbivores, a hole of 3 mm in diameter was cut with a cork borer in the middle of each segment (Fig. 1). Two segments of each individual were left uninjured. Structural changes around the hole were studied in freshly wounded segments (0-day control) and 1, 2, 3, 5, 7 and 9 days after wounding. On day 9, the uninjured segments were freshly wounded (9-day control). At each sampling time, two segments of each individual were removed. In each segment a 7-mm square was cut out around the hole first and then a triangle with the wound area in one corner. Some triangles were investigated directly by light and fluorescence microscopy and some were fixed and used for light and transmission electron microscopy. In general, all sections were cut along the shortest edge of the triangle, which is parallel to the longitudinal axis of the blade.

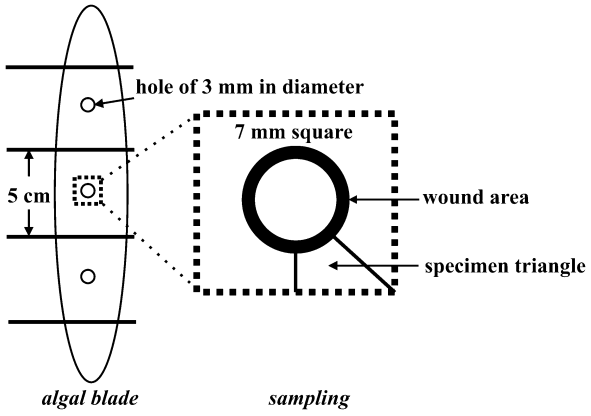


Fig. 1 Experimental design for wounding of *E. radiata*. A hole of 3 mm in diameter is made in each segment of 5 cm length. The triangle is the precisely shaped sample used for this study

Light and fluorescence microscopy of living material

Living tissue was sectioned with a razor blade and was examined under bright-field illumination and for autofluorescence using a light microscope operating in an epifluorescence mode (Axiophot photomicroscope; Zeiss). Phlorotannins were imaged as green fluorescence using excitation filter BP 450–490 nm (blue light), dicroic beam splitter FT 510 nm, and barrier filter BP 515–565 nm. Phlorotannins were imaged as white/aqua fluorescence together with an orange fluorescence of chlorophyll using excitation filter BP 395–440 nm (violet–blue light), FT 460 nm and long-bandpass barrier filter LP 470 nm.

Light and transmission-electron microscopy of fixed material

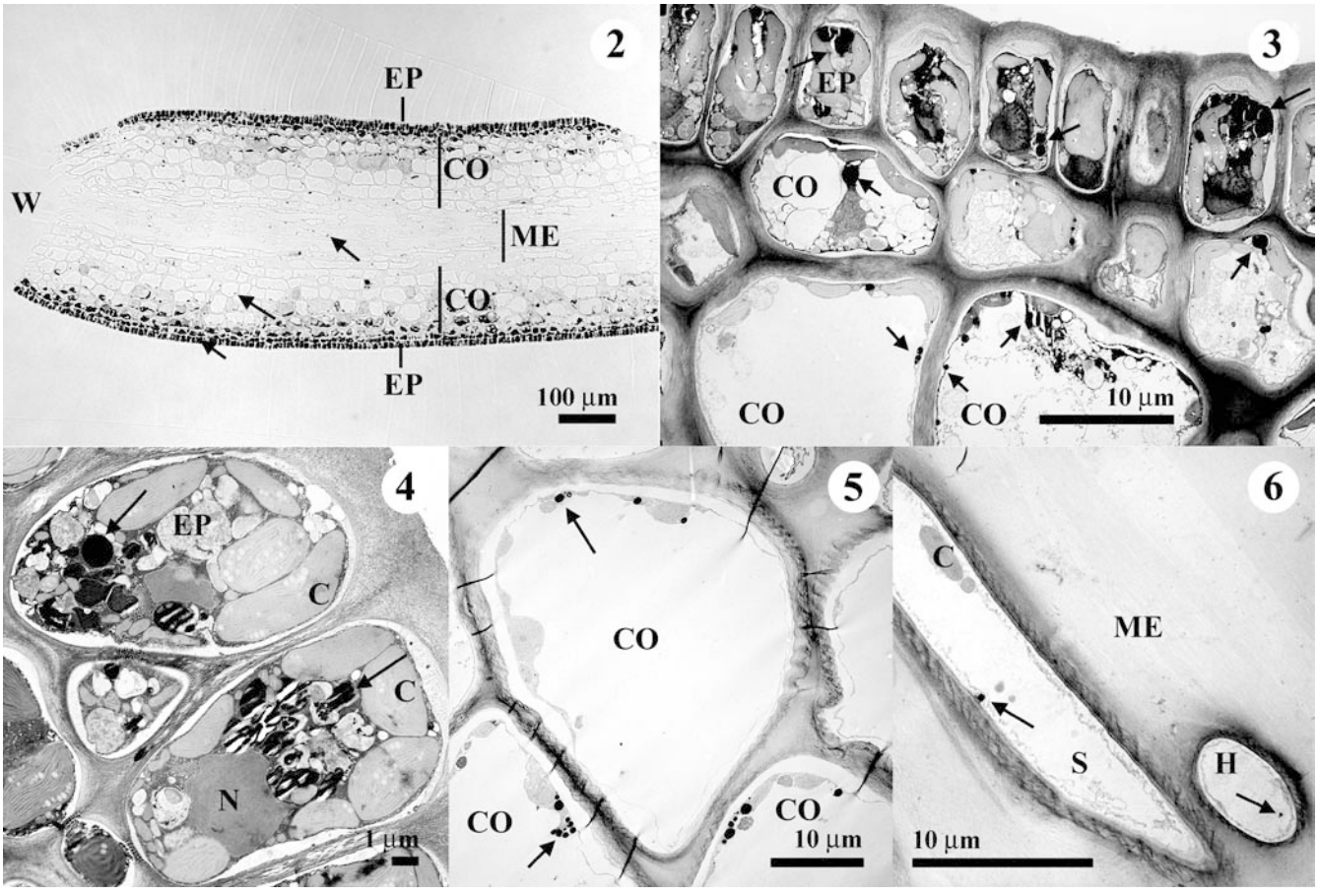
Specimen triangles were fixed as described by Schoenwaelder and Clayton (1998b). Immediately after cutting, triangles were transferred into 2% (w/v) glutaraldehyde, 1% (w/v) paraformaldehyde, 1% (w/v) caffeine, 2% (w/v) sodium chloride and 0.1% (w/v) calcium chloride dihydrate in 0.1 M sodium cacodylate buffer (pH 7.2), and fixed for 3 h. Specimens were post-fixed in 1% (w/v) osmium tetroxide in buffer for 2 h and dehydrated through an acetone series. After infiltration of medium-grade Spurr's resin, specimens were polymerised at 70°C in flat embedding moulds.

Overview sections (2 μm) were collected on slides and examined in the light microscope under bright field. Ultrathin sections (90 nm) were collected on Formvar-coated slotted copper grids, post-stained with saturated uranyl acetate in 50% (w/v) methanol and saturated lead citrate for 15 min each and examined in the transmission-electron microscope (JEM 200CX; Jeol).

Results

Natural distribution of phlorotannins in *E. radiata*

Three types of tissue could be distinguished in the blades of *E. radiata*: (i) a central filamentous medulla, consisting of a loose network of longitudinally directed cells (sieve elements) interconnected by transverse files of cells (hyphal cells), both of which are surrounded by a mucilaginous matrix; (ii) a parenchymatous cortex; and (iii) a thin epidermis, which is both photosynthetic and meristematic, and sometimes known as meristoderm. Figures 2, 3, 4, 5, and 6 show the distribution of



Figs. 2–6 Distribution of physodes in freshly wounded *E. radiata* (0d control). Physodes (*arrows*) are stained black and appear round to ovoid. *C* Chloroplast, *CO* cortex, *EP* epidermis, *H* hyphal cell, *ME* medulla, *N* nucleus, *S* sieve element, *W* wound site

Fig. 2 Bright-field image of a cross-section of thallus as an overview. Most physodes are located in the epidermis, some in the cortex and a few in the medulla

Fig. 3 Electron micrograph showing epidermal and outer cortical cells

Fig. 4 Electron micrograph showing details of epidermal cells

Fig. 5 Electron micrograph showing details of inner cortical cells

Fig. 6 Electron micrograph showing a sieve element and a hyphal cell

physodes in these tissue types in freshly wounded *E. radiata* (0-day control). Physodes were stained black, round to ovoid in shape and appeared in the overview section as black dots (Fig. 2). Most of the physodes were located in the epidermis, but some occurred in the cortex and very few were visible in the medulla. The electron micrographs showed typical epidermal cells filled with large physodes, which were up to 1.4 µm in diameter, and with chloroplasts located close to the thallus surface (Figs. 3, 4), typical outer cortical cells with some large and smaller physodes (Fig. 3), and typical inner cortical cells with small physodes (up to 0.7 µm in diameter; Fig. 5). Occasionally, small physodes (up to 0.7 µm in diameter) were observed in sieve elements and in medullary hyphae (Fig. 6). Sieve elements were connected end-to-end by cross walls perforated by many pores and formed structures similar to the sieve tubes of higher plants (see also Figs. 16, 24). The osmium staining of physodes as an indication of the presence of phlorotannins was validated by stainings with toluidine blue O and fast red GG according to Schoenwaelder and Clayton (1998b; data not shown).

In *Ecklonia*, the distribution of physodes was easily visible in living tissue (Fig. 7, first line, 0d control). The large amount of phlorotannins in the epidermis appeared brown to dark brown under bright field (first column), fluoresced white and aqua under violet–blue light excitation together with an intensively orange fluorescence of chlorophyll (column 2), and fluoresced intensively green under blue light excitation (column 3).

Accumulation of phlorotannins after wounding

Bright field and fluorescence microscopy of living tissue showed clearly an accumulation of phlorotannins at the wound area (Fig. 7). On days 1 and 2 after wounding, slight phlorotannin fluorescence was visible at the wound surface. On day 3 after wounding, phlorotannin fluorescence became conspicuous in the medulla adjacent to the wound surface. In bright field, a brown spot of phlorotannins was visible at the wound area. In the following days, especially at days 7 and 9 after wounding, there was a further increase in phlorotannin

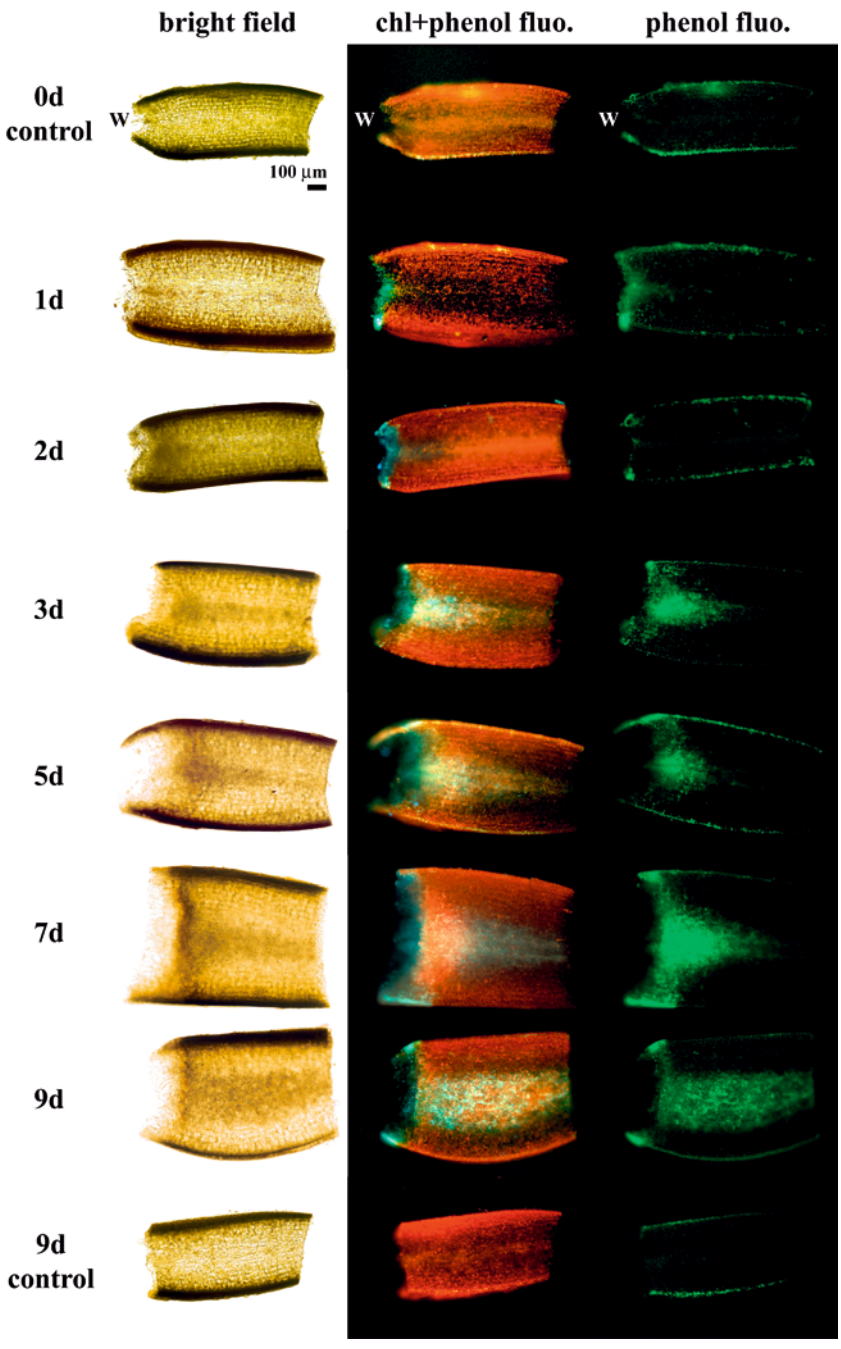


Fig. 7 Time course of wounding and the distribution of phlorotannins in living tissue of *E. radiata*. Phlorotannins are brown to dark brown under bright field (*column 1*), fluoresce white and aqua under violet–blue light excitation together with an orange fluorescence of chlorophyll (*column 2*), and fluoresce green under blue light excitation (*column 3*). In all images, the wound site is on the *left*. *W* Wound site

fluorescence in the medullary tissue further away from the wound surface. In bright field, a brown track of phlorotannins was visible in the medulla. At the end of the time course, the '9d control' showed no accumulation of phlorotannins at the wound area or in the medulla. The same results were found in the three individuals studied.

Ultrastructural changes after wounding

Light and electron micrographs of fixed material showed more details of wounded tissues. On day 1 after wounding, some cell layers away from the wound surface, the innermost cortical cells, started to generate additional medullary cells (Fig. 8). These new cells contained several physodes and were initially much longer than wide and underwent transverse divisions (Fig. 9). Later, these cells differentiated into sieve elements or hyphal cells, as described by Schmitz and Srivastava (1974, 1975). On day 2 after wounding, the wound surface at the medulla was completely closed with new medullary cells (Fig. 10, compare with Fig. 8) showing cross wall formation and containing a few scattered physodes (Fig. 11). On day 3 after wounding (Fig. 12), the number and size of physodes in the new

medullary cells at the wound area increased conspicuously; physodes measured up to 2.2 μm in diameter (Fig. 13, compare with Fig. 11). These cells showed evidence of cell division (Fig. 13) with conspicuous Golgi bodies (Fig. 14) and many new cross walls (Fig. 15). Along the cross walls, there was a layer of small physodes and the cross walls themselves contained two dark-stained layers of what appeared to be phlorotannin deposits (Fig. 15). At the wound site, the cortical cells also started to accumulate physodes (Fig. 12, compare with Fig. 2). The number and size of physodes in the medullary cells further away from the wound also increased and became more conspicuous (Figs. 12 and 16, compare with Figs. 2 and 6, respectively). On day 5 after wounding, a layer of cells filled with physodes had formed across the wound surface (Fig. 17). The cortical cells showed evidence of cell divisions and increasing accumulation of physodes (Fig. 18). The medullary cells at the wound surface started to differentiate into new epidermal cells (Fig. 19). On day 7 after wounding, the formation of a new epidermal cell layer filled with physodes was in full progress (Fig. 20). Figure 21 shows new epidermal cells that are formed from medullary cells and are rich in physodes, Golgi bodies and other organelles. New epidermal cells were also differentiated from cortical cells (Fig. 22) and the incorporation of phlorotannins into the cell walls was common (Fig. 23). Furthermore, there was a great increase in number and size of physodes in the medullary cells further away from the wound site; physodes were up to 2.4 μm in diameter (Fig. 24, compare with Figs. 6 and 16). On day 9 after wounding, the formation of a new epidermis across the wound surface was completed, and physodes were up to 2.8 μm in diameter (Figs. 25, 26, 27).

Discussion

Wound-healing

The wound-healing process in *E. radiata* can be divided into three stages. First, the medulla was 'closed' by new medullary cells that were initiated from the innermost cortex cells and then underwent cell division. Then, a local accumulation of physodes occurred at the wound area, first in the medullary tissue, then in the cortical tissue, but also in the medullary tissue further away from the wound site. The third stage was the formation of a new epidermis across the total wound surface by differentiation of new epidermal cells directly from medullary and cortical cells. In *Fucus* and *Sargassum* a re-differentiation of cortical and medullary cells into meristematic tissue and directly into new epidermal cells was also observed during regeneration of wounded stipes, when new tissue resulted from the continued mitotic growth of medullary cells (Fulcher and McCully 1969, 1971; Fagerberg and Dawes 1976, 1977). As in our study, Fagerberg and Dawes (1976) described in *Sargassum* a central role of the medullary tissue during wound-healing and the formation of a new epidermis covering the regenerated tissue.

►

Figs. 8–15 Ultrastructural changes in *E. radiata* after wounding. *CO* Cortex, *CW* cross wall, *ME* medulla, *G* Golgi apparatus, *W* wound site. *Arrows* indicate physodes

Fig. 8 Day 1 after wounding. Generation of new medullary cells from innermost located cortical cells

Fig. 9 Day 1 after wounding. Several physodes in a regenerated medullary cell

Fig. 10 Day 2 after wounding. 'Wound-closing' by newly generated medullary cells

Fig. 11 Day 2 after wounding. Newly generated medullary cells containing a few scattered physodes

Fig. 12 Day 3 after wounding. Cross-section of thallus showing accumulation of physodes in newly generated medullary cells at the wound site and in medullary cells further away from the wound. Sections shown in Figs. 13, 14, 15 and 16

Fig. 13 Newly generated medullary cells with increased number of physodes

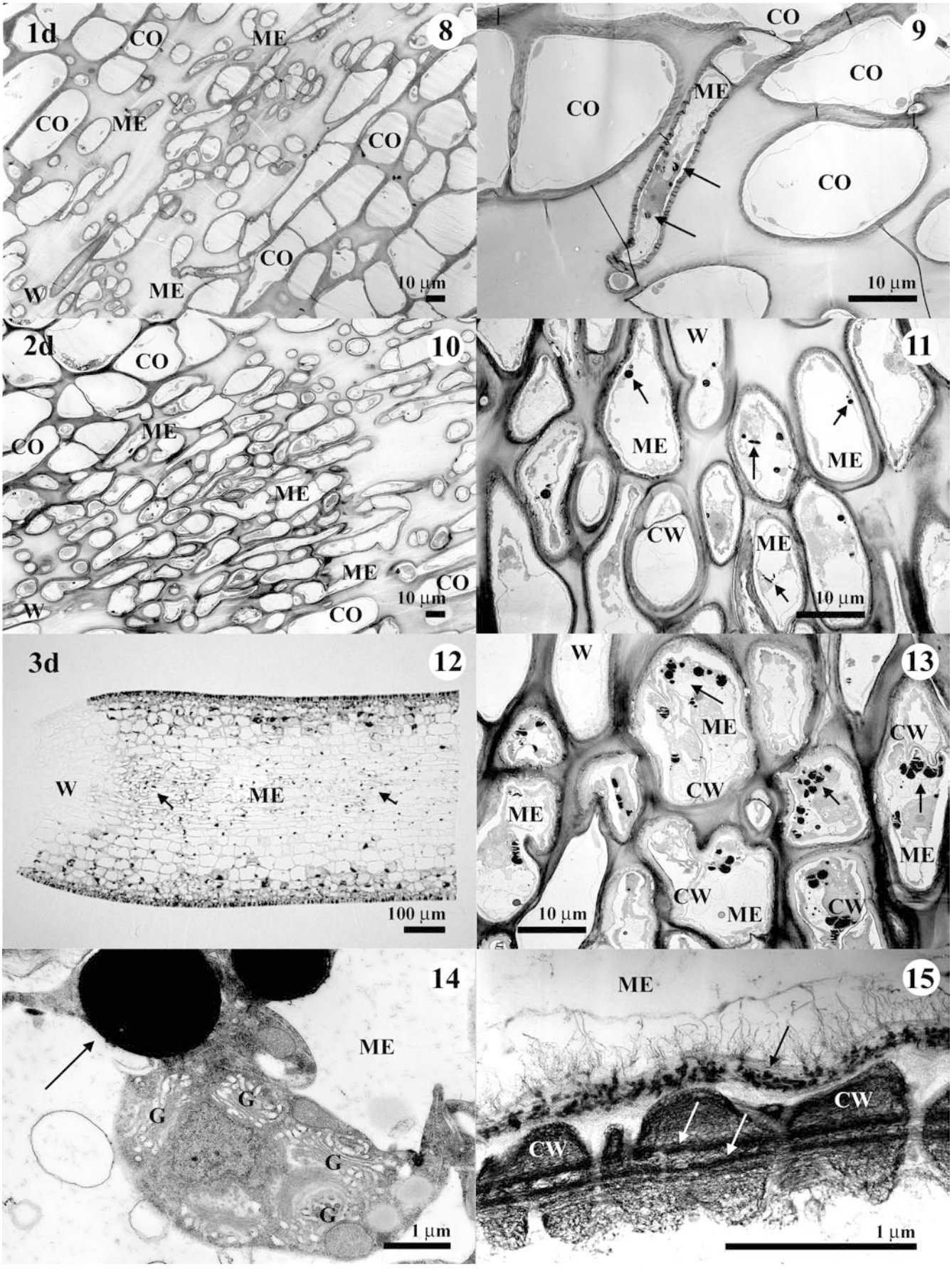
Fig. 14 Newly generated medullary cells with increased numbers of Golgi bodies

Fig. 15 Newly generated medullary cells with increased numbers of cross walls showing the incorporation of phlorotannin deposits

Induction of phlorotannins

The lack of phlorotannin accumulation in the '9d control' (i.e. the uninjured segment) shows that the induction of phlorotannins in *E. radiata* was a wound response and not initiated by the experimental culture conditions, and also that the algal segments were still healthy.

The first indications of phlorotannin accumulation on days 1 and 2 after wounding were the appearance of scattered small physodes in the new medullary cells and phlorotannin autofluorescence at the wound surface. It is possible that the fluorescence at the wound surface was the result of leakage from damaged cells. Medullary and cortical cells did not contain many physodes, but there should be sufficient 'free' phlorotannins to react and bind with other macromolecules such as the cell walls exposed at the wound. Another possible explanation might be an extracellular release of phlorotannins from intact cells. Fulcher and McCully (1971) described the loss of phlorotannins from undamaged sieve elements that were within 5–10 cell lengths of the wound, during the first 24 h after wounding in *Fucus*. Extracellular phlorotannin release also occurs from healthy algae in response to stress (Ragan and Glombitza 1986; Jennings and Steinberg 1994) and in algal zygotes after fertilisation (Clayton and Ashburner 1994; Schoenwaelder and Clayton 1998a, 1998b). A further possible explanation of the autofluorescence at the wound surface could be sealing of the wound by phlorotannins in a manner similar to that observed for $^{35}SO_4$-labeled material in *Fucus* (Fulcher and McCully 1971). Newly synthesised $^{35}SO_4$-labeled material was deposited at the cross walls, separating wounded and unwounded med-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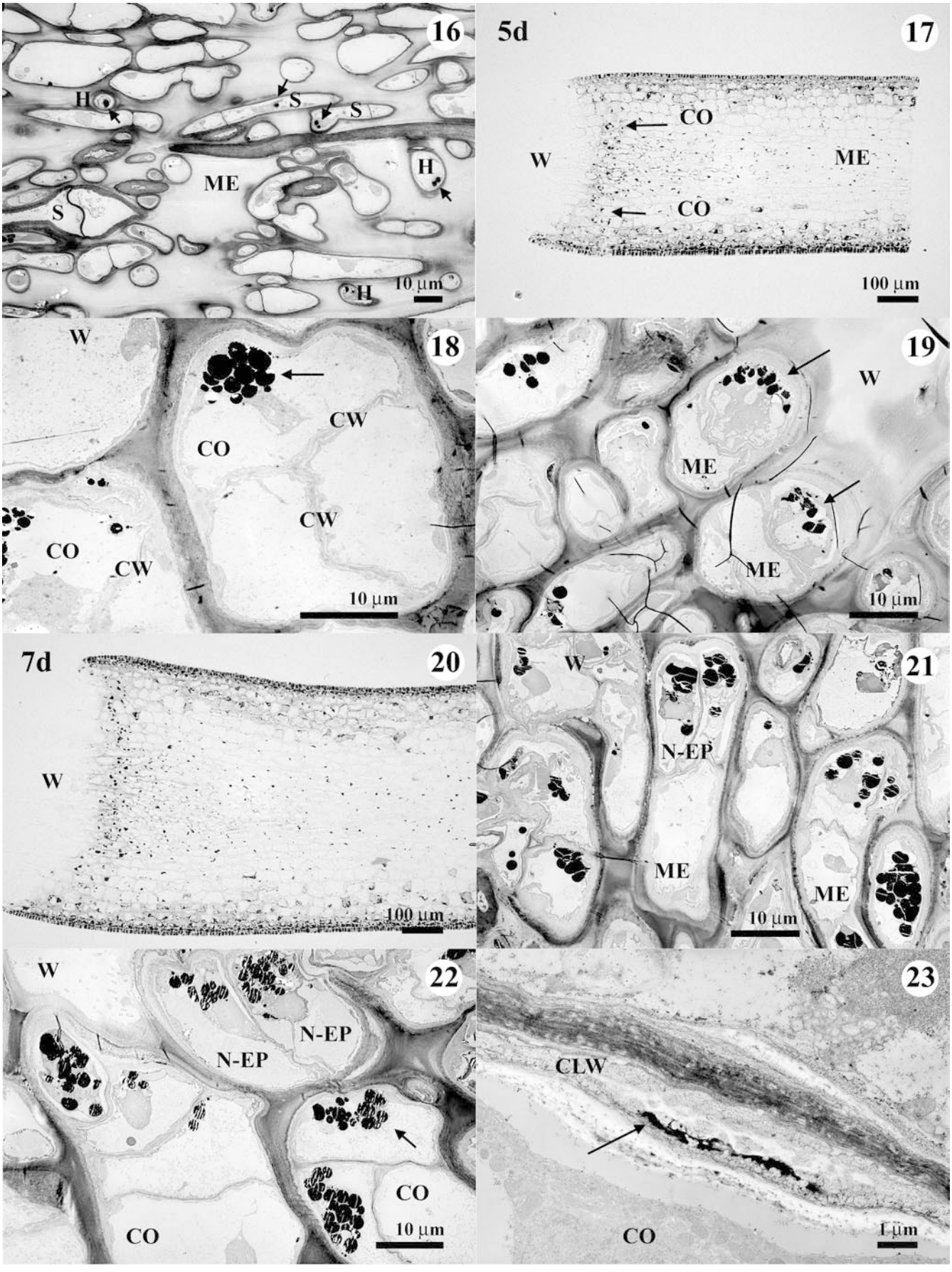
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Figs. 16–23 Ultrastructural changes in *E. radiata* after wounding. *CLW* Cell wall, *CO* cortex, *CW* cross wall, *H* Hyphal cell, *ME* medulla, *N-EP* new epidermis, *S* sieve element, *W* wound site. *Arrows* indicate physodes

Fig. 16 Day 3 after wounding. Accumulation of physodes in medullary cells further away from the wound area

Fig. 17 Day 5 after wounding. Cross-section of thallus showing accumulation of physodes in cortical cells at the wound site. Sections shown in Figs. 18 and 19

Fig. 18 Accumulation of physodes and cross wall formations in cortical cells near the wound surface

Fig. 19 The beginning of re-differentiation of newly generated medullary cells into epidermal cells

Fig. 20 Day 7 after wounding. Cross-section of thallus showing a layer of cells filled with physodes across the wound surface. Sections shown in Figs. 21, 22, 23 and 24

Fig. 21 Re-differentiation of medullary cells into new epidermal cells

Fig. 22 Re-differentiation of cortical cells into new epidermal cells

Fig. 23 Large deposits of phlorotannins in the cell wall

ullary cells, and at the end walls adjacent to the wound surface, thereby closing the plasmodesmata. Fulcher and McCully (1971) identified the $^{35}SO_4$-labeled material as sulfated polysaccharides [using periodic acid–Schiffs (PAS) stain], but there are also sulfated phenolic compounds, and some phenolic compounds react positively with PAS in the same manner as polysaccharides (Geier 1980).

In our study, the induction of phlorotannins was obvious on day 3 after wounding. Fagerberg and Dawes (1977) also found an accumulation of physodes at the wound surface on day 3 after wounding in *Sargassum* stipes. A similar rapid induction of phlorotannins in response to mechanical wounding was measured using chemical analysis in blades of *Agarum fimbriatum* and *Laminaria groenlandica*, in stipes of *L. complanata* and in holdfasts of *Pleurophycus gardeneri* (Hammerstrom et al. 1998) and in *Fucus vesiculosus* (Yates and Peckol 1993; Peckol et al. 1996).

In contrast, Steinberg (1994) found no induction of phlorotannins in *E. radiata* in response to simulated wounding. Seasonal variation in phlorotannin levels (Ragan and Glombitza 1986; Steinberg 1995) can be excluded as a cause, since in both Steinberg's and our studies the algae were harvested in April. A more probable explanation for this anomalous result is the natural distribution of phlorotannins in this species. We have shown that most of the physodes (possibly almost 90% of the total phlorotannin content) are located in the thin outer epidermal cell layer, which represents only about 10% of the total algal tissue. Furthermore, the surface of *E. radiata* is not uniformly thick, and the epidermis varies between one to three layers over the total blade (compare Fig. 20 with Fig. 25). Thus, our observed induction of phlorotannins, especially the accumulation of phlorotannins in the medulla more distant from the wound site, would not be detectable by measuring the total phlorotannin concentration in whole segments of *E. radiata*; instead it would be lost in the variation between samples. Our study strongly demonstrates the value and importance of detailed microscopic studies in addition to chemical analysis. Probably for similar reasons, several other studies have failed to show phlorotannin induction in response to wounding. The abundance of physodes in epidermal and outer cortical cells in brown algae has been described in several other microscopic studies (McCully 1966, 1968; Evans and Holligan 1972; Fagerberg and Dawes 1977; Kaur and Vijayaraghavan 1992) and in one chemical analysis (Tugwell and Branch 1989). All of these studies also mentioned the presence of a few physodes in the medullary tissue. Studies on phenolics in plant defence have also shown that quantification of total phenolic compounds is not enough to determine the specific effect of phenolics in plant defence. Heil et al. (2002) argue that knowledge of molecule structures and mixtures is also required.

There is no evidence of translocation of pre-existing phlorotannins (e.g. from the phlorotannin-rich epidermal cells to the medullary cells) during the wound-healing process in *Ecklonia radiata*. The chemical character of phlorotannins necessitates their inclusion in vesicles. Physodes are produced in the perinuclear region, in vesicles derived from the ER and the Golgi bodies (Schoenwaelder and Clayton 2000). We have shown that each cell type (epidermal, cortical and medullary cell) generally contained physodes and was able to increase the size and number of physodes; thus the formation of physodes is a highly dynamic process. Furthermore, cells with high physode accumulation even showed a high accumulation of Golgi bodies. New medullary cells containing physodes were generated from the innermost located cortical cells and then by cell division.

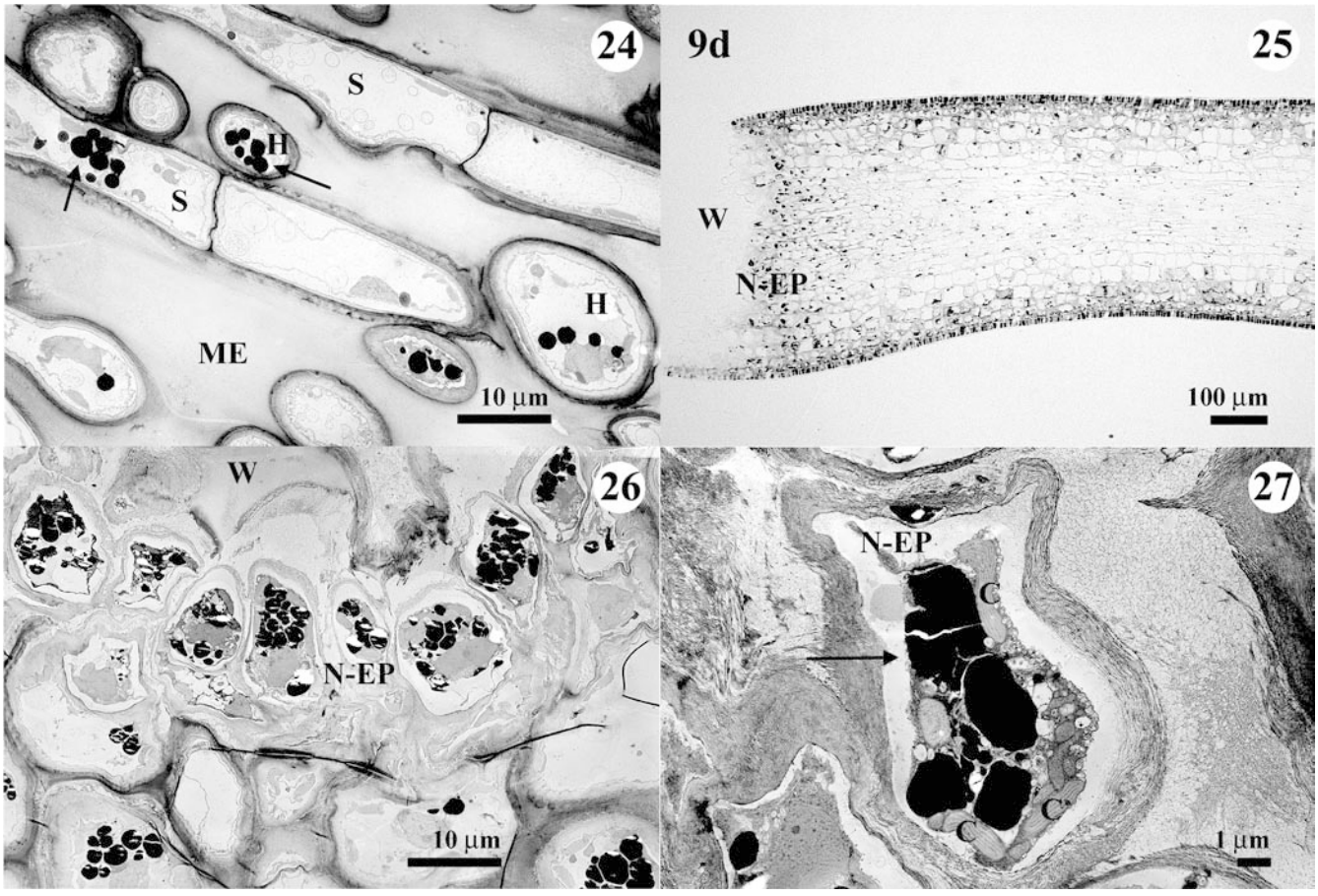
The role of phlorotannins during wound-healing

Our results suggested three different kinds of function for phlorotannins during wound-healing: wound-sealing, structural wound-healing, and anti-herbivore defence.

The presence of phlorotannins at the wound surface in the early stage of wounding in *E. radiata* corresponds with a sealing function of phlorotannins and would also help to prevent microbial attack. Phlorotannins are known to precipitate proteins (Ragan and Glombitza 1986; Stern et al. 1996) and thereby are able to 'clot' the wound by precipitation of proteins as suggested by Faberberg and Dawes (1976).

The deposition of phlorotannins into cell walls, including into new cross walls, from day 3 of wounding implies that phlorotannins have a structural function during wound-healing. This is consistent with the findings of Schoenwaelder and Clayton (1998a, 1998b, 1999) that phlorotannins are secreted into the primary zygote wall during fucoid development.

In *E. radiata* the induction of phlorotannins is primarily a local response restricted to the wound site, but we have also found a general accumulation of physodes in the medullary tissue further away from the wound



Figs. 24–27 Ultrastructural changes in *E. radiata* after wounding. *H* Hyphal cell, *ME* medulla, *N-EP* new epidermis, *S* sieve element, *W* wound site. *Arrows* indicate physodes
Fig. 24 Day 7 after wounding. Additional physode accumulation in medullary cells further away from the wound
Fig. 25 Day 9 after wounding. Cross-section of thallus showing a new epidermis across the wound surface. Sections shown in Figs. 26 and 27
Fig. 26 New epidermal cells covering the total wound surface
Fig. 27 Higher magnification of new epidermal cells

site, which would seem to be more consistent with an anti-herbivore response than a wound-healing response. Van Alstyne (1988) also found an accumulation of phlorotannins at the wound site and within adjacent undamaged branches as a response to mechanical wounding. The retention of a high phlorotannin level over 9 days and probably even longer also would support an anti-herbivore function, as suggested by Hammerstrom et al. (1998). A local accumulation of phlorotannins is an efficient form of defence, as shown by the findings of van Alstyne (1988). In grazer-preference experiments, herbivorous snails preferred freshly wounded *Fucus distichus* over uninjured thalli, but with the accumulation of phlorotannins at the wound area, the snails shifted their preference towards the uninjured controls. Thus, in *E. radiata*, phlorotannins might be considered as an inducible chemical defence agent that deters herbivores from further eating and prevents infection by microbes such as bacteria or fungi.

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