*Botanica Acta 103 (1990), 1–130, No. 1, February 1990*

# Botanica Acta

Berichte der Deutschen Botanischen Gesellschaft
Journal of the German Botanical Society

**Georg Thieme Verlag**
Rüdigerstraße 14
D-7000 Stuttgart 30

Postfach 104853
D-7000 Stuttgart 10

**Thieme Medical Publishers, Inc.**
381 Park Avenue South
New York, NY 10016

**Reprint**

© Georg Thieme Verlag Stuttgart · New York

The inhibition of glutathione synthesis by PPT could lead to an impoverishment, on a long-term scale, of sulfur in the plant. Besides being the only free sulfur amino acid, glutathione is also the major free thiol compound in plants (Giovanelli et al., 1980).

Glutathione also acts as an intermediate substance in the pathway of removal of superoxide radicals (Law and Halliwell, 1983). Considerable increases in total glutathione have been observed as plants react to stress (Lay and Consida, 1976; Smith et al., 1985), for example catalase inhibition. A treatment with PPT prevents the increase of glutathione and consequently the removal of toxic oxygen radicals is decelerated. However, plants contain substantial amounts of glutathione (mmole) (Schupp and Rennenberg, 1988), so the herbicidal effectiveness of PPT may not be established by the inhibition of glutathione synthesis.

The target enzyme of PPT is GS. As shown in previous investigations (Ziegler et al., 1989) the herbicide causes a quick inhibition of photosynthesis under atmospheric conditions. Under conditions in which photorespiration could not occur, there was no inhibition of photosynthesis. An ammonium accumulation, because of the inhibition of GS through PPT, takes place under atmospheric conditions as well as under non-photorespiratory conditions. This indicates that ammonium accumulation cannot be the primary cause for photosynthetic inhibition by PPT. The investigations indicate that the effectiveness of PPT on photosynthesis, in connetion with the inhibition of photorespiration, plays the essential role (Ziegler et al., 1989).

The inhibition of GS by PPT causes an ammonium accumulation and an amino acid deficiency (Wendler et al., 1989). At the same time photosynthesis is inhibited by the herbicide. These disturbances of cell metabolism cause the phytotoxic symptoms and finally kill the plants and not the inhibitional effect of PPT on γ-GCS.

## Acknowledgements

We gratefully acknowledge Hoechst Inc. (Frankfurt/Main) for kindly supporting this work by donation of bialaphos and PPT.

## References

Benson, J. R. and Hare, P. E. – *o*-Phthaladehyde-fluorogenic detection of primary amines in the picomole range. Comparism with fluorescamine and ninhydrin. Acad. Sci. USA 72 (1975), 619–622.

Carringer, J., Rieck, C. E., and Bush, L. P. – Effect of R-25788 on EPTC metabolism in corn *(Zea mays)*. Weed Science 26 (1978), 167–171.

Del Ric, L. A., Ortega, M. G., Lopez, A. L., and Corge, J. L. – A more sensitive modification of the catalase assay with the clark oxygen electrode. Anal. Biochem. 80 (1977), 409–415.

Giovanelli, J., Mudd, H. S., and Datko, H. – Sulfur amino acids in plants. In: The biochemistry of plants. Vol. 5, Amino acids and derivatives. Chapter 12 (1980), 453–505.

Griffith, O. W. and Meister, A. – Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine. J. Biol. Chem. 254 (1979), 7558–7560.

Griffitz, O. W. – Determination of GSH and GSSG using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106 (1980), 207–212.
– Mechanism of action, metabolism and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. J. Biol. Chem. 157 (1982), 13704–12712.

Köcher, H. – Wirkungsweise des Herbizids Hoe 039866. 44. Deutsche Pflanzenschutztagung in Gießen: In: Mitteilungen aus der Bundesanstalt für Land- und Forstwirtschaft. Verlag P. Parey, Berlin 1984.

Lamoureux, G. L. and Rusness, A. G. – Catabolism of glutathione conjugates of pesticides in higher plants. In: Sulfur in pesticide action and metabolism. ACS-Symposium Serie 152 (1981), 132–164.

Law, C. and Halliwell, B. – Glutathione and ascorbic acid in spinach chloroplasts. Biochem. J. 216 (1983), 899–903.

Lay, M. M. and Cosida, J. – Dichloroacetamide antidotes enhance thiocarbamate sulfoximide detoxification by elevating corn root glutathione content and glutathione-S-transferase activity. Pestic. Biochem. Physiol. 6 (1976), 442–456.

Leason, M., Cunliffe, D., Parkin, D., Lea, P. J., and Miflin, B. J. – Inhibition of pea leaf glutamine synthetase by methionine sulphoximine and other glutamate analogues. Phytochemistry 21 (1982), 855–857.

Manderscheid, R. and Wild, A. – Studies on the mechanism of inhibition by phosphinothricin of glutamine synthetase isolated from *Triticum aestivum* L. J. Plant Physiol. 123 (1985), 135–142.

Meister, A. and Orlowski, M. – Partial reactions catalyzed by γ-glutamylcysteine-synthetase and for an activated glutamate intermediate. J. Biochem. 246 (1971), 7095–7105.

Meister, A., Richman, B., and Orlowski, M. – Inhibition of γ-glutamylcysteine synthetase by methionine sulfoximine. J. Biol. Chem. 218 (1973), 6684–6690.

Meister, A. and Sekura, R. – γ-Glutamylcysteine-synthetase. J. Biol. Chem. 252 (1977), 2599–2605.

Platt, S. G. and Anthon, G. E. – Ammonia accumulation and inhibition of photosynthesis in methionine sulfoximine treated spinach. J. Plant Physiol. 678 (1981), 509–513.

Rennenberg, H., Schmitt, K., and Bergmann, L. – Long-distance transport of sulfur in *Nicotiana tabacum*. Planta 147 (1979), 57–62.

Rennenberg, H. and Uthemann, R. – Effect of L-methionine-S-sulfoximine on growth and glutathione synthesis in tobacco suspension cultures. Z. Naturforsch. 35 c (1980), 945–951.

Roa, E. N. and Meister, A. – *In vivo* formation of methionine sulfoximine-phosphate, a protein metabolite of methionine sulfoximine. Biochem. 11 (1972), 1123–1127.

Schupp, R. and Rennenberg, H. – Diurnal changes in the glutathione content in spruce needles. Plant Science 57 (1988), 113–117.

Sedmak, J. J. and Grossberg, S. E. – A rapid sensitive and versatile assay for protein using Coomasie Brilliant Blue G 250. Anal. Biochem. 79 (1977), 544–552.

Smith, J. K., Kendall, A. G., Keys, J. C., Turner, J. C., and Lee, P. F. – The regulation of the biosynthesis of the glutathione in leaves of barley (*Hordeum vulgare* L.). Plant Science 41 (1985), 11–17.

Smith, J. K. – Stimulation of glutathione synthesis in photorespiring plants by catalase inhibiton. Plant Physiol. 79 (1985), 1044–1047.

Wendler, C., Barniske, M., and Wild, A. – Effect of PPT (glufosinate) on photosynthesis and photorespiration of $C_3$ and $C_4$ plants. Photosynth. Res., in press.

Wild, A. and Manderscheid, R. – The effect of phosphinothricin on the assimilation of ammonia in plants. Z. Naturforsch. 39 c (1984), 500–506.

Wild, A., Sauer, H., and Rühle, W. – The effects of phosphinothricin (glufosinate) on photosynthesis. I. Inhibition of photosynthesis and accumulation of ammonia. Z. Naturforsch. 42 c (1987), 23–29.

Wild, A. and Ziegler, C. – The effect of bialaphos on ammonium-assimilation and photosynthesis. I. Effect on the enzymes of ammonium-assimilation. Z. Naturforsch. 44 c (1989), 97–102.

Ziegler, C. and Wild, A. – The effect of bialaphos on ammonium-assimilation and photosynthesis. II. Effect on photosynthesis and photorespiration. Z. Naturforsch. 44 c (1989), 103–108.

*Aloysius Wild*

Institute of General Botany
of the Johannes Gutenberg-University
Saarstr. 21
D-6500 Mainz
Federal Republic of Germany

# A Virus Infection in the Marine Brown Alga *Ectocarpus siliculosus* (Phaeophyceae)

*D. G. Müller, H. Kawai*, B. Stache, and S. Lanka*
Fakultät für Biologie der Universität, D-7750 Konstanz, FRG
* Permanent address: Department of Botany, Faculty of Science, Hokkaido University, Sapporo 060, Japan

Received: June 6, 1989; Accepted: July 29, 1989

### Abstract

Laboratory cultures of *Ectocarpus siliculosus* originating from New Zealand showed a defect in gametangium formation. Nuclear divisions in gametangium initials are not followed by cell wall formation. In the resulting multinucleate cells nuclear DNA increases dramatically, and nuclear membranes disintegrate. Eventually, the entire structure is filled with hexagonal particles of approximately 130 nm diameter. These were isolated and shown by EM to consist of a dense core surrounded by a 3-layered shell. They are released into the culture medium when the host cells burst. *Ectocarpus* gametes from healthy cultures could be infected by these particles. The resulting partheno-sporophytes developed pathological symptoms, suggesting that the particles are viruses.

The expression of the defect is temperature dependent. At 10 °C all gametangia are abnormal, while between 15 and 20 °C defective and normal gametangia and gametes are formed on the same plant. Partheno-sporophytes developing from such gametes carry the viral particles expressed in deformed unilocular and plurilocular sporangia.

### Key words

*Ectocarpus*, marine virus, brown algae.

### Introduction

*Ectocarpus siliculosus* is a filamentous cosmopolitan brown alga inhabiting temperate coasts. It shows a sexual life history, with a succession of gametophytes and sporophytes connected by fertilization and meiosis. Since it can be easily cultured under laboratory conditions, many details of its development and reproduction have been worked out (Müller, 1967, 1988).

Upon confrontation with abnormal plants in field collections, we became interested in pathogens affecting *Ectocarpus*.

"Virus-like particles" have been reported in electron microscopic studies of several brown algae: *Chorda tomentosa* Lyngb. (Toth and Wilce, 1972), *Ectocarpus fasciculatus* Harvey (Clitheroe and Evans, 1974), *Pilayella littoralis* (L.) Kjellmann (Markey, 1974), and *Streblonema uvaeformis* (Lyngb.) Pringsh. (La Claire and West, 1977). *Sorocarpus uvaeformis* was studied in more detail, and the infectivity of a virus could be demonstrated in this species (Oliveira and Bisalputra, 1978). Likewise, experimental details were reported on a virus infection of the unicellular fresh water green alga *Chlorella* (Meints et al., 1988; Reisser, 1989; Van Etten et al., 1987; reviews by Lemke, 1976; Dodds, 1983; and Ushiyama, 1985).

In field material of *Ectocarpus siliculosus* collected in New Zealand we encountered plants with aberrant morphological features. Affected specimens were sterile, and microscopic examination showed that gametangium initials had entirely lost their cellular organization and were filled with an amorphous mass.

We studied cultures of this material under well defined laboratory conditions and present here the first report on an infective pathogen in *Ectocarpus siliculosus*. Combined evidence from light and electron microscopic observations, infectivity of the pathogen and biochemical data point to a DNA virus.

### Materials and Methods

Fertile gametophytes of *Ectocarus siliculosus* (Dillw.) Lyngb. were collected near Kaikoura, New Zealand in August 1988. Tufts of plants with plurilocular gametangia (Fig. 1) were used to establish uni-algal cultures by isolation and regeneration of filament fragments. Sterile specimens with abnormal structures (Fig. 2) were also isolated.

Cultures were maintained in plastic Petri or glass dishes in Provasoli medium (Starr, 1978), based on natural sea water (German Bight, salinity 28 ‰). They were kept at temperatures ranging from 10 ± 2 to 20 ± 2 °C and illuminated with daylight-type fluorescent lamps at a photon irradiance of 11 µmol $m^{-2}s^{-1}$ for 16 h per day.


© Georg Thieme Verlag Stuttgart · New York

*Fluorescence microscopy*

4′,6′-Diamidino-2-phenylindole 2HCl (DAPI, Serva) was used for epifluorescence studies. Materials were fixed in 1 % glutaraldehyde, washed in distilled water and stained in a solution of 1 μg/ml in NS buffer (Kuroiwa et al., 1984). Observations were made on a Zeiss epifluorescence microscope with UV excitation at 365 nm. Quantitative measurements of DAPI fluorescence intensity were made with a Zeiss Microscope Photometer SF and Photomultiplier HTV R 928 at 100× objective magnification. A KP 500 filter was used to cut off red chlorophyll fluorescence. Measuring areas were 4 μm in diameter, roughly corresponding to the diameter of *Ectocarpus* nuclei.

DNase treatment (Serva, 10 mg/ml, 24 h incubation at 37 °C) combined with DAPI staining was applied to material fixed in 1 % glutaraldehyde.

*Electron microscopy*

Cultured *Ectocarpus* material was fixed for 2 h on ice with a solution of 3 % glutaraldehyde in ¼ strength seawater, which contained 0.25 % caffeine and 0.05 M Na-cacodylate at pH 7.7. Washing in ½ strength seawater with 0.5 % caffeine and 0.1 M Na-cacodylate was followed by post-fixation in 1 % $OsO_4$. The material was washed in distilled water, dehydrated step-wise in acetone and embedded in Spurr's resin (Spurr, 1969). Sections were stained with 2 % aqueous uranyl acetate followed by 0.5 % lead citrate in 0.1 N NaOH. Observations were made on a Zeiss EM9S instrument.

*Isolation of viral particles*

3 g gametophytes (fresh weight) with infected gametangia were ground with 2 g quartz sand under liquid nitrogen. The resulting powder was dissolved in a solution of 0.1 M NaCl, 0.005 M $MgCl_2$ and 0.04 M Tris at pH 7.0. Cell debris was removed by low-speed centrifugation. Particles in the supernatant were precipitated by addition of 10 % PEG 6000 (Serva) and 0.6 M NaCl on ice and pelleted by low speed centrifugation.

The pellet was dissolved in 2 ml of the Tris buffer described above and layered onto a CsCl step gradient (Maniatis, 1982) with steps of 1.50, 1.45, 1.35, 1.30 and 1.20 g/ml. Centrifugation followed in a SW 40 rotor at 4 °C with 20 000 rpm for 2 h in a Beckman ultracentrifuge. A band enriched for the virus-like particles appeared at the density of 1.35 g/ml and was collected by punctation. The preparation was dialysed stepwise to 0.02 M NaCl, 0.005 M $MgCl_2$ and 0.04 M Tris at pH 7.0.

Negative staining of the particles was obtained with a buffered 5 % uranyl-acetate solution after Plattner and Zingsheim (1987). Nucleic acid from the particle fraction was isolated according to Maniatis (1982).

## Results

*Normal gametophytes*

The unialgal cultures derived from the typical field material were male or female gametophytes of *Ectocarpus siliculosus* (Dillw.) Lyngb. (Fig. 1). They can be propagated by fragmentation and regeneration of filaments. Sexual fusion of iso-gametes and zygote formation occurs between male and female plants from Kaikoura as well as in crosses with various isolates from Australia and the northern hemisphere. Single, non-mated gametes develop into partheno-sporophytes with a different morphology, which reproduce by uni- and plurilocular sporangia in the same way as described for isolates from other areas (Müller, 1967, 1988).

*Abnormal gametophytes*

The field collections contained some plants with malfunctional gametangia (Fig. 2). Among many isolates of this material, several fast-growing strains were selected for culture studies. Their filament cells appear normal on the light microscopical level. At 10 °C in white light with an irradiance of 11 μmol $m^{-2}$ $s^{-1}$ the plants grow rapidly by intercalary mitotic divisions. When the illumination is reduced to white light of 1.7 μmol $m^{-2}$ $s^{-1}$ or changed to red fluorescent light (Philips TL 20W15) at 0.9 μmol $m^{-2}$ $s^{-1}$ at 10 °C formation of defective gametangia is induced.

Gametangia in healthy *Ectocarpus* are initiated by a series of 10 to 20 transverse walls in a side branch. This is followed by longitudinal walls and additional mitoses until a densely packed plurilocular structure is formed, which contains several hundred gametes in individual loculi (Fig. 1). In the abnormal plants cell divisions in gametangium initials end early, and none or only few longitudinal walls are formed. Instead, the initial cells swell, and their pigment content vanishes. The development ends with a series of turgescent hyaline cells filled with a whitish mass (Figs. 3, 4, 23).

DAPI staining shows that, in the early stages of defective gametangium initials, 1 to 8 nuclei per host cell are present. These nuclei are significantly larger than those in somatic cells of the host, and show much stronger DAPI fluorescence. Such stages are predominantly seen in the basal part of young gametangia (Figs. 20, 21), where the fluorescence of chloroplast DNA is still clearly detectable. Subsequently, the nuclear membranes appear to disintegrate, and intense DNA fluorescence extends throughout the cell (Fig. 22). In this stage the chloroplast DNA is no longer detectable.

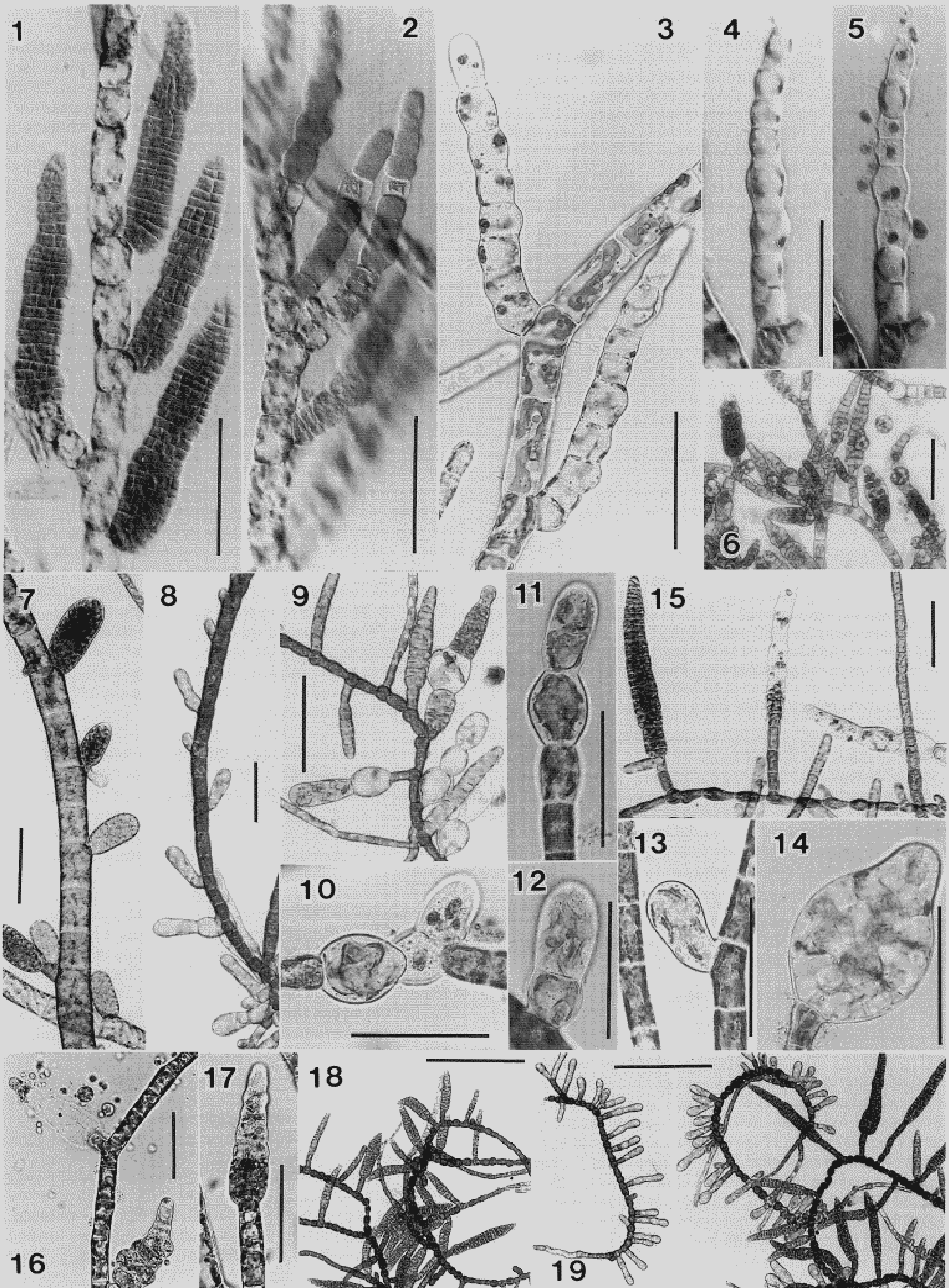
Occasionally, intense fluorescence is observed in filament cells (Fig. 37), suggesting that the pathological symptoms can also be expressed in vegetative cells.

*Release of virus particles from the living host*

Environmental stimuli such as a temperature increase or the procedure of preparing a light microscopic mount stimulates the swollen hyaline cells to burst laterally (Figs. 4, 5, 27). The expulsion of a finely granulated whitish mass can be followed easily under the microscope, although the size of the particles derived from electron microscopic measurements is below the resolution limit of light microscopy. The particles become invisible after they have dispersed in the surrounding sea water.

*Temperature dependence of disease manifestation*

The abnormal structures just described are formed when the host is cultured at 10 °C. When infected *Ectocarpus* is grown at 15 to 20 °C, the defect is reduced, and a small proportion of normal gametangia are formed (Fig. 6). Similar effects were observed, when infected plants were exposed for several days to 20 °C and subsequently


1
2
3
4
5
6
7
8
9
11
15
10
12
13
14
16
17
18
19

cultured at 10 to 15 °C. Under this treatment various transitions from defective structures to normal gametangia are observed (Fig. 16). Even within the same gametangium defective and normal sections may be found side by side (Fig. 17). Intact gametangia of such plants produce normal zoids as well as aggregates containing multiple sets of organelles (Fig. 16). The zoids of our infected cultures are male gametes. They react in the typical manner and form zygotes with female gametes of healthy *Ectocarpus siliculosus* isolates from Kaikoura and Australia.

### *Infected sporophytes*

Unfertilized gametes of healthy plants develop into partheno-sporophytes, which form uni- and plurilocular sporangia. The same occurs with functional gametes of the infected plants described above. In unilocular sporangium initials (Fig. 7) development proceeds up to the 4- or 8-nucleate stage, but ends in the transformation of the sporangium content into an amorphous mass, which is eventually discharged into the surrounding sea water (Figs. 8, 12, 13, 28).

Infected partheno-sporophytes also form plurilocular sporangia, which are either normal, or contain the pathogen (Figs. 9, 15). Zoids from intact plurilocular sporangia of these sporophytes are normal, but the disease is expressed in the next sporophyte generation. Partheno-sporophytes occasionally show pathological manifestations, intercalary or terminal, in filament cells (Figs. 10, 11, 14).

DAPI-staining reveals essentially the same sequence of events as in gametophytes. The increase in DNA content in normal uni- and plurilocular sporangia is shown in Figs. 29, 34 and 35. Sessile or stalked initials of unilocular sporangia contain 1 to 8 nuclei (Fig. 25), which are much larger than somatic nuclei, and show intense DAPI fluorescence. After disintegration of the nuclear membrane, the DNA fluorescence occupies the entire cell (Figs. 24–26). Plurilocular sporangia of the sporophyte either appear normal, or show similar expression of the pathogen as seen in gametophytes (Figs. 30–32). Occasionally, they appear in a mosaic arrangement (Fig. 33), suggesting a delay of disease manifestation to a later stage of sporangium development. In the sporophyte, intense accumulations of DNA are occasionally seen in swollen filament cells (Figs. 24, 27).

Partheno-sporophytes were used for an estimate of DNA multiplication during pathogen expression. Infected DAPI-stained material was examined by epifluorescence combined with photometry, and the following relative values were obtained:

- nuclei in vegetative filament cells: 0.020 to 0.039 (10 counts)
- nuclei in early stages of pathogen expression in plurilocular sporangia (1 to 8-nucleate stage): 0.120 to 0.194 (18 counts)
- final stage after disintegration of nuclei: 0.390 to 0.672 (14 counts).

Abnormal *Ectocarpus* cells, when treated with DNase, lost their DAPI fluorescence (Figs. 36, 38), while control runs incubated in water maintained their intense fluorescence (Fig. 37).

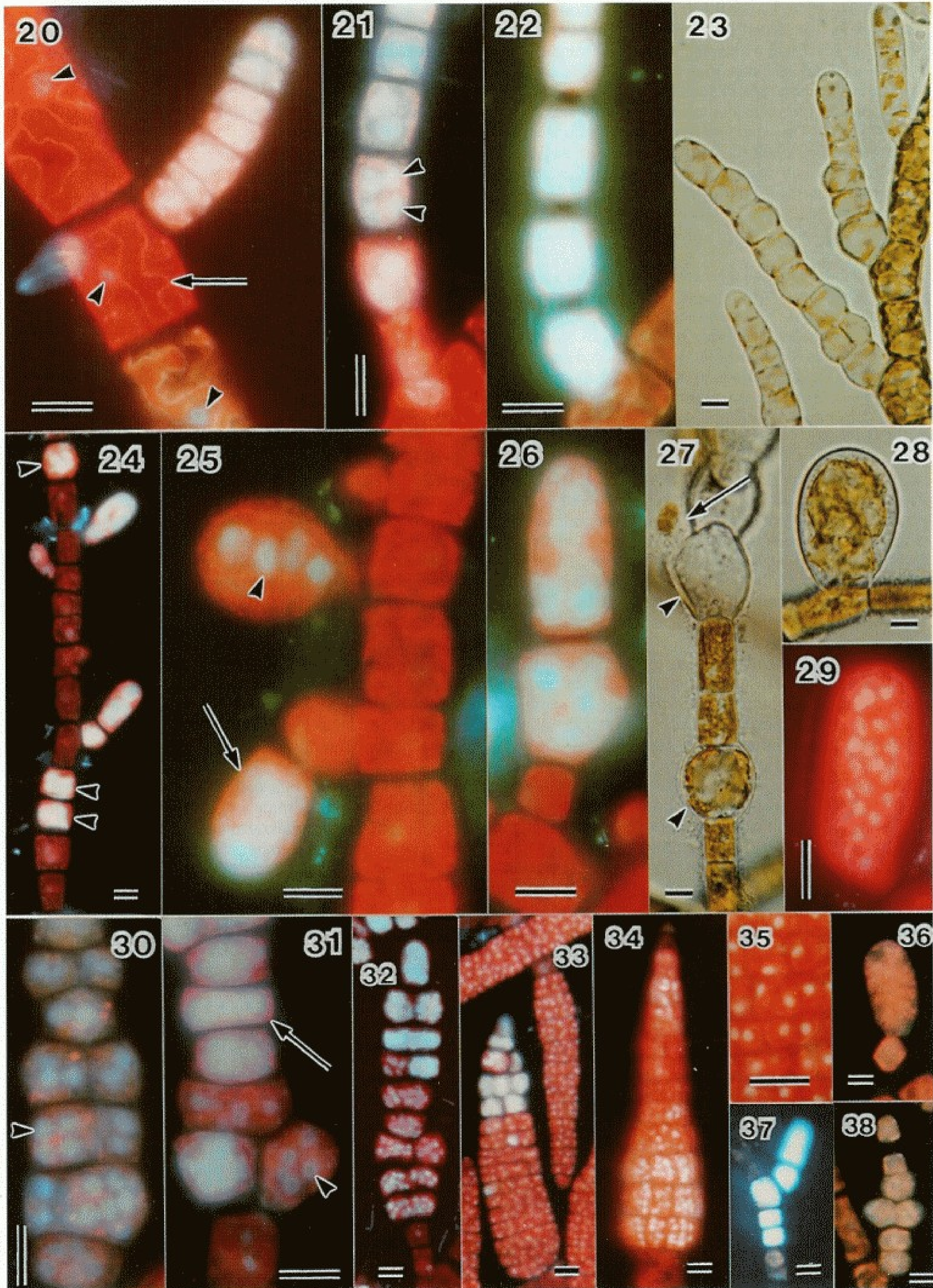
### *Transmission of the pathogen to new hosts*

Co-incubation of particles released from infected plants with filaments of healthy *Ectocarpus* gametophytes did not result in transmission of the disease. However, the following type of experiment showed that the released particles are able to infect new host cells. Fragments of an infected gametophyte carrying 20 to 40 deformed gametangia were combined with those of a healthy gametophyte with mature gametangia in 0.3 ml of culture medium in a plastic Petri dish. This resulted in release of viral particles from affected cells in the presence of healthy *Ectocarpus* gametes. Microscopic observation showed that zoids swimming near a cloud of freshly released material from an infected cell became instantly paralysed. Their flagellar activity ceased, and the inactivated cells sank to the bottom. After 1 h all filaments were removed, and the inoculant incubated in Provasoli medium for 35 days.

With this technique the disease could be transmitted to healthy male and female cultures of *Ectocarpus siliculosus* from Kaikoura and Australia. The progeny of female gametes in 4 experiments contained normal fertile partheno-sporophytes with plurilocular sporangia, and 59 to 79% infected plants (Fig. 19). DAPI-staining confirmed that all lateral vesicular structures on these affected germlings were massively filled with DNA. Parallel experiments with untreated gametes resulted in completely normal progeny (Fig. 18).

**Figs. 1–19** Light microscopy of gametophytes and partheno-sporophytes of *Ectocarpus siliculosus*.

**Figs. 1–2** Field material as collected at Kaikoura, New Zealand. Syrup mounts, scale bars 50 µm. **1:** normal gametophyte with healthy gametangia; **2:** specimen with aberrant plurilocular structures, as used for the establishment of virus-infected gametophyte cultures.
**Fig. 3** Abnormal plurilocular structures containing virus particles and rudimentary plastids in a cultured male gametophyte. Scale bar 50 µm.
**Fig. 4, 5** Release of virus masses from lateral openings in deformed gametangial cells in cultivated gametophyte. Same structure after 5 min interval. Scale bar 50 µm.
**Fig. 6** Infected gametophyte cultured at 20 °C with partial normalization of gametangia. Scale bar 100 µm.
**Fig. 7** Functional unilocular sporangia on healthy sporophyte. Scale bar 50 µm.
**Figs. 8–15** Various virus manifestations appearing as hyaline vesicular structures in partheno-sporophytes derived from gametes of infected gametophyte. Scale bars: **8:** 50 µm; **9:** 100 µm; **10–14:** 50 µm; **15:** 100 µm.
**Figs. 16, 17** Partly normalized infected gametophyte. **16:** defective (below) and empty functional gametangium (above), with normal zoids and aggregates of unseparated cells. Scale bar 50 µm. **17:** male gametangium with normal functional section (below) and virus-containing section (above). Scale bar 50 µm.
**Figs. 18, 19** Infection experiment. Partheno-sporophytes originating from female gametes of normal healthy gametophyte. **18:** untreated control; **19:** progeny of gametes exposed to virus particles contains normal plants with plurilocular sporangia and others with vesicular structures containing virus. Scale bar 200 µm.


20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38

## Electron microscopic observations

### *Infected gametophytes*

Vegetative cells of the infected *Ectocarpus* gametophyte cultures show no abnormal characteristics (Figs. 39, 40). Their general features agree with the findings of a previous study on *Ectocarpus* sp. by Oliveira and Bisalputra (1973).

First fine structural details characteristic for the development of pathological symptoms become visible in gametangium initials. They contain several nuclei with nucleoli. Sometimes a protuberance from the cell wall can be located, but wall formation is not completed (Figs. 43, 44). Some mitochondria are degenerated. In later stages large numbers of hexagonal particles appear in the cytoplasm (Fig. 41). Their diameter is about 130 nm in Spurr's medium. Embedding in LR White (Agar Scientific, Essex, UK) gave larger sizes of about 170 nm. The darkly staining core of these particles measures 80 nm diameter in Spurr's and appears granular (Fig. 52). The shell is about 20 nm thick and consists of 3 dark layers. A thicker outer and thinner inner layers are separated from each other by translucent spaces. In earlier stages the core of many particles is also translucent, and only few electron – dense cores are found.

The cytoplasm is inhomogeneous and shows fibrillar material with a translucent halo (Figs. 45, 52). Long tubular structures of ca 50 nm diameter with varying size and shape, and many vesicles and membraneous elements are scattered between the particles (Figs. 49–51). Sometimes such tubular structures are seen attached to particles or incomplete shells (Fig. 49). In a later stage, the chloroplasts of the host cells appear degenerated (Fig. 48) and remnants of nuclei can be seen (Figs. 41, 45). The nuclear membrane ruptures, and particles appear in the nuclear area (Figs. 46, 47).

Mitochondria are located between the degenerated chloroplasts at the periphery of the gametangial loculi, or grouped in particle-free areas of the cytoplasm, which possibly represent the former nuclear regions. Typical dictyosomes or endoplasmic reticulum are only rarely found, and it seems likely that the many vesicles and membraneous structures represent dictyosome cisternae. In more advanced stages the entire cytoplasm is filled with hexagonal particles. The chloroplasts are severely degenerated, and the mitochondria are now also affected and restricted to the periphery of the cell.

### *Infected partheno-sporophyte*

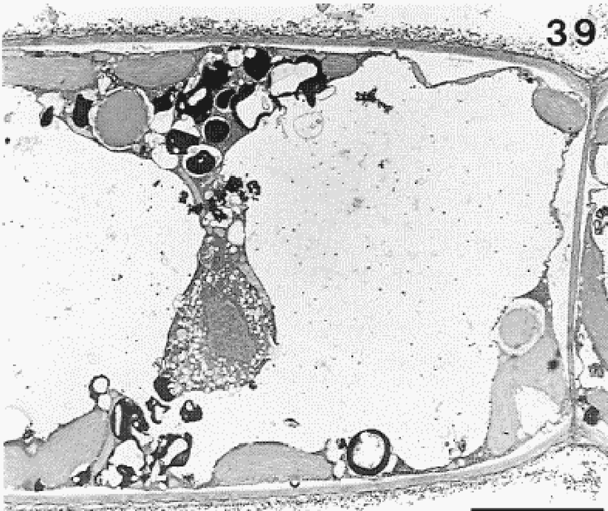
Infected sporophytes have normal filament cells and can develop normal plurilocular sporangia. These are indistinguishable on the electron microscopic level from those of healthy uninfected sporophytes. The cytological events in sporophyte cells in which the virus-like particles appear closely resemble those described for gametangia. Particles formed in sporophyte cells are the same in size and structure, and also in their development and association with fibrillar and tubular material. The degeneration of host cell organelles appears similar, but no evidence for disturbed cytokinesis and wall formation could be detected (Fig. 42).

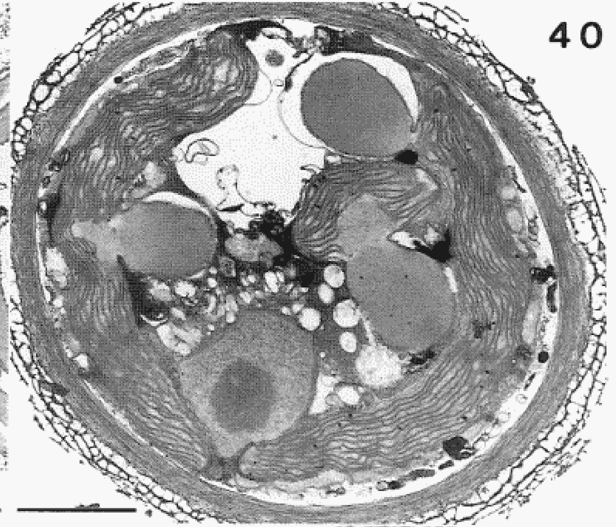
### *Isolated pathogen particles*

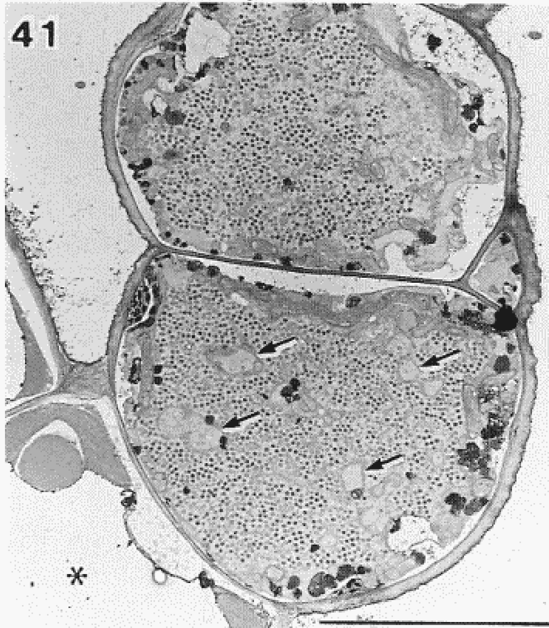
Particles isolated from infected gametophytes, when studied in negative staining preparations (Fig. 53), show the same structure as in EM sections *in situ*. They appear as hexagonal bodies coated by three laminae and have a diameter of about 150 nm.

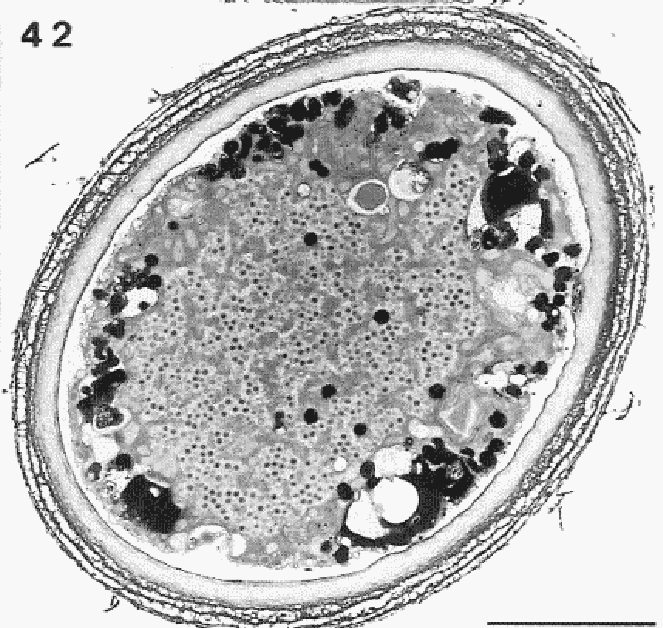
◁ **Figs. 20–38** Epifluorescence observations on healthy and virus-infected *Ectocarpus siliculosus* stained with DAPI. All scale bars 10 µm.

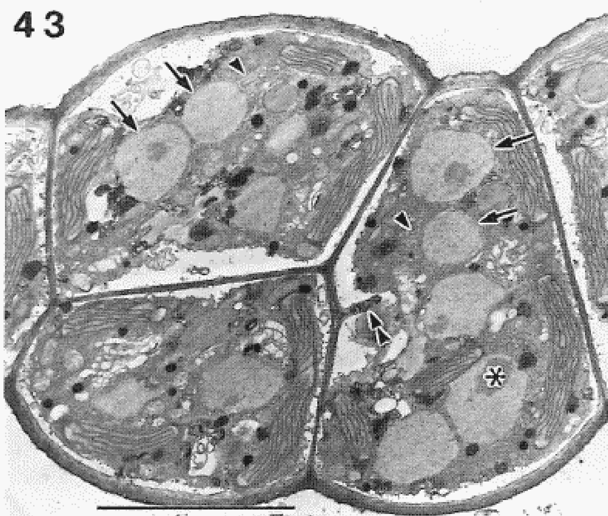
**Fig. 20** Early stage of virus expression in gametangium initial. Arrowheads: vegetative nuclei; arrow: chloroplast DNA.
**Fig. 21** 2 to 4 nuclei in gametangium initial containing increased amount of virus DNA (arrowheads).
**Fig. 22** Later stage. Nuclear membranes are disintegrated and virus DNA occupies the entire host cell.
**Fig. 23** Living gametophyte with virus expression in modified gametangia. Bright field observation.
**Fig. 24** Virus formation in presumptive unilocular sporangium of sporophyte. Arrowheads: filament cells showing virus production.
**Figs. 25, 26** Successive stages of virus expression in unilocular sporangium initial of sporophyte. Degenerating chloroplasts show weak red fluorescence. Arrowhead: expanded host nucleus; arrow: later stage of virus expression.
**Figs. 27, 28** Filament cells (Fig. 27, arrowheads) and unilocular sporangium initial producing virus. Arrow: remnant material after extrusion of virus from host cell. Living material, bright field observation.
**Fig. 29** Normal unilocular sporangium on sporophyte of healthy, uninfected culture.
**Figs. 30–32** Successive stages of virus expression in plurilocular sporangia of sporophyte. Fig. 30: Early stage with 2 to 8 nuclei per cell (arrowhead); Figs. 31, 32: early stage (arrowhead) and later stage showing DAPI fluorescence throughout the cells (arrow).
**Fig. 33** Plurilocular sporangia, apparently normal, or with virus expression in mosaic manner.
**Figs. 34, 35** Nuclear and chloroplast DNA in plurilocular sporangia of healthy, uninfected material.
**Figs. 36–38** DNase digestion experiment of *Ectocarpus* structures containing virus. Fig. 37: control. Gametophyte segment incubated in distilled water. Figs. 36, 38: unilocular and plurilocular sporangium after DNase treatment showing loss of virus DNA.

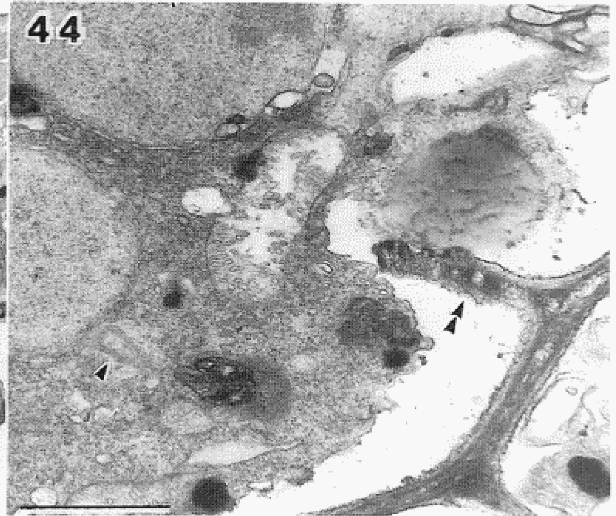

39


40


41
*


42


43
*


44

## Discussion

The reproduction and life history of *Ectocarpus siliculosus* have been studied in culture experiments (Müller, 1967, 1979, 1988) and are well known. Gametophyte and sporophyte generations are connected by sexual fusion of gametes and meiosis. Unfertilized gametes develop into haploid partheno-sporophytes, which can propagate independently. Fig. 54 summarizes that part of the *Ectocarpus* life history which is involved in the expression and transmission of the pathological symptoms.

Our studies on infected cultures described here give convincing evidence that these symptoms are caused by a virus:

- Abnormal gametangial and sporangial cells lose their structural differentiation and become filled with hexagonal particles not found elsewhere in the plants.
- DAPI-staining, DNase treatment and biochemical methods show that these cells contain extraordinary amounts of DNA.
- Masses of particles are released from infected cells into the surrounding medium. Infection experiments show that these particles can infect healthy *Ectocarpus* zoids and transmit the pathogen to the next generation.
- Biochemical characterization of isolated virus particles indicates that they contain double stranded DNA. Its size, as determined by adding up the fragments after restriction endonuclease digestion and pulse field gel electrophoresis, is ca. 350 kilobase pairs (unpublished observations).

Virus propagation seems to start by replication of virus DNA in the nuclei of gametangium or sporangium initials. Such nuclei contain 5 to 10 times more DNA than those of somatic cells. In early stages of virus propagation nuclear divisions do occur, but not wall formation. The resulting compartments contain several expanded nuclei and abundant amounts of DNA. The assembly of virus shells occurs mainly in the cytoplasm after degeneration of the nuclear membrane, although some virus particles are occasionally seen inside the remnant nuclei.

The morphological appearance of the virus in our cultures agrees with the virus-like particles described for *Ectocarpus fasciculatus* (Clitheroe and Evans, 1974) and *Chorda tomentosa* (Toth and Wilce, 1972), having three laminae. The other virus particles reported in brown algae show two laminae (*Streblonema*, La Claire and West, 1977; *Sorocarpus*, Oliveira and Bisalputra, 1978; *Pilayella*, Markey, 1974). The tubular structures observed in our material around virus particles agree with those reported for *Ectocarpus fasciculatus* (Clitheroe and Evans, 1974), *Sorocarpus* (Oliveira and Bisalputra, 1978), and *Pilayella* (Markey, 1974). They are considered to be involved in the assembly processes of the virus. Their connection with empty virus shells, as seen in our material (Figs. 49–51), supports this conclusion.

In previous reports on virus-affected brown algae "virus-like particles" were observed in vegetative or reproductive cells. Our isolates of *Ectocarpus siliculosus* show virus propagation in uni- and plurilocular reproductive structures. Virus particles are not encountered in normal somatic filament cells, but do occasionally occur in swollen cells interspersed in normal filaments (Figs. 10, 37).

Cardinal (1964) illustrated, under the name *Ectocarpus dimorphus* Silva, structures closely resembling the defective gametangia in our material. He discussed the possibility that various *Ectocarpus* species could be affected by a pathogen, possibly a virus, which causes this abnormality.

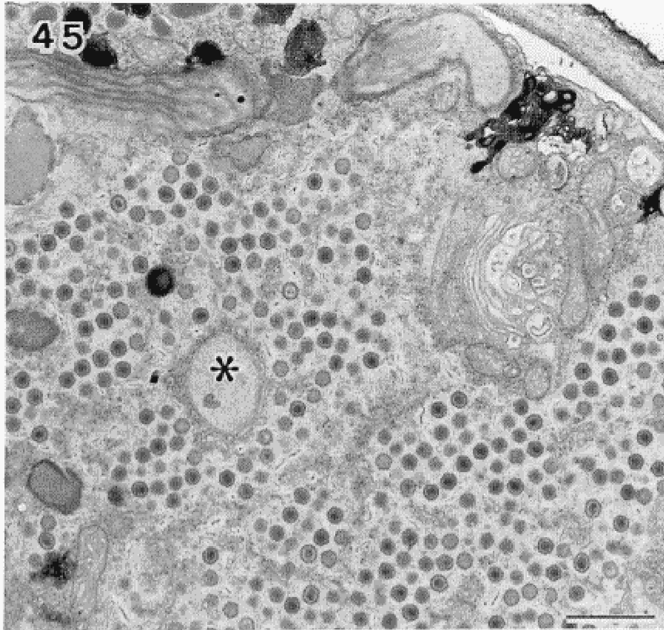
Our infection experiments demonstrate that the virus is not capable of infecting somatic cells which are surrounded by a solid cell wall. Swimming zoids however, with their plasmalemma directly exposed, can be attacked by the virus. The instant paralysis of gametes swimming into a cloud of freshly released virus particles points to a dramatic effect of the virus on swimming *Ectocarpus* zoids.

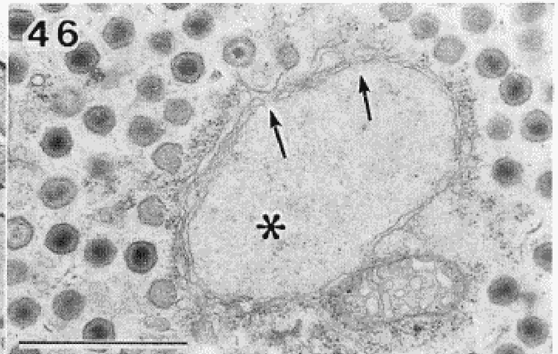
The progeny of virus-exposed zoids in our infection experiments consists of two types, normal germlings and others with the virus expression. Likewise, isolates from field material are either completely healthy, or irreversibly virus-infested. This observation suggests that the virus is replicated and reliably transmitted to the daughter cells of the host during mitosis. The virus genome could be either integrated in the genome of the host or behave as an episomal element.

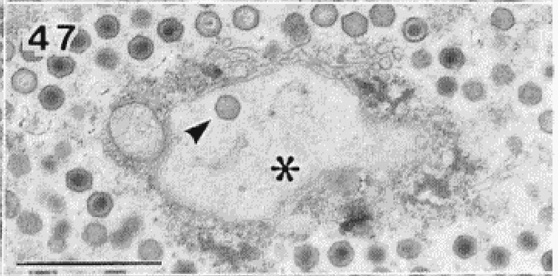
The infection experiments described here are still incomplete. It will be necessary to examine the infective potential of isolated virus particles, and to study details of recognition and attachment between virus and susceptible *Ectocarpus* zoids. Although the unialgal status of our *Ectocarpus* cultures implies that epiphytic bacteria are present in all experiments, it seems unlikely that they act as a vector for the infection process.

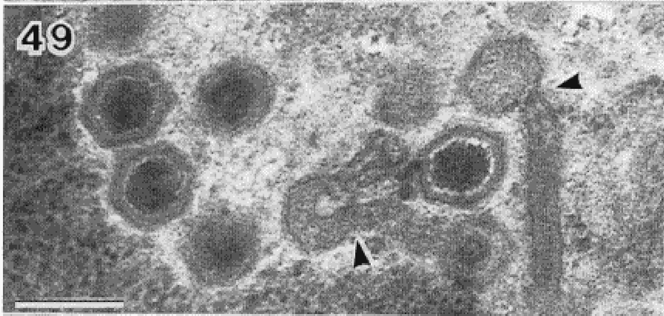
◁ **Figs. 39–44** Fine structure of virus infected *Ectocarpus siliculosus* in culture.

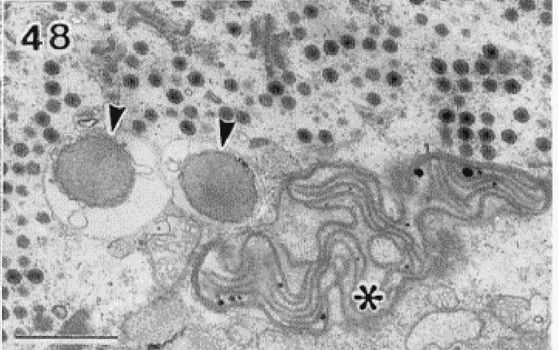
**Fig. 39** Vacuolated cell of gametophyte filament in longitudinal section. Scale bar 5 µm.
**Fig. 40** Young vegetative filament cell in cross section. Scale bar 2 µm.
**Fig. 41** Gametangial initial cells in longitudinal section with abundant virus particles. Arrow: remnant nuclei; asterisks indicate vegetative filament cell. Scale bar 10 µm.
**Fig. 42** Unilocular sporangium of partheno-sporophyte with abundant virus particles in cross section. Virus particles occupy the whole central region and many chloroplasts are seen in parietal position. Scale bar 5 µm.
**Figs. 43, 44** Early stage of virus propagation in gametangial initials in longitudinal section. Several nuclei (arrows) with nucleoli (asterisk) are seen. Double arrowhead shows incomplete wall formation. Arrowhead points to centriole. Scale bars: **43:** 5 µm, **44:** 1 µm.

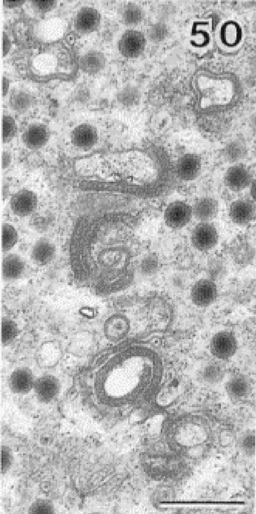

45

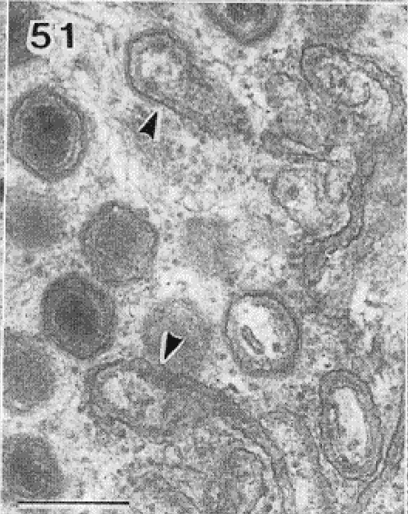

46

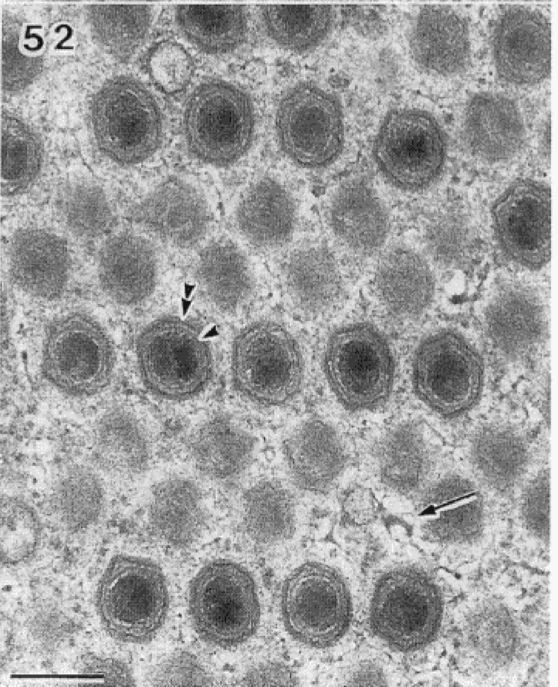

47

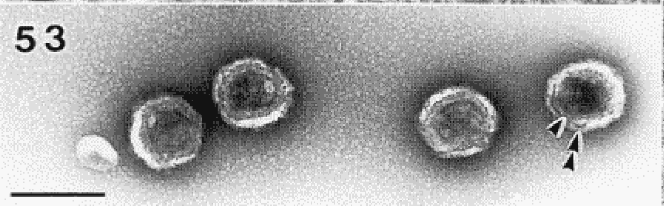

49


48


50


51


52


53

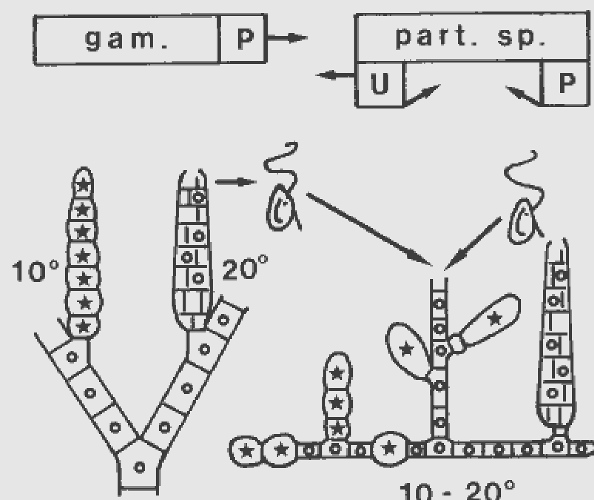



**Fig. 54** *Ectocarpus siliculosus*. Part of the life history relevant to the present study on virus-infected plants. gam = gametophyte. Partheno-sporophytes (part. sp.) originate either from gametes or propagate by zoids from plurilocular zoidangia (P). U = unilocular sporangia are characteristic for *Ectocarpus* sporophytes, but are non-functional in virus-infected material. Circles indicate morphologically normal cells; asterisks: host cells and organs producing and releasing virus.

So far, we have succeeded in transmitting the virus to male and female gametes, which develop into new partheno-sporophytes. This is a developmental dead end, since the virus infection prevents the formation of intact unilocular sporangia and thus the origin of new gametophytes. So far we have no explanation of how the virus infection enters a new gametophyte of *Ectocarpus*. Culture experiments, such as raising zygotic sporophytes containing one healthy and one virus-affected genome can be expected to give more information on this question.

The *Ectocarpus* virus system appears to be a well balanced, non-lethal arrangement. The host can flourish by profuse vegetative development, but it is forced to devote its entire reproductive potential to virus propagation. On the population level, in natural habitats, only a small proportion of the plants was seen to be infected by the virus.

The *Ectocarpus* virus is an interesting system for further studies since its expression is temperature-sensitive and linked to specific differentiation steps of the host. Furthermore, availability of virus-infected and healthy cultures, including gametophytes of both sexes from many localities, makes it possible to study infection processes, host range and the fate of the virus during syngamy and meiosis of the host. Further studies will establish the size of the virus genome and its possible integration into the genome of the host. Isolated virus particles can be used for infection-experiments, including host-specificity, and to study the composition and structure of their protein-shell.

## Acknowledgements

Thanks are due to Deutsche Forschungsgemeinschaft for travel funds, T. Motomura, Hokkaido, R. Wessel and K. Mendgen, Konstanz for technical support, R. Knippers and H. Stahl, Konstanz for valuable discussions.

## References

Cardinal, A. – Etude sur les Ectocarpacées de la Manche. Beih. Nova Hedwigia 15 (1964), 1–86.
Clitheroe, S. B. and Evans, L. V. – Viruslike particles in the brown alga *Ectocarpus*. J. Ultrastr. Res. 49 (1974), 211–217.
Dodds, A. J. – New viruses of eucaryotic algae and protozoa. In R. Matthews, ed. A critical appraisal of viral taxonomy, pp. 177–188. CRC Press Inc. Boca Raton, Florida, 1983.
Kuroiwa, T., Kojima, H., Miyakawa, I., and Sando, N. – Meiotic karyotype of the yeast *Saccharomyces cerevisiae*. Exp. Cell Res. 153 (1984), 259–265.
La Claire II, J. W. and West, J. A. – Virus-like particles in the brown alga *Streblonema*. Protoplasma 93 (1977), 127–130.
Lemke, P. A. – Viruses of eucaryotic microorganisms. Ann. Rev. Microbiol. 30 (1976), 105–145.
Maniatis, T. – Molecular cloning. A laboratory manual. Cold Spring Harbor, 1982.
Markey, D. R. – A possible virus in the brown alga *Pilayella littoralis*. Protoplasma 80 (1974), 223–232.
Meints, R. H., Burbank, D. E., Van Etten, J. L., and Lamport, D. T. A. – Properties of the *Chlorella* receptor for the virus PBCV-1. Virology 164 (1988), 15–21.
Müller, D. G. – Generationswechsel, Kernphasenwechsel und Sexualität der Braunalge *Ectocarpus siliculosus* im Kulturversuch. Planta 75 (1967), 39–54.
Müller, D. G. – Genetic affinity of *Ectocarpus siliculosus* (Dillw.) Lyngb. from the Mediterranean, North Atlantic and Australia. Phycologia 18 (1979), 312–318.
Müller, D. G. – Studies on sexual compatibility between *Ectocarpus siliculosus* (Phaeophyceae) from Chile and the Mediterranean Sea. Helgoländer Meeresuntersuch. 42 (1988), 469–476.
Oliveira, L., and Bisalputra, T. – Studies in the brown alga *Ectocarpus* in culture. J. Submicr. Cytol. 5 (1973), 107–120.
Oliveira, L. and Bisalputra, T. – A virus infection in the brown alga *Sorocarpus uvaeformis* (Lyngbye) Pringsheim (Phaeophyta, Ectocarpales). Ann Bot. 42 (1978), 439–445.
Plattner, H. and Zingsheim, H. P. – Elektronenmikroskopische Methodik in der Zell- und Molekularbiologie. Gustav Fischer, Stuttgart 1987.

◁ **Figs. 45–53** Virus particles and their formation in *Ectocarpus siliculosus*.

**Figs. 45–47** Virus particles and remnant nuclei in gametangium. Asterisks show degenerating nuclei with extended nuclear pores (arrows). Arrowhead: virus particle inside nuclear region. Scale bar 1 µm.
**Fig. 48** Degenerated chloroplast (asterisk) and pyrenoids (arrowheads) in gametangium. Scale bar 1 µm.
**Fig. 49–51** Tubular structures associated with virus particles (arrowheads). Scale bars **49, 51:** 200 nm; **50:** 500 nm.

**Fig. 52** Regular arrangement of virus particles in gametophyte. Arrow shows fibrillar structure surrounded by a translucent halo. Double arrowhead marks outer and arrowhead middle laminae of virus particles. Scale bar 200 nm.
**Fig. 53** Negative staining of isolated virus particles, showing the same structure as in situ. Markings as in Fig. 52. Scale bar 200 nm.

Reisser, W. – *Chlorella* viruses: A new group of plant viruses. Botanica Acta 102 (1989), 117–118.
Spurr, A. R. – A low-viscosity epoxy resin-embedding medium for electron microscopy. J. Ultrastr. Res. 26 (1969), 31–43.
Starr, R. C. – The culture collection of algae at the University of Texas at Austin. J. Phycol. 14, (Suppl. 1978), 47–100.
Toth, R. and Wilce, R. T. – Viruslike particles in the marine alga *Chorda tomentosa* Lyngbye (Phaeophyceae). J. Phycol. 8 (1972), 126–130.
Ushiyama, R. – Viruses in fungi and eucaryotic algae: Their possible origins and evolution. Microbiol. Sci. 2, (1985), 181–184.
Van Etten, J. L., Xia, Y., and Meints, R. H. – Viruses of a *Chlorella*-like green alga. In Kosuge, T. and Nestes, E. W., eds. Plant-microbe interactions II. Macmillan Publishing Co. New York 1987.

*D. G. Müller*

Universität Konstanz
Fakultät für Biologie
Postfach 55 60
D-7750 Konstanz 1