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Influence of plant growth regulators, polyamines and glycerol interaction on growth and morphogenesis of carposporelings of *Grateloupia* cultured *in vitro*

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Abstract

The influence of the plant growth regulators 2,4-D, GA_3, BA and kinetin, and the polyamines putrescine, spermidine and spermine were tested on axenic *in vitro* cultures of carposporelings of *Grateloupia doryphora*. The auxin 2,4-D (10^{-3} M) and the polyamine spermine (10^{-6} M and 10^{-3} M) induced a callus (disorganised cell mass that arose from the organised tissue of the carposporeling, as demonstrated by microscopic monitoring of the tissue). Putrescine and spermidine (10^{-3} M) transformed the carposporelings into cell masses that produced shoots. BA (10^{-3} M) and kinetin (10^{-6} M and 10^{-3} M) were inhibitory. In 10^{-1} M glycerol-containing culture medium, which is known to induce the formation of morphogenic cell masses, the addition of GA_3 M) resulted in the inhibition of the morphogenesis (i.e. shoot emission) in the cell mass. The kinetin at 10^{-6} M inhibited morphogenesis, whilst at 10^{-3} M inhibited even the formation of the cell masses. The combination of glycerol (10^{-1} M) and the auxin 2,4-D (10^{-6} and 10^{-3} M) or the polyamines putrescine, spermidine and spermine (10^{-6} and 10^{-3} M) resulted in a bigger size of the cell masses that led to a higher amount of shoots per cell mass than in glycerol alone.

Abbreviations: 2,4-D – 2,4 dichlorophenoxyacetic acid; GA_3 – gibberellic acid; BA – benzylaminopurine; kin – kinetin; put – putrescine; spd – spermidine; spm – spermine

Introduction

Organic carbon sources and plant growth regulators are basec tools for the *in vitro* propagation of macroalgae. It is normally understood that the organic carbon source supports carbon requirements for plant growth and development, and that the plant growth regulators may allow direction of the pattern of growth and development of the cells and tissues.

The carbon source by itself may alter the pattern of growth and development. In the red alga *Grateloupia doryphora*, we reported (Robaina et al., 1990b) that glycerol stimulated morphogenesis (shoot emission) in thallus explants. Glycerol also promoted growth (elongation and high cell division rate) and morphogenesis that transforms carposporelings of *G. doryphora* into morphogenic cell masses (Garcia-Jimenez et al., 1996).

Auxins, cytokinins and gibberellins have been reported to occur endogenously in algae (Bradley, 1991; Evans & Trewavas, 1991). Polyamines, like putrescine, spermidine and spermine, are ubiquitous substances that can be found in algae, animals and plants. In plants they have an effect resembling that of the plant growth regulators (Galston & Kaur-Sawhney, 1982; Smith, 1982 and references therein, 1985, Tiburcio et al., 1993), being associated with cell division in tissue (Evans & Malmberg, 1989).

The aim of this work was to study the influence of the addition of plant growth regulators (auxin, cytokinins, gibberellins) and the polyamines putrescine, spermidine and spermine on the growth

and morphogenesis of carposporelings cultured in glycerol-containing media.

Materials and methods

Thalli of *Grateloupia doryphora* (Sheet 129 of the LPA herbarium) were collected in the upper and middle part of the coastal zone of Gran Canaria (Canary Islands). Fertile material bearing cystocarps were cut to 3 mm diameter, disinfected and tested for sterility following methods which ensured that explants and the carpospores subsequently released were axenic. Sterile and fertile disc fragments were then cultured in agarised Provasoli Enriched Seawater (PES, Provasoli, 1968) for 1 month until carpospores were liberated (Garcia-Jimenez et al., 1996). Culture conditions were 30 μmol photon m^{-2} s^{-1} light intensity from cool white fluorescent lamps (Sylvania grolux), a 18:6 h light: dark cycle, 18 ± 2 °C.

All experiments were carried out with 1-month old carposporelings (12 to 20 per Petri dish, ca. 45 per treatment) which were recultured every 15 d (3 recultures). Glycerol-containing media were prepared with an enriched seawater medium based on PES which was supplemented with 10^{-1} M of glycerol + 0.8% agar in water made by dilution of the seawater with distilled water (90% seawater, PES90- glycerol: Robaina et al., 1990b). The osmolality of the medium was 1 osmol kg^{-1} as checked in an Autostat^{TM} osmometer (Daiichi kogaku Co. Ltd, Tokyo, Japan).

To examine the effects of plant growth regulators, we used cytokinins, benzylaminopurine (BA) and kinetin (kin); gibberellins and gibberellic acid (GA_3); auxins as 2,4 dichlorophenoxyacetic acid (2,4-D) and polyamines putrescine (put), spermine (spm) and spermidine (spd) at final concentrations of 10^{-3} and 10^{-6} M. They were added individually to autoclaved cultured medium as filter-sterilised stock solutions. All chemicals were from the Sigma Chemical Company. To ensure that results were caused by the addition of plant growth regulators, control assays with PES, PES + plant growth regulators (or plus polyamines) and PES90-glycerol without growth regulators (or polyamines) were run simultaneously.

Three specimens from each treatment were fixed with 2.5% glutaraldehyde in 10^{-1} M sodium cacodylate buffer containing 0.3 M NaCl (pH 7.4) for 4 h at room temperature. This was followed by washing in the same buffer containing 0.3 M NaCl (2 × 30 min) and embedded in glycol methacrylate (GMA, Historesin TM, Reichert-Jung, Gerrids & Smid, 1983). Serial sections 5-μm thick were cut on a Reichert-Jung 2050 microtome and stained for the observation of general morphology with toluidine blue and haematoxylin-eosin (Tsekos, 1983).

The experiments were repeated twice with three replicates of each treatment. Two quantitive indices were used: i) mass growth index as diameter of the cell mass in mm, determined by using an electronic caliper (Mitutoyo Digimatic Caliper, Mitutoyo Co.); and ii) morphogenic index as the number of protrusions or shoots regenerated per carposporeling (Robaina et al., 1990a, b; Robaina et al., 1992). Mean values of the index in the different treatments were compared to the control (PES90-glycerol) using student's t-test.

Results

The addition of the plant growth regulators or polyamines to the PES medium influenced the growth and development of the carposporelings: calli were observed in 10^{-3} M 2,4-D and in 10^{-6} M and 10^{-3} M spermine. Calli extended from the surface of the carposporeling as a disorganised mass of tissue to cover it almost entirely. Microscopy showed that a disorganised dense cellular mass formed the calli (Figure 1). Mass growth index reached 0.50 mm in callus in 10^{-3} M 2,4-D as the maximum value observed. 10^{-3} M BA and 10^{-6} and 10^{-3} M kinetin were inhibitory as the carposporeling did not develop further (even months after the experiments had finished. The carposporelings grew and transformed into small morphogenic cell masses (Figure 2) in the treatments with putrescine and spermidine alone at both concentrations tested. The morphogenic index ranged from 1.70 to 6.83 shoots per morphogenic carposporeling observed in media with polyamines. The cell masses resembled these typical of carposporelings grown in glycerol. In treatments with GA_3 the carposporelings formed new shoots (morphogenic index 3.5 ± 0.53 in 10^{-3} M). In PES, the carposporelings emitted 1–2 shoots (actually just the protrusion on the surface of the carposporeling, morphogenic index = 0–2 and mass growth index was 0.20 ± 0.1).

Table 1 shows the results as values of mass growth and morphogenic indices obtained from the combination of the carbon source (glycerol) and the plant growth regulators tested. When carposporelings of *G. doryphyora* were cultured in a glycerol-containing medium, glycerol first increased cell division, produc-

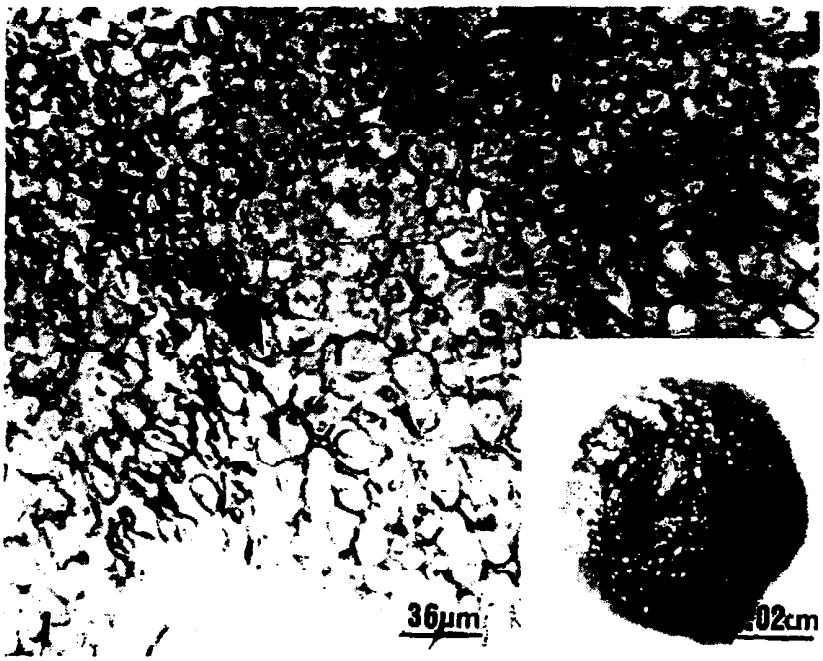

Figure 1. Callus formed by the carposporeling of *G. doryphora* cultivated in 10^{-3} M 2,4-D (lower right corner). Note the disorganised mass (arrow). Semithin sections (5 µm) stained with toluidine blue.

Table 1. Effect of plant growth regulators on carposporelings of *Grateloupia doryphora* when combined with glycerol. Data are the mean (± SD) from two experiments, each with three replicates.

Treatment	Diameter of the cell mass (mm)	Shoots per morphogenic carposporeling
10^{-1} M Glycerol	0.72 ± 0.08	9.83 ± 1.25
10^{-1} M Glycerol + 10^{-6} M 2,4-D	0.90 ± 0.15*	9.50 ± 4.34
10^{-1} M Glycerol + 10^{-6} M 2,4-D	1.36 ± 0.31*	23.50 ± 4.68*
10^{-1} M Glycerol + 10^{-6} M BA	0.75 ± 0.13	10 ± 4.9
10^{-1} M Glycerol + 10^{-3} M BA	Inhibitory	Inhibitory
10^{-1} M Glycerol + 10^{-6} M kin	0.72 ± 0.09	Non-morphogenic
10^{-1} M Glycerol + 10^{-3} M kin	Inhibitory	Inhibitory
10^{-1} M Glycerol + 10^{-6} M GA_3	0.74 ± 0.07	Non-morphogenic
10^{-1} M Glycerol + 10^{-3} M GA_3	0.64 ± 0.05	Non-morphogenic

* $P < 0.001$ as compared to control. Inhibitory = carposporeling appeared as they were at the beginning of the experiment.

ing the cell masses, and then the shoots were formed. The values of mass growth and morphogenic index in the glycerol-containing medium are higher than those obtained in plain PES or PES + plant growth regulators. The highest value for mass growth and morphogenic indices was observed in glycerol + 10^{-3} M 2,4-D among all treatments tested, included those of polyamines described below. The cell masses produced by this treatment were fully covered by sprouting shoots. The addition of 10^{-6} M kinetin or 10^{-6} and 10^{-3} M GA_3 to glycerol-containing media inhibited morphogenesis. The 10^{-3} M kinetin and 10^{-3} M BA inhibited both growth and morphogenesis.

Table 2 shows results obtained with glycerol plus polyamines. Except for 10^{-6} M putrescine and the slight increase in mass growth observed in 10^{-3} M spermidine, all the treatments with polyamines significantly increased cell growth and morphogenesis over the control with glycerol, as seen in the values of mass growth and morphogenic indices in Table 2. The effect

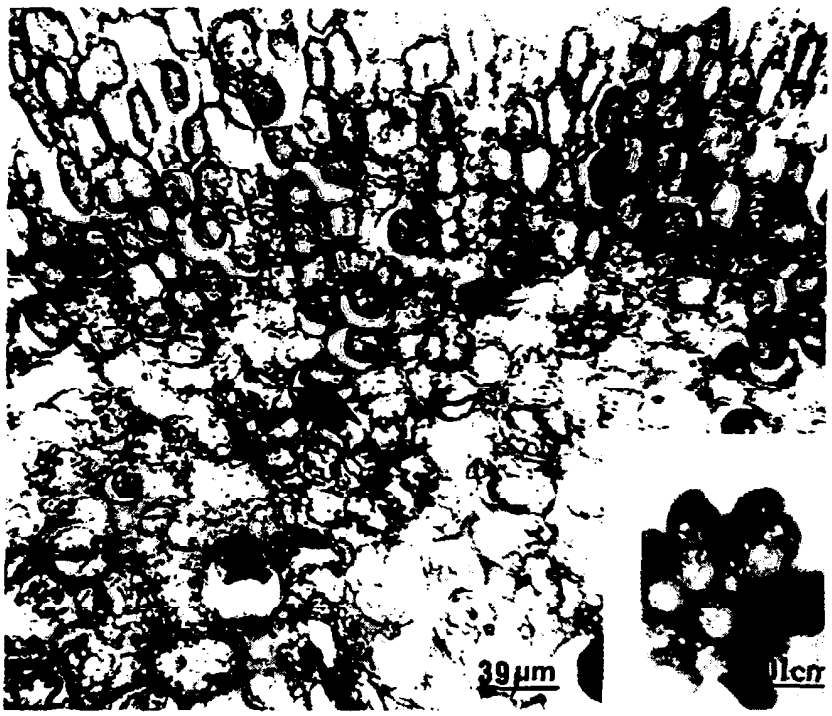

Figure 2. Cell mass formed by the carposporeling of *G. doryphora* in media with the polyamines, glycerol (10^{-1} M) and in glycerol + plant growth regulators or plus polyamines. Example shown is that observed in PES + 10^{-3} M put. Note the cells in organised arrangement (arrow). *Semithin section (5 µm) stained with haematoxylin-eosin.*

Table 2. Effects of polyamines on carposporelings of *Grateloupia doryphora* when combined with glycerol. Data are the mean (± SD) from two experiments each with three replicates.

Treatment	Diameter of cell mass (mm)	Shoots per morphogenic carposporeling
10^{-1} Glycerol	0.72 ± 0.08	9.83 ± 1.25
10^{-1} M Glycerol + 10^{-6} M put	0.63 ± 0.10	Non-morphogenic
10^{-1} M Glycerol + 10^{-3} M put	0.91 ± 0.15*	14.7 ± 4.65*
10^{-1} M Glycerol + 10^{-6} M spd	1.05 ± 0.32	16 ± 3.51*
10^{-1} M Glycerol + 10^{-3} M spd	0.83 ± 0.13	12 ± 1.86*
10^{-1} M Glycerol + 10^{-6} M spd	1.21 ± 0.17	18 ± 4.14*
10^{-1} M Glycerol + 10^{-3} M spd	1.18 ± 0.21*	18.5 ± 2.20*

* $P < 0.001$ as compared to control.

of the combination of polyamines and glycerol on growth and morphogenesis increased from the lowest concentration of putrescine to spermine. In addition, the calli observed when spermine was tested alone was not observed when combined with glycerol.

Discussion

The 2,4-D is a synthetic auxin able to induce cell division and growth even in the more recalcitrant plant tissues (Evans et al., 1983). It has been reported to induce filament growth in *Ascophyllum* (Fries, 1991) and the growth of callus-like structures in *Grateloupia dichotoma* (Yokoya & Handro, 1996). In the present study, a callus sprouting from the carposporelings of *G. doryphora* was observed as a result of treatment with 10^{-3} M 2,4-D (Figure 1). The 2,4-D combined with glycerol increased growth and morphogenesis over glycerol and glycerol and the other plant regulators tested (Table 1). It should be mentioned that calli induced by 2,4-D and cell masses obtained in 2,4-

D + glycerol were further propagated in PES, where they regenerated shoots and apparently normal thalli in PES (26 calli of about 20 mg fresh weight produced up to 200 mg fresh weight thalli in 4 months). A combination of glycerol and plant growth regulators was reported to induce growth of the callus in *Gracilaria verrucosa* (Kaczyna & Megnet, 1993) although the auxin 2,4-D had no significant effect.

Result on Table 1 also show that cytokinins and gibberellins influenced on the effects of glycerol on growth and morphogenesis of the carposporelings. The 10^{-6} M kinetin and 10^{-6} and 10^{-3} M GA_3 tested inhibited the morphogenic effect of glycerol. At higher concentration (10^{-3} M), BA and kinetin inhibited both growth and morphogenesis.

Glycerol also influenced the effects of the plant growth regulators as compared when they were tested alone. Thus, callus was not observed when 2,4-D was combined with glycerol since the carposporelings became organised cell masses (compare Figure 1 to Figure 2), and the presence of glycerol relieved the inhibitory effect of the lowest (10^{-6} M) concentration of kinetin.

The causes of the interaction of glycerol and plant growth regulators (and polyamines, discussed below) remain to be explored. Nevertheless, it is interesting to note that when algae are supplied with organic substrates, the latter altered basic metabolic features and redirected them towards different end-products (Antia, 1980; Robaina et al., 1995). Carposporelings of *G. doryphora* accumulate floridean starch, phenolic and other unknown compounds in large electron- dense vesicles when cultured in glycerol (Garcia-Jimenez et al., 1996). Metabolic differences as the algae are grown with or without glycerol may result in different endogenous concentrations of plant growth regulators in the carposporelings, hence the exogenous addition of the same amount of a particular plant growth regulator would produce different effects in each case. Mooney and Van Staden (1986) argued explicitly that exogenous applied kinetin inhibited growth in algae due to the fact that supraoptimal concentrations were achieved.

There are few reports on the occurence of polyamines in algae. Uptake and transport within the thallus were reported in *Ulva rigida* (Badini et al., 1994). The unicellular *Porphyridium* absorbed polyamines with the polysaccharides in the cell wall (Scoccianti et al., 1989; Scoccianti & Bagni, 1992). Cohen et al. (1984) found evidence that the polyamines promoted cell division in *Chlorella*. In the present paper, carposporelings cultured in the presence of putrescine and spermidine grew and became cell masses (Figure 2) similar to those reported for glycerol (though smaller, see Figure 14 in Garcia-Jimenez et al., 1996). The polyamines combined to glycerol interacted to significantly increase growth and morphogenesis of the carposporelings in a concentration- dependent manner (Table 2). The strongest effect was obtained with the polyamine spermine, with which we have found the highest increase in mass growth and morphogenic indices. In fact, the results obtained with spermine were closed to those of the auxin 2,4-D, considering that both substances induced callus or enhanced cell growth and morphogensis when combined with glycerol (Tables 1 and 2).

Several authors (Seraffini-Fracassini, 1991; Kotzabasis, 1996) have related the effects of polyamines to their contribution to cellular carbon and nitrogen. Although this possibility cannot be discarded as an explanation for our results, it is unlikely that the sole addition of 10^{-3} or 10^{-6} M of polyamines to 10^{-1} M glycerol would increase by itself the amount of organic carbon available for growth and morphogensis. Our previous experiences have proved that carbon sources were ineffective below 10^{-1} M (Robaina et al., 1990b).

In conclusion, the cytokinins kinetin and BA and gibberellins GA_3 were inhibitory for growth and/or morphogenesis induced by glycerol in carposporeling cultured *in vitro*. The auxin 2,4-D (10^{-6} and 10^{-3} M) or the polyamines putrescine, spermidine and spermine (10^{-6} and 10^{-3} M) resulted in bigger cell masses that emitted higher amount of shoots per cell mass than in glycerol alone.

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