

Light and glycerol driven development of *Grateloupia doryphora* (Rhodophyta) *in vitro**

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SUMMARY: The implications of light in structural changes during growth and development of a carpospore seedling from *Grateloupia doryphora* cultivated in glycerol were studied by changing photon flux density from the standard white light of $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ to 20, 60 and $100 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ and spectra from white to both blue and red light ($30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ of 450 and 640 nm respectively). The growth rate as well as morphogenesis (bud, shoot or simply production of new axes) increased with the photon fluence rate of white light, as well by changing cultures from white to both blue and red light. Friability was higher in blue and red light and when carpospore-seedlings were cultivated under the highest photon fluence rates. At structural-ultrastructural level it was observed that glycerol and light (in any light condition tested) promoted a short term induction of cell division and biosynthesis in the former cells of the carpospore-seedling. As a result, new cell layers were formed concentrically arranged with respect to former cells. The division of these cells lead to the formation of the new axes (morphogenesis). The accumulation and later degradation of starch and intense proliferation of endomembranes accompanied morphogenesis.

Key words: culture, glycerol, *Grateloupia doryphora*, light, macroalgae.

INTRODUCTION

Inorganic carbon is the major source of carbon for algae. Nevertheless, the ability of algae and in particular microalgae for taking up and growing with organic carbon as a carbon source in light is well documented (Cheng and Antia, 1970; Saunders, 1972; Bennet and Hobbie, 1972; Neilson and Lewin, 1974 and references therein; Ukeles and Rose, 1976; Schwelitz *et al.*, 1978; Nicolas *et al.*, 1980; Kirst and Bisson, 1983; Schwelitz *et al.*, 1987; Markager and Sand-Jensen, 1990; Lewitus *et al.*, 1991).

Organic substrates enhance algal growth (Lewitus and Caron 1991 a, b; Lewitus *et al.*, 1991). They also

change normal physiological performance i.e. carbon allocation, photosynthesis, respiration, etc. (Antia 1980; Dubinsky *et al.*, 1986; Falkowski *et al.*, 1985; Wymann, 1992; Robaina *et al.*, 1994) and/or cell structure (Cheng and Antia 1970; Antia *et al.*, 1973, 1979; Monroy and Schwartzbach, 1984).

A few references have reported heterotrophic activity in macroalgae (Neilson and Lewin, 1974; Markager and Sand-Jensen, 1990). Organic carbon has been included in some algal culture media (Polne-Fuller *et al.*, 1984, 1986; Gusev *et al.*, 1987). Glycerol has been an effective organic substrate in axenic culture, perhaps because it is a part of floridoside and isofloridoside (galactosyl-glycerol heterosides), photosynthetic products in red and other algae (Fries 1973; Ekman *et al.*, 1991).

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In the light, glycerol enhanced vegetative growth and induced morphogenesis in explants and carpospore-seedlings of *G. doryphora* (Robaina *et al.*, 1990a,b; Robaina *et al.*, 1991).

In this paper we approach to the role of light on the structural and ultrastructural changes that follows the cultivation of carpospore-seedling of *G. doryphora* in glycerol.

MATERIAL AND METHODS

Carposporophytic thalli of *Grateloupia doryphora* (Montagne) Howe were collected in the upper and middle part of the littoral zone at S. Cristóbal (Las Palmas G.C., Canary Islands). They were immediately transported to the laboratory and disc fragments of 3 mm diameter bearing cystocarps were excised within 2 hours after collection. These disc fragments or explants were disinfected and tested for sterility following previously described methods (Robaina *et al.*, 1990a,b). Explants were then cultured in agarised Provasoli Enriched Seawater (PES, Provasoli, 1968) until carpospores were liberated under 18:6 light:dark photoregime for 30 days at $19 \pm 2^\circ\text{C}$ and $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ provided by cool white fluorescent tubes (Sylvania grolux) at the Petri dish level.

When carpospores had been liberated they were transferred to agarised glycerol-containing medium. The culture medium was an enriched seawater medium based on PES which was supplemented with 0.3 M glycerol and made by dilution of the seawater with distilled water (70% seawater) (PES70 + 0.3M glycerol) to reach 1 osmol kg^{-1} , the same osmolality of seawater. The osmolality was checked in an Autotast TM osmometer (Daiichi kogaku Co. Ltd, Tokyo, Japan) (Robaina *et al.*, 1990a). Light ($30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, 18:6) and temperature ($19 \pm 2^\circ\text{C}$) were as described above for controls. The effect of photon flux density was studied by changing the standard condition of white light of $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ to 20, 60 and $100 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. The cultivation under blue (Mazda TF 36/18-Plexi blue filter combination producing hundred nm broad peak at 450 nm) and red light (same broad peak at 640 nm by combination of Mazda TF 35/15-plexi red filter) were used to monitor "light quality" effects. Photon flux density in these experiments was adjusted to $30 \mu\text{mol m}^{-2}\text{s}^{-1}$.

To follow structural changes, one month old carpospore-seedlings in PES were cultured in PES70 + 0.3M glycerol as described, then they were collected within any of 12-14 days in which morphogenesis (bud, shoot or axe regeneration as "differentiated" algal structures) had concluded (Robaina *et al.*, 1991).

Specimens from the different developmental stages and experiments were prepared for light microscopy and transmission electron microscopy (TEM). They were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.3 M NaCl (pH 7.4) for 4 hours at room temperature. This was followed by washing in the same buffer containing 0.3 M NaCl (2 x 30 minutes) and embedded in glycol methacrylate (GMA, Historesin™, Reichert-Jung, Gerrids and Smid, 1983). Serial sections of 5 μm thick, cut on a Reichert Jung 2050 microtome, were floated on to distilled water on clean microscope slides, air dried and treated as follows for light microscopic observations:

(1) for observation of neutral polysaccharides, periodic acid-Schiff reaction (PAS) after treatments with periodic acid and sodium borohydride to block background and active aldehyde groups (McCully, 1966).

(2) for localization of sulphated polysaccharides, alcian blue pH 1.0 (Parker and Diboll, 1966).

(3) for localization of proteins, mercury bromophenol blue (Tsekos, 1983).

(4) for detection of starch, lugol (I_2/IK) (Lea, 1990).

(5) for observation of general morphology, toluidine blue (Tsekos, 1983).

Metilation, deamination and amylases treatments of control specimens confirmed the specificity of the sulphated polysaccharides, proteins and starch grains stains respectively (Kiernan 1990).

For electron microscopy, samples were postfixed in 2% OsO_4 also in cacodylate buffer containing NaCl solution for 2 hours at 4°C . The material was then dehydrated in graded series of ethanol, gradually transferred to propylene oxide and embedded in Epon. Sections of 1 μm thick cut on a Reichert Jung Ultracut E with a diamond knife were mounted on formvar-coated copper grids and poststained with aqueous uranyl acetate and lead citrate for photography in a Zeiss EM 9 S-2 electron microscope.

All experiments were repeated twice and included at least 5 replicates (a petri dish with 20-30 carpospores was considered a replicate) of each culture stage.

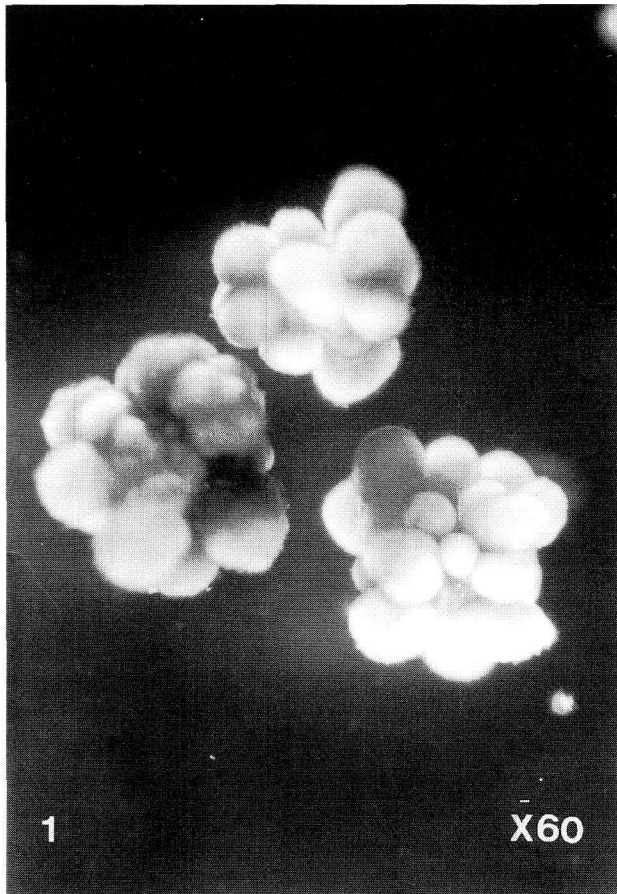


FIG. 1. – Carpospore-seedling cultivated in glycerol and $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$. The morphology, compactness and ability to regenerate new axes changed when photon fluence changed ($\mu\text{mol photons m}^{-2}\text{s}^{-1}$).

RESULTS

The carpospore-seedling cultivated in glycerol-containing medium and $30 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ (18:6) light emitted buds at 3-9 days in culture to become a budding cell mass up to 2 mm diameter in 12-14 days. This cell mass lost pigments unless transferred to new media after 8-10 days. They also originated new cell masses if split when transferred to new media (Fig. 1).

The changes in photon fluence and light quality did not vary the pattern described above (i.e. growth followed by morphogenesis) but accelerated, retarded, increased or decreased some of the events. Thus, carpospore-seedling cultivated to high photon fluence ($60\text{-}100 \mu\text{mol m}^{-2}\text{s}^{-1}$) became highly and spontaneously friable, the growth and morphogenesis was also faster than in the control. By contrast, reduction of PFD to $20 \mu\text{mol m}^{-2}\text{s}^{-1}$ retarded growth and reduced morphogenesis.

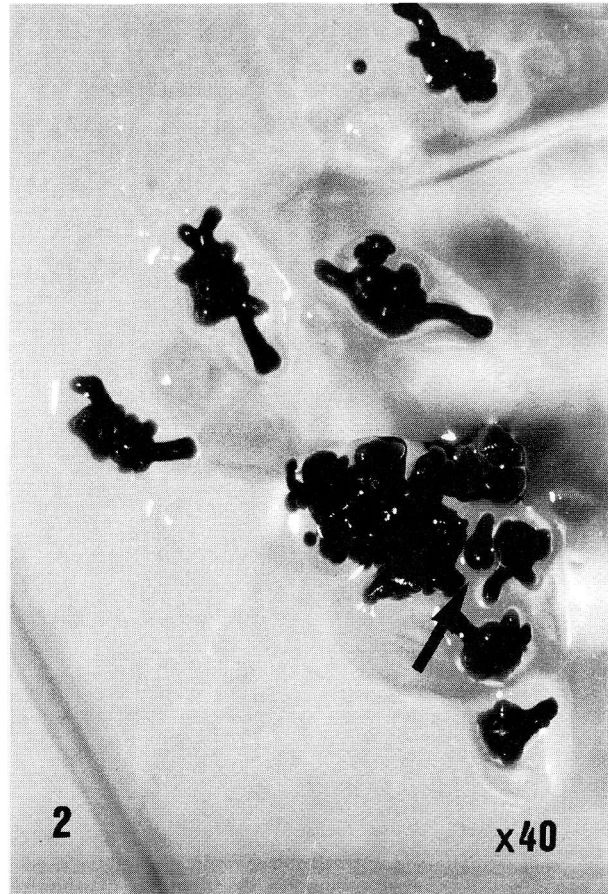


FIG. 2. – Friable cell mass with morphogenic-nodules (arrow) obtained from carpospore-seedling cultivated under blue light.

Friability (i.e. the tendency for plant cells to separate from one another; Schaeffer, 1979) increased under blue and red light treatments whose cells masses were formed by “morphogenic nodules” that could be separated quite easily (Fig. 2). If continuously supplied, both red and blue light gave such morphogenic cell masses as soon as 5 days, 4 to 7 days before material under continuous white light.

Ultrastructural changes during growth and morphogenesis

One month old carpospore-seedlings were constituted of two cellular layers: large, internal cells (medulla-like) and small, peripheral cells (cortical-like) (García-Jimenez *et al.*, 1994).

After only 2 hours in glycerol, the internal cells of the carpospore-seedlings showed a high cellular division activity. They divided rapidly (Fig. 3) to produce rows of small and rounded cells and the proliferation of new cellular layers (Fig. 3.) through

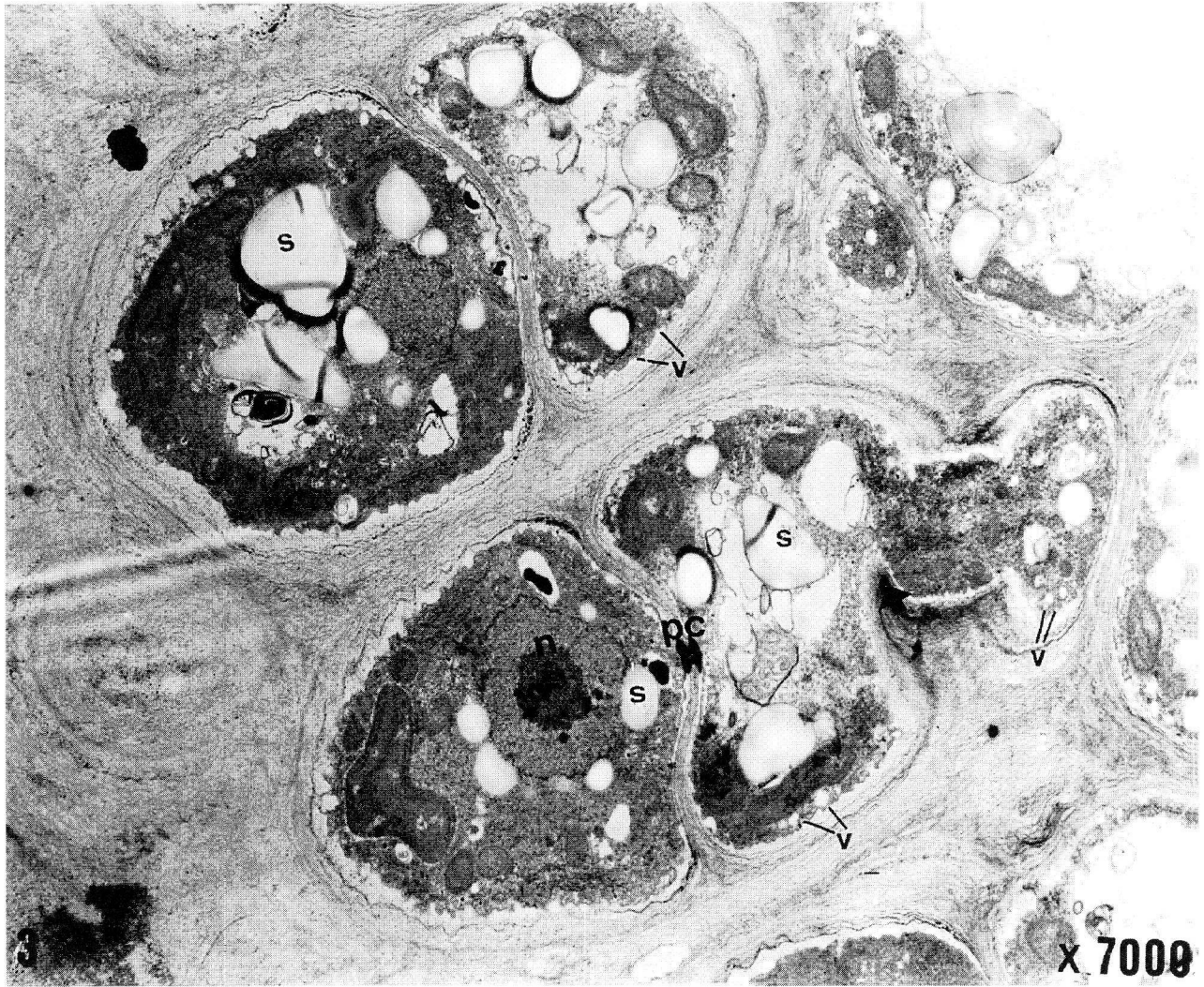


FIG. 3. – In 2 h the cells of the carpospore-seedling in glycerol and any light (i.e. photon fluence white, red or blue) divided. Starch accumulated and small Golgi vesicles secreted. The final step in cytokinesis was the presence of pit connections. n : nucleus; pc = pit connections; s = starch.

the following 2 days. Biosynthesis was evident as starch grains and lipid droplets accumulated in the cytoplasm.

In 3 to 9 days (i.e. at morphogenetic stage) cell division had produced new cell layers concentrically arranged with respect to the former internal cells. Then, cell division activity was seen in the new layers. Mercury bromophenol stained a big concentration of protein in this middle layer, as well as high neutral polysaccharides (PAS stain) in these cells. Lugol stain reacted positively giving a deep reddish-brown colour with the starch grains which disappeared as buds sprouted. This intense accumulation of compounds was revealed by TEM as an intense accumulation of starch and an increase the number of dictyosomes and secretory vesicles from Golgi (Fig. 4).

DISCUSSION

Culture conditions have been shown to control growth and development of macroalgae. Solidity (agar concentration) and osmolality (e.g. seawater or glycerol concentration) can switch callus into shoot regeneration in several red algae (Robaina *et al.*, 1990 a; Robaina *et al.*, 1992).

In this work the pattern of “growth followed by morphogenesis” of the cell masses from carpospores of *G. doryphora* changed as light (photon fluence, blue-red) changed. The growth rate as well as morphogenesis increased with increasing photon fluence, as well when blue and red replaced white light at a given fluence. Friability was higher in blue and red light and when carpospore-seedlings were cultivated under the highest photon fluence (Fig. 2).

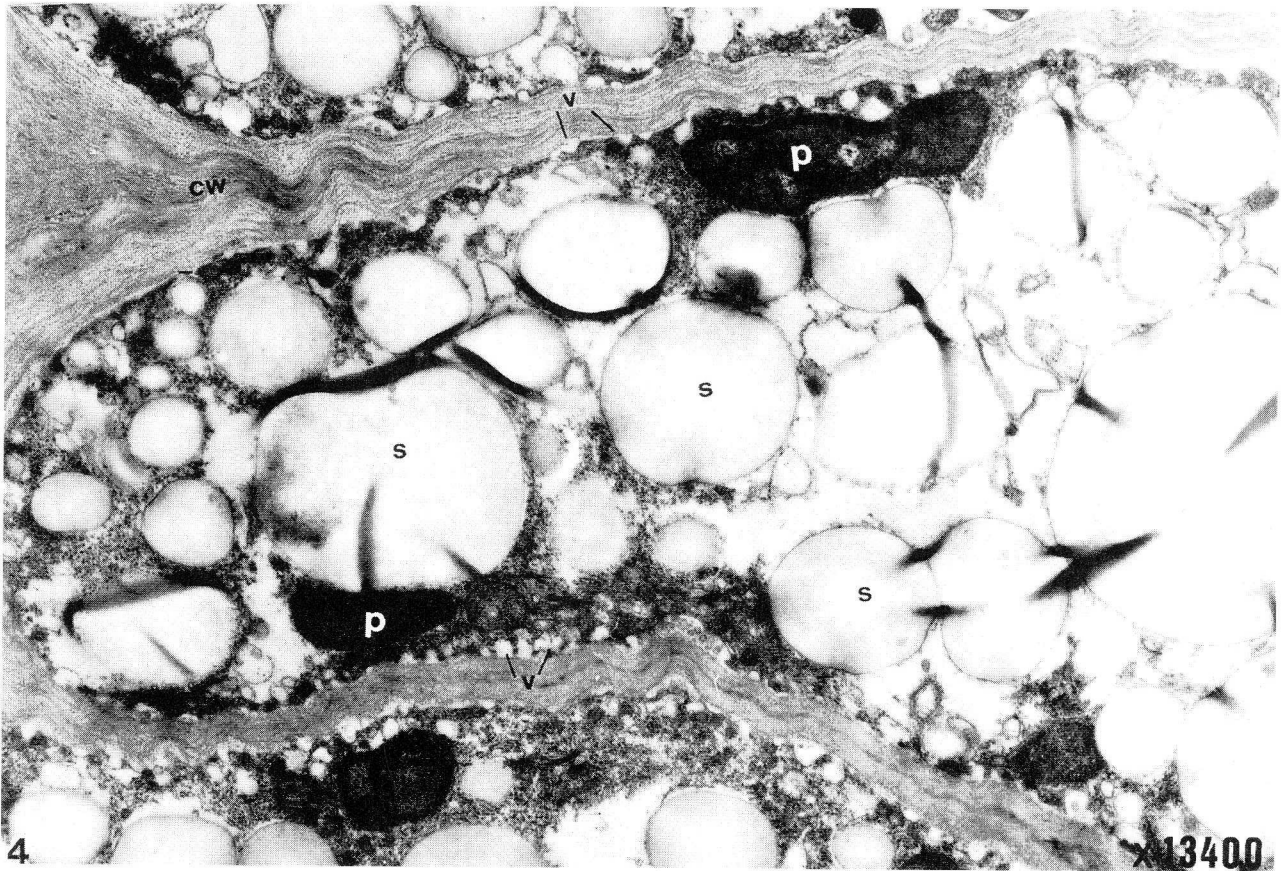


FIG. 4. – A cell from the area of starch accumulation and shoot protrusion. Note also the intense deposition of cell wall from vesicles. cw = cell wall; p = plastid; s = starch; v = vesicles.

Whereas chemical plant growth regulators do not seem to be effective, the results with *G. doryphora* and other algae suggest that an appropriate combination of culture conditions, including light and carbon sources, would constitute a tool to control growth and development in *in vitro* cultures.

The results also show that light and glycerol interferes with cell metabolism. The first outstanding effect of glycerol and light (in any light condition) was to promote cell division and biosynthesis (Fig. 3). To promote growth an organic carbon source should provide the cells with carbon and energy through metabolic enhancement. Glycerol and light (PFD and quality) enhanced respiration and changed metabolic performance in thalli (Robaina *et al.*, 1994). Probably, this also occurred with carpospore-seedlings.

Morphogenesis was the second event in culture (3 to 9 days). It correlated with the formation of the new concentric cell layers and triggering cell division and biosynthetic metabolism in their constituent cells (i.e. starch accumulation and proliferation of endomembranes, Fig. 4).

Starch has been reported to be involved in the morphogenesis of tobacco callus (Thorpe, 1977; Thorpe and Murashige, 1970; Thorpe and Meier, 1972; Brown *et al.*, 1979). We have observed a large amount of starch grains before shoot emission from the callus of the red macroalgae *Laurencia* (Robaina *et al.*, 1992). The presence of hypertrophied dictyosomes is linked with a rich supply of biosynthetic products. The fusion and discharge of Golgi vesicles in the plasma membrane contribute to cell expansion (Juniper and Roberts, 1966; Burns *et al.*, 1984). It has been also reported (Cleland, 1967, 1971; Zimmerman, 1978; Brown *et al.*, 1979) that the turnover of starch grains and the fusion of vesicles to produce large vacuoles may provide complementary plasmatic pressures for the extension of plasma membrane during growth and morphogenesis.

Although we have not observed ultrastructural differences among light treatments, further research (e.g. quantitative cytology) would explain the necessary structural changes that made the cell masses more friable as PFD increased or under blue and red light.

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