Morphological Study on the Inhibitory Effect of Germanium Dioxide on Growth and Development of Brown Algae[1)]

By

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Introduction

Germanium dioxide (GeO_2) has been generally used in unialgal cultures of various seaweeds as an agent for eliminating diatoms. The concentration which would control diatom growth while allowing other algae to grow normally was suggested to be 1-10 mg GeO_2/l (LEWIN 1966). In fact, many studies on the inhibitory effect of GeO_2 on various algae and other organisms have been done from very early. Especially, the toxicity of germanium (Ge) has been recognized at very low concentrations in many diatoms (WERNER 1966, 1967a, LEWIN 1966, TATEWAKI and MIZUNO 1979), and Ge is generally recognized as a specific inhibitor of diatoms because no inhibitory effects at low doses were found in Chlorophyta (WERNER 1966, 1967b, LEWIN 1966), Rhodophyta (LEWIN 1966, KIRITA 1970), Cyanophyta (LEWIN 1966), various fungi, bacteria (LEWIN 1966, WERNER 1967b) and protozoa (LEWIN 1966). However, since MCLACHLAN *et al.* (1971) reported the inhibition by GeO_2 at concentrations of 5 mg/l in their culture experiment of four *Fucus* species, many species of brown algae have been found to be affected at low germanium concentrations (HOPKIN and KAIN 1978, TATEWAKI and MIZUNO 1979, MARKHAM and HAGMEIER 1982). In their studies, TATEWAKI and MIZUNO (1979) found that regardless of the constitution of thallus, all the species of brown algae examined showed smaller growth quantity and some damage in the formation of thallus at less than 1-5 mg GeO_2/l concentrations. This response to Ge was found to be significant in the brown algae and not observed in species of other macroalgae, such as red and green algae, under the same Ge concentrations.

The mechanism of the inhibition of Ge on diatoms has been investigated in detail by several workers and there are good descriptions of the effects on frustule morphology and silicate uptake and incorporation. These workers observed that Ge prevents cell division in diatoms generally by altering wall formation (AZAM *et al.* 1973, CHIAPPINO *et al.* 1977), and specifically, Ge prevents Silicon uptake and also prevents the metabolism of Si already taken up, which in turn seems to affect DNA, protein or chlorophyll synthesis (DARLEY and VOLCANI 1969, WERNER 1966, 1967a). But most Ge toxicity in diatoms was found to be reversible by

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addition of Si (WERNER 1967b, LEWIN 1966). All these results show that germanic acid is an analogue of silicic acid; it has been thought to act as a competitive inhibitor of Si metabolism in the diatom. This hypothesis has gained wide acceptance to become a matter of general knowledge.

As to the germanium toxicity to brown algae, however, there are only a few explanations and speculations so far (TATEWAKI and MIZUNO 1979, MARKHAM and HAGMEIER 1982). MARKHAM and HAGMEIER (1982) explained the brittleness due to germanium inhibition of the *Laminaria* plants as a result of the competitive action between Si and Ge elements, because PARKER (1969) had reported the presence of Si in brown and green algae and suggested that silica is localized in the cell wall in brown algae. However, MCLACHLAN *et al.* (1971) and MCLACHLAN (1977) have found that the omission of silicon from culture medium had no effect on growth and development of embryos of *Fucus edentatus*, and excess silicon was not effective in suppressing the effects of germanium toxicity in the species. Similarly, TATEWAKI and MIZUNO (1979) performed an experiment with several brown algae including *Fucus evanescens, Scytosiphon lomentaria* and *Desmarestia viridis*, to see whether the brown algae show any dependence on Si and whether additional Si will reduce the inhibition of Ge. Their results did not show any effect of Si on the brown algae. Therefore, it can be thought that the brown algae do not require Si as an essential nutrient, and it is possible that another mechanism of Ge inhibition exists rather than competing with Si as an analogue in the brown algae. So the questions are: Why are the brown algae are more sensitive to GeO_2 than other macroalgae, how do the brown algae respond to germanium treatment and then how are they affected.

In living organisms, all the physiological and biochemical processes are eventually reflected in their morphological features. In this way, the inhibitory processes of Ge on brown algae can be expected to appear also morphologically. Therefore, investigating the changes in morphology after treating with Ge will be helpful in deducing the possible mechanism of Ge inhibition. In order to make the mechanism of germanium inhibition on brown algae clear, an examination focused on the morphology of plants and cells has been carried out in the present study.

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Chapter 1. Inhibition of GeO_2 in brown algae and their morphology under the light microscope

Introduction

A significant growth inhibition due to small amounts of germanium has been observed in many diatoms (LEWIN 1966, WERNER 1966, 1967a, TATEWAKI and MIZUNO 1979) and brown algae (MCLACHLAN *et al.* 1971, MCLACHLAN 1977, HOPKIN and KAIN 1978, TATEWAKI and MIZUNO 1979, MARKHAM and HAGMEIER 1982). Both the groups of algae show higher sensitivity to germanium compounds. However, differing from the diatoms, in which germanium function has been clarified as a competing analogue with nutrient silicon (Si), the brown algae show no dependence on Si and additional Si does not work in reducing the inhibitory effect of Ge. So, it is difficult to assume that germanium inhibits the brown algae in the same way as in the diatoms.

In the brown algae examined so far, the damage to the thallus due to Ge treatment has been observed together with the inhibition of the growth rate (TATEWAKI and MIZUNO 1979, MARKHAM and HAGMEIER 1982). From those reports it can be seen that the morphological change in thallus varies greatly according to the species, which is probably because of the fact that there is a large variation of the system of thallus organization and the way of growth even in the same group of brown algae. It is reasonable to think that relatively earlier affected place is most sensitive to Ge toxicity and this position in the thallus depends on the species. This possible difference in Ge sensitivity location might cause the different appearance of thallus when altered by germanium. However, all the brown algae examined have in common higher sensitivity to Ge compared with other macroalgae, no matter how different they appear (TATEWAKI and MIZUNO 1979). This generality in brown algae is suggestive of some way of reacting applicable to all the species in the group.

The morphological change is thought likely to provide some information about the inhibitory mechanism of GeO_2. To know the ways in which various brown algae respond to germanium, and further find the general character in their morphological reaction to germanium, observations were done on several brown algae under the light microscope.

Materials and Methods

Four species of the brown algae, *Fucus distichus* subsp. *evanescens* (C. AG.) POWELL, *Pelvetia wrightii* OKAMURA, *Analipus japonicus* (HARV.) WYNNE and *Scytosiphon lomentaria* (LYNGB.) LINK, appears from the upper tide zone to the lower tide zone at Charatsunai, Muroran, Hokkaido. Fertile plants are commonly found from February to June in *Analipus japonicus* and *Scytosiphon lomentaria*, from April-May to July in *Fucus distichus* and from September to December in *Pelvetia wrightii*. The strains of *Sphacelaria* sp. and *Pilayella littoralis* (L.) KJELLMAN were from the culture collection (see Table 1).

Mature plants of *Fucus distichus* and *Pelvetia wrightii* were rinsed with autoclaved

Table 1. Species used for the experiment

Species	Place of collection	Date of collection
Phaeophyta		
Fucus distichus subsp. *evanescens*	Charatsunai, Muroran, Hokkaido	1986-1988.
Pelvetia wrightii	Charatsunai, Muroran, Hokkaido	1986-1988.
Analipus japonicus	Charatsunai, Muroran, Hokkaido	1986. 12.-1988. 7.
Scytosiphon lomentaria	Charatsunai, Muroran, Hokkaido	1987. 2.-1988. 7.
Sphacelaria sp.	Culture collection of Texas Univ.	
Pilayella littoralis	Culture collection at the Institute of Algological research, Hokkaido Univ.	

seawater and stored at 18°C in constant illumination of 3000-4000 lx (12-14 W/m²) overnight. After about 4 hr incubation at 4°C in a dark condition, eggs and sperms were released from the receptacles by immersion of the plants in cold seawater. Around the released eggs, many swimming sperms could be observed under the light microscope. Fertilization was allowed to proceed over a 30-60 min period. The medium used for egg and sperm release was either unenriched seawater or a complex of seawater mixed with a small amount of GeO_2 (5-10 mg GeO_2/l); zygotes for the culture of control or GeO_2 treatment were washed for several times with these respective media. A stock solution of GeO_2 was usually prepared by dissolving 500 mg GeO_2 in 200 m*l* boiling aqueous 1N NaOH in a glass beaker, adjusting the solution to pH 7.8-8.0 with 1N HCl and finally diluting it into 500 m*l* with distilled water (TATEWAKI and MIZUNO 1979). The fertilization ratio was measured after staining with Evans blue solution (1%) by which the cell wall of only fertilized eggs was stained to judge. After fertilization examined, the zygotes were collected and placed randomly and sparsely in 60 mm x 30 mm Petri dishes containing 30 m*l* PESI medium (TATEWAKI 1966).

For the experiment on *Fucus distichus* and *Pelvetia wrighii*, a serial range of GeO_2 of 0, 5, 10, 20, 30, 50 and 70 mg GeO_2/l was set up. High concentrations of GeO_2 were used to determine the lethal dose with the species. Germination ratios under various GeO_2 conditions were measured after 18-24 hr incubation. The observation on the morphology was done with a light microscope.

After culturing under the different Ge conditions for a certain period of time, plants

which had already been affected to different degrees were moved into the fresh medium without GeO_2 to see whether and how they would recover from the damage.

In *Analipus japonicus* and *Scytosiphon lomentaria*, zoospores of *A. japonicus* and gametes of *S. lomentaria* were released by immersing the matured thalli into cold seawater after placing them at 4°C in the dark for several hours. Droplets containing swarmers were pipetted onto cover glasses. When the swarmers settled, cover glasses were placed in 85 mm x 18 mm glass Petri dishes containing 30 m*l* PESI medium. GeO_2 concentrations used for these two species were 0, 2, 5, 10 and 20 mg GeO_2/l for each group.

Filaments of *Sphacelaria* sp. and *Pilayella littoralis* were cultured in PESI medium with GeO_2 of 0, 0.5, 2 and 5 mg GeO_2/l.

All of the cultures of brown algae were kept under the condition of 14°C with a 14: 10 LD photoregime. Light intensity ranged from 3000 lx to 4000 lx (12-14 W/m^2).

Results

1. *Fucus distichus* and *Pelvetia wrightii*.

F. distichus zygotes incubated for 8 hr were still round shaped, and no germination was observed. After 18-24 hr incubation, the rhizoid protrusions were found appearing at one side of the zygotes in control culture (Plate 1, A), and the zygotes became attached to the substrate (Petri dish). The germination rates of *Fucus distichus* and *Pelvetia wrightii* zygotes in various conditions were measured. The results are shown in Figure 1. Under 2 mg GeO_2/l, the germination rate of *Fucus distichus* zygotes decreased to 82% of the control, and under 5 mg GeO_2/l, to 72%. When Ge concentration was 20 mg GeO_2/l, the rate was down to 35%. Similar tendencies were seen with the zygotes of *Pelvetia wrightii*. Within the

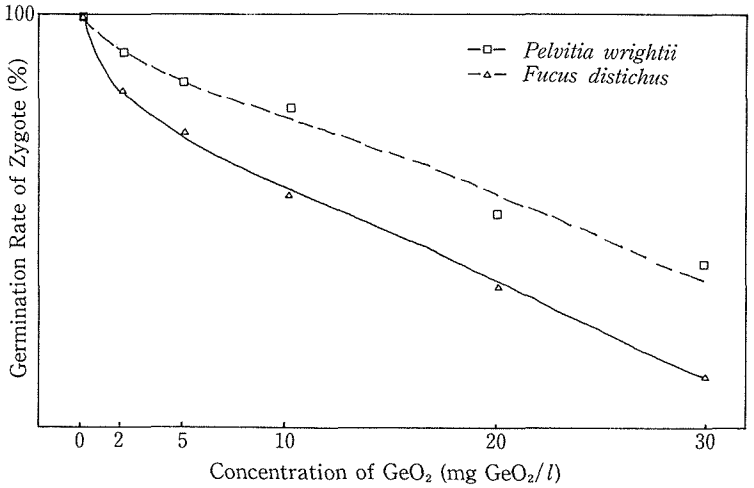

Fig. 1. Effect of GeO_2 on germination of *Fucus distichus* and *Pelvitia wrightii* zygotes

extent of 5-30 mg GeO_2/l, the zygotes could germinate, although more slowly (Plate 1, B). When GeO_2 concentration was as high as 50-70 mg GeO_2/l, the germination of zygote ceased completely (Plate 1, C). In the culture with GeO_2 treatment, the zygotes seemed to have difficulty in attaching to the substrate (Petri dish), and as the concentration of GeO_2 became higher, the ratio of zygotes unable to adhere increased. In the higher concentrations of GeO_2 (20-30 mg GeO_2/l), germinated zygotes were sometimes found to be broken at the end of developing rhizoid protrusion, and cytoplasms flew out of the hole.

A week later, embryos of *Fucus distichus* developed into a multicellular plant with primary and secondary rhizoids (Plate 1, D). As the GeO_2 concentration was increased, this rhizoid formation was affected to different degrees. Secondary rhizoids were rarely observed when GeO_2 concentration was 5-10 mg GeO_2/l (Plate 1, E). In the culture with 30 mg GeO_2/l, the zygotes could not produce any rhizoids and showed an obvious abnormal appearance (Plate 1, F). All of them looked somewhat loose due to cell swelling. The zygotes in the cultures of 50 mg GeO_2/l could divide, but no differentiation of the thalli or rhizoids was found (Plate 1, G). As the time of treatment lengthened, the damage became increasingly obvious. Although the embryos developed almost as large as in the control culture after 16 days, some cleavages of the thallus were detectable at the apical and lower parts of the embryos cultured in 5 and 10 mg GeO_2/l conditions (Plate 1, H,I). The rhizoid was also lacking in the thallus. The embryos in 30 mg GeO_2/l condition could not develop into a complete thallus but a mass of cells even after one month in culture (Plate 1, J). The cells at the broken points of such embryos had a rounded shape and were not connected with each other as strongly as those of control. Irregular masses of cells in 50 mg GeO_2/l slowly underwent division and under the concentration of 70 mg GeO_2/l, the zygotes kept a swollen rounded shape and sometimes they could divide. Most of the cells were able to remain alive for a long time.

Affected cells generally looked swollen with lighter color. Their cell walls became very thick and their thick parts were translucent (Plate 1, K). Such a thickening did not necessarily occur through all the cell wall, but more heavily at the surface part. Similar inhibitory effects were also observed in *Pelvetia wrightii*.

As described above, an obvious damage occurred after long culture in media with GeO_2. In *Fucus distichus*, affected thalli in 30, 50 and 70 mg GeO_2/l conditions were transferred back into the fresh medium without GeO_2. Twenty days later, certain recoveries were first detected in samples from 50 to 70 mg GeO_2/l conditions; small masses of cells produced many protrusions and rhizoids grew out in almost the same direction (Plate 1, L). This recovery seemed more obvious in samples in 70 mg GeO_2/l medium. After 1-month culture, new thalli appeared from the the mass of cells to an opposite direction with the rhizoids and several protrusions of these thalli could be produced from different places at the same time. These protrusions could develop into complete plants after about 45 days (Plate 1, M). However, the embryos inhibited in 30 mg GeO_2/l somehow took much more time to recover from the

damage. Thalli of *Pelvetia wrightii* treated with 10 mg GeO_2/l for about 3weeks showed similar recovering ability as well.

2. *Analipus japonicus* and *Scytosiphon lomentaria*.

Similar inhibitory effects were observed in *S.lomentaria* and *A.japonicus*. At the beginning (within about 1 week), a delay of germination was recognized after GeO_2 treatment and the formation of germ tubes was stopped (Plate 2, A,B ; Plate 2, E,F). About 2 weeks later, germlings of *S.lomentaria* developed into uniseriate filaments in control culture (Plate 2, C), while the inhibitions appeared in the formation of filaments in the culture treated with GeO_2. The filaments were much shorter in 5-10 mg GeO_2/l medium, and cells of filaments became swollen and round in shape (Plate 2, D). All the filaments seemed to be weakly formed. When GeO_2 concentration became as high as 20 mg GeO_2/l, no filamentous growth occurred, but only small masses of several cells were formed. In the case of *A.japonicus*, germlings developed into a prostrate disc in about 3-week-old culture (Plate 2, G). In the presence of 2 mg GeO_2/l, the disc became smaller due to Ge inhibition, and at 5 mg and 10 mg GeO_2/l concentrations, the disc could not even be formed (Plate 2, H). The appearance of the affected cells was the same as that of *S.lomentaria*.

3. *Sphacelaria* sp. and *Pilayella littoralis*.

These two filamentous species seemed to be more sensitive to Ge treatment. They were affected by very low Ge concentration from 0.5 mg GeO_2/l. The inhibition was firstly characterized by an enlargement of the apical cell and new bud cells in *Sphacelaria* sp. (Plate 2, J), sometimes these cells burst at the high Ge concentration. In comparison with the long and straight filaments in the control, affected filaments had more small branches which were not well grown (Plate 2, I, K). The lower parts of the filaments turned to dark in color.

In the case of *Pilayella littoralis*, a cell swelling was not limited in the apical and budding cells. The cells could become abnormally larger in any random part of the filament (Plate 2, L, M). The swollen cells usually looked transparent and because of inhibition in these cells, the filaments were crooked.

Discussion

Inhibition from low doses of GeO_2 (0.5, 2 and 5 mg GeO_2/l) was recognized in six brown algae under the present culture condition. Most of the morphological changes due to GeO_2 treatment are similar to those described by previous workers (TATEWAKI and MIZUNO 1979, MARKHAM and HAGMEIER 1982). In *Fucus distichus* and *Pelvetia wrightii*, GeO_2 delays embryonic germination from the beginning. At that stage, *P.wrightii* is somehow less influenced than *F.distichus*. In addition, the first visible inhibition is clearly seen in the elongation of the rhizoid under the light microscope. Zygote cell breakage from the tip of the rhizoid protuberance happens at concentrations of 20-30 mg GeO_2/l, which allow embryo

germination. Meanwhile those zygotes which do not germinate due to the high GeO_2 concentrations (50-70 mg GeO_2/l) are not destroyed. The facts suggest a closer connection between the action of germination and GeO_2 inhibitory effect. The cells become especially sensitive to the attack by GeO_2 when they are tending to change shape or structure.

A longer treatment (within 1 week) with 5-30 mg GeO_2/l demonstrated the same inhibition in the rhizoid formation. The damages were obvious after 1-3 weeks treatment and the degree of this inhibition of rhizoid depended on the GeO_2 concentration. However, the growth at the thallus side could proceed to some extent in the same conditions. Thus, a thallus, if it can still be called a thallus, can form without any rhizoid development (Plate 1, F,J). The imperfection and the lack of rhizoid at this stage have the same implications as seen above in embryo germination and GeO_2 inhibition.

Detectable thallus damage appears after 1-3 weeks culture with GeO_2. It happens later than at the rhizoid part. The thallus collapses at some place, often in the lower part. At that stage, the embryo should have developed into a multicellular thallus, perhaps having started to differentiate into cortical and medullar tissues. This definite point in time from which the thallus begins to collapse might mean that there is some relation between GeO_2 inhibition and differentiation of the thallus. The abnormally swollen thallus suggests that some changes are taking place within it.

The embryos of *F.distichus* and *P.wrightii* can tolerate GeO_2 concentration as high as 50 mg GeO_2/l without all dying. Although the embryos cannot germinate, they slowly continue cell division. That there is no cell breakage seen at concentrations of 20-30 mg GeO_2/l seems to be because of the cessation of germination. When the number of cells have increased to some extent, the masses of cells in the presence of 50 mg GeO_2/l will eventually break. Cells usually have thickened walls and abnormally swollen shapes. The appearance is the same as that in the other lower GeO_2 conditions of 5-30 mg GeO_2/l. In addition, ability to recover from GeO_2 inhibition has been testified in these two species. Ungerminated cells in 50-70 mg GeO_2/l conditions usually have vigorous recovery. Therefore, from the initial changes in embryos under the various GeO_2 conditions, it becomes clear that GeO_2 is not a vital inhibitor within the range of 5-70 mg GeO_2/l. It does not inhibit cell division, but rather a component necessary for embryo germination and tissue differentiation.

For *Analipus japonicus* and *Scytosiphon lomentaria* as well, GeO_2 causes a delay in spore germination. In the later culture, a similar inhibitory effect appears in almost all cells. Affected filaments consisted completely of swollen cells from one end to another. This result might be explained by the way of growth in the species; they perform diffuse growth, producing a prostrate disc. Every cell is thought to be in a constant state of growth, and therefore more sensitive to GeO_2 treatment.

The morphological changes of thalli of *Sphacelaria* sp. and *Pilayella littoralis* also show a relation between Ge inhibition and their growing patterns. *Sphacelaria* sp. performs a typical apical growth and the damage occurs in the apical and budding cells. Similarly, this

damage happens randomly in cells of any place in *P. littoralis* filaments. Actually, these cells first affected by GeO_2 might be growth cells, because the species has a intercalary growth.

A generality can be summarized from all the observations on brown algae of various constitutive type: Regardless of the thallus organization, cells ready for movement are more sensitive to GeO_2. This result led to the conclusion that there is a close connection between Ge inhibition and the physiological state of the cell.

This conclusion obviously emphasizes the importance of cell movement in the inhibition process. In general, cell movement, such as spore or embryo germination, vegetative cell growth, bud initiation and development, and cell differentiation are certain to be involved in a serial change of the cell itself, eventually the expansion and constituent specialization of cell wall. As the motive force for cell growth, several factors in cell proper have been generally related (WADA 1981): The first is high osmotic pressure of the cell and cytoplasmic pressure (turgor), which is derived from the former. These pressures are regulated chiefly by the vacuoles in cytoplasm and they control the rate of cell growth. Secondly, the tension put on the cell may assist elongation. Thirdly, continued synthesis of new wall materials and incorporation of these materials into cell wall are indispensable for irreversible expansion. The incorporation of new materials not only occurs at the inner parts of the cell wall, but also within the wall. And fourthly, to accommodate the new material deposition, a certain change in the existing old wall is necessary. This process might cover some structural and chemical changes of the wall.

With some species of algae, there are also studies about cell expansion. The cytoplasmic pressure has been verified to be necessary by the experiments in brown algae, *Sphacelaria furcigera* (BURNS *et al.* 1982b), *Fucus vesiculosus* (TORRY and GALUN 1970, VREUGDENHIL *et al.* 1976) and *Fucus gardneri* (NOVOTNY and FORMAN 1974). It has been observed that there is a loss of vacuoles and increase in cytoplasmic mass and density before bud initiation in *S. furcigera*, which might serve in raising cell osmotic pressure. In the same way, a high osmotic sucrose solution can cause the cessation of germination in *Fucus* embryo and make an 'apolar' embryo, although it has no effect on the establishment of axis and new wall material production for germination (TORRY and GALUN 1970, NOVOTNY and FORMAN 1974, VREUGDENHIL *et al.* 1976). Rhizoid formation of *Fucus* embryo has been found to biochemically necessitate the synthesis of protein (QUATRANO 1968). This rhizoid formation of *Fucus* embryo and the bud initiation of *Sphacelaria furcigera* are always accompanied with the supply of various polysaccharides (QUATRANO and STEVENS 1976, BURNS *et al.* 1982a). BURNS *et al.* (1982b) have found that bud initiation and development involve loosening of the outer cell wall and change in the layering pattern. They have furthermore suggested that the abundance of protein within the bud cell wall may be the enzymes which participate in cell wall loosening and/or synthesis (BURNS *et al.* 1984). In other words, every process happening during cell growth is a part in the metabolism and they are closely related with each other. The lack of any of these preparations will result in some destruction in cell

expansion.

In the brown algae examined during this study, the swelling of Ge affected cell has been found to be the most apparent and common characteristic. From this, firstly, the existence of cytoplasmic pressure can be strongly suggested. Also, the breakage of germinating spores and elongating apical cells shows some outward force inside the cytoplasm. Secondly, the continuing cell division might be indicative of the production of new materials for increase in numbers of cells. Although it can not be concluded that there is no inhibition in synthesis of cell wall material, it appears likely, since there is no tendency for inability to carry out cell division due to lack of synthesis of some materials. The implication of this is that the cell's basic mechanism of material synthesis still functions. Thus, thirdly, the possible site where germanium acts can easily be thought to be the cell wall proper. In general, cell shape and direction of growth are controlled by the way in which structural microfibrils are arranged in the cell wall. Any damage in the construction of this cell skeleton will bring about the deformation cell and the loss of a definite direction of growth (BUGGELN and GALUN 1981). In fact, an obvious thickening of cell wall in affected cells and a loss of intercellular connection have been observed in addition to the change of the cell shape in the brown algae examined here. The appearance of affected cells is somewhat similar to ones treated with some cellulose-formation-inhibitory dye, such as colchicine (HOGETSU and SHIBAOKA 1978). Therefore, it seems that the formation of the cell wall is affected because of the difficulty in some wall material deposition. The swelling and breaking of the cell also strongly suggest that the cell wall loses its strength when attacked by GeO_2.

In the brown algae, cellulose, alginic acid and sulphated glucuronoxylofucan have been generally defined as the structural polysaccharides (MCCANDLESS 1981). Among these three polysaccharides, alginic acid is specific to the group, which has been found in all species of the brown algae examined though the amount differs in different species, but not in any other plant tissues. It is known that cellulose, the fibrous structural element of most plants, forms quantitatively only a minor component of brown algae (CRONSHAW *et al.* 1958), while the crystalline compounds of alginate has been suggested to play an important structural role in addition to its ion exchange function in cell walls (FREI and PRESTON 1962). During this study, the observation on several brown algae with the light microscope has put the focus of the problem on the cell wall. Therefore, considering the special cell wall component of brown algae together with these results, the chain of alginate fibrils can be thought of as the possible site of germanium attack, and the structural aberration might come from the destruction of the chain. To ascertain the possibility more investigation is warranted, perhaps on the ultrastructure of cell wall.

Chapter 2. Electron microscopic investigation on the effects of GeO_2 on several brown algae

Introduction

Under the light microscopy, obvious morphological damage due to GeO_2 treatment was recognized in many brown algae (TATEWAKI and MIZUNO 1979, present study, chapter 1). In the present study (chapter 1), it is found that there are some certain places easily attacked by germanium and there are morphological changes of the cells and thalli in the different species. The generality in having high sensitivity to GeO_2 at growth regions suggests the connection between the physiological states and the movement of cells and germanium inhibition. Meanwhile, the similarities in morphological changes focuses the possible reason of thallus damage on some structural inhibition, such as the difficulty in cell wall formation. All these results make it necessary to carry out a more detailed study of the ultrastructure because the observation by the light microscope can not give direct evidence for this speculation.

With the diatoms, there are some reports about the electron microscopic details of germanium inhibition on cell wall formation (AZAM *et al.* 1973, AZAM 1974, AZAM and VOLCANI 1974, CHIAPPINO *et al.* 1977). An incorporation of Ge into the cell wall was detected at low Ge/Si ratios without toxicity, and a morphological manifestation of Ge inhibition at higher Ge/Si ratio was observed to be cell wall aberration (AZAM 1974, CHIAPPINO *et al.* 1977). The competitive inhibition of Ge as an analogue of Si in the diatom observed in cultural experiments was relevantly explained by the ultrastructural investigation.

In brown algae, however, nothing is known so far about the ultrastructure of the cells and thallus affected by germanium compounds. Now that there is such a morphological aberration under the light microscope, some details in the ultrastructural change must be detectable under the electron microscope at the same time. In order to obtain more information to explain the phenomenon under the light microscope and to verify the hypothesis suggested in chapter 1, an examination of ultrastructure of GeO_2 affected brown algae was made with the electron microscope.

Materials and Methods

Materials

The six species of brown algae described in chapter 1 were examined with an electron microscope. Specimens in different stages of development and treated with GeO_2 for definite time were chosen and fixed for electron microscopy.

Methods

1. *Fucus distichus* and *Pelvetia wrightii*.

Zygotes of 3-4 hr and 8-9 hr after fertilization were round shaped and not attached to the

substratum. They were easily collected by pipetting. Germinated embryos were usually firmly attached to the bottom of the dishes in control culture. In order to keep the embryos from being injured, glutaraldehyde fixation was carried out before scraping them off from the dish with a pipette. The embryos were accumulated in 15 mm x 100 mm centrifuge tubes and fixed in 3% glutaraldehyde, made up in 0.1M cacodylate buffer with 2% NaCl and 0.1% $CaCl_2$. Fixing was generally done for about 3 hr at 4°C, then specimens were washed with the buffer several times during a couple of hours. The specimens were post-fixed with 2% OsO_4 in the same buffer for 3 hr at 4°C, rinsed in the buffer, *en-bloc* stained with 1-2% uranyl acetate for 20-30 min and dehydrated in graded series of acetone (10-100%). Finally, they were infiltrated and embedded in SPURR's epoxy resin (SPURR 1969). Polymerization was done at 70°C for about 15 hr. Sections were made with a diamond knife, stained with uranyl acetate and REYNOLDS' lead citrated (REYNOLDS 1963). Observations were carried out with a Hitachi H-300 electron microscope.

In order to avoid the artifacts that might occur during the conventional chemical fixation and obtain images with more realistic representation of cell structure, the method of rapid freeze fixation and freeze substitution was also tried. Specimens were placed on the formvar-film-covered thin gold wire loops (ϕ5 mm) and then the excess medium was removed with a wedge of filter paper. These specimen holders were rapidly plunged into a well of liquid propane which was kept at −196°C in a liquid nitrogen bath for a few seconds, and then quickly transferred into liquid nitrogen. Freeze fixation was followed by freeze substitution where the specimens were exposed to a chemical fixative during dehydration. The chemical fixative used was made up of 1% OsO_4 in acetone with or without 0.2% uranyl acetate and kept in acetone together with dry ice (about −80°C). The loops with fixed specimens were placed in precooled Pyrex vials containing 2 m*l* fixative. Substitution was carried out at −80°C for about 18 hr. After that the vials were allowed to warm up slowly in stages to room temperature, pausing at both −20°C and 4°C for 2 or 3 hr. After several rinses in acetone, the specimens having fallen from or attaching to the formvar film were infiltrated and embedded in SPURR's resin (SPURR 1969).

An extraction of the sulphated polysaccharides was carried out by immersing the glutaraldehyde fixed samples in 0.01N HCl with 1% $CaCl_2$ at room temperature for 6 hr and then at 30°C for 18 hr. One percent Na_2CO_3 was used to extract alginic acid. This treatment was carried out at 60°C for about 18 hr. Both treatments were followed by post-fixation with OsO_4.

2. *Analipus japonicus* and *Scytosiphon lomentaria*.

For TEM fixation, swarmers of *A. japonicus* and *S. lomentaria* were inoculated on 18 mm x 18 mm cover glasses and formvar-film-covered gold loops and then incubated under the same condition as described in chapter 1. After a period of culture, the cover glasses and the loops were directly fixed by means of chemical fixation and rapid freeze fixation respective-

ly. The procedure was similar to what has been described above. To reduce the effects of phenolic material, 1% caffeine was added in the buffer before the post-fixation during the chemical fixation. The cover glasses with samples were embedded in SPURR's resin and finally glasses alone were peeled off from the resin by immersion in liquid nitrogen.

3. *Sphacelaria* sp. and *Pilayella littoralis*.

The fixation of *Sphacelaria* sp. and *Pilayella littoralis* was similar to *Fucus* and *Pelvetia*. Freeze fixation and substitution were also tried on the two species, but it seemed more difficult than with other species. The filaments were placed on formvar-film-covered loops as it was necessary to keep them as separate from each other as possible. Other procedures were the same as those mentioned above.

Results

1. *Fucus distichus* and *Pelvetia wrightii*.

F. distichus: Fertilized zygotes of 4 hr were round in shape and had a very thin cell wall surrounding the cells. The wall was of uniform thickness and there were no individual layers being detectable at the stage (Plate 3, A). The cell wall appeared to consist of fibrous materials. Zygotes cultivated for 4 hr in the presence of GeO_2 (10 mg GeO_2/l) showed little difference from the controls. A thin cell wall surrounded the zygote and other cellular organelles stood the same way as those of the control (Plate 3, D).

In the zygotes cultured for 18-20 hr, rhizoids became apparent as a protuberance on the shaded side of the cell. The thickness of the cell wall increased and it was differentiated into individual layers. Two layers could be more clearly observed (Plate 3, B). The inner layer was composed of bundles of fibrils which chiefly ran parallel to the cell surface and was at the inner part less organized, interrupted by some amorphous inclusions. The deposits of such material were frequently seen between the plasma membrane and the inner fibrous layer, especially at the rhizoid tip. An amorphous layer formed the outermost surface of the cell (Plate 3, B), but it was frequently eroded during fixation and dehydration procedures and therefore could not often be well seen. There was always an extension of this amorphous layer at the rhizoid tip (Plate 3, C). The first cell division occurred about 24-30 hr after fertilization, cutting off a rounded thallus cell and an elongated rhizoid cell which continued in its attachment to the substratum.

In the culture with GeO_2 (5-20 mg GeO_2/l), the zygote germination rates were retarded, and sometimes the zygote broke from the tip of the protuberance. The observation on 24-hour-old zygotes by electron microscopy revealed some structural damages in the constitution of the cell wall. The cell had a nucleus in the central region and many Golgi bodies and ER could be observed throughout the cytoplasm, especially near the nucleus. Chloroplasts, mitochondria, vesicles and physodes were distributed as in cell of control culture. No obvious change could be recognized in the structure of these organelles. An extraordinarily

thickened cell wall was observed in the full view of the affected zygote. This increase in cell wall thickness occurred often at the thallus side, while on the other hand, the wall at the rhizoid tip became very thin. A magnification of the cell wall at the upper part of thallus was shown in Plate 3-E. From the inner side of the cell wall, the constitution of the wall began to become loose and the organization of the layers was affected to be irregularly arranged. It seemed that the fibrils mixed with each other in a disorderly fashion instesd of organizing into a bundle. As a result, many hollow-like spaces were formed and they scattered among the fibrous layer. These hollow-like spaces seemed to be full of amorphous materials, but sometimes they more or less disappeared, likely due to the fixation and dehydration procedures. At the low Ge concentration (5 mg GeO_2/l), the outer layers could remain to some extent in a quasi-normal state. A high Ge concentration (20 mg GeO_2/l), however, caused damage throughout the wall. The observation on the rhizoid showed a loss of some wall materials after having been affected by Ge. As shown in Plate 3-F, the cell wall became very thin. Apparently, only the inner fibrous layer of the wall remained, while there was a complete lack of outer layers of the wall, especially the amorphous layer.

After 2 or 3 weeks culture in the control condition, the embryo developed into a multicellular thallus. The differentiantion of cortical and medullar cells became obvious (Plate 4, A). Cortical cells were relatively small and had dense cytoplasms. There were many chloroplasts and a large number of physodes in the cytoplasm. A large nucleus could be seen in the central region of each cell and there were many mitochondria located chiefly at the surface of the cell in longitudinal section (Plate 4, B). Medullar cells were larger than cortical cells and characterized by having large vacuoles. Chloroplasts were evenly distributed along the cell wall, and not many physodes could be observed.

TEM observations verified the ultrastructural pattern of the cell wall. The outer walls of cortical cells was distinctive, being composed of four layers (Plate 4, B). The innermost one (CW4) was continuous with the basal walls of the cells and consisted of bundles of fibrils. The next layer (CW3) had abundant amorphous material within a fibrillar network. These two layers (CW4 and CW3) were relatively thick. Outside the CW3, there was a layer (CW2) of mixed fibrous and amorphous substances, which was very thin, so that it was hardly detectable. The outermost layer (CW1), covering the whole thallus surface, appeared almost amorphous. This layer was also thin at the thallus side and was also sometimes not visible, but became thicker near the rhizoid.

The medullar cell wall and intercellular matrix ultrastructures were clearly observed in the medulla, where two regions could be identified (Plate 4, C). The first region formed the real wall around the cell. It was continuous with the matrix part and composed of fibrillar bundles which sometimes extended from the wall into the surrounding matrix. The second one contained abundant amorphous materials and appeared to be crossed by fine, individual fibrils in its middle part. The area close to the cell wall was consisted of more uniform amorphous substance.

In the affected multicellular embryos grown in the presence of GeO_2 (5-20 mg GeO_2/l), the damage appeared mainly in the cell wall. Many swollen spaces were found in affected cell walls, especially in the fibrous layers (Plate 4, G). The alignment of fibrils was altered to become random, and among the fibrils there were large amounts of irregularly arranged materials. Therefore, the entire wall became abnormally thick and very loosely organized. In the innermost part of the cell wall, especially that part on the side towards the cell surface, obvious anomalous wall thickening was frequently observed (Plate 4, E, F). Sometimes it occupied half of the cell, and in some cases physode-like materials were included in the thickened parts. In these anomalously thickened parts, weakly crystallized fibers could be seen. As exposed to GeO_2 for a long period, the affected outer layers tended to break away from the cell. In the medullar part of the thallus, the formation of intercellular matrix was severely affected and no intact matrices could be formed (Plate 4, H). The cells then lost their interconnection and became separated from each other, due to which the thallus collapsed (Plate 4, D). However, even when cells partly separated from each other, the innermost layer of the walls didn't break away. The cells became round shaped and stay alive for a long time.

Sulphated polysaccharide extract removed both the outermost wall layer of the thallus and the amorphous materials in the intercellular matrix (Plate 5, A, B). The disorder of fibrils in the wall and accumulation of the amorphous materials within the fibrous layer due to GeO_2 treatment were suggested by results of the extraction procedure (Plate 5, C, D). Extraction of alginate showed the same results (Plate 5, E-G).

Other than the changes in cell wall, it seemed that there were more vesicles in Ge affected cells, in which some fibers existed (Plate 6, C, D). But no obvious difference in structure was recognized in Golgi bodies compared with those in the control cells (Plate 6, A, B).

The ultrastructure of newly developed thalli from affected cells was observed to be the same as normal thallus cells, while the old affected parts remained in a damaged state (Plate 6, E). The layers of the cell wall materials in the new thallus were regularly arranged. However, the old affected cells seemed to have some difficulty in recovering quickly and completely from the inhibition. These cell walls still looked abnormal even with long term cultures. Especially, the matrix region seemed to have difficulty in recovering from the damage (Plate 6, F).

P. wrightii: Similar inhibition by GeO_2 was also observed in *Pelvetia wrightii*. Zygotes of 4 hr in the presence of GeO_2 (10 mg GeO_2/l) showed little change and damage in the organelles including the cell wall. A single, thin cell wall formed around the cytoplasm. However, in the zygote of about 18-hr culture with GeO_2, disintegration of fibrils and the abnormal thickening of the whole wall were observed. Compared with the cell wall of control, in which three layers were recognized (Plate 7, A-C), the wall after GeO_2 treatment had a loosened fibrous layer, and the wall became abnormally thin at the rhizoid tip without the outer amorphous layer (Plate 7, D-F). Two or three weeks later, embryos developed into

multicellular thalli and the constitution of the cell wall both at the thallus surface and the matrix region were the same as in *F. distichus* (Plate 8, A-D). However, the thalli appeared to different degrees in the culture with GeO_2 (5-20 mg GeO_2/l). They tended to break from the inner medullar regions (Plate 9, A). Under the electron microscope, heavy damage was recognized in the cell wall and the intercellular matrix regions. The layers of microfibrils became very loose and the wall looked abnormally thick. Within the loosened fibrous layers, a large amount of amorphous materials could be observed (Plate 9, B-E). The results of sulphated polysacchride extraction showed disordering of the fibrous layers and accumulation of amorphous materials (Plate 10, A-D). However, not any inclusion of physode into the abnormally thickened cell wall was detected in *P. wrightii*. Moreover, the appearance of Golgi bodies did not change markedly compared with those of the control, though it seemed that there were more vesicles throughout the cytoplasm.

2. ***Analipus japonicus*** and ***Scytosiphon lomentaria*.**

A. japonicus: Two-day-old germlings of *A. japonicus* had germinated by a germ tube and had a two-layered wall (Plate 11, A). The inner layer of the cell wall was mainly composed of fibrils while the outer layer was of amorphous material. In the case of treatment with GeO_2, formation of the germ tube was affected and stopped at the concentration of 20 mg GeO_2/l, and the cell was heavily swollen (Plate 11, D). With the electron microscope, a distinct inhibition was recognized in the cell wall. The fibrils in the wall became loosened and irregularly arranged compared with the control. Within the loose fibers, there were many amorphous materials. However, at the position from where the swarmer would germinate, the wall was remarkably thin. It looked like that only a few fibers existed. At this stage, other organelles of the cell including Golgi bodies did not show much difference from the control cell.

After 2 weeks culture, germlings developed into prostrate disc and in each cell, there were a nucleus, some mitochondria, several Golgi bodies around the nucleus and several chloroplasts in the periplasm. The cell wall could be approximately recognized as having two layers (Plate 11, B). The inner layer was composed of fibrils and the outer layer was amorphous. In the internal cell of the prostrate disc, the wall was relatively thin and between the cells was a matrix region (Plate 11, C). Many fibrils were extending into the amorphous matrix.

The presence of GeO_2 (5-20 mg GeO_2/l) caused the disordering in the constitution of the cell wall. The fibrils became loose and amorphous materials tended to break away from the thallus surface (Plate 11, E). The same damage also appeared in the internal cell wall and the matrix could not normally formed (Plate 11, F). In this way, the formation of the cell wall was inhibited, thereby resulting in a malformation of the thallus. No obvious changes were seen in other organelles except that more small vesicles seemed to be appearing in the cytoplasm of Ge affected cells than in the control cells (Plate 11, G-I).

S. lomentaria: Similar inhibition was observed in *S. lomentaria.* The damage in the cell wall was recognized in 2-day-old germlings. At the germinating tip, the wall became extremely thin and the loosening of the fibrils occurred at the other part of the wall. But no obvious change of other organelles including Golgi bodies was detected. Two or three weeks later, germlings developed into the multicellular thalli and the same inhibition of Ge in the cell wall could be obviously observed everywhere as in *A. japonicus.*

3. *Sphacelaria* sp. and *Pilayell littoralis*.

Sphacelaria sp.: In control culture, the apical cell of the filament had one nucleus in the central region and the cell had a dense cytoplasm with many chloroplasts and mitochondria, especially in the distal part (Plate 12, A). A large number of Golgi bodies scattered throughout the cytoplasm randomly. There were no obviously large vacuoles in the apical cell, while in other cells prominent vaculoes and fewer chloroplasts existed. The cell wall near the apex of the apical was relatively thin. It consisted of a very thin outer layer and thicker inner layer in which some fibrils could be seen (Plate 12, B). At the lower region of the filament, however, four layers were recognized (Plate 12, C). From the outermost to the innermost layer they were designated CW1, CW2, CW3, and CW4. The layers CW2 and CW4 were composed mainly of fibrils, while CW1 and CW3 were amorphous. The layers CW3 and CW4 extended into the cross walls of the axis cells, and the outer layers CW1 and CW2 were spread over the filaments.

The present examination revealed some structural changes in the cell wall when the filaments were exposed to Ge. When the inhibition was not very serious, fibrous layers (CW2 and CW4) became a little wavy in arrangement and the wall began to become loose (Plate 12, E). With a long term culture in the GeO_2 medium, the cell wall lost general constitution. The fibrils in the wall tended to break and separated from each other. Within the abnormally rounded apical and budding regions, several misshapen cells were found sometimes, and the septum between them were also irregularly formed (Plate 12, F, G). In general, the cells of the basal region of the filament seemed stronger, and they were affected only by long term treatment with GeO_2 (Plate 12, H). Within the disordered fibrils, inclusions of electron-dense granules were sometimes observed.

However, no distinct damage was detected in organelles in the cells affected by GeO_2. A large number of chloroplasts, mitochondria and Golgi bodies were located in the apical part as in the control cells, except that more vacuoles appeared (Plate 12, D).

P. littoralis: In *Pilayella littoralis,* the normal cell had a nucleus in the medial position and there were several Golgi bodies and vesicles around the nucleus (Plate 13, A). Chloroplasts located in the peripheral region of the cell and most areas of the cell were occupied by large vacuoles. The cell wall near the apex of the apical cell was thin and made up of two layers; the inner fibrous layer and outer amorphous one (Plate 13, B). The basal region of the filament had a four-layered wall as in that of *Sphacelaria* (Plate 13, C).

A long term treatment with GeO_2 caused a similar inhibitory effect on cell wall. An abnormal thickening occurred everywhere in the wall, especially at the corners of the cell (Plate 13, D). In the detail, the fibrils were detected to be loose and the spaces between the disordered fibrils were full of amorphous materials (Plate 13, E). These damages could be observed at any region of the cell wall and any cells of the filament.

Discussion

1. *Fucus distichus* and *Pelvetia wrightii*.

The observation on ultrastructure revealed obvious damage in cell wall structure of *Fucus distichus* and *Pelvetia wrightii*. The disorder of fibrils and the random accumulation of amorphous materials happened both in newly germinated embryos and multicellular thalli.

As for the composition and development of cell walls of *Fucus* embryos, it has already been reported that a fibrous wall with the same property is deposited around the naked zygote soon after fertilization. This wall is mainly composed of alginic acid and cellulose. Wall thickness increases gradually by deposition of new wall materials (QUATRANO and STEVENS 1976). From 4 to 12 hr after fertilization, a lot of amorphous materials, sulphated polysaccharide, begin to deposit preferentially in the presumptive rhizoid pole. This deposition usually causes the outer layer of the wall to extend in that region (QUATRANO and STEVENS 1976, NOVOTNY and FORMAN 1974, 1975, CALLOW *et al.* 1978). These sulphated polysaccharides are thought to have the function of assistance to the zygote in attaching to the substrate. The present study shows that in newly germinated embryos of 18–20 hr cell wall at the thallus side becomes loose due to GeO_2 effect, and fibrils are in a disorderly arrangement. Within that fibril arrangement there exist many hollow-like spaces. This disorder in the wall organization causes the swelling of the whole wall and probably makes the wall very weak. In contrary to the normal embryo, an extreme thinning of cell wall occurs at the rhizoid tip when cultured with GeO_2; the lack of amorphous layer there is especially evident. From this structural change in rhizoid tip wall the following can be explained: Under the cytoplasmic turgor, the rhizoid tends elongate, and this process requires rapid deposition of new wall materials and continuing formation of new wall. Thus, when due to GeO_2 inhibition this deposition and formation can not be accomplished normally and timely, the results are thinning of the wall and tip breakage. Moreover, the lack of the outer amorphous layer might be the reason for the embryos having not attached to the substrate.

By electron microscopy and light microscope histochemical techniques, cell walls of vegetative thallus and those of mature reproductive cells of many brown algae have been observed to be composed of three to four well-defined layers, mainly composed of alginic acid, sulphated polysaccharide and a mixture of these substances (MCCULLY 1968, EVANS and HOLLIGAN 1972, MARIANI *et al.* 1985). Alginic acid is confirmed as a major constituent of the cell wall and sulphated polysaccharide is chiefly located in the matrix region between medullar cells. The present observation by electron microscopy is in agreement with those

previously reported. In the 2-3-week-old multicellular thallus, the most serious damage happens at the surface part of cortical cell walls, medullar cell walls and intercellular matrix regions. The prominent accumulation of amorphous materials inside the innermost cell wall of cortical cell occurs together with the loss of the outer layers. At the same time, separation of the cells inside the thallus appears because of the inability to perfect the matrix formation (Plate 9, A). This result well explains what has been observed under the light microscope (chapter 1). The thickening of cell wall and the separation of medullar cells cause the thallus to appear swollen, be weakly constructed and eventually break at some place. Both the phenomena of the loss of outer layers of cortical cell wall and the imperfection of intercellular matrix suggest an inhibition in new wall material transportation and deposition. This is due to inhibition becoming especially strong at the stage of tissue differentiation when cells are changing greatly in volume, nature and connection with each other. All these processes usually occur during the period of cell wall formation.

In the section made by conventional chemical fixation, many hollow-like spaces are detectable in disordered fibrous layer. These hollow-like spaces are actually not empty, but rather full of amorphous materials. The observation from the section prepared by the freeze substitution method has verified the existence of those amorphous materials. However, the nature of these amorphous materials, to where they must transfer, and by what mechanism they accumulate there, are still unknown.

2. *Analipus japonicus* and *Scytosiphon lomentaria*.

With respect to the delay of initial germination of spores observed in these two species by the light microscope (chapter 1), a prominent damage due to Ge inhibition was recognized accordingly in the cell wall by electron microscopic examination. The germ tube can not be produced normally and the cell wall itself is in a disorderly arrangement. The thin wall at the place from where the spore would germinate seems ready to break at any time. It might be considered that the loss of cell wall organization reduces the strength of the wall that makes the cell swell when it bears cytoplasmic pressure. On the other hand, the secretion of new wall materials necessary for rapid germination is affected. Therefore, new wall and germ tube formation cease.

The aberration in the cell wall appears also in the multicellular thallus. The disorder of the wall occurs not only in the surface cells but also in the inner cells of the thallus and intercellular region. Although there is still little reporting about the cell wall of the species, it seems that the cell wall is formed by fibrous layer and amorphous materials. It is the same as *F. distichus* and *P. wrightii,* GeO_2 causes disorder in fibril arrangement and the accumulation of amorphous materials within it. Because of the swelling of the cell due to wall aberration and the lack of normal intercellular connection seen under the light microscope, the thallus can not be seen as well constructed.

3. *Sphacelaria* sp. and *Pilayella littoralis*.

Ultrastructural, histochemical and cytochemical studies of bud initiation have been well done in *Sphacelaria* species (BURNS *et al.* 1982a,b, 1984). The process of bud initiation involves at least three changes: (1) the loss of cytoplasmic vacuoles and increase in the mass and density of organelles; (2) cytoplasmic pressure and the loosening of the cell wall; (3) incorporation of new wall material into cell wall. In addition, the composition and constituents of the cell wall are clearly defined; the cell wall is composed primarily of alternating layers of sulphated polysaccharides (fucans) and acidic polysaccharides (alginates). The present observation reveals that the fibrils in cell wall are loosened and mixed with amorphous materials. In this way, Ge inhibits cell wall formation, especially the apical cell wall formation, and eventually causes cessation of filament elongation. The ultrastructural observation also show the higher sensitivity of active apical and bud cells in this species.

The disorder of fibrous layer in cell wall and the occupation of the amorphous material inside the cell wall similarly occur in *P. littoralis*. These cell wall aberrations can be detected equally in any cell. The results are consistent with those obtained from light microscope examination (chapter 1).

From the ultrastructural investigation of these six species of brown algae, a common feature in reaction to Ge toxicity becomes clear: Wall synthesis and morphogenesis are altered by germanium dioxide exposure; anomalous wall thickening, structural and developmental aberrations of the wall of an actively changing region or cell, such as the rhizoid tip of *Fucus* embryo and germination tip of *Analipus* spore, occur in every species, though there are some differences in detail. Although it can not be stated with certainty that there is no inhibitory effect on other processes during the cell growth, these findings strongly support the previous speculation from the ultrastructural aspect.

Both light and electron microscope investigations have revealed the weakening of the cell wall due to the inhibition by GeO_2. It primarily involves the change of fibrous structural components in the wall. During this ultrastructural studies on the six brown algae, cytoplasmic organelles have been found to show no obvious structural aberration due to GeO_2 treament, except the apparent larger number of vesicles. But, it does not necessarily mean that there is surely no change inside those organelles. In brown algae, in fact, Golgi body has been thought to be the site where the polysaccharides are synthesized (CALLOW *et al.* 1978), and these polysaccharides, including alginate and fucoidan, might be transferred and secreted to the cell wall by some certain vesicles (EVANS and HOLLIGAN 1972). Certainly, to know whether germanium inhibits the synthesis, polymerization or transportation of these polysaccharides, biochemical investigation is needed. But, the seemingly increase of the vesicle in cytoplasm, observed in some species during the present study, might imply some trouble in the processes mentioned above. In other words, the inhibition in the incoporation of new wall materials and cell wall structure might cause the abnormal accumulation of the related cell organs or vesicles. So from the overall view point, the structural aberration of the brown

algae might be thought to stem from the trouble in the formation of some structural component, such as alginate crystalline structure, as related in chapter 1.

Chapter 3. Histochemical investigation of GeO_2-affected cell wall of *Fucus distichus* and *Pelvetia wrightii*

Introduction

Brown algae characteristically contain alginate and fucoidan in cell walls and as intercellular substances. It is well known that acidic polysaccharide (alginic acid) and sulphated polysaccharide (fucoidan) can be identified and distinguished histochemically, and the histochemical studies have been well done with the species of the genus *Fucus,* both on vegetative plant and reproductive tissue (McCULLY 1965, 1966, 1968, 1970, VREUGDENHIL *et al.* 1976, CALLOW *et al.* 1978, MARIANI *et al.* 1985) and *Sphacelaria furcigera* (BURNS *et al.* 1982 b). Alginate has been proved to characterize the fibrous layers in the cell wall, while fucoidan is mainly detectable in the amorphous regions. Those negatively charged polysaccharides are claimed to be responsible for a major part of the cell skeleton and the uptake of certain metals by a simple ion-exchage reaction. In fact, a predominant localization of cations has been proved to correspond mainly with the sulphated polysaccharide distribution in the cell wall of the surface cellular layer in *Fucus virsoides* (MARIANI *et al.* 1985). In addition, some heavy metals, such as cadmium, have been found to be absorbed and chiefly located in the cell wall, physode and some other parts of cells in *Fucus vesiculosus* (LIGNELL *et al.* 1982).

As described and discussed in chapter 1 and chapter 2, the observations with light and electron microscopes have revealed that germanium dioxide heavily inhibits the morphogenesis of cell wall in the brown algae. In the presence of GeO_2, cell division continues, though slowly, but because of the damage in cell wall formation, cells become misshapen and lose their interconnection. This loss gives rise to a collapsing of thallus. Under the electron microscope, the arrangement between fibrous components and amorphous substance became randomly mixed and in addition, the amount of amorphous substance seemed to have increased. It is reasonable to think that the structural damage must have involved some change in cell wall component. Thus, the focus of the problem should be on how the distribution of these cell wall components changes when affected by GeO_2. Moreover, the absorption of Ge into cells might be possible as other cations, and becomes the reason of the inhibition in cell wall formation. Therefore, based on the results from previous studies, further investigation on histochemistry of the cell wall, changes of cell wall components, and the localization of possible absorbed Ge were considered to be necessary, and might be helpful in answering the question.

In this chapter, concerning with a general description of cellular and intercellular constituents of *Fucus distichus* and *Pelvetia wrightii* vegatative tissue, emphasis is laid on the changes of every layer in Ge-affected cell wall.

Materials and Methods

Materials

Plants of *Fucus distichus* and *Pelvetia wrightii* from both control and Ge treatment cultures (5-10 mg GeO_2/l) were used for the examination. They were the same as those used in electron microscopic observations.

Methods

Fixation and sectioning.

Fixation was mainly done as described previously in chapter 2. But, for the staining of some dyes, for example periodic acid-Schiff's (PAS) stain, glycol methacrylate resin was used for embedding. Specimens were placed in gelatin capsules filled with the resin, then the capsules were capped and polymerized for 24 hr at 60°C. Sections (*ca.* 1 μm) were cut with glass knives on a MT-1 ultramicrotome. Some were placed in drops of distilled water on clean glass slides and allowed to dry as heated with an alcohol lamp. Dried section were then stained with various histochemical stains. The others were directly transferred to the staining solution with a forceps.

Staining.

Stains used in this experiment are listed in Table 4. The preparation of solutions and the stainings are related as below.

(1) Toluidine blue O: A 0.05% solution was made in benzoate buffer (benzoic acid 0.25 g, sodium benzoate 0.29 g in 200 ml water) at pH 4.4. The solution was filtered with a glass fiber filter and stored in a brown vial. Sections were stained for several seconds and washed in running distilled water until the stain had washed out of the glass.

(2) Alcian blue: A 3% solution was prepared in 0.1 N sodium acetate buffer (pH 5.6). The solution was adjusted to pH 2.4 with acetic acid. The staining of sections was the same as with toluidine blue O.

(3) Periodic acid-Schiff's (PAS) stain: To 200 ml boiling distilled water was added 1 g basic fuchsin followed by shaking. Upon cooling to 50°C, the solution was filtered, 30 ml HCl and 3 g potassium metabisulfite were added, and the solution was stored at 4°C in the dark for 24-48 hr. After the red color disappeared, the solution could be used. Before staining, sections were placed in a saturated solution of DNPH (2,4-dinitrophenylhydrazine) for 30 min to block the aldehydes in the tissue. After being washed with distilled water for two times, the sections were moved to 1% periodic acid with a platinum ring letting them oxidized for 5-10 min and placed in Schiff's reagent for 30 min. Finally, the sections were transferred to 3 successive baths of 0.5% sodium bisulfite (2 min each), washed for two times (5 min each) and dried on a clean glass slide.

(4) Carboxyl methylation and saponification: A solution for methylation was made by

putting 0.8 m*l* concentrated HCl into 100 m*l* methanol. Sections were directly placed in the solution, incubated at 60°C for 6 hr and then washed with distilled water. Saponification treatment was done by placing the methylated sections in 1% KOH made in 70% ethanol. Reaction was allowed for more than 1 hr at the room temperature, then washed and stained with toluidine blue O (pH 4.4). For control test, methanol without HCl was used in the step of methylation.

(5) Ruthenium red: A 0.02% aqueous solution of the stain was used. Sections were stained for 20-30 min and then washed with distilled water.

(6) Calcofluor white stain: A 100 μg/m*l* solution was prepared by dissolving the stain in the water and shaking. The solution should be made before used. Staining was done directly on the glass slide with sections for several minutes, then washed and observed under an Olympus BH-2 microscope.

Results

1. Histochemical observations.

Similarity in histochemistry of the cell wall was found between *Fucus distichus* and *Pelvetia wrightii*. Previous electron microscopy of thallus tissue has distinguished two kinds of components in the cell wall—microfibrils which chiefly surround all the cell and exist within the intercellular matrix, and amorphous materials which principally fill the intercellular spaces and cover the surface of the thallus. In the present study, several stainings were used for histochemical tests of the cell wall components. The indications the stainings and the reactions of different layers of the cell wall are summarized in Table 2. The layers of the cell wall from the outermost to the innermost part were denoted as CW1, CW2, CW3 and CW4 as in chapter 2.

As shown in Table 2, all the cell wall components stained metachromatically with toluidine blue (pH 4.4), alcian blue (pH 2.4) and ruthenium red. The amorphous materials of both the cell wall (CW3) and the intercellular matrix, in addition to the outermost wall layer of the surface cell (CW1), showed purple color with toluidine blue and more intense stains with alcian blue and ruthenium red, while pinkish color and lighter stains were observed in cell wall proper and fibrous layers respectively. In the fibrous layer which is mixed with some amorphous materials, such as CW4, purple staining spots could be detected throughout the layer. The PAS reaction and Calcofluor white stain, however, gave the differential staining of the fibrillar and amorphous layers—the former (CW2 and CW4) being PAS- and Calcofluor white-positive, and the latter (CW1 and CW3) either PAS- and Calcofluor white-negative or only weakly stained. Extraction with Na_2CO_3 reduced the Calcofluor fluorescence in the cell wall due to the removal of alginic acid, but no obvious effect was detected after dilute HCl treatment which is thought to extract sulphated polysaccharides. With the treatment of methylation, cell walls lost their metachromatic staining to toluidine blue, but they recovered the staining, especially at the fibrous layers, after being saponificated. This

Table 2. Cell wall histochemistry of *Pelvetia wrightii* embryo

Stain	Indication	Reaction					
		Cell wall of germinated zygote		Cell wall of multicellular embryo			
		CW1	CW2	CW1	CW2	CW3	CW4
Toluidine blue O, pH 4.4	Carboxyl & sulphate groups	Pu.+	Pi.+	Pu.+	Pi.+	Pu.+	Pi.+
Toluidine blue O (pH 4.4) after methylation and saponification	Carboxyl groups			Pi.±	Pi.+ Blue	Pi.±	Pi+ Blue
Alcian blue 8GX, pH 2.4	Carboxyl & sulphate groups	++	+	++	+	++	+
Periodic acid-Schiff's	Polysaccharides with vicinal hydroxyl groups	−	+	−	+	−	+
Ruthenium red 0.02%	Polyanions	++	+	++	+	++	+
Calcofluor white	Beta-1.3 & -1,4 linked polysaccharides			−	+	−	+
Calcofluor white after "Fucan" extraction	〃 〃			−	+	−	+
Calcofluor white after "Alginate" extraction	〃 〃			−	±	−	±

is because that the treatment of saponification restored the affinity of methylated polyuronides with toluidine blue. Moreover, the metachromasy of the matrix region showed that both the two components were located in that region and the central part was mainly composed of the substance which was stained pink by toluidine blue (pH 4.4).

On the basis of metachromasy and reaction to the stains and the procedures listed in Table 2, it is clear that in the thallus the alginate is mainly confined to the fibrous layers of the wall, whereas the fucoidan is the main component of the amorphous matrix. In the

central part of the matrix, there are also many alginates. This distribution of the polysaccharides has been examined previously by several workers (McCULLY 1966, 1968, 1970, CALLOW *et al.* 1978, MARIANI *et al.* 1985).

Compared with the controls, some changes in the distribution of cell wall components were recognized in the thallus cultured with the presence of GeO_2 in the medium. The disordered fibrous layers were stained pink with toluidine blue (pH 4.4), lightly stained with alcian blue (pH 2.4) and ruthenium red. These disordered fibrils were also PAS- and Calcofluor white-positive, though the reactions were more or less weak. Therefore the layers can be thought to be alginate-rich. Within the thickening area, some substances, which were stained purple by toluidine blue (pH 4.4), were detected. These substances were mingled with the microfibrils from the innermost to the outermost area of the cell wall. Moreover, the histochemical study also showed the lacking of the outer alginate layer, sulphated polysaccharide layer, and intercellular matrix when the thallus broke into separated cells.

Discussion

Metachromatic staining by thiazine dyes such as toluidine blue occurs with high molecular weight compounds having free carboxyl, sulphate or phosphate groups (BARKA and ANDERSON 1963). The color shift is in particular characteristic of sulphated polysaccharides, polyuronic acids and polyphosphates. On the other hand, the PAS reaction is considered quite specific for polysaccharides having free hydroxyl groups on two vicinal carbon atoms (HOTCHKISS 1948). The fluorescent stain of Calcofluor is demonstrated to react with the β-1,4- and β-1,3- linked polysaccharides (e.g. alginate), but not with the α-linked polysaccharides (e.g. fucans)(HAIGLER *et al.* 1980). Moreover, by methylating at 60°C, the basophilia of both sulphated and carboxylated mucins is blocked, but on subsequent saponification, the basophilia of the carboxylated but not the sulphated substances is restored (OKAZAKI *et al.* 1984). Therefore, staining after methylation-saponification treatment with toluidine blue allows the discrimination between alginate or alginate-like polysaccharides and sulphated polysaccharides.

The principal polysaccharides of high molecular weight in the brown algae are the polyuronic acid, alginic acid, the sulphated polysaccharides and cellulose. In the wall of *Fucus* and *Pelvetia*, both alginate and fucoidan should stain metachromatically by toluidine blue (pH 4.4) and alcian blue (pH 2.4) because of the free carboxyl groups in alginic acid and the sulphate esters in fucoidan. Moreover, alginic acid still has free adjacent vicinal hydroxyl groups and therefore must be PAS- and Calcofluor white-positive.

In the present study, the distribution of alginate-rich and sulphated polysaccharide-rich components was recognized both in control cell walls and Ge affected walls. Control cells showed the regular layers of the two components as previously reported results (McCULLY 1966, 1968, 1970, MARIANI *et al.* 1985). However, alginate and sulphated polysaccharide have

been observed to be obviously distributing irregularly throughout the entire cell wall from the overall observation of the wall. The fact that many sulphated polysaccharides accumulated within the swollen alginate fibrils suggested the difficulty in the transportation of these polysaccharides. It becomes clear that because of the inhibition by GeO_2, fibrous components of the cell wall, alginate or cellulose, were not able to organize normally, and thereby the sulphated polysaccharides which play the role of the matrix within the wall and between the cells, localized throughout the different layers irregularly, especially within the inner part of the wall. Thus, the outer layers of the wall on the thallus surface and the intercellular matrix of the thallus could not be formed well as usual and this caused the thallus collapsing.

Together with the observation on the structural damage, it is more reasonable to imagine that because of the disorder in fibrils formation of alginate-rich layer, the incorporation of amorphous sulphated polysaccharides is inhibited and tends to accumulate on the way of being transported to their proper sites.

Conclusion

The present study on the morphology has revealed a generality in the response to germanium toxicity in the brown algae. All the species examined have been found to lose their normal cell wall formation, which results in the damage to the growth and development of the algae. Considering the speciality in the cell wall constitution of the brown algae, in which alginate is characteristically the main structural component, the same mechanism can be surmised to exist within all the brown algae.

The swelling of GeO_2 affected cell wall of several brown algae has been observed, and especially the appearance of the thickened cell wall as seen with the electron microscope suggests the loss of the firm constitution of the fibrils. A similar response has been examined in yeast *Saccharomyces cerevisiae* and green alga *Oocystis apiculata* when they are interfered with by congo red and Calcofluor white (VANNINI *et al.* 1983, ROBERTS *et al.* 1982). Both of these dyes are known as inhibitors of cellulose and chitin microfibril assembly because they bind to fibrous glycan (BENZIMAN *et al.* 1980, HERTH 1980, ROBERTS *et al.* 1982, QUADER *et al.* 1983, VANNINI *et al.* 1983). They interfere with the crystallization of cell wall constituents and result in anomalous wall thickening (ROBERTS *et al.* 1982, VANNINI *et al.* 1983). The aberration in the cell wall formation has been explained by the gap between the cellulose and the chitin synthesis and the their polymerization, and then crystallization. The structural similarities in cell wall caused by the inhibition of these two dyes and germanium dioxide suggest some possibility of damage in the structural component alginate.

Alginic acid consists of unbranched chains comprising blocks of β-1, 4-linked D-mannuronic acid and α-1, 4-linked L-guluronic acid (HAUG *et al.* 1967) and the proportions of the two acids have been found to vary in the extracts from different species and different parts of the same thallus (HAUG *et al.* 1974). Alginate is present in the plant as salts of different metals, primarily sodium and calcium. It is known that guluronic acid-rich alginates have

a high affinity for Ca^{2+}, and these ions are responsible for the gel strength of the alginate. By Ca ions fitting into the cavities between residues in the G-blocks, a three-dimensional structure is firmly formed. Alginic acid enriched in polymannuronic acid has more flexibility and has been reported to be located in young cell wall and/or intercellular regions, whereas polyguluronic-rich alginic acid has high mechanical strength and appears chiefly in the cell wall proper (Haug *et al.* 1969).

A number of possible steps have been elucidated about the biosynthesis of alginates. GDP-D-mannuronic acid, a precursor of D-mannuronic acid, has been isolated from *Fucus gardneri* (LIN and HASSID 1966), and the poly-D-mannuronic acid is synthesized through the polymerization reaction. In the process of conversion from polymannuronic acid into a mixed poly-D-mannuronic-L-guluronic polymer, the enzyme epimerase has been regarded as necessary. This enzyme is reported to require Ca^{2+} for activity (LARSEN 1981). Therefore, a factor which interrupts any of these processes may affect the formation of the alginate fibril structure.

Although the direct proof is still lacking, from all the present research it can be speculated that GeO_2 may affect the conversion from polymannuronic acid into polyguluronic acid, thereby reducing the cell wall's mechanical strength.

Summary

The inhibitory effects of germanium dioxide on the growth and development of six brown algae, *Fucus distichus* subsp. *evanescens, Pelvetia wrightii, Analipus japonicus, Scytosiphon lomentaria, Sphacelaria* sp. and *Pilayella littoralis,* were investigated by light and electron microscopy and histochemical staining. The results are summarized as follows.

A close connection between cell growth and the effect of germanium was revealed throughout the culture experiment and observation with the light microscope; actively growing cells and the germinating tip or rhizoid protuberance were most sensitive to germanium toxicity. This connection was recognized in all the species examined regardless of the differences in thallus constitution. At the same time, the aberration of cell shape (the swelling and tendency to break) and the loss of interconnection between cells had focused the possible site of germanium attack on the cell wall.

Electron microscopy verified that germanium treatment causes the malformation of fibrils in the cell wall. In GeO_2-affected thalli, the cell walls appeared in a loosened and irregularly arranged way, and the lack of intercellular matrix was observed everywhere. Within the affected cell wall many amorphous substances existed. Moreover, the cell wall at the region of tip in germinating zygotes and spores was found to be abnormally thin. These changes in the structure of cell wall were consistent with what was observed with the light microscope and they well explained the damage in the growth and development of cell and thallus.

In addition, the histochemical study further confirmed that the disordered fibrous compo-

nent mainly consisted of alginate, and along with the disordering in the layer of these alginate fibrils, the amorphous fucoidan became located randomly throughout the affected cell wall. The fucoidan were also found in the inner part of the wall.

It appears clear from the overall investigation that germanium dioxide affects the growth and development of brown algae by interfering with the morphogenesis of the cell wall. It reduces the cell wall's mechanical strength, thereby causing the aberration of the cell shape, loss of cell connection and finally deformation of the thallus. As the main as well as the special structural component of brown algae, the crystalline structure of alginate was supposed to be the possible site where germanium attacks easily. The present study has provided some information useful in approaching the mechanism of germanium toxicity on the brown algae.

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PLATE 1

Light micrographs of *Fucus distichus* subsp.*evanescens*
Development of zygotes and embryos under the various concentration of GeO_2 and growth recovery of affected embryos in GeO_2-free medium

A. Zygotes of 24 hr in the control culture.
B. Zygotes of 24 hr in the culture with GeO_2 at 30 mg/*l*.
C. Zygote of 24 hr in the culture with GeO_2 at 50 mg/*l*.
D. Embryos of 1 week in the control culture with primary and secondary rhizoids.
E. Embryos of 1 week in the culture with GeO_2 at 5 mg/*l*.
F. Embryos of 1 week in the culture with GeO_2 at 30 mg/*l*.
G. Cells in embryos of 1 week in the culture with GeO_2 at 50 mg/*l*.
H. Embryos of 16 days in the control culture.
I. Embryos of 16 days in the culture with GeO_2 at 10 mg/*l*. Note the cleavages of the thallus occurring at apical and lower parts.
J. Embryos of 16 days in the culture with GeO_2 at 30 mg/*l*. Thallus is not formed normally.
K. Thickening in the affected cell wall (arrows), especially at the surface part.
L. Recovery of 15-day-affected zygotes (by GeO_2 at 50 mg/*l*) after 20 days culture in GeO_2-free medium. Note many rhizoids developing from one side of the mass of cells.
M. New thalli developed from affected zygotes after 45 days culture in GeO_2-free medium.

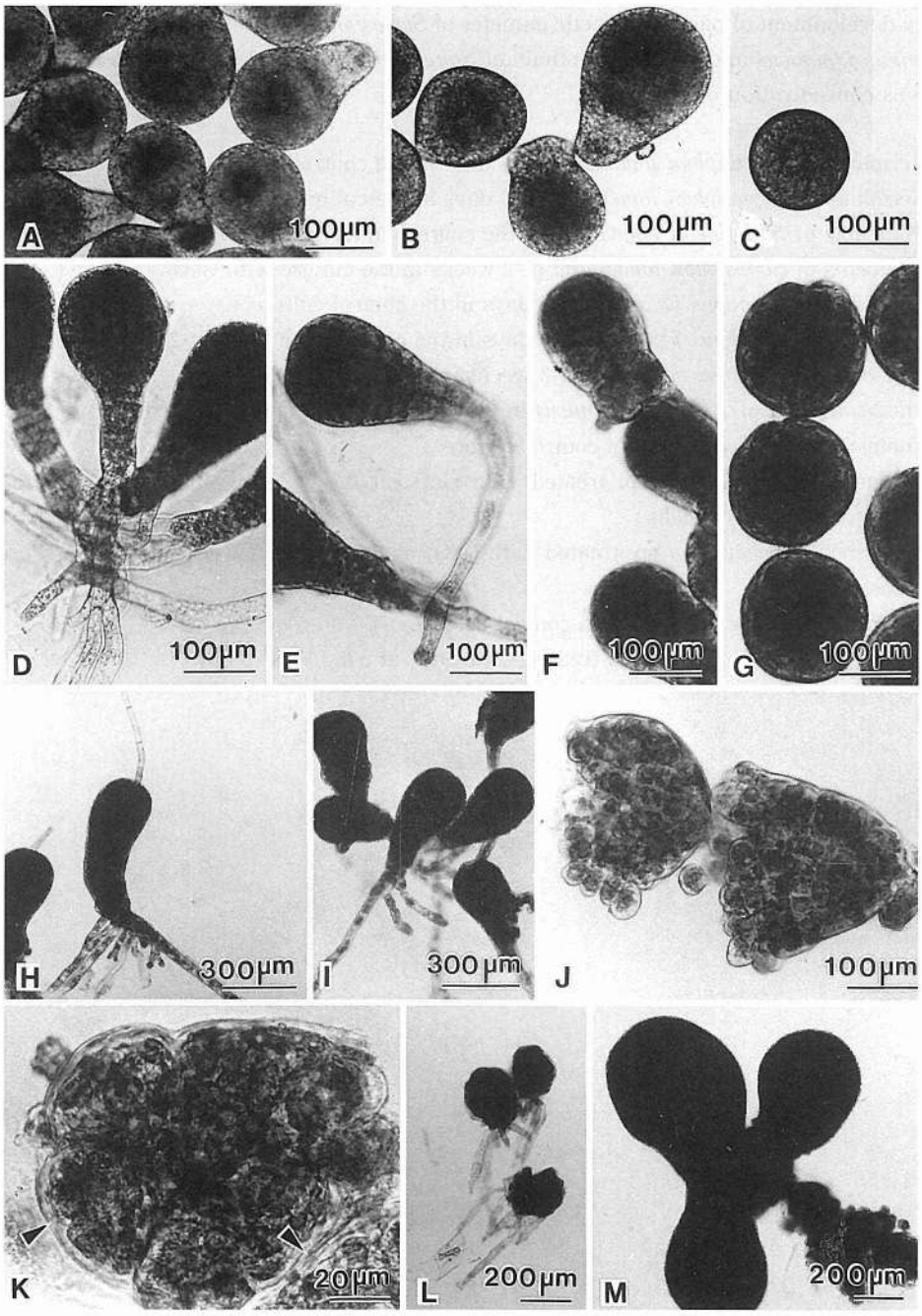
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PLATE 2

Early development of parthenogenetic gametes of *Scytosiphon lomentaria* and zoospores of *Analipus japonicus* and filamentous thalli of *Sphacelaria* sp. and *Pilayella littoralis* under various concentration of GeO_2

A. Germlings of *Scytosiphon lomentaria* of 3 days in the control culture.
B. Germlings of *Scytosiphon lomentaria* of 3 days in the culture with GeO_2 at 20 mg/*l*.
C. Filaments of *Scytosiphon lomentaria* in the control culture.
D. Filaments of *Scytosiphon lomentaria* of 2 weeks in the culture with GeO_2 at 5 mg/*l*.
E. Germlings of *Analipus japonicus* of 6 days in the control culture.
F. Germlings of *Analipus japonicus* of 6 days in the culture with GeO_2 at 20 mg/*l*.
G. Disc-thalli of *Analipus japonicus* of 3 weeks in the control culture.
H. Masses of cells of *Analipus japonicus* in the culture with GeO_2 at 5 mg/*l* for 3 weeks.
I. Filaments of *Sphacelaria* sp. in control culture.
J. Filaments of *Sphacelaria* sp. treated with GeO_2 at 0.5 mg/*l* for 2 weeks. Note the swelling of the apical cells.
K. Filaments of *Sphacelaria* sp. treated with GeO_2 at 2 mg/*l* for 2 weeks. Note the short branches.
L. Filaments of *Pilayella littoralis* in control culture.
M. Filaments of *Pilayella littoralis* treated with GeO_2 at 5 mg/*l* for 5 days.

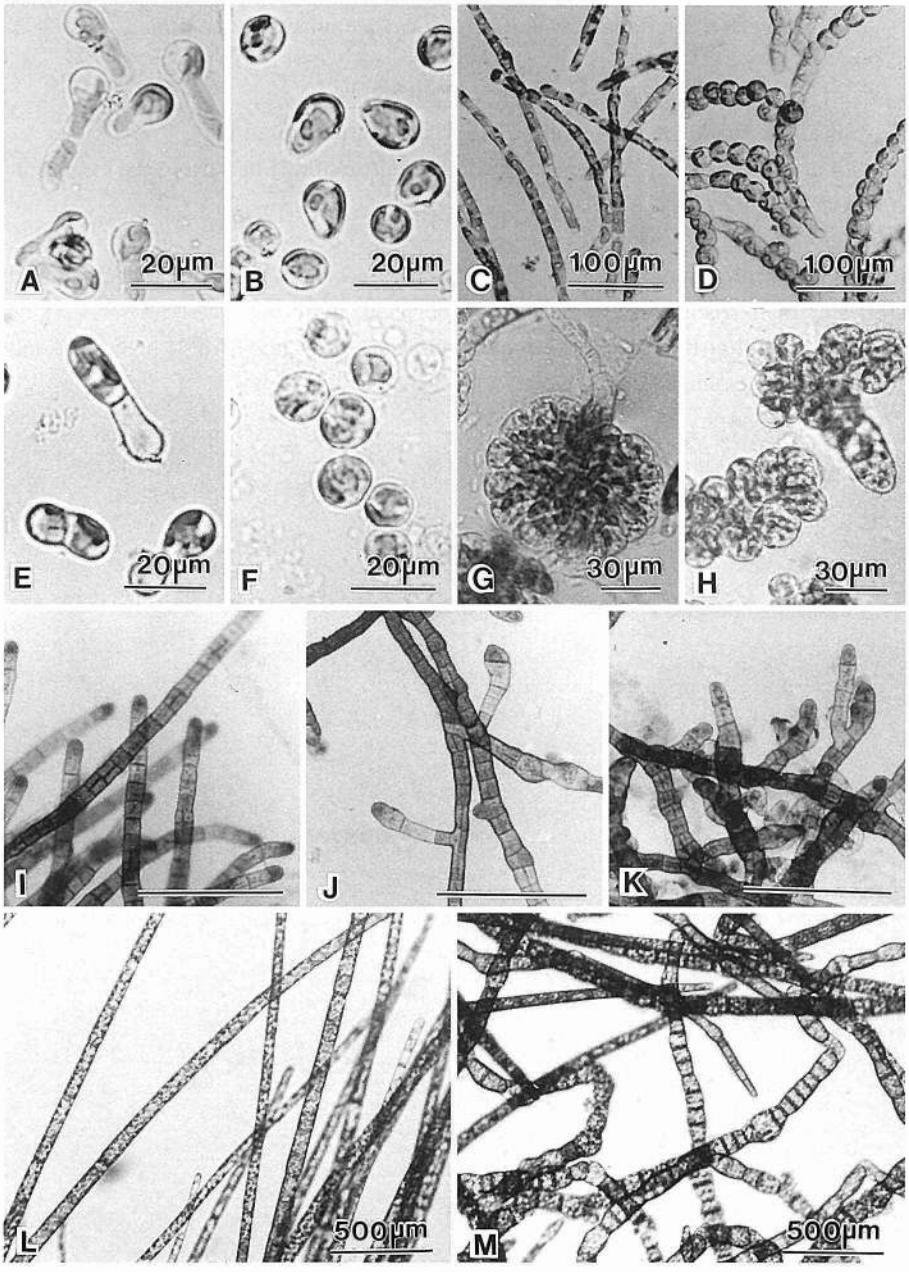
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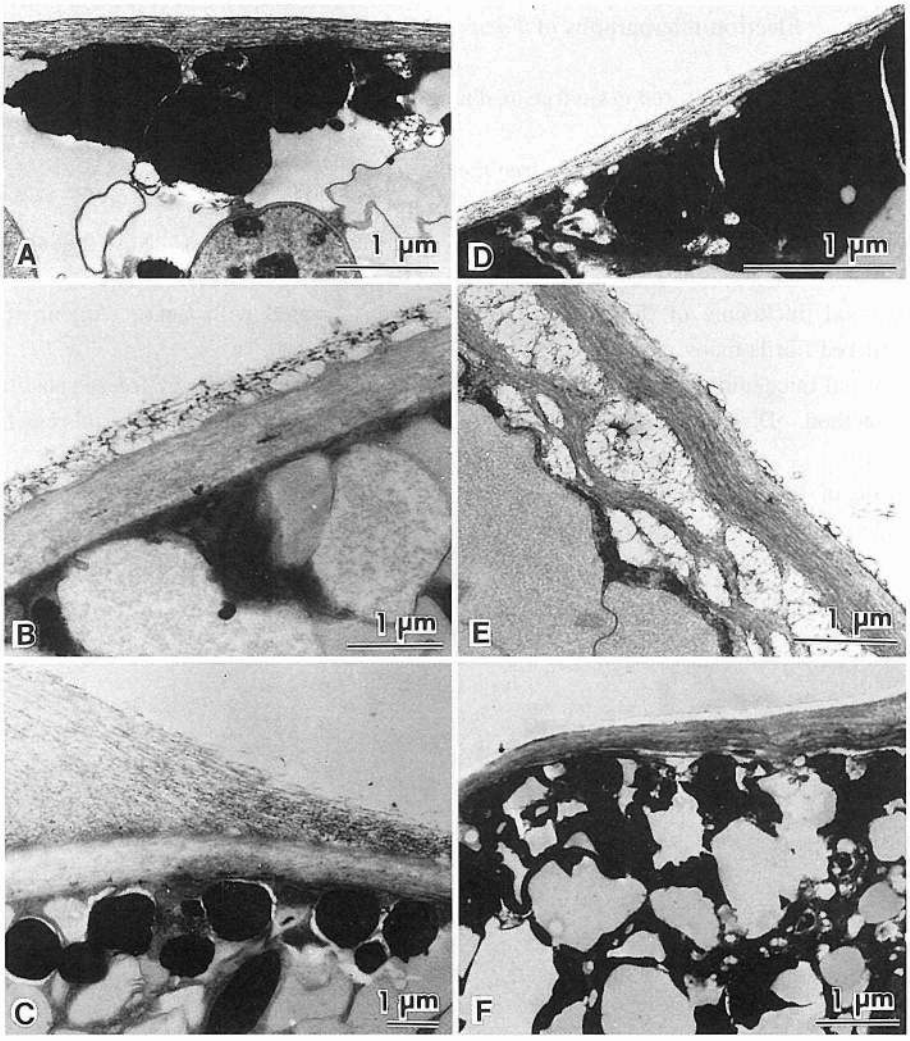
PLATE 3

Electron micrographs of *Fucus distichus* subsp. *evanescens*

A. Zygote of 4 hr in the control culture with a thin cell wall.
B. Cell wall at the thallus part of embryo in control culture for 24 hr.
C. Cell wall at the rhizoid tip of embryo in control culture for 24 hr. Note the extension of the outermost amorphous material.
D. Zygote of 4 hr treated with GeO_2 at 10 mg/l.
E. Affected cell wall at the thallus side treated with GeO_2 at 10 mg/l for 24 hr. Note the disordering of fibrils and accumulation of amorphous materials.
F. Affected cell wall at the rhizoid tip treated with GeO_2 at 10 mg/l for 24 hr. Note only a thin fibrous layer remained.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1 μm
D
1 μm
B
1 μm
E
1 μm
C
1 μm
F
1 μm

PATE 4

Electron micrographs of *Fucus distichus* subsp. *evanescens*

A. Thallus of 2 weeks cultured in Ge-free medium. There is a differentiation of cortical and medullar cells.
B. Outer wall of the cortical cell in Ge-free medium.
C. Medullar cell wall and the intercellular matrix in Ge-free medium.
D. Collapse of thallus after treatment with GeO_2 (at 20 mg/l) for 3 weeks. Note the loss of the connection between the cells.
E. Abnormal thickening of the cell wall of the thallus treated with GeO_2. Among the disordered fibrils many electron-dense inclusions exist.
F. Abnormal thickening of the cell wall of the thallus treated with GeO_2 by freeze substitution method. Disordered fibrous layer and accumulation of amorphous materials can be seen.
G. Section of the surface of affected cell wall by GeO_2 treatment, showing irregularly arranged fibrils.
H. Affected intercellular matrix by GeO_2 treatment. Note the breakage from the middle region of the matrix.

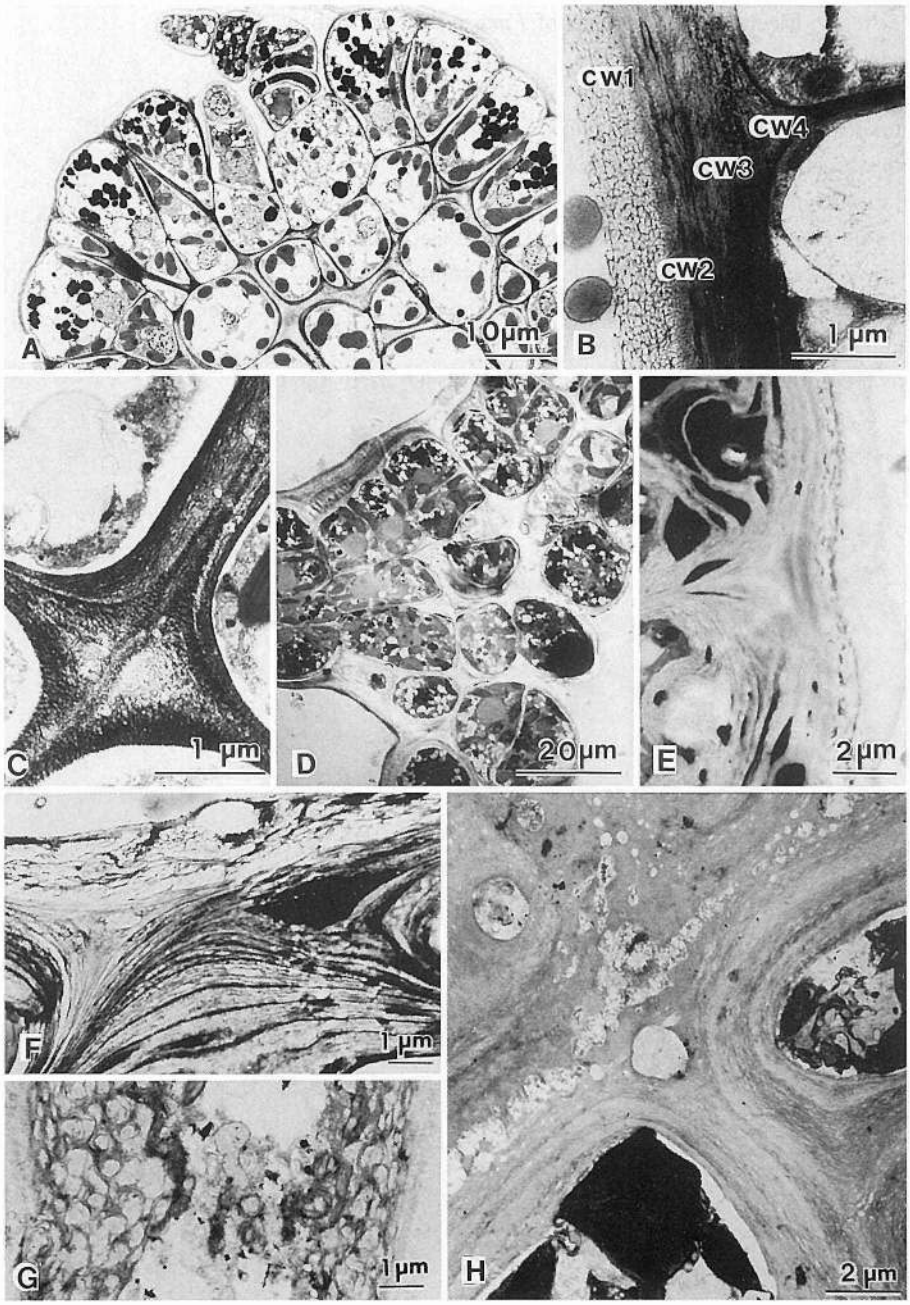

A
10μm
B
CW1
CW4
CW3
CW2
1 μm
C
1 μm
D
20μm
E
2μm
F
1μm
G
1μm
H
2 μm

PLATE 5

Electron micrographs of *Fucus distichus* subsp. *evanescens*

Two-week-old embryo extracted with dilute acid

A. Outer wall of the cortical cell, showing the movement of amorphous materials.
B. Wall of medullar cells and intercellular matrix.
C. Outer wall of the cortical cell affected with GeO_2 at 10 mg/*l*.
D. Affected medullar cell wall and matrix with GeO_2 at 10 mg/*l*.

Two-week-old embryos extracted with dilute alkali

E. Outer wall of the cortical cell. Note the movement of fibrous layers.
F. Medullar cell walls and intercellular matrix.
G. Affected outer wall of the cortical cell with GeO_2 at 10 mg/*l*.

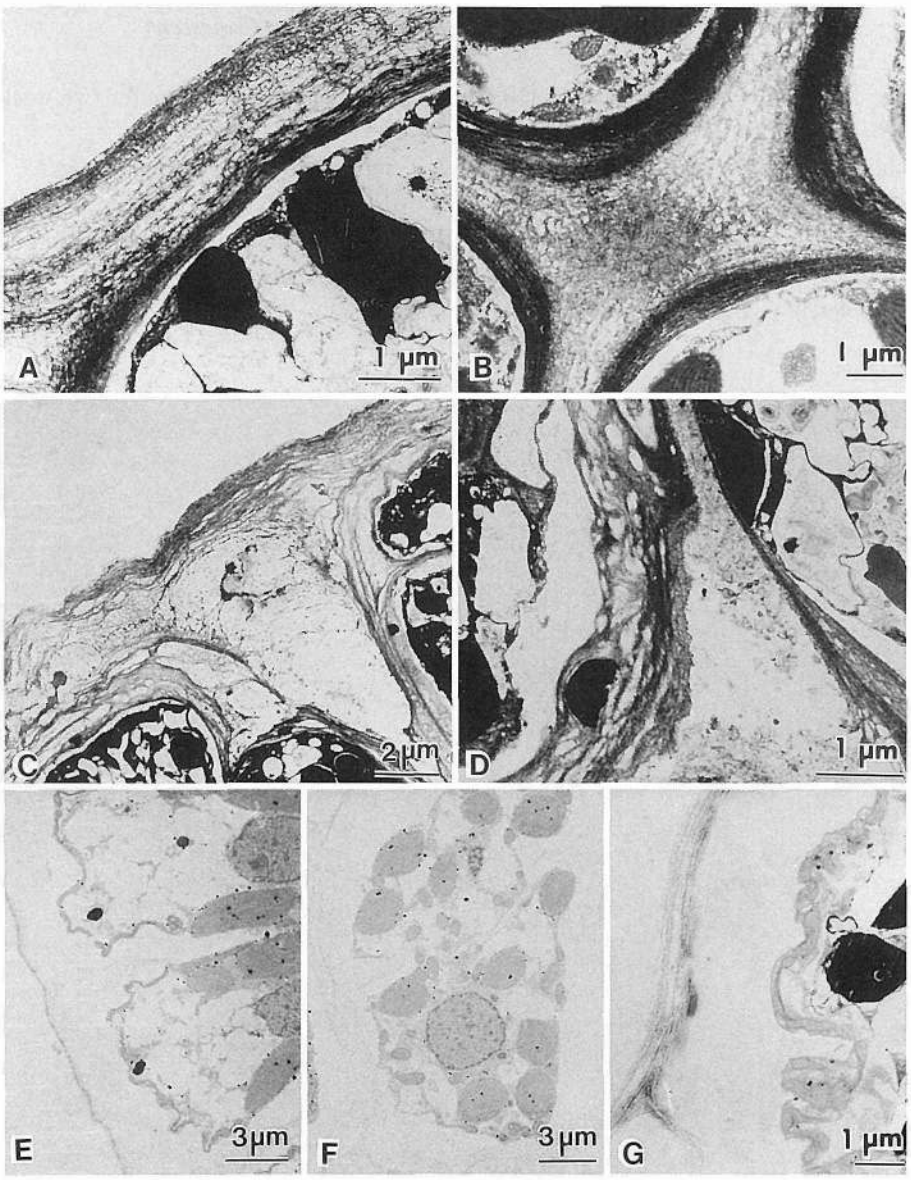

A
1 μm
B
1 μm
C
2 μm
D
1 μm
E
3 μm
F
3 μm
G
1 μm

PLATE 6

Electron micrographs of *Fucus distichus* subsp. *evanescens*

A. Golgi bodies (G) around the nucleus (N) of embryo cell in control culture for two weeks.
B. Higher magnification of A.
C. Golgi bodies and related vesicles (V) in affected embryo cell with GeO_2 at 10 mg/*l*.
D. Higher magnification of C.
E. Longitudinal section of newly developed thallus from affected cells (arrow). Note the difference between new and old tissues, especially in the matrix part.
F. Incompletely recovered matrix and cell wall.

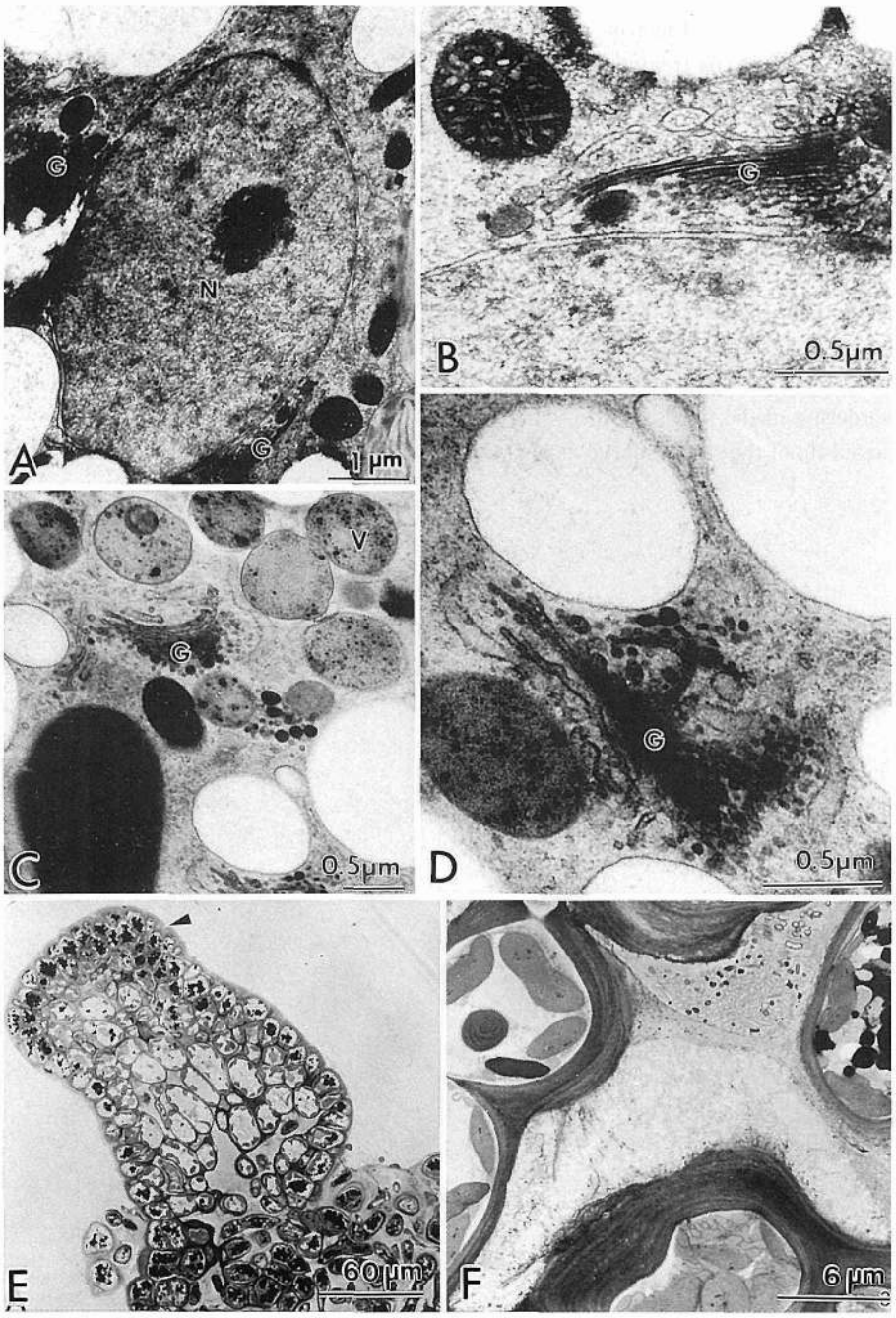

G
N
G
A
1 μm
G
B
0.5μm
V
G
C
0.5μm
G
D
0.5μm
E
60μm
F
6 μm

PLATE 7

Electron micrographs of *Pelvetia wrightii*
Embryos treated with or without GeO_2 (at 10 mg/*l*) for 18 hr

A. Longitudinal section of 18-hr-old embryo in the control culture.
B. Cell wall at the thallus side of the same embryo as in A. The outer amorphous layer (CW1) tends to erode during the fixation and dehydration. CW2 and CW3 denote outer and inner fibrous layers.
C. Cell wall at the rhizoid tip of the same embryo as in A. Three layers can be detected.
D. Longitudinal section of 18-hr-old embryo treated with GeO_2. Note the thickening of the cell wall, except the rhizoid tip.
E. Cell wall at the thallus side of the same embryo as in D treated with GeO_2. Note the disordering of the fibrils.
F. Rhizoid tip of the same embryo as in D treated with GeO_2, showing the affected thin wall.

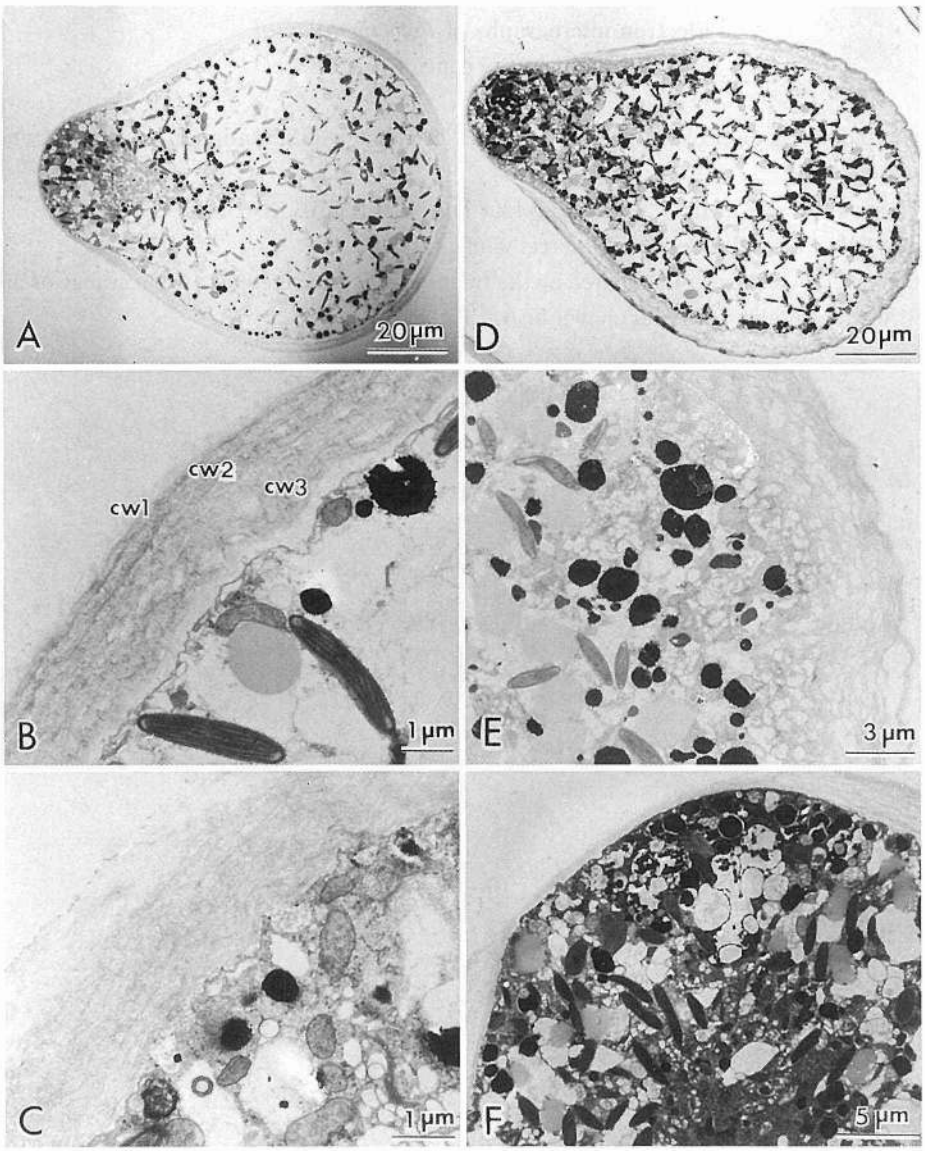

A
20μm
D
20μm
cw1
cw2
cw3
B
1μm
E
3μm
C
1μm
F
5μm

PLATE 8

Electron micrographs of *Pelvetia wrightii*
Embryos in the control culture

A. Longitudinal section of 15-day-old embryo. There is a differentiation of cortical and medullar cells.
B. Outer wall of the cortical cell, having four layer(CW1, CW2, CW3, and CW4).
C. Cell wall of medullar cells and intercellular matrix.
D. Outer wall of cortical cell prepared by the freeze substitution method. Appearance of the cell wall is similar with that shown in A.

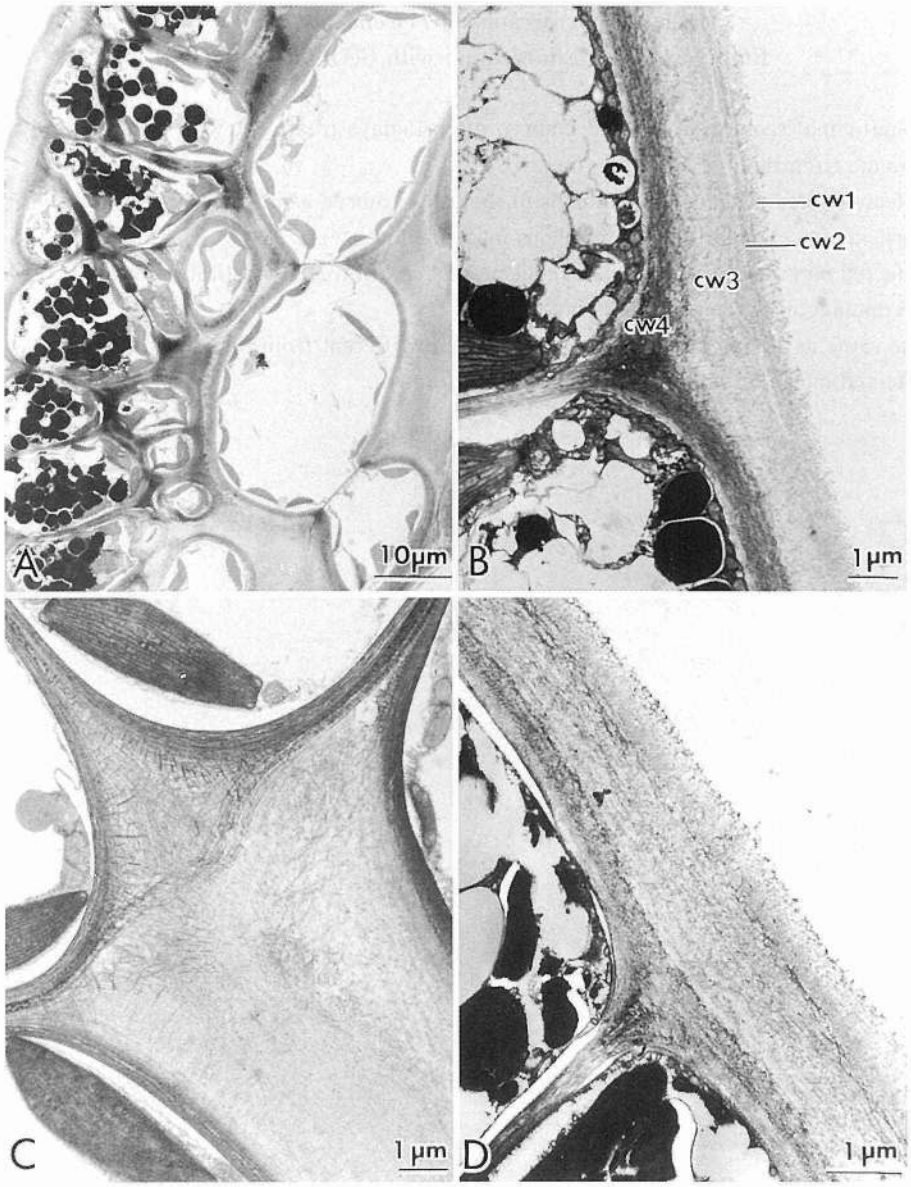

A
10μm
B
cw1
cw2
cw3
cw4
1μm
C
1μm
D
1μm

PLATE 9

Electron micrographs of *Pelvetia wrightii*
Embryos cultured in medium with GeO_2 (at 10 mg/*l*)

A. Longitudinal section of affected embryo after 15-days treatment with GeO_2. Note the damage from the matrix region (arrow).
B. Affected outer walls of cortical cells of the same embryo as in A. Note the disordering fibrils and accumulation of amorphous materials (arrow).
C. Affected outer cell wall of cortical cells prepared with freeze substitution method. Note the damage in the inner fibrous layer.
D. The same as C. Appearance of the cell wall is different from that shown in A.
E. Affected medullar cell wall and intercellular matrix.

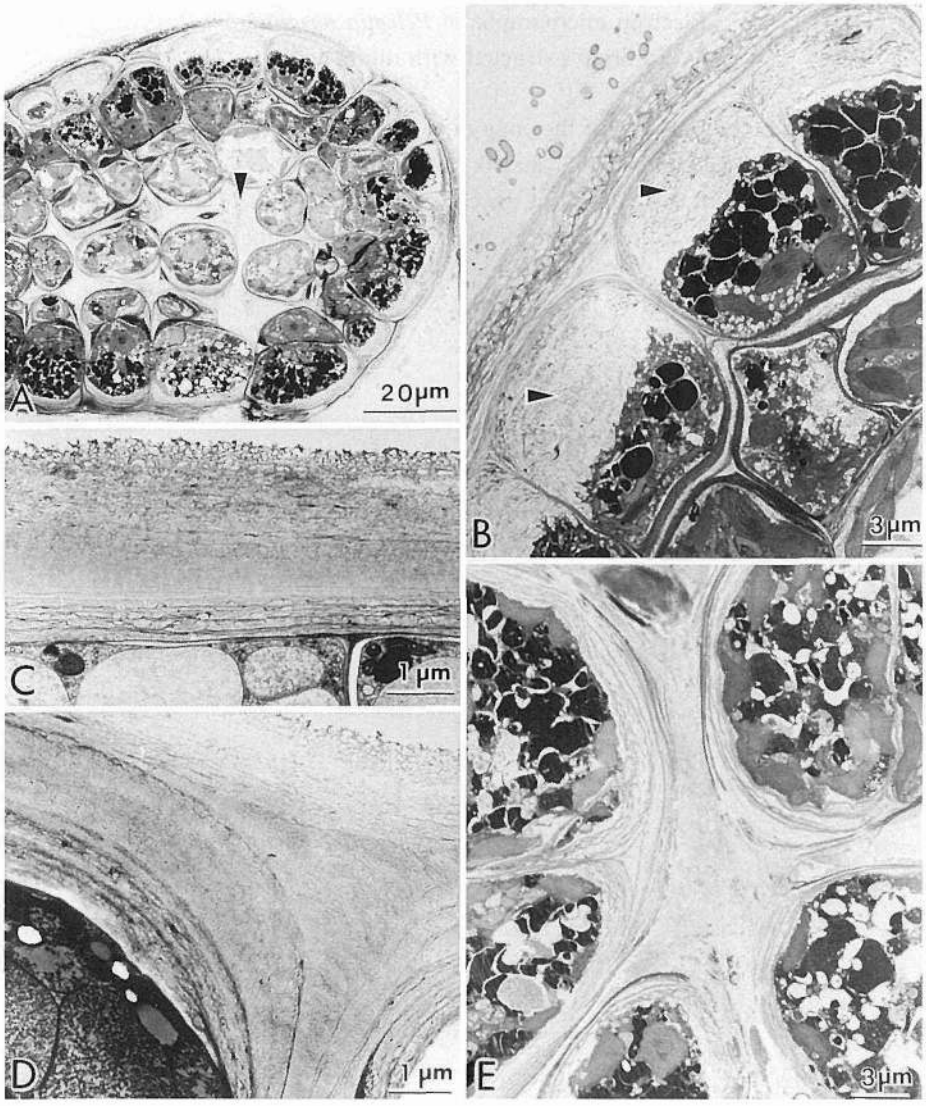

A
20μm
B
3μm
C
1μm
D
1μm
E
3μm

PLATE 10

Electron micrographs of *Pelvetia wrightii*
Embryos extracted with dilute acid

A. Outer wall of the cortical cell of the embryo in the control culture.
B. Medullar cell wall and intercellular matrix of the same embryo as in A.
C. Affected outer wall of cortical cell of the embryo cultured with 10 mg GeO_2/l.
D. Affected medullar cell wall and intercellular matrix of the same embryo as in C.

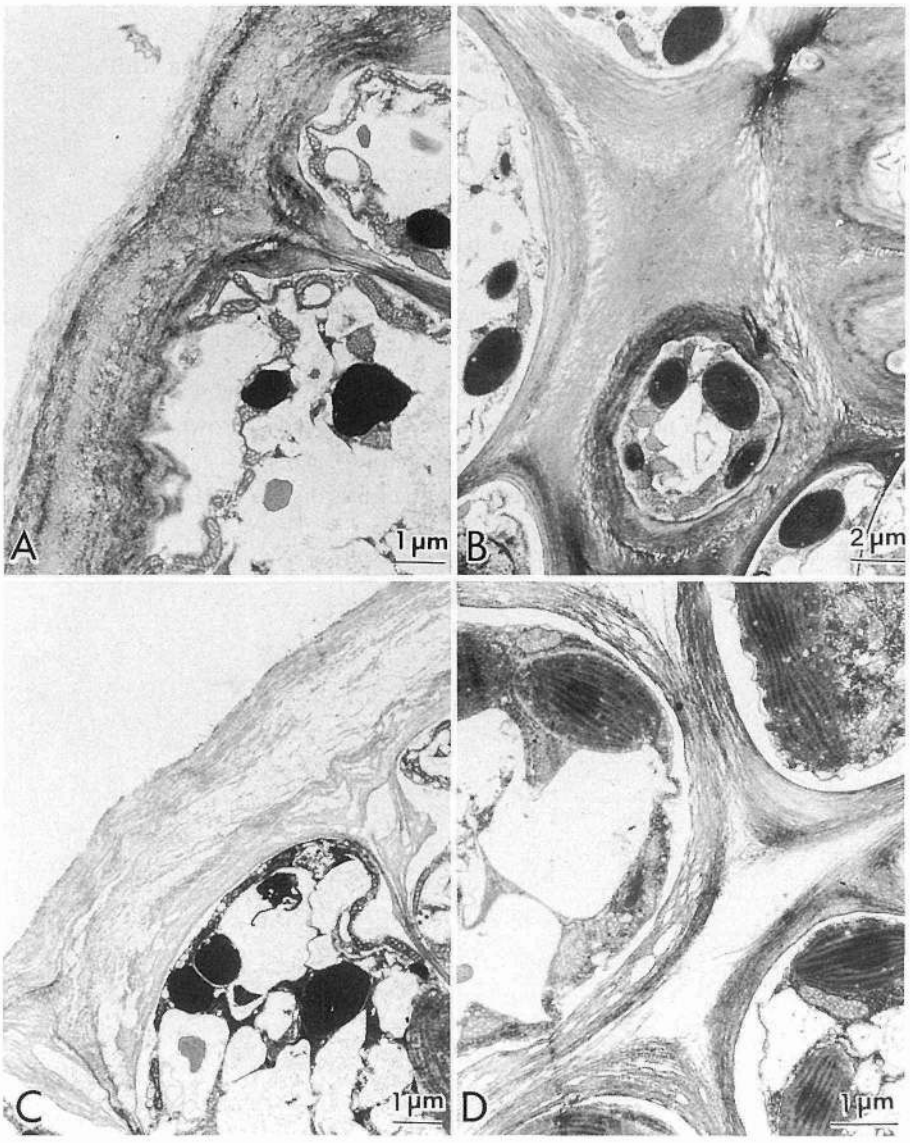

A
1 μm
B
2 μm
C
1 μm
D
1 μm

PLATE 11

Electron micrographs of *Analipus japonicus*
Germlings and cell walls of prostrate disc cultured in media with or without GeO_2 (at 20 mg/*l*)

A. Longitudinal section of 2-day-old germling in control culture.
B. Cross sections of 2-week-old disc, showing the outer wall.
C. Wall of internal cell and intercellular matrix of the same disc as in A.
D. Longitudinal section of germling of 2 days in culture with GeO_2 at 20 mg/*l*.
E. Affected outer cell wall of 2-week-old disc (cross section). Outer layer breaks away sometimes.
F. Affected wall of internal cell and intercellular matrix of the same disc as in E.
G. Golgi bodies (G) near a nucleus (N) in the cell of 2-week-old disc grown in control culture.
H. Golgi bodies in the cell of 2-week-old disc affected by GeO_2.
I. Vesicles (V) in the cytoplasm of the cell from the same disc as in H.

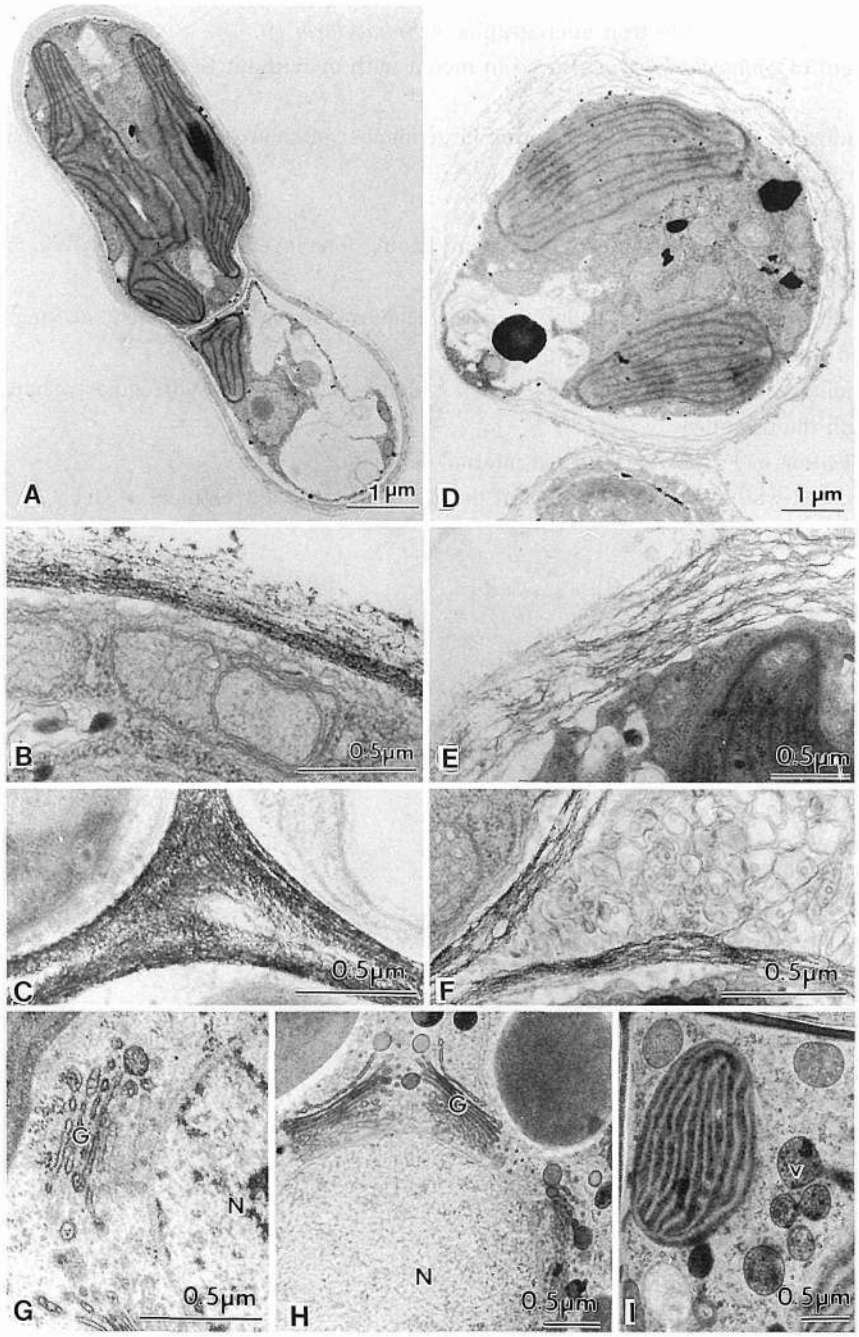

A
1 μm
D
1 μm
B
0.5μm
E
0.5μm
C
0.5μm
F
0.5μm
G
N
0.5μm
G
H
G
N
0.5μm
I
V
0.5μm

PLATE 12

Electron micrographs of *Sphacelaria* sp.
Filament of *Sphacelaria* sp. cultured in media with or without GeO_2 (at 5 mg/*l*)

A. Longitudinal section of apical cell with a large number of chloroplasts in the distal part in control culture.
B. Wall of apical cell in control culture.
C. Cell wall at the lower region of the filament, having four layers (CW1, CW2, CW3, and CW4) in control culture.
D. Longitudinal section of apical cell with more vacuoles (Va) treated with GeO_2 at 5 mg/*l*.
E. Affected wall of apical cell with GeO_2.
F. Longitudinal section of swollen apical cell or budding cells, with many irregularly shaped cells with the treatment of GeO_2.
G. Magnification of F, showing affected internal cell wall.
H. Affected cell wall at the lower region of the filament with the treatment of GeO_2.

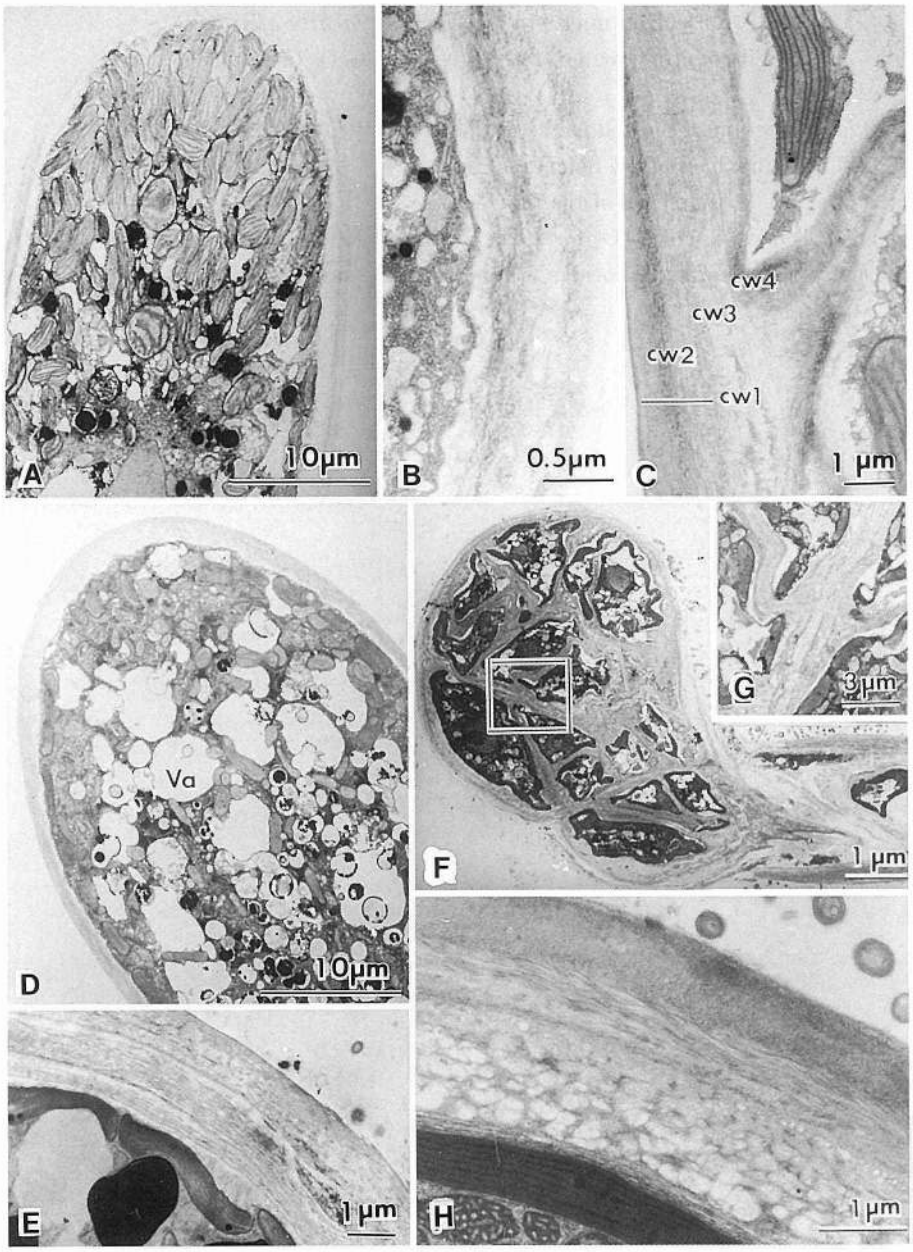
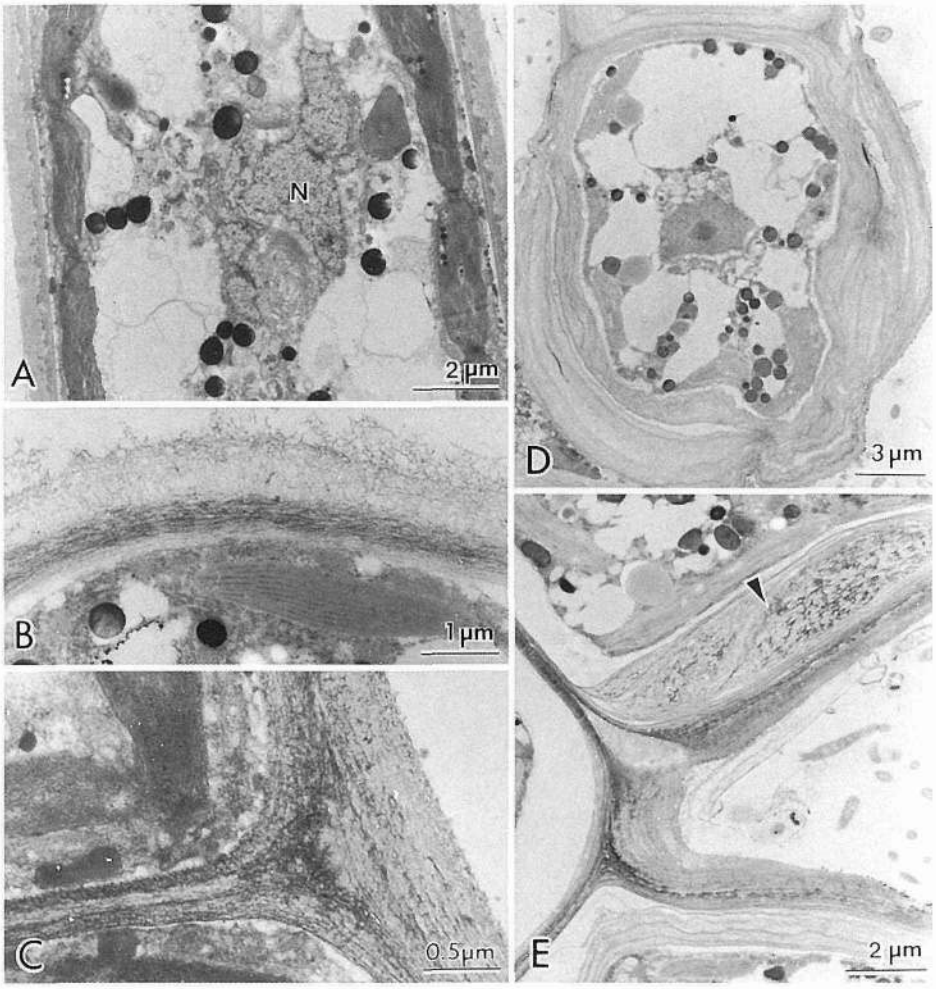

A
10μm
B
0.5μm
C
cw4
cw3
cw2
cw1
1 μm
D
Va
10μm
E
1 μm
F
1 μm
G
3μm
H
1 μm

PLATE 13

Electron micrographs of *Pilayella littoralis*
Filament of treated with or without GeO_2 (at 5 mg/*l*)

A. Longitudinal section of the cell of the filament in control culture. N: nucleus.
B. Cell wall near the apex of the apical cell.
C. Cell wall at the lower region of the filament.
D. Affected cell treated with GeO_2 for 2 weeks. Note the abnormally thickened wall.
E. Abnormal thickening at the corners of the cell of the same filament as in D (arrow).


N
A
2 μm
B
1 μm
C
0.5μm
D
3 μm
E
2 μm