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Morphogenetic effect of glycerol on tissue cultures of the red seaweed *Grateloupia doryphora*

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Abstract

Explants of *Grateloupia doryphora* were cultivated in Provasoli Enriched Seawater culture medium (PES) supplemented with glycerol (0.1, 0.3, 0.5 or 0.8 mol l^{-1}) or carbohydrates (0.1 or 0.3 mol l^{-1} mannose, glucose and galactose) and agar (3, 8, 15 g l^{-1}). The osmolality of the medium was adjusted by dilution of the seawater (70 or 100%, v v). The increase in fresh weight of explants cultivated in liquid medium with glycerol (0.3 mol l^{-1}) and without glycerol was compared. All experiments were carried out in the light, except for one assay in which the explants were cultivated in the dark. Glycerol was an effective carbon source for the vegetative propagation of *G. doryphora* in solid and liquid media. Mannose, glucose and galactose all had no effect on growth or morphogenesis of the explants. In solid media the main effect of glycerol was as a morphogenetic inductor, with PES70 (70% seawater) + 0.1 or 0.3 mol l^{-1} glycerol + 3 or 8 g l^{-1} agar the best formulation. An increase in the concentration of agar in glycerol-containing medium reduced the morphogenetic capacity of the explants, which developed into compact cell masses. The effects of glycerol were observed only in explants cultivated under light.

Abbreviations: PES, Provasoli Enriched 100% Seawater; PES70, Provasoli-Enriched 70% (v v) seawater in distilled water.

Introduction

Carbon sources effective for culture of seaweeds and tissue cultures seem to be those that are accumulated by seaweed cells (Saga *et al.*, 1982; Fries, 1984). Red seaweeds accumulate glycerol in the form of isofloridoside, floridoside (isomeric compounds of galactosyl-glycerol) or digeneaside (mannosyl-glycerol (Lobban *et al.*, 1985), all of which appear to be involved in cellular osmo-adaptation (Kauss, 1967; Reed *et al.*, 1980; Reed, 1985). Fries (1973) reported that glycerol is an effective carbon source for the vegetative propagation of several red seaweeds.

Robaina *et al.* (1990) reported a strong effect of high osmolality and solidity (agar concentration) on bud regeneration and callus formation in *Grateloupia doryphora*. They pointed out that the addition of glycerol to the culture medium modifies the response of the expants by the carbon

source effect of glycerol and also by the modification of the osmolality.

The aim of the present work was to study the effects of glycerol and other carbon sources on tissue cultures of *G. doryphora* with controled osmolality and solidity of the culture medium.

Material and methods

Grateloupia doryphora (Montagne) Howe was collected in Gran Canaria (Canary Islands). Two h after collection, disc fragments (3 mm diameter) were excised from the middle-lower thallus. Disc fragments were rinsed for 5 min in distilled water and mechanically cleaned with brushes and sonication (3 times, 1.5 min each). The explants were then immersed in Betadine (5% v/v of Betadine, 10% polyvinylpyrrolidone-iodine complex commercial solution. Sarget Lab. Barcelona. Spain) for 7 min. Sterilization was continued by incubation for 5 days in 10 ml autoclaved seawater with penicillin (300 mg l^{-1}) (Sigma), ampicillin (30 mg l^{-1}) (Sigma), nystatin (25 mg l^{-1}) (Sigma) and germanium dioxide (5 mg l^{-1}) (Aldrich). The fragments were tested for sterility by cultivation for 10 days in Provasoli Enriched Seawater (PES, Provasoli 1968) supplemented with glucose (0.5 g l^{-1}), sucrose (1 g l^{-1}), casein hydrolysate (0.5 g l^{-1}) and lactose broth (1 g l^{-1}, Difco). Disc fragments apparently free of contaminants (under stereomicroscope) were transferred to agarized PES medium. After 30 days, semicircular-shaped explants (ca 1 mm long) were excised from the disc fragments.

Explants were cultivated in Petri dishes with 20 ml of culture media. Cultures were placed at 22 ± 2 °C in a growth chamber adjusted to a day length of 18 h and 27 μmol m^{-2} s^{-1} (Sylvania Grolux) at the level of the Petri dishes.

The culture medium for semicircular explants was an enriched seawater medium based on PES, which was supplemented with 0.0, 0.3, 0.5 and 0.8 mol l^{-1} glycerol (Merck). The seawater was diluted and the appropiate amount of glycerol was added to reach the osmolalities shown in Table 1. The osmolality was checked in a Autostat TM osmometer (Daiichi Kogaku Co. Ltd, Tokyo, Japan). All experiments were carried out with solid culture medium (8 g l^{-1} agar), but the fresh weight increased of semicircular explants cultivated in liquid PES70 + 0.3 mol l^{-1} glycerol was also monitored during 45 days (reculture every 15 days). A control assay was made in liquid PES.

After 20 days, explants were transferred from solid culture media with high concentration of glycerol – high osmolality (1.5 Os kg^{-1}) (Table 1) to solid medium with 0.3 mol l^{-1} glycerol (1.0 Os kg^{-1}).

The effects of glycerol, mannose, glucose and galactose were compared by cutting and cultivating the buds (ca 0.5 mm long) that sprout at the border of initially cultivated disc fragments. The buds (explants; 15 per treatment) were cultivated for 30 days in PES70 + 0.1 or 0.3 mol l^{-1} of the carbon sources + 8 g l^{-1} agar (Table 1).

Table 1. Concentration of seawater, glycerol and carbohydrates (mannose, glucose or galactose) in the different culture media.

Culture medium	Seawater (%, v/v)	Osmolality (Os Kg^{-1})	Glycerol (mol l^{-1})	Carbohydrates (mol l^{-1})
PES70	70	0.7	0	0
		0.8	0.1	0
			0	0.1
		1.0	0.3	0
			0	0.3
		1.5	0.8	0
PES	100	1.5	0	0
			0.5	0

Agar concentation experiments were carried out varying the usual concentration (8 g l^{-1}) of the PES70 + 0.3 mol l^{-1} glycerol to 3 or 15 g l^{-1} agar. Semicircular explants were used in these experiments (15 explants in each medium).

An experiment was made to see the effects of light on growth. Fifteen semicircular explants were cultivated in darkness in PES70 + 0.3 mol l^{-1} glycerol and 8 g l^{-1} agar.

The morphogenetic response of the explants was quantified by 'number of buds/number of cultivated explants' and/or 'number of buds/number of morphogenetic explants' (Garcia-Reina & Luque, 1988). The increase in length of the bud explants was used to quantify experiments with this material.

Results

Effects of glycerol on growth and regeneration of the explants

Glycerol had a positive effect on explant growth and morphogenetic capability. Within 45 days of cultivation, liquid PES70 + 0.3 mol l^{-1} glycerol increased fresh weight of explants by more than 400% over PES (Fig. 1). In solid PES70 + 0.3 mol l^{-1} glycerol (3 or 8 g l^{-1} agar), the semicircular explants turned from reddish to orange and became rounded and bigger than those in the other culture media. After 25 days, the growth of the explants made it necessary to subculture them to avoid desiccation and death.

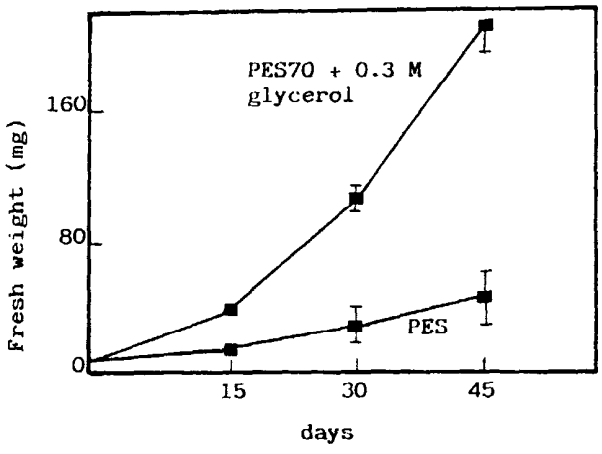

Fig. 1. Variation of the fresh weight of explants cultivated in liquid PES70 + 0.3 mol l^{-1} glycerol and liquid PES (average fresh weight from three replicates with 15 explants in each. Vertical bars = ± SD).

The index 'number of buds/number of cultivated explants' decreased from culture media without glycerol (PES70) or with 0.3 mol l^{-1} glycerol (0.7 and 1.0 Os kg^{-1} respectively) to media with 0.5 or 0.8 mol l^{-1} glycerol (1.5 Os kg^{-1}) (Table 2).

Explants cultivated on media with PES + 0.5 mol l^{-1} and PES70 + 0.8 mol l^{-1} glycerol were transferred to the 0.3 mol l^{-1} glycerol + 8 g l^{-1} agar medium. After 20 days, 70% of the explants recultivated from PES + 0.5 mol l^{-1} glycerol, and 90% of those recultivated from PES + 0.8 mol l^{-1} glycerol showed bud regeneration. Explants recultivated from PES70 + 0.5 mol l^{-1} glycerol showed higher value of the index 'number of buds/number of morphogenetic explants' than explants coming from PES only (Table 3). Explants coming from PES70 + 0.8 mol l^{-1} glycerol showed higher values of both 'number of buds/number of cultivated explants' and 'number of buds/number of morphogenetic explants' than those coming from PES only (Table 3).

After 30 days, the bud explants were larger and regenerated new buds, which sprouted around them. The bud explants cultivated in PES70 and PES showed a significantly higher longitudinal growth than hose cultivated in PES70 + 0.3 mol

Table 2. Values of the index 'number of buds/number of cultivated explants' (B/C) observed in semicircular explants cultivated in different concentrations of glycerol and agar. (n = 15).

Osmolality (Os kg^{-1})	Glycerol (mol l^{-1})	Agar (g l^{-1})	B/C index
0.7	0.0	8	7.1
1.0	0.0	8	7.2
	0.3	3	18.3
	0.3	8	16.1
	0.3	15	6.2
1.5	0.5	8	1.5
	0.8	8	2.6

Table 3. Values of the indices 'number of buds/number of cultivated explants' (B/C) and 'number of buds/number of morphogenetic explants' (B/M) 20 days after transfer from PES70 + 0.8 mol l^{-1} glycerol and PES + 0.5 mol l^{-1} glycerol to PES70 + 0.3 mol l^{-1} glycerol (agar = 8 g l^{-1}; n = 15 semicircular explants).

Transfer from	B C index	B M index
PES to PES70 + 0.3 mol l^{-1} Glycerol (control)	16.2	17.3
PES + 0.5 mol l^{-1} glycerol to PES70 + 0.3 mol l^{-1} glycerol	17.4	61
PES70 + 0.8 mol l^{-1} glycerol to PES70 + 0.3 mol l^{-1} glycerol	52	87

l^{-1} glycerol (Table 4). Bud explants cultivated in 0.1 and 0.3 mol l^{-1} turned orange, as observed for semicircular explants, and showed the highest morphogenetic response (Table 5). Mannose, glycose and galactose did not affect pigmentation or morphogenesis; no differences were observed among explants cultivated in PES70 or PES and those cultivated in media supplemneted with carbohydrates (Table 5).

Effects of agar concentration

The 'number of buds/number of cultivated explants' was negatively correlated with the agar concentration (Table 2). The explants cultivated on media with 3 g l^{-1} agar showed bud formation on the border, and the initial explant morphology was maintained. Ten% of the explants cultivated on media with 8 g l^{-1} agar did not regenerate buds: their initial morphology was lost and they developed into compact cell masses larger than

Table 4. Increase in length (average ± SD) of buds cultivated in solid media (8 g l^{-1} agar) with and without glycerol, osmolality 1.0 Os kg^{-1} (**, $0.05 > P > 0.01$, n = 15).

Culture medium	Length increase (mm)
PES70 + 0.3 mol l^{-1} glycerol	6.5 ± 2.2
PES	21.7 ± 6.0(**)

Table 5. Values of the index 'number of buds/number of cultivated explants' observed in buds after 30 days of culture in media with and without carbon source. Agar 8 g l^{-1} in all media (n = 15).

Culture medium	Carbon source	Concentra. (mol l^{-1})	B/C index
PES	–	0	2.8
PES70	–	0	2.6
PES70	glycerol	0.1	9.8
		0.3	7.6
PES70	mannose	0.1	1.7
		0.3	1.1
PES70	glucose	0.1	4.4
		0.3	2.4
PES70	galactose	0.1	1.5
		0.3	2.1

the explants cultivated on media without glycerol. When the agar concentration was increased to 15 g l^{-1}, 47% of the cultivated explants showed a disorganized pattern of growth (Fig. 2a). Filamentous callus-like masses sprouted from the explants cultivated on 8 and 15 g l^{-1} agar.

Effects of darkness

Explants cultivated in PES70 + 0.3 mol l^{-1} glycerol (8 g l^{-1} agar) in darkness did not show growth or regeneration.

Discussion

Bud regeneration in *Grateloupia doryphora* is observed only when the osmolality of the culture medium is 0.7 to 1.0 Os kg^{-1} (Robaina *et al.*, 1990). Explants cultivated in a high concentration of glycerol did not regenerate (Table 2), but they maintained the morphogenetic capability as shown after reculture in 0.3 mol l^{-1} glycerol (Table 3). The results in Table 3 suggest that explants accumulated glycerol and when they were recultivated to a non-inhibitory culture medium (0.3 mol l^{-1}) the morphogenetic effect of glycerol was even higher. Consequently, the inhibiting

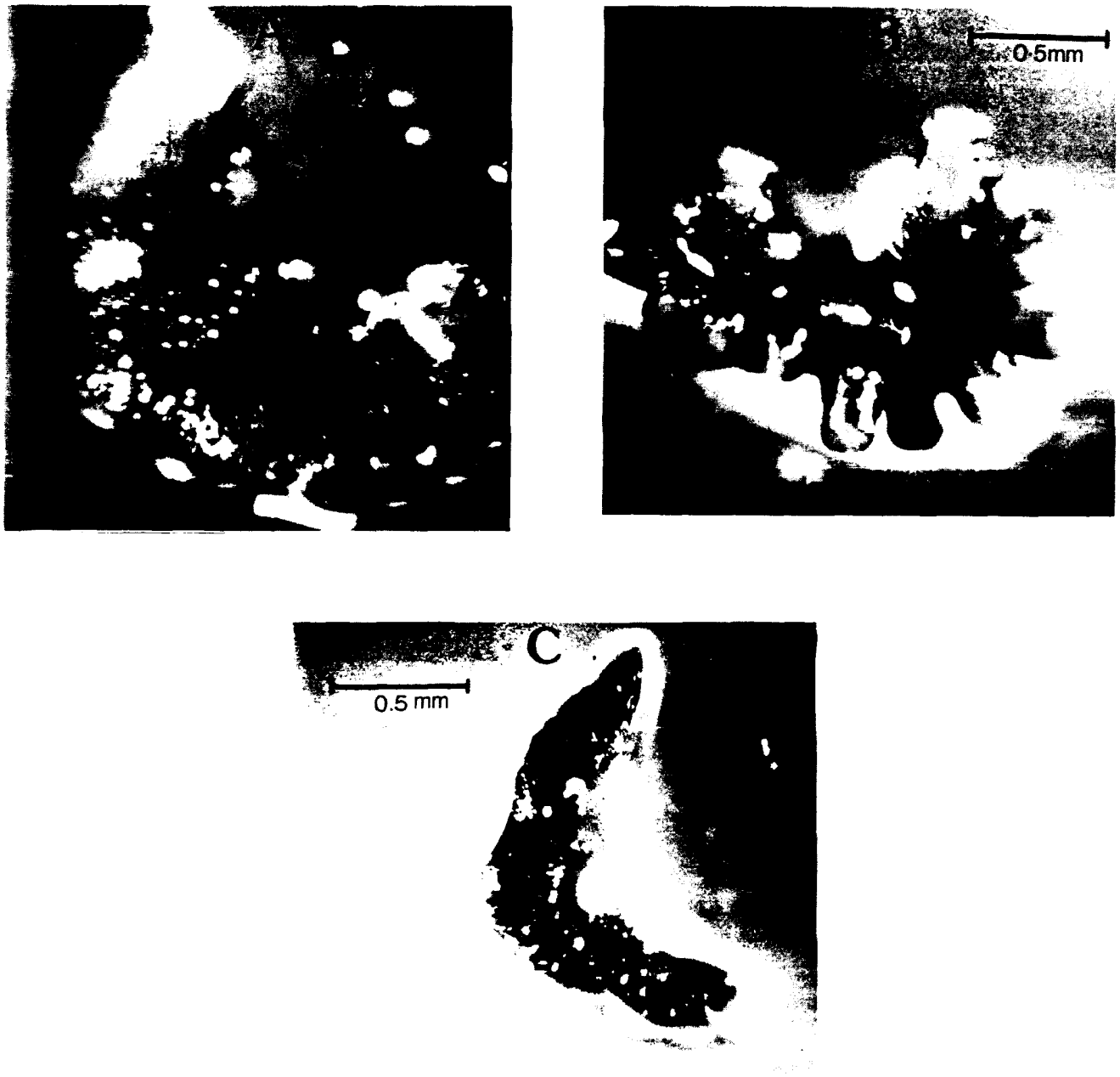

Fig. 2. Explant morphology after 20 days of culture in PES70 + 0.3 mol l^{-1} glycerol + 15 g l^{-1} agar (A), PES70 + 0.3 mol l^{-1} glycerol + 8 g l^{-1} agar (B), and PES + 8 g l^{-1} agar (C).

effect of high concentrations of glycerol is due to the high osmolality of the culture medium (Table 1) rather than an inhibitory effect of glycerol. Mannose, glucose or galactose did not promote growth or morphogenesis of the explants. Galactose and mannose appear to be somewhat inhibitory for bud formation (Table 5). Since the osmolality of these culture media ranged from 0.8 to 1.0 Os kg^{-1}, an osmotic effect of the mentioned carbon sources is discarded. Glucose, fructose, mannitol and other carbon sources were not effective in *Ecklonia* in the light (Lawlor *et al.*,

1988). The absence of a co-factor or inhibition of enzymes required for carbon metabolism were suggested by Lawlor *et al.* (1989) as a cause of the failure of several carbon sources, including glycerol and mannitol, to induce growth in the dark. The results obtained with glycerol (Fig. 1 & 2, Tables 2 & 5) clearly show that carbon metabolism is not inhibited in *Grateloupia doryphora*. Perhaps *G. doryphora* prefers glycerol as a carbon source or the enhancement of the viscosity of the culture medium promoted by glycerol contributes not only to facilitate the diffusion of ions, as suggested by Fries (1985), but also glycerol uptake. Carbon metabolism in *G. doryphora* harbours interesting problems and requires further research.

The main effect of glycerol in solid medium appears to be as a morphogenic inductor, since the higher the glycerol content of the culture medium the stronger the morphogenic response of the explants after transfer (Table 3). Moreover, bud cultures change from longitudinal growth to morphogenesis when they are cultivated in a glycerol-containing medium (Table 4). Glycerol compounds are involved in osmoadaptation in seaweeds (Kauss, 1967; Reed *et al.*, 1980; Reed, 1985; Macler, 1988) and intracellular osmotic compounds have been implicated in shoot induction in higher plant tissue cultures (Brown *et al.*, 1979). Still, the fact that galactose and mannose are not so effective as glycerol obscures the implication of glycerol compounds in the morphogenetic effect of glycerol.

The agar concentration of the culture medium affects morphogenesis in higher plants (Brown *et al.*, 1979; Debergh 1983). In *G. doryphora*, bud regeneration is also reduced by high agar concentration in the culture medium (Robaina *et al.*, 1990). The data in Table 2 show that an increase in the concentration of agar reduced the morphogenetic effect of glycerol. The capacity of glycerol to promote growth does not seem to be reduced as the explants reached a similar size in all media containing glycerol, regardless of their agar concentration. The increase in explants developing into compact cell masses when the agar concentration is increased, points to the implication of agar not only in the induction of filamentous callus-like cell masses, but also in the disorganization of the whole explant (Fig. 2a).

Glycerol did not substitute for photosynthetic activity as explants cultivated in the dark did not grow or renegerate. Other effects of the light could be involved, since light controls the metabolism of some carbon compounds in algae (Lüning, 1981), and light has been implicated in the regulation of floridoside synthesis in *Gelidium* (Macler, 1986).

In conclusion, glycerol is an effective carbon source for *Grateloupia doryphora* tissue culture, promoting growth and morphogenesis. The effects of glycerol are influenced by the osmolality of the culture medium (glycerol content), agar concentration and light.

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