PRODUCCION CIENTIFICA DEL

INSTITUTO DE ALGOLOGIA APLICADA

1990-1996

ULPGC/ITC

El **Instituto de Algología Aplicada** (IAA) es un proyecto que siempre hemos considerado de **interés estratégico** para Canarias.

La consideración del concepto "*isla*" como un territorio rodeado de algas por todas partes y de la "*agronomia marina*" como un sistema agro-industrial que no consume los recursos escasos (agua, tierra fértil, mercados establecidos) y explota las ventajas estratégicas locales (irradiación solar, temperatura, aguas no contaminadas) es la idea fundamental que nos ha impulsado a crear una infraestructura y un equipo humano que pueda abordar un proyecto tan innovador y competitivo para Canarias.

Hemos desarrollado nuestra actividad en tres frentes:

- formacion de personal (científicos, doctores y personal técnico)
- investigación fundamental
- investigación aplicada

La carencia en Gran Canaria de la infraestructura material y humana imprescindible para desarrollar un proyecto de I+D tan ambicioso como el planteado se ha podido ir paliando con voluntarismo, contactos internacionales y capacidad de aguante durante los últimos años. La pertinaz carencia de criterios, de organización y de planificación de la I+D continúa siendo un lastre que termina corroyendo toda capacidad de aguante, sobre todo en un entorno social tan poco conocedor de su imporancia y de sus reglas.

La reciente demanda de la Biblioteca Central para recopilar la producción científica que se genera en la ULPGC nos ha motivado a presentar y tabular la **cantidad y calidad** de la produccion científica que ha generado el IAA, baremada conforme a los **criterios internacionales de calidad**. Su estudio comparativo permitiría obtener conclusiones que posibilitarían el saneamiento y clarificación del sistema actual y, quizás, abrir la posibilidad para alcanzar los niveles de calidad y rentabilidad que decimos desear para nuestra Institución.

Dr. Guillermo Garcia-Blairsy Reina
Director del IAA/ULPGC

Resumen de la calidad de la producción científica del Instituto de Algologia Aplicada (1988-96).

I.I.= Indice de impacto recogidos en el Journal Citation Reports (1994, ISI).
(*) Revistas no recogidas en el JCR (1994, ISI).
Pos/N°Rev= Posición que ocupa la revista en el ranking de calidad de las revistas de su Area Científica y número total de revistas del área (entre paréntesis).

AÑO	AREA CIENTIFICA	REVISTA	N° ART	I.I.	POS./ N° REVISTA	I.I. ACUMUL
1988	PLANT SCIENCES	J. Plant Physiol.	2	1.088	37 (130)	2.176
		Plant Cell Tiss Org	1	0.745	53 (130)	0.745
		Total	3			2.921
1990	MARINE BIOLOGY	Hydrobiologia	1	0.592	43 (62)	0.592
		J. Applied Phycology	1	0.803	33 (62)	0.803
		Total	2			1.395
1991	MARINE BIOLOGY	Hydrobiologia	1	0.592	43 (62)	0.592
		Total	1			0.592
1992	PLANT SCIENCES	Planta	2	3.300	12 (130)	6.600
		Plant Phys Biochem.	1	0.967	42 (130)	0.967
		British Phycol Journal	1	0.844	48 (130)	0.844
	MARINE BIOLOGY	Marine Biology	2	1.359	9 (62)	2.718
		J. Applied Phycology	1	0.803	33 (62)	0.803
		Total	8			11.932
1993	PLANT SCIENCES	Plant Cell Reports	1	1.590	24 (130)	1.590
		Physiol. Plantarum	1	1.507	26 (130)	1.507
	MARINE BIOLOGY	J. Phycology	1	1.932	4 (62)	1.932
		Marine Biology	1	1.359	9 (62)	1.359
		Hydrobiologia	2	0.592	43 (62)	1.184
	MICROBIOLOGY	Arch. Microbiology	1	2.125	20 (61)	2.125
		Current Microbiology	1	0.953	42 (61)	0.953
		Total	8			10.650
1994	PLANT SCIENCES	Planta	1	3.300	12 (130)	3.300
		Eur. J. Phycology	1	0.600	62 (130)	0.600
	MARINE BIOLOGY	Scientia Marina	2	(*)		
		Total	4			3.900
1995	PLANT SCIENCES	Planta	3	3.300	12 (130)	9.900
	MARINE BIOLOGY	Marine Biology	1	1.359	9 (62)	1.359
		J. Appl. Phycol.	1	0.803	33 (62)	0.803
	FOOD SCIENCE & TECHNOLOGY	J. Sci. Food Agr.	1	0.866	17 (70)	0.866
		Total	6			12.928
1996	PLANT SCIENCES	Planta	1	3.300	12 (130)	3.300
		Eur. J. Phycology	1	0.600	62 (130)	0.600
	MARINE BIOLOGY	J. Appl. Phycol.	1	0.803	33 (62)	0.803
		Hydrobiologia	1	0.592	43 (62)	0.592
		Scientia Marina	4	(*)		
	ANALYTICAL CHEMISTRY	Fresen J. Anal. Chem.	1	0.975	28 (50)	0.975
		Total	9			6.270
		TOTAL	**41**			**50.588**

Científicos, estudiantes de doctorado y personal laboral que ha colaborado con el equipo de Algologia Aplicada desde el año 1988.

(*En itálica el personal que actualmente compone el Equipo de Algología Aplicada*)

Rosa Artiles Bolaños
Juana R. Betancort Rodríguez
Mats Björk
Elena del Campo Fernández
Jonas Collen
Yolanda Freile Pelegrín
Guillermo García Reina
Juan Luis Gómez Pinchetti
Kurt Haglund
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Roberto Jiménez del Rio
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1987

REGENERATION OF THALLICLONES[1] FROM *LAURENCIA* SP. (RHODOPHYTA)

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INTRODUCTION

Tissue culture techniques could be applied to improve the genetic qualities of seaweeds. In order to apply those techniques, efficient methods for obtaining and regenerating calli are needed. The term "callus" or "calluslike" has been applied to define very different structures in seaweeds (Dixon 1963; Fries 1980; Chen 1982; Saga et al. 1982; Tsekos 1982; Polne-Fuller et al. 1984; Yan 1984). Histological studies have been performed on "tumour-like" growths induced by bacteria in *Gigartina teedii* (Tsekos 1982), and to our knowledge nothing is known on the cytological events preceding differentiation from true callus. In a previous paper (Garcia-Reina et al. 1987), we reported the spontaneous formation of morphogenetic calli, the different calligenic potentials among *Laurencia* species and primary explants, and the lack of necessity for axenicity. Callus growth was drastically reduced after its organogenetic trigger, and the organogenetic potential seemed to decrease with time in culture.

The objective of this paper was: (1) to study the effect of different physical states of the culture media and its supplementation with several hormones to determine their effect on *Laurencia* callus induction, growth and in vitro regeneration, and (2) the histological study of the callus, in an attempt to clarify the callus versus calluslike composition.

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[1] Thalliclones = thalli regenerated from callus.

MATERIAL AND METHODS

Apical branched segments (5 mm long) from Laurencia sp. were used as the primary explant. Two hours after collection they were subjected to the "sonication - 1% Betadine - GAN" treatment described previously (Garcia-Rheina et al. 1987) and cultured in petri dishes with 20 ml Provasoli-enriched seawater media (PES) (Provasoli 1968). Seawater (not the enrichment) was diluted 70 and 50% with distilled water in the "seawater assay". PES was supplemented with GEO_2 (0.5 mg l^{-1}), Ampicillin (10 mg l^{-1}), Nyastatin (2 mg l^{-1}), (GAN) and agar (Bacto Difco, 8 g l^{-1} agar were tested; also 45 explants were cultured in PES ± GAN liquid media. Two or 5 mg l^{-1} kinetin, BA or 2,4-D was added to the media in the "hormone assay". The effect of 5 mg l^{-1} Na-Naphtenate (Pfalz and Bauer, Inc., NO0910) was also tested.

The cultures were incubated in a growth chamber adjusted to 21 + 2°C, 18 h light and 1500 lx. Transfers of cultures to fresh media were done at 15-day intervals. After 45, 60, 75 and 90 days the number of buds arising from the calli were evaluated. Calli with a homogeneous phenotype were transferred to an aerated liquid medium (PES + GAN) after 60 days in culture. The histological study of calli was performed following previously described methods (Devilopoulos and Tsekos 1986). Semi-thin sections (0.5 μ) were stained with toluidine blue and post-stained with lugol.

RESULTS

After 45 days in culture (uni-algal, non-axenic), we found different types of growth: (1) callus arising from the cut surface or from the disorganization of the apical neogrowth, with or without thalliclonal regeneration; (2) finger like growths of buds from the cut or apical zone; (3) thallus arising from the cut end or developing from the buds of the primary explant.

Reducing seawater concentration (osmotic potential) to 70 and 50% halted development of any type of growth (Table 1).

Reducing agar concentration (matric potential) produced an increase in the number of viable explants, but a decrease in the number of explants producing callus (Table 1) None of the explants in liquid media produced callus.

Table 1. Effects of the different assays on the type of growth after 45 days in culture

Assay[b]		N	V	C	Type of growth[a] F	T
Seawater	50%	45	0	-	-	-
	70%	45	2	0	0	2
	100% (*)	45	16	10	2	4
Agar (g l^{-1})	3	45	43	0	0	43
	8 (*)	45	17	7	4	6
	20	45	17	17	0	0
2 mg l^{-1} Kinetin		30	21	15	4	2
2 mg l^{-1} BA		30	17	8	6	3
2 mg l^{-1} 2,4-D		30	28	18	7	3
5 mg l^{-1} Kinetine		30	0	-	-	-
5 mg l^{-1} BA		30	0	-	-	-
5 mg l^{-1} 2,4-D		30	0	-	-	-
5 mg l^{-1} Na-Naph.		30	0	-	-	-
Control (*)		30	11	7	0	4

[a]V= Viability (number of explants showing any type of growth after 45 days), C= callus, F= finger, T= tallus. [b](*)= control = PES + GAN + 8 g l^{-1} agar.

Two mg l^{-1} kinetin, BA or 2,4-D increases viability and number of explants forming callus. Five mg l^{-1} kinetin, BA, 2,4-D or Na-Naphtenate has an inhibitory effect upon any type of growth (Table 1). Morphogenetic calli have an average of three to four buds after 45 days. No differences in callus growth and number of regenerating buds were observed among agar assay, hormone assay and the control. Differences were among individual calli. Later controls (60, 75 days) of the highest morphogenetic calli (at 45 days) show a decrease in the number of buds. The decrease is related to the fusion of thalliclones. Calli can be cultured for more than 5 months in agarized media, thus retaining their morphogenetic potential, and producing "air-growing" thalliclones.

In liquid media some calli developed many fast-growing thalliclones around the callus, while others developed a few slow-growing thalliclones, despite showing a homogeneous phenotype

at the transfer. Fast- and slow-growing lines have retained their phenotype for at least 4 months in culture.

Histological study under light microscopy shows a clear difference between the cells from the explant and the callus. Callus cells are small, meristematic and filled with florid granules, which became brown-black after being post-stained with lugol. Enlarged cells and wide intercellular spaces seem to be oriented to the regenerating areas on the surface of the calli. Toluidine blue stained the calli metachromatic.

DISCUSSION

The physical state of the media (agarized) seems to be a key factor in the induction of callus in seaweed (Fries 1980; Chen 1982; Saga et al. 1982; Saga and Sakai 1983; Neushul 1984; Polne-Fuller et al. 1984; Lee 1985). Our results show that the decrease of the hydric potential (more negative) enhances callus formation related to the matric and osmotic potentials (percent agar and seawater respectively). However, Polne et al. (Polne-Fuller et al. 1984) reported callus formation in several species of Porphyra in PES supplemented with 0.3% agar, and callus from protoplasts of several green species when grown on 1% agar, while reporting only thalli when grown on 0.5% agar (Polne-Fuller et al. 1986). Differences between species (and their habitats) may be involved.

The effect of the addition of hormones to seaweed media in order to obtain or regenerate callus is confusing with regards to the effects and type of hormones and concentrations (Bradley and Cheney 1986; Chen 1982). Na-Naphtenate (5 mg l^{-1}) has been described as a "crucial hormone" for inducing callus in Laminaria japonica (Yan 1984). Our results suggest that great differences in the in vitro uptake, metabolism and effect of plant hormones may exist between seaweed species.

The high facility of the buds arising from the calli cultured in agarized media to fuse together, and of the apex of the thalliclones to differentiate into a disc, indicates the high platicity of the apex of Laurencia sp. The fusion among regenerated buds shows that no loss of the morphogenetic potential, nor a mechanism of dominance, is involved in the apparent mor-

phogenetic potential decline. High plasticity and ano dominance seem to be desirable characteristics for selecting a primary explant to start tissue cultures.

The spontaneous apparition of fast- and slow-growing lines and its apparent stability indicate the usefulness of seaweed tissue culture for selecting superior strains.

The dimensions and high concentration of starch grains in callus cells resemble apical cells and carpospores (Devilopoulos and Tsekos 1986) of tip regions of Laurencia and other tubular branched Rhodophyta. Thalliclones show the normal histologly of Laurencia thalli. Copious deposition of starch in plastids has been described as the first visible change accompanying callus differentiation of the moss Psicomitrium coorgense (Lal and Narang 1985). Laurencia calli are cell systems closely related to the calli of higher plants, but different from structures referred to as calli in brown seaweeds.

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Selection for NaCl Tolerance in Cell Culture of Three Canary Island Tomato Land Races. I. Recovery of Tolerant Plantlets from NaCl-Tolerant Cell Strains

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Summary

Seeds from two cultivated land races of *Lycopersicon esculentum* Mill. from the island of Fuerteventura and one wild *Lycopersicon* from the volcanic mountains of the island of Gran Canaria were germinated in MS media + 0, 1.2, 2.4, 3.6, and 5.8 g/l NaCl. Organogenetic calli from cotyledon, leaf and shoot apex were generated directly in MS + 5 mg/l BA and 0.5 mg/l IAA in the same NaCl concentrations. NaCl did not inhibit callus production but reduced organogenesis. After 210 days, NaCl concentration was proportionally increased to 5.8, 10, 15, and 20 g/l. Different tolerant strains appered in the same callus, allowing us to obtain organogenetic calli which tolerated 20 g/l NaCl in all genotypes, and plantlets from callus grown on 15 g/l in all genotypes.

Key words: Lycopersicon, NaCl tolerance, organogenesis, callus, tissue culture.

Abbreviations: BA = benzyladenine, IAA = indoleacetic acid.

Introduction

The possibility of improving tomato NaCl-tolerance by *in vitro* selection exists because salt resistant mechanism/s are thought to be cell based (Bhaskaran et al. 1983, Daines and Gould 1985, Orton 1980, Warren and Gould 1982), and do not depend on whole plant organization in *Lycopersicon* (Tal et al. 1978, Tal and Katz 1980, Taleinsk et al. 1983). Stable NaCl-tolerant cell strains have been described in *Citrus* (Ben-Hayyim and Kochba 1982), *Nicotiana* and *Capsicum* (Dix and Street 1975, Watad et al. 1983), *Solanum* (Garciglia et al. 1985), *Pennisetum* (Rangan and Vasil 1983), and *Distichlis* (Warren and Gould 1982). NaCl-tolerant somaclones have been obtained in *Saccharum* (Liu and Yeh 1984). NaCl-tolerant phenotype was found to be heritable to some degree at least in the case of tobacco (Nabors et al. 1980).

In an *in vitro* NaCl-selecting programme, it is preferable if the genotypes used exhibit some degree of NaCl tolerance *in vivo* and have a high organogenetic potential. NaCl-tolerant genotypes often show a correlation with their *in vivo* and *in vitro* responses to salt stress (*Distichlis* Daines and Gould 1985, Warren and Gould 1982; *Hordeum* Orton 1980; *Medicago* Smith and McComb 1983; *Suaeda* and *Salicornia* Von Hendeström and Breckle 1974; *Lycopersicon* Tal et al. 1978, Rosen and Tal 1981), and the organogenetic potential declines with time in culture. The common methodological steps for selecting NaCl-tolerant cell strains involved long time in culture. An important improvement would be to develop an alternative protocol maintaining the organogenetic potential of the selected plant material.

The aim of our work was to select NaCl-tolerant, and organogenetic, cell lines of three land races of tomato (*Salvaje, Rusa* and *Especial*) which show a high organogenetic potential (Garcia-Reina and Luque 1988) and a certain degree of salt or drought tolerance.

Material and Methods

Plant material

Two *L. esculentum* land races from the island of Fuerteventura (*Rusa* and *Especial*), cultivated by the farmers for more than 60 years in the semi-arid environment of the island and irrigated with brackish water (over 3 g/l salinity), and one wild genotype from volcanic

fields of the island of Gran Canaria (*Salvaje*). The *Salvaje* genotype tolerates several months without any rainfall.

Culture media

Murashige and Skoog (1962) inorganic medium and the following in mg/l: inositol 100, panthotenic acid 1, nicotinic acid 1, piridoxine-HCl 1, thiamine 10, biotin 0.01, adenine 30, caseine hydrolysate 50, citric acid 200, sucrose 20000, agar (Bacto Difco) 8000. The pH was adjusted to 5.8 before adding agar; the general procedures were the same as previously described (Garcia-Reina and Luque 1988).

Sterilized seeds of *Salvaje*, *Rusa* and *Especial* were germinated in basal MS (as above) supplemented with 0, 1.2, 2.4, 3.6, 5.8, 7.5, and 10 g/l NaCl. Seven weeks later roots, cotyledons, leaves and shoot apices were directly cultured in MS medium containing 5 mg/l BA plus 0.5 mg/l IAA and in the same NaCl concentration in which the seeds germinated. Fifteen samples of each explant were used for each genotype and NaCl concentration (900 cultures).

After 35 days a control of the cultures was made to determine:

a) the percentage of explants forming calli (as a viability index).
b) callus growth. Measurement of the callus growth was done under stereomicroscope. Values from 1 (minimum) to 3 (maximum) were given depending on their relative growth.
c) the average number of buds or plantlets per morphogenetic callus.
d) the number of calli with an organogenetic development/number of viable calli.

Shoots producing calli areas were subcultured at 35 day intervals. Shoots were isolated and further cultured on MS without hormones, supplemented with the same NaCl concentration. The same procedures were followed with plant material after an increase in NaCl (see below).

After 210 days (6 subcultures) the NaCl concentration of the cultures was increased proportionally (in g/l): Control (0), 1.2 to 5.8, 2.4 to 10, 3.6 to 15, and 5.8 to 20. Also 26 *Salvaje* calli growing in medium 0 were subcultured in 5.8 g/l NaCl as well as those that survived, after 35 days, to 20 g/l NaCl.

Results

Germination in salinized media

Increasing NaCl concentration clearly decreases germination at 3.6 and 5.8 g/l NaCl, at 7.5 is highly reduced and at 10 g/l NaCl germination becomes completely inhibited (Table 1).

Callus growth and morphogenesis in saline media

Root callus growth was practically uninhibited by NaCl in the three genotypes after 70 days in culture, but was incapable of regenerating any organized structure. So root calli were discarded and not considered in the tables and figures.

Table 1: Seed germination (%) of Salvaje, Rusa and Especial in NaCl supplemented MS media. Data after 35 days in culture. Average of 87 seeds by genotype and NaCl concentration. Three repetitions.

NaCl (g/l)	0	1.2	2.4	3.6	5.8	7.5	10
Salvaje	91±11	93± 9	56±4	42±37	13±19	0.7	0
Rusa	80± 6	80± 3	75±3	68± 4	16± 4	0	0
Especial	23±19	37±15	38±3	34± 2	11± 9	0	0

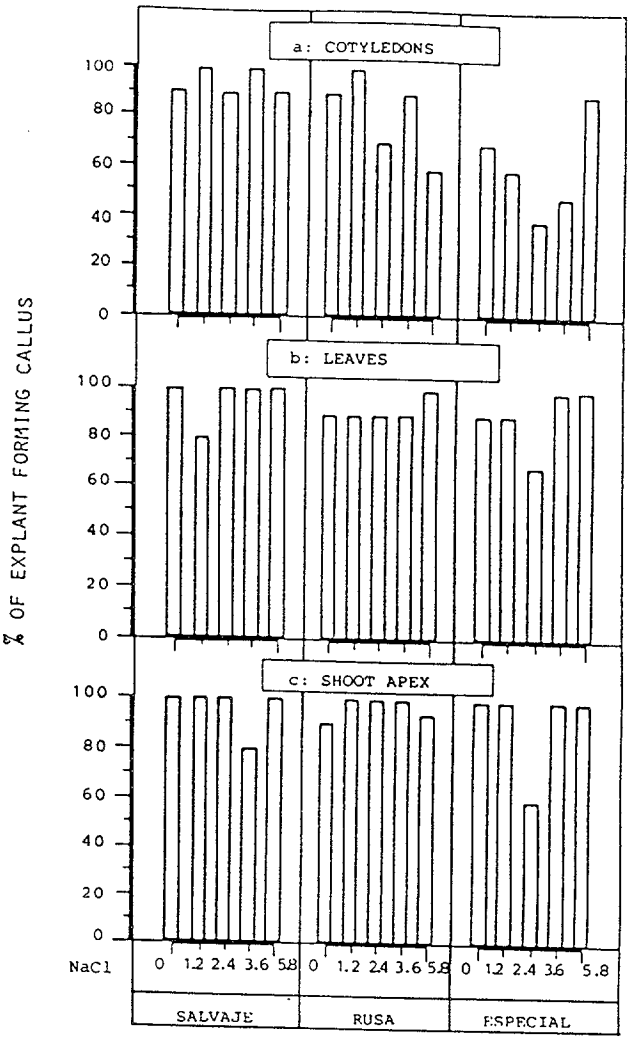

Fig. 1: NaCl concentration effect on the % explants forming callus from cotyledon, leaves and shoots apex after 35 days in culture.

NaCl increment did not affect callus formation of the cotyledons, leafes and shoot apices of the three genotypes (Fig. 1). Callus growth was not affected by NaCl increments, when we compare 0 and 5.8 g/l of NaCl (Fig. 2). Low NaCl concentrations (1.2 and 2.4 g/l) generally increased callus growth, decreasing at higher NaCl concentrations (Fig. 2).

NaCl increments reduced the incidence of organogenetic cultures. Cotyledon calli are more affected in this respect than leaf and apex derived calli (Fig. 3). The decrease in the number of buds or plantlets formed per callus shows at 5.8 g/l NaCl similar values in the explants of the three genotypes (except for *Rusa* leaf calli), showing that NaCl affects the organogenetic triggering mechanism (Fig. 3). In some explants and genotypes, the number of buds per calli at low NaCl concentrations (1.2, 2.4 g/l) is slightly higher than the control (Fig. 3).

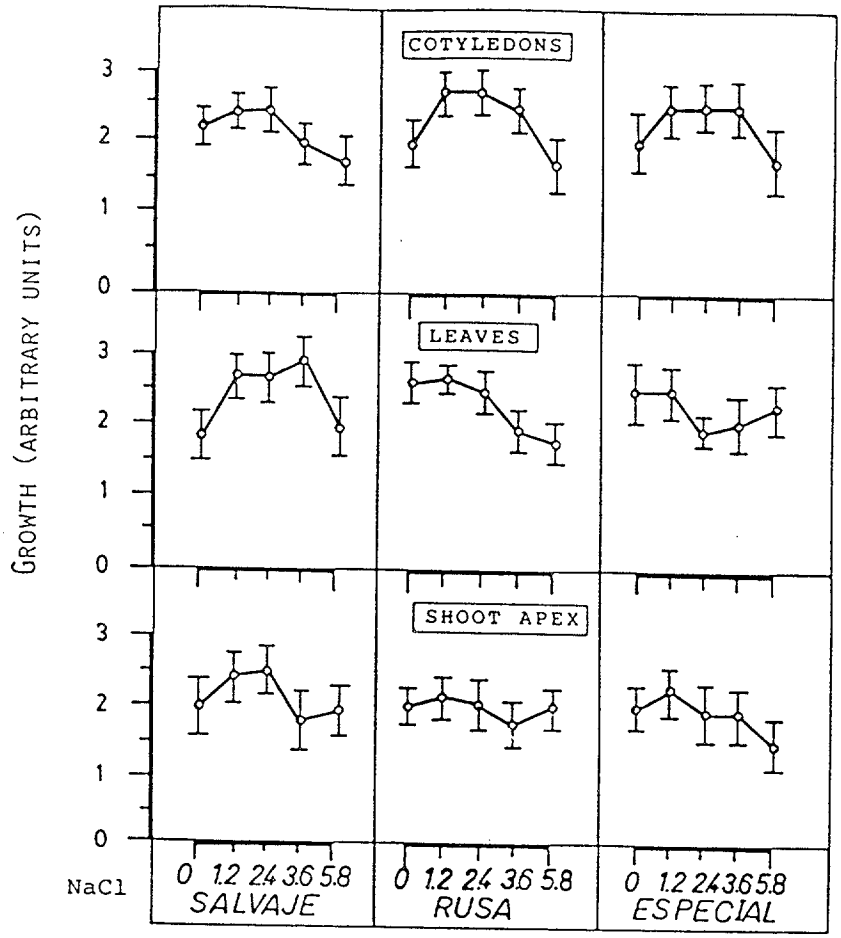

Fig. 2: NaCl concentration effect on the growth of cotyledon, leaf and shoot apex calli. Data after 35 days in culture.

Established calli (which were sometimes partially covered by less compact green callus) show a globular and microglobular appearance and did not lose their regeneration potential over six subcultures (210 days).

The plantlets obtained from calli grown on 0 to 5.8 g/l NaCl grew quite normally, producing a basal morphogenetic callus, and rooted without difficulty irrespective of preculture, genotype or explant type.

Callus growth and morphogenesis at increased NaCl concentrations

Seven months after callus formation in the five initial NaCl concentrations, we increased the salinity. Excess of NaCl in the medium over 5.8 g/l had a strong effect on the viability and organogenetic potential of the calli. Table 2 shows the evolution of the number of cultures containing shoot regenerating calli until 180 days (5 subcultures) after the NaCl concentration increase.

The initial response to NaCl increase varies from one callus to another. Many calli became completely necrotic, others retained pigmented areas as described in other species (Bhaskaran et al. 1983, Crougham et al. 1978), and some calli wholly retained their green colour (only at 5.8 and 10 g/l NaCl). No differences between explant types or genotypes could be detected. Callus and shoot growth was inversely related to NaCl concentrations.

The predominant callus growth type after NaCl increments were the globular and «microglobular» callus areas. The less compact callus areas became necrotic.

Plantlets (1.5–2 cm) isolated from calli grown on up to 15 g/l NaCl, were further cultured *in vitro* in the same NaCl concentration. After two months, they showed slow growth and some developed roots. Transfer to soil (peat : vermiculite, 1 : 1) showed no success. Despite being grown in the same growth chamber and under a transparent plastic box, the plantlets suffered strong dessication after 20–30 min. *In vitro* developed roots (fragile and without root hairs) were non-functional, and the plantlets seems to have lost their hydric regulation capacity.

Discussion

By preselecting seedlings on NaCl and direct callus formation on selective media, we have avoided the use of previously formed calli in a non-selective medium for *in vitro* NaCl-selection. Direct callus formation in selective media appears to make selection more economical and seems to select for NaCl tolerance rather than adaptating to progressive

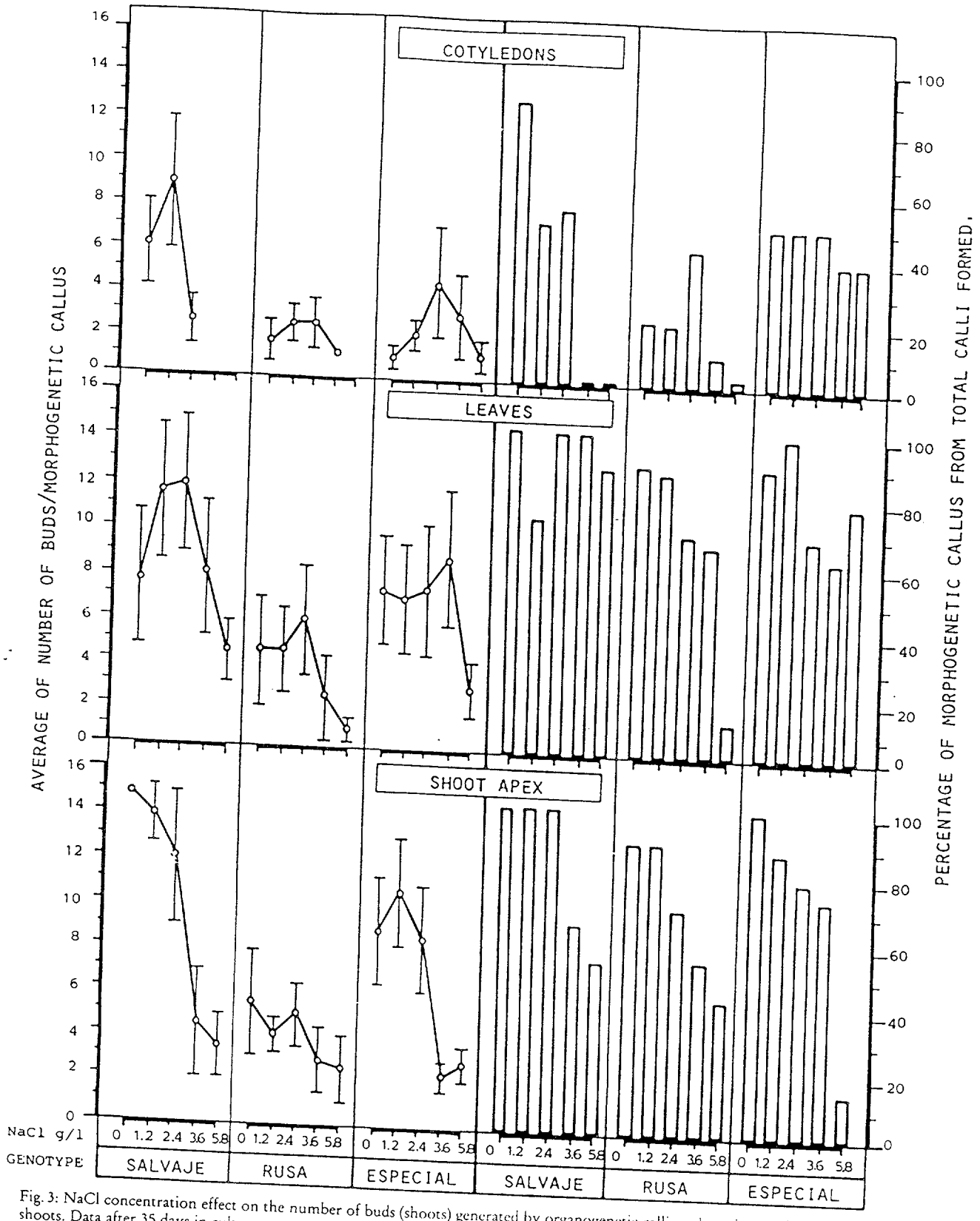

Fig. 3: NaCl concentration effect on the number of buds (shoots) generated by organogenetic calli, and on the number of calli regenerating shoots. Data after 35 days in culture.

Table 2: Selection of NaCl tolerant calli on increased NaCl levels. Figures represent the number of cultures containing organogenetic callus after NaCl increment (day 0). () = NaCl concentration before NaCl increment. S. = Salvaje; R. = Rusa; E. = Especial.

NaCl	5.8(1.2)			10(2.4)			15(3.6)			20(5.8)(g/l)		
Days	S	R	E	S	R	E	S	R	E	S	R	E
0	38	42	47	47	47	43	44	25	24	22	16	21
60	15	28	13	14	10	9	6	2	4	7	7	2
100	8	8	2	8	5	3	2	1	1	3	3	0
180	6	2	0	6	4	1	2	1	1	1	1	0

NaCl increments. This procedure appears to maintain the morphogenetic potential, thus allowing us to obtain morphogenetic calli tolerant to 20 g/l NaCl and somaclones tolerant to 15 g/l NaCl.

This procedure generates NaCl tolerant cell strains in the initial steps of the culture, avoiding the wait for genetic (or epigenetic) variations associated with long term culture, in some cases related with the loss of its organogenetic potential. Variability in the initial steps of the culture has been described by several authors (Larkin and Scowcroft 1981, Larkin et al. 1983, Sibi 1980).

NaCl increase reveals different NaCl tolerant levels between areas from the same callus. Higher tolerance is related to microglobular callus development, suggesting it to be a morphological marker for tolerance. Taleinsk et al. (1983) concluded that NaCl tolerance in the tomato is related to the meristematic status of the cells. Similar results could be interpreted from the NaCl resistant callus obtained by Kurtz (1982), which obtained better results if the tomato callus was induced in an organogenetic medium previous to the NaCl selection. In a previous paper we described how the globular strúctures are surrounded by meristematic nodules (Garcia-Reina and Luque 1988). It suggests that the hormonal balance employed (BA : IAA = 5 : 1), is of importance in the tomato, not only as an efficient organogenetic trigger, but as a generator of a callus system with a high meristematic/surface ratio. Reducing the globular diameter increases this ratio and the tolerance to NaCl, in agreement with our results and Taleinsk et al. (1983).

Several calli retained their organogenetic potential even after drastic NaCl increase, independent of the initial and final NaCl concentrations. As the somaclones retain tolerance, the possible roles of epigenetic variation (Hasegawa et al. 1980, Cocking and Riley 1981, Orton 1980) are discarded. And even if they are involved this does not necessarily imply the impossibility of transmitting the *in vitro* selected phenotype (Meins 1980).

The number of shoots per organogenetic callus obtained in 5.8 g/l NaCl is very similar in all the explants and genotypes, and is independent of the values in media without NaCl (Fig. 3). This data suggests that a very high organogenetic potential is not a fundamental requisite in initiating a NaCl-selection programme in species with a certain organogenetic potential.

In a collateral experiment 26 *Salvaje* calli, generated in a medium without NaCl, were subcultured in 5.8 g/l NaCl. After 35 days 16 calli presented, in a greater or lesser proportion, green globular structures. Subcultured in 20 g/l NaCl some calli remained green after 100 days in culture. The rapid NaCl-tolerance of the 26 *Salvaje* calli shows that «pre-adapting steps» are not necessary, at least with the wild genotype.

The rapid and high NaCl tolerance of our callus cannot be explained by the particular degree of NaCl tolerance in the genotypes, neither by the pre-selecting seedling step or by mutations occurring in the initial steps of the culture. We suggest (as a hypothesis) that the genetic information codifying NaCl tolerance is canalized. Cell culture (as a stressful environment) allowed the expression of a cryptic genetic variability which is repressed in natural environments. If our hypothesis is valid neither strict mutations nor sexual or somatic hybridization are required to induce NaCl-tolerance.

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Selection for NaCl Tolerance in Cell Culture of Three Canary Island Tomato Land Races. II. Inorganic Ion Content in Tolerant Calli and Somaclones

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Summary

The concentration of inorganic ions (Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-, and NO_3^-) in seedlings, morphogenetic calli and somaclones from three Canary Island (Spain) tomato land races was analyzed. MS medium supplemented with 1.2, 2.4, 3.6, and 5.8 g/l NaCl were used. Raising the NaCl concentration of the media increased the endogenous concentration of Na^+ and Cl^- in all plant material analyzed. Na^+ and Cl^- concentration were higher in morphogenetic calli than in seedlings and higher in somaclones than in the morphogenetic calli from which they were derived.

The decrease in NO_3^- and K^+ did not correlate with the increase in Na^+ and Cl^-. Calcium and Mg^{++} contents did not vary significantly with NaCl increase. The tolerance of the selected phenotypes seems to be related to a mechanism that controls the internal osmotic potential by increasing Cl^- and Na^+ concentrations while preventing the cells from their toxic effect.

Key words: Lycopersicon, NaCl-tolerance, callus, ionic contents.

Abbreviations: BA = benzyladenine, IAA = indoleacetic acid, MS = Murashige and Skoog (1962).

Introduction

In vitro NaCl selection in several plant species has suggested different mechanisms underlying salt tolerance: single gene (Jia-Ping et al. 1981), polygenetic inheritance (Nabors et al. 1980, Simmons et al. 1984), non-medelian transmission (Nabors et al. 1975), or epigenetic changes (Larkin and Scowcroft 1981, Larkin et al. 1983, Watad et al. 1983).

Overproduction of organic acid has been suggested as the mechanism exerted by salt tolerant tomato genotypes (Rush and Epstein 1976). However Tal and Katz (1980) and Rosen and Tal (1981) have demonstrated only a minor role of proline in NaCl tolerance at the cellular level in several *Lycopersicon* species. They have suggested a better osmotic control mechanism as the key process of NaCl tolerance in *Lycopersicon* (Tal et al. 1978, Tal and Katz 1980).

In a previous paper (Garcia-Reina et al. 1988) we reported the generation of NaCl-tolerant calli and somaclones of three Canary Island *Lycopersicon* genotypes. Here we report on the variation of ions in seedlings, morphogenetic calli and somaclones, analyzing theoretically the variation of the inorganic component of the osmotic potential.

Material and Methods

Three *Lycopersicon* genotypes were studied. Two land races from the island of Fuerteventura (*Rusa* and *Especial*) adapted to their semiarid and saline environment. One wild relative (*Salvaje*) growing on volcanic salinized land on the island of Gran Canaria.

Plant material analyzed

1. Axenically grown seedlings (leaves + stem) growing in MS medium supplemented with 0, 1.2, 2.4, 3.6, and 5.8 g/l NaCl. Plants were analyzed ninety days after germination

2. Calli induced from cotyledons (15 days old) on MS medium supplemented with 0, 1.2, 2.4, 3.6, 5.8, 10, and 15 g/l NaCl (Garcia-Reina et al. 1988). Calli were subcultured at 35 day intervals. At the end of the second subculture (105 days) the calli were taken for analysis.

3. Somaclones (stem and leaves) previously isolated from the calli and cultured (at 35 day intervals) in the same NaCl concentration. At the end of the second (105 days) or third (140 days) subculture the somaclones (between 2–3 cm long) were taken for analysis. Roots were not included in the analysis.

Analytical techniques

Seedlings, calli and somaclones were rinsed in distilled water (approx. 1 minute) blotted dry with a cotton towel and dried at 80 °C for 48 hours. Dried plant material was ground and then analyzed.

Three samples of each plant material, genotype and NaCl concentration were analyzed. Chloride was extracted with 0.1 N HNO_3 and the extract analyzed with an automatic Cl^- tritator (Adriano et al. 1973).

Nitrate was extracted from the ground samples using distilled water and then the NO_3^- concentration of the extract was determined colorimetrically by nitration of salicylic acid (Cataldo et al. 1975).

Sodium, K^+, Ca^{++}, and Mg^{++} ions were determined after wet mineralization with a mixture of nitric-perchloric acids (2:1 v/v) for 36 hour at 80 °C (Gange and Page 1974). Sodium and K^+ were determined by flamefotometry (emission) and Ca^{++} and Mg^{++} by atomic absorption with an atomic absorption spectrophotometer (Variant-Techtron AA6).

Osmotic potential

The inorganic component of the osmotic potential in plant material was theoretically calculated applying Vant Hoff's equation. The osmotic potential of the culture media was calculated according to the equation and data given by Debergh et al. (1981). The osmotic potential of our basal media was −6.03 bars, and in the different NaCl concentration (in bars): 1.2 g/l = −6.97; 2.4 g/l = −7.87; 3.6 g/l = −8.74; 5.8 g/l = −10.44; 10 g/l = −13.66 and 15 g/l = −17.48.

Results

Chlorine and Sodium content

The endogenous Cl^- concentration increased in all plant material and genotypes analyzed as the NaCl concentration of the media increased (Table 1).

In seedlings the wild genotype (*Salvaje*) showed a significantly lower Cl^- content. However the three genotypes have similar Cl^- contents in calli and somaclones, increasing with the NaCl of the media. This increase was evident after the first saline treatment, showing the highest values in the somaclones (Table 1).

The sodium concentration of the basal medium was 51 mM. Increasing the Na^+ level in the media resulted in an increase in the endogenous Na concentration in seedlings, calli and somaclones from all three genotypes studied (Table 1).

Chlorine and Na^+ content of calli continued the general increase at 10 and 15 g/l NaCl in all the genotypes (Table 3).

Nitrate and Potassium content

Increased NaCl levels resulted in a concomitant decrease of NO_3^- and K^+ in all the genotypes and plant material analyzed (Table 2 and 3). The decrease of NO_3^- is more pronounced in seedlings than in calli or somaclones in all the genotypes. The concentration of potassium was higher in all plant material than the potassium concentration in the culture media (20 meq/l).

Calcium and Magnesium content

In seedlings and calli, increased levels of NaCl did not affect the Ca^{++} and Mg^{++} content in all the genotypes analyzed. No differences were found among genotypes. Seedlings show double amounts of Ca^{++} and Mg^{++} to calli in all the genotypes and NaCl treatments. Somaclones were not analyzed for Ca^{++} and Mg^{++}. The average content of Ca^{++} and Mg^{++} (in $mM \times g^{-1}$ DW) in seedlings was: 0.28 ±

Table 1: Cl^- and Na^+ contents in seedlings, calli and somaclones of tomato cultured in MS media supplemented with NaCl (each value represent the mean of three samples).

Genotype	NaCl $g \cdot l^{-1}$ Culture media	Cl^- ($mM \cdot g^{-1}$ d.w.)			Na^+ ($mM \cdot g^{-1}$ d.w.)		
		Seedlings	Calli	Somaclones	Seedlings	Calli	Somaclones
Salvaje	0	0.09±0.00	0.25±0.06	0.34±0.01	0.24±0.03	0.42±0.02	0.36±0.04
	1.2	0.18±0.01	0.89±0.04	1.08±0.03	0.35±0.03	0.92±0.14	1.28±0.06
	2.4	0.30±0.01	1.19±0.06	1.64±0.03	0.63±0.06	1.84±0.08	2.10±0.10
	3.6	0.35±0.01	1.22±0.07	1.92±0.05	0.88±0.05	2.30±0.16	3.01±0.15
	5.8	0.54±0.04	1.67±0.03	3.41±0.09	1.62±0.05	3.04±0.18	4.39±0.26
Rusa	0	0.09±0.00	0.08±0.00	0.28±0.03	0.38±0.03	0.33±0.04	0.42±0.05
	1.2	0.59±0.02	0.69±0.02	1.47±0.05	0.54±0.05	0.83±0.09	1.59±0.11
	2.4	0.99±0.10	0.95±0.07	1.71±0.05	1.12±0.05	1.14±0.09	1.66±0.08
	3.6	1.27±0.04	1.29±0.03	2.72±0.07	1.26±0.05	1.48±0.11	2.97±0.10
	5.8	1.63±0.05	1.48±0.15	4.03±0.11	1.39±0.05	2.15±0.18	4.51±0.20
Especial	0	0.21±0.01	0.15±0.03	0.41±0.02	0.27±0.02	0.48±0.05	0.41±0.05
	1.2	0.67±0.02	0.89±0.06	1.08±0.03	0.50±0.04	1.00±0.04	1.11±0.05
	2.4	0.73±0.02	1.09±0.07	1.41±0.05	0.56±0.03	1.56±0.05	1.51±0.07
	3.6	1.30±0.04	1.17±0.04	1.88±0.04	0.96±0.07	1.65±0.09	2.17±0.16
	5.8	1.84±0.05	1.59±0.03	3.04±0.10	1.30±0.04	2.01±0.15	3.75±0.29

Table 2: K^+ and NO_3^- contents in seedlings, calli and somaclones of tomato genotypes cultured in MS media supplemented with NaCl (each value represents the mean of three samples).

Genotype	NaCl g · l^{-1} Culture media	K^+ (mM · g^{-1} d.w.) Seedlings	Calli	Somaclones	NO_3^- (mM · g^{-1} d.w.) Seedlings	Calli	Somaclones
Salvaje	0	0.87±0.06	1.10±0.04	0.69±0.06	0.95±0.05	1.00±0.09	0.28±0.02
	1.2	0.57±0.15	0.90±0.04	0.77±0.07	0.50±0.03	0.70.07	0.29±0.01
	2.4	0.52±0.19	0.82±0.06	0.71±0.07	0.30±0.03	0.44±0.05	0.17±0.02
	3.6	0.46±0.15	0.56±0.07	0.59±0.04	0.20±0.06	0.37±0.02	0.15±0.02
	5.8	0.34±0.03	0.43±0.04	0.46±0.04	0.14±0.02	0.27±0.02	0.14±0.02
Rusa	0	1.13±0.10	0.84±0.11	1.14±0.12	0.50±0.09	0.43±0.01	0.27±0.01
	1.2	1.14±0.05	0.77±0.05	1.12±0.10	0.50±0.09	0.38±0.05	0.26±0.02
	2.4	1.23±0.08	0.68±0.08	1.11±0.08	0.52±0.07	0.33±0.04	0.25±0.03
	3.6	1.17±0.02	0.49±0.02	1.07±0.10	0.37±0.05	0.31±0.09	0.16±0.02
	5.8	0.86±0.04	0.32±0.01	0.84±0.07	0.25±0.05	0.26±0.05	0.14±0.02
Especial	0	1.22±0.08	0.96±0.10	1.10±0.06	1.10±0.10	0.65±0.05	0.94±0.04
	1.2	1.17±0.07	0.84±0.03	1.00±0.07	0.83±0.08	0.53±0.12	0.87±0.05
	2.4	0.90±0.05	0.72±0.04	0.75±0.10	0.68±0.05	0.35±0.10	0.78±0.02
	3.6	0.89±0.03	0.71±0.06	0.64±0.06	0.41±0.04	0.32±0.03	0.59±0.03
	5.8	0.80±0.02	0.62±0.03	0.63±0.04	0.25±0.03	0.28±0.02	0.25±0.02

Table 3: Cl^-, Na^+, K^+ and NO_3^- content in calli of tomato cultured in MS medium supplemented with 10 and 15 g · l^{-1} NaCl (each value represent the mean of three samples).

Genotypes	NaCl g · l^{-1} Culture media	mM · g^{-1} (d.w.) Cl^- Calli	Na^+ Calli	K^+ Calli	NO_3 Calli
Salvaje	10	1.80±0.03	3.04±0.28	0.35±0.05	0.16±0.02
	15	1.89±0.06	3.90±0.26	0.33±0.04	0.16±0.02
Rusa	10	1.98±0.06	2.65±0.16	0.31±0.03	0.17±0.03
	15	2.35±0.08	3.40±0.17	0.31±0.03	0.16±0.03
Espécial	10	2.07±0.04	2.70±0.15	0.47±0.05	0.20±0.02
	15	2.16±0.09	2.90±0.21	0.39±0.05	0.15±0.03

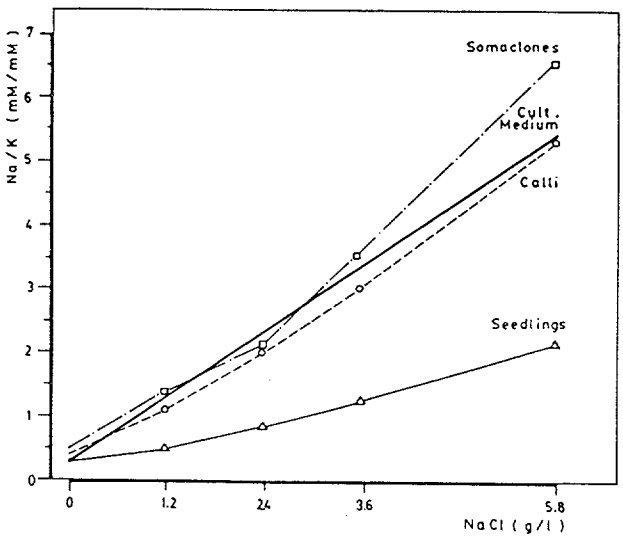

Fig. 1: Na/K ratio in seedlings, calli and somaclones of tomato cultured in MS media supplemented with NaCl. Each value represent the mean of 9 samples (three from each tomato genotype).

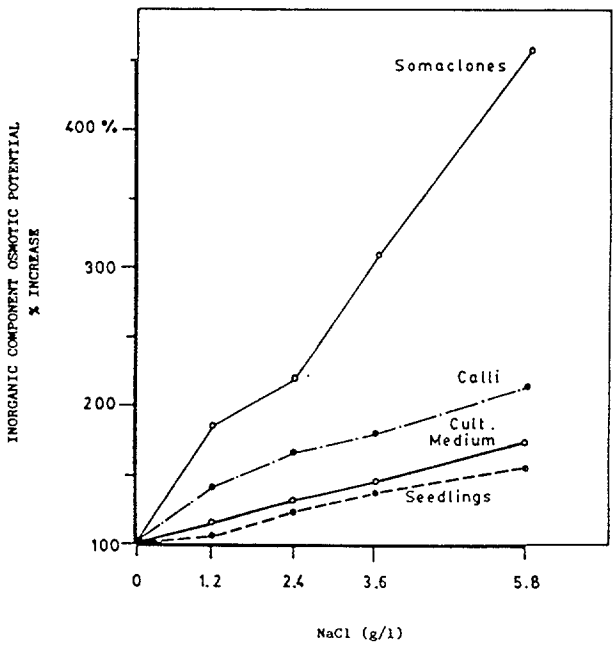

Fig. 2: Percentage of increase of the theoretical inorganic component of the osmotic potential. 100% seedlings = −4.60 bars, 100% calli = −4.34 bars, 100% somaclones = −4.32 bars. Each value represent the mean of 9 samples (three from each tomato genotype).

0.02 and 0.22 ± 0.01, and in calli: 0.15 ± 0.01 and 0.08 ± 0.01 respectively.

Na/K ratio

Fig. 1 shows the evolution of the Na/K ratio in seedlings, calli and somaclones of the three genotypes. A higher ratio

was found in somaclones than in calli at all NaCl levels, and callus ratios exceeded those found in seedlings. This response was seen in all the genotypes (data not shown).

Osmotic potential

Fig. 2 shows the evolution of the theoretical osmotic potential in seedlings, calli and somaclones. The increase (absolute values) of the osmotic potential of the culture medium rises from −6.03 at 0 g/l NaCl (0%) to −10.44 at 5.8 g/l NaCl (172%). The results indicate a clear difference between the three types of plant material analyzed. Somaclones (408% increase) have quite a higher osmotic potential increase than seedlings (135% increase) at 5.8 g/l NaCl. The osmotic potential increased in seedlings at a similar rate to the culture media (Fig. 2).

Discussion

The internal increase of Cl^- and Na^+ in seedlings and calli in our genotypes as a function of external NaCl concentration agree with the results of other authors (Tal 1971, Rush and Epstein 1976, Tal et al. 1978, Taleinsk et al. 1983). As opposed to other wild tomato genotypes (Tal et al. 1971) *Salvaje* seedlings show a comparatively low Cl^- increase, while their content is similar to the other genotypes at the callus level (Table 1). These data suggest the presence of an efficient mechanism in the roots of *Salvaje* preventing the absorption of Cl^-.

Rush and Epstein (1981) pointed out that the Na^+ content of leaves from tomato cultivars becames toxic at levels over 5% (d.w.), while wild halophytic species can contain up to 20% (d.w.). The highest Na content in seedling was 1.62 mM Na/g (d.w.) equivalent to 3.72% of Na (*Salvaje* in 5.8 g/l NaCl, Table 1). At the same NaCl concentration, calli of all the genotypes contained around 5% of Na (d.w.), and the somaclones 7.6% of Na in *Especial*, and around 10% Na (d.w.) in *Salvaje* and *Rusa*. These results could be an indication of the degree of NaCl tolerance selected in the somaclones.

Another indication could be the higher Na/K ratio of the somaclones compared to seedlings (Fig. 1). Several authors have described the relationship between high Na/K ratio and NaCl tolerance (Liu and Yeh 1984, Phillips et al. 1979, Tal and Shannon 1983, Watad et al. 1983). The Na/K ratio increase together with the NaCl concentration in the calli of the three genotypes is in accordance with the results of Tal et al. (1978) in *Lycopersicon* and Croughan et al. (1978) with alfalfa.

In the three plant materials, Cl^- and Na^+ increase (Table 1 and 3) was greater than the K^+ and NO_3^- decrease (Table 2 and 3). The theoretical organic acid content $[(Na^+ + K^+) - (Cl^- - NO_3^-)$ = organic acid] did not increase with NaCl (data not shown). Our results suggest a better osmotic adjustment by inorganic ions (Cl^- and Na^+) and not by organic acids. These results agree with the results of Heyser and Nabors (1981) who described an increase in the inorganic component of the osmotic potential from 25–30% to 56–72% in salt tolerant cell lines of *Nicotiana*. Our results also agrees with the suggested minor role of proline in NaCl tolerance at the cellular level in several *Lycopersicon* species (Tal and Katz 1980, Rosen and Tal 1981).

Considerably higher Cl^- and Na^+ concentrations (and Na/K ratio) were found in somaclones than in calli at all NaCl levels, and calli concentrations exceeded those found in seedlings. This response was observed in all genotypes alike. The expression of a cryptic mechanism allowing the cells to tolerate the stress seems more probable than a mutation in the initial steps of the culture. The information which allows the tolerance of Cl^- and Na^+ toxic levels, and which leads to a better osmotic control mechanism, could be canalized.

The somaclones retained the same mode of tolerance selected at the cellular level, which permits them, but not seedlings, to grow in saline media. These results are in accordance with those reported by other authors (Liu and Yeh 1984, Yano et al. 1982). The higher Cl^- and Na^+ levels in somaclones with respect to the calli (from which they were generated) suggest their origin in callus cells of a higher tolerance.

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Enhancement of Berberine Production in Suspension Cultures of *Coptis japonica* by Gibberellic Acid Treatment

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Summary

The production of berberine by cell cultures of *Coptis japonica* was enhanced by the addition of gibberellic acid (GA_3 10^{-8}–10^{-5} M) to the medium, particularly in the early stages of the culture period. After 14 days of culture the berberine yield of GA_3 treated cells (10^{-8} M) was 1660 mg · l^{-1} suspension, controls 1230 mg · l^{-1}. Also, the addition of gibberellic acid suppressed the starch synthesis in the late exponential growth phase. Furthermore, the addition of gibberellic acid altered the rates of incorporation of sucrose and nitrogen into the cells during the early stage of culture. The results suggest that the change in the incorporation rate of main nutrients triggered by gibberellic acid has a marked effect on the synthesis and accumulation of berberine.

Key words: Coptis japonica, alkaloid production, berberine, cell culture, gibberellic acid, inhibition of starch synthesis.

Abbreviation: GA_3: gibberellic acid.

Introduction

Berberine is an important pharmaceutical alkaloid that has antibacterial, stomachic and anti-inflammatory activity. In the roots of the source plant, *Coptis japonica*, the berberine content is very low, and more than 5 years are required to produce roots big enough to harvest. A few publications only report the production of berberine in cell cultures to be higher than that found in plants (Yamada and Sato, 1981; Fukui et al., 1982; Sato and Yamada, 1984). Yamada and Sato helped pioneer the industrial production of berberine by establishment of *Coptis* cell cultures and selection of highly productive cell lines by a small cell aggregate selection method.

Cell growth of the *Coptis* cells employed here ceased between the 7th and 10th day after inoculation, and berberine production ended between the 14th and 17th day (Morimoto et al., 1988). The sucrose in the medium (3%, initially) was incorporated into the cells over a period of 14 days, after which starch grains appeared in the cytoplasm (unpublished data). Accumulation of starch at this stage indicated depletion of the carbon source. Decreased production of berberine may also have resulted from depletion of carbon in the medium. The observation suggested that the cells would retain their potential for additional growth and berberine production, provided starch formation is inhibited.

A switch of carbon flow from starch synthesis to cell growth or berberine synthesis might enhance berberine accumulation. GA_3 was reported to reduce the starch contents of cultured cells of sweet potato (Sasaki and Kainuma, 1984). Therefore we tested its action on cell division and berberine production. There are no reports in the literature on GA_3 enhancing secondary metabolite production in undifferentiated cells except observations of stimulation of metabolite production in tissue or organ culture by Ohlsson et al. (1986) and Chung and Staba (1986). Here we show that GA_3 enhances berberine production.

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Analysis of the organogenetic potential of calli of three Canary Island *Lycopersicon esculentum* land races

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Key words: *Lycopersicon*, callus, organogenesis, shoot regeneration

Abstract. The organogenetic potential from callus of three tomato land races from the Canary Islands adapted to semi-arid environment ('Salvaje', 'Rusa' and 'Especial'), and one tomato cultivar ('Meltine'), were examined. The response of four explant types (cotyledon, shoot apex, hypocotyl and root) to nine PGR regimes (BAP at 1 or 2 or 5 mg/l) + either IAA (0.5 mg/l) or 2,4-D (0.5 or 1 mg/l) were measured. BAP at 5 mg/l + IAA at 0.5 mg/l induced most organogenesis in all the explant types for all genotypes. 'Salvaje' has one of the highest organogenetic potentials described in tomato.

Abbreviation: OP = organogenetic potential

Introduction

A high intraspecific OP variability has been described in the more than 100 *L. esculentum* genotypes studied. Within the primary explants used, differences in callus growth and organogenetic responses have been recorded. Genotype-specific [1, 5, 10, 12, 13], and explant-specific hormonal requirements have been described [1, 12] with similar methodologies leading to contradictory results.

Cell culture techniques in the selection of salt-tolerant somaclones require plant material with a high organogenetic potential (OP), as NaCl reduces callus growth [3, 8] and organogenesis in several species [11, 14] including tomato [9, 16].

The objective of this work was to determine the callus growth and organogenetic response of different explant types to several PGR concentrations and combinations in four genotypes, three of them identified as potential salt/drought-tolerant breeding lines.

Material and methods

Four genotypes were used. Two land races 'Rusa' and 'Especial' of the island of Fuerteventura (Canary Islands, Spain), where they have been cultivated for at least 60 years, a third land race 'Salvaje' from the volcanic soil of the island of Gran Canaria and a cultivar 'Meltine' (Van de Berg, Holland).

Seeds were disinfected and cultured on Murashige and Skoog medium [18]. Seedlings germinated in a growth chamber at 25 ± 2 °C, with 16 h light photoperiod at 2000 lux. Tissue cultures were similarly incubated.

An average of 46 cotyledons, 29 shoot apices (3–5 mm), 50 hypocotyl segments (1.5–2 cm) and 17 roots (1.5–2 cm), from 13-day old seedlings, were used by genotype and PGR regime. Nine PGR regimes were used, BAP at 1, 2 or 5 mg/l + either IAA at 0.5 mg/l or 2,4-D at 0.5 or 1 mg/l. Cotyledons were implanted with the adaxial surface in contact with the medium, shoot apices implanted perpendicularly, and hypocotyl and root segments implanted parallel to the medium surface. Subculture to fresh medium was made at 35 days interval.

Indices were determined after 15, 35 and 85 days: Morphogenetic Index (IM): average of the number of buds or plantlets/number of morphogenetic calli; Morphogenetic Percentage (%M): number of calli with an organogenetic development/number of viable calli.

Results and discussion

IAA-containing media

The IM and %M increased with increasing BAP concentration, supporting the concept that BAP is an organogenetic trigger in *Lycopersicon* [1, 5, 6, 7, 10, 12, 16]. The four genotypes gave similar organogenetic responses to changing BAP concentrations (Fig. 1). In some cases the increase was linear, in agreement with some results of Kurtz and Lineberger (1983) with similar BAP concentrations.

The greatest IM and %M in any explant source were from callus from cotyledons (Fig. 2). 'Salvaje' induced the greater OP (Fig. 1), comparable with results obtained with other wild genotypes [4, 6, 15].

Shoot apices generated a nodular green basal callus, which developed buds in the neighbourhood of the stem apex. Only apical hypocotyl segments formed organogenetic callus. Roots developed a white friable non-morphogenetic callus, independently of genotype and hormonal treatment

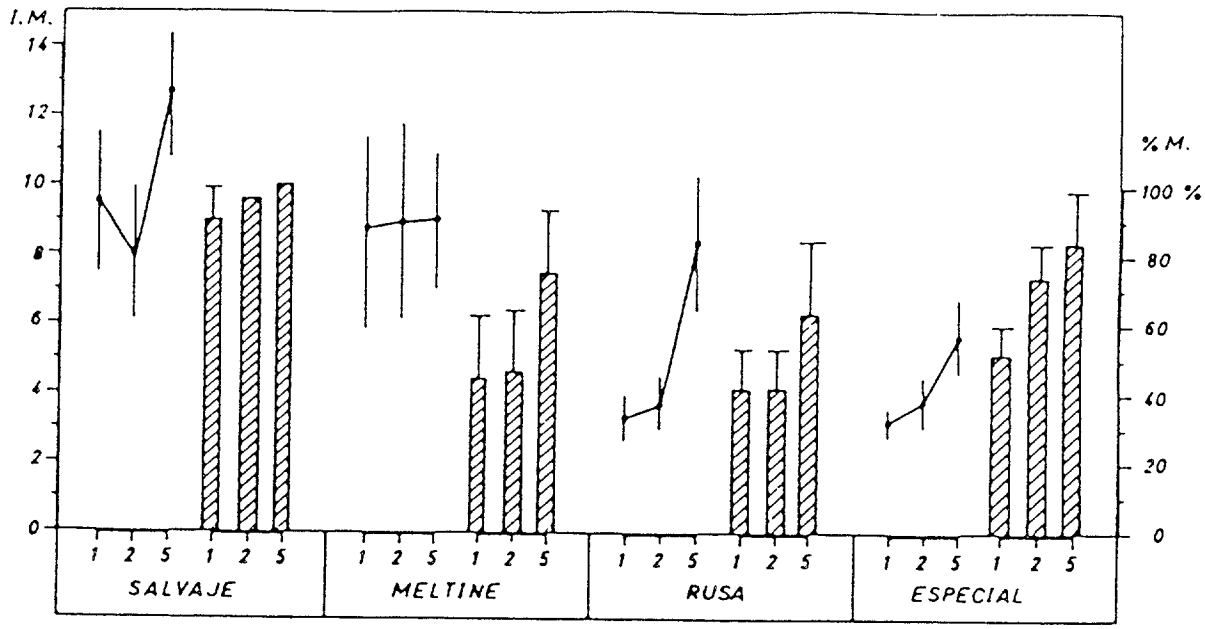

Fig. 1. Mean Morphogenetic Index (IM) and Morphogenetic Percentage (%M) for all cultivars in media with IAA (0.5 mg/l) and BAP (1, 2 and 5 mg/l) after 35 days in culture.

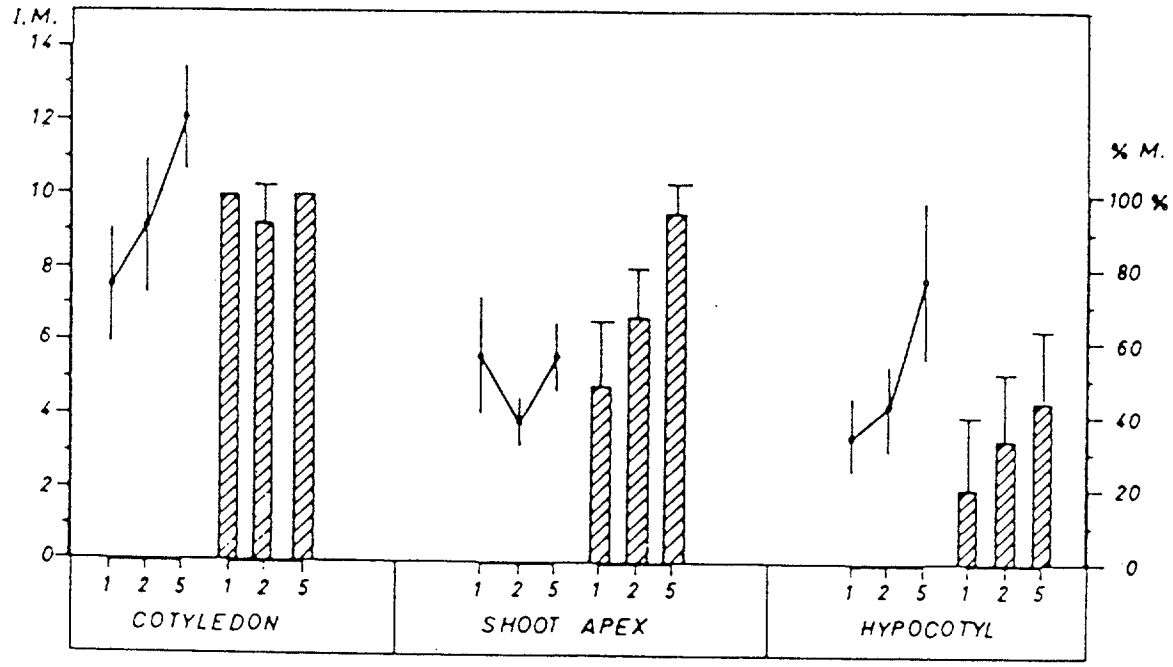

Fig. 2. Mean Morphogenetic Index (IM) and Morphogenetic Percentage (%M) for all explants in media with IAA (0.5 mg/l) and BAP (1, 2 and 5 mg/l) after 35 days in culture.

The incapacity of the roots to produce morphogenetic callus in tomato and other Solanaceae has been described [6]. As the roots are the sites of cytokinin synthesis in tomato [17], different PGR requirements are probably needed.

After 85 days, at the lowest BAP concentration (1 and 2 mg/l) the IM values decreased to half of the values recorded after 35 days in all the organogenetic calli. The organogenetic decrease may be related to a 'dominance effect' exerted by the first formed shoots [2]. TIBA addition (0.2 μM) does not increase or sustain the OR

At the higher BAP concentration (5 mg/l) the IM values did not decrease, being similar to those obtained after 35 days in culture. Media with 5 mg/l BAP promoted an even growth-rate of the regenerated plantlets with a globular structure of the callus. This suggests a blockade of the 'dominant effect'. A histological study of the globular calli shows nodular meristematic cell areas, always close to a disorganized vascular system, distributed around the globular surface.

2,4-D media

Plant material in 2,4-D media initiated a large, white and friable non-morphogenetic calli. Only in 5 mg/l BAP + 0.5 mg/l 2,4-D four 'Salvaje' and 'Meltine' cotyledon-derived calli developed 2–3 buds. 2,4-D calli did not induce any vascular system. Changing from 2,4-D to IAA after 35 days produced the organogenetic reversion only in 33% of 'Salvaje' calli induced in 5 mg/l BAP.

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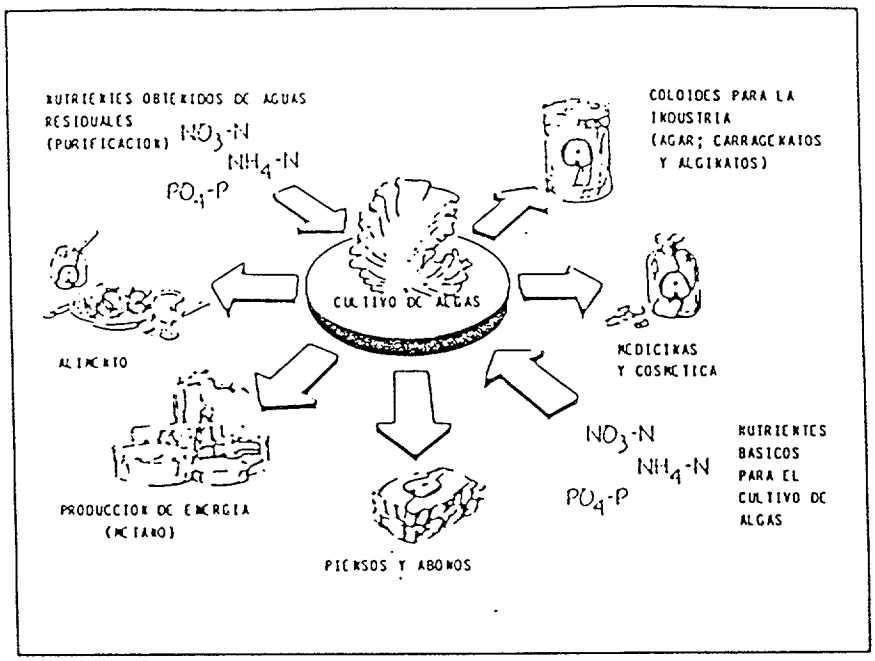
LAS MACROALGAS MARINAS, UN RECURSO DESCONOCIDO

En este país y en Occidente en general, cuando se habla de algas, aún en Congresos dedicados a cultivos marinos, poca gente sabe exactamente cual es su importancia en el sector. No pretendemos idealizar las posibilidades de este recurso, ni el describir su cultivo como una panacea, pero si divulgar y destacar el potencial y aplicaciones del cultivo de algas como una actividad agrícola alternativa, de especial interés para Canarias.

A nivel mundial, el cultivo de algas es la actividad de acuicultivo que ocupa a más gente y mueve más tonelaje y dinero. Estimaciones del año 1.982 cifraban en un billón de dólares el montaje de la actividad de cultivo de macroalgas marinas.



Esquema de productos y aplicaciones de las macroalgas marinas

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I.- ¿Qué son las macroalgas marinas?

Las macroalgas marinas son plantas de una altura que oscila entre unos pocos centímetros y más de sesenta metros de longitud, clasificadas en tres tipos generales en función de su color: algas verdes, algas rojas y algas pardas. Se distinguen de las plantas terrestres porque no poseen ni sistema vascular, ni flores, ni raíces (sólo estructuras de fijación al sustrato, que no sirven para absorber nutrientes) y no pueden vivir fuera del agua.

II.- ¿Para qué sirven las macroalgas marinas? (Fig. I)

La respuesta inmediata sería: como alimento para los "chinos", y ciertamente la utilización como alimento humano es una de sus aplicaciones más antiguas (Japón S.X), debido al elevadísimo contenido en vitaminas (diez veces más vitamina A que las espinacas), sales minerales (más calcio que la leche, 1.500 veces más Iodo que el pescado), ácidos grasos poliinsaturados (previenen contra enfermedades coronarias), proteínas de alto valor (mejores que las de cualquier fuente vegetal y comparable a la ovoalbúmina), y bajo contenido calórico (no engordan y son laxantes), que aumentan la longevidad de las comunidades que las consumen habitualmente. En Las Palmas de Gran Canaria ya existen tres tiendas que se dedican a la venta de algas empacadas para alimentación humana (importada de Corea, con una sorprendente aceptación por parte del público. En EE UU. se están poniendo de moda las "hamburguesserías" de algas marinas como sustitutivo de la "comida-basura".

Tras el desastre de Chernobyll se están comercializando pastillas de macroalgas como tratamiento de desintoxicación radiactiva en los países escandinavos, ya que fijan rápidamente los elementos radioactivos ingeridos y, al ser laxantes, los eliminan rápidamente.

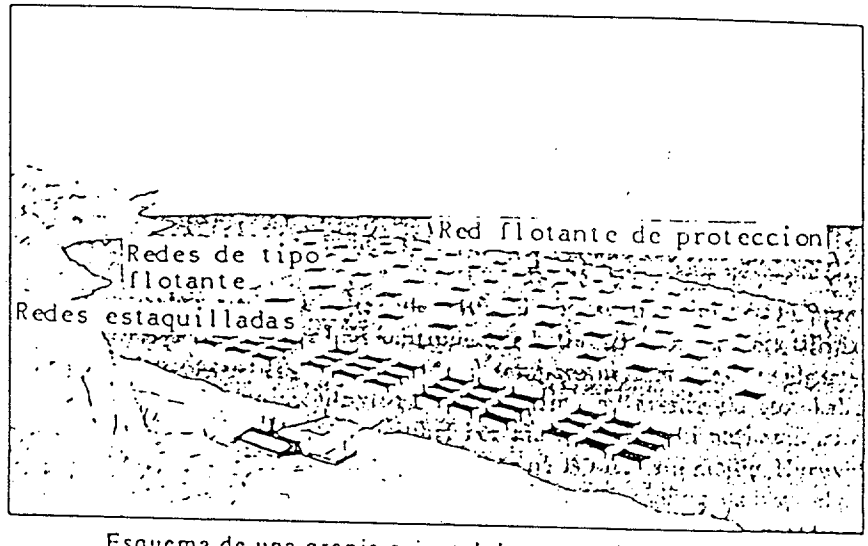
No sólo los humanos se benefician de las ventajas dietéticas de las macroalgas, ya que también se emplean como aditivo de piensos para el ganado (entre el 10 y 25%). Las harineras de algas marinas noruegas son actualmente las que dominan este sector y su producción no cubre la demanda. Debido a las vitaminas, sales y otros compuestos, la adición de algas a la dieta de ovejas, vacas y gallinas se ha comprobado que aumenta la cantidad y calidad de la lana, leche y

huevos, respectivamente, aumenta la fertilidad y reduce las enfermedades infecciosas. Teniendo en cuenta que también es consumido por caballos, visones y hasta zorros, no hay porque destacar el que pueda ser consumido por la cabaña caprina de nuestro Archipiélago, adaptada a comer "piedras" (a decir de los majoreros).

La utilización de las macroalgas marinas en la agricultura es la aplicación española más antigua de estos vegetales. Desde Galicia al País Vasco se empleaban los arribazones de algas como correctores de suelos, abono y mejoradores de las estructura del suelo agrícola desde tiempo inmemorial. Hoy en día hay una importante industria de abonos foliares y fertilizantes selectos producidos de algas, radicada fundamentalmente en Gran Bretaña, Nueva Zelanda y Africa del Sur. Estos abonos algales (Maxicrop, Alginure, Seagro, SM3, etc.), tienen un alto contenido en hormonas vegetales (además de nutrientes básicos) que han incrementado, en algunos casos más del 20%, las cosechas de plátanos, piña, tomate y papas (por citar algunos cultivos canarios), además de reducir significativamente las infecciones por Botrytis y Verticillium y reducir los problemas de almacenamiento de frutas y verduras.

No obstante, la utilización más rentable de las algas marinas es para extraer los exclusivos coloides que producen. Estas sustancias (agar, carragenatos y alginatos), se emplean como gelificantes en la industria alimentaria, cosmética, agrícola, detergentes, farmacéutica, biotecnológica y bioquímica. El agar y el carragenato se extraen de determinadas especies de algas rojas y las estamos ingiriendo y empleando todos los días (mermeladas, repostería, comidas preparadas, aceitunas rellenas, pomadas, sprays, jabones, telas estampadas e hidrófobas, cápsulas, detergentes, cerveza, champú, pasta de dientes, análisis clínicos y bacteriológicos, etc.). Tampoco es muy conocido que la industria española es líder mundial en el campo de la extracción de estos coloides y que estos productos son de importancia estratégica para cualquier economía mínimamente desarrollada.

Además de las aplicaciones que hemos mencionado, las macroalgas también se emplean en cosmética y actualmente se están invirtiendo fuertes sumas (General Electric, Co., California) en investigar su aplicación como fuente de energía alternativa (metano), mediante su cultivo en océano abierto y posterior fermentación de la biomasa. Otra aplicación importante en desarrollo la constituye la extracción de fármacos con actividades antibacterianas, antifúngicas y reductoras del colesterol.



Esquema de una granja oriental de macroalgas marinas

III.- ¿Cómo se obtienen las macroalgas marinas?

Hay tres formas de obtener macroalgas marinas:

1- Explotación de poblaciones naturales
2- Cultivo en el mar
3- Cultivo en tierra

1.- La explotación de poblaciones naturales es insuficiente para cubrir la demanda. Además, la sobreexplotación del recurso y la contaminación del litoral han disminuído o eliminado las poblaciones de algas de interés industrial en muchas zonas del planeta (incluída España, que es la mayor reserva ficológica de algas de interés industrial de Europa).

En Canarias contamos con una gran diversidad de algas marinas de interés industrial, aunque, según un estudio subvencionado por la Consejería de Agricultura y Pesca y realizado por el Departamento de Botánica de la Universidad de La Laguna, la extensión y densidad de poblaciones de algas de interés industrial en Canarias no son lo suficientemente abundantes como para rentabilizar su explotación, si bien diversas empresas han mostrado interés por realizar prospecciones (acuciadas por el agotamiento de los recursos).

Conclusión: la explotación de poblaciones naturales no permite cubrir el constante aumento en la demanda de macroalgas marinas.

2.- El cultivo de macroalgas en el mar está muy extendido en Oriente (China, Filipinas, Corea, Thailandia, Japón, etc.). Este tipo

de cultivo consiste en fijar las algas a cuerdas o a redes que se extienden como auténticas granjas marinas por todo el ambiente determinado en cuanto a: corrientes, mareas, profundidad, transparencia del agua, luz, temperatura, nutrientes y libre de contaminaciones, además de disponer de una ingente mano de obra barata. En cuanto al ambiente físico en Canarias se podría pensar en desarrollar granjas similares a las orientales en determinadas zonas costeras, pero la competencia por el uso del litoral (turismo y actividades lúdicas), riesgos de contaminación (limpieza incontrolada de petroleros, urbanización incontrolada, ausencia de informes previos de impacto ambiental submarino), ausencia de zonas acotadas para cultivos marinos y el comparativamente elevado coste de mano de obra, no hacen muy prometedor este tipo de cultivo en Canarias.

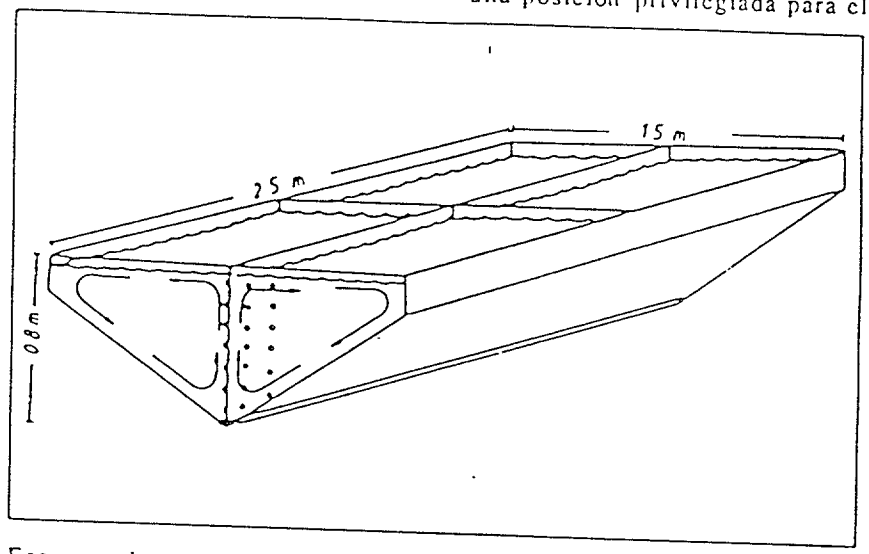
Conclusión: los sistemas de cultivo en el mar no son aplicables en Canarias.

3.- El cultivo de algas marinas en tierra es una actividad agrícola intensiva que se realiza en estanques (Fig. 3), canales, "bidones" transparentes, etc.

Los requisitos para desarrollar el cultivo intensivo de algas son básicamente cuatro: agua de mar, tierra árida e inservible, sol y temperatura, cuatro recursos son abundantes y baratos en Canarias.

Las ventajas de esta actividad agrícola son:

1.- No compiten por tierra fértil (escasa en Canarias y disminuyendo de forma alarmante).
2.- No compiten por agua dulce (sin comentarios).
3.- No compiten por mercados agrícolas establecidos (crean nuevos mercados)
4.- El acceso y control del cultivo es sencillo y permite un elevado grado de automatización.
5.- La demanda de algas no cubre la oferta y experimenta un constante aumento.
6.- No existe competencia industrial o biotecnológica que pueda producir los productos extraídos de algas, sólo se pueden obtener de ellas.



Esquema de un estanque de cultivo. Las algas se cultivan a altas densidades y sin fijación a sustrato alguno (flotando). Por el fondo del tanque se inyecta aire para generar movimiento y mantener unas condiciones de cultivo adecuadas.

7.- En España hay compañías que absorberían fácilmente la producción.

8.- El cultivo puede ser de multiuso, (p.e. aprovechar aguas residuales y gases de combustión (descontaminación), producir algas agarofitas (extracción de coloides) y el residuo de la extracción emplearlo como abono.

Los inconvenientes de este tipo de cultivo intensivo radican fundamentalmente en dos aspectos:

1- Saber cómo sacar más dinero (producción) del que se necesita para sostener estos sistemas de cultivo de alta sofisticación y 2- obtener especies selectas que den mayor valor a la producción.

Ambos aspectos están celosamente guardados por las (aún) pocas compañías que se dedican a esta actividad (en Suecia, EE.UU., Israel, Canadá y Japón).

Por sus características y situación geográfica, Canarias tiene una posición privilegiada para el desarrollo de este tipo de cultivos y en este empeño estamos un grupo de investigadores de la Universidad Politécnica de Canarias.

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ALGAL BIOTECHNOLOGY

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ATTEMPTS TO ESTABLISH AXENIC CULTURES AND PHOTOAUTOTROPHIC GROWTH OF *GELIDIUM VERSICOLOR*, *GRACILARIA FEROX* AND *LAURENCIA* SP. CELL CULTURES

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ABSTRACT

Combinations of sterilizing treatments described as effective in recovering axenic cultures were unsuccessful in the species tested. Direct photoautotrophic cell growth and regeneration were achieved in simple inorganic enriched seawater devoid of hormones. Fast and slow growing lines were recovered. Endophytic bacteria may play a role in callus induction and regeneration.

INTRODUCTION

Tissue culture techniques have been recently applied to seaweeds in order to improve the genetic qualities or as a system for mass culture. Callus formation in seaweeds can appear spontaneously and has been described to be dependent on: (i) the physical state of the media (semisolid) (1, 2, 3), (ii) the type of tissue from which the cultures were initiated (3, 4), and (iii) bacterial infections (5, 6).

Axeny is not a requisite for autotrophic cell culture (7, 8, 9). However for long cultivation periods axeny is necessary, as the initially controlled growth of contaminants frequently overgrowth the cultures (9).

The objective of our work was to determine the possibility to obtain axenic (or aseptic) cultures, starting from apical segments of economically important seaweeds, regeneration and calligenic potential.

MATERIAL AND METHODS

elidium versicolor, Gracilaria ferox and Laurencia sp., ollected from intertidal shores along the NW coast of land of Gran Canaria (Canary Islands) between November- er 1986. Two hours after collection, healthy and appa- epiphyte-free apical segments (1-1.5 cm long) were d and subjected to the 13 sterilizing treatments des- in table 1. Sonication was performed in a ultrasonic ng device, 30 s in sterile distilled water followed by 5 min in sterile seawater. Commercial Hibitane (5% xidin bigluconate, ICI Farma Lab) was diluted 1:1000 5000 in sterile seawater, and commercial Betadine (10% ne iodine, Sarget Lab) was used pure (100%) or diluted with sterile seawater. They were used alone or in ation with GAN (GeO_2 0.5 mg/l, Ampicillin 10 mg/l, and n 2 mg/l) or GAP solutions (GAN plus Streptomycin 100 Penicillin 300 mg/l). An average of 15 explants were treatment and species. Sterilizing treatments were flasks containing 6-8 explants. After sterilization ends were excised (aprox. 3mm) and the explants d in Petri dishes with PES media (13) solidified with gar (Bacto Difco). pH was previously adjusted to 8. tures were incubated in a growth chamber at 23 ± 2 °C, x, and 16 h ligth. Controls and transfers to fresh were done at 15 days intervals. Apparent sterile s were tested in Axenity Test Media (glucose 0.05%, 0.1%, casein hydrolyzate 0.05% and bacteriological broth 0.1% in sterile seawater). Identification of a was done using API (Difco) standard procedures. mples for electron microscopy were processed following ods described by Devilopoulos and Tsekos (11).

RESULTS

Axeny

Contaminants are gradually diminished by increasing biocide treatments and concentrations, as inversely did the viability of the explants (table 1). Explants from Gracilaria ferox show a high sensitivity to the treatments, while Gelidium versicolor and Laurencia sp. explants are more tolerant and show similar results. Axeny was obtained only in two treatments (table 1), which kill the explants. Sterilizing treatments killing the explants still allowed the survival and growth of contaminants (table 1). Endoderma viride and less frequently Phaeophila sp. appeared from dead (or viable) explants.

Regeneration and Calligenic Potential

After 30 days in culture, 90 -95% of the apical segments from Gelidium versicolor showed a healthy and vigorous development of preexisting or neoformed buds over the whole explant. Similar results were obtained in other assay using stipe fragments (1 cm long). In this case buds arose from medullary cells of the cut surfaces. Gelidium versicolor showed the highest regenerative potential of the three species tested. The neoformed thalli showed a strong phototropism, growing erect away from the medium. One depigmented explant produced a highly pigmented (brown-red) non-morphogenetic callus. The callus was highly friable, formed by "independent" nodules (0.5 mm diameter) and appeared on the middle of the explant. The reculture of the callus was problematic as it easily desintegrated in nodules. After reculture the callus progressively was depigmented and died after 15 days.

After 30 days in culture, 70 - 75% of the viable explants from Gracilaria ferox showed the development of preexisting and neoformed buds overall the explant surface.

TABLE 1

rage and types of contaminants after the sterilizing treatments

	LAURENCIA SP.		G. VERSICOLOR		G. FEROX	
	ALIVE (%)	CONTAM.	ALIVE (%)	CONTAM.	ALIVE (%)	CONTAM.
zed	100	NFB,Pas	100	Fla,F,Sta	100	Vib
	100	Vi,F,Ps	100	NFB,F,Vi,St	100	F,Vi,Pa
:5000	100	F,Sta,Fla	100	NFB,Pas	0	F
:1000	100	F	100	NFB,Vib	0	F, Pse
:5000 + GAN	100	Pse	100	NFB (*)	0	Bact
:1000 + GAN	100	NFB (*)	100	NFB (*)	0	Bact
:5000 + GAP	90	EndoA	100	Bact	0	EndoA
:1000 + GAP	100	Bact	100	Bact	0	EndoA
)%	100	F,Sta, Fla	100	NFB,F	0	EndoA,F
)0%	0	F,Pas	0	Bact	0	EndoA,F
)% + GAN	80	Pse	100	Bact	0	EndoA
)0% + GAN	0	Pse	0	Bact	0	AXENIC
)% + GAP	100	EndoA	0	D	0	EndoA
)0% + GAP	0	EndoA	0	AXENIC	0	EndoA

lentified bacteria, D = Diatoms, EndoA = Endophytic algae, F = = Flavobacterium odoratum, NFB = non fermentative bacteria, urella pneumotropica, Ps = Pseudomonas sp., St = Staphyllo- Vi = Vibrio alginolyticus. (*) resistant to Penicillin and n; sensible to Gentamycin, Cefoxitin, Cefamandole, Cephalori- xmycin, Erithromycin, Tobramycin and Dibekacin.

elly half of the new and preexisting buds became zed in the apical area after growing 2-3 mm in ne disorganization promotes a friable yellowish non- etic callus nodule (0.5 - 1 mm). After reculture the enerated a callus mass (3-4 mm diameter). In some econdary callus, more pigmented and organogenetic, n the primary callus. Subcultured isolated calli ely bleached and died.

30 days in culture, 40 - 45% of the viable explants encia sp. produced a highly pigmented (red) compact allus formation took place in the cut and apical the explant. The reculture of callus-producing was successful. Regeneration (4-5 thalli per callus) n all the calli. Thalliclones (thallus regenerated t) showed a high phototrophism. Differential thalli- vth allowed the cloning of slow and fast growing

lines. During the next passages the calli were isolated and successfully cultured during the successive 4 recultures, and after then recultured in PES liquid media. Contamination (mostly fungi) proliferated in liquid media and the slow and fast growing thalliclonal variants were overcrowded.

Callus histology

Electron microscopy of the callus from Laurencia sp revealed the presence of mycoplasma-like bacteria. Bacteria with a double membrane and without cell wall appeared filled with starch (?) granules, like the callus cells (9), and they were localized intracellulary.

DISCUSSION

The results from the sterilizing treatments suggest that axeny starting from thallus fragments may be obtained more by chance than by the effectiveness of a specific treatment. Endophytes are a widespread phenomenon in seaweeds, and surface sterilizing treatments are innocuous. Endophytes tolerate lethal treatments of the host cells. The best strategies seems to be starting from spores (12), isolated cells (13) or protoplasts (14). Another question is wether axenic cultures could sustain the growth of cell and tissue cultures. From the earlier experiments of Provasoli (15) it seems that the bacteria furnish a requirement for normal morphology and growth. Cheney et al (14) related the failure of growing axenic cell clusters derived from protoplasts of Gracilaria, to a shortage or absence of essential morphogenetic substances released by bacteria. As pointed out by Morita (16) "bacterial endosymbionts may be more common than we realize in marine macroorganisms". A role played by the ectocarpoid endophytes has also been suggested in the formation of callus (5). However some authors have reported cell culture and

iclonal regeneration in axenic cultures (1, 2). A better standing of the organic requirements of seaweed cells is sary to run axenic cell cultures.

A more speculative, but fascinating, effect of bacteria be related to a tumour-like effect analogous to the Tiids of Agrobacterium. Dixon (5), reviewing the literaon "galls and tumour-like growths" in the Rhodophyta, references from 1892 relating the bacteria with seaweed . Agrobacterium tumefaciens induced "crown-gall disea- in red algae (references in 5). Tsekos (6) suggested a ır-like effect when he found bacteria filled with starch ıles in naturally-induced calli of Gigartina teedii. The finding we report now. However Apt (17) found no evidenf inter- or intracellular bacteria in callus-like growths racilaria.

The absence of any carbon source in the medium (if agar not provide it) shows a direct induction of photosynthegrowth in cell cultures without CO_2 enrichment. The capaty of cell cultures to be initiated, cultured and regened under normal atmospheric conditions opens up the possity to select for higher productivity strains. Higher rise biphosphate carboxylase efficiencies may be the explaon for higher growths. If our "fast growing strains" are ted with such an effect still remains speculative.

Some authors have described that callus from several iophyta do not survive the transfer to fresh medium (18, Chen (19) related this "transfer-effect" to a failure in absortion of nutrients from the agarized media. Our ılts with Gelidium versicolor and Gracilaria ferox agree ;ome manner with those observations, while the results the calli of Laurencia sp. suggest other explanations those proposed by Chen. A different "physiological capaty" of the callus cells, which could be related to the ture of the callus (friable / compact), seems more proba- . As no histological study of the calli of Gelidium and cilaria could be performed, the question wether the endoous bacteria plays a role remains unresolved.

Another unresolved question arising from our results is how the erect-growing thalliclones and regenerated plants (until 1.5 cm heigth, sometimes with profuse branching) obtain their nutrients. A possible apoplasmic transport of nutrients guided by evapotranspiration could be the explanation.

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PROTOPLAST PRODUCTION, CELL WALL REGENERATION AND PHOTOPOLARIZATION IN ZYGOTES OF *FUCUS DISTICHUS* (L.) POWELL (PHAEOPHYTA)

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ABSTRACT

We report here on the isolation and regeneration of protoplasts from young zygotes (6-8h) of *Fucus distichus*. Using commercial cellulases and alginate-lyases from marine molluscs, protoplasts were isolated either as a suspension or attached to a substratum. Protoplasts yields were greater than 95% of the cell population. Over 80% of the protoplasts synchronously regenerated a wall, germinated and divided into a polar, multicellular embryo. Cell wall polysaccharide deposition and localization was studied in normal zygotes and protoplasts by labelling with monoclonal antibodies specific for alginates or fucans. Protoplast wall regeneration was slower but similar to native wall assembly following fertilization. Pulses of unilateral light were given to protoplasts before wall removal or at various intervals during wall regeneration to investigate the role of the cell wall in photopolarization of zygotes. Wall removal had no effect upon either the maintenance or the establishment of the embryonic axis. By contrast, fixation of the polar axis was dependent upon the presence of a cell wall.

INTRODUCTION

The *Fucus* zygote is an established experimental system to study plant cell development [1]. It is also a convenient natural system to study the assembly and localization of cell wall polymers [2]. Large populations of fertilized eggs can be obtained that develop synchronously [3]. The unfertilized egg is a symmetrical, unpolarized cell and large populations of fertilized eggs can be obtained that develop synchronously [3]. During the first hours of development, the young zygote establishes an embryonic axis, i.e., determines the site of the future rhizoid. Up to 10-12h after fertilization orientation of the axis is labile and can be experimentally manipulated by external vectors such as unilateral light (axis formation) [4]. At 10-12h the axis becomes irreversibly

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The effects of the physical characteristics of the culture medium on the development of red seaweeds in tissue culture

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Key words: callus, *Gelidium versicolor*, *Grateloupia doryphora*, *Laurencia* sp., osmolality, regeneration, solidity, tissue culture

Abstract

Explants of *Gelidium versicolor*, *Grateloupia doryphora* and *Laurencia* sp. were cultivated in Provasoli enriched seawater culture medium (PES) adjusted to several osmolalities (0.5, 0.7, 1.0 and 1.5 Os kg^{-1}) and solidities (agar concentration = 3, 8 and 15 g L^{-1}). Osmolality was adjusted by dilution of seawater with distilled water (50, 70 and 100% seawater) and by NaCl addition. Explants of *Laurencia* sp. and *Grateloupia doryphora* showed bud regeneration and callus formation. Explants of *Gelidium versicolor* only showed bud regeneration. Osmolalities of 0.5 and 1.05 Os kg^{-1} inhibited or drastically reduced bud regeneration and callus formation. The highest callus formation and bud regeneration were observed at 0.7 to 1.0 Os kg^{-1}. An increase in the agar concentration of the culture medium was positively correlated with callus formation and negatively correlated with bud regeneration. An increase in the percentage of seawater increased the solidity of the culture medium and was positively correlated with callus formation. Glycerol was an effective carbon source for the vegetative propagation of axenic explants of *Grateloupia doryphora*, promoting growth and bud regeneration. An increase in glycerol concentration in the culture medium increased its osmolality, inhibiting the growth of the explants and their morphogenetic development.

Introduction

The control of cell growth and development is still a problem in seaweed cell and tissue cultures (Saga *et al.*, 1986; Polne-Fuller & Gibor, 1987). Some authors have reported the formation of callus and callus-like structures induced by the semi-solid (agarized) state of the culture medium (Fries, 1980; Saga *et al.*, 1982; Saga & Sakai, 1983; Polne-Fuller & Gibor, 1984; Garcia-Reina *et al.*, 1988). The solidity of the culture medium seems to be involved in callus induction, since other gelling compounds produced the same effects as agar (Polne-Fuller & Gibor, 1987).

The role of carbohydrates in the growth and organogenesis of seaweed cell cultures remains equivocal (Lawlor *et al.*, 1988). The addition of carbohydrates to the culture medium, without controlling osmolality, can obscure their role in seaweed growth.

The aim of the present work was to study the effects of solidity and osmolality of culture medium on callus induction and bud regeneration of several red seaweeds, as well as the effects of

glycerol addition on the osmolality of the culture medium and as a carbon source.

Materials and methods

Gelidium versicolor (S. G. Gmelin) Lamouroux, *Grateloupia doryphora* (Montagne) Howe and *Laurencia* sp., an intertidal alga similar to *Laurencia obtusa* (Hudson) Lamouroux, were collected in Gran Canaria (Canary Islands). Voucher specimens are deposited in the herbarium of the Jardin Canario, Gran Canaria, Canary Islands, Spain (LPA), as sheets LPA 30 (*Laurencia* sp.), LPA 110 (*Gelidium versicolor*) and LPA 129 (*Grateloupia doryphora*).

Two hours after collection, explants were excised from apparently epiphyte-free, highly pigmented and sterile thalli. *Laurencia* sp. explants were apical secondary branches 1 cm long; *Gelidium versicolor* explants were cylinder-shaped fragments (0.5 cm long) excised from the middle zone of the long primary branches. *Grateloupia doryphora* explants were disc fragments (0.3 cm diam.) excised from the middle lower thallus. Explants were cleaned following the methods described by Garcia-Reina *et al.* (1988). After cleaning, *Laurencia* sp. explants were cut again to obtain cylinder-shaped explants (0.5 cm long).

The culture medium was PES (Provasoli, 1968) adjusted to the different osmolalities and solidities in Table 1. Explants (195 per treatment) were cultivated in Petri dishes (15 explants in each) with 20 mL of the culture medium. Cultures were placed in a growth chamber at 20 ± 2 °C with a day length of 18 hours and 27 μmol m^{-2} s^{-1} at the level of the Petri dishes provided by Sylvania Grolux daylight lamps.

Quantitation was made using the indices % callus (percentage of cultivated explants forming disorganized structures) and % bud (percentage of cultivated explants regenerating buds). For *Laurencia* sp., quantitation was made at 30 days of cultivation; for the other two species, at 15 days. The results were expressed as a percentage of those in the control treatment.

Axenic cultures of *Grateloupia doryphora* were established following the methods described by Polne-Fuller *et al.* (1984). Semicircular-shaped explants (approx. 1 mm long) were excised from the axenic disc. Explants (15 semicircular explants per treatment) were cultivated in different culture media enriched with glycerol (Table 1) under the same culture conditions described before. Quantitation was made using the index 'number of buds/number of cultivated explants'. The fresh weight increase was monitored for semicircular explants cultivated in liquid PES70 + 0.3 M glycerol and in PES.

Table 1. Percent seawater and NaCl or glycerol addition to the culture media used in osmolality variation exeriments (agar concentration in all the media = 8 $g\,l^{-1}$).

Culture media notation	Percent seawater	NaCl addition mol m^{-3}	Glycerol addition mol m^{-3}	Osmolality Os Kg^{-1}
PES50	50	–	–	0.5
		0.10	–	0.7
		0.27	–	1.0
		0.51	–	1.5
PES70	70	–	–	0.7
		0.17	–	1.0
		0.41	–	1.5
		–	0.30	1.0
		–	0.80	1.5
PES	100	–	–	1.0
		0.25	–	1.5
		–	0.50	1.5

Results

After 15 days calli (filamentous callus-like structures) and buds were regenerated by *Grateloupia doryphora* explants. During this time, the regenerating explants of *Gelidium versicolor* only produced buds; callus formation was not recorded even in explants cultivated for 45 days. The explants of *Laurencia* sp. developed callus (a compact cell mass in the wounded area of the explants) and buds after 30 days.

Callus and bud morphology were the same regardless of the osmolality or solidity of the culture medium.

Effects of osmolality

The highest % callus and % bud were obtained in culture media at 0.7 to 1.0 Os kg^{-1}. Culture media at 0.5 Os kg^{-1} or 1.5 Os kg^{-1} inhibited or drastically reduced regenerator (Figs. 1 & 2).

Effects of solidity

The agar content of the culture media was positively correlated to callus formation (% callus) and negatively correlated to bud regeneration (% bud; Fig. 3).

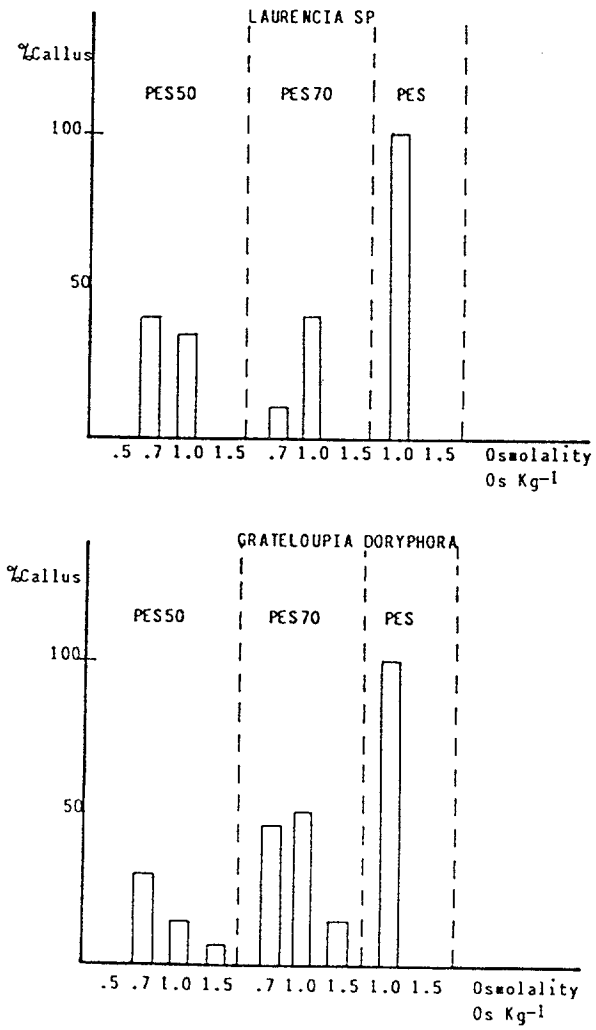

Fig. 1. Values of the index % callus observed in explants cultivated in different osmolalities (agar concentration = 8 g L^{-1} in all media, n = 195).

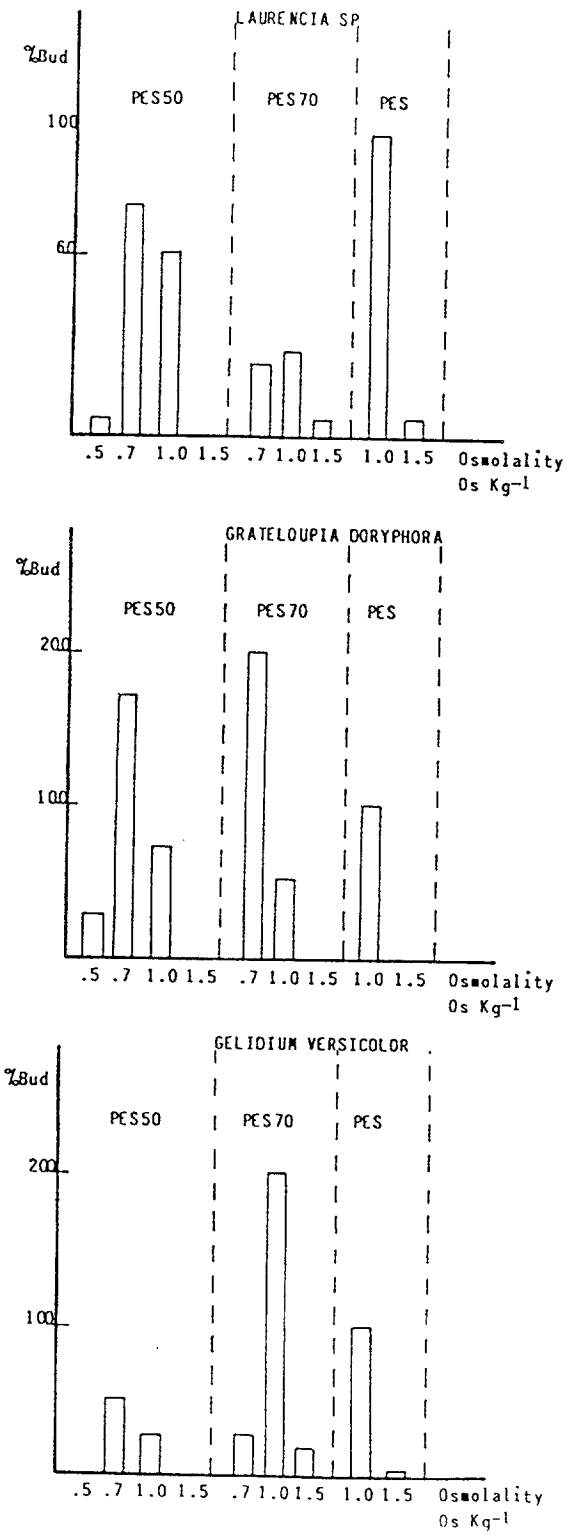

Fig. 2. Values of the index % bud observed in explants cultivated in different osmolalities (agar concentration = 8 g L^{-1} in all media, n = 195).

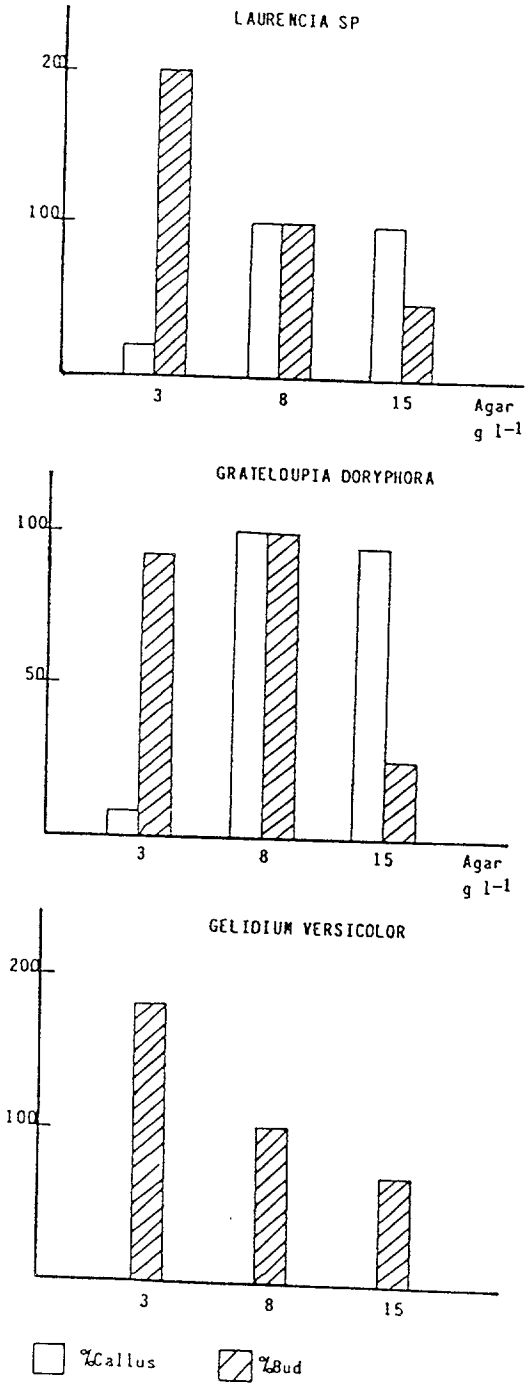

Fig. 3. Values of the indices % callus and % bud observed in explants cultivated in PES culture medium with 3, 8 or 15 g L^{-1} agar (osmolality = 1.0 Os kg^{-1} in all media, n = 195).

Effects of seawater

A decrease in the solidity of the culture medium was observed from PES (100% seawater) to PES50 (50% seawater).

Callus formation in *Laurencia* sp. and *Grateloupia doryphora* decreased from PES100 to PES50 (Fig. 1). No direct relation between dilution of seawater and % bud formation was found, but the highest % bud formation in *Gelidium versicolor* and *Gracidaria doryphora* was observed in PES70. For *Laurencia* sp. (Fig. 2), the highest % bud formation was in PES100, followed by PES50, then PES70.

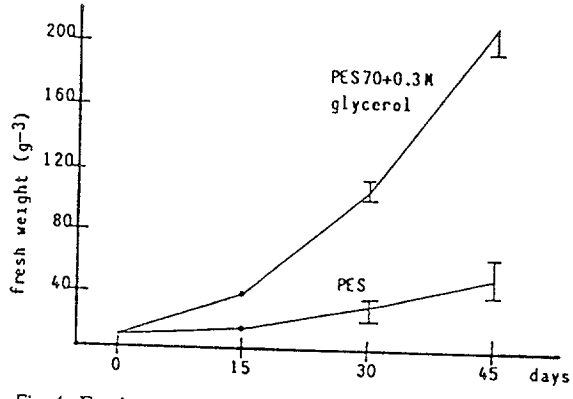

Fig. 4. Fresh weight increase of *Grateloupia doryphora* explants cultivated in liquid PES70 + 0.3 M glycerol and in PES culture media.

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

Osmolality Os kg^{-1}	Agar g L^{-1}	Culture medium	Glycerol mol m^{-3}	B/C index
0.7	8	PES70	–	7.10
1.0	3	PES70	0.3	18.25
	8	PES70	0.3	16.25
	15	PES70	0.3	6.25
	8	PES	–	7.20
1.5	8	PES70	0.8	2.60
	8	PES	0.5	1.51

Effects of glycerol

Liquid PES70 + 0.3 M glycerol gave a greater increase in fresh weight than liquid PES alone (Fig. 4). In solid media, the best results were obtained with a PES70 + 0.3 M glycerol and 3 or 8 g L^{-1} agar; the explants became round, turned from reddish to orange, and reached a larger size than those in other culture media. Also, the highest values of the index 'number of buds/ number of cultivated explants' was observed in explants cultivated in that medium (Table 2). The value of this index decreased with increasing glycerol concentration and agar content of the culture medium (Table 2).

Discussion

The best morphogenetic response of an explant (callus and bud regeneration) was obtained at the 'natural osmolality' of 1 Os kg^{-1}, the osmolality of the seawater, or slightly lower, 0.7 Os kg^{-1} (Figs. 1 & 2). Variation in the osmolality of the culture medium to 0.5 and 1.5 Os kg^{-1} inhibited or drastically reduced bud regeneration and callus formation.

When the explants were cultivated in media with optimum osmolality (0.7 to 1.0 Os kg^{-1}), the solidity of the culture medium affected the development of the explant, reducing bud regeneration and increasing callus formation (Fig. 3). The solidity of the culture medium is not only involved in callus induction (Polne-Fuller & Gibor, 1987), but, in fact, in switching development from organized (bud) to disorganized (callus or callus-like) structures.

Lawlor *et al.* (1988) reported that an increase in salinity (% seawater) of the solid medium promotes callus formation and growth in *Ecklonia*. The results in Fig. 1 show the same effect of seawater on callus formation in *Laurencia* sp. and *Grateloupia doryphora*. Also, the highest % bud formation in *G. doryphora* and *Gelidium versicolor* was observed in PES50 or PES70 medium (Fig. 2). The fact that the solidity increased with the percentage of seawater in the PES medium clearly shows that the effect of seawater on callus and bud formation is due to the alteration of the solidity.

Glycerol is an effective carbon source for the vegetative propagation of *Grateloupia doryphora*. Liquid PES70 culture medium enriched with 0.3 M glycerol gave an increase in fresh weight 400% higher than PES medium alone after 45 days. This result agrees with those reviewed by Fries (1973) on the effect of glycerol as a carbon source on red seaweeds.

The inhibiting effect of high concentrations of glycerol (0.5 and 0.8 M) on morphogenesis of the explants (Table 2) is primarily due to the increase in osmolality. This result demonstrates the need to control osmolality when testing the addition of carbohydrates or other osmotically active compounds on seaweed cell and tissue cultures.

Conclusions

Osmolality and solidity are physical characteristics of the culture medium that affect the vegetative propagation of *Laurencia* sp., *Grateloupia doryphora* and *Gelidium versicolor* by tissue culture. The alteration of the culture medium, such as dilution of seawater or addition of glycerol modifies the response of the explants by its own effect (i.e. carbon source effect of glycerol) and also by the modification of the solidity or osmolality of the culture medium.

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

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Key words: carbon-source, glycerol, *Grateloupia doryphora*, morphogenesis, osmolality, solidity, tissue culture

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.

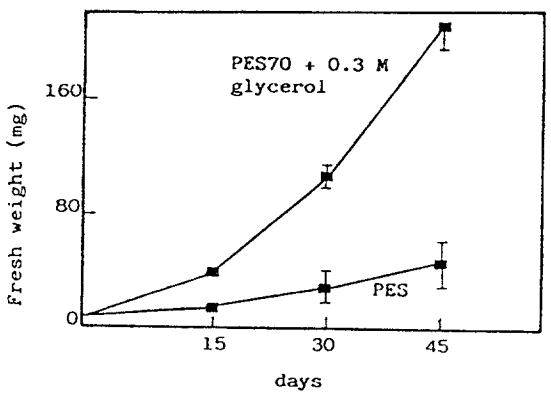

Fig. 1. Variation of the fresh weight of explants cultivated in liquid PES70 + 0.3 mol l^{-1} glycerol and liquid PES (average fresh weight from three replicates with 15 explants in each. Vertical bars = ± SD).

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

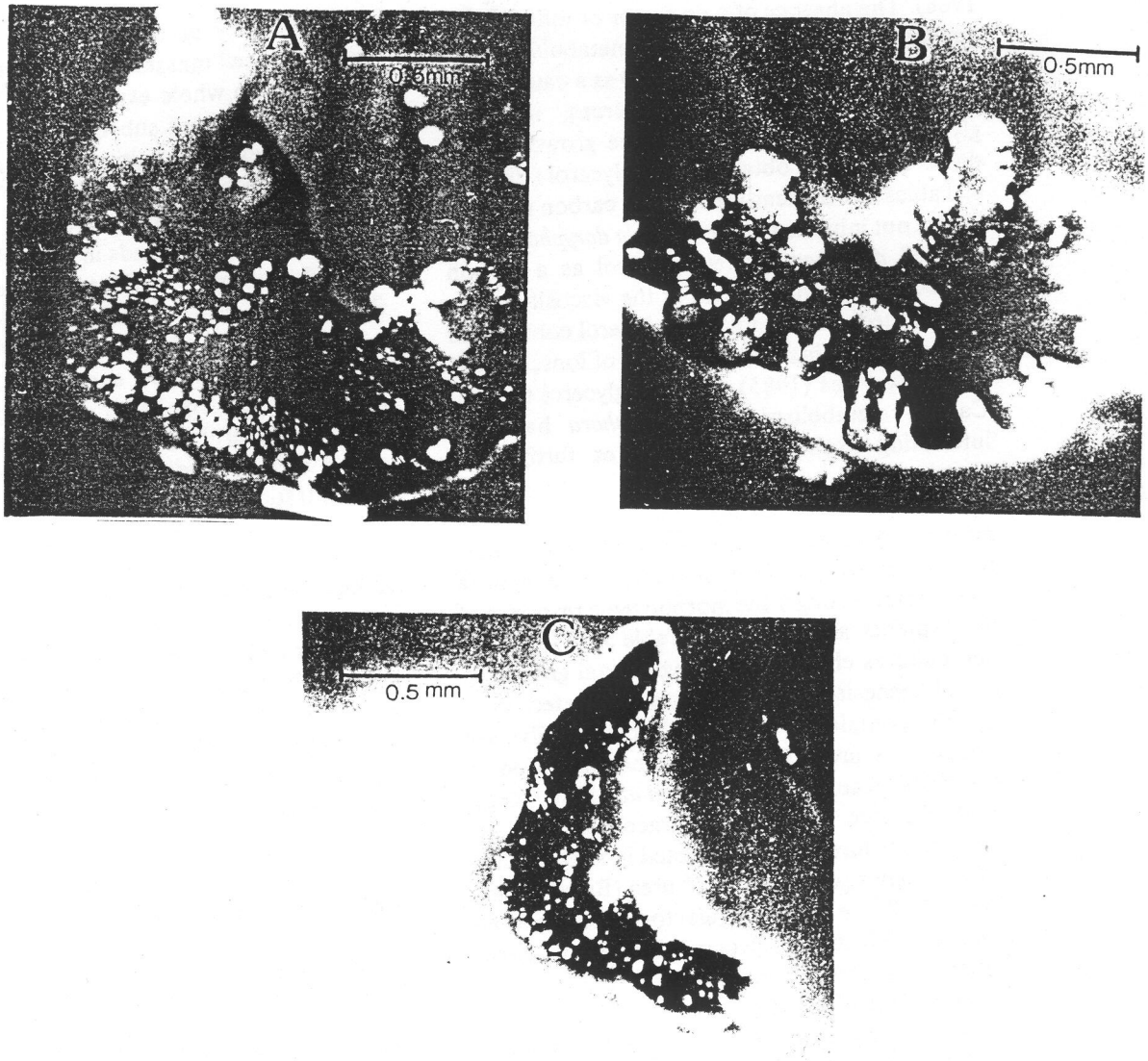

Fig. 2. Explant morphology after 20 days of culture in PES70 + 0.3 mol l^{-1} glycerol + 15 g l^{-1} agar (A), PES70 + 0.3 mol l^{-1} glycerol + 8 g l^{-1} agar (B), and PES + 8 g l^{-1} agar (C).

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 [illegible]

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.

Acknowledgements

We are grateful to Dr Nieves Gonzalez for assistance with the taxonomy and collection of the algae. Grants from the Spanish Ministerio de Education y Ciencia to R.R.R. and from the Fundacion Universitaria de Las Palmas to P.G. are gratefully acknowledged.

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Canarias cuenta con todos los requisitos necesarios para la agricultura marina.

IMPORTANCIA ECONOMICA Y ESTRATEGICA DE LAS ALGAS MARINAS

Su producción en Canarias: objetivo básico del Laboratorio de Biotecnología Vegetal Marina de la Universidad de Las Palmas

GUILLERMO GARCIA

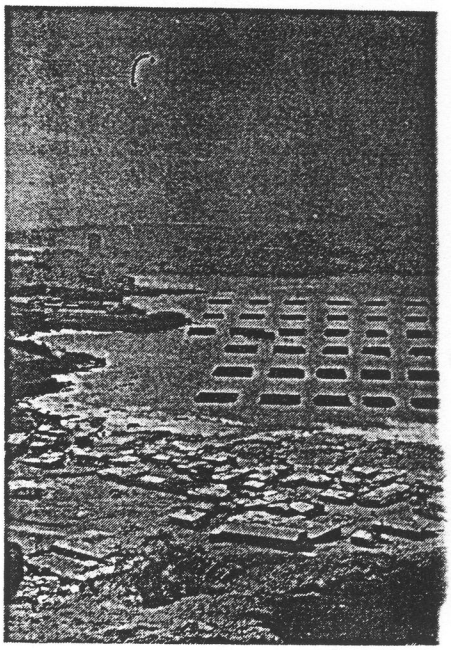
"En general, cuando se habla de algas, aun en Congresos dedicados a cultivos marinos, poca gente sabe exactamente cuál es su rol en este sector". Con esta frase comenzaba el Director del Departamento de Algología del Instituto Español de Oceanografía de Santander su disertación en un Congreso de Acuicultura celebrado en 1985. Esta frase sigue vigente hoy en día, aunque cada vez más va calando en la comunidad científica europea y nacional, así como en la Administración Nacional y de algunas Comunidades Autónomas, el interés por las algas marinas, un recurso que se puede calificar como desconocido.

Este creciente interés por las algas marinas no es puramente científico. El cultivo de algas a nivel mundial es la actividad de acuicultivo que ocupa a más gente y mueve más tonelaje y dinero. En 1988 se estimaba que la maricultura vegetal generaba empleo directo a cerca de un millón de personas (Japón, Corea, Indonesia, China) y datos de 1982 cifraban en un billón de dólares el mercado mundial de las macroalgas de cultivo.

La idea general de que las algas sólo sirven como comida para los "chinos" es totalmente errónea. La actividad cotidiana de cualquier europeo está imperiosamente relacionada con productos algales; detergentes para la ropa, pasta de dientes, espuma de afeitar, repostería industrial, mermeladas, natillas, batidos, cerveza, telas estampadas, aceitunas rellenas, sprays, tejidos hidrófobos, comidas preparadas, champús, pinturas, elementos de cosmética y un largo etcétera de productos cotidianos se elaboran con productos extraídos de algas marinas. La producción de antibióticos, análisis clínicos y microbianos y prácticamente toda la industria biotecnológica está basada en la utilización de coloides extraídos de algas marinas. Estos coloides en general (agar, caragenatos y alginatos), y fundamentalmente el agar, están considerados como productos de interés estratégico en las economías occidentales. La falta de suministro de estos coloides provocaría un colapso total para algunas líneas industriales y de la industria farmacéutica, clínica y biotecnológica.

La utilización de algas como alimento humano es la aplicación más antigua de estos vegetales. Aunque es una tradición centenaria en Oriente (se empleaban incluso como moneda para el pago de impuestos -interesante alternativa a sugerir-), hasta hace poco tenía un mercado casi nulo en Europa. No obstante, el constante aumento del interés y conocimiento de la población europea por la dietética y los alimentos naturales (biológicos) está propiciando el crecimiento de este mercado. Tomando como ejemplo la ciudad de Las Palmas de Gran Canaria, es de destacar el establecimiento en los últimos tres años de seis tiendas que importan algas de Japón, Corea y China para su venta como alimento humano.

Teniendo en cuenta el elevadísimo contenido en vitaminas (diez veces más vitamina A que las espinacas), sales minerales (más calcio que la leche, 1.500 veces más iodo que el pescado), ácidos grasos poliinsaturados (previenen enfermedades coronarias), proteí-

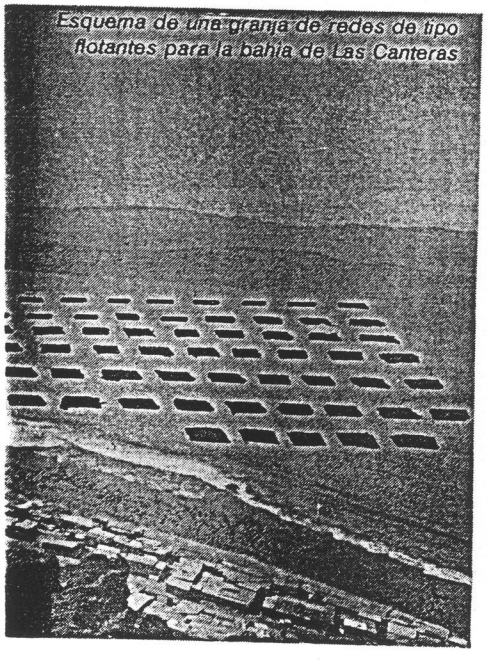


nas de alto valor (similares a la ovoalbúmina) y bajo contenido calórico (no engordan y son laxantes), no es de extrañar la fácil penetración de esta nueva fuente alimentaria en la dieta europea, favorecida además por su status de producto "biológico" (no se emplean pesticidas ni fertilizantes químicos) y sus recientemente probadas propiedades anticancerosas.

Otras aplicaciones industriales de las macroalgas marinas las constituye la producción de piensos y fertilizantes agrícolas. Los fertili-

zantes líquidos producidos de macroalgas reducen las infecciones fúngicas y la incidencia de plagas de araña roja. La plaga de "trip" californiano que estamos padeciendo podría ser, al igual que la araña roja, controlable con la aplicación de extractos algales.

El problema principal radica en la obtención de un tipo, calidad y garantía de suministro de materia prima que las cada vez más exiguas y sobreexplotadas poblaciones naturales no pueden cubrir. La solución a este problema tiene diez mil años de antigüedad, cultivar en vez de depredar. Cultivar algas marinas no es ningún problema, es muy fácil... Lo que no es fácil es hacerlo de forma rentable



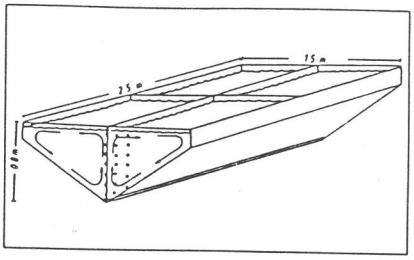
Esquema de una granja de redes de tipo flotantes para la bahía de Las Canteras

en una economía occidental.

Existen dos formas de cultivar macroalgas marinas: fijándolas a cuerdas y cultivando éstas en el mar (modelo oriental) o cultivándolas en estanques en tierra. El modelo oriental tiene la desventaja de la considerable cantidad de mano de obra que se necesita, no sólo para el mantenimiento del cultivo en el mar, sino también para mantener durante meses el "semillero" de las microplántulas que se emplean para injertarlas en las cuerdas. El problema del cultivo en tierra radica en el coste de las instalaciones de cultivo que, como todo cultivo intensivo, aunque tiene una mayor producción por superficie que el semiextensivo (oriental), consume mucha más energía. El objetivo que se fijó hace unos años el Equipo de Algología Aplicada de la Universidad de Las Palmas fue el de intentar desarrollar en Canarias la agricultura marina. Canarias cuenta con todos los requisitos necesarios para esta actividad, sin competencia posible de otras regiones europeas: tasa de irradiación anual, fluctuación anual de la temperatura ambiental y del agua de mar, transparencia e hidrodinamicidad de las aguas costeras, riqueza de flora marina de interés industrial... Lo que nos faltaba era la "herramienta" con la que poder desarrollar las ideas, un laboratorio adecuado.

Gracias a la cesión de 1.500 metros cuadrados en la explanada del muelle de Taliarte por el Cabildo Insular, a la financiación inicial aportada por la Universidad de Las Palmas y la Consejería de Agricultura y Pesca, a la colaboración del Ayuntamiento de Telde y a la reciente obtención de un Proyecto de Infraestructura del Plan Nacional de Recursos Marinos y Acuicultura, la "herramienta", el Laboratorio de Biotecnología Vegetal Marina, será una realidad en Marzo de 1990. Estas instalaciones permitirán además ofertar a los alumnos de la Facultad de Ciencias del Mar la posibilidad de formarse en una biotecnología emergente. Los convenios firmados con la primera multinacional extractora de agar (HISPANAGAR), y con los equipos de Algología Aplicada de la Universidad de Uppsala (Suecia) y del Instituto de Oceanología (República Popular China), nos permiten iniciar la andadura con optimismo. Los contactos establecidos con otros grupos de investigación europeos nos han permitido obtener la organización de un "Workshop" del Programa Europeo de Colaboración e Intercambio Científico (COST) a principios de 1991. Esta reunión permitirá evaluar la posibilidad de que nuestro Laboratorio se convierta en un Centro abierto a todos los grupos que en Europa están desarrollando Programas de Maricultura Vegetal.

Esquema de un estanque de cultivo. Las algas se cultivan a altas densidades y sin fijación a sustrato alguno (flotando). Por el fondo del tanque se inyecta aire para generar movimiento y mantener unas condiciones de cultivo adecuadas.



Tres becarios de nuestro equipo están trabajando desde hace varios meses en la Universidad de Uppsala gracias al convenio suscrito con la compañía AIR EUROPA. A su regreso, que coincidirá con las "estancias de sabático" de varios profesores de la Universidad de Uppsala y con la terminación parcial de las obras del Laboratorio de Biotecnología Vegetal Marina, esperamos iniciar programas conducentes al estudio de nuevas estrategias de cultivo tanto intensivo como semiintensivo de macroalgas marinas de interés industrial. Para entonces, la colaboración con los equipos existentes en esta Universidad de oceanografía costera, contaminación marina, química orgánica, dinámica de costas, ingeniería y automatización será vital para poder abarcar un campo tan multidisciplinar.

El sol, la costa y el mar de Canarias pueden aprovecharse para algo más que sembrar hoteles; un grupo de profesores, becarios y alumnos de esta Universidad tendremos en breve la herramienta que nos permita evaluar si las algas marinas pueden ser una buena zafra complementaria.

1991

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Actual, potential and speculative applications of seaweed cellular biotechnology: some specific comments on *Gelidium*

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Key words: callus, cell culture, domestication, protoplast, tissue culture

Abstract

Cellular biotechnology is a promising application in the propagation and selection of superior strains of seaweeds. Although axenic cultures, organogenetic tissue cultures, vegetative micro-propogation, callus induction and high yields of agar from calli have been described for several species of *Gelidium*, a number of basic problems remain to be solved. These include standardized methods for obtaining axenic cultures, identification of requirements for organic nutrients, PGR's, cellular disorganization and reorganization, somaclonal variation and somatic incompatibilities. Future progress in seaweed biotechnology will depend on the resolution of many of these problems.

Introduction

Cellular biotechnology was introduced into the phycological field at the beginning of the 1980's (Gibor, 1980), and is considered important in future development of seaweed cultivation. The four major techniques in seaweed cellular biotechnology, tissue-, callus-, cell- and the protoplast-culture, offer means in understanding seaweed domestication (propagation and selection) germplasm storage and for production of biomass. We first discuss seaweed cellular biotechnology as highly effective techniques, and then consider some still unresolved questions, which limit their potential and actual applications.

Seaweed biotechnology

Vegetative micropropagation

The possibility of using small thallus fragments (tissue culture) to establish cultures, instead of spores, exploits the *organogenetic potential* of explants. Chen and Taylor (1978) described the regeneration of whole plants from decorticated explants of *Chondrus crispus* Stackh. Waaland (1982) and Sylvester and Waaland (1983) obtained plants following inoculation of finely-chopped fragments of the thallus of *Gigartina* into braided rope. Their data indicated that the best strategy for commerical purposes is to maximize the amount of propagules per donor plant. During the early 1980's, similarly successful experiments were done with *Gelidium nudifrons* Gardner and *G. robustum* (Gardner) Hollenberg & Abbott (Polne-Fuller, 1988).

In China, Luqin *et al.* (1988), described a tech-

nique, using small explants of *Gelidium pacificum* Okamura, that permitted the firm attachment of small explants to ropes through the development of adventitious rhizoids and the regeneration of new plantlets. Garcia-Reina *et al.* (1988a) reported on organogenetic development from wounded explants of *Gelidium versicolor* (Gmel.) Lamouroux, and Robaina *et al.* (1990a) discussed the effects of solid culture medium and its osmolality on organogenetic potential.

If the best commercial strategy for vegetative micropropagation is to maximize propagules per donor-plant ratios, the logical strategy would be to reduce the plant to single, somatic cells, using these as inocula. 'Somatic spores' implies totipotentiality, as pointed out by Saga *et al.* (1978). Single somatic cells, obtained by enzymatic digestion of the thalli of *Porphyra* and *Ulvaria*, have been inoculated onto ropes and, from these, attached plants have developed (Polne-Fuller *et al.*, 1984; Kapraun, 1987; Kapraun & Sherman, 1989). This technique has been tested in commercial *Porphyra* farms (Mumford, 1987).

Somatic-cell isolation in seaweeds is not a difficult task as it has been achieved by partial enzymatic digestion of thalli (in *Porphyra*: Tang, 1982; Chen, 1986; Wang *et al.*, 1986, 1987a, 1987b; Tait *et al.*, 1990; Wang & Yan, 1990; *Sphacelaria*: Ducreux *et al.*, 1988), by cell-wall regeneration from protoplasts (*Porphyra*: Chen, 1989) or spheroplasts (*Undaria*: Kapraun & Sherman, 1989), by mechanical disruption of thalli (*Prasiola*: Schiff *et al.*, 1972; Bingham & Schiff, 1973; *Griffithsia*: Duffield *et al.*, 1972; *Porphyra*, Tait *et al.*, 1990), or of frozen thalli (*Porphyra*: Zhao & Zhang, 1984), by mechanical disruption of calli (*Laminaria*: Saga *et al.*, 1978; McLean & Connolly, 1989; *Alaria*: Saga *et al.*, 1978; *Undaria*: Zhang, 1982) or by combinations of mechanical and enzymatic treatments of both thallus and callus (*Pterocladia*: Liu & Gordon, 1987).

There are advantages to vegetative micropropagation over conventional propagation from spores. It is unnecessary to know or control the life-history of the species; 'seedlings' can be produced in quantity, both when they are needed and from a small number of donor plants, requiring cheap storage and handling.

These advantages are directly related to propagation, while the techniques are applicable to selection. Mono-phase crops (*i.e.* monoculture of *Gelidium* sporophytes or of male or female gametophytes) can be produced. Mutant plants (natural or induced) can be propagated rapidly, even if sterile, and even if the selected phenotype has a non-genetic basis (epigenetic) but is stable.

Selection

Tissue culture of seaweeds is based on natural genetic variability of the somatic cells; for example, *Gracilaria* species (van der Meer, 1986) have been shown to have a high degree of somatic recombination. Genetic diversity among cells of the thallus, normally masked by more common non-variant cells, is exploited, as described for higher plant tissue culture (D'Amato, 1978). This 'island effect' (retention of pigmentation, growth, *etc.*, by some cells or cell aggregates) is a common phenomenon in seaweed tissue culture (Fries, 1980; Polne-Fuller *et al.*, 1986; Garcia-Reina *et al.*, unpubl. data).

Polne-Fuller and Gibor (1986) have described the isolation and growth to callus on solid medium of a few living cells from *Porphyra* tissue cultures contaminated by *Pythium*. The callus was enzymatically digested to cells and small clumps of cells and co-cultured with *Pythium*, where healthy, normal callus developed. Although no plant regeneration was reported, this simple experiment shows how quickly and effectively tissue-culture techniques can yield results by selection.

Selected strains of *Sargassum* and *Enteromorpha* (showing differences in temperature tolerance, orphology and vitamin auxotrophy) have been established through tissue culture (Polne-Fuller *et al.*, 1986), apparently owing to intercellular variability among explants. Tissue culture (*i.e.* callus culture) is not, however, considered the best technique for cultivar improvement. The application of callus culture for selection is based on a process referred to as

somaclonal variation by Larkin and Scowcroft (1981). This term covers genomic, chromosomic, genetic and even epigenetic variations associated with or induced by the disorganized callus state, and is also expressed in the reorganized plants (Karp, 1989). Callus is associated with a high degree of genetic (or epigenetic) variability. Fang *et al.* (1983) have described genomic alteration in the callus cells of *Laminaria*, and Lee (1986), varying levels of polyploidy.

Callus culture has many advantages over conventional selection procedures. Within a small space (*e.g.* Petri dish incubated in growth chamber), large numbers of 'plants' (assuming cell = plant), having natural or induced genetic variabilities, can be screened. The stability of selected phenotypes can be determined in a short time, as the following generation is the next mitotic division, not the new adult sporophytic phase as in *Laminaria*. Any selected phenotype can be easily propagated to large amounts (using cell culture), even if it is an epigenetic but stable character.

In seaweeds, obtaining haploid cultures through cell culture is easy, whereas with higher plants the only way to obtain haploid cultures is by anther or pollen culture (Han & Hongyuan, 1986). For the latter group of plants, diploid cells can proliferate and few haploid cells remain viable, so that the efficiency is low. In seaweeds, haploid cultures depend only on the type of explant (gametophyte) chosen. Thus, exploitation of recessive information, haploid somatic recombination and gametoclonal variation (somaclonal variation in haploid cells, Evans *et al.*, 1984), can be readily employed as can the production of fully-homozygous cells and plants by colchicine treatment or by spontaneous chromosome doubling. All such characteristics (haploid culture, gametoclonal variation, chromosome doubling) have been demonstrated and utilized for *Laminaria*-improvement programs (Fang, 1984; Wu & Lin, 1988). Reports of regeneration of variant plants (altered morphology and life-history: monospore producing) from isolated cells or protoplasts of *Porphyra* (Tang, 1982; Fujita & Migita, 1985; Chen, 1987) and *Monostroma* gametophytes, with different developmental patterns and apogamy (Saga & Kudo, 1989), may be explained by differential regenerative potential or different variant cell strains pre-existing in the haploid thallus.

Selection through callus culture has some drawbacks, as the techniques allow selection only of cellular-based characteristics. It is impossible to screen for stipe-length or frond-thickness at the cellular level or for sulphate content in the cell walls of *Gelidium*. Monoclonal antibodies (Vreeland *et al.*, 1987) can be useful markers. There are also possibilities of selecting variant cells with characteristics expressed only at the cellular level and not in fully-differentiated plants.

The regeneration of plants from calli can yield surprises, some of which could be useful, even in the absence of directionally selective pressures. Several reports indicate inadvertent selection resulting from seaweed somaclonal variation. Yan (1984) described plants, reorganized from calli of *Laminaria* and *Undaria*, with more rapid growth and tolerance of high temperatures for a longer period than the normal sporophytes. Garcia-Reina *et al.* (1988b) selected two types of callus of *Laurencia* obtained from the same plant and similar in appearance (pigmentation, texture). These differed markedly in organogenetic potential (number of plantlets/callus) and growth-rate of the regenerated plants.

A major advantage of selection using cellular biotechnologies is in protoplast culture. This allows for somatic hybridization (through the breakdown of sexual barriers, through interspecific or intergeneric fusion of somatic cells, and exclusively maternal extrachromosomal inheritance). There is also improved efficiency of *genetic transformation* by the removal of physical barriers, and host-vector recognition specificities through elimination of the cell wall.

Protoplast isolation and regeneration of whole plants have been successful (Table 1). The same techniques as applied to higher plant protoplasts induce somatic hybridization in seaweeds. These include electrofusion (*Enteromorpha*: Saga *et al.*, 1986; *Porphyra*: Fujita & Saito, 1989) and PEG (*Ulva* × *Monostroma*: Zhang, 1982; *Enteromor-*

pha, Saga *et al.*, 1986; *Porphyra*: Fujita & Migita, 1987; Fujita & Saito, 1989; *Gracilaria*: Cheney, 1989). True somatic hybrids have been obtained in microalgae (*Chlamydomonas*: Matagne *et al.*, 1979; *Dunaliella* × *Porphyridium*: Lee & Tan, 1988). Recently, Fujita & Migita (1987), with wild × mutant *Porphyra* and Kapraun (1987) *Enteromorpha*, in anatomically-simple species, and Cheney (1989), with anatomically more complex species (*Gracilaria tikvahiae* McLachlan ×

Table 1. Seaweeds from which protoplasts have been isolated, types of isolation (Iso; M = mechanical, F = enzymes obtained from phycophages, C = commercial enzymes, B = enzymes obtained from bacteria, A = enzymes obtained from amoeba) and types of further development (Dev; R = cell wall regeneration, R = without cell wall regeneration, R + = plant regeneration, Ca = callus formation, Ca + = callus and plants).

	Iso.	Dev.	
Chlorophyta			
Bryopsis plumosa	M	R +	Tatewaki & Nagata 1970
Enteromorphalinza	C	R +	Fujita & Migita 1985
Enteromorphalinza	C	R	Saga 1984
E. intestinalis	C	R +	Millner *et al.* 1979
E. intestinalis	C	R +	Saga *et al.* 1986
Monostroma angicava	C + F	R +	Zhang 1982
Monostroma angicava	C	R	Saga & Kudo 1989
Monostroma nitidum	C	R +	Fujita & Migita 1985
Monostroma zoostericola	C	R +, Ca	Saga *et al.* 1986
Ulva conglobuta	C + F	R +, Ca	Reddy *et al.* 1989
Ulva fasciata	C + F	R +	Reddy *et al.* 1989
Ulva linza	C + F	R +	Zhang 1982
Ulva pertusa	C	R +	Fugita & Migita 1985
Ulva pertusa	C	R +	Saga 1984
Ulva pertusa	C	R +, Ca	Fujimura & Kajiwara 1989
Ulva pertusa	C + F	R +	Reddy *et al.* 1989
Ulvaria oxysperma	C	R +	Kapraun & Sherman 1989
Uronema gigas	F		Gabriel 1970
Phaeophyta			
Fucus distichus	C + F	R +	Kloareg *et al.* 1988
Laminaria	F		Saga & Sakai 1984
Laminaria digitata	F		Butler & Evans 1988
Laminaria digitata	C + F	R	Butler *et al.* 1989
Laminaria japonica	F	R −	Saga & Sakai 1984
Laminaria saccharina	F		Butler & Evans 1988
Laminaria saccharina	C + F	R	Butler *et al.* 1989
Macrocystis pyrifera	C + F		Saga *et al.* 1986
Macrocystis pyrifera	C + F	R, Ca	Kloareg *et al.* 1989
Macrocystis pyrifera	M + F	R −	Davison & Polne-Fuller 1990
Sargassum echinocarpum	C + F		Fisher & Gibor 1987
Sargassum muticum	F	R	Neushul 1984
Sargassum muticum	C + F		Saga *et al.* 1986
Sargassum muticum	C + F		Fisher & Gibor 1987
Sargassum muticum	A		Polne-Fuller & Gibor 1987c
Sargassum polyphyllum	C + F		Fisher & Gibor 1987
Sphacelaria	C + F	R +	Ducreux & Kloareg 1988
Undaria pinnatifida	B	R −	Fujita & Migita 1985
Undaria pinnatifida	F	R	Tokuda & Kawashima 1988

Table 1. (Continued)

	Iso.	Dev.	
Rhodophyta			
Chondrus crispus	B	R −	LeGall *et al.* 1989
Chondrus crispus	B		Smith & Bidwell 1989
Gracilaria lemaneiformis	C	Ca	Cheney *et al.* 1986
Gracilaria lemaneiformis	C		Björk *et al.* 1990
Gracilaria secundata	C		Björk *et al.* 1990
Gracilaria tenuistipitata	C		Björk *et al.* 1990
Gracilaria tikvahiae	C	Ca	Cheney *et al.* 1986
Gracilaria verrucosa	C		Björk *et al.* 1990
Palmaria palmata	C	R	Liu 1989
Porphyra	F	R +	Liu *et al.* 1984
Porphyra	C + B		Fujita & Saito 1989
Porphyra haitanensis	C + F		Wang *et al.* 1986
Porphyra leucosticta	C + F	Ca, R +	Chen 1987
Porphyra linearis	C + F	R +, Ca	Chen *et al.* 1988
Porphyra maculosa	C + F		Waaland & Dickson 1987
Porphyra nereocystis	C + F	Ca	Waaland & Dickson 1987
Porphyra perforata		R +	Saga *et al.* 1986
Porphyra perforata	C + F	R +, Ca +	Polne & Gibor 1984
Porphyra perforata	C + F		Waaland & Dickson 1987
Porphyra pseudolanceolata	C + F		Waaland & Dickson 1987
Porphyra suborbiculata	F	R	Tang 1982
Porphyra yezoensis	F	R +	Araki *et al.* 1987
Porphyra yezoensis	F	R +, Ca +	Polne *et al.* 1984
Porphyra yezoensis	F	R +, Ca +	Polne & Gibor 1984
Porphyra yezoensis	B	R −	Saga & Sakai 1984
Porphyra yezoensis	C + F	R +	Fujita & Migita 1985
Porphyra yezoensis	F	R +	Saga *et al.* 1986
Porphyra yezoensis		R +	Dai 1987

G. chilensis Bird, McLachlan & Oliveira), have described the recovery of somatic hybrids. These results suggest that the production of 'agarophytic corn' (*Gelidium* × *Zea mays*), of 'agarageenan' (*Gelidium* × *Eucheuma*) or other imaginative hybrids, can be realized.

There are a few somewhat speculative and distantly related reports on genetic engineering in seaweeds. Thes include *Agrobacterium tumefaciens*, used genetically to engineer algae, at least in *Chlamydomonas* (Ausich, 1983); the possible existence of *Agrobacterium*-like bacteria that promote the induction and growth of seaweed tumours, galls and calli (Cantacuzene, 1930; Apt, 1988; Tsekos, 1982; Garcia-Reina *et al.*, 1988a); DNA-transferring parasitic seaweeds, possibly usable as vectors (Goff & Coleman, 1984); chromosomal location and molecular biological regulatory studies of alginate-related genes in *Pseudomonas*, with characteristics similar to those in *Fucus* alginates (Deretic *et al.*, 1987); development of cloning vectors in photosynthetic microorganisms (Chauvat *et al.*, 1988).

Germplasm storage

Tissues and callus cultures can be highly efficient techniques for germplasm storage in selection programs or 'seed' storage for propagation as they avoid expensive whole-plant management. The longest, 5-year-old culture of seaweed calli

(Fang, 1983; Polne-Fuller & Gibor, 1987b), and culture of somatic cells (Chen, 1989) parallels the immortality shown by higher plant-cell cultures (Gautheret, 1985). Cryopreservation will require even less handling, and van der Meer and Simpson (1984) have described techniques for *Gracilaria* tissue.

Production of biomass or metabolites

The cultivation of tissues, calli or cells in photobioreactors, bioreactors or immobilized bioreactors are alternative techniques for direct production of biomass or of economically-significant metabolites. The best example (Misawa, 1977) is described in Japanese patent No. 74-101561 obtained by Nakamura in 1984 (Kureha Chem. Co. Ltd). This patent protects the right to produce agar from calli of *Gelidium amansii* Lamouroux and *G. subcostatum* Okamura (among other agarophytes). Callus growth increased 11.3 times in 20 days and, from 100 g of dried callus, 75 g of agar was obtained.

In phycocolloid production in cell culture, Cheney *et al.* (1987) did not find a significant difference between iota-type carrageenan extracted from tissue culture of *Agardhiella subulata* (C. Ag.) Kraft & Wynne compared with extractive from field-collected material. Tait *et al.* (1990) obtained comparative spectra for polysaccharides produced by *Porphyra* cell-cultures and the native plants, but, as the authors concluded, 'it should be demonstrated whether specific growth conditions can affect the structure and properties of the polysaccharides produced by cell cultures'... or by disorganized cells.

Bryhni (1978) found highly-significant quantitative and structural differences between polysaccharides extracted from organized strains of *Ulva mutabilis* Føyn and those from a strain of disorganized aggegates of undifferentiated cells. Liu *et al.* (1989) obtained 50% agar yield from dried callus of *Pterocladia capillacea* (Gmel.) Born. & Thur., 25% less than Nakamura's patent, but the agarose molecule contained less sulfate and fewer methyl groups. The callus of *Laurencia* consists of small photoautotrophic, pseudomeristematic cells, filled with starch-like granules, and with cell walls at least twice the width of normal cells (Garcia-Reina *et al.*, unpub. data). These data indicate that phycocolloid production, with high quality and distinct characteristics, is possible through cell and callus culture.

In addition to phycocolloid production, Chen (1989) has suggested the possibility of producing *Porphyra* for human consumption through cell culture. Fujimura and Kajiwara (1989) produced a bioflavor (released into the culture media) from *Ulva pertusa* Kjellman cell-suspension cultures.

Some critical considerations on applicability

All the applications described above are possible. The question is whether application on a commercial scale will be practical. *Laminaria* selection by monoploid cell-culture has yielded industrial results, and propagation by somatic cell-inoculation of morphologically simple species could be achieved commercially in a few years. There remain, however, many unresolved basic questions, limiting types of applications and species to which these processes can be applied.

Axenic cultures

Owing to seasonal variation of contaminants, differential interspecific sensitivities to biocides and the widespread phenomenon of endophytism in seaweeds and other algae, few generalizations can be made on obtaining axenic cultures. Evaluation of axenic cultures do not include tests for viruses, mycoplasma, rickettsia and so forth. Even if judged axenic, contaminants can appear in the cultures after months or years (Fries, 1963; Lee, 1986; Cheney, 1986; Tait *et al.*, 1990), and even with morphologically simple species, there are contradictory results of the efficacy of sterilization treatments (Bonneau, 1976).

Callus, callus control and PGR's

Gall, tumour, cancer, tumour-like, callus and callus-like are all terms used to describe proliferative, disorganized growth in seaweeds (Apt, 1988). The term 'callus-like' has been widely-used to qualify filamentous, crustose, hyperplasic and other abnormal outgrowth of the explant. If 'callus-like' is not true callus, as some authors claim, the literature on seaweed callus culture can be dramatically reduced. A consensus of what a callus in seaweed is has not been achieved.

Callus control implies callus induction, growth (without the explant) and regeneration. Although auxins and cytokinins exist in seaweeds, their effects when applied *in vitro* are not clear. Based on the literature of cell and callus culture of agarophytes (*Gelidium* and *Pterocladia*), three types of responses have been described for the effects of these hormones: 'success', although not compared with hormone-free media (Nakamura, 1974; Gusev *et al.*, 1987); 'no effect' (Polne-Fuller & Gibor, 1987a; Garcia-Reina *et al.*, 1988a); 'not clear' (Liu & Gordon, 1987).

Ranking of calligenic potential (Table 2) can be done (Gusev *et al.*, 1987; Garcia-Reina *et al.*, 1988a; Polne Fuller & Gibor, 1987a), indicating genotypic differences in disorganization (Fang *et al.*, 1979). 'Wounding-response' seems to be the key factor in initiating callus growth in seaweeds.

True callus culture (excised from the explant) has been described for a few species (*Gelidium* and *Gracilaria*: Nakamura, 1974; *Laurencia*: Garcia-Reina *et al.*, 1988b; *Grateloupia*: Robaina *et al.*, 1990b; *Phyllophora*: Gusev *et al.*, 1987; *Sargassum*, *Cystoseira*, *Macrocystis*, *Ulva*, *Enteromorpha*, *Eucheuma* and *Porphyra*: Polne-Fuller & Gibor, 1987a, 1987b; *Ecklonia*: Lawlor *et al.*, 1989; *Pterocladia*: Liu & Gordon, 1987). Only the Nakamura (1974) patent describes (true) callus culture of *Gelidium amansii* and *G. subcostatum*. Calli from *Gelidium vagum* Okamura (Gusev *et al.*, 1987), *G. nudifrons*, *G. robustum* (Polne-Fuller & Gibor, 1987a) and *G. versicolor* (Garcia-Reina *et al.*, 1988a) have not been described, either excised from the explant or with organogenetic potential.

Table 2. Ranking of 'calligenic potential' expressed as percent of tissue explants which developed callus (data from Gusev *et al.* 1987; Kawashima & Tokuda, 1989; Garcia-Reina, unpubl.; Polne-Fuller & Gibor, 1987a).

Chlorophyta	
88.0%	*Enteromorpha intestinalis*
86.0%	*Ulva augusta*
Phaeophyta	
70.0%	*Ecklonia cava*
29.0%	*Macrocystis pyrifera (gametophyte)*
20.0%	*Laminaria sinclairii (gametophyte)*
17.0%	*Sargassum muticum*
17.0%	*Pelvetia fastigiata*
10.0%	*Sargassum hystrix*
10.0%	*Cystoseira osmundacea*
9.2%	*Laminaria sinclairii (sporophyte)*
9.0%	*Sargassum fluitans*
7.8%	*Macrocystis pyrifera (sporophyte)*
Rodophyta	
87.0%	*Porphyra lanceolata*
84.0%	*Porphyra perforata*
81.0%	*Porphyra nereocystis*
75.0%	*Smithora naiadum*
33.0%	*Laurencia sp*
18.0%	*Phyllophora nervosa*
16.0%	*Furcellaria fastigiata*
15.0%	*Gelidium vagum*
10.0%	*Gracilaria ferox*
7.0%	*Eucheuma alvarezii*
4.0%	*Gracilaria verrucosa*
2.0%	*Ceramium kondoi*
1.0%	*Gigartina exasperata*
0.9%	*Eucheuma uncinatum*
0.6%	*Gelidium robustum*
0.5%	*Gracilaria papenfusii*
0.3%	*Gelidium versicolor*

Neither the physiological status of the explant (differential nutritional levels yielding differences in calligenic potential (Fries, 1980) seasonal differences in endogenous hormones (Mooney & van Staden, 1984; Featonby-Smith & van Staden, 1984) nor seasonally-different calligenic potentials, (Polne-Fuller & Gibor, 1987b) could be key factors for success. With so many unknown variables, it is difficult to project a productive future in the short term for the application of callus culture to seaweeds.

Culture media

As organic requirements for axenic or aseptic seaweed cultures are largely unknown, the more common culture media are undefined (enriched seawater). The addition of organic complexes (coconut milk, algal and yeast extracts, *etc.*) to increase low growth rates (indicating suboptimal conditions) of seaweed tissue (Fries, 1973; 1984) and callus cultures (Nakamura, 1974; Saga & Sakai, 1983) has resulted in both stimulatory effects and no effects (Robaina, 1988; Lawlor *et al.*, 1988). Nutrient depletion could be the reason for callus and 'callus-like' inductions or reversions (Pedersén, 1968; Fries, 1980; Bradley & Cheney, 1986; Polne-Fuller & Gibor, 1986; Lawlor *et al.*, 1989). Sugars have been reported to be unnecessary or even inhibitory to tissue (*Fucus spiralis*: Fries, 1984), callus (*Ecklonia radiata*: Lawlor *et al.*, 1989) and cell culture (*Porphyra umbilicalis*: Tait *et al.*, 1990) growth or organogenesis. Our studies on the interrelationship between the osmolality of the culture medium and the effect of osmotically active sugar supplementation has shown that, as in higher plants (van Rensburg & Vcelar, 1989), the effects of carbohydrates in seaweed tissue and callus culture seem osmotic or metabolic or both (Robaina *et al.*, 1990a; 1990b). Solidity of culture media promotes disorganization (Robaina *et al.*, 1990a; 1990b).

Heterotrophic growth is required for biomass production in bioreactors, but heterotrophic frowth of seaweed tissue remains to be demonstrated (Fries, 1973; Robaina, 1988; Lawlor *et al.*, 1989; Robaina *et al.*, 1990b). Although a callus of *Gelidium* has been obtained with the addition of sucrose (Nakamura, 1974), 20 g l^{-1} mannitol (Gusev *et al.*, 1987), or in the absence of sugars in the culture media (Polne-Fuller & Gibor, 1987a; Garcia-Reina *et al.*, 1988a), callus production has not occurred in darkness. However, as seaweed tissues, (Fries, 1977; 1980), cell (Saga *et al.*, 1978; Polne-Fuller *et al.*, 1987; Tait *et al.*, 1990) and callus cultures (Polne-Fuller *et al.*, 1986; Polne-Fuller & Gibor, 1987b; Garcia-Reina *et al.*, 1988a; Lawlor *et al.*, 1989) are phototrophic, including *Gelidium* species, photobioreactors remain a possibility for biomass production.

True cell culture

Porphyra is the only genus for which continuous cell cultures have been established (Chen, 1989; Tait *et al.*, 1990). Other reports on 'cell culture' refer to dynamic steps between protoplasts, pseudoprotoplasts or enzymatically/mechanically-isolated cells and their development to callus or to plants. The application of cell culture of other species for selection and for the production of biomass or metabolites requires confirmation of true cell culture.

Viable protoplasts

Protoplast isolation is not a problem in some seaweeds (Table 1), whereas the isolation of *large* numbers of *highly-viable* protoplasts is. Trial and error assays with cocktails of commercial (Table 3), phycophage-extracted (Table 4) or microorganism-extracted (Table 5) enzymes are common approaches to obtain protoplasts. Basic knowledge of composition and structure of cell walls of seaweeds and enzymatic activity of cell-wall digesting enzymes are scarce. Until more information becomes available, possible benefits from protoplast culture as applied to *Gelidium*, for example, are likely limited.

Somatic incompatibility

Somatic incompatibility needs to be overcome to realize the potential for somatic hybridization. In somatic incompatibility chromosomes, chloroplasts and mitochondria from one of the fused protoplasts degenerate, as noted in heterokaryons of *Zygnema* × *Porphyridium*, *Zygnema* × *Mougeotia* (Ohiwa, 1978; 1980; 1981) and *Daucus* × *Chlamydomonas* (Fowke *et al.*, 1979). Other undesirable processes could result from the

Table 3. Commercial enzymes commonly used for the digestion of seaweed cell walls.

Abalone acetone powder	Sigma A7514
Agarase	Sigma A6162
Cellulase	Sigma C2415
Cellulisin	Sigma C2274
Laminarinase	Sigma L7758
Limpet I	Sigma L1251
Limpet II	Sigma L0630
Limpet III	Sigma L8755
Hemycelulase	Sigma H2125
Papain	Sigma P3125
Pectinase	Sigma P2401
Pectolyase	Sigma P3026
Protease XXIV	Sigma P8163
Lysozyme	Sigma L6876
Cellulase onozuka RS	Yakult
Cellulase onozuka R-10	Yakult
Macerozyme R-10	Yakult
Celluiase onozuka R-10	Serva
Maceroyme R-200	Serva
Cellulysin	Calbiochem 219466
Macerase	Calbiochem 441201
Laminarinase	BDH 39120 2G
Driselase	Kyowa hakko
Pronase E	Merck 7433

formation of different types of chimaera in plants regenerated from the heterokaryon. This occurred in the first seaweed somatic hybrid; irregularly-variegated chimeral thalli from the fusion of wild (red) and mutant (green) *Porphyra* protoplasts occurred (Fujita & Migita, 1987). Cheney (1989) claimed to verify true hybridization in green and red plants regenerated from the fused green mutant (*Gracilaria tikvahiae*) and red (*G. chilensis*) protoplasts by isoenzyme analysis. Although suggestive of true hybridization, this technique cannot distinguish chimaeric plants from true somatic-hybrid plants.

As with somatic hybridization in microalgae (Matagne *et al.*, 1979), the first recovery of a stable seaweed heterokaryon (Kapraun, 1987) has been achieved using haploid cells, although these were zoospores, not somatic protoplasts. If the reduction of somatic incompatibility from haploid state of the fusants can be confirmed, potential applications of seaweed somatic hybridization can be reexamined with some likelihood of success.

Table 4. Phycophages from which have been isolated seaweed cell wall digestive enzymes.

Aplysia dactylomela	Gómez-Pinchetti *et al.* 1989
Aplysia depilans	Boyen *et al.* 1990
Aplysia punctata	Kloareg & Quatrano 1987a, 1987b
Aplysia vaccaria	Kloareg *et al.* 1989
Aplysia kurodai	Tokuda & Kawashima 1988
Crassostrea gigas	Onishi *et al.* 1985
Diadema antillarum	Gómez-Pinchetti *et al.* 1989, Lewis 1964
Diadema setosum	Benitez & Macaranas 1979
Dolabella auricula	Nisizawa *et al.* 1968
Haliotis cracherodii	Dai 1987
Haliotis rufescens	Dai 1987
Haliotis tuberculata	Ducreux & Kloareg 1988
Haliotis corrugata	Nakada & Sweeny 1967
Katherina tunicata	Kloareg & Quatrano 1987a
Littorina sp	Elyakova & Favorov 1974
Littorina striata	Gomez-Pinchetti *et al.* 1989
Littorina littorea	Chen 1986
Littorina brevicula	Onishi *et al.* 1985
Lunella cornata	Zhu 1982
Melagraphia aethiops	Liu & Gordon 1987
Monodonta labio	Zhu 1982
Mytilus edulis	Onishi *et al.* 191985
Nordotis discus	Onishi *et al.* 1985
Patella vulgata	Ducreux *et al.* 1988
Purpura clavigera	Zhu 1982
Strongylocentrotus intermedius	Saga & Sakai 1984
Strongylocentrotus purpuratus	Neushul 1984
Tegula funebralis	Galli & Giese 1959
Turbo coronatus	Tang 1982
Turbo sp	Liu *et al.* 1984
Turbo cornutus	Muramatsu *et al.* 1977

Genetic engineering

Few data are available on seaweed gene-identification, location and cloning, vectors, effectiveness of DNA injection techniques or possible expression of foreign genes. The genetic engineering of seaweeds is thus largely speculative at this time. When, for instance, the genes codifying agar synthesis are transferred, the beginning of phycocolloid-producing bacterial biotechnology will be a real possibility.

Table 5. Microorganisms from which has been isolated seaweed cell wall digestive enzymes.

Achromobacter	Quatrano & Caldwell 1978
Aeromonas sp. F-25	Araki *et al.* 1987
Alcaligenes	Quatrano & Caldwell 1978
Alteromonas	Quatrano & Caldwell 1978
Amoeba (Am I-7)	Polne-Fuller & Gibor 1987c
Arthrobacter	Quatrano & Caldwell 1978
Cytophaga flevensis	van der Meulen 1975
Flavobacterium	Quatrano & Caldwell 1978
Pseudomonas atlantica	Yaphe 1957, Morrice *et al.* 1983
Pseudomonas sp. strain P-1	Fujita & Migita 1987
Pseudomonas carrageenovora	Smith & Bidwell 1989
Vibrio sp. AP-2	Araki *et al.* 1987
Vibrio sp. AX-4	Araki *et al.* 1987

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Effect of inhibitors of carbonic anhydrase activity on photosynthesis in the red alga *Soliera filiformis* (Gigartinales: Rhodophyta)

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Abstract. The effect of light intensity, pH and carbonic anhydrase (CA) inhibitors on photosynthesis of the red marine macroalgae *Solieria filiformis* (Kützing) Gabrielson, collected from Taliarte (Gran Canaria, Canary Islands) in 1991, has been investigated. Plants taken from the sea ("wild phenotype") developed spherical morphology ("ball phenotype") after 2 mo culture in aerated tanks. The photosynthetic oxygen evolution in the wild phenotype was saturated at 100 µmol photons $m^{-2}s^{-1}$, while the "ball" phenotype displayed saturation at 200 µmol photons $m^{-2}s^{-1}$. The inhibitors of total CA activity (6-ethoxizolamide) and extracellular CA activity (dextran-bound sulfonamide) inhibited photosynthesis at pH 8.2, to 90 and 50% respectively, in both phenotypes. No inhibition of the photosynthetic oxygen evolution was detected at pH 6.5. CA activity was associated with both supernatant and pellet fractions of crude extracts of *S. filiformis*. The rate of alkalization of the medium by the algae was dependent on light intensity. We suggest that carbon dioxide is the general form of inorganic carbon transported across the plasmamembrane in *S. filiformis*. HCO_3^- transport into the cell takes place simultaneously by an "indirect" mechanism (dehydration to CO_2 catalyzed by CAext) and by direct uptake. Extracellular (CAext) and intracellular (CAint) CAs are involved in the mechanisms of inorganic carbon assimilation by *S. filiformis*.

Introduction

The concentration of HCO_3^- in natural seawater at pH 8.2 is ~2.2 m*M*, while dissolved CO_2 represents <12 µ*M*. Aquatic plants can use both CO_2 and HCO_3^- as an inorganic carbon (C_i) source for photosynthesis (Sand-Jensen and Gordon 1984, Bidwell and McLachlan 1985, Smith and Bidwell 1987, 1989). The mechanism of CO_2 and HCO_3^- uptake from the medium into the cell and its transport over the chloroplast envelope are not fully understood, and several transport mechanisms have been suggested. CO_2 can be transported from the medium into the cell and through the cell compartments by diffusion. In the unicellular green alga *Chlamydomonas reinhardtii*, an "active CO_2-transporting system" (after adaptation to low CO_2 conditions) is considered an important component of the total C_i-concentrating mechanism (Sültemeyer et al. 1989). However, a mechanism of "active" CO_2 transport in seaweeds has not yet been demonstrated.

In aquatic plants, HCO_3^- can enter the cells by a specific plasmamembrane ATPase-dependent HCO_3^--transporter, by a H^+/HCO_3^- symport, or by an OH^-/HCO_3^- antiport mechanism (Lucas 1983, Raven and Lucas 1985). However, a specific protein for such transport of HCO_3^- has not been identified (Marcus et al. 1984, Goyal and Tolbert 1989).

Recently, Smith and Bidwell (1987, 1989) described, in the red seaweed *Chondrus crispus*, an "indirect" mechanism of HCO_3^- assimilation; i.e., after dehydration of HCO_3^- to CO_2, catalyzed by an extracelullar carbonic anhydrase CAext). However, Cook et al. (1986, 1988) did not find CAext activity in several species of red seaweeds, and proposed an active mechanism for the direct transport of HCO_3^- in marine plants. These contradictory results may reflect species-specific capabilities related to growth conditions.

The present study presents evidence of the involvement of CAext and CAint (intracellular CA) in a dual mechanism of HCO_3^- uptake in *Solieria filiformis* by (a) an "indirect" mechanism after dehydration to CO_2 (catalyzed by CAext) and (b) by direct HCO_3^- uptake from seawater.

Materials and methods

Plant material

Solieria filiformis (Kützing) Gabrielson [formerly *Agardhiella tenera* (J. Agardh) Schmitz or *S. tenera* (J. Agardh)-Wynne et Taylor

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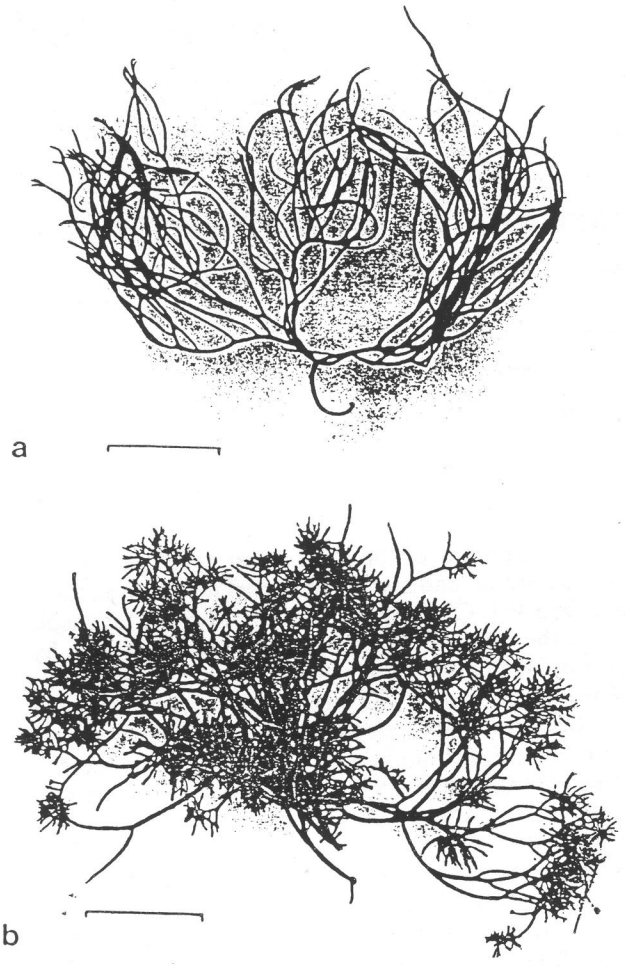

Fig. 1. *Solieria filiformis*. Morphology of plants freshly collected from in situ conditions ("wild" phenotype) (a) and after 2 mo in tank culture ("ball" phenotype) (b). Scale bars = 2 cm

(Gigartinales: Rhodophyta)] was collected from Taliarte, on the east coast of Gran Canaria, Canary Islands, Spain, in 1991. In nature, *S. filiformis* grows in a free-floating population at 3 to 4 m depth where maximum irradiance is < 100 µmol photons m^{-2} s^{-1}. Plants grow erect from a fibrous basal system and may attain a height of 10 to 15 cm ("wild phenotype"; Fig. 1a).

Healthy material was cleaned thoroughly with UV-filtered seawater and cultivated for 2 mo in 300-litre tanks, under greenhouse conditions, with continuous aeration. The seawater was enriched with minerals according to Provasoli (1968), but no vitamins, and changed once a week. Maximum irradiance ranged between 1100 and 1300 µmol photons m^{-2} s^{-1}. After the 2 mo culture period, plants exhibited spherical morphology ("ball phenotype"; Fig. 1b).

Fresh healthy material was collected from the sea every day during the experimental period. Both, wild and ball-type phenotypes were used in all experiments.

Irradiance

Photosynthesis and rate of alkalization were measured by white light from a slide projector (Reflecta, Germany). Light was measured with a radiometer LI-1000 Data Logger using a spherical quantum sensor, LI-193SA (LI-COR, Nebraska, USA).

Photosynthetic oxygen evolution in plant fragments

Photosynthetic oxygen evolution was measured by a Clark-type electrode fitted with a measuring chamber (Hansatech Instruments Ltd., Norfolk, UK) thermostated to 25 °C. The chamber was filled with buffered seawater at pH 8.2 [50 m*M* Tris (Tris[hydroxymethyl]-aminomethane)] or at pH 6.5 [50 m*M* Mops (3-[*N*-morpholino]-propanesulphonic acid)] and plant fragments (25 mg fresh wt) were added. A flatbed recorder was used to follow oxygen evolution. Sequential experiments were performed with the same thallus fragments after changing the incubation buffer.

Alkalization measurements

Alkalization rates were measured on thallus pieces of 2.0 cm in length in fresh natural seawater at different light irradiances. The change in the pH of the medium was measured with a pH-meter (Orion 701, USA). The electrode tip was immersed in a sealed 50 ml flask containing medium and algae at a density of up 1.0 g fresh wt/l. The spectral composition of the light was varied with blue (500 to 700 nm with peak at 640 nm) and red (400 to 600 nm with peak at 540 nm) Plexiglas filters.

Carbonic anhydrase measurements

Carbonic anhydrase (EC 4.2.1.1.) activity was measured according to Ramazanov and Semenenko (1988). Algal thalli were ground in liquid nitrogen, extracted in a buffer containing 50 µ*M* Tris (pH 8.5), 5 µ*M* dithiothreitol (DTT), 25 m*M* isoascorbic acid and 5 m*M* EDTA (CA buffer), and centrifuged at $13\,000 \times g$ for 60 min. CA activities of the supernatant and pellet were measured potentiometrically by determining the time required for the pH of the enzyme solution to change from 8.5 to 7.4 at 2 °C, in a 2 ml sample. The reaction was started by rapidly introducing 2 ml of ice-cold CO_2-saturated distilled water into the enzyme solution. The decrease in pH was measured with a digital pH-meter (Orion 701, USA), connected to a flatbed recorder.

One unit of activity (Wilbur Andersen, WA unit) was defined as $10\,(T_0/T) - 1$, where T_0 and T = the times for changes in the pH of the nonenzymatic and the enzymatic reactions, respectively. Nonenzymatic reaction was established by the same procedure in the absence of algal material.

Inhibitors

6-ethoxizolamide (EZ; Sigma, St. Louis, USA) and dextran-bound sulfonamide (DBS; kindly supplied by Professor G. Samuelsson, Department of Plant Physiology, University of Umeå, Sweden) dissolved in 50 m*M* NaOH were used as total and extracellular inhibitors of CA activity, respectively. 100 µ*M* DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea; Sigma, St Louis, USA] were used to inhibit photosynthesis.

Pigments and protein analysis

Chlorophyll was measured according to Wintermans and De Mots (1965), and phycoerythrin according to Beer and Eshel (1985). Protein content was determined using a modification of the Bradford method as described by Peterson (1983). Results are the means of 4 to 5 independent experiments in each case.

Results

Effect of light intensity and pH on photosynthesis

The rate of photosynthetic oxygen evolution in both phenotypes of *Solieria filiformis* was dependent on light in-

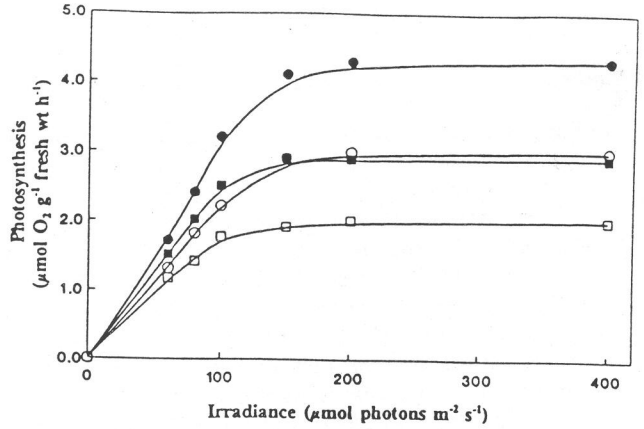

Fig. 2. *Solieria filiformis*. Photosynthetic oxygen evolution by wild (■, □) and ball (●, ○) phenotypes (25 mg fresh wt/ml) in buffered seawater at pH 8.2 (50 m*M* Tris; □, ○) and 6.5 (50 m*M* Mops; ■, ●)

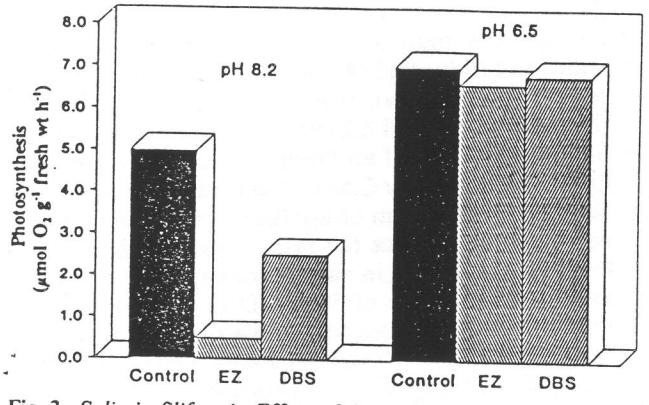

Fig. 3. *Solieria filiformis*. Effect of 6-ethoxizolamide (EZ; 50 μ*M*) and dextran-bound sulfonamide (DBS; 40 μ*M*) on photosynthetic oxygen evolution by wild phenotype (25 mg fresh wt/ml) in buffered seawater at pH 8.2 (50 m*M* Tris) and 6.5 (50 m*M* Mops). Data are maximum rates of linear portions of the O_2-evolution kinetics; means of 5 measurements. Light intensity = 100 μmol photons $m^{-2} s^{-1}$

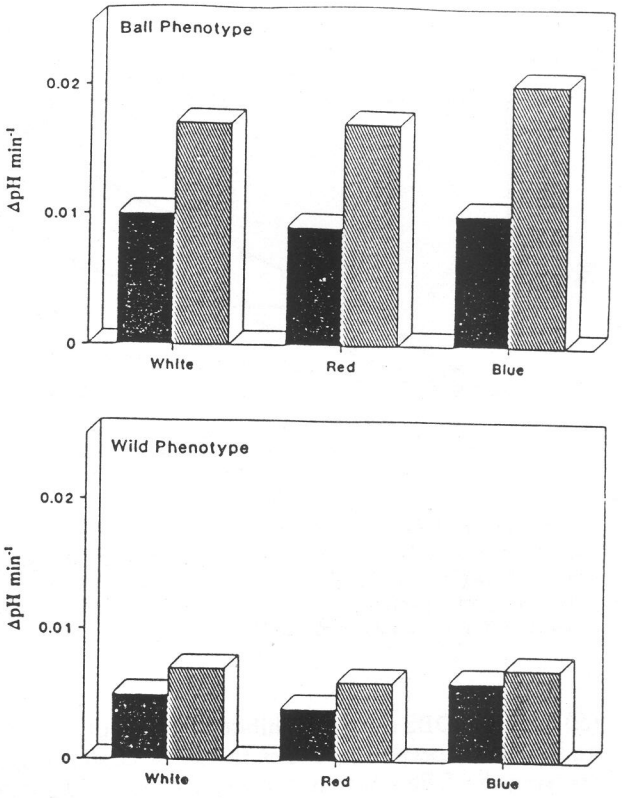

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Table 1. *Solieria filiformis*. Chlorophyll (chl) and phycoerythrin (phy) concentrations and ratio of wild and ball phenotypes

Phenotype	Chlorophyll (μg/ml)	Phycoerythrin (μg/ml)	chl:phy
Wild	24.23 ± 0.13	14.96 ± 0.65	1.62
Ball	15.26 ± 0.66	16.84 ± 0.39	0.91

tensity and the pH of the medium (Fig. 2). In the wild phenotype, photosynthetic saturation was observed at 100 and 200 μmol photons $m^{-2} s^{-1}$ at pH 8.2 and 6.5, respectively. In the ball phenotype, photosynthetic saturation was observed at 150 and 200 μmol photons $m^{-2} s^{-1}$ at pH 8.2 and 6.5, respectively. However, the rate of oxygen evolution was 25% higher than in the wild phenotype at both pHs. The difference in oxygen evolution was more significant at higher light intensities (400 μmol photons $m^{-2} s^{-1}$; Fig. 2).

Table 1 shows chlorophyll (chl) and phycoerythrin (phy) concentrations and their ratios for wild and ball phenotypes. Chlorophyll concentration and the chl:phy ratio were higher in the wild than in the ball phenotype.

Carbonic anhydrase activity

CA activity in *Solieria filiformis* plants was associated with both the supernatant and the pellet of the crude extracts. CA activity in the supernatant was 7 U/mg protein and in the pellet 117 U/mg protein. Low CA activity in the supernatant could have been caused by the relatively high protein concentration of this fraction (135 μg ml^{-1}) compared with that of the pellet (34 μg ml^{-1}).

Effect of inhibitors of carbonic anhydrase activity on photosynthesis

The wild phenotype of *Solieria filiformis* exhibited high photosynthetic rates in buffered seawater at pH 8.2 and 6.5 (Fig. 3). However, at pH 6.5 the rate of oxygen evolution was ~25% higher than at pH 8.2. At pH 8.2, EZ

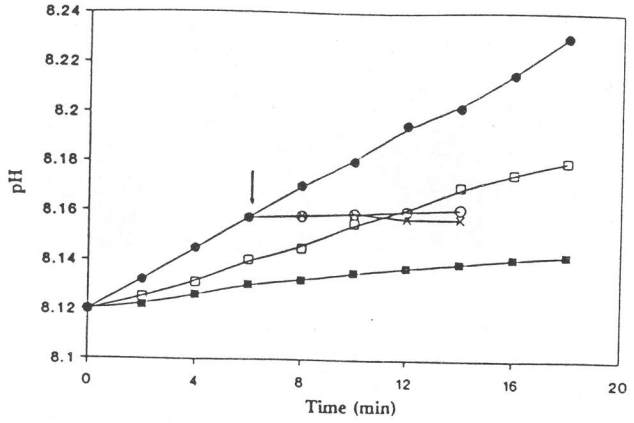


Fig. 5. *Solieria filiformis*. Effects of 100 μ*M* of 6-ethoxizolamide (■), dextran-bound sulfonamide (□), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (○) and darkness (×) on alkalization rate of seawater by wild phenotype (50 mg fresh wt/ml); ●: control. Light intensity 400 μmol photons m^{-2} s^{-1}

(50 μ*M*) and DBS (50 μ*M*) inhibited photosynthesis to 90 and 50%, respectively. Under the same conditions, increasing the DBS concentration to 100 and 200 μ*M* did not change the percentage of inhibition. At pH 6.5, inhibition was not observed. The same results were exhibited by the ball phenotype (data not shown).

Effect of light intensity on alkalization rate of medium

Fig. 4 shows the rate of alkalization of the medium by wild and ball phenotypes. Alkalization rates were dependent on light intensity and spectral composition. The ball phenotype displayed higher alkalization rates than the wild phenotype. When light intensity was increased from 100 to 230 μmol photons m^{-2} s^{-1}, the rate of alkalization by the ball phenotype increased by almost twice. In the wild phenotype, alkalization was saturated at low light intensity (100 μmol photons m^{-2} s^{-1}) regardless of spectral composition. Although there were no evident differences in alkalization rates as a function of spectral composition, there was a slight increase in all measurements when blue light was used.

Effect of inhibitors of carbonic anhydrase activity on alkalization rate of medium

Fig. 5 shows the rate of alkalization of the medium by the wild phenotype of *Solieria filiformis*. Alkalization took place only in the light and was inhibited by DCMU (100 μ*M*) and also by EZ and DBS. However, as in the case of photosynthesis, EZ inhibited the alkalization rate to a stronger degree. The same results were obtained with the ball phenotype (data not shown).

Discussion

The results reveal important changes in the morphological and photosynthetic characteristics of *Solieria filiformis* cultivated in aerated tanks. The morphological changes (ball phenotype) would seem to be related to high water turbulence. The lower light-saturation point of photosynthetic oxygen evolution and the lower alkalization rate of the wild phenotype are probably due to adaptation of the light-harvesting system to low light intensities characteristic of "shade-growing" plants (Falkowski and LaRoche 1991). The ball phenotype is protected from high light intensities by its morphology (self-shading) and the resulting decrease in chlorophyll concentration; this explains the absence of photoinhibition at low light intensities in this phenotype (Fig. 2).

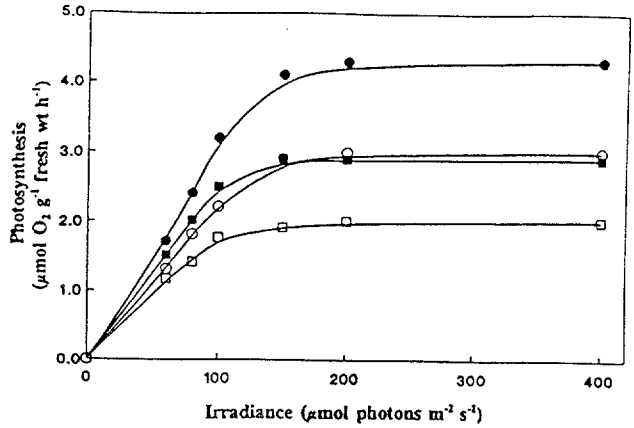
When bound to dextran, sulfonamide cannot penetrate the plasma membrane, and selectively inhibits CAext (Moroney et al. 1985, Palmqvist et al. 1990). It has been suggested that the role of CAext is to speed up the conversion of HCO_3^- to CO_2 in the extracellular space (Smith and Bidwell 1989). CO_2 can then either diffuse through the plasma membrane (Simpson et al. 1978, Smith and Bidwell 1989), or be actively transported to the cell (Sültemeyer et al. 1989). The inhibition of photosynthesis by DBS at pH 8.2 (Fig. 3) indicates the existence in *Solieria filiformis* of an "indirect" mechanism of HCO_3^- uptake catalyzed by CAext. However, the importance of the direct mechanism of uptake of inorganic carbon cannot be ignored, since the rate of photosynthetic oxygen evolution at pH 8.2, in natural seawater (2.2 m*M* HCO_3^-) and in the presence of 50 μ*M* DBS (Fig. 3), was higher than theoretical values based on a spontaneous supply of CO_2 from HCO_3^- (Axelsson 1988). Thus, active HCO_3^- transport in *S. filiformis* may constitute a significant contribution to the total uptake of inorganic carbon.

The stronger inhibition of photosynthetic oxygen evolution by EZ (Fig. 3) indicates the existence of intracellular CA in *Solieria filiformis*.

The non-inhibitory effect of EZ and DBS at pH 6.5 (Fig. 3) evidences a high CO_2-assimilation efficiency by *Solieria filiformis*, CO_2 being the main form of inorganic carbon entering through the cell wall and plasmalemma.

Alkalization of the medium by algae has been taken as evidence of the presence of active HCO_3^- transport (Cook et al. 1988). Recently, it has been shown that alkalization by the green and red seaweeds *Ulva rigida* and *Gracilaria tenuistipitata* is performed by an extracellular mechanism requiring CAext (Björk et al. 1992, Haglund et al. 1992). The efficiency of this mechanism is dependent on the concentration of inorganic carbon in the medium (Beer and Israel 1990, Björk et al. 1992, Haglund et al. 1992). Several authors have postulated that the process of alkalization of the growth medium by aquatic plants is effected by the release of 1 mol OH^- for each molecule of CO_2 transported (Simpson et al. 1978, Morgan et al. 1980, Lucas 1983). If OH^- production and efflux to the medium is an exclusively intracellular mechanism, as maintained by Lucas, then DBS would not affect the rate of alkalization by *Solieria filiformis*, as the present results show that it does (Fig. 5).

In conclusion, the different effects of EZ and DBS on the alkalization rate (and photosynthetic oxygen evolution) of *Solieria filiformis* indicate the involvement of external carbonic anhydrase in the uptake of inorganic carbon.



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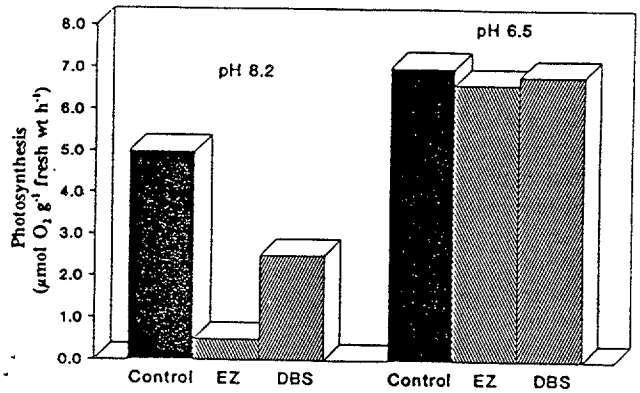

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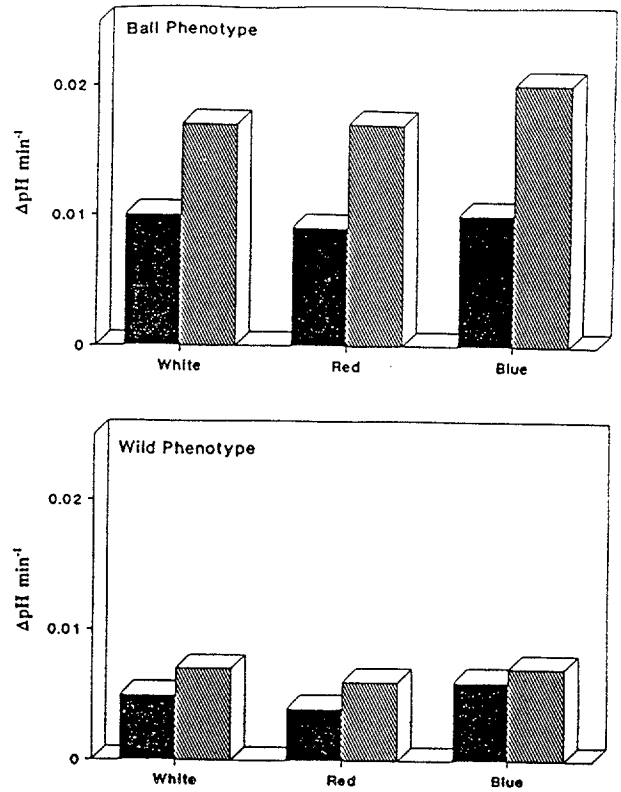

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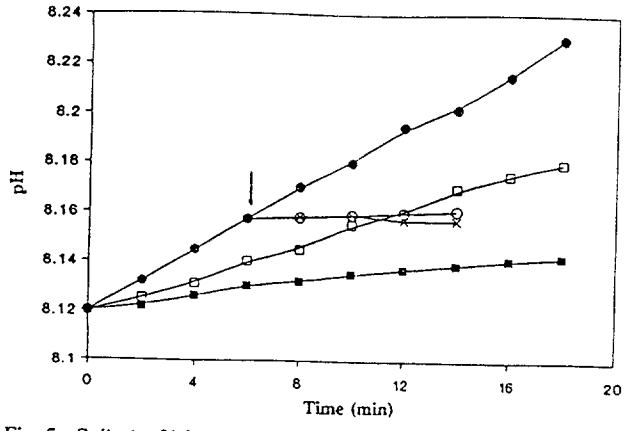

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Acknowledgements. The authors wish to thank to D. Robledo Ramírez and M. Jiménez del Rio for providing the cultured material. This work was supported by a scholarship from the University of Las Palmas of Gran Canaria to J. L. Gómez-Pinchetti and CICYT (project MAR-1237/91 and a sabbatical to Z. Ramazanov, invited Professor of Ministerior de Educación y Ciencia, Spain). Support by Fundación Universitaria de Las Palmas and by Air Europa is gratefully acknowledged.

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Protoplast Isolation from *Ulva rigida* (Chlorophyta)

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High numbers of protoplasts were isolated from wild and cultivated thalli of *Ulva rigida*. Optimal conditions for protoplast release were obtained with 1·5% Abalone Acetone Powder and 1·5% Cellulysin in 0·4 M mannitol. Treatment for 30 min with hypotonic (0·8 M) solution more than doubled the yield. Growth conditions prior to enzyme treatment also influenced the yield greatly. Protoplasts were photosynthetically active, and 70–90% of the protoplasts were estimated to be viable.

Protoplasts of higher plants are valuable for physiological, genetic, and biochemical studies (Eriksson, 1985). The photosynthesis of protoplasts has been studied for a number of species, like spinach (Nishimura & Akazawa, 1975) and wheat (Edwards *et al.*, 1978). The photosynthetic activity of protoplasts from the mesophyll compared to that of bundle sheath cells has been used to study the localization of photosynthetic enzymes in several C_4 plants (Kanai & Edwards, 1973; Gutierrez *et al.*, 1974). However, there are few reports of physiological studies using seaweed protoplasts. Protoplasts from the red macroalga *Chondrus crispus* have been used to study inorganic carbon uptake (Smith & Bidwell, 1989). It has also been suggested that protoplasts from *Macrocystis pyrifera* can be used as a model for physiological research (Davison & Polne-Fuller, 1990), and protoplasts from this species have recently been used for studies on bromoperoxidases (Butler *et al.*, 1990).

Ulva rigida is a common, widely distributed, green seaweed that has been used in many physiological studies (Zavodnik, 1987; MacFarlane & Smith, 1984; Fujita, Wheeler & Edwards, 1988), but to our knowledge no reports on protoplast isolation from this species have been published. However, protoplasts have been isolated from *Ulva linza* (Zhang, 1983), *Ulva pertusa* (Saga, 1984), and *Ulva lactuca* (Chou & Lu, 1989). Complete thalli have been obtained from protoplasts of *Ulva fasciata, U. conglobata* and *U. pertusa* (Reddy, Migita & Fujita, 1989; Fujimura *et al.*, 1989a). Experiments on immobilization (Fujimura *et al.*, 1989b), fusion (Reddy & Fujita, 1989) and production of bioflavour compounds (Fujimura & Kajiwara, 1990) of *Ulva* protoplasts have been reported recently. However, no physiological experiments have been reported for these protoplasts. In the present study we describe a method to isolate large amounts of photosynthetically active protoplasts from *Ulva rigida*. Factors such as standardized precultivation conditions, and preplasmolysis that influence the yield of protoplasts are considered.

MATERIALS AND METHODS

Ulva rigida C.Ag. was collected in May 1991 from the harbour of Taliarte, Gran Canaria, (Canary Islands, Spain). Plants were taken from populations growing on ropes near the surface under constant immersion. After collection, the plants were thoroughly cleaned in sterile sea-water and then maintained in 20 l tanks with running sea-water or cultured in sea-water in 4 l bottles with aeration. Fluorescent lamps (Thorn

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Polylux 4000) were used to illuminate the plants, which were cultured in a medium of filtered sea-water (0·2 μm pore size) enriched according to Provasoli (1968) with NO_3^- as nitrogen source. Nutrients were added after determining the fresh weight (fw) of the algae, the additions being adjusted to algal growth rate. Unless otherwise indicated, temperature were maintained at 25 ± 1°C, at a continuous photon flux density of 150 μmol m^{-2} s^{-1}, at a salinity of 38‰. The density of the cultures was maintained at 1–2 g fw l^{-1} medium. Algae were weighed each day after gentle centrifugation to remove excess water, and growth rate was calculated as percent increase in fresh biomass per day.

Cellulysin (Calbiochem AG, Lucerne, Switzerland) and abalone acetone powder (Sigma, St. Louis, MO, USA) were dissolved in sea-water at a salinity of 38‰ with addition of 0·4 M mannitol and 20 mM Bis-Tris. The enzyme solution was left on ice with stirring for 30 min, centrifuged at 1000 *g* for 10 min, the pH was adjusted to 6·0 and the solution filtered through 0·8 μm and 0·2 μM filter (Sartorius). Prior to the experiments the enzyme solution was stored frozen in 10 ml portions at −20°C.

The tissue was fragmented into millimeter pieces using a razor blade. The fragmented thallus was then rinsed five times in wash buffer (0·2 M mannitol and 20 mM Hepes in sea-water, pH 7). To test the effect of preplasmolysis on protoplast yield, samples of thallus were incubated for 30 min in sea-water with addition of 20 mM Hepes and 0·8 M mannitol, final pH 7·0, and compared with untreated material.

One gram fragmented thallus was immersed in 10 ml enzyme solution in a 90 mm diameter petri dish. Incubation in enzyme solution was at 20°C under constant shaking (60 rpm). After cell wall digestion, the protoplast suspension was filtered through 100 μm and 50 μm nylon meshes to remove cell wall debris and non-digested material, rinsed with wash buffer and centrifuged in a swing our rotor at 100 *g* for 5 min. The pellet was then resuspended in test buffer and re-centrifuged. The resulting protoplast pellet was resuspended in a buffer for photosynthesis tests or the culture medium of Provasoli enriched sea-water (Provasoli, 1968) supplied with 0·2 M mannitol.

After purification, protoplasts were allowed to settle on the bottom of a petri dish and counted directly with an inverted microscope. Newly isolated protoplasts were stained with 0·01% Calcofluor-White, and examined in UV-light for absence of cell wall by fluorescence microscopy (Olympus IMT2-RFL).

Fluorescein diacetate (FDA, Sigma) was added to the protoplast suspension to a final concentration of 35 μg ml^{-1}. After 5 min incubation, the sample was washed by centrifugation (100 *g*, 5 min.) and the pellet resuspended in wash medium. Protoplasts were examined in UV-light by fluorescence microscopy.

Oxygen evolution was measured with an oxygen electrode (Hansatech Ltd, UK). Algal samples were transferred to the measuring chamber, irradiated at a photon flux density of 400 μmol m^{-2} s^{-1} at 25 ± 0·1°C, and allowed to consume the remaining inorganic carbon of the buffer and the intracellular pool of inorganic carbon until no net oxygen evolution was observed. Inorganic carbon was then added as bicarbonate. Two test buffers of low dissolved inorganic carbon content were used, one for thalli consisting of sea-water with addition of 100 mM Bis-Tris Propane (Sigma), and the same for protoplasts with addition of 0·2 M mannitol. Low inorganic carbon contents were obtained by acidifying buffers to pH 2, bubbling for a minimum of 12 h with CO_2-free air to remove dissolved inorganic carbon, and adjusting the pH to 6·5, 7·5, and 8·5 respectively with carbonate-free NaOH solution. Protoplasts used in these experiments were produced from cultured plants, growing at 18–23% per day, by isolation in 1·5% AAP and 1·5% Cellulysin, using preplasmolysis.

All solutions were filtered through a 0·22 μm sterile-filter before use (Sartorius Minisart N).

OBSERVATIONS

A mixture of 1·5% Cellulysin and 1·5% Abalone Acetone Powder gave a high yield of protoplasts, up to more than 10^7 per gram fresh weight, of plants from natural populations. Increasing the enzyme concentration further did not give a corresponding increase in protoplast yield. Neither Cellulysin nor abalone acetone powder were by themselves sufficient to release large numbers of protoplasts from *Ulva rigida* (Table I). Release of

TABLE I. Effect of enzyme composition on protoplast yield from thalli of *Ulva rigida* from a natural population. + + + = > 10^6, + + = 10^5–10^6, + = 10^4–10^5, − = < 10^4 protoplast per g fw. Values represent at least three repeated experiments

Enzymes	Relative yield	
	young parts	old parts
3·0% Cellulysin	+	−
3·0% AAP (abalone acetone powder)	+	−
3·0% Cellulysin, 3·0% AAP	+ + +	+
1·5% Cellulysin, 1·5% AAP	+ + +	+
0·75% Cellulysin, 0·75% AAP	+ +	+

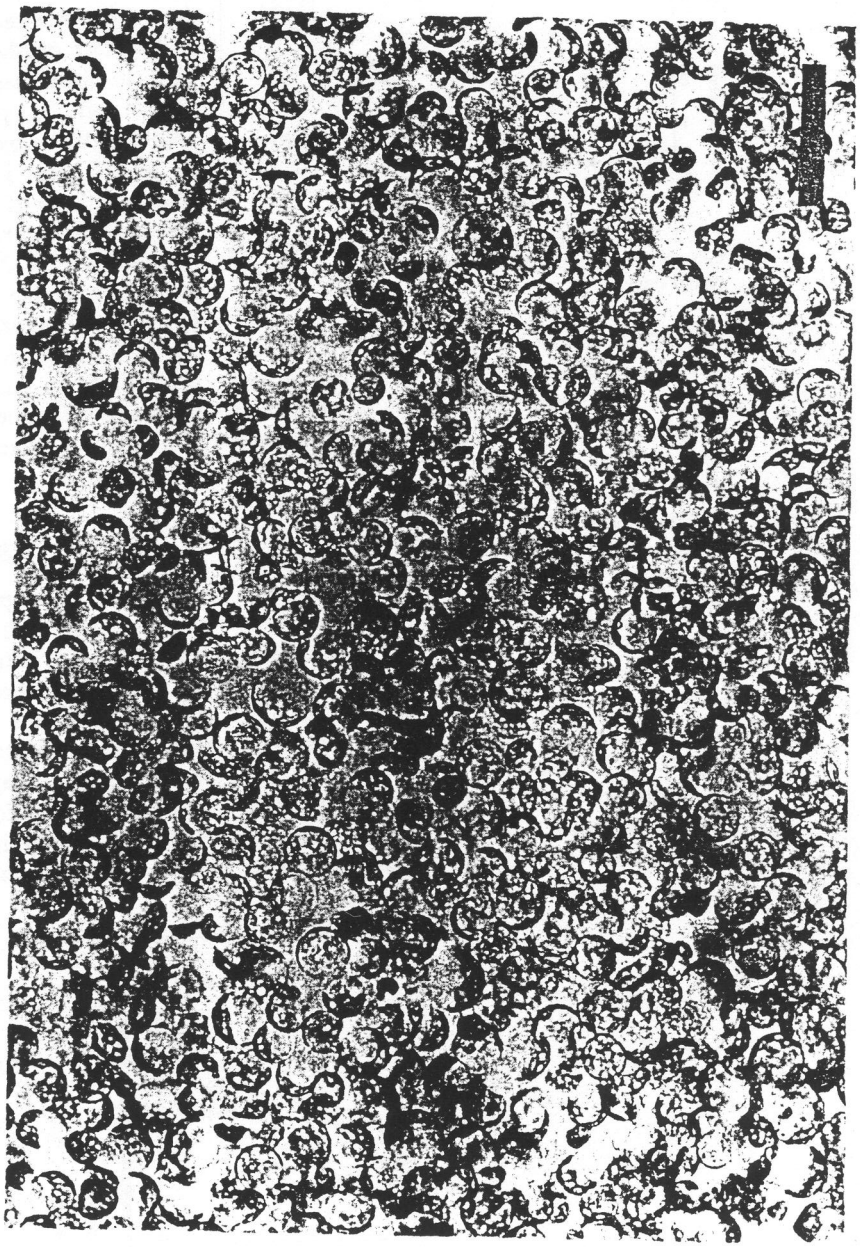

FIG. 1. Freshly isolated protoplasts of *Ulva rigida*. Scale bar = 100 μm.

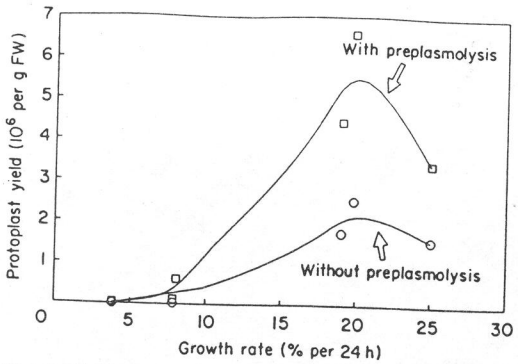

FIG. 2. Yield of protoplasts from *Ulva rigida* from plants cultured at different growth rates. Each growth rate was tested once.

protoplasts started after about 1–2 h of incubation. The protoplasts were spherical and ranged between 10–30 μm in diameter (Fig. 1). The release of protoplasts continued for up to 24 h, but after 8–12 h large numbers of protoplasts started to lyse (visual observation). No cell wall material was found when protoplasts were observed with Calcofluor in UV light. Preplasmolysis prior to the enzyme incubation was effective in enhancing the yield by a factor of about 2·5 (Fig. 2), and there was no observed difference in viability by this treatment. The growth rate of cultured algae was also important for the yield of protoplasts, with an optimum growth rate of about 20% biomass increase per day (Fig. 2). Only the young parts from natural populations plants were used for protoplast isolation. The yield decreased rapidly when plants were stored in tanks, so that after 3 days of storage without growth, except on one occasion, no protoplasts were released from this plant material.

The rate of photosynthetic oxygen evolution in protoplasts from *Ulva rigida* was shown to be pH dependent (Fig. 3b). Photosynthetic oxygen evolution was higher at pH 6·5 than in 7·5 and 8·5. At pH 6·5, photosynthesis of protoplasts as well as thalli was close to saturation at a concentration of 400–500 μM HCO_3^- [Fig. 3(a)]. Oxygen concentration in the medium decreased in the dark. Incubation in FDA resulted in 70–90% fluorescing protoplasts. After 24 h, protoplasts in culture had regenerated cell walls and still appeared viable after five days. No attempts were made to further culture the protoplasts.

DISCUSSION

The toxicity to plant cells of crude enzyme extracts such as abalone acetone powder has been discussed extensively (e.g. Tribe, 1955; Cocking, 1972; Berliner, 1981). Extracts of digestive enzymes from herbivorous organisms contain substances potentially harmful to protoplasts. Therefore low concentrations and short incubation times should be used. Addition of inhibitors of proteases during isolation has also been suggested to increase

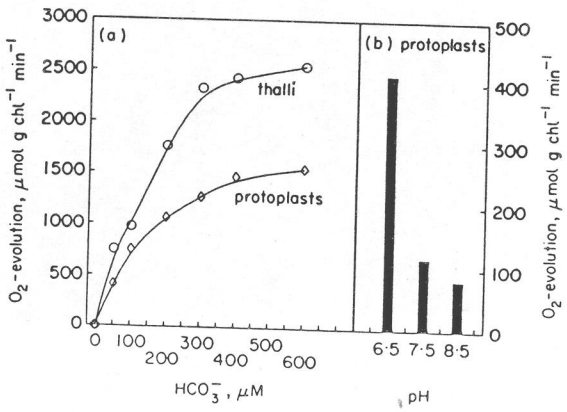

FIG. 3. Photosynthetic oxygen evolution of protoplasts and cultured thalli of *Ulva rigida*. (a). pH 6·5 with different concentrations of added HCO_3^-. (b). pH 6·5, 7·5 and 8·5, after addition of 50 μm HCO_3^-. Irradiance was 400 μmol m^{-2} s^{-1}, and temperature 25°C.

protoplast viability. This may of course not always be compatible with an optimizing initial yield. In this study high yield was obtained using enzyme concentrations of 1·5% Cellulysin and 1·5% AAP. This is lower than the total enzyme concentrations previously used to obtain corresponding yields of protoplast from other species of *Ulva* (Reddy, Migita & Fujita, 1989; Fujita & Migita, 1985; Zhang, 1983). Improved protoplast yield was obtained in all material tested by the use of pretreatment with a plasmolytic solution. However, plasmolysis may be harmful to the cells. Premecz *et al.* (1978) showed a decrease in RNA and protein synthesis in tobacco protoplasts exposed to osmotic stress, and inhibition of photosynthesis under such conditions has been shown (Fleck *et al.*, 1982). Nevertheless, pretreatment with a plasmolysing solution prior to enzyme treatment may increase both yield and viability of plant protoplasts. Tribe (1955) showed that plasmolysis greatly increased the viability of potato cells incubated in crude enzymes. Butler *et al.* (1989) reported a marked increase in yield and viability of protoplasts of *Laminaria saccharina* and *L. digitata*. Cocking (1972) suggested preplasmolysis of the tissue before enzyme treatment as a way to prevent uptake of crude enzymes solutions into the cytoplasm. In this study protoplasts obtained with the use of preplasmolysis were healthy and demonstrated photosynthetic activity (Fig. 3). The status of the plant material was important for isolation of protoplasts. Mature parts of collected thalli, and thalli in stagnant culture, were not suitable for protoplast isolation, whereas actively growing plants yielded high numbers. Cultured *Gracilaria tenuistipitata* and *G. sordida* showed the same characteristics (Björk *et al.*, 1990), and Kloareg, Polne-Fuller & Gibor (1989) demonstrated that higher yields of protoplasts of *Macrocystis* could be obtained from younger tissue than from old. Standardized culture conditions in this study produced reliable source tissue for protoplast isolation, yielding constant high numbers of protoplasts. However, variations in protoplast yield at the same growth rates indicate that factors other than the growth rate of the plant have to be considered during preculture. More work is needed to establish culture procedures for obtaining plants optimal for protoplast isolation. This may well prove as useful as the development of improved cell wall degrading enzymes.

The retention of photosynthetic and respiratory activity in protoplasts is additional proof of viability. If the photosynthetic rate of protoplasts in Fig. 3, is compared to that of intact thalli, it is shown that protoplasts retain about 65% of the photosynthetic capacity of source tissue. In the unicellular green alga *Chorella*, oxygen evolution rate of protoplasts was a third of that of intact cells (Webb, Berliner & Carlsson, 1980). *Enteromorpha* protoplasts had a slightly higher rate of oxygen evolution than the intact thallus (Millner, Callow & Evans, 1979), whilst the photosynthetic capacity of *Macrocystis* protoplasts was 40% of the intact tissue (Davison & Polne-Fuller, 1990). The higher rate of oxygen evolution of protoplasts at pH 6·5 than at 7·5 and 8·5 is to be considered in future experiments with protoplast culture. This decrease at higher pH, may be caused by the associated decrease in CO_2, and/or a decreased capacity by the protoplasts to utilize HCO_3^-. Calculation of the spontaneous dehydration of HCO_3^- to CO_2 (Johnson, 1982) at the different pH conditions in this study, reveals that the photosynthetic rate of protoplasts did not exceed what can be expected by the formation of CO_2. Different species of *Ulva* have been reported to utilize HCO_3^- (Larsson *et al.*, 1990; Drechsler & Beer, 1991; Johnston, 1991), indicating that the HCO_3^- utilization mechanism may be destroyed when the cell wall is removed, and that freshly isolated protoplasts are dependent solely on the CO_2 in the medium.

High yields and results from measurements of photosynthesis, as well as the result of viability tests, indicate that protoplasts isolated from actively growing *Ulva rigida* plants in good condition, preplasmolysed

prior to enzyme treatment with 1·5% Cellulysin and 1·5% Abalone Acetone Powder, may prove useful in studies of algal physiology.

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Photosynthetic characteristics of *Dunaliella salina* (Chlorophyceae, Dunaliellales) in relation to β-carotene content

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Abstract

Photosynthetic characteristics of *Dunaliella salina* with high (red form) and low β-carotene (green form) concentrations were studied. *D. salina* growing in brine saltworks exhibited a high level of β-carotene (15 pg cell^{-1}). The rate of oxygen evolution as a function of irradiance was higher in the red than in the green form (on chlorophyll basis). Photosynthetic inhibition of the green form was observed above 500 μmol m^{-2} s^{-1}. The red form appeared more resistant to high irradiance and no inhibition in O_2 evolution was observed up 2000 μmol m^{-2} s^{-1}. However, when these results are expressed on a cell number basis the rate of oxygen evolution was significantly higher in the green form.

Carbonic anhydrase (CA) activity (total, soluble, membrane bound) was found in red and green forms. CA was higher in the red form on a chlorophyll basis, but lower if expressed on a protein basis. The light dependent rate of oxygen evolution and photoinhibition depends on the concentration of β-carotene in *D. salina* cells.

Introduction

The halotolerant unicellular alga *Dunaliella salina* Teodoresco exhibits the ability to accumulate large amounts of β-carotene (up to 10–14% dry weight) under extreme environmental conditions, such as high salt concentration, high irradiance or nitrogen deficiency (Massyuk, 1973; Ben-Amotz & Avron, 1982; Borowitzka *et al.*, 1984). This property is being exploited in *D. salina* farms for the production of β-carotene, a natural additive used to enhance the red colour of a wide range of products (Borowitzka & Borowitzka, 1988). The accumulation rate of β-carotene is directly dependent on the total irradiance to which the cells are exposed during a division cycle (Ben-Amotz, 1987; Borowitzka *et al.*, 1990). β-Carotene is usually associated with the chlorophyll in the thylakoids where it acts as a light harvesting pigment. However, in extreme growth conditions, β-carotene is localizated mostly in the chloroplast periphery forming lipoidal globules ('carotene droplets') playing a dual role as light-harvesting pigment and as a photo-protector of the photosynthetic apparatus from high irradiance.

The aim of this work was the study of the pho-

tosynthetic adaptive capabilities of β-carotene-rich *D. salina* cells.

Material and methods

Algal material and culture conditions

Dunaliella salina was isolated from a brine (5.5 M total salts) saltwork from the island of Gran Canaria, Canary Islands, Spain, according to Massyuk (1973). Cells exhibited a strong red colour (red form). The red form was stepwise adapted during two months from its natural medium (5.5 M NaCl) to seawater supplemented with 2 M NaCl, 8 mM KNO_3, 2 mM $MgSO_4$, 1.9 mM $MgCl_2$, 0.01 mM $Ca(NO_3)_2$, 4 mM K_2HPO_4 and micronutrients after Surzycki (1971). During this period the chlorophyll content increased and, as a consequence the alga took on a green colour (green form). This process was reversible. When the green form was re-adapted to the initial growing conditions, the cells again took a red colour.

The green form was grown at 26 ± 1 °C under continuous illumination with white light (50 W m^2) in 1 l glass bottles sparged with a CO_2:air (5:95, v/v) mixture. Three days before experiments cells were adapted to low C_i condition by transferring cultures to seawater supplemented with 2 M NaCl and sparged with air only.

Enzyme assays

Carbonic anhydrase (EC 4.2.1.1.) activity was determined electrochemically according to Ramazanov and Semenenko (1986). The measurements were performed in the cell-free homogenate (total CA activity) and cell fractions after centrifugation of the homogenate at 20000 × *g* for 30 min (supernatant and pellet) in 60 mM ice-cold phosphate buffer pH 8.1, containing 1 mM EDTA and 2 mM DTT.

Measurements of photosynthetic oxygen evolution

Photosynthetic O_2 evolution was measured with an oxygen electrode (Hansatech Ltd, UK) in algal samples resuspended in 1 mL seawater supplemented with 2 M NaCl at pH 8.2. White light was obtained from a slide projector lamp (Reflecta, Germany). Light measurements were carried out with a radiometer LI-1000 DataLogger using a spherical quantum sensor LI-193SA (LI-COR, Nebraska, USA).

Pigment assays

Chorophyll was extracted with absolute ethanol and quantified using the absorption coefficients given by Wintermans and De Mots (1965). The β-carotene concentration was calculated according to Ramazanov *et al.* (1988).

Statistical analysis

Results are the mean of 5 independent determinations. Variability in an experiment never exceeded 10% of the mean.

Results

Figure 1 shows the spectral characteristics of the pigments extracted from red and green forms. The ratio of maximum carotene:chlorophyll absorbances (A_{450}/A_{665}) in the red form was considerably higher than in the green form (Table 1). The β-carotene content in red and green forms was 15 and 0.5 pg $cell^{-1}$, respectively. The chlorophyll content in red and green *D. salina* forms was 0.4 and 4 pg $cell^{-1}$, respectively.

Figure 2a shows the rate of photosynthetic oxygen evolution as a function of irradiance in green and red forms. Presented results have demonstrated that the rate of photosynthetic oxygen evolution on chlorophyll basis is higher in the red than in the green form. The rate of photosynthesis was inhibited in the green form above

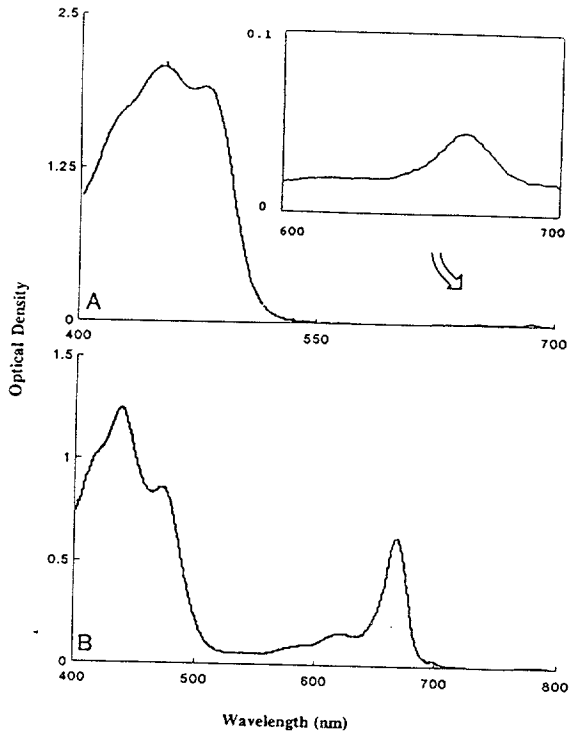

Fig. 1. Spectral characteristics of the pigments extracted from red and green forms of *Dunaliella salina*: (A) red; (B) green.

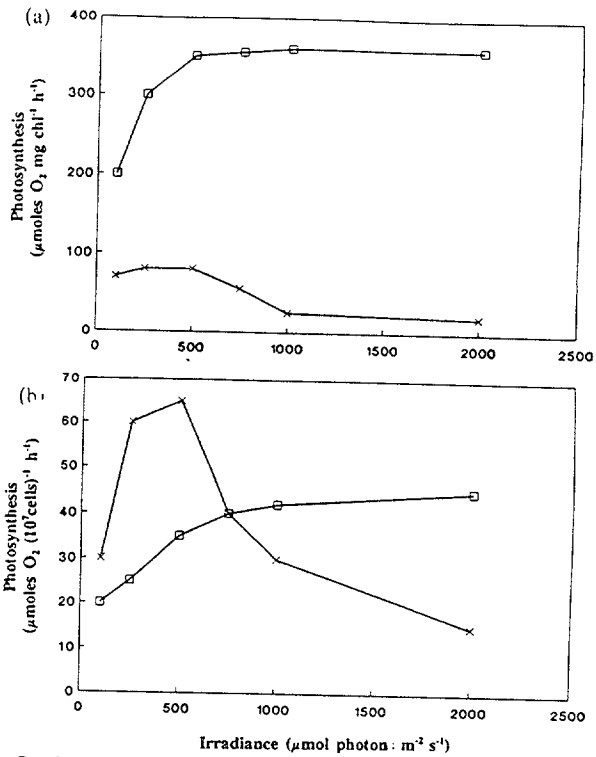

Fig. 2. Rate of photosynthetic oxygen evolution as a function of irradiance in green and red forms: (a) expressed on chlorophyll basis (□ red form; × green form); (b) expressed on cell number basis (□ red form; × green form).

Table 1. Carbonic anhydrase activity, ratios of chlorophyll-protein concentrations and maximum carotene-chlorophyll absorbances of red and green forms of *Dunaliella salina*. [1] Total CA activity in homogenate; [2] CA activity in supernatant after centrifugation of the homogenate; [3] CA activity associated with membrane fraction (in pellet).

Form	CA units μg protein^{-1}			CA units μg chlorophyll^{-1}			Chl/Prot	A_{450}/A_{665}
	T[1]	S[2]	M[3]	T	S	M		
Green	190	93	97	137	73	68	1.12	1.55
Red	36	17	23	266	92	122	0.13	40

500 μmol m^{-2} s^{-1}, whereas carotene-rich cells of *D. salina* appeared more resistant to high irradiance. However, when these results are expressed on a cell number basis (Fig. 2b) the rate of photosynthesis was significantly higher in the green form at irradiances below 500 μmol m^{-2} s^{-1}. Nevertheless, the photosynthetic inhibition by high light was more pronounced in the green form.

We found CA activity (total, soluble and membrane-bound CAs) associated with both red and green forms (Table 1). On a chlorophyll basis, CA activity was always higher in the red form. However, as it was the case of the photosynthetic rate, on a protein basis this pattern of CA activity was virtually the opposite and the green form showed significantly higher CA activity. CA activity of the green form was associated with both, a soluble protein fraction and the pellet.

Discussion

Previous authors (Ben-Amotz & Avron, 1983) have shown that β-carotene-rich *D. salina* has a

marked capability for photosynthetic adaptation to extreme environmental conditions. High light intensity and salinity induces carotenogenesis in *D. salina* as an adaptative reaction to an environmental stress (Borowitzka *et al.*, 1990) and during carotenogenesis the concentration of chlorophyll decreases (Ben-Amotz & Avron, 1982; Ramazanov *et al.*, 1988), although other authors have described higher carotenoid and chlorophyll content of cells grown at high salinities (Booth & Beardall, 1991). It seems evident from our results that high levels of β-carotene imply more resistance to photoinhibition at the expense of lower photosynthetic O_2 evolution, i.e. the ability to synthesize high levels of β-carotene correlates with higher photoinhibitory thresholds at the expense of a lower photosynthetic O_2 evolution. The chlorophyll concentration, which has been decreased by increased light intensity, may perhaps be an intracellular signal for the induction of β-carotene synthesis.

A major limiting environmental factor for photosynthetic productivity of aquatic plants in natural environments or in cultivation is the low inorganic carbon concentration (C_i) of the medium. *Dunaliella salina*, like other microalgae, adapts to low C_i, inducing a C_i-concentrating mechanism, which shows a Km (CO_2) below 5 μM (Zenvirth and Kaplan, 1981; Aizawa and Miyachi, 1984; Palmqvist *et al.*, 1990). It has been suggested that the low Km (CO_2) of *D. salina* cells may be due to the presence of carbonic anhydrase (CA). In addition it has been shown that there is a direct relationship between CA activity and salinity in *D. salina* (Latorella & Vadas, 1973; Booth & Beardall, 1991). Booth and Beardall (1991) have shown that the relationship between photosynthesis of *D. salina* and the dissolved inorganic carbon concentration is strongly affected by the NaCl content of the medium. Cells grown at high salinity show higher affinity in photosynthesis for CO_2 than those grown at low salinity. At increased salinities, the concentration of CO_2 decreases markedly, from 12 μM in freshwater to approximately 5.8 μM at 2.2 M NaCl (Booth & Beardall, 1991). In our case, CA activity was measured in the red form of *D. salina* isolated directly from a hypersaline evaporation pond (total salinity approximately 5.5 M) where the concentration of dissolved CO_2 should be lower than 5.8 μM. The lower C_i concentration is probably responsible for the higher CA activity (on chlorophyll basis) of the β-carotene-rich-cells.

Values for the comparison of photosynthetic rate and CA activity in red and green forms of *D. salina* should be expressed on a chlorophyll basis. In contrast to our data, Booth and Beardall (1991) obtained similar results whether photosynthesis was expressed on a per chlorophyll on per cell basis. Their results can be explained as due to the surprisingly high chlorophyll content of the β-carotene-rich cells of *D. salina* described by these authors.

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Inorganic-carbon assimilation in the green seaweed *Ulva rigida* C.Ag. (Chlorophyta)

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Abstract. Mechanisms of carbon assimilation were investigated in thalli and protoplasts of *Ulva rigida* by measuring HCO_3^--dependent O_2 evolution at pH 6.5 and 8.6. In thalli, dextran-bound azetazolamide (DBAZ), a specific inhibitor of extracellular carbonic anhydrase (CA), inhibited the rate of O_2 evolution at pH 8.6 when HCO_3^- was the only available form of inorganic carbon (C_i) in the medium. At pH 6.5 when CO_2 is accessible, DBAZ did not affect photosynthetic O_2 evolution. Inhibition of total CA activity (extracellular and intracellular) by ethoxyzolamide (EZ) inhibited photosynthesis at pH 6.5 and 8.6. During illumination of thalli the medium was alkalized at a rate which increased with increasing light. This alkalization decreased during inhibition of extracellular CA by DBAZ. Protoplasts at pH 6.5 exhibited a higher rate of O_2 evolution than in pH 8.6. Addition of CA to protoplasts at pH 8.6 increased the rate of O_2 evolution, whereas EZ was inhibitory at both pH 6.5 and 8.6, and DBAZ did not affect photosynthesis at either pH. We suggest that both extracellular and intracellular CA are present and that *Ulva rigida* assimilates HCO_3^- by an indirect mechanism. A theoretical scheme for carbon utilization is suggested.

Key words: Carbon-assimilation – Carbonic anhydrase – Photosynthesis (*Ulva*) – Protoplast (photosynthesis) – *Ulva*

Introduction

Marine macroalgae have been shown to utilize both CO_2 and HCO_3^- from the surrounding seawater (Smith and Bidwell 1989; Raven 1990); CO_2 can enter through membranes by diffusion, or by active transport as in microalgae (Sültemeyer et al. 1988). Carbon dioxide is the most accessible form of inorganic carbon (C_i), but considering the greater availability of HCO_3^- in seawater, this latter carbon source could well be more important in natural systems (Smith and Bidwell 1987, 1989). The mechanisms for uptake of HCO_3^- are still not clear. It has been proposed that HCO_3^- is transported through the plasmalemma by an HCO_3^-/OH^- antiporter or HCO_3^-/H^+ symporter mechanism (Lucas 1983) which may involve ATPases (Raven and Lucas 1985). Bicarbonate could also be assimilated by an indirect mechanism, i.e. after dehydration to CO_2 in the cell wall (Smith and Bidwell 1987). This mechanism would require an extracellular carbonic anhydrase (CA) (Smith and Bidwell 1987, 1989). Carbonic anhydrase catalyzes the reversible hydration and dehydration of CO_2 and HCO_3^-, and has been shown to be involved in active C_i transporting and accumulating mechanisms in seaweeds (Smith and Bidwell 1987, 1989; Beer and Israel 1990). Another possibility is the spontaneous low-pH-dependent dehydration of HCO_3^- in "acidic regions" of the cell wall of aquatic plants (Lucas 1983). It has been suggested that extracellular CA can increase the rate of conversion of HCO_3^- to CO_2 in the extracellular space; however, CA analyses on several seaweeds have shown no indications of extracellular CA activity (Cook et al. 1986, 1988).

In the present work we demonstrate that the green seaweed *Ulva rigida* can assimilate both CO_2 and HCO_3^-, but that protoplasts isolated from this plant take up only CO_2. We suggest that *U. rigida* assimilates HCO_3^- by an indirect mechanism, that extracellular CA is involved in C_i uptake, and the evidence for the presence of extracellular and intracellular CA is presented.

Material and methods

Algal material and culture. Healthy [illegible] plants collected from Laharte, Gran Canaria, Spain and cleaned thoroughly in sterile seawater [illegible] were then kept in 20 l tanks with running seawater for a maximum time of 12 h

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Abbreviations: CA = carbonic anhydrase; C_i = inorganic carbon; DIC = dissolved inorganic carbon; DBAZ dextran-bound acetazolamide; EZ = 6-ethoxyzolamide

Protoplast isolation. Cellulysin (Calbiochem AG, Lucerne, Switzerland) and Abalone Acetone Powder (Sigma, St. Louis, Mo., USA) were dissolved in seawater at a salinity of 38 ‰ with addition of 0.4 M mannitol and 20 mM 2,2-bis(hydroxymethyl)-2,2',2''-nitrilo triethanol (Bis)-Tris. The enzyme solution was stabilized by stirring on ice for 30 min, centrifuged at 1000 · *g* for 10 min, the pH adjusted to 6.0, and then filtered through a 0.8-µm sterile filter. The enzyme solution was frozen in 10-ml portions and kept at −20° C prior to use. The tissue was chopped into 1- to 2-mm² pieces using a razor blade. The fragmented thallus was then rinsed five times in wash buffer (0.2 M mannitol and 20 mM 4-(2-hydroxyethyl)-1 piperazine-ethanesulfonic acid (Hepes), in seawater, pH 7), and incubated for 30 min in seawater with addition of 20 mM Hepes and 0.8 M mannitol, final pH 7.0. One gram of fragmented thallus was immersed in 10 ml enzyme solution and incubated at 20° C under constant shaking (60 rpm). After digestion the protoplast suspension was filtered through a 100-µm nylon mesh to remove cell-wall debris and non-digested material, rinsed with wash buffer and centrifuged in a swing-out rotor at 100 · *g* for 5 min. The pellet was resuspended in test buffer and centrifuged again, the resulting pellet of protoplasts was resuspended in a test buffer for photosynthesis experiments.

Preparation of inhibitor and CA solutions. Two inhibitors of CA were used, 6-ethoxyzolamide (EZ) which penetrates through the cell wall and membranes, and dextran-bound acetazolamide (DBAZ). When bound to dextran, DBAZ cannot penetrate into the cell and inhibits only the extracellular CA (Moroney et al. 1985; Palmqvist et al. 1990). Stock solutions were prepared as follows: EZ was dissolved in 0.05 N NaOH to a concentration of 10 mM; DBAZ, kindly provided by Prof. Göran Samuelsson (Umeå, Sweden), was dissolved in 0.05 N NaOH to a concentration of 0.1 g · ml^{-1}. Bovine CA (Sigma) was dissolved in ultra-pure water (milliQ) to a concentration of 0.1 mg · ml^{-1}.

Measurement of photosynthetic O_2 evolution. When thalli were used they were chopped with a razor blade to approx. 10 mm² pieces and rinsed several times in seawater. The fragments were transferred to seawater medium and maintained with continuous air bubbling and 100 µmol · m^{-2} · s^{-1} light for at least 6 h before use. Protoplasts were suspended in test buffer and transferred to the measuring chamber. Photosynthesis was measured in 1-ml samples with an oxygen electrode (Hansatech, Kings Lynn, Norfolk, UK). Two test buffers were used, consisting of seawater, 100 mM 3-(N-morpholino)ethanesulfonic acid (Mops) (Sigma) or 2-(N-cyclohexylamino)ethane-sulfonic acid (Ches) (Sigma) adjusted to pH 6.5 and 8.6 respectively. When protoplasts were measured, an addition of 0.2 M mannitol was used to prevent lysis. Buffers of low dissolved-inorganic-carbon content (low-DIC buffer) were obtained by acidifying natural seawater to pH 3, bubbling for 12 h with CO_2-free air to remove DIC and then adjustment of pH with carbonate-free NaOH. Before HCO_3^- was added, samples were allowed to consume the remaining C_i of the buffer and the intracellular pool of C_i until no net O_2 evolution was observed. Final inhibitor concentrations used here were 100 µM EZ and 100 µM DBAZ. The final CA concentration used was 1 mg · l^{-1}. The experiment was repeated four times with material from the same batch of cut thalli or protoplasts, then repeated up to six times with new material.

Photosynthetic alkalization in whole thalli. Photosynthetic alkalization was measured with a pH meter (CKC UC-23 with UC-502E electrode; Central Kagaku Co., Tokyo, Japan). An Erlenmeyer flask was filled to 55 ml with medium and 0.5 g seaweed thallus (fresh weight) was placed in the flask together with a bar magnet. The medium consisted of natural seawater (pH 8.12). A closed system was obtained with the electrode tip immersed in the medium in a 50 ml Erlenmeyer flask by sealing the electrode and flask together with Parafilm and covering the seal with paraffin to prevent CO_2 exchange with the atmosphere. The pH change was recorded at photon irradiances of 75 and 200 µmol · m^{-2} · s^{-1} and at 23 ± 1° C. The experiment was repeated three times with material from the same batch of cut thalli or protoplasts, then repeated up to six times with new material.

Extraction of algal material. A 2-g sample of algal thallus was homogenized carefully in liquid nitrogen and extracted with a buffer containing 50 mM Tris (pH 8.5), 15 mM dithiothreitol (DTT), 25 mM isoascorbic acid and 5 mM EDTA. Buffer was added to a total volume of 20 ml.

Assay of carbonic anhydrase. Carbonic-anhydrase activity was measured potentiometrically at 0–2° C by determining the time taken for a linear drop of 0.4 pH units in the pH range 8.1 to 7.1 in the reaction mixture, which consisted of 2 ml sample and 2 ml substrate. Samples used were intact thalli, protoplast suspensions, and algal extracts centrifuged at 12000 · *g* for 15 min. The sample buffer used was the same as the extraction buffer. The reaction was started by rapidly introducing 2 ml of the substrate, ice-cold CO_2-saturated distilled H_2O. The method was modified after Ramazanov and Semenenko (1988). One unit of relative enzyme activity (REA) was defined as (t_0/t_c)-1 where t_0 and t_c are the times for pH change of the nonenzymatic (sample buffer) and the enzymatic reactions, respectively. When measuring CA activity in thallus fragments, 0.16–0.29 g fresh weight was transferred directly from seawater to 2 ml of sample buffer. Prior to the measurements with protoplasts, they were lysed in the hypo-osmotic test buffer. The fragments were maintained in suspension by magnetic stirring. Results shown are means of three parallel determinations in two independent repetitions of the experiment.

Measurement of chlorophyll. Chlorophyll was measured spectrophotometrically after ethanol extraction according to Wintermans and de Mots (1965).

Results

Photosynthesis in thalli. The photosynthesis of *U. rigida* thalli fragments in seawater of pH 8.6 is shown in Fig. 1. The rate of photosynthetic O_2 evolution was inhibited by both DBAZ and EZ; however, the inhibition by EZ was 40% higher than that of DBAZ. After subsequent addition of CO_2, the rate of O_2 evolution recovered and under these conditions the inhibition by EZ was lower than in the presence of only HCO_3^- (Fig. 1). In the presence of CO_2 in the medium, DBAZ had no effect on the rate of O_2 evolution (data not shown). The photosynthesis of thalli fragments in seawater at pH 6.5 is also shown in Fig. 1. At this pH the photosynthetic rate was about three times higher than at pH 8.6 at the same concentration of C_i, and EZ strongly inhibited the rate of O_2 evolution whereas DBAZ had no effect.

The rate of pH change in natural seawater caused by thalli of *U. rigida* is shown in Fig. 2. The highest alkalization rate was obtained at the highest irradiance. Addition of DBAZ to the medium caused a decrease in the alkalization rate. When DBAZ was removed by washing the thallus in fresh medium the rate of alkalization increased again. No attempts have been made to correlate the rate of photosynthetic O_2 evolution with the rate of photosynthetic alkalization.

Photosynthesis in protoplasts. The rate of O_2 evolution in protoplasts of *U. rigida* was also dependent on the pH in the medium (Fig. 3), and was higher at pH 6.5 than at pH 8.6. At pH 6.5, photosynthesis was saturated at a

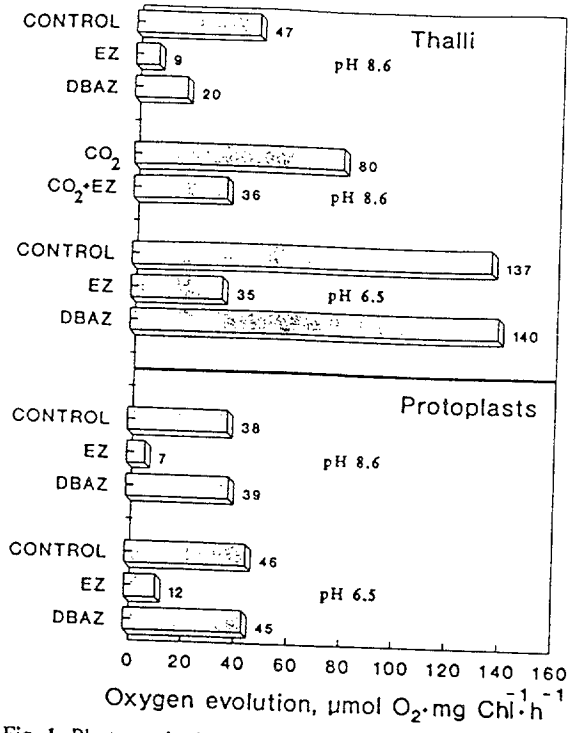

Fig. 1. Photosynthesis of *Ulva rigida* thalli and protoplasts at pH 6.5 and 8.6 In low-DIC medium composed of natural seawater depleted of C_i, with addition of HCO_3^- to a final concentration of 100 µM (for thalli at both pH values, and protoplasts at pH 6.5) or 600 µM (for protoplasts at pH 8.6). Fresh weight of thalli was 0.5 g/50 ml medium, light irradiance was 400 µmol photons · m^{-2} · s^{-1}. *EZ*, 6-ethoxyzolamide (100 µM); *DBAZ*, dextran-bound acetazolamide (100 µM); CO_2, addition of 330 µM CO_2. Mean values (n = 4); SD not exceeding 7%.

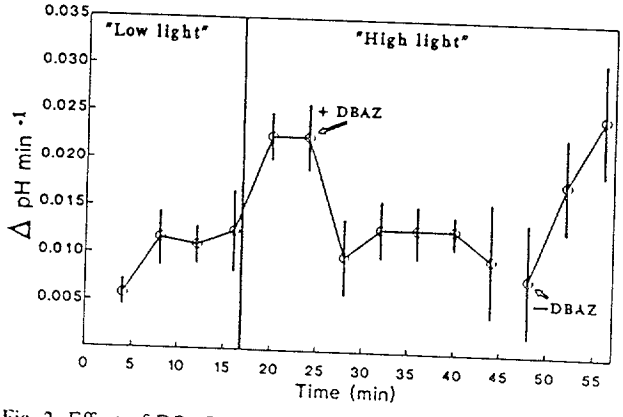

Fig. 2. Effect of DBAZ on the rate of light-dependent alkalization of the medium by *Ulva rigida* thalli. *Arrows* indicates the addition of DBAZ to a final concentration of 100 µM (+ *DBAZ*) and the removal of DBAZ by washing with new medium (− *DBAZ*). *Low light*, 75 µmol photons · m^{-2} · s^{-1}; *High light*, 200 µmol photons · m^{-2} · s^{-1}. Values are means ± SD (n = 3)

concentration of 400-500 µM HCO_3^-, while at pH 8.6 no saturation was observed. Addition of CA to the medium at pH 8.6 increased the rate of O_2 evolution. The effect of CA-inhibitors, EZ and DBAZ, on the rate of O_2

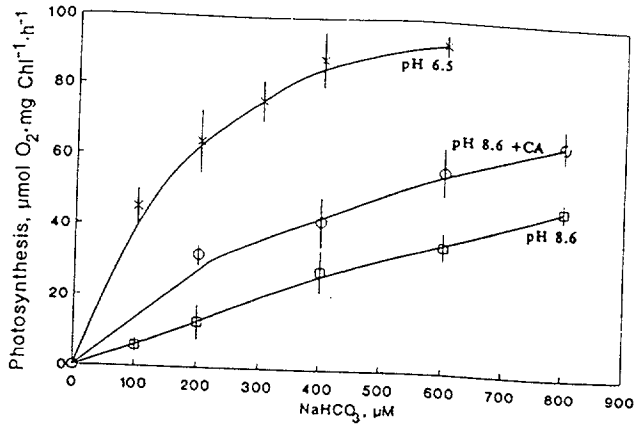

Fig. 3. Photosynthesis of *Ulva rigida* protoplasts at pH 6.5 and 8.6 in low-DIC medium composed of natural seawater depleted of C_i, which was then added as HCO_3^-. Light irradiance was 400 µmol photons · m^{-2} · s^{-1}. *+CA*, experiment performed with medium containing bovine CA at a final concentration of 1 µg · ml^{-1}. Values are means ± SD (n = 4)

Table 1. Relative enzyme activity (REA) of CA in an extract of 1 g of *Ulva rigida* thallus tissue. Activity was measured in the homogenate before centrifugation and in the supernatant and the pellet fractions after centrifugation. Protoplasts were lysed in buffer prior to measurement. Whole thallus was submersed in buffer and measured directly. Data are from two separate experiments, each with three parallel sample measurements. The protoplast fraction was measured only once. Values are means ± SD

	REA in 1 g thallus	
Homogenate	5 ± 0.5	(n = 6)
Supernatant	2 ± 0.7	(n = 6)
Pellet	1 ± 0.6	(n = 5)
Protoplast (lysed)	4	(n = 1)
Living thalli	4 ± 1.1	(n = 6)

evolution by protoplasts at pH 6.5 and pH 8.6 is shown in Fig. 1. The effects of the inhibitors were similar in both pH conditions. The rate of photosynthetic O_2 evolution was not affected by DBAZ, which does not enter into the protoplast as it cannot penetrate the membrane, whereas EZ decreased the rate.

Carbonic-anhydrase activity. Measurements of the activity of CA in thallus extract showed a higher activity in the soluble fraction than in the insoluble pellet (Table 1), although a comparison with the total CA activity in the homogenate shows that some activity is lost during fractionation. Lysed protoplasts and living intact thalli also posessed CA activity, but these values cannot be directly compared with those of the extracts because of the methodological differences involved in obtaining the measurements.

Discussion

The results in this study demonstrate [illegible] of the green alga *Ulva rigida* the predominant form of C [illegible]

into the cell through the plasmalemma is CO_2. Bicarbonate is dehydrated to CO_2 in the cell wall, an event most likely catalysed by an external CA.

The higher photosynthetic rate of thalli at pH 6.5 compared with pH 8.6, at the same concentration of C_i (Fig. 1), demonstrates the uptake of CO_2. In a closed chamber, in seawater at pH 8.6, the only available form of C_i is HCO_3^-, while at pH 6.5 CO_2 is readily accessible to the plant. If *U. rigida* has the ability to assimilate HCO_3^- directly, it is difficult to explain the effect of DBAZ on the rate of photosynthesis at pH 8.6 since DBAZ specifically inhibits extracellular CA and, accordingly, the catalysed dehydration of HCO_3^- to CO_2 in the cell wall. At pH 6.5, when the relative CO_2 concentration is higher, inhibition of external CA does not affect photosynthetic O_2 evolution. That C_i is mainly assimilated into the cell in the form of CO_2 is also supported by the observation that addition of CO_2 to the medium at pH 8.6 increased photosynthesis and countered the inhibitory effect of EZ (Fig. 1).

However, the mechanism of C_i assimilation in *U. rigida* cannot be explained only by the presence of a extracellular CA. The inhibition by EZ at both pH values demonstrates that the C_i assimilation mechanism in *Ulva*, as in green unicellular algae (Moroney et al. 1985), requires both extra- and intracellular CA catalysis. Smith and Bidwell (1987) showed that inhibition of carbon uptake in the red seaweed *Chondrus crispus* by CA inhibitors was largest at air levels of CO_2, when HCO_3^- absorption predominates, and least when CO_2 was readily available. This supports our results that indicate the involvement of CA in HCO_3^- uptake in *U. rigida*.

The CA-activity assay shows the presence of active enzyme in *U. rigida*. The higher activity found in the supernatant compared with the pellet indicates that a high proportion of the enzyme might be in solution inside the cell, or that the enzyme can be easily removed, still intact, during extraction. The variability of these measurements is however high and, as shown by comparison with the activity of the homogenate, some activity is lost during fractionation, making it difficult to compare the values. The different extraction methods also make it pointless to compare the activities of the homogenized tissue with the intact thalli. Nevertheless, the detection of CA activity at the surface of living thalli is evidence for the existence of cell-wall-bound CA.

The inhibition of alkalization by DBAZ provides additional evidence that *Ulva* assimilates HCO_3^- by an indirect mechanism. Lucas (1983) and Axelsson and Uusitalo (1988) reported that aquatic plants assimilated HCO_3^- by HCO_3^-/OH^- antiport or HCO_3^-/H^+ symport mechanisms and that the alkalization of the medium resulted from the efflux of OH^-. Accordingly, DBAZ would have no effect on the rate of alkalization at pH 8.6. The DBAZ inhibition of light-dependent alkalization in this study indicates that alkalization of the medium by *U. rigida* involves a mechanism requiring extracellular CA. This mechanism is different from the one suggested by Axelsson and Uusitalo (1988) and Lucas (1983). However, a contribution of active ion transport in C_i uptake cannot be excluded from the results presented here.

The pH dependence of protoplast photosynthesis demonstrates that HCO_3^- does not pass through the protoplast plasmalemma. Similar results were obtained for protoplasts from the red seaweed *Chondrus crispus* (Smith and Bidwell 1989). This may reflect a stress effect on the protoplasts in in-vitro pH conditions. Such an explanation does not, however, explain the observation that photosynthetic O_2 evolution by protoplasts was increased by addition of CO_2 and CA. Adding CA to the medium at pH 8.6 did not restore the rate of O_2 evolution to levels matching those at pH 6.5. However, CA dissolved directly in the medium can not be expected to act as efficiently as a CA incorporated into the wall of a intact cell. As mentioned above, there is also a stress upon the protoplasts in vitro, when exposed to pH 8.6, that might effect O_2 evolution negatively. At both pH values, DBAZ had no effect on the rate of photosynthesis which demonstrates that intact protoplasts have no extracellular CA. However, extracellular CA activity would be removed during protoplast preparation.

It is important to note that CA activity was detected in solutions of lysed protoplasts, indicating an intracellular CA. Further investigations are needed to clarify the intracellular localization of CA in *Ulva* and other seaweeds. Decreased photosynthesis in protoplasts during inhibition by EZ at both pHs demonstrated that intracellular CA plays an important role in the C_i-uptake system of *U. rigida*.

The fact that seaweeds have a low photorespiratory rate indicates that the chloroplastic CO_2/O_2 ratio may be high enough to suppress photorespiration (Bidwell and McLachlan 1985). Low photorespiratory levels are typical of green unicellular algae grown in low-C_i conditions which reportedly induce C_i-concentrating mechanisms (Moroney et al. 1985). *Chlamydomonas reinhardtii* grown in low-C_i conditions accumulates C_i to a concentration 40 times that of the environment (Badger et al. 1980). However, no such mechanism has been found in *Chondrus crispus* which does not accumulate C_i above the concentration of its incubation medium, and probably acquires C_i through a diffusive rather than an active mechanism (Smith and Bidwell 1987). The ability of *Ulva lactuca* to assimilate HCO_3^- is induced at high pH (Carlberg et al. 1990). The degree of HCO_3^- utilization is also stimulated in calm waters, where dissolved CO_2 is more limited (Larsson et al. 1990), indicating an induced increase of extracellular CA activity.

We propose an indirect mechanism for C_i uptake and transport in *U. rigida* (Fig. 4, I). This mechanism for C_i transport requires extracellular CA, which catalyzes the dehydration of HCO_3^- to CO_2 in or at the surface of the cell wall. Our model agrees with that previously proposed by Smith and Bidwell (1989). Figure 4(II) also shows a scheme for an ATP-ase dependent C_i-transport mechanism in aquatic plants, as suggested by Lucas (1983 review). In this scheme acid regions are produced in the cell wall by H^+ extrusion, and this local acidification may stimulate pH-dependent conversion of HCO_3^- to CO_2. In both model I and II, internal CA regulates the CO_2 concentration inside the plasmalemma, although the details of this regulation are not known.

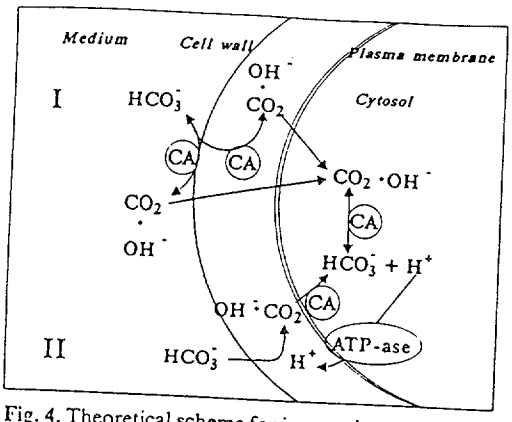

Fig. 4. Theoretical scheme for inorganic-carbon assimilation in *Ulva rigida*. *I*, Carbonic-anhydrase-requiring indirect mechanism of C_i assimilation. *II*, ATP-ase-dependent indirect mechanism of C_i assimilation

We suggest that, with the help of an extracellular CA, *Ulva rigida* has the ability to utilize the HCO_3^- pool present in seawater as a source of inorganic carbon for photosynthesis. Bicarbonate is dehydrated to CO_2 in the cell wall, and C_i crosses the plasmalemma in the form of CO_2. However, the possibility cannot be excluded that a ATP-ase dependent H^+-extrusion system as described above, co-exists with this system. The two systems might combine to enhance the conversion of HCO_3^- to CO_2.

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Marine Biology

Genetic variability and differentiation of sporophytes and gametophytes in populations of *Gelidium arbuscula* (Gelidiaceae: Rhodophyta) determined by isozyme electrophoresis

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Abstract. Genetic differentiation and genetic variability of sporophytic and gametophytic populations of *Gelidium arbuscula* (Bory) from three localities sampled in 1989 and 1990 in the Canary Islands (Spain) were examined by isozyme electrophoresis. Twenty-three to 29 putative alleles corresponding to 22 gene loci, were compared. High deviations in Hardy-Weinberg equilibrium, and significant differences between allelic frequencies of sporophytic and gametophytic subpopulations at the same locality were found, suggesting a predominant asexual reproduction of *G. arbuscula*. The genetic variability (percentage of polymorphic loci, mean number of alleles per locus and average gene diversity) of haploid subpopulations was lower than that of diploid subpopulations at all three localities, being the lowest described for seaweeds. No correlation between genetic and geographical distance was found. The high genetic differentiation coefficient between all subpopulations suggests a very reduced genetic flow between subpopulations of the same and of different localities. These results suggest that the genetic structure of the populations of *G. arbuscula* from the Canary Islands is due to a founder-effect combined with a predominance of asexual reproduction. This is the first report comparing allelic frequencies between sporophytic and gametophytic subpopulations of seaweeds.

Introduction

Very little is known about genetic variability and differentiation among populations of marine algae (Innes 1984, Cheney 1985). Quantitative estimates of genetic variability and differentiation have been greatly facilitated by isozyme electrophoresis (Innes 1984). Studies of isozyme variability in seaweeds have been extensive for systematic purposes (Mallery and Richardson 1971, 1972, Miura et al. 1978a, Marsden et al. 1981, 1984a, b, Blair et al. 1982, Vilter and Glombitza 1983, Rice and Crowden 1987, Lindstrom and South 1989, Lindstrom and Cole 1990), whereas the application of isozyme electrophoresis to analyze genetic differentiation in populations of seaweeds has been very limited compared with other organisms (Innes 1984). Malinowsky (1974) analyzed ten populations of *Codium fragile* and found no differentiation between them. Innes (1987, 1988) described patterns of differentiation among populations of *Enteromorpha linza*, and suggested its maintenance by factors operating on a microgeographic scale. Data on red seaweed are very scarce. Cheney and Mathieson (1979) analyzed eight populations of *Chondrus crispus*, and also suggested the existence of considerable genetic differentiation over relatively short distances. Cheney and Babbel (1978) analyzed four species of *Eucheuma* and found a high genetic identity within *E. isiforme* and *E. nudum*. High genetic differentiation by isozyme electrophoresis was found between Japanese populations of *Porphyra yezoensis* (Miura et al. 1979, Fujio et al. 1985, 1987), but no data are available on agarophytes, the most commercially valuable of the seaweeds.

Fujio et al. (1985, 1987) reported the genetic differentiation between gametophytic populations of *Porphyra yezoensis* to be different from that of diploid organisms, but the amount of genetic variability did not differ much from that of diploid organisms including plants. No study has so far analysed and compared sporophytic and gametophytic subpopulations of seaweeds.

Materials and methods

Sampling

Three populations of *Gelidium arbuscula* (Bory) from three locations in the Canary Islands were examined. Sampling sites are shown in Fig. 1. Plants from a population were collected over a 15 to 20 m^2 area throughout the intertidal zone, at least twice a year during 1989 and 1990. In order to avoid sampling the same individual, single collections were made from aggregations which were at least 20 cm apart, usually on separate rocks. Each individual was transported to the laboratory in a separate plastic bag in an ice cooler. In the laboratory the samples were carefully cleaned of epiphytes and sorted into two subpopulations: tetrasporophytic

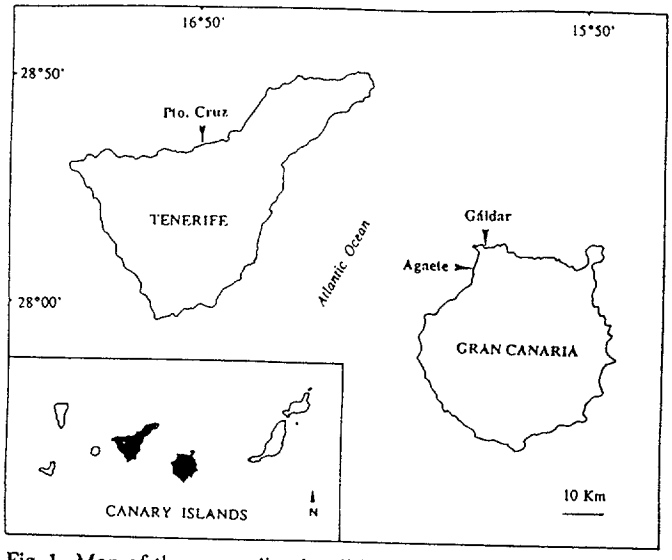

Fig. 1. Map of three sampling localities (arrowed) of *Gelidium arbuscula* in the Canary Islands

(bearing tetrasporangia) and gametophytic (bearing cistocarps). Prior to extraction of enzymes, plants were cultured in 10-litre tanks, under uniform culture conditions: 18 plants per 10 litres of Provasoli-enriched seawater (Provasoli 1968); 21 °C; 18 $\mu E\ m^{-2}\ s^{-1}$ (Gro-Lux, 1:1 cool-white fluorescent lamps); 16 h light:8 h dark; for periods ranging from 1 to 20 d. After 2 mo, the plants were still healthy, and showed no alteration in their enzyme-banding patterns.

Explants (0.30 to 0.5 g) were ground with mortar and pestle in liquid nitrogen until the sample was reduced to a fine powder, before the addition (1:1 w/v) of the grinding buffer. Preliminary experiments to determine the most effective procedure for grinding revealed the best grinding buffer assay to be 0.1 *M* Tris-HCl, pH 7.4, 0.2 *M* L-ascorbic acid, 1 m*M* Na_2 EDTA, 3% (w/v) polyvinylpolypyrrolidone (PVPP).

The tissue-buffer mix was allowed to thaw and the crude extracts were absorbed onto paper wicks (Whatman N°.3; 1 × 0.3 cm) which were inserted vertically into the starch gel (7.5 × 22.5 × 4.5 cm).

Electrophoresis

Proteins were separated by horizontal starch-gel electrophoresis. Starch gels were prepared using 12% (w/v) Sigma starch in 0.015 *M* Tris, 0.003 *M* citric acid, pH 7.8, gel buffer. The electrode buffer consisted of 0.304 *M* boric acid, 0.1 *M* NaOH, pH 8.6 (Poulik buffer system, Cheney 1985). Tris-EDTA-borate, pH 8.6, and Tris-citrate, pH 7.2, buffer systems were also tested, but did not prove as efficient.

The gels were run at 250 V for 10 min, after which the wicks (18 to 20 per gel) were removed. The run was then continued in the same electrical conditions until the tracking dye (bromophenol blue) was 10 cm from the origin (after 3 to 3.5 h).

Following electrophoresis, the gels were cut horizontally into 3 or 4 slices and assayed (anodical and cathodical migration) for the following enzymes (to 100 ml final volume): alkaline phosphatase (ALP; E.C. 3.1.3.1) 0.1 g Fast Blue RR salt, 1.0 g NaCl, 0.1 g $MgCl_2$, 0.05 g $MnCl_2$, in 0.1 *M* Tris-HCl, pH 8.0 (modified from Ayala et al. 1972); NADH-diaphorase (DIA; E.C. 1.6.4.3) 12 mg NADH, 3 ml dimethylthiazoldiphenyl-tetrazolium (MTT) 1% (w/v), 5 mg dichlorophenol indophenol (DCPIP) in 0.1 *M* Tris-HCl, pH 8.0 (modified from Vallejos 1983); esterase (EST; E.C. 3.1.1.1) 0.1 g Fast Blue RR salt, 10 ml β-naphthyl acetate (1% (w/v) in acetone), in 0.1 *M* Tris-HCl, pH 7.1 (modified from Shaw and Prasad 1970); glutamate dehydrogenase (GDH; E.C. 1.4.1.2) 25 ml 1.5 *M* L-glutamic acid, pH 8.0, 40 mg NAD, 3 ml MTT 1% (w/v), 0.5 ml phenazine methosulphate (PMS) 1% (w/v) in 0.1 *M* Tris-HCl, pH 8.0 (modified from Cheney 1985); malate dehydrogenase (MDH; E.C. 1.1.1.37), same conditions as for GDH, but with 1.5 *M* L-malic acid substituted for L-glutamic acid; phosphoglucose isomerase (PGI; E.C. 5.3.1.9) 0.1 g fructose-6-phosphate, 10 mg NADP, 0.1 g $MgCl_2$, 3 ml MTT 1% (w/v), 0.5 ml PMS 1% (w/v), 40 U glucose-6-phosphate dehydrogenase, in 0.1 *M* Tris-HCl, pH 8.0 (modified from Soltis et al. 1983); phosphoglucose mutase (PGM; E.C. 2.7.5.1), same conditions as PGI, but with 0.1 g glucose-1-phosphate substituted for fructose-6-phosphate; superoxide dismutase (SOD; E.C. 1.15.1.1) 0.1 g Na_2 EDTA, 3 ml MTT 1% (w/v), 5 mg riboflavin, in 0.1 *M* Tris-HCl, pH 7.4 (20 min in the dark, and then between 1 to 2 h in the light until bands appear; modified from Vallejos 1983).

Other enzymatic systems were tested: alcohol dehydrogenase, aldolase, amylase, glutamate oxalacetate transaminase, catalase, malic enzyme (NADP-dependent), acid phosphatase, galactose dehydrogenase, α-glycero phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, isocitrate dehydrogenase, lactate dehydrogenase, leucine aminopeptidase, peroxidase, sorbitol dehydrogenase, shikimate dehydrogenase and xantine dehydrogenase; but, after several modifications, no activity was detected.

Individuals from different localities were run together, side by side on the same gel, to estimate whether corresponding electromorphs had similar mobilities. Stain solution without substrate served as controls. After staining, the gels were fixed in 50% ethanol and 3.5% acetic acid.

Banding-pattern interpretation

As formal genetic conclusions were not possible (sexual crosses were not performed) genetic interpretations of non-artefactual banding-patterns were inferred directly from isozyme phenotypes. A tentative interpretation of banding patterns was possible through identification of haploid (gametophytic) and diploid (tetrasporophytic) plants (Cheney and Babbel 1978).

Allelic designations were based primarily upon whether a banding-pattern displayed polymorphic or monomorphic characteristics; i.e., bands in haploid individuals were assumed to be the products of different loci, since only one allele (of the same locus) can occur in haploid individuals. Likewise, bands in the same activity zone which occurred together in diploid plants (heterozygotic) were considered to be alleles of the same locus (Cheney and Babbel 1978). The subunit structure of enzymes was also considered in the interpretation of banding-patterns; heterozygotes for a locus coding a dimeric enzyme will produce three bands, while heterozygotes for a locus coding a monomeric enzyme will produce two bands (Innes 1984).

Phycoerythrin (biliprotein, BP) was visible as a fluorescent band under ultraviolet light, and was used as an internal standard for comparative studies of band mobility. Presumed loci were labelled with the abbreviation for the enzyme name and numbered beginning with the locus displaying the highest anodal migration. Alleles of the same locus were designated with lowercase letters, starting with "a" for the highest anodal electrophoretic migration. Relative mobility, R_f (mobility of the band/mobility of bromophenol blue) was calculed for each electromorph.

Data analyses

The proportion of polymorphic loci (P), mean number of alleles per locus (A/L) and average heterozygosity or average gene diversity (*H*) (Nei 1987), were calculated to determine the amount of genetic variability for each subpopulation.

For each locus in Hardy-Weinberg equilibrium, the expected genotypic frequencies were calculated assuming sexually reproducing populations (panmixia). Since *Gelidium arbuscula* displayed alternation of generations, the allelic frequencies of gametophytes were treated as allelic frequencies of gametes in the population. Under conditions of panmixia, the allelic frequencies of male and female gametophytic subpopulations are identical, and the expected genotypic frequencies of sporophytic subpopulations, assuming mating crosses between male and female gametophytes, must be identical to those theoretically predicted by the Hardy Weinberg law. Therefore, expected genotypes of sporophytic subpopulations were calculated from the allelic frequencies of female gametophytic subpopulations from the same locality. Levene's formula for small samples (Levene 1949) was used to estimate the expected number of sporophytic individuals in Hardy-Weinberg equilibrium.

Chi-square tests (Nei 1987) were used to determine differences in allelic frequencies between haploid and diploid subpopulations for each locus. Chi-square values derived from independent loci were added together following the method of Workman and Niswander (1970).

Genetic identity, I, and genetic distance, D (Nei 1972), were computed for pairwise comparisons of the three localities and for pairwise comparisons of subpopulations within each locality. A dendrogram of genetic distances between subpopulations was constructed by UPGMA (unweighted pair-group method of analysis) cluster-analysis (Nei 1987).

Genetic differentation (and therefore gene flow) between subpopulations was estimated using Nei's coefficient of differentiation (G_{ST}) (Nei 1973). G_{ST} is a measure of the amount of differentiation among subpopulations, and indicates the genetic differentiation between alleles of different populations. G_{ST} can vary from 0 (= populations with same allele frequencies at locus in question) to 1 (= populations fixed for different alleles).

Results

Table 1 lists electromorphs (alleles) and shows their relative mobility (R_f) for each loci in *Gelidium arbuscula*. Fig. 2 presents typical banding patterns for ALP, DIA, PE, GDH, MDH and PGM. All bands migrated anodally. *ALP-1**, *EST-2**, *GDH-1** and *DIA-2** conformed to a monomeric structure enzyme, with two bands occurring in heterozygotic sporophytes (Fig. 2), whereas *MDH-1**, *MDH-2** and *PGI-2** conformed to two allele, dimeric enzymes, with three bands in heterozygotic sporophytes. We identified 22 gene loci, 7 of which (*ALP-1**, *DIA-2**, *EST-2**, *GDH-1**, *MDH-1**, *MDH-2** and *PGI-3**) were polymorphic in at least one subpopulation (Table 1).

The allelic frequencies of polymorphic loci are listed in Table 2. *DIA-2** displayed the greatest amount of variability (all subpopulations exhibited polymorphism), while *ALP-1** and *PGI-3** each exhibited polymorphism in one subpopulation only (Agaete sporophyte and Puerto Cruz sporophyte, respectively). Three alleles were observed for *DIA-2** and *GDH-1**, and two alleles for the remaining loci.

Table 3 summarizes the genetic variability in *Gelidium arbuscula* subpopulations. The diploid subpopulation (tetrasporophytic) displayed higher variability than the haploid subpopulation for all parameters in each locality. The percentage of polymorphic loci ranged from 4.5 to 27.3%, the average for the combined sporophyte subpopulations was 21.2%, and for the combined gametophytic subpopulations 6%. The mean for all subpopulations combined was 13.6%. The average number of alleles per locus ranged from 1.04 to 1.32; a mean of 1.06 for haploid subpopulations, and 1.24 for diploid subpopulations. 1.15 being the average for the species. The average gene diversity for sporophytic subpopulations was 0.070, for gametophytic subpopulations 0.018; 0.044 was the average for the species.

Table 1. *Gelidium arbuscula*. Relative mobilities (R_f) of alleles detected in sporophytic and gametophytic subpopulations from Canary Islands

Invariable loci		Polymorphic loci	
Alleles	R_f	Alleles	R_f
*PE**	0.38	*ALP-1* a*	0.43
*DIA-1**	0.85	*ALP-1* b*	0.38
*DIA-3**	0.59	*DIA-2* a*	0.68
*DIA-4**	0.50	*DIA-2* b*	0.64
*EST-1**	0.82	*DIA-2* c*	0.62
*EST-3**	0.35	*EST-2* a*	0.60
*PGI-1**	0.62	*EST-2* b*	0.58
*PGI-2**	0.60	*GDH-1* a*	0.44
*PGM**	0.79	*GDH-1* b*	0.38
*SOD-1**	0.90	*GDH-1* c*	0.36
*SOD-2**	0.85	*MDH-1* a*	0.73
*SOD-3**	0.77	*MDH-1* b*	0.62
*SOD-4**	0.75	*MDH-2* a*	0.55
*SOD-5**	0.73	*MDH-2* b*	0.53
*SOD-6**	0.70	*PGI-3* a*	0.55
		PGI-3 b*	0.49

Table 2. *Gelidium arbuscula*. Allelic frequencies of polymorphic loci detected in sporophytic (S) and gametophytic (G) subpopulations from three locations in Canary Islands. (*n*): no. of individuals analyzed

Allele	Gáldar		Agaete		Puerto Cruz	
	S	G	S	G	S	G
ALP-1 a*	0.00	0.00	0.09	0.00	0.00	0.00
ALP-1 b*	1.00	1.00	0.91	1.00	1.00	1.00
	(42)	(12)	(49)	(16)	(38)	(13)
DIA-2 a*	0.00	0.00	0.03	0.00	0.70	0.80
DIA-2 b*	0.37	0.10	0.88	0.73	0.30	0.20
DIA-2 c*	0.63	0.90	0.09	0.27	0.00	0.00
	(31)	(20)	(48)	(22)	(46)	(15)
EST-2 a*	0.50	1.00	0.64	1.00	0.79	1.00
EST-2 b*	0.50	0.00	0.36	0.00	0.21	0.00
	(45)	(12)	(38)	(15)	(31)	(16)
GDH-1 a*	0.39	0.17	0.00	0.00	0.00	0.00
GDH-1 b*	0.52	0.83	0.87	1.00	1.00	1.00
GDH-1 c*	0.09	0.00	0.13	0.00	0.00	0.00
	(33)	(12)	(39)	(19)	(35)	(11)
MDH-1 a*	1.00	1.00	0.79	1.00	0.69	1.00
MDH-1 b*	0.00	0.00	0.21	0.00	0.31	0.00
	(46)	(15)	(48)	(14)	(31)	(13)
MDH-2 a*	0.00	0.00	0.02	0.00	0.19	0.00
MDH-2 b*	1.00	1.00	0.98	1.00	0.81	1.00
	(43)	(14)	(48)	(18)	(31)	(11)
PGI-3 a*	0.00	0.00	0.00	0.00	0.07	0.00
PGI-3 b*	1.00	1.00	1.00	1.00	0.93	1.00
	(39)	(12)	(49)	(19)	(37)	(14)

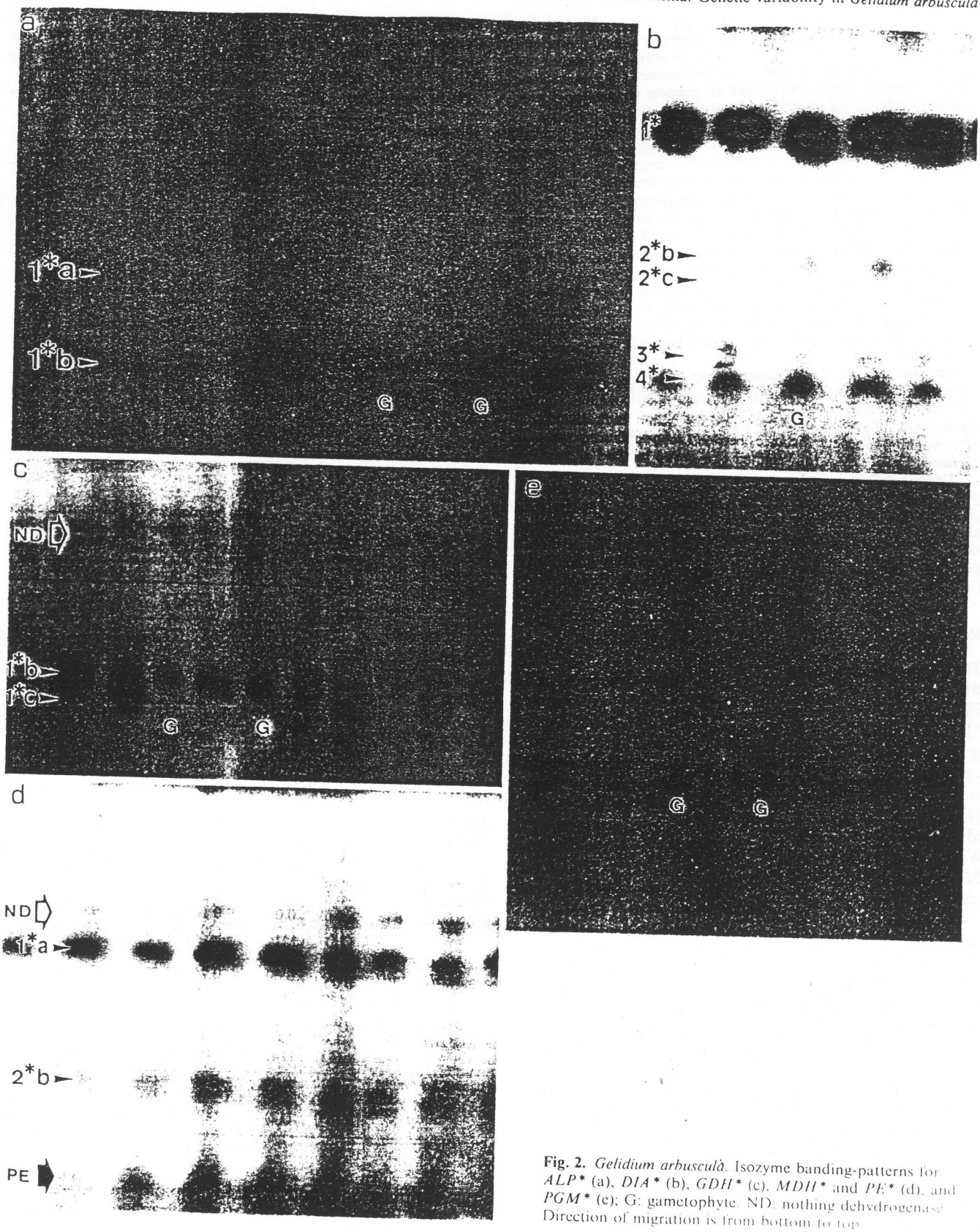

Fig. 2. *Gelidium arbusculà*. Isozyme banding-patterns for *ALP** (a), *DIA** (b), *GDH** (c), *MDH** and *PE** (d), and *PGM** (e); G: gametophyte. ND: nothing dehydrogenase. Direction of migration is from bottom to top

Table 3. *Gelidium arbuscula*. Number of alleles (n), proportion of loci polymorphic (P), average number of alleles per locus (A/L), and average gene diversity (H), recorded in sporophytic (S) and gametophytic (G) subpopulations from three locations in Canary Islands

Location	(n)	A/L	P	H
Gáldar				
S	(26)	1.18	0.136	0.070
G	(24)	1.09	0.091	0.021
Agaete				
S	(29)	1.32	0.273	0.065
G	(23)	1.04	0.045	0.018
Puerto Cruz				
S	(27)	1.23	0.227	0.074
G	(23)	1.04	0.045	0.015
$\bar{x}$ S	(27)	1.24	0.212	0.070
$\bar{x}$ G	(23)	1.06	0.060	0.018
$\bar{x}$ S+G	(25)	1.15	0.136	0.044

Table 4. *Gelidium arbuscula*. Observed, O, and expected (E) genotypes for each polymorphic loci at three locations studied

Genotype	Gáldar		Agaete		Puerto Cruz	
	O	(E)	O	(E)	O	(E)
ALP-1 a/a*	0	(0)	3	(0)	0	(0)
ALP-1 b/b*	42	(42)	43	(49)	38	(38)
ALP-1 a/b*	0	(0)	3	(0)	0	(0)
DIA-2 a/a*	0	(0)	0	(0)	20	(29)
DIA-2 b/b*	6	(0.3)	36	(25.5)	2	(2)
DIA-2 c/c*	14	(25)	0	(3.4)	0	(0)
DIA-2 a/b*	0	(0)	3	(0)	24	(15)
DIA-2 a/c*	0	(0)	0	(0)	0	(0)
DIA-2 b/c*	11	(5.7)	9	(19.1)	0	(0)
EST-2 a/a*	6	(45)	11	(38)	18	(31)
EST-2 b/b*	6	(0)	0	(0)	0	(0)
EST-2 a/b*	33	(0)	27	(0)	13	(0)
GDH-1 a/a*	6	(1)	0	(0)	0	(0)
GDH-1 b/b*	9	(23)	33	(39)	35	(35)
GDH-1 c/c*	2	(0)	4	(0)	0	(0)
GDH-1 a/b*	14	(9.4)	0	(0)	0	(0)
GDH-1 a/c*	2	(0)	2	(0)	0	(0)
GDH-1 b/c*	0	(0)	0	(0)	0	(0)
MDH-1 a/a*	46	(46)	32	(48)	12	(31)
MDH-1 b/b*	0	(0)	4	(0)	0	(0)
MDH-1 a/b*	0	(0)	12	(0)	19	(0)
MDH-2 a/a*	0	(0)	0	(0)	0	(0)
MDH-2 b/b*	43	(43)	46	(48)	19	(31)
MDH-2 a/b*	0	(0)	2	(0)	12	(0)
PGI-3 a/a*	0	(0)	0	(0)	0	(0)
PGI-3 b/b*	39	(39)	49	(49)	32	(37)
PGI-3 a/b*	0	(0)	0	(0)	5	(0)

Table 4 compares the observed and expected numbers of each genotype for each locality. As <1 degree of freedom was recorded for each polymorphic locus, no chi-square test could be performed, but important differences were nevertheless apparent. Although *EST-2*a/a* was the predominant genotype expected for all localities, *EST-2*a/b* was the predominant genotype observed. Similarly, *DIA-2** exhibited clear differences in observed and expected genotypes for all localities. The genotype *GDH-1*b/b* was expected to be predominant in Gáldar samples, but *GDH-1*a/b* was the more predominant genotype observed. Similar results were obtained for *MDI-1** and *MDH-2** loci from Puerto Cruz. In most cases, the allele fixation in gametophytic subpopulations was higher than in sporophytic subpopulations.

Table 5. *Gelidium arbuscula*. Chi-square comparison of allelic frequencies in sporophytic and gametophytic subpopulations from same locality in Canary Islands. Values are means (degrees of freedom) –: no test possible (invariable locus)

Locus	Locality		
	Gáldar	Agaete	Puerto Cruz
*ALP-1**	–	3.1 (1)	–
*DIA-2**	7.3 (1)**	8.8 (2)*	1.1 (1)
*EST-2**	19.8 (1)***	14.3 (1)***	7.8 (1)**
*GDH-1**	7.9 (2)*	4.3 (1)*	–
*MDH-1**	–	7.0 (1)**	10.3 (1)***
*MDH-2**	–	0.7 (1)	4.9 (1)*
*PGI-3**	–	–	2.1 (1)
Total	35.0 (4)***	38.2 (7)***	26.2 (5)***

* $P<0.05$; ** $P<0.01$; *** $P<0.001$

Table 6. *Gelidium arbuscula*. Nei's genetic distance, D (above diagonal) and genetic identity, I (below diagonal) (Nei 1972), between sporophytic (S) and gametophytic (G) subpopulations from Canary Islands. $D = 0.028 \pm 0.011$, $D = 0.030 \pm 0.009$, $D = 0.026 \pm 0.007$, and $D = 0.028 \pm 0.003$, between sporophytic plus gametophytic subpopulations, between sporophytes, between gametophytes, and between sporophytic and gametophytic subpopulations, respectively

	Gáldar (S)	Gáldar (G)	Agaete (S)	Agaete (G)	Puerto Cruz (S)	Puerto Cruz (G)
Gáldar (S)		0.019	0.024	0.027	0.043	0.047
Gáldar (G)	0.981		0.040	0.019	0.043	0.036
Agaete (S)	0.976	0.961		0.010	0.024	0.035
Agaete (G)	0.973	0.981	0.990		0.026	0.023
Puerto Cruz (S)	0.958	0.958	0.976	0.974		0.009
Puerto Cruz (G)	0.954	0.965	0.966	0.977	0.991	

Table 5 shows the results of chi-square tests testing for differences in allelic frequency at each polymorphic locus. Most comparisons between independent loci revealed significant differences at each locus, and χ^2 tests were, as a whole, significant at the 0.001% level for all comparisons.

Table 6 shows Nei's genetic identity, I (below diagonal), and genetic distance, D (above diagonal), between subpopulations of *Gelidium arbuscula*. The average genetic distance between all subpopulations was 0.028, while the average genetic distance between sporophytic subpopulations was 0.030, and between gametophytic subpopulations 0.026.

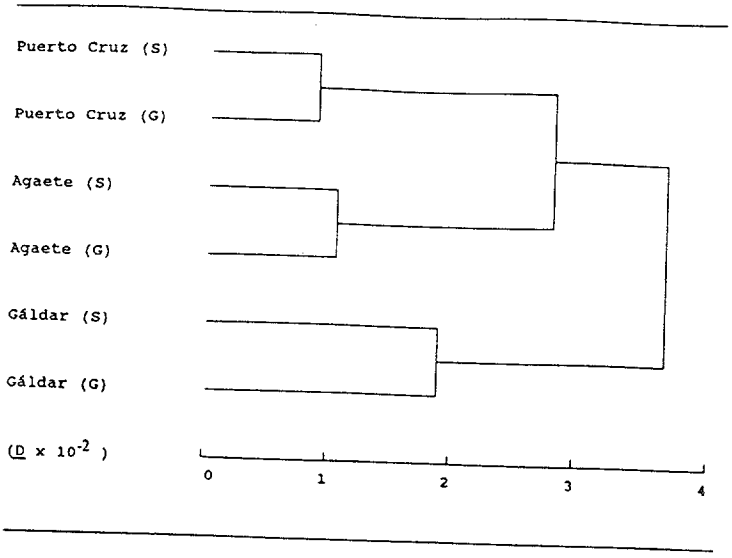

Fig. 3. *Gelidium arbuscula*. UPGMA dendrogram of genetic distances, *D* (Nei 1972), between sporophytic (S) and gametophytic (G) subpopulations from the Canary Islands

Table 7. *Gelidium arbuscula*. Coefficient of genetic differentiation (Nei 1973) between all sporophytic and gametophytic subpopulations for each polymorphic loci. G_{ST}: measure of amount of differentiation among subpopulations (indicates genetic differentiation between alleles of different populations)

Sporophytic subpopulations		Gametophytic subpopulations	
Locus	G_{ST}	Locus	G_{ST}
*ALP-1**	0.062	*GDH-1**	0.120
*DIA-2**	0.404	*DIA-2**	0.548
*EST-2**	0.061	$\bar{x}$	0.334
*GDH-1**	0.227		
*MDH-1**	0.116		
*MDH-2**	0.112		
*PGI-3**	0.048		
$\bar{x}$	0.147		

Fig. 3 is an UPGMA dendrogram of genetic distances, *D*, between subpopulations. Subpopulations of the same locality (independent of their ploidy) displayed the least genetic distance. No relationship was found between genetic and geographic distance. Subpopulations from different islands (Agaete and Puerto Cruz, Fig. 1) exhibited less genetic distance than subpopulations from the same island (Agaete and Gáldar, Fig. 1).

Table 7 shows the coefficient of genetic differentiation for the individual polymorphic loci, and the average differentiation between sporophytic subpopulations, and that between gametophytic subpopulations. The degree of differentiation was higher between haploid subpopulations ($G_{ST} = 0.334$) than between sporophytic subpopulations ($G_{ST} = 0.147$).

Discussion

This study on *Gelidium arbuscula* is the first to compare allelic frequencies between sporophytic and gametophytic subpopulations of a seaweed. Differences in allelic frequencies within each locality (Tables 2 and 5) and departures from Hardy-Weinberg equilibrium in sporophytic subpopulations (Table 4) indicate significant differences among the gene pool of sporophytic and gametophytic subpopulations. Sporophytes and gametophytes of *G. arbuscula* coinhabit, with no along-shore differences. Although we cannot dismiss the possible existence of selective forces acting in each subpopulation to maintain a given gene frequency, the predominance of asexual reproduction in both states could be the main reason of the genetic differentiation within each locality. Stoloniferous outgrowths of creeping axes is a common way for the propagation of *Gelidium* spp. on hard substrate (Oliveira 1989, Rueness and Fredriksen 1989, Santelices 1989, 1990, Gorostiaga 1990). Vegetative propagation has been interpreted as a conservative mechanism of adaptation in seaweeds (Stebbins and Hill 1980, Santelices 1990). When populations are able to reproduce asexually for several years, Hardy-Weinberg deviations and non-random associations of genotypes develop (Black and Johnson 1979, Hebert and Crease 1983, Innes and Yarish 1984, Gallagher 1986, Innes 1987, 1988). A predominance of vegetative propagation will decrease the genetic flow between sporophytes and gametophytes, increasing the differences between gene pools of both within the same population. Innes and Yarish (1984) described large deviations from expected genotypes for populations of sexually reproducing individuals in five populations of *Enteromorpha linza* (L.) J. Ag. from Long Island, USA. These deviations were attributed to the demonstrated asexual reproduction of *E. linza*, with no evidence of segregation by laboratory-culture experiments (Innes and Yarish 1984). Cheney and Babbel (1978) described heterozygote deficiences and Hardy-Weinberg deviations in two ACPH loci of *Eucheuma nudum*, which exhibited a propensity for vegetative reproduction (Cheney and Babbel 1978). Malinowsky (1974) has suggested asexual reproduction to be the cause of the high rate of fixed heterozygotes detected in populations of *Codium fragile* and

the inherent deviations of expected genotypes (Malinowsky 1974).

Gelidium arbuscula is genetically depauperate in the three populations studied. Gametophytic subpopulations have the lowest genetic variability described for seaweeds (Table 8), with the exception of cultivated Japanese populations of *Porphyra yezoensis* (Miura et al. 1979). The cultivated strains of *P. yezoensis* have been subjected to selection over a period of years, which is probably the cause of their low level of genetic variability. The genetic variability of the sporophytic subpopulations of *G. arbuscula*, although higher than that of the gametophytic subpopulations, is also the lowest described for diploid populations of seaweeds (Table 8).

Gelidium spp. are slow-growing perennial rodophytes, which undergo very few changes in biomass, and regenerate new individuals from an established holdfast (Silverthorne 1977, Akatsuka 1986, Oliveira 1989, Santelices 1989, 1990, Gorostiaga 1990). A recent "bottleneck" is therefore unlikely to be the cause of the low levels of genetic variation observed in these populations; were most of the available substrate occupied by perennial individuals with long life-spans, then recruitment would be severely limited and the genetic composition of a given year would remain relatively stable (Innes 1987). Their location – volcanic islands – suggests that these populations may have arisen through a founder effect. This, combined with predominantly asexual propagation, could explain the low genetic variability in *G. arbuscula* from the Canary Islands. It has been reported that asexual reproduction can result in reduced genetic variability in a population by magnifying the effects of selection, random-drift or founder effects (Black and Johnson 1979, Innes and Yarish 1984). In fact, of the factors determining the genetic structure of a population, none has a more profound effect than the mode of "breeding strategy", with the degree of allozyme variation being a strong indicator of the type of breeding strategy employed by a species (Selander and Ochman 1983, Baur and Klemm 1989). The effects of genetic drift arising from founder events have been estimated to result in a 65% reduction in heterozygosity (Nei et al. 1975) and a simultaneous important reduction in the average number of alleles per locus (Systma and Schaal 1985). This appears to be the case for *G. arbuscula* from the Canary Islands. A similar interpretation has been made for natural populations of *Porphyra yezoensis*, in which selfing and asexual propagation have been hypothesized to be responsible for the low genetic variability and the high number of fixed alleles detected (Fujio et al. 1985, 1987), and similar results have been reported for other selfing organisms (Selander and Hudson 1976, Systma and Schaal 1985, Soltis and Soltis 1987, Baur and Klemm 1989).

In the present study, analysis of phylogenetic relations between subpopulations by the UPGMA method has revealed the absence of a correlation between genetic and geographic distances, and the existence of a higher genetic identity between subpopulations at the same locality (independent of the ploidy of the individuals). This indicates that, in addition to high genetic differentiation between subpopulations at the same locality, genetic flow between localities is very reduced or non-existent. *Gelidium* spp. have been described as a genus which release spores, and free-floating fragments of this seaweeds do not seem to be very successful in establishing new populations (Coon et al. 1972, Alvarez et al. 1978, Amsler and Searles 1980, Okuda and Neushul 1981, Gorostiaga 1990, Santelices 1990), supporting the high values for the coefficient of differentiation detected between all localities in the present study (Table 7).

The coefficient of differentiation between localities was higher between gametophytic subpopulations (G_{ST} = 0.334) than between sporophytic subpopulations (G_{ST} = 0.147). Similar results have been reported for wild populations of the haploid laver *Porphyra yezoensis*, which had similar and even higher differentiation coefficients (0.623, 0.125) between populations only a few meters apart (Fujio et al. 1985, 1987). High genetic differentiation has even been described in areas separated by only a few hundred meters for *Enteromorpha linza*, suggesting that geographic microdifferentiation plays some role in generating such differences (Innes 1987, 1988).

It is difficult to explain the low genetic variability and the genetic structure of populations of *Gelidium arbuscula* from the Canary Islands. An increased founder effect, due to a lower number of gametophytes than sporophytes during the colonizing process, combined with predominant asexual reproduction would magnify genetic differentiation, fixation of alleles, and lower the genetic variation of gametophytes. If the same capacity for reproduction and recruitment is assumed for both sporophytes and gametophytes, then the population structure of *G. arbuscula* would be the consequence of the colonizer step; i.e., when populations originated. In the three populations analyzed, the number of mature gametophytic individuals detected was always lower than the number of mature sporophytes (S:G = 7:1; own unpublished data), independent of season or sampling locality. A predomi-

Table 8. *Gelidium arbuscula*. Comparison of genetic variability of natural populations. A/L: Average number of alleles per locus; P: average proportion of polymorphic loci. *H*: Average gene diversity. (h): haploid individuals. (d): diploid individuals. (h, d): haploid and diploid individuals. pop.: populations; sub.: subpopulations; –: no data

Species	A/L	P	*H*	Source
Codium fragile (d)	–	0.310	0.150	Malinowsky (1974)
Eucheuma nudum (h, d)	1.31	0.313	–	Cheney and Babbel (1978)
E. isiforme (h, d)	1.36	0.364	–	
E. gelidium (H, d)	1.29	0.286	–	
E. acanthocladum (h, d)	1.25	0.250	–	
Porphyra yezoensis (h)	1.64	0.333	0.127	Fujio et al. (1985)
P. yezoensis (h)	–	0.583	0.197	Fujio et al. (1987)
P. yezoensis (h)				
Wild pop.	1.50	0.398	–	Miura et al. (1979)
Cultured pop.	1.00	0.000	–	
Gelidium arbuscula				
Sporophytes sub. (d)	1.24	0.212	0.070	Present study
Gametophytes sub. (h)	1.06	0.060	0.018	

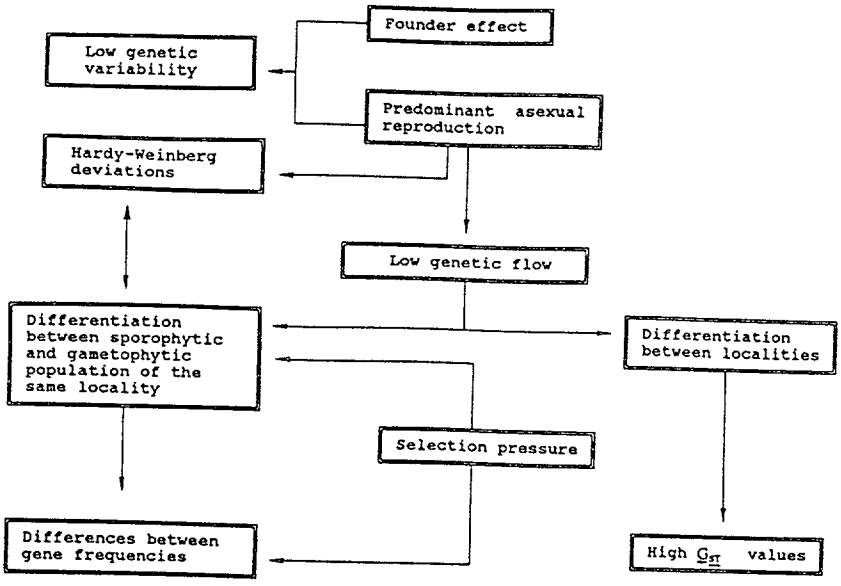

Fig. 4. *Gelidium arbuscula*. Schematic of genetic structure and genetic differentiation of the Canary Islands populations

nance of sporophytes has been described for several populations of *G. sesquipedale* (Seoane-Camba 1965, 1969, Gorostiaga 1990), *G. robustum* (Guzmán del Proó and de la Campa de Guzmán 1969), *G. pristoides* (Carter 1985, Robertson et al. 1985), and *G. amansii* (Akatsuka 1986). This disproportion supports the idea that alternating generations and consequent sexual reproduction is not as important as other ways of propagation.

On the other hand, the higher genetic variability of isozymes recorded for the sporophytic subpopulations of *Gelidium arbuscula* could reflect a higher variability of the global diploid genome, which could promote higher adaptation of diploids and explain the predominance of sporophytes in the populations. Evidence that natural selection could directly or indirectly influence the allele frequencies is suggested from the observations of significant differences in allelic frequency between sporophytes and gametophytes at one and the same locality.

Although it is difficult to estimate fitness differentials associated with loci coding for enzymes, particularly in natural populations (Hilbish and Koehn 1985), genetic factors have been hypothesized to be involved in the predominance of the diploid phase in the life-history of seaweeds (Hansen and Doyle 1976, Litler et al. 1987). Relationships between the polymorphic loci of isozymes and fitness or differentiation processes have been reported. Innes (1988) described significant differentiation between high and low intertidal positions with respect to the *GOT-2* locus in *Enteromorpha linza*. One of the clones (Clone 6, *GOT-2* FM) was usually associated with the high intertidal zone. Miura et al. (1978 b) reported a relationship between the catalase genotype and morphology in *Porphyra yezoensis*. Those thalli with the CAT^A gene were wider and had smooth edges, whilst those thalli with the CAT^B gene were narrower and had crinkled edges. Okumura and Fujino (1986) described a close relationship between the water temperature of the habitat and frequencies of thermostable alleles at the PGM, PGI and GDH loci in two populations of *P. yezoensis*, suggesting that these three loci act as a polygenic system that contribute to temperature tolerance of the seaweed. For the marine mussel *Mytilis edulis*, Hilbish and Koehn (1985) described differences in catalytic efficiences of the LAP locus, and suggested that an allele-frequency cline operates by natural selection at this locus. Similar results have been reported for *Crassostrea virginica* (Singh and Zourous 1978, Zouros et al. 1980) and for *Drosophila* spp. (reviewed by Lewontin 1985).

Fig. 4 summarizes the events that could explain the genetic differentiation and genetic structure of the populations of *Gelidium arbuscula* in the Canary Islands.

Additional studies are needed to determine the importance of ploidy to the relative fitness of sporophytic and gametophytic subpopulations of isomorphic species, but the interaction of drift with natural selection could explain the genetic structure seen in *Gelidium arbuscula*.

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Role of carbonic anhydrase in photosynthesis and inorganic-carbon assimilation in the red alga *Gracilaria tenuistipitata*

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Abstract. The mechanism of inorganic-carbon (C_i) accumulation in the red seaweed *Gracilaria tenuistipitata* Zhang et Xia has been investigated. Extracellular and intracellular carbonic-anhydrase (CA) activities have been detected. Photosynthetic O_2 evolution in thalli and protoplasts of *G. tenuistipitata* were higher at pH 6.5 than at pH 8.6, where HCO_3^- is the predominant form of C_i. Dextran-bound sulfonamide (DBS), a specific inhibitor of extracellular CA, reduced photosynthetic O_2 evolution at pH 8.6 and did not have any effect at pH 6.5. After inhibition with DBS, O_2 evolution was similar to the rate that could be supported by CO_2 from spontaneous dehydration of HCO_3^-. The rate of photosynthetic alkalization of the surrounding medium by the algal thallus was dependent on the concentration of C_i and inhibited by DBS. We suggest that the general form of C_i that enters through the plasma membrane of *G. tenuistipitata* is CO_2. Bicarbonate is utilized mainly by an indirect mechanism after dehydration to CO_2, and this mechanism involves extracellular CA.

Key words: Carbon assimilation – Carbonic anhydrase – Carbon dioxide uptake – *Gracilaria* – Photosynthesis

Introduction

Seawater at normal pH (8.0–8.2) contains 2 mM HCO_3^- and only 10 μM CO_2 (Skirrow 1975) for which reason marine red seaweeds have been proposed to take up both these forms of inorganic carbon (C_i) (Sand-Jensen and Gordon 1984; Bidwell and McLachlan 1985; Beer and Israel 1990; Maberly 1990). Carbon dioxide can enter through lipid membranes by a diffusion process (Gutknecht et al. 1977) and, in addition to this uncatalyzed diffusion of CO_2, several other models for C_i acquisition from the medium into the cell in aquatic plants have been proposed: (i) ATPase-dependent HCO_3^- transport (Raven and Lucas 1985); (ii) H^+/HCO_3^- cotransport or OH^-/HCO_3^- antiport systems (Lucas 1983); (iii) HCO_3^- dehydration in the cell wall dependent on acidic regions created by H^+ extrusion (Lucas 1983); (iv) an "indirect mechanism" of HCO_3^- transport after dehydration to CO_2, i.e. a mechanism requiring extracellular carbonic anhydrase (CA) (Smith and Bidwell 1989a); and (v) active CO_2 transport (Sültemeyer et al. 1989). A specific protein for the transport of HCO_3^- has not yet been identified. Also, the mechanism by which an ATPase is involved in C_i transport is still unknown.

Alkalization of the medium resulting from uptake of C_i has been suggested as being caused by OH^- efflux simultaneous with active influx of HCO_3^- (Lucas 1983; Cook et al. 1988), H^+/HCO_3^- symport (Lucas 1983) or simultaneous uptake of CO_2 and H^+ (Axelsson 1988). The processes involving HCO_3^- transport imply that the dehydration reaction of HCO_3^- to CO_2, which is the substrate for the carbon-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), is intracellular. In the reaction, OH^- is produced and intracellular pH is maintained by efflux of OH^- or influx of H^+. However, for many seaweeds, it has not been determined if the mechanism of alkalization is extra- or intracellular. The extracellular conversion of HCO_3^- to CO_2 and OH^-, and the following uptake of CO_2 would result in direct alkalization of the surrounding medium.

In *Chondrus crispus*, extracellular CA was found to be involved in C_i uptake (Smith and Bidwell 1989a). Involvement of CA was greatest at high pH and low CO_2 concentration, and no evidence of any active or facilitated mechanism for the transport of HCO_3^- in *C. crispus* was detected. However, it has been demonstrated for a wide variety of seaweeds that no extracellular CA activity

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Abbreviations: C_i = inorganic carbon ($CO_2 + HCO_3^-$); CA = carbonic anhydrase; DIC = dissolved inorganic carbon (total); DBS = dextran-bound sulfonamide; EZ = ethoxyzolamide; NSW = natural seawater; PPFD = photosynthetic photon flux density; REA = relative enzyme activity; Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase

is present (Cook et al. 1988; Giordano and Maberly 1989).

In this work we demonstrate the presence of extracellular and intracellular CA in the red seaweed *Gracilaria tenuistipitata*. The role of CA in carbon assimilation is discussed and we propose a model in which seawater HCO_3^- at alkaline pH is converted by external CA to CO_2. The rate of alkalization of the surrounding medium is inhibited by dextran-bound sulfonamide (DBS), which specifically inhibits extracellular CA. We suggest that the alkalization of the medium by *G. tenuistipitata* is an extracellular mechanism.

Material and methods

Cultivation. *Gracilaria tenuistipitata* var. *liui* Zhang et Xia was collected in Hainan Island, south China and has since then been kept in unialgal culture in the laboratory. Plant material was brought to the Marine Plant Biotechnology Laboratory (MPBL), Gran Canaria, where the experiments reported in this paper have been carried out. *Gracilaria tenuistipitata* was cultivated in Plexiglas cylinders as described by Lignell et al. (1987). The photon fluence rate followed natural daily irradiances and was at midday maximum 1200–1350 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The seawater salinity was 38‰ and temperature varied between 20 and 30° C during the day. pH and growth rates were measured daily and algal biomass was harvested down to initial level. Nutrients were applied according to Provasoli (1968) with the following exceptions: nitrogen was added as NH_4NO_3 at 410 µM, phosphorus was added as NaH_2PO_4 at 30 µM and vitamins were excluded. Daily additions were made according to the produced biomass in order to restore the nitrogen and phosphorus concentrations. Fresh weight was determined after mild centrifugation to remove excess water.

Extraction and fractionation of algal material. Algal thallus (10 g) was carefully ground in liquid nitrogen and extracted with a buffer containing 50 mM Tris (pH 8.5), 25 mM dithiothreitol (DTT), 25 mM isoascorbic acid and 5 mM EDTA. Buffer was added to a total volume of 60 ml. Fractionation of the obtained homogenate was then carried out according to Palmqvist et al. (1990b) with some modifications. The procedure is outlined in Fig. 1. The homogenate was first centrifuged at 100 · *g* for 15 min. Pelleted material contained cell debris including cell-wall material and was not used for assay in this study. The 100 · *g* supernatant was centrifuged at 100 000 · *g* for 40 min and the supernatant from this centrifugation was saved for assay of CA. The pellet from this centrifugation contained chloroplast and plasma membranes and was bright green. This pellet was resuspended in 15 ml extraction buffer and spun down at 10 000 · *g* for 10 min. The new pellet was resuspended in 10 ml extraction buffer and contained chloroplast membranes while plasma membranes could be found in the supernatant. Both these fractions were saved for determination of CA activity.

Preparation of thallus fragments. Algal thallus was chopped to 1-mm pieces with a razor blade and rinsed several times in seawater to get rid of soluble protein that may have leaked out from the cut surfaces. Fragments were then transferred to seawater medium and kept with continuous air bubbling and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for at least 6 h before use.

Protoplast isolation. Protoplasts were isolated following the procedure of Björk et al. (1990) with some modifications. Cellulysin 2% (Calbiochem AG, Switzerland) and Agarase 0.01% (Sigma, St. Louis, Mo., USA) were dissolved in seawater at a salinity of 38‰ with addition of 0.2 M mannitol and 20 mM bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane (Bis-Tris), final pH-6.0. The algal thallus was chopped into 1-mm pieces using a razor blade. The fragmented thallus was then rinsed five times in washing buffer

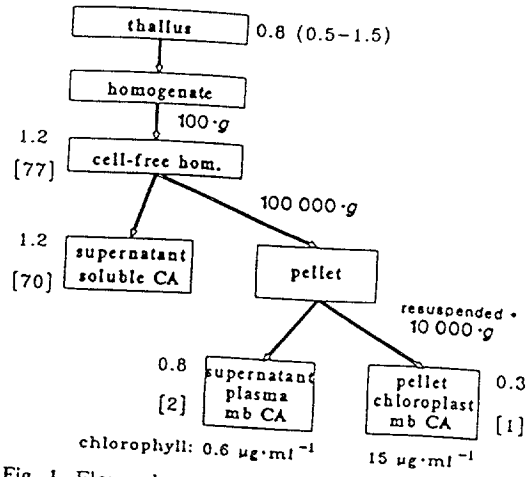

Fig. 1. Flow scheme showing the procedure for fractionation of homogenate from *G. tenuistipitata*. The enzyme activities are denoted beside the boxes of the different fractions and are expressed as REA units · mg^{-1} protein and also as total REA units in each fraction [in *square brackets*]. For thallus, CA activity is expressed per g FW (range indicated in parentheses, $n = 9$) and should not be directly compared with CA activities in the fractions. The fractionation experiment was repeated three times with similar results. The supernatant from the 100 000 · *g* centrifugation had a strong violet colour from phycobiliproteins and the washed pellet was bright green. Chlorophyll concentrations in the two membrane-containing fractions are denoted at the bottom of the figure. *mb*, membrane-bound

(0.2 M mannitol and 20 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid (Hepes) in seawater, pH 7), then incubated for 30 min in seawater with additions of 20 mM Hepes and 0.8 M mannitol, final pH-7.0. One gram of fragmented thallus was immersed in 10 ml enzyme solution and incubation in enzyme solution was performed at 25° C under constant shaking (60 · min^{-1}). After digestion the protoplast suspension was filtered through a 50-µm nylon mesh filter to remove cell-wall debris and non-digested material, and rinsed with washing buffer. The suspension was layered on top of a centrifugation medium consisting of equal parts of Percoll (Pharmacia, Uppsala, Sweden) and 1.2 M mannitol in seawater buffered with 20 mM Bis-Tris (pH 7). This was then centrifuged in a swingout rotor at 100 · *g*, 15 min. The band containing *G. tenuistipitata* protoplasts was resuspended in buffered seawater of pH 6.5 or 8.6 with addition of 0.2 M mannitol to prevent lysis of the protoplasts and centrifuged again at 100 · *g* for 15 min. Yield of protoplasts ranged from 10^6 to $2 \cdot 10^6$ protoplasts per g FW. The resulting pellet of protoplasts was resuspended in the same buffered seawater of pH 6.5 or 8.6 for photosynthesis experiments, as described below.

Preparation of inhibitors. Two inhibitors specific for CA have been used in this study. Stock solutions were prepared as follows: EZ (ethoxyzolamide, Sigma) was dissolved in 0.05 N NaOH to a concentration of 10 mM. Dextran-bound sulfonamide (synthesized according to Tinker et al. (1981), kindly provided by Professor Göran Samuelsson, Department of Plant Physiol., University of Umeå, Sweden) was dissolved in 0.05 N NaOH to a concentration of 0.1 g · ml^{-1} corresponding to 80 mM active inhibitor. Dextran-bound sulfonamide does not penetrate cell membranes and specifically inhibits extracellular CA (Moroney et al. 1985).

Assay of CA in extracts and in thallus fragments. [illegible] drase activity was measured potentiometrically, [illegible] timing the time taken for a linear drop of 0.1 pH unit [illegible] range 8.1 to 7.1 in a cuvette containing [illegible] ml of sample and [illegible] chopped [illegible] range [illegible] [illegible] extract or fresh, chopped

thallus suspended in sample buffer. The sample buffer used was the same as the extraction buffer. The reaction was started by rapidly introducing 2 ml of ice-cold CO_2-saturated distilled H_2O (substrate). The method was modified after Ramazanov and Semenenko (1988). One unit of relative enzyme activity (REA) was defined as $(t_0/t_e)-1$ where t_0 and t_e are the times for pH change of the nonenzymatic (buffer) and the enzymatic reactions, respectively.

For measurement of CA activity in thallus fragments, 0.16–0.29 g FW was transferred directly from seawater to 2 ml of sample buffer. The same method was used as above with the addition of a magnetic stirrer to keep the fragments in suspension. Final inhibitor concentrations used in CA assay were 50–100 µM EZ and 1 g · l^{-1} DBS (800 µM inhibitor).

Photosynthetic O_2 evolution in thallus fragments and protoplasts. Photosynthetic O_2 evolution was measured with a Clark-type electrode (Hansatech Instruments Ltd., Kings Lynn, Norfolk, UK) with the measuring-chamber thermostat set to 25° C. Thallus fragments (0.2–0.3 g FW) or protoplasts (7–8 · 10^5 ml^{-1}) were transferred to the measuring chamber, which contained buffered seawater with a low content of dissolved inorganic carbon (low-DIC) of pH 6.5 (100 mM 3-[N-morpholino]propanesulphonic acid (Mops)) or pH 8.6 (100 mM 2[N-cyclohexylamino]ethanesulphonic acid (Ches)), or fresh natural seawater (NSW). Low-DIC seawater was obtained by acidifying NSW to pH 3 and bubbling for 12 h with CO_2-free air to remove DIC, and then adjusting to the desired pH with carbon-free NaOH. Samples were allowed to consume any remaining C_i and $NaHCO_3$ was then added to desired concentration. The volume was adjusted to 1 ml or 0.5 ml when using protoplasts. Oxygen evolution at a photosynthetic photon flux density (PPFD) of 500 µmol · m^{-2} · s^{-1} was followed on a flatbed recorder. In sequential experiments with thalli the same thallus fragments could be used while changing the solutions.

Measurement of pH change in the medium caused by whole thalli. Alkalization of the medium was measured with a pH meter (CKC UC–23 with UC–502 E electrode; Central Kagaku Co., Tokyo, Japan). A closed system was obtained with the electrode tip immersed in the medium in a 50-ml Ehrlenmeyer flask by sealing the electrode and flask together with Nescofilm and covering the seal with paraffin to prevent CO_2 exchange with the atmosphere.

Before sealing the Ehrlenmeyer flask it was filled to 55 ml with medium and 0.5 g FW *G. tenuistipitata* was placed in the flask together with a bar magnet. The medium consisted of natural seawater (pH 8.12) or low-DIC seawater (pH 8.12) obtained as described above. The change of pH was recorded after turning on the light. The PPFD used was 440 µmol · m^{-2} · s^{-1} and the temperature was 22–24° C.

Measurement of chlorophyll and protein. Chlorophyll was measured after extraction in absolute ethanol according to Wintermans and De Mots (1965), and protein concentration was measured using Serva blue reagent (Serva, Heidelberg, FRG) as described by Peterson (1983). Both chlorophyll and protein were measured in triplicate.

Calculations of spontaneous dehydration of HCO_3^-. The rate of uncatalyzed formation of CO_2 from HCO_3^- in seawater was calculated according to Johnson (1982) using the values of C_i ratios in seawater from Skirrow (1975). The assumption was made that in alkaline pH conditions the CO_2 present in the seawater at the start, as well as the CO_2 formed by dehydration, was consumed almost instantly in photosynthesis and that the CO_2 concentration thus tends to zero. The photosynthetic quotient, i.e. the ratio between O_2 evolution and CO_2 uptake, was not calculated for *G. tenuistipitata*, but the value of 1.17 reported for the O_2/CO_2 ratio of several red, green and brown macroalgae (Axelsson 1988) has been used here.

Results

Carbonic-anhydrase activity. In Fig. 1 we indicate total REA and REA · mg^{-1} protein in thallus fragments and different fractions of homogenized thalli after centrifugation. The results show the presence of extracellular and intracellular CA in *G. tenuistipitata*. Most of the activity was found in the soluble fraction of the cell-free homogenate, although some activity was also found in the green fraction containing chloroplast and plasma membranes. Carbonic-anhydrase activity in fractions of homogenate from *G. tenuistipitata* was inhibited by EZ (DBS not tested); CA activity in *G. tenuistipitata* thallus was inhibited by EZ and DBS

Photosynthetic O_2 evolution in thallus and effect of specific CA inhibitors. The recorded rate of O_2 evolution of *G. tenuistipitata* thallus in unbuffered NSW (pH 8.1, DIC 2 mM) was 400 nmol O_2 · g FW^{-1} · min^{-1} (Fig. 2). In buffered seawater the rate of photosynthetic O_2 evolution was highest at pH 6.5 where $NaHCO_3$ had been added to a DIC concentration of 0.5 mM. At pH 8.6 with a DIC concentration of 2 mM, O_2 evolution was one-third of the rate at pH 6.5. When the thallus was transferred from pH 8.6 (2 mM DIC) to pH 6.5 (0.5 mM DIC), photosynthetic O_2 evolution increased to the level recorded first at pH 6.5 (data not shown). The rates of photosynthetic O_2 evolution recorded at pH 8.1 (NSW, 2 mM DIC) and at pH 8.6 (buffered seawater, 2 mM DIC) (Fig. 2) are both higher (1.7– to 3-fold) than the theoretical rate that can be supported by the CO_2 derived

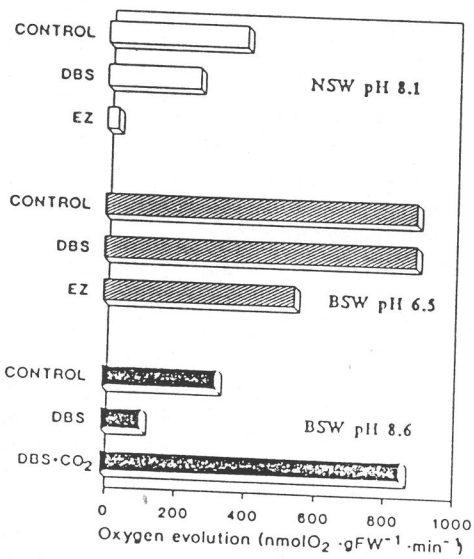

Fig. 2. Photosynthetic O_2 evolution in small thallus pieces of *G. tenuistipitata* and the effect of specific CA inhibitors. Oxygen evolution was measured in three different media, in NSW (pH 8.1, DIC 2 mM), buffered seawater of pH 6.5 (DIC 0.5 mM) and in buffered seawater of pH 8.6 (DIC 2 mM). Inhibitor concentrations: EZ = 100 µM; DBS = 1 g · l^{-1} (800 µM inhibitor). The PPFD was 500 µmol · m^{-2} · s^{-1}. Values are means of ten measurements and variability for the datum points never exceeded 10% of the mean results. *NSW* natural seawater, *BSW* buffered seawater

Table 1. Comparison of the observed rates of photosynthetic O_2 evolution at alkaline pH in *G. tenuistipitata* with the O_2 evolution that could, in the present experimental setup, theoretically be supported by the CO_2 derived from uncatalyzed, spontaneous dehydration of HCO_3^- (according to Johnson 1982), assuming a photosynthetic quotient of 1.17 (Axelsson 1988). pH and HCO_3^--concentration both determine the theoretical dehydration rate. The observed photosynthesis rates were calculated from the values shown in Fig. 2. *NSW*, natural seawater; *BSW*, buffered seawater

Treatment	O_2 evolution (nmol · ml^{-1} · min^{-1})			
	NSW pH 8.1, 2 mM DIC		BSW pH 8.6, 2 mM DIC	
	Observed	CO_2-supported	Observed	CO_2-supported
Control	100	56	83	27
DBS 800 µM	67	56	29	27

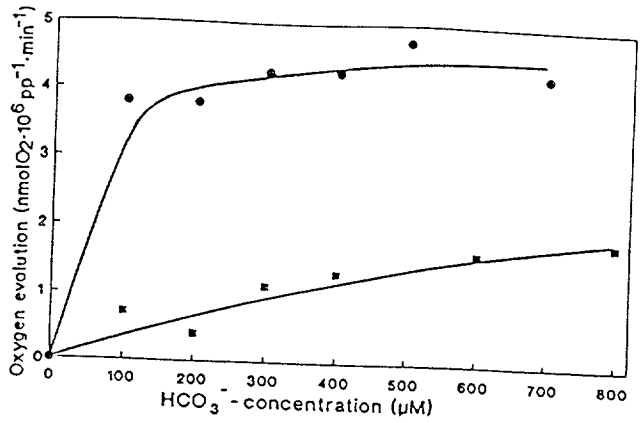

Fig. 3. Photosynthetic O_2 evolution by protoplasts isolated from *G. tenuistipitata* in buffered seawater of pH 6.5 (•) and pH 8.5 (■) with increasing concentration of HCO_3^-. Values on the x-axis show added concentrations of HCO_3^-. 1 g FW thallus yields approx. 10^6 protoplasts, i.e. the amount used per ml in the experiments. The PPFD was 500 µmol · m^{-2} · s^{-1}. Datum points represent one experiment. The experiment was repeated twice with similar results. *pp.* protoplasts

from uncatalyzed, spontaneous dehydration of HCO_3^- (see Table 1).

In natural seawater (pH 8.1, DIC 2 mM) EZ inhibited O_2 evolution by 90% while the inhibition with DBS was approx. 30%. At pH 8.6 (2 mM DIC) in buffered seawater, DBS reduced O_2 evolution by 65%. The rates of O_2 evolution after inhibition by DBS were, at both pHs, similar to the theoretical rates that could be supported by spontaneous dehydration of HCO_3^- (Table 1). Photosynthetic O_2 evolution at pH 6.5 (0.5 mM DIC) was not affected by DBS but was reduced by 40% by EZ (Fig. 2). At pH 8.6 (2 mM DIC) upon addition of 10 µl CO_2-saturated water (340 µM CO_2) the rate of O_2 evolution increased to the same level as in pH 6.5 conditions.

Photosynthetic O_2 evolution in protoplasts. In Fig. 3 we show photosynthetic O_2 evolution of *G. tenuistipitata* protoplasts at pH 6.5 and pH 8.6 in the presence of various concentrations of HCO_3^-. At pH 6.5, O_2 evolution increased strongly upon addition of 100 µM HCO_3^- and remained fairly constant at higher concentrations. However, at pH 8.6, O_2 evolution increased slowly and at a more linear rate with increasing HCO_3^- concentration. The O_2 evolution rates at both pH values are lower than can be supported by spontaneous dehydration of HCO_3^-, indicating that the measuring conditions induce some stress to the protoplasts. Oxygen evolution is expressed per 10^6 protoplasts which is the average yield from 1 g FW thallus and also the number of protoplasts present in the measuring chamber (1 ml). Assuming a density of the protoplasts of 1.1 g · cm^{-3} and an average diameter of 11 µm, $1.3 \cdot 10^9$ protoplasts would have the biomass of 1 g FW.

Effect of DBS on the rate of pH change. During cultivation the growth rate of *G. tenuistipitata* was 18–37% · d^{-1} and the pH increased from 8.2 to 9.2 in 7 d. The alkalization only took place during the light period of cultivation; in darkness the medium was slowly acidified.

The pH change caused by photosynthetic alkalization of natural seawater by *G. tenuistipitata* was recorded with and without addition of DBS. The results are shown in Fig. 4A where pH is plotted against time after turning on the light. Initially, we observed a slight acidification and, after 4–5 min, a linear alkalization. The alkalization rate in the presence of DBS was less than half the rate of the control. The alkalization rate was steady for longer periods than indicated in the figure.

In seawater with low DIC (see *Material and methods*) a slow acidification continued for 3 min and the following alkalization was slow and not linear (Fig. 4B). Upon addition of 1 mM HCO_3^-, alkalization increased to a rate similar to the control experiment in Fig. 4A. This demonstrates that the alkalization rate is dependent on the concentration of DIC. Subsequent addition of DBS strongly inhibited this alkalization. After rinsing the algal thallus to wash away DBS, the alkalization rate was restored to the rate before addition of DBS (data not shown).

Discussion

To our knowledge this is the first report on the presence of CA in *Gracilaria*. The presence of CA in marine macroalgae has previously been demonstrated by several authors, both in homogenates (Bowes 1969; Graham and Smillie 1976; Smith and Bidwell 1987; Beer and Israel 1990) and extracellularly (Giordano and Maberly 1989; Smith and Bidwell 1989a). The presence of CA in microalgae, including the red microalga *Porphyridium cruentum*, both intra- and extracellularly, is also well documented (e.g. Yagawa et al. 1987; Burns and Beardall 1988). Its role in carbon assimilation and photosynthesis is best characterized in the green algal genus *Chlamydomonas*. Studies of C[illegible] accumulation in [illegible] *hardtii* (Moroney et al. 19[illegible] Primavesi et al. 1990) [illegible] shown that external [illegible] time pH catalyzes HCO_3^- to CO_2 which then [illegible] plasmalemma

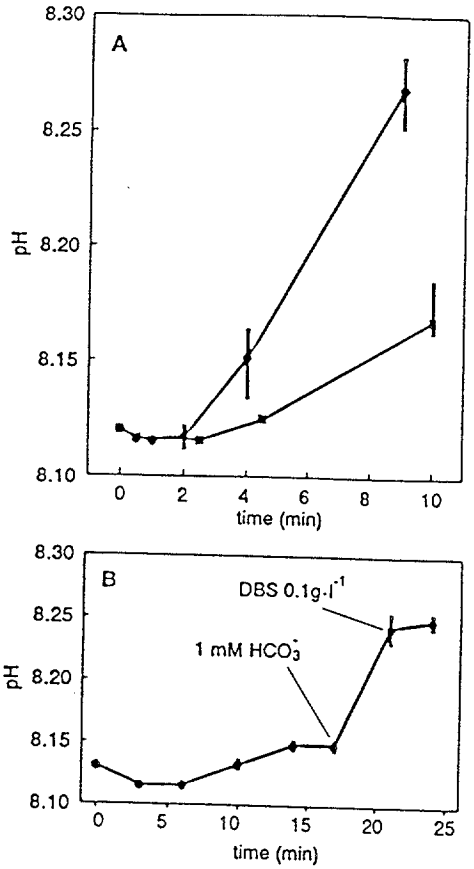

Fig. 4. A Influence of the CA inhibitor DBS on the rate of alkalization by whole thallus of *G. tenuistipitata* in natural seawater. Light (PPFD = 440 μmol · m^{-2} · s^{-1}) was turned on at time 0. Datum points are obtained from calculating the average slope of *n* separate pH-change curves (•, control, $n = 7$; ▪, DBS 0.1 g · l^{-1} (80 μM inhibitor), $n = 4$). B Rate of alkalization by whole thallus of *G. tenuistipitata* in low-DIC seawater and the influence of HCO_3^- and DBS. Additions of HCO_3^- (1 mM) and DBS (0.1 g · l^{-1}, 80 μM inhibitor) are indicated by *arrows*. Light (PPFD = 440 μmol · m^{-2} · s^{-1}) was turned on at time 0. Datum points are obtained from calculating the average slope of 2 separate pH-change curves. *Vertical bars* indicate range. When bars are absent the range is smaller than the size of the symbols

The present report of CA activity in chloroplast membranes in *G. tenuistipitata* is the first for red macroalgae. It is in accordance with the presence of CA in the chloroplasts of microalgae reported by many authors (e.g. Sültemeyer et al. 1990; Coleman et al. 1991). Also, Palmqvist et al. (1990b) demonstrated the presence of CA activity in thylakoid and plasma membranes of four different green algae. The chloroplast of microalgae has been suggested to be the site for a C_i-concentrating mechanism (Beardall 1981; Moroney et al. 1985, 1987). Carbonic-anhydrase activity has also been found in the chloroplasts of the green macroalgae *Codium* and *Udotea* (Reiskind et al. 1988). The presence of CA in different fractions of homogenized *G. tenuistipitata* could involve the presence of different isoenzymes of CA.

Several results in this work indicate that *G. tenuistipitata* possesses extracellular CA and that this enzyme is involved in photosynthesis. Both DBS, which does not penetrate membranes, and EZ, which penetrates easily (Moroney et al. 1985), inhibit photosynthetic O_2 evolution in thallus fragments. However, EZ has a stronger inhibitory effect than DBS since it inhibits both extra- and intracellular CA activity. Smith and Bidwell (1987) have reported similar results with *Chondrus crispus*. Photosynthetic C_i uptake in this alga was inhibited by both acetazolamide and DBS. Inhibition by acetazolamide was greater and the effect was greatest at low CO_2 concentrations.

Since DIC in natural seawater of pH 8.0–8.2 predominantly consists of HCO_3^-, it seems likely that marine algae would be able to utilize this form of C_i. Carbon-acquisition strategies vary among marine algae, as well as the form of C_i utilized for growth and photosynthesis. Marine algae have been shown to use only CO_2 (Tseng and Sweeney 1946; Jolliffe and Tregunna 1970; Maberly 1990), or to have also the ability to utilize HCO_3^- (Sand-Jensen and Gordon 1984; Bidwell and McLachlan 1985; Smith and Bidwell 1987, Lignell and Pedersén 1989; Beer and Israel 1990; Maberly 1990). Results are sometimes conflicting. Thus *Chondrus crispus* has been shown to utilize HCO_3^- (Bréchignac et al. 1986; Smith and Bidwell 1987; Maberly 1990) but Smith and Bidwell (1989a) reported the absence of an active HCO_3^--transporting mechanism in this species. Instead they found CA at the cell surface and proposed a model whereby CA converts HCO_3^- to CO_2 which then passively diffuses into the cell. The same mechanism has been suggested by Surif and Raven (1989) for brown algae of the Fucales and the Laminariales, although they state that diffusive entry of CO_2 into the cell is not enough to supply the Rubisco of these algae and therefore imply that a CO_2-concentrating mechanism operates even when extracellular CA is present.

By contrast, the absence of extracellular CA has been reported in several (including red) macroalgae (Cook et al. 1986, 1988; Maberly 1990). Giordano and Maberly (1989) reported CA to be absent in *Chondrus crispus*, both externally and internally, contrary to the findings of Smith and Bidwell (1989a). In several algae reported to lack extracellular CA, photosynthesis rates exceeding the rate that could be supported by uncatalyzed conversion of HCO_3^- to CO_2 have been found, indicating an uptake of HCO_3^- by these algae (Cook et al. 1986, 1988; Maberly 1990).

Changes in pH have been used as a measure of carbon uptake in macroalgae (Lindahl 1963; Haglund et al. 1987; Axelsson 1988). Moreover, the photosynthetic alkalization of the medium has been suggested as being caused by an energy-driven process involving OH^- efflux or H^+ influx coupled with active transport of HCO_3^- (Kerby and Raven 1985; Cook et al. 1988). In this case, the alkalization takes place inside the cell, and intracellular pH is maintained through efflux of OH^- or influx of H^+.

In *G. tenuistipitata*, we have found extracellular CA and have observed that specific inhibition of this CA considerably reduces photosynthesis, both measured as O_2 evolution and as pH change. We suggest that this alga

uses HCO_3^- and that extracellular CA catalyses the conversion to CO_2 at the surface of the plasmalemma. Carbon dioxide then enters into the cell where its further transport to the site of CO_2 fixation is enhanced by interconversion between CO_2 and HCO_3^- catalysed by intracellular CA (Enns 1967; Kerby and Raven 1985).

This hypothesis is supported by our observation that at alkaline pH photosynthetic O_2 evolution in *G. tenuistipitata* is higher than could be supported by spontaneous conversion of HCO_3^- to CO_2 (see Table 1 and Fig. 2). Also, DBS, a specific inhibitor of extracellular CA, reduces photosynthetic O_2 evolution both in natural seawater (pH 8.2) and in buffered seawater (pH 8.6) to levels similar to those that could be supported by spontaneous dehydration of HCO_3^- to CO_2. These findings imply the involvement of extracellular CA in the photosynthetic carbon uptake of this alga. Photosynthetic O_2 evolution in *G. tenuistipitata* was higher in buffered seawater of pH 6.5 (DIC 0.5 mM), when approx. 30% of the C_i exists as CO_2, than at pH 8.6 (DIC 2 mM), when the concentration of CO_2 is close to zero. This indicates a preference for CO_2 as a carbon source in this alga (Fig. 2). As a further indication of CO_2 preference, addition of CO_2 to *G. tenuistipitata* at pH 8.6 increased O_2 evolution to the same rate as at pH 6.5. This CO_2-addition took place in the presence of DBS, which shows that the inhibition of extracellular CA does not affect CO_2 uptake. Addition of EZ at pH 6.5, however, inhibited the O_2 evolution by 40%, showing the importance of the interconversion between CO_2 and HCO_3^- catalyzed by intracellular CA. Protoplasts of *G. tenuistipitata* exhibited a similar preference for CO_2 as carbon source when O_2 evolution was measured in response to an increased concentration of DIC. The rates of photosynthesis for protoplasts are lower than the rates recorded for thalli if the FW per protoplast is calculated according to our method, indicating that the protoplasts are subjected to stress in vitro. This is in accordance with the results reported by Smith and Bidwell (1989b) for protoplasts of *Chondrus crispus*. The CO_2-preference of *G. tenuistipitata* thalli is in agreement with a report by Sand-Jensen and Gordon (1984) that photosynthetic O_2 evolution declined at pH values above 8.0–8.4 for two red algae also able to use HCO_3^-. As early as 1946, Tseng and Sweeney showed that photosynthetic O_2 evolution in newly collected *Gelidium cartilagineum* was higher at pH 6.5 than at pH 8.5 in normal seawater.

A further indication that extracellular CA is involved in photosynthesis and C_i uptake is the observation that photosynthetic alkalization of the seawater medium by *Gracilaria tenuistipitata* is inhibited by DBS. Such an extracellular-CA-dependent alkalization has recently been reported for the chlorophyte *Ulva rigida* by Björk et al. (1992).

Conclusions. We assume that extracellular CA in *G. tenuistipitata* converts HCO_3^- in the surrounding medium to CO_2, which is the form of C_i entering through the plasma membrane. The high amount of soluble CA in *G. tenuistipitata* indicates that intracellular CA is involved in the subsequent transport and assimilation of C_i. The presence of CA in the fraction containing chloroplast membranes indicates the involvement of CA in interconversion and transport of CO_2 and HCO_3^- also in the chloroplast.

Furthermore, we suggest that the CA-catalyzed dehydration of HCO_3^- followed by CO_2 uptake causes alkalization of the surrounding medium, i.e. the photosynthetic alkalization is an extracellular process in *G. tenuistipitata*. From the presented data we cannot entirely exclude the presence of an additional HCO_3^--transporting system in this alga, but such a contribution would be minor. Investigations of the role of the chloroplast in C_i acquisition in *G. tenuistipitata* are needed and further experiments to clarify the C_i-uptake mechanisms close to the site of Rubisco are in progress.

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Osmotic-adjustement in the cyanobacterium *Spirulina platensis:* Presence of an α-glucosidase

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Cell-free extracts of *Spirulina platensis* grown under a hypersaline condition showed substantial levels of in vitro α-D-glucosidase activities. The activity of this enzyme increased upon transfer to hyposaline medium. The increase in *in vitro* activity of the α-D-glucosidase in the down-NaCl-shock cells was prevented by addition of an inhibitor of either RNA biosynthesis (streptomycin or methyl-purine), or protein biosynthesis (chloramphenicol). A possible involvement of the cyanobacterial α-D-glucosidase in the osmotic adjustement of *S. platensis* is discussed.

Additional key words – Cyanobacteria, glycosyl-glycerol, saline stress.

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INTRODUCTION

The utilization of cyanobacteria as a source of biomass and for the extraction of economically important metabolites has received great attention (Dubinsky *et al.*, 1978; Shefel and Soeder, 1980; Aaronson and Dubinsky, 1982; Vonshak, 1990). These organisms occur in environments which show dramatic changes in various environmental parameters such as salinity. Cyanobacteria exposed to salt accumulate osmotically active compounds to generate a positive hydrostatic pressure (Stal and Reed, 1987). The accumulation of various osmolytes makes it possible for the organisms to adjust to different osmotic pressure, the salinity of the medium, growth conditions, temperature and the light/dark conditions (Müller and Wegmann, 1978 *a* and *b;* Setter and Greenway, 1983; Warr *et al.*, 1985 *a* and *b*). Among the most studied cyanobacteria are *Spirulina* spp., a filamentous, non-heterocystous organism which has the unique capacity to grow at high temperature in highly alkaline waters (Ciferri, 1983; Richmond, 1988; Vonshak, 1990). Cultivation of *Spirulina* species in sea-water has become an alternative to improve industrial biomass production. Arid areas where the climatic conditions are favorable for outdoor cultivations and with available sea-water are ideal for massive culture of this halotolerant organisms (Materassi *et al.*, 1984; Tredici *et al.*, 1986). To determine the suitability of sea-water for mass culture of different *Spirulina* species/strains, it is necessary to understand the mechanism(s) of adaptation to high concentrations of NaCl, and its physiological and biochemical effects, on the exposed cells (Materassi *et al.*, 1984; Warr *et al.*, 1985 *a;* Vonshak *et al.*, 1988).

Earlier studies with *S. platensis* have demonstrated an intracellular osmotic adjustement of low-

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molecular-weight carbohydrates, synthesis and accumulation of glycosyl-glycerol [*O*-α-D-glucopyranosyl-(1 → 2)-glucerol] and trehalose, in response to high concentrations of NaCl (Warr *et al.*, 1985 *a* and *b*, 1987). Glucosyl-glycerol is a nine carbon heteroside (Kollman *et al.*, 1979), a structural analog to floridoside (galactosyl-glycerol) found in Rhodophyta (Reed *et al.*, 1984). Glucosyl-glycerol, the major osmoticum, is accumulated in proportion to the external salinity (Warr *et al.*, 1985 *a*) When cells of *S. platensis* were subjected to a hyperosmotic shock (transferred from low to high salt concentration) the accumulation of glucosyl-glycerol was accompanied by a decrease in glycogen content. Similarly, a hypo-osmotic shock resulted in a decrease in glucosyl-glycerol content and an increase in the glycogen level, indicating that glucosyl-glycerol/glycogen interconversions may be partially responsible for the changes in glucosyl-glycerol content of *S. platensis* (Warr *et al.*, 1985 *a*).

In red algae it has been demonstrated that the concentration of the osmoticum floridoside (galactosyl-(1 → 2)-glycerol) increased upon exposure to an elevated external osmotic pressure (Kauss, 1968; Kirst and Bisson, 1979; Reed *et al.*, 1980; Reed, 1985). Floridoside is degraded by an α-galactoridase. The activity of this enzyme, regulated at the level of its synthesis, increased when the algae were transferred from hypersaline to hyposaline medium and decreased when it was transferred back to hypersaline medium (Yu and Pedersén, 1990 *a* and *b*).

Isofloridoside (galactosyl-(1 → 1)-glycerol), an isomeric form of floridoside, has been described as the osmolite in the chrysophyte *Poterioochromonas malhamensis*, an unicellular fresh-water flagellate (Dey, 1980). An increase in external concentration of NaCl caused the algal cells to shrink within 1-2 min. However, the cell volume recovered within 2 h. This recovery was accompanied by an increase in the internal level of isofloridoside (Kauss, 1973). Reducing the concentration of NaCl resulted in a rapid decrease in the internal level of isofloridoside (Kauss, 1979). An α-galactosidase isolated from *P. malhamensis* is involved in regulating the level of isofloridoside (Dey and Kauss, 1981). Activity of this α-galactosidase has been shown to be regulated at the transcription level as the addition of a protein synthesis inhibitor abolished the induction of this enzyme under high concentrations of NaCl (Dey, 1980).

We have examined the presence of an α-glucosidase in cell-free extracts of the filamentous halotolerant cyanobacterium *S. platensis*, and determined its responses to hypo-osmotic shock.

MATERIALS AND METHODS

Organism and growth condition. Axenic *Spirulina platensis* Geitler (Sammlung von Algenkulturen, Göttigen Universität, Germany) cells were maintained in 50 ml Erlenmeyer flasks containing 25 ml of Zarrouk medium (Zarrouk, 1966). Experimental cells were culturel in Zarrouk medium supplemented with 1 or 4 % NaCl (Zarrouk medium contains 0.1 % NaCl), on a shaker, at 25°C, and under continuous light [THORN Polylux 4000, and OSRAM Warmton Warm White (400-700 nm), providing 220 μmol m^{-2} s^{-1} at the surface of the flasks]. After 4 days of growth (*i.e.* cells in exponential growth phase), the cultures were harvested and resuspended in fresh Zarrouk medium (*i.e.* 0.1 % NaCl). Samples were taken after 5, 10, 15, 20 and 25 h for the measurements of *in vitro* α-glucosidase activity.

Preparation of cell-free extracts. Cells of *S. platensis* were harvested (glassfilter; Whatman GF/C), and ground in 3 ml chilled (4°C) phosphate buffer [50 mM NaH_2PO_4-NaOH (pH 7.4) containing 14 mM β-mercaptoethanol], at room temperature, using a chilled (4°C) mortar-pestle. After centrifugation (2000 × *g*, 4°C, 10 min) the supernatants were immediately used for *in vitro* α-glucosidase activity measurements.

Analytical determinations. The *in vitro* activity of α-glucosidase (EC 3.2.1.20) was assayed by incubating 150 μl of cell-free extract with 100 μl of 13.5 mM *p*-nitrophenyl-α-D-glucopyranoside, 100 μl of phosphate buffer (see above) and distilled water to a final volume of 540 μl. After incubating at 30°C for 30 min, the reaction was terminated by the addition of 360 μl of 1 M Na_2CO_3. Liberated nitrophenol was measured at 405 nm using a Hitachi U-2000 spectrophotometer. Specific activity of α-glucosidase was expressed as pkat mg^{-1} protein. Total amount of proteins in the cell-free extracts were calculated using the method described by Peterson (1983), and bovine serum albumin as a standard.

Inhibitor studies. Cells of *S. platensis* grown in Zarrouk medium with the addition of 4 % NaCl were harvested and transferred to 25 ml fresh Zarrouk medium (0.1 % NaCl) without or with the addition of either a transcription inhibitor [streptomycin (75 μg ml^{-1}) or methylpurine (250 μg ml^{-1})], or a protein synthesis inhibitor [chloramphenicol (50 μg ml^{-1})]. Samples were then taken for measurements of *in vitro* α-glucosidase activities after 5 h. Chemicals used were from Sigma (USA).

RESULTS

General properties

High *in vitro* activity of α-glucosidase was obtained when the cell-free extract from *S. platensis*

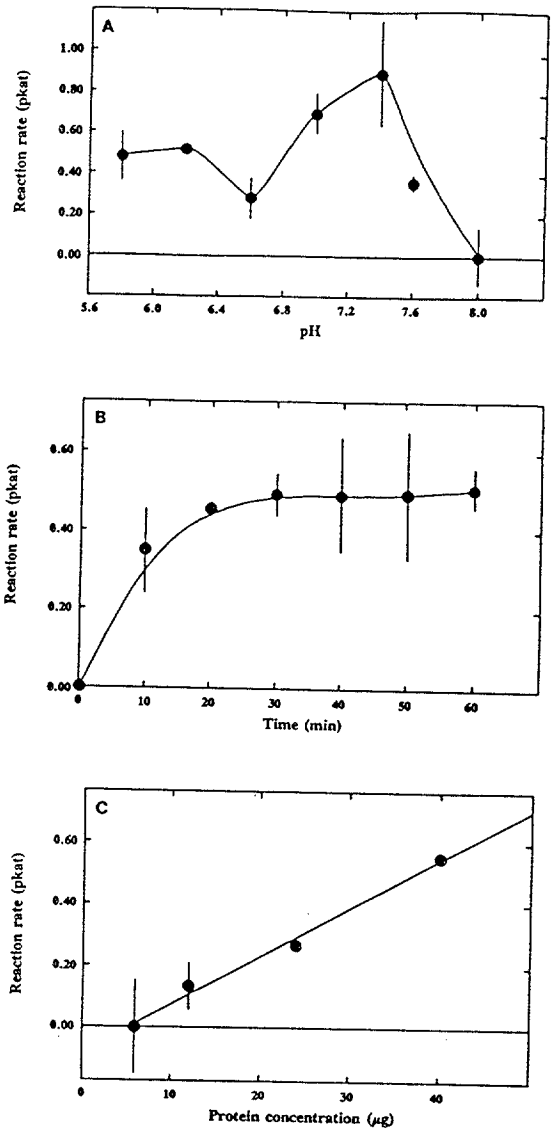

Figure 1. *Effect of pH* (A), *incubation time* (B) *and total protein content* (C) *on the* in vitro *activity of α-glucosidase in cell-free extracts from* Spirulina platensis *grown in the light.* The results presented in A and B correspond to two separate cell-free extracts used in each of the pH or time incubations, respectively. Values given are means ± S.D. (n = 4).

was prepared and assayed in a phosphate buffer (50 mM NaH_2PO_4-NaOH). Early experiments using 50 mM Bicine-NaOH (pH 8.2) did not show any appreciable *in vitro* activities (data not shown). The optimal pH for activity of α-D-glucosidase was found to be 7.4 (*fig.* 1 A), and the cell-free extracts could be stored at +4°C for 24 h without losing activity. Figure 1 B shows the activity of α-glucosidase as a function of reaction time at 30°C and a pH of 7.4. Using a phosphate buffer with a pH of 7.4 and incubation time of 30 min, we checked the relation between total amount of protein in the cell-free extract versus the enzyme activity. Ten μg, or less, of total protein content in the *in vitro* assay did not show any detectable level of α-glucosidase activity. However, a linear response was observed when using total protein concentrations between 10 and 40 μg (*fig.* 1 C).

Substrate specificity of α-glucosidase

The enzyme α-glucosidase showed activity only when using *p*-nitrophenyl-α-D-glucopyranoside as the substrate. Alternative substrates, methyl-α-D-glucoside, trehalose, and maltose, did not support any α-glucosidase activity (data not shown).

Effect of down-shock on the *in vitro* enzyme activity

Cell-free extracts of *S. platensis* grown under a hypersaline condition [Zarrouk medium (0.1 % NaCl) supplemented with either 1 or 4 % NaCl] showed detectable levels of *in vitro* α-glucosidase activities. When cells cultured with the addition of 4 % NaCl were transferred to a hyposaline condition [Zarrouk medium (0.1 % NaCl)] the *in vitro* α-glucosidase activity started to increase and reached a maximal level approximately 10 h after the transfer. Thereafter,

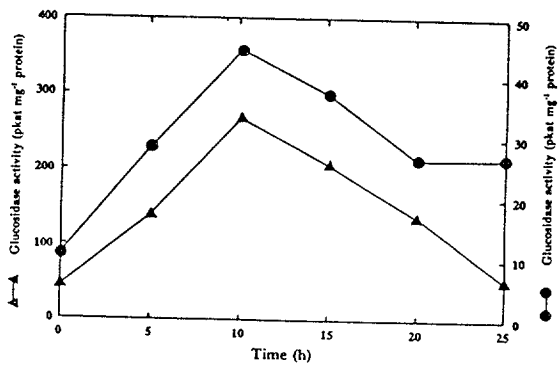

Figure 2. *Effects of down-shock on the* in vitro *activity of α-glucosidase in cell-free extracts from* Spirulina platensis *grown in the light.* At time zero, a culture of *S. platensis* grown at the salinity of 4 % NaCl (▲), or 1 % NaCl (●), was transferred to a medium containing 0.1 % NaCl (Zarrouk medium). Samples were then taken for measurements of *in vitro* α-glucosidase activities during 25 h.

the activity decreased and restored the initial level after 25 h (*fig.* 2). A similar response was observed in cultures down-shocked from 1 % to 0.1 % NaCl. The α-glucosidase activity increased over 10 h of hypo-osmotic shock before starting to decrease and reach a new level after 20 h (*fig.* 2). Considerable higher *in vitro* α-glucosidase activities were found in cells transferred from 4 % NaCl to Zarrouk medium compared with cells transferred from 1 % NaCl to Zarrouk (*fig.* 2).

Effect of inhibitors of RNA and protein biosynthesis on the activity of α-glucosidase

The increase in *in vitro* activity of α-glucosidase in the down-NaCl-shock was prevented by addition of either an inhibitor of RNA biosynthesis (streptomycin or methyl-purine), or an inhibitor of protein biosynthesis (chloramphenicol) (*tab.* 1).

Table 1. *Effect of addition of a transcription inhibitor (streptomycin or methyl-purine), or a protein synthesis inhibitor (chloramphenicol) on the* in vitro *activity of α-glucosidase from* Spirulina platensis *grown in the light.* At time zero, a culture of *S. platensis* grown at the salinity of 4 % NaCl was transferred to medium containing 0.1 % NaCl (Zarrouk medium) without or with the addition of an inhibitor. Samples were then taken for measurements of *in vitro* α-glucosidase activities after 5 h of incubation. Values given, in pkat mg^{-1} protein, are means ± S.D. (n = 3).

Experimental conditions	α-Glucosidase activity *(pkat mg^{-1} protein)*
Control (no addition)	85.3 ± 0.4 (1204)[a]
+ Streptomycin *(75 μg ml^{-1})*	11.2 ± 0.5 (159)
+ Methyl-purine *(250 μg ml^{-1})*	8.8 ± 0.5 (125)
+ Chloramphenicol *(50 μg ml^{-1})*	0.7 ± 0.06 (10)

[a]Relative activity compared to that observed at time zero. 100 %, 7.1 ± 0.3 pkat mg^{-1} protein (n = 3) corresponds to cultures grown at salinity of 4 % NaCl.

DISCUSSION

This is, to our knowledge, the first report describing the existence of an α-glucosidase in the filamentous halotolerant cyanobacterium *S. platensis*. The enzyme α-glucosidase showed no activity with a variety of glucosidase substrates with the exception of *p*-nitrophenyl-α-D-glucopyranoside. The optimal pH profile showed a peak of 7.4. This contrasts to that of higher plants which lie in the acidic range, pH 4-5 (Dey and Campillo, 1984). However, in agreement with α-glucosidase from other sources (Dey and Campillo, 1984), no cofactors were necessary for the catalytic activity of α-glucosidase from *S. platensis*.

It is known that the intracellular pool of the osmoticum glucosyl-glycerol in *S. platensis* increases at high external osmotic pressure; in response to a hypo-osmotic shock this glucosyl-glycerol content is converted into glycogen (Warr *et al.*, 1985 *a*). Interestingly, higher *in vitro* activities of α-glucosidase were detected in cultures transferred from 4 % NaCl compared to those transferred from 1 %, indicating a correlation between the pool size of glucosyl-glycerol and α-glucosidase activity. Similar results have been found with the red macroalgae *Gracilaria teniuistipitata* and *G. sordida*, when transferred from a medium of 5 % salinity to hyposaline medium of 1 % salinity, the activity of the enzyme α-galactosidase increased steadily over 24 h and thereafter remained constant (Yu and Pedersén, 1990 *a*). In contrast, the activity of α-glucosidase in the cells of *S. platensis* began to decrease rapidly after 10 h of increase, and a new level, close to the initial level of activity, was reached. Our data, and those obtained from the red algae (Yu and Petersén, 1990 *a*), differ from those reported for *Poterioocromonas malhamensis* (Kreuzer and Kauss, 1980). In this microalgae, high activities of α-galactosidase was always present in cells exposed to high salinities.

In *S. platensis*, the increase in α-glucosidase induced by down-shock was prevented by incubating the cells with an inhibitor of either protein or RNA biosynthesis; chloramphenicol, streptomycin or methyl-purine (*tab.* 1). The results suggest that α-glucosidase is a *de novo* synthesed enzyme rather than a pre-existing form(s) which can be either active or inactive. In agreement with this, the osmoregulatory enzyme α-galactosidase in *P. malhamensis* also seems to be regulated, based on inhibitor studies, on the level of its synthesis (Kreuzer and Kauss, 1980). Moreover, the activity of the osmoregulatory enzyme α-galactosidase from the red algae *G. tenuistipitata* and *G. sordida* decreased by addition of either inhibitors of RNA or protein synthesis. However, the responses to theses inhibitors were not immediate (Yu and Pedersén, 1990 *a*).

In conclusion, the present study clearly demonstrates the presence of a functionnal α-glucosidase in the filamentous halotolerant cyanobacterium *S. platensis*. Moreover, a substantial stimulation in *in vitro* activity, regulated on the level of transcription, is observed upon transfer from a hypersaline to a hyposaline condition.

Acknowledgments. We thank Dr. Ziydin Ramazanov for constructive discussions. Antera Martel is grateful to the Swedish Institute for a European Guest Scholarship. The planning of this cooperation was facilitated by COST 48. This study was financially supported by grants from the Fundación Universitaria de Las Palmas (to AM), Cabildo Insular de Gran Canaria (to AM), Air Europe (to AM), CITYT/SPAIN (SAB 89-0091 and Mar 91-1237; to MP), the Swedish Council for Forestry and Agricultural Research (to MP and SY), and from the Swedish Natural Science Research Council (to PL).

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PRELIMINARY RESULTS ON THE NUTRITIONAL VALUE FOR POULTRY OF *ULVA LACTUCA* PRODUCED IN BIOFILTERING TANKS

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INTRODUCTION

The use of microalgae (including cyanobacteria) in poultry nutrition have been extensively studied in comparison with seaweeds (reviewed by Indergaard & Minsaas, 1991). The main differences between micro and macroalgae are the high protein (more than 50%) and low ash (less than 10%) content of microalgae ; otherwise, crude fiber content (about 5%) is similar in both kind of algae.

The objective of this study was the study of the basic nutritional parameters of *Ulva* biomass cultivated in running through enriched nitrogen seawater, and the effect of different seaweed supplements on the true (TME) and apparent metabolizable energy (AME) and growth trials of chick and cockerel.

MATERIAL AND METHODS

Weekly harvest of *Ulva rigida* cultivated, at 2 and 4 g/l initial density, in 750 l aerated tanks with a daily continuous flow (exchange rates of 4 volumes per day) of 105 mM NH_4^+ from *Sparus aurata* waste waters, and a ammonium efficiency removal of 90%, was washed with fresh water and sun-dried to 15% relative humidity, before powder-grinded, analyzed and used as feed supplement.

Basic nutritional values, true and apparent metabolizable energy of N-enriched *Ulva* were determined. Two standard diets (A and B) with different seaweed inclusion percentages (10, 20 and 50%) were comparatively studied in chicks and cockerels.

RESULTS AND DISCUSSION

Basic nutritional parameters analysed in the powder-grinded biomass are given in Table 1.

Table 1 : Basic nutritional values of N-enriched biomass of *Ulva rigida* ($g.kg^{-1}$ DM).

Nitrogen	28	CA	5
Oil	14	P	1
Crude Fiber	40	Cl	39
NDF	35	Na	9
ADF	30	K	24
Ash	190	Mg	15
Gross energy	1.1 Mj.Kg.DM		

(NDF= Neutro Detergent Fiber; ADF= Acid Detergent Fiber)

The TME of *Ulva rigida* as the sole diet or as 10, 20 or 50% supplement to two standard diets or glucose, are given in Table 2. The TME of *Ulva rigida* as the exclusive diet was 5.7 Mj.Kg DM for chicks and 4.3 $Mj.Kg^{-1}$ DM for cockerels. In general (except for chicks with diet A), the addition of *Ulva* meal (up to 20%) to chick and cockerels standard diets did not modify the TME values (Table 2). Significative differences between chicks and cockerels with standard B diet may be related to the addition of seaweed, but only at 20% supplementation.

Table 2.- True Metabolizable Energy Values (Mj.Kg^{-1} DM) of N-enriched *Ulva rigida* and *U. rigida* supplements to standard diets, on chick and cockerels (n= 40 per treatment).

	Chick	Cockerels	
Seaweed	5.7± 0.31	4.3± 0.37	*
Standard A diet :			
0% seaweed	15.3± 0.35	14.4± 0.23	NS
50% seaweed	13.8± 0.33	14.6± 0.47	NS
	*	NS	
Standard B diet :			
0% seaweed	13.6± 0.16	13.1± 0.10	NS
10% seaweed	13.8± 0.31	12.6± 0.27	NS
20% seaweed	13.9± 0.16	12.3± 0.26	***
	NS	NS	
Glucose (MJ/kg air dried) :			
0% seaweed	(Gross energy) 15.7		
10% seaweed	15.7± 0.20	15.5± 0.06	NS
20% seaweed	15.3± 0.30	15.3± 0.19	NS
	NS	NS	

(*= significative; NS= not significative)

Growth trial was performed with chicks (N=40 per treatment) feeded during 10 day with standard diet and 10, 20 or 30% *Ulva* meal supplements. Résults are given Table 3. The AME values did not exceed 3.2 Mj.Kg^{-1} DM, being too low for poult diets. The inclusion of *Ulva* decreased the metabolizable energy (ME) of diets (MI 10.6-0.08 x %inclusion, R2=0.73). However, addition of *Ulva* did not modify tl metabolizable energy of the standard diet, indicating the absence of antinutrition factors by *Ulva* supplements.

Although, the decrease of weight gains is clearly related with the increase in *Ul* supplements (non quantified), a negative relation with *Ulva* supplementation ai the amount of feed consumed by chicks chould be considered. The lower acceptance seaweed supplemented diets is probably due to organoleptic factors. Adaptation seaweed meals have been described in longer-living animals, but are impossible performe in poultry.

Table 3.- Weight gain (g) and apparent metabolizable energy (AME) (Mj.Kg^{-1} DW) of chicks feed on standard and *Ulva* -supplemented diets after 10 days, (N=40).

	Weight gain	AME
Standard diet	102 g	10.5 Mj/Kg
Standar + 10% seaweed	90 g	10.0
Standar + 20% seaweed	85 g	8.3
Standar + 30% seaweed	77 g	8.3

CONCLUSIONS

Diets containing 10% or higher supplements of crude meal of N-enriched *Ulva rigid* are not convenient for chicks and cockerels. Valorization of seaweed supplemente feed for monogastric animals based only on the values of total metabolizable energ can lead to wrong conclusions.

The effect of additives (enzymes, yeast, etc.) to improve the nutritional value of *Ulva*-supplemented feed, as well as digestibility trials with ruminants, should be evaluate in order to assess the suitability of *Ulva* as feed supplement.

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Cuadernos da Área de Ciencias Mariñas

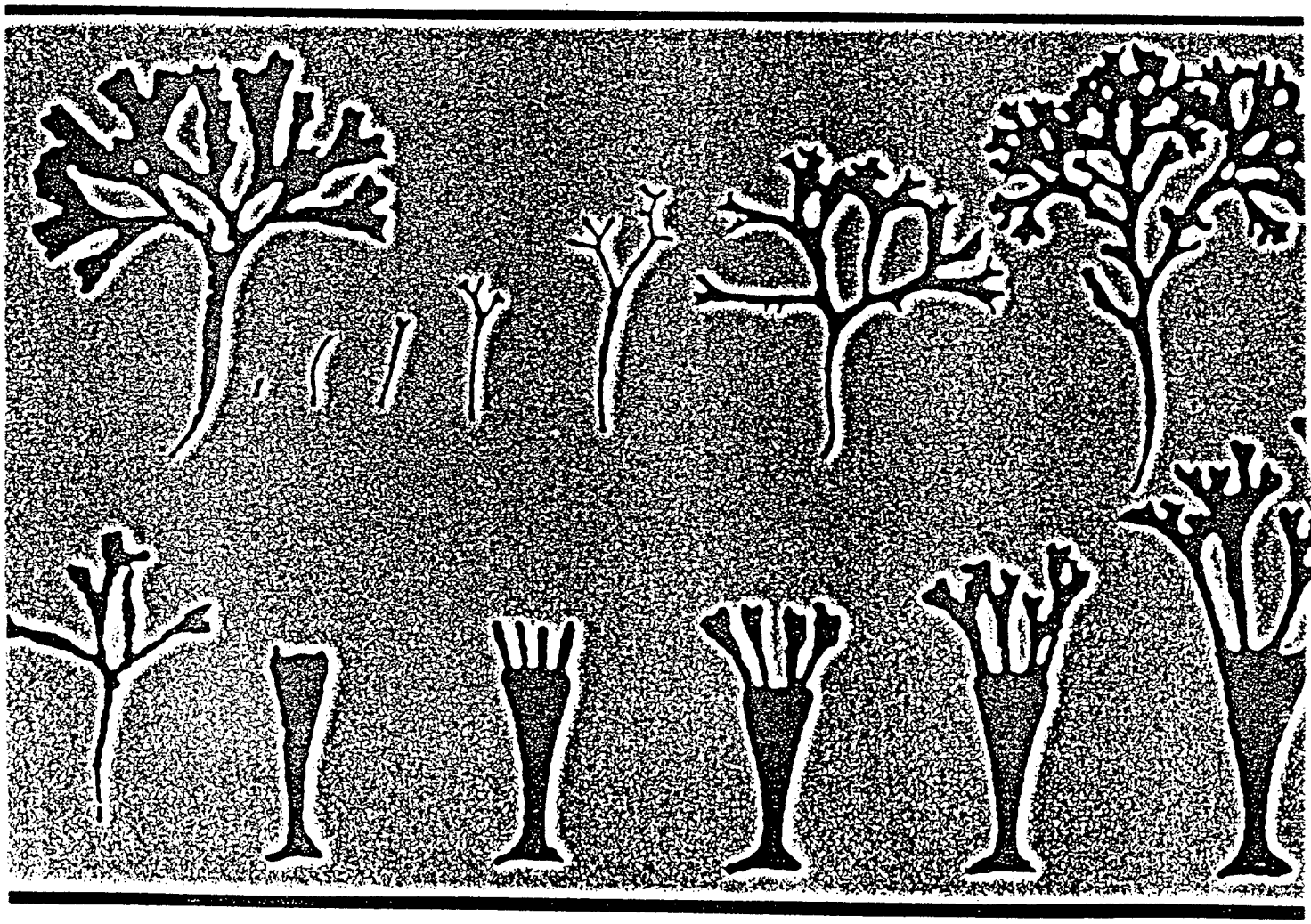
5

SEMINARIO SOBRE CULTIVO E APROVEITAMENTO INDUSTRIAL DAS ALGAS MARIÑAS

Sargadelos, 17 e 18 de Marzo de 1988

SEMINAR ON CULTURE AND INDUSTRIAL USE OF SEAWEEDS

Sargadelos, 17 and 18 of March, 1988



Cuadernos da Área de Ciencias Mariñas, Seminario de Estudos Galegos, 5: 65-79 (1992)

Aplicación de procesos de biotecnología a la selección y propagación de macrofitos marinos

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El desarrollo y aplicación de biotecnologías a macrofitos marinos de interés industrial puede ser una de las herramientas de trabajo más eficaces para solventar los problemas básicos que restringen la expansión de la maricultura vegetal como actividad industrial en Occidente. Antes de analizar el estado actual y potencialidad de la aplicación de biotecnologías a macrofitos marinos vamos a exponer brevemente la problemática de los sistemas de producción de biomasa macroalgal que, como veremos, estará centrada en la selección de clones de alta producción. Una vez establecida la necesidad de seleccionar clones de alto rendimiento pasaremos a analizar brevemente los métodos convencionales de mejora genética aplicados a macrofitos, analizando los resultados y los problemas que plantean, para finalmente desarrollar el estado actual de la aplicación de procesos de biotecnología vegetal a la selección y propagación de macrofitos marinos.

Existen tres sistemas de producción de biomasa algal, que por orden de aparición y de importancia son:

I— Explotación de poblaciones naturales.

II— Cultivo en granjas marinas (sistemas outdoor).

III— Cultivo en tierra (sistemas indoor-tanques, canales fotobioreactores, etc.).

I.— *La explotación de poblaciones naturales* es una de las industrias más antiguas de la humanidad y sigue constituyendo la fuente más importante de obtención de biomasa en términos globales. La ventaja de la recolección es la nula inversión en el sistema productor, aunque actualmente existen sofisticados sistemas de recolección, como los barcos-segadoras utilizados en California para cosechar *Macrocystis* y *Laminaria*. Pero frente a esta ventaja existen numerosos inconvenientes que podríamos resumir en una absoluta falta de control, tanto de la calidad (no se puede garantizar la monoespecificidad de la biomasa ni su contenido en compuestos orgánicos de interés) como de la cantidad, ya que el número de poblaciones naturales explotables es muy limitado, están sometidas a variaciones estacionales y al ataque por ficófagos, y en general se encuentran sobreexplotadas.

II.— Vista la insuficiencia e ineficacia de la explotación de poblaciones naturales,

y debido al gran aumento de la demanda de productos extraídos de macroalgas, se empezaron a desarrollar en Oriente *granjas marinas* basadas en las experiencias del cultivo de *Porphyra* de los japoneses. Estos sistemas de cultivo se fundamentan en el anclaje de las algas a un sistema de redes que, bien de forma suspendida o flotante, se cultivan en zonas costeras. La metodología de este tipo de cultivo la podemos clasificar en dos tipos:

— cultivos de propagación vegetativa y
— cultivos de propagación sexual

Los cultivos de propagación vegetativa consisten en la fijación a las redes oceánicas de fragmentos de talo. Esta metodología está obviamente restringida a especies con un alto potencial regenerativo.

Los cultivos de propagación sexual presentan la desventaja de que requieren el cultivo de las dos fases del ciclo biológico. La primera se realiza en tierra y consiste en la manipulación muy precisa de temperatura e intensidad lumínica de los tanques de cultivo donde se induce la fijación de las esporas a las redes. Una vez obtenida la emisión, fijación y germinación de las esporas, se trasladan las redes al mar. Por tanto, para este tipo de especies la propagación es un factor que encarece considerablemente su cultivo.

La ventaja del cultivo en granjas marinas es que permite un mayor control de la biomasa en un doble aspecto: garantiza la monoespecificidad de la biomasa y da una cierta garantía de suministro continuado. Los inconvenientes son básicamente tres:

— La susceptibilidad de estos sistemas de cultivo monespecífico y de alta estabulación a las infecciones y contaminaciones por ficófagos y patógenos, problemas que han generado la pérdida de cosechas enteras.
— La baja productividad de estos sistemas, puesto que no se pueden abonar.
— El elevado coste de la mano de obra que requieren estos sistemas. Datos económicos referidos a una granja marina en Taiwan estimaban el coste de la mano de obra en más de un 60% de los gastos totales. La aplicación de estos sistemas es pues impensable en una economía occidental.

III.— La aproximación al cultivo de macrófitos en Occidente, fundamentalmente para la producción de ficocoloides de alta calidad, se ha realizado sobre *sistemas de cultivo indoor*. Estos sistemas de cultivo de alta sofisticación —tanques, canales y fotobioreactores— permiten un control total de la especie, nutrición, producción cuanti/cualitativa de ficocoloides, parasitismo, etc., pero a costa de una elevada inversión de energía y capital que hace impensable el cultivo de especies de reproducción sexual, ya que el cultivo de todas las fases del ciclo biológico requeriría una inversión que dispararía los costes de producción.

La resolución de lo que podríamos considerar como factores tecnológicos del cultivo (diseño del sistema, tipo y forma de fertilización, procesos de extracción de mayor eficacia, etc.) no ha permitido, en general, obtener cosechas suficientes como para rentabilizar a nivel comercial estos sistemas de cultivo.

El factor limitante en la producción de algas marinas no es tecnológico; es puramente biológico y radica en la selección de clones de alta producción, en la domesticación de las especies. Lo que ha quedado claro tras cerca de dos décadas de estudio de sistemas experimentales de cultivo es que, haciendo una comparación con la agricultura terrestre, por muchos tractores, abonos, pesticidas e invernaderos que se apliquen a un sistema de cultivo, si lo que se está cultivando es trigo salvaje... no hay pan.

La principal limitación de la expansión de la maricultura vegetal es la mejora genética.

Una vez establecido que el objetivo es la

mejora genética, la siguiente pregunta que se plantea es *Cómo* abordar un programa de selección y, en segundo lugar, *Qué* características interesan, o mejor dicho, qué características son susceptibles de mejora. Como veremos, la metodología que se emplee en el proceso selectivo va a condicionar las características que se pueden seleccionar.

En principio , y como declaración de ideales, las características prioritarias a seleccionar son:

— Variedades de alto crecimiento.
— De reproducción vegetativa.
— De alto contenido cuanti/cualitativo del producto a extraer.
— De alta resistencia a infecciones y estrés ambiental.
— Que produzcan más de un compuesto de alto valor añadido.

Abordar un programa de selección en macroalgas es realmente difícil, debido a la complejidad del ciclo biológico de estos organismos, en los que la alternancia de gametofito-esporofito no es más que una simplificación de un ciclo vital, que cuanto más se estudia más ramificaciones y complejidades presenta. Otra gran dificultad de partida la constituyen las enormes lagunas en el conocimiento del metabolismo y genética de estos organismos.

La contestación al *Cómo* seleccionar la podemos clasificar en dos respuestas: por *métodos convencionales* o por aplicación de *procesos de biotecnología vegetal*.

1.— Vamos a describir brevemente las metodologías, resultados y problemas de los *procesos convencionales de mejora*:

Los trabajos pioneros en la selección de macrofitos fueron los iniciados a finales de la década de los 50 en el Shandong College of Oceanography en China, (TSENG; T.C. FANG) con *L. japonica*. Las características que se pretendía seleccionar eran altas tasas de crecimiento, así como tamaño y grosor del fronde. La metodología seguida fue la de autocruzamiento; es decir, se seleccionaron esporofitos con las características antes mencionadas, se cultivaban en tanques individuales, se inducía la emisión de esporas, el desarrollo y maduración de gametofitos, la producción de esporofitos, se seleccionaban los más adecuados y vuelta a empezar.

Los resultados fueron muy alentadores, ya que se obtuvieron no sólo variedades de mayor tasa de crecimiento y grosor de fronde, sino también con un mayor contenido en iodo, lo que permitió erradicar los problemas de bocio endémico en la China continental.

Como veremos a continuación, y tal como lo han expuesto los propios investigadores que llevaron a cabo este programa, hay que introducir muchas matizaciones a la eficacia de la hibridación sexual por autocruzamiento como sistema eficaz de mejora en macrofitos.

En primer lugar, la caracterización de un fenotipo óptimo con el que iniciar un programa de selección, está muy condicionada por la enorme plasticidad que presentan los macrofitos.

En segundo lugar, el grado de heredabilidad de las características deseadas es muy bajo, lo cual alarga enormemente los programas de selección y obliga a un control muy ajustado de las características ambientales, a fin de poder determinar la base genética o epigenética de los fenotipos.

En tercer lugar, las características deseables de selección (alto crecimiento, grosor, tamaño, etc.) son caracteres cuantitativos, ligados por tanto a las normas que regulan su transmisión, es decir, lentos de sumar y en todo caso con un límite que su propia interacción delimita.

En cuarto lugar, no se conocen bien los mecanismos que inducen la esporogénesis y sobre todo la gametogénesis, lo que implica que no se puede reducir en cultivo el tiempo en que se completa el ciclo biológico.

En quinto lugar y relacionado con el punto anterior, la necesidad de cultivar hasta su madurez ambas fases del ciclo para po-

der identificar los fenotipos óptimos con los que continuar el proceso selectivo.

En sexto lugar, y relacionado con la herencia cuantitativa, está el hecho de que los genes, o mejor dicho el sistema de poligenes que regula las características deseables por el hombre, no tiene por qué ser dominante, ya que normalmente no tiene por qué presentar una ventaja selectiva en la naturaleza, y obliga realizar infinidades de cruces para que se vayan sumando genes recesivos en homozigosis.

Y como conclusión final y consecuencia de los problemas expuestos, la larga duración de estos programas de selección, que en el caso de *L. japonica* duraron más de 30 años.

Los trabajos de hibridación interespecífica e intergenérica realizados en las dos últimas décadas en Japón (Saito, Tokida y Yabu, Migita), Canadá (Chapman, Lúnning) y EE.UU. (Sabonsunga y Neushul), a pesar de estar más bien centrados en dilucidar problemas de relaciones filogenéticas, han permitido un cierto avance en el conocimiento del potencial de la hibridación sexual aplicado a la mejora en Laminarias.

Estos avances han permitido:

— Establecer métodos de control de la gametogénesis y de la emisión de gametos.

— Establecer técnicas para obtener cultivos de gametofitos unisexuales.

— Desarrollar sistemas de cultivo e identificación de los esporofitos híbridos.

Pero estos avances en la manipulación de la hibridación sexual han puesto de manifiesto nuevos problemas, como:

— La gran mortalidad de los híbridos tanto en cultivo en laboratorio como en condiciones protegidas en el mar.

— El descubrimiento de la gran importancia que tiene la herencia materna en características de alto crecimiento.

— La esterilidad de los esporofitos híbridos, que impide el estudio de la estabilidad de la transmisión de los fenotipos variantes de interés.

De los trabajos de selección por hibridación sexual se extraen dos conclusiones: la primera es que no se puede plantear un programa de selección a medio plazo si se trabaja con especies de reproducción sexual, e incluso plantearlo a largo plazo es sumamente arriesgado si previamente no se conoce la heredabilidad de las características que se pretende seleccionar, y la segunda es que como única alternativa viable queda la selección de especies capaces de propagarse vegetativamente.

Esta segunda alternativa de los métodos convencionales de selección tuvo un gran impulso en Occidente tras las experiencias de los sistemas de cultivo indoor, ya que en estos cultivos aparecían espontáneamente mutantes que presentaban, aparentemente, mayores tasas de crecimiento, un elevado potencial de reproducción vegetativa y un mayor contenido en ficocoloides.

La primera referencia fue la del clon T-4 de *Chondrus crispus* obtenido en Canadá por el equipo de VAN DER MEER en 1973 y patentado en 1978. A partir de los trabajos de la escuela canadiense no ha habido año en que no se publicaran nuevos mutantes.

Las grandes expectativas qe despertaron estos clones se fueron diluyendo cuando, al pasar los clones a cultivo industrial, se comprobó que las características estaban relacionadas con las condiciones de cultivo en laboratorio (baja densidad, pequeño tamaño de las plantas, nutrientes de pureza analítica, irradiación y temperaturas óptimas, etc.) y no con su constitución genética.

La conclusión de todos estos trabajos es que la mejora genética continúa siendo el cuello de botella de la expansión de la maricultura vegetal, ya que no se dispone de una metodología eficaz con que abordarla. Otra conclusión es que, a menos que no se obtenga un sistema alternativo de propaga-

ción, los sistemas de cultivo occidentales están restringidos a especies de reproducción vegetativa.

2.— La alternativa a los métodos convencionales de mejora surge de la potencialidad de aplicar las *técnicas de biotecnología vegetal* que se vienen aplicando a plantas vasculares. Estas técnicas permitirían solventar tanto la selección de clones como su propagación, haciendo innecesario el cultivo de todas las fases del ciclo vital de las especies con reproducción sexual.

La aplicación de los procesos de biotecnología vegetal a la mejora y propagación de macrofitos se puede clasificar en dos tipos:

A.— Selección y propagación por *cultivo celular.*

B.— Selección por *ingeniería genética.*

A.— Las ventajas de la aplicación del *cultivo celular* a la selección son:

— Permite trabajar con millones de células en un espacio reducido y con un ambiente totalmente controlado.

— Cada célula se puede considerar una planta en potencia ya que en teoría, y como se ha demostrado en diversas especies, cada célula es totipotente (capaz de regenerar una planta completa).

— El callo es un sistema celular generador de una gran cantidad de variabilidad genética y sobre el que se puede aumentar la variabilidad por tratamientos con mutágenos físicos o químicos.

— Se puede efectuar una presión selectiva directa sobre una población de millones de «plantas» (células) e identificar rápidamente los fenotipos variantes.

— Como célula equivale a planta, la estabilidad del fenotipo variante se puede controlar por el número de divisiones mitóticas.

Estas ventajas del cultivo celular aplicado a macrofitos son las mismas si se aplican a plantas vasculares, pero hay dos características de los macrofitos que hacen que estas técnicas tengan aún mayor potencial que en plantas vasculares, y son las siguientes:

— En primer lugar, la alternancia de generaciones que caracteriza a estos organismos permite trabajar indistintamente con gametofitos o esporofitos isomórficos. Esta característica diferencial con plantas vasculares es muy importante, pues permite establecer el cultivo celular de la fase haploide. Esta posibilidad es de gran interés aplicada a la mejora genética ya que:

— La información codificada en alelos recesivos se manifestará y se podrán clonar líneas con características que de otra forma habría sido muy difícil o imposible conseguir, y

— permite obtener clones diploides enteramente homozigóticos mediante tratamientos suaves con colchicina a los clones haploides seleccionados.

Esta posibilidad, que en plantas vasculares está supeditada a la compleja manipulación de polen y anteras, constituye una gran ventaja del cultivo celular de macrofitos que, como hemos visto, constituye uno de los principales inconvenientes en una aproximación convencional a la mejora.

— La segunda ventaja específica de macrofitos referida a la selección por cultivo celular es su comparativamente elevada tasa de recombinación mitótica, lo que implica que se parte, ya desde el explante primario, con una considerable variabilidad genética en las células que inician el cultivo celular.

En resumidas cuentas, el cultivo celular aplicado a la mejora genética de macrofitos permitiría reducir los programas de selección de 30 años a unos cuantos meses. Esta enorme ventaja del cultivo celular presenta un único inconveniente:

Ya habíamos mencionado al principio que el *Cómo* seleccionar iba a condicionar el *Qué* seleccionar. Hemos visto que los pro-

gramas de selección convencionales permitían seleccionar características morfológicas tales como grosor, tamaño, etc; es decir, rasgos anatómicos a nivel de planta entera. El cultivo celular presenta el inconveniente (inconveniente muy matizable, como veremos), de que únicamente se podrán seleccionar características codificadas a nivel celular, no características a nivel de planta entera.

No obstante, esta salvedad está referida a los programas de selección en sentido estricto, es decir, aplicando presiones selectivas dirigidas, y no implica que por la variabilidad generada en cultivo aparezcan espontáneamente mutantes, que siempre lo harán con una frecuencia muy superior a la variabilidad natural.

La aplicación del cultivo celular a la selección y propagación de macrofitos marinos fue sugerida por primera vez por un científico californiano, el Dr. A. GIBOR, en una conferencia sobre maricultura vegetal en el Pacífico celebrada en 1980. Su sugerencia fue recibida con bastante escepticismo, pero sólo siete años despues de aquella elucubración ya existen varias compañías dedicadas a la aplicación de biotecnologías a macrofitos.

En su conferencia, el Dr. GIBOR expuso claramente que antes de especular acerca de las características a seleccionar por estas técnicas, lo primero que había que hacer era desarrollar la herramienta metodológica que permitiera acceder a la manipulación celular de los macrofitos. Para el desarrollo de esta herramienta metodológica se han de cumplir los siguientes requisitos:

a) Obtener cultivos axénicos.

b) Obtener medios de cultivo adecuados para el cultivo celular.

c) Desarrolar métodos que permitan la desorganización celular y la formación de callo, es decir, el establecimiento del cultivo celular propiamente dicho.

d) Mantener la totipotencia celular y por tanto la capacidad de regenerar planta a partir del cultivo celular desorganizado.

a/b.— Los dos primeros pasos, la *metodología de la obtención de cultivos axénicos* y la *formulación de medios de cultivo adecuados*, ya estaban dados antes de la conferencia de GIBOR. L. PROVASOLI *y col.* en EE.UU., y posteriormente L. FRIES en Suecia, venían desarrollando desde mediados de los años 50 estudios de nutrición de macroalgas en cultivos axénicos. Estos estudios básicos de nutrición ya habían sentado las bases de los protocolos de desinfección y obtención de cultivos axénicos, requerimientos nutritivos y orgánicos, formulaciones artificiales de agua de mar y metodología de cultivo.

No obstante, fueron solamente las bases, ya que cuando se intentó establecer el cultivo axénico en otras especies se encontraron numerosas dificultades que hasta el presente sólo se han solucionado en su totalidad para un número muy reducido de especies.

La necesidad de establecer el cultivo axénico es doble:

— Primero, porque para estudiar los requerimientos nutritivos y los reguladores que inducen la desorganización y la morfogénesis del cultivo celular hay que evitar toda interferencia con microorganismos que mediatizarían los resultados.

— En segundo lugar, porque estos microorganismos impedirían el desarrollo del cultivo celular o acabarían destruyéndolo.

El problema de la obtención de cultivos axénicos radica básicamente en 3 puntos:

— El primero de ellos es que los tratamientos desinfectantes no se pueden generalizar. Es decir, las características diferenciales en cuanto a pared celular, difusión y tolerancia del agente biocida en algas verdes, rojas

y pardas hacen que éstas respondan de forma muy diversa frente a un mismo agente biocida. Un tratamiento que funcione muy bien para un grupo o para una especie determinada puede ser completamente tóxico o ineficaz para otra, lo cual obliga a desarrollar un tratamiento específico para cada especie.

— El segundo problema lo constituyen las relaciones simbióticas establecidas entre las macroalgas y sus endo-epifitos, que provocan malformaciones y detención del crecimiento cuando se establece el cultivo axénico de macroalgas.

— Y en tercer lugar, el problema que plantea la eliminación de endofitos que quedan protegidos de los tratamientos de desinfección.

El establecimiento del cultivo axénico en macroalgas requiere unos tratamientos muy sofisticados, en comparación con plantas vasculares, que podemos esquematizar en el siguiente protocolo:

— En primer lugar se precultivan fragmentos juveniles, liberados de epifitos, por raspado con cepillo en un medio con una concentración ligera de una mezcla de antibióticos durante un par de días.

— Sucesivos tratamientos de sonicación de los fragmentos más vitales en agua de mar estéril.

— Tratamiento con compuestos orgánicos iodados, alcohol, hipoclorito cálcico o sódico, desinfectantes clínicos, etc.

— Post-tratamiento de los fragmentos más vitales con una mezcla de antibióticos de amplio espectro, fungicidas y dióxido de germanio.

De los trabajos iniciales de PROVASOLI y FRIES surgieron diversas formulaciones artificiales de agua de mar que en mayor o menor grado de definición permitían el crecimiento «in vitro» de macroalgas. No obstante, las formulaciones artificiales de agua de mar no daban los mismos resultados que las formulaciones de agua de mar enriquecida. Los trabajos de M. PEDERSEN demostraron la presencia de citoquininas en aguas someras cercanas a poblaciones densas de macroalgas. Estos resultados, conjuntamente con los aportados por PROVASOLI y FRIES de la auxotrofía macroalgal frente al complejo vitamínico B, han llevado a que en el desarrollo del cultivo celular se emplee el agua de mar como medio base. La estrategia ha sido la de posponer el conocimiento y definición exacta de los requerimientos nutritivos y orgánicos frente al objetivo de establecer el cultivo celular.

c.— Y entramos en el tercer requisito del desarrollo de esta herramienta de trabajo, el *establecimiento del cultivo celular*, que se entiende por el desarrollo de un sistema celular desorganizado, un tumor a fin de cuentas, que es lo que se denomina callo en estas técnicas.

El primer problema que se plantea es el de la definición de callo en organismos como los macrofitos, que se caracterizan por un bajo nivel de organización celular. Así como en el cultivo celular de plantas superiores una estructura de callo es fácilmente reconocible, la escasa organización celular y la gran plasticidad morfológica de los macrofitos ha llevado al abuso del término «callus-like» para referirse al desarrollo, al parecer desorganizado, de los explantes en cultivo, y a un ambiente de crítica y escepticismo entre los diversos grupos de trabajo en cuanto a la consideración de los resultados ajenos como un auténtico establecimiento del cultivo desorganizado. Esta situación se ha visto potenciada por los escasos datos histológicos aportados en los trabajos.

La primera referencia que se suele citar en el desarrollo de la biotecnología de macrofitos marinos es la de CHEN y TAYLOR (1978), aunque estos autores no obtienen la

desorganización de los fragmentos de médula de *Chondrus crispus* que emplean como explante primario. No obstante es la primera referencia en la que se describe el efecto de hormonas sobre la regeneración «in vitro» de fragmentos de macroalgas empleando la metodología del cultivo celular. El mismo año SAGA en Japón describe la regeneración de esporofitos a partir de células aisladas, lo que denomina callus-like, en Laminarias. Estos trabajos sentaron las bases de la posibilidad de que células o pequeñas agrupaciones celulares podían expresar su totipotencia en cultivo «in vitro» y por tanto confirmaban la posibilidad de aplicar las técnicas del cultivo celular a macrofitos marinos. En 1980 FRIES publica la inducción de callo y su regeneración en medio artificial semisólido.

A partir de 1980 aumentan los grupos de trabajo interesados en el establecimiento del cultivo celular de macrofitos. CHENEY y col. en Bostón, BRINKHUIS en Nueva York, GIBOR y POLNE FULLER en California, VAN STADEN en Sudáfrica, GORDON en Nueva Zelanda, YAN en China y BOROWITZKA en Australia, constituyen los grupos más importantes que surgen a partir de 1983.

La aproximación que hacen todos estos grupos al desarrollo de la «herramienta» del cultivo celular es bastante coincidente en cuanto a los parámetros básicos que parecen controlar la desorganización celular:

— Identificar los genotipos de mejor respuesta.
— Seleccionar el tipo de explante más adecuado.
— Determinar la composición del medio de cultivo y su estado físico más adecuado para la inducción de la desorganización celular.
— Determinar el efecto que presentan diversos reguladores del crecimiento sobre la desorganización y regeneración del callo.

Tras seis años de trabajo, cada grupo había desarrollado la metodología necesaria para establecer el cultivo celular en las especies particulares en las que trabajaban, que en su mayoría eran algas pardas. Los medios de cultivo se fueron complicando cada vez más con la introducción de compuestos orgánicos, formulaciones modificadas de los medios de cultivo de plantas vasculares, diversos tipos de azúcares, reguladores del crecimiento convencionales y no convencionales, etc.; es decir, se siguió el mismo slogan de «adiciona que algo hará» que sufrió el cultivo celular de plantas vasculares veinticinco años antes.

Asimismo se descubrió el efecto-herida en macrofitos como inductor de desorganización y la influencia de las concentraciones hormonales endógenas y su variación estacional entre las fases haploide y diploide en la formación de callo, así como la importancia del potencial osmótico en la desorganización celular. En resumidas cuentas, se avanzó en cuatro años lo que en el desarrollo del cultivo celular de plantas superiores había costado veinte. Aunque eran pocos los parámetros controlados y muy diversos los modos de acción entre diferentes especies, la «herramienta» ya estaba construída, para algunas especies en 1984, en lo que se refiere a los tres primeros puntos que antes habíamos mencionado: obtención de cultivo axénico, medio de cultivo adecuado y tumorogénesis.

d.— El cuarto punto, la *regeneración de planta*, es decir el mantenimiento de la totipotencia de la célula desorganizada, es el que mayor problema plantea.

La regeneración de planta del cultivo celular se ha conseguido solventar en parte jugando con dos factores:

— La *adición de hormonas* al medio de cultivo y
el *estado físico* del medio de cultivo.
— La *utilización de hormonas* para inducir la reversión organo-genética en el cultivo celular de macrofitos ha

dado lugar a resultados bastante contradictorios. La importancia de la regulación hormonal endógena en macrofitos está bien establecida, al menos en algunas especies, pero lo cierto es que dentro de un amplio rango de tipos de auxinas y citoquininas, concentraciones y balances relativos experimentados, no se ha conseguido dar con una formulación que de forma eficaz revierta el crecimiento desorganizado hacia la formación de una planta completa. Haciendo una comparación con el cultivo celular de plantas superiores, la utilización de hormonas para inducir organogénesis probablemente no será eficaz hasta que se obtenga con macrofitos un sistema celular análogo al de *Nicotiana* en plantas vasculares, cuya gran facilidad de cultivo «in vitro» permitió dar con formulaciones hormonales que permitían «dirigir» a voluntad la morfogénesis del cultivo celular.

Algunos autores han descrito el efecto de compuestos orgánicos inéditos en la fisiología vegetal, tales como el naftenato sódico (YAN, 1984), como reguladores esenciales para la inducción de organogénesis en callos de Laminariales. Nuestra experiencia al respecto es que esta sustancia no tiene efecto aparente en la organogénesis de callos de algas rojas.

— El *estado físico del medio de cultivo* parece ser un sistema más eficaz para controlar a voluntad un crecimiento desorganizado o inducir organogénesis. Según los resultados de la escuela californiana, la formación de callo tiene lugar en medio sólido y la regeneración se induce sin problemas, simplemente subcultivando callos o agregados celulares en medio líquido. Al igual que en el cultivo celular de plantas superiores, el potencial osmótico parece jugar un importante papel como regulador del crecimiento en el cultivo celular de macrofitos.

Lo cierto es que actualmente la regeneración del cultivo celular constituye el principal problema en la expansión de estas técnicas; prueba de ello es que de las 30 especies en que se ha obtenido el establecimiento del cultivo celular, menos de la mitad se han conseguido regenerar. Pero lo que es igualmente cierto es que este contratiempo no ha desalentado la continua aparición de nuevos grupos de trabajo en el campo de la biotecnología aplicada a los macrofitos marinos.

Los programas de selección aplicando estas técnicas, iniciados en el Shangdong College of Oceanography en China y en la Universidad de California, comprenden la selección de cuatro características:

— Selección de estirpes de alto crecimiento.
— Selección de estirpes resistentes a antibióticos y fungicidas.
— Selección de estirpes resistentes a elevadas temperaturas.
— Selección de estirpes resistentes a toxinas bacterianas.

El común denominador de estas características es que están codificadas a nivel celular, y no precisan de marcadores morfológicos para seleccionar. El indicativo de su resistencia es que continúan creciendo o lo hacen a mayor velocidad que otros agregados celulares. Es decir, son características que no precisan de marcadores especiales para ser detectadas.

Los resultados prácticos más espectaculares hasta el momento los constituyen los trabajos realizados en China con *Laminaria japonica*. La selección de clones resistentes a elevadas temperaturas llevados a cabo por YAN ZUO-MEI (1984), le han permitido regenerar plantas de callos resistentes que no sólo retenían la resistencia manifestada en el callo, sino que además mostraban una tasa de crecimiento superior a la especie sal-

vaje. La obtención de clones resistentes a elevadas temperaturas ha permitido el desarrollo de la maricultura de esta especie en el Mar de China, zona muy rica en nutrientes, donde las especies salvajes no se podían cultivar por las elevadas temperaturas que alcanzaba el agua de mar.

Con este ejemplo práctico de la aplicación del cultivo celular a la selección vamos a introducir otro aspecto aplicado muy importante de estas técnicas: la posibilidad de emplear el cultivo celular como un sistema muy eficaz de propagación.

La base de esta aplicación es muy sencilla. Consiste simplemente en disgregar un talo y provocar que cada célula o agregado celular origine una planta completa, con lo que se podrían obtener de golpe millones de plantas idénticas a la planta disgregada sin necesidad de pasar por todo el ciclo biológico de la especie. Por lo tanto, se podrían ahorrar las costosas instalaciones en tierra y la compleja manipulación de las fases alternantes de las especies con reproducción sexual que habíamos expuesto anteriormente, y la producción de material para la siembra se podría efectuar a partir de una única planta o grupo de plantas que presentara las características más deseadas, que estarían exactamente reflejadas en su descendencia, ya que se trata de un sistema vegetativo de propagación.

El último avance en esta aplicación del cultivo celular se publicó el pasado año en la revista «Genetic Engineering News» (1987) con un trabajo realizado por el Dr. KAPRAUN con la especie *Ulvaria oxisperma*, en la que obtiene la producción de planta cosechable seis semanas después del aislamiento de células y su fijación en cuerdas.

Con el nivel actual de conocimiento en el campo de la biotecnología vegetal, las aplicaciones que hemos mencionado —en cuanto a las posibles características a seleccionar y su aplicación a la propagación somática— son las que presentan una expectativa de aplicación comercial más próxima. No obstante, el desarrollo del cultivo celular y otras biotecnologías han permitido abordar otros estudios que, aunque en un plazo más largo, tendrán un gran impacto en la maricultura vegetal. De entre estos campos a medio-largo plazo citaremos dos:

— la *producción directa de compuestos orgánicos* de interés industrial en biorreactores y

— la aplicación de *anticuerpos monoclonales* a ficocoloides para la identificación y selección de estirpes celulares de agarofitas.

Paradójicamente, la producción de metabolitos secundarios de algas eucariotas a partir del cultivo celular desorganizado en biorreactores está citada desde 1974, cuando al parecer ni tan siquiera se había planteado en el mundo la posibilidad de aplicar el cultivo celular a las algas. Y si ello constituye una paradoja, lo que resulta sorprendente es la base misma de esta referencia, que no es ni más ni menos que una patente japonesa para la producción de agar a partir de callos de *Gelidium* cultivados en un biorreactor estático. Y si la paradoja es sorprendente, los datos aportados en esta patente han sido calificados de exageraciones comerciales por la comunidad científica, ya que la producción de agar en biorreactor era tres o cuatro veces superior al equivalente de biomasa en cultivo en tanque. No se conoce ninguna empresa que disponga de esta tecnología y, en general, esta patente se ha relacionado con la posibilidad de patentar ideas en el campo de la biotecnología y con el interés en destacar el potencial de la técnica, más que con el desarrollo real del sistema.

No obstante, un reciente estudio microscópico del callo de algas rojas nos ha permitido descubrir dos aspectos que hacen suponer que el cultivo en biorreactor puede dar un rendimiento de ficocoloides muy superior al de la planta organizada.

Estas dos observaciones son:

— De una parte, el considerable grosor

de las paredes celulares del callo, lo que unido al pequeño tamaño de las células podría aumentar un orden de magnitud el contenido en ficocoloides en relación a una planta completa.

— En segundo lugar, el descubrimiento de una desproporcionada concentración de amiloplastos en las células del callo. Este predominio del metabolismo de los hidratos de carbono bien podría ser el responsable de una sobreproducción de polisacáridos estructurales.

La posibilidad de aplicar las técnicas del cultivo celular a la selección de estirpes con un mayor contenido cuantitativo y cualitativo de ficocoloides depende de un requisito: la obtención de un marcador; un marcador morfológico, fisiológico o bioquímico, pero que nos permita identificar en una población de billones de células, aquellas que manifiestan las características deseadas. Este aspecto de la aplicación del cultivo celular a la selección genética tiene un gran interés económico, ya que los ficocoloides de alta calidad constituyen el producto de mayor interés actual extraído de los macrofitos.

Si no se dispone de marcadores, el procedimiento es generar variabilidad en el cultivo desorganizado, regenerar planta y analizar la «descendencia» una por una intentando obtener un variante somaclonal, es decir un proceso tedioso y sin ninguna garantía de éxito.

El segundo ejemplo que habíamos mencionado como biotecnologías que tendrán impacto en el campo de la biotecnología y maricultura vegetal es el de los anticuerpos monoclonales a ficocoloides. La importancia del desarrollo y perfeccionamiento en la producción de anticuerpos monoclonales a ficocoloides radica en que es la «miniherramienta» que precisa la «gran herramienta» del cultivo celular para abordar un programa de selección cuanti y cualitativa de ficocoloides.

Los primeros trabajos de obtención de anticuerpos monoclonales a ficocoloides se realizaron en Canadá a mediados de los 7 por el grupo de la Dra. McCANDLESS. Pc co a poco se fue aumentando la especifici dad de estos marcadores moleculares, desa rrollándose anticuerpos específicos a iota lambda y kappa carrageno y, tras la incorpo ración del equipo californiano de la Dra VREELAND a principios de los 80, se desa rrollaron las técnicas de hibridación de poli sacáridos con marcadores fluorescentes.

A mediados de los 80 ya se disponía dc la «miniherramienta» que permitía identifi car a nivel celular la longitud y composición de carragenatos y agar, ya que este últimc también mostraba una respuesta inmunogénica. Los resultados presentados en 1987 en el Congreso «Recent Progress in Algal Biotechnology» (Lille, Francia), han sido espectaculares en cuanto a la variabilidad en contenido y composición que presentan las células de especies carragenofitas sometidas a tratamientos mutágenos. Por el momento no se han regenerado plantas de los clones selectos, pero lo que es importante tener presente es el impresionante avance que suponen estas técnicas si las comparamos con los métodos de hibrización sexual iniciados en China. La aplicación conjunta del cultivo celular y los anticuerpos monoclonales permite, además de las ventajas que ya mencionamos del cultivo celular, seleccionar casi a nivel genético, porque lo que sí ha quedado claro tras los trabajos de McCANDLESS y VREELAND es que la cantidad de unidades estructurales y su grado de sulfatación, es decir la cantidad y calidad de los ficocoloides, está sujeta a una codificación genética. El desarrollo de estas técnicas nos ha llevado pues a las puertas de la manipulación genética, es decir al máximo grado posible de control... pero sólo a las puertas.

Porque para hablar estrictamente de manipulación genética hay que llegar a eso, a manipular, y no a identificar y seleccionar variabilidad que no sabemos de dónde ni cómo ha surgido... por muy útil que nos pueda resultar esta variabilidad.

B.— Esto nos lleva al punto final del desarrollo de la biotecnología aplicado a la selección de macrofitos, *los procesos de ingeniería genética*. Los requisitos para el desarrollo de la ingeniería genética en estos organismos pasan por:

— la obtención de protoplastos
— la obtención de métodos eficaces de hibridación somática y producción de heterocariones
— la identificación de plásmidos
— la obtención de vectores
— y finalmente por la identificación, aislamiento y clonado de genes en plásmidos.

Las metodologías para manipular la información genética en macrofitos las podemos clasificar en dos:

a.— por *hibridación somática* y

b.— por *transformación genética*.

a.— La aplicación de la *hibridación somática* es la metodología más sencilla, pero la menos fiable de las dos. Esta técnica se basa en la obtención de protoplastos y su fusión con protoplastos de otras especies o géneros, dando lugar a una célula que presenta el genoma de ambos. En teoría esta metodología permite romper cualquier barrera genética y generar una «especie» nueva, que expresará las características codificadas en ambos genomas. Como es fácilmente imaginable, la potencia de esta técnica es muy superior a la simple hibridación sexual de los sistemas convencionales de mejora. La potencialidad de esta técnica ha hecho que científicos de gran prestigio dentro del campo de productos bioactivos de macrofitos como W. FENICAL (1983), hayan sugerido la posibilidad de «ingeniar» plantas terrestres que produzcan ficocoloides o antibióticos característicos de la flora marina.

La gran expectación que causó la posibilidad de hibridación somática en el mundo de los ficólogos, estuvo en parte condicionada por la similar expectación [illegible] cha mayor base científica y metodológica, que se había levantado en el campo de la biotecniología de plantas vasculares. No obstante es muy probable que, al igual que ha sucedido en el campo de la hibridación somática en plantas vasculares, se descubra que esta posibilidad aplicada a macrofitos presenta muchos más problemas de los esperados.

El primer requisito para realizar la hibridación somática es obtener protoplastos, y aquí aparece el primer problema, porque la aplicación de la metodología y sobre todo, de los sistemas enzimáticos que se han desarrollado para plantas vasculares, no funcionan en algas rojas y pardas, que son las de mayor interés. Las paredes celulares de estas algas son diferentes y mucho más complejas que las plantas vasculares, y no digieren las mezclas de celulasas, pectoliasas y hemicelulasas que funcionan perfectamente con plantas vasculares. Para la digestión de las paredes celulares de macrofitos marinos se han empleado dos sistemas:

El primero es la utilización de auténticos cócteles enzimáticos en los que se añade una muestra de la mayoría de los enzimas existentes en el mercado que actúan sobre pared celular. De esta forma ha surgido un considerable número de formulaciones enzimáticas. Este recetario de formulaciones enzimáticas plantea dos problemas:

— El primero es que la idoneidad de una formulación está restringida a una especie determinada, no sirve para todas las especies, incluidas las de un mismo género. Es más, su efectividad puede estar restringida a una fase determinada del ciclo celular; es decir, el que digiera las células gametofíticas no significa que digiera las células del esporofito.

— El segundo problema es el que plantea la toxicidad de estos cocteles enzimáticos en lo que se refiere a la viabilidad del protoplasto para regenerar pared celular y posteriormente dividirse y dar lugar a una

tenido con estos cocteles, pero la obtención de plantas sólo se ha descrito en muy pocas especies. En las demás especies o no se ha conseguido la regeneración de pared, o como máximo un par de divisiones mitóticas.

El segundo sistema empleado para la obtención de protoplastos consiste en la extracción de los sistemas enzimáticos de ficófagos. La utilización de extractos crudos, o ligeramente purificados, del sistema digestivo de *Aplysia, Diadema* o *Littorina* han permitido la regeneración de callo y en algunos casos la regeneración de plantas a partir de éstos. Este sistema ha demostrado ser mucho más eficaz en cuanto a la repetibilidad, ausencia de toxicidad, número de especies a las que se puede digerir la pared y, sobre todo, cantidad de protoplastos obtenidos.

Realmente el «estado del arte de la ingeniería genética en macrofitos» no ha pasado de este punto. A lo largo de 1986 y 1987 se han publicado diversos trabajos y ha habido varios congresos en los que se han descrito técnicas rutinarias para el aislamiento de protoplastos. El primer paso se puede considerar dado. Los siguientes están en su mayoría por dar, pero es muy probable que se efectúen en la presente década.

Lo que vamos a exponer a continuación son los trabajos que se están realizando actualmente en diversos laboratorios del mundo en cuanto a las posibilidades que el desarrollo de sistemas eficaces de obtención de protoplastos han abierto en el campo de la ingeniería genética aplicada a macrofitos.

Los trabajos de hibridación somática se están llevando a cabo en la Universidad de California bajo la Dirección del Dr. GIBOR, y en la Universidad de Nottingham y la de Boston, que llevan a cabo el proyecto conjuntamente. Los resultados publicados hasta el momento sólo hacen referencia a la facilidad con que los protoplastos se fusionan espontáneamente, por lo que en principio no parece que este aspecto vaya a presentar graves problemas. Donde es muy probable que surjan es en la formación de auténticos heterocariones, es decir, que realmente se fusionen los núcleos, y sobre todo, en que los genomas de distintas especies puedan coexistir en el híbrido. Este último aspecto es el que ha producido el abandono, o al menos ralentización en los programas de hibridación somática en vegetales superiores.

Los más optimistas en cuanto al potencial de la hibridación somática en macrofitos suelen citar el trabajo de MATAGNE *y col* que, en 1979, describieron la obtención de un auténtico heterocarion estable al fusionar protoplastos de *Chlamydomonas reinhardi*. Lo que no suelen mencionar es que se empleó un mutante que carece de pared celular. También los trabajos de OHIWA en Japón con hibridaciones interespecíficas e intergenéricas con otras especies de algas unicelulares o filamentosas (*Zygnema, Mougeotia, Spirogyra*), que dieron lugar rápidamente a procesos de incompatibilidad somática y nuclear. Los mismos procesos de incompatibilidad que en un trabajo precoz y muy olvidado, publicó L. FOWKE en 1979 al fusionar protoplastos de *Chlamydomonas* y *Daucus carota*. Ciertamente la bibliografía es muy escasa, pero si se extrapolan los resultados obtenidos en la hibridación somática de plantas vasculares, algas unicelulares o filamentosas y algas y plantas vasculares, la profecía de FENICAL de fabricar plantas terrestres que produzcan compuestos bioactivos característicos de macrofitos, parece que tardará algún tiempo en hacerse realidad.

b.— La otra gran vía que ha abierto la obtención de protoplastos es la de la ingeniería genética propiamente dicha, es decir, la *transformación genética*.

Démonos cuenta de cuántos prerrequisitos para aplicar esta tecnología se han superado en apenas siete años:

- Se han obtenido cultivos axénicos; no hay pues interferencia con otros organismos.

— Se han obtenido medios de cultivo que pueden hacer crecer en forma organizada o desorganizada a las células de macrofitos.

— Se pueden cultivar en medio sólido, con lo que posibilitan la identificación y aislamiento de mutantes.

— Se ha descrito la existencia de plásmidos en macrofitos de interés comercial.

— Se pueden obtener protoplastos, con lo que la especificidad de los procesos de transformación (o conjugación) se ve reducida a un mínimo, y potenciadas las eficacias de transformación. Es decir, podrían servir los mismos vectores o plásmidos que se emplean en plantas vasculares.

El cuello de botella radica en la obtención de vectores naturales en macrofitos.

Aunque es un hecho poco conocido, la existencia de bacterias marinas generadoras de tumores en macrofitos está descrita desde el siglo pasado. Desde los trabajos de BROWN en 1983, diversos autores han descrito a las bacterias como las causantes de los tumores, agallas o callos (se han empleado indistintamente estos tres términos) que aparecen en poblaciones naturales de macrofitos. Un trabajo reciente de TSEKOS (1983) mostraba la morfología de estas bacterias en tumores de *Gracilaria*. El estudio de la microscopía electrónica de los callos inducidos en nuestro Departamento ha puesto de manifiesto la presencia de bacterias similares a las descritas por TSEKOS y otros autores en tumores de poblaciones naturales. De la identificación y aislamiento de estas bacterias puede que venga ese vector, esa «llave maestra» que nos permita introducirnos en el código genético de los macrofitos.

Otro cuello de botella, mucho más difícil de resolver, es la identificación, aislamiento y clonación de genes que confieran una mejora cuantitativa o cualitativa a la especie en particular. Ciñéndonos al caso específico de ficocoloides, la pregunta sería, ¿qué genes codifican una mayor producción de agarosa, de k-carrageno?, ¿qué genes codifican un menor grado de sulfatación en estas moléculas? y la segunda pregunta sería, ¿dónde están localizados? En macrofitos se desconoce la respuesta a estas preguntas. No obstante, unos trabajos recientes de DERETIC *y col* (1987) en *Pseudomonas aeruginosa*, una bacteria productora de alginatos iguales a los producidos por *Fucus gardneri*, ha permitido identificar los genes involucrados en su síntesis y ha abierto la posibilidad de comenzar a identificar los genes relacionados con la síntesis de alginatos en macrofitos.

Aunque la alternativa de la transformación genética en macrofitos parece muy lejana, hay que tener presente que el penúltimo número de la revista «High Technology» dedica un artículo a la biotecnología en algas marinas en el que se citan diversas empresas de ingeniería genética que están investigando el desarrollo de estos procesos en especies de algas marinas de interés industrial.

Para finalizar haremos un resumen historiado-especulativo de lo expuesto. Hemos visto cómo a partir del estancamiento de la recolección como método de obtención de biomasa, el hombre ha ido desarrollando la maricultura vegetal en sucesivos escalones hacia un objetivo muy concreto: máximo control.

El primer escalón fue el cultivo en granjas, primer paso de cazador a agricultor. Vimos las razones que le impulsaron a subir un escalón más y desarrollar los sistemas de cultivo indoor. En el sistema indoor el cultivo es muy fácil, incluso se puede automatizar, pero descubre que la cantidad de energía que consumen estos sistemas de cultivo en tierra en relación a la cantidad de dinero que recibe por su producto no es suficiente, y que si cultiva especies de reproducción sexual la explotación es ruinosa. Sabe que la demanda y el precio de las algas van en aumento, que esas

que pueden producir el producto que necesita (no existen sistemas industriales o biotecnológicos alternativos), y que la solución es hacerlas más productivas. Pasa entonces a aplicar los métodos convencionales de selección; trata de hibridar especies, trata de cultivar los mutantes que han aparecido en el cultivo, pero descubre que esas herramientas selectivas no le van a garantizar el material vegetal que necesita. Aprende que el único éxito descrito por esa vía es puntual y muy limitado a unas características que no son exactamente las que anda buscando.

Decide subir un escalón más y llegar al control de la célula, la máquina que produce los compuestos que necesita. Entonces, mientras intenta controlar el comportamiento de la célula, descubre un gran abanico de posibilidades. No sólo es posible seleccionar células resistentes a diversos estrés ambientales, que crezcan muy rápido, resistentes a toxinas, etc., sino que puede identificar y seleccionar células que sintetizen una mayor cantidad del producto que le interesa, o una mayor calidad, o incluso nuevos productos. Puede también cruzar especies que divergieron en la evolución hace millones de años, y puede montar un sistema de producción de «semilla» que produzca millones de plantas idénticas a un fenotipo determinado, en un espacio de cultivo muy reducido. Ha superado la esclavitud de la reproducción sexual y las barreras genéticas.

Al mismo tiempo se da cuenta de que el cultivo celular abre la posibilidad de saltar al siguiente escalón y controlar el mecanismo que regula la maquinaria celular. En este momento está intentando encontrar el vector que le permita manipular el ADN. Este camino probablemente le llevará bastante tiempo, pero mientras tanto dispone de la herramienta del cultivo celular, que ya le está permitiendo producir las plantas que necesita.

Además, el cultivo celular le ha enseñado otra cosa: que puede hacer cambiar drásticamente la maricultura vegetal, y es que realmente no necesita la planta. Cultivando las células ha descubierto que obtiene mayor cantidad del producto que le interesa y de una forma mucho más controlable y cómoda. Si pudiera desarrollar un biorreactor con agregados celulares que le produzcan mucho más que la mejor de las plantas y encima no necesite empleados, ni espacio, ni mar, su decisión no es difícil de imaginar. Probablemente la historia no acabará aquí. Para cuando haya conseguido identificar las rutas metabólicas que conducen al producto que le interesa y los enzimas que las regulen, se dará cuenta de que no necesita a las células, ni tan siquiera manipular a voluntad el ADN; lo único que necesita es sujetar los enzimas en un biorreactor de inmovilización, introducir el sustrato adecuado por un extremo y recoger el producto por el otro. Al final llegará al Control Total.

Como hemos visto, las rutas biosintéticas de ficocoloides y los enzimas que las controlan se están empezando a conocer, y en el mercado ya existen varios modelos de biorreactores normales y de inmovilización desarrollados para la producción y biotransformación de metabolitos secundarios de plantas vasculares. El tiempo que se tarde en emplearlos y el tipo de biorreactores que se empleen dependerán de la velocidad a la que se vaya resolviendo el actual «estado del arte» del cultivo celular, y en vez de «arte» se convierta en ciencia.

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Apical callus formation in *Solieria filiformis* (Gigartinales, Rhodophyta) cultured in tanks

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Key words: callus, disorganization, *Solieria filiformis*, Rhodophyta, seaweed, tank culture, turbulence

Abstract

Loose-lying wild plants of the carragenophyte *Solieria filiformis* (Kützing) Gabrielson were cultivated under greenhouse conditions in 600 l tanks in stationary and turbulent cultures, produced either by air bubbling or water jets at the bottom of the tanks. One week after inoculation 90.3% of the apices of the plants grown in air turbulent cultures initiated the formation of callus. The apices were not broken and apparently non-wounded. No callus formation were observed from a few accidentaly broken apices in any culture. Only 4% of the apices in water turbulent cultures induced callus. Reorganization of branches from the calli took place after three weeks. Organogenetic calli detached from the mother plant after four weeks and formed spherical masses of 3 cm in diameter growing as unattached balls. Cellular disorganization (*i.e.* callus formation) in *S. filiformis* seems to be a consequence of intermittent abrasion or contact stimuli against tank walls produced by turbulence.

Introduction

Studies on callus induction, culture and reorganization have played an important role in the development of higher plant tissue culture techniques and applications (Yeoman & Forche, 1980). *In vitro* attempts to induce and culture seaweed calli have been hitherto performed with little success, basically due to the lack of knowledge on the factors promoting callus formation and reorganization. Although the addition of plant growth regulators to the culture media has been described as an important factor for callus induction in seaweeds (Fries, 1973; Gusev *et al.*, 1987; Bradley & Cheney, 1990; Liu & Kloareg, 1991), there are some reports emphasizing the efficacy of physical parameters (*i.e.*, solid *vs* liquid medium, osmolality) or species specific capabilities (Polne Fuller & Gibor, 1987; Garcia Reina *et al.*, 1991).

Even the concept of what is a callus in seaweeds is controversial due to the utilization of the term callus-like to define a wide range of abnormal neo-growths obtained *in vitro* and the absence of histological studies (Garcia-Reina *et al.*, 1991). On the other hand, various types of warty outgrowths (galls, tumours) have been reported for seaweeds found in natural habitats or in laboratory cultures (reviewed in Apt, 1988).

The high capacity of *Solieria* species to form secondary attachment (Shintani, 1988; Floc'h *et al.*, 1987; Perrone & Cecere, 1991), and previous observations in our laboratory, lead us to

speculate on the potential ability of *Solieria filiformis* apices to form calli in a response to an inductive physical factor (*i.e.* turbulence).

Materials and methods

Round-shaped loose-lying plants of *Solieria filiformis* growing in a sheltered sandy bottom at the east-coast of Gran Canaria (Canary Islands) were collected, hand cleaned of epiphytes and precultured for one week with running filtered seawater. The effect of turbulence on the cellular disorganization of the plants (*i.e.* callus formation) were tested by growing the unattached plants (10–14 cm in diameter) for three months in three fiberglass tanks (600 l) at a density of 2–2.5 g wet wt l^{-1}. The first tank was a 'stationary' culture without any agitation, but with a gentle water flow provided by an aquarium pump to prevent water stratification. The turbulence of 'air-turbulent' and 'water-turbulent' tanks were generated either by injecting air or recirculating water through a PVC pipe (1/2 inch diameter), with 1 mm diameter holes drilled at 10 cm intervals centered at the bottom of the tank. This created a circulation in the turbulent cultures that maintained the round-shaped plants in constant motion, rolling from the bottom to the top of the tanks in both turbulent cultures at approximately 3 rpm. However, the plants grown in the 'air-turbulent' tanks had a higher self-rotatory speed.

All cultures were performed in a greenhouse exposed to daylight conditions. Highest irradiance (measured with a spherical sensor below the water surface) was in the range of 160–200 μmol m^{-2} s^{-1}, approximately the same irradiance the plants receive in their natural environment. Water temperature at noon was slightly lower in 'air-turbulent' (17.5 °C) than in 'water-turbulent' and 'stationary' cultures (19.9 and 19.2 °C respectively). Seawater (37‰) enriched with $NaNO_3$ (8 mM) and NaH_2PO_4 (0.8 mM) was renewed weekly. Daily fluctuations in pH were similar in the three tanks (8.2 ± 0.11).

Table 1. Frequency of apical callus in *Solieria filiformis* after one week in culture under different turbulent conditions. Number of plants examined per treatment = 30

Treatment	% Apices disorganized into callus	Number of apices examined
Stationary	0	7113
Air turbulent	90.3	5528
Water turbulent	4.0	6305

Thirty plants were collected randomly from each tank at weekly intervals and the status (normal or callus) of the apices of each plant (100–250 per plant) were examined under a stereomicroscope. Results were represented as the percentage of disorganized apices (of thirty plants) per culture system. The regeneration of accidentally broken apices were also examined.

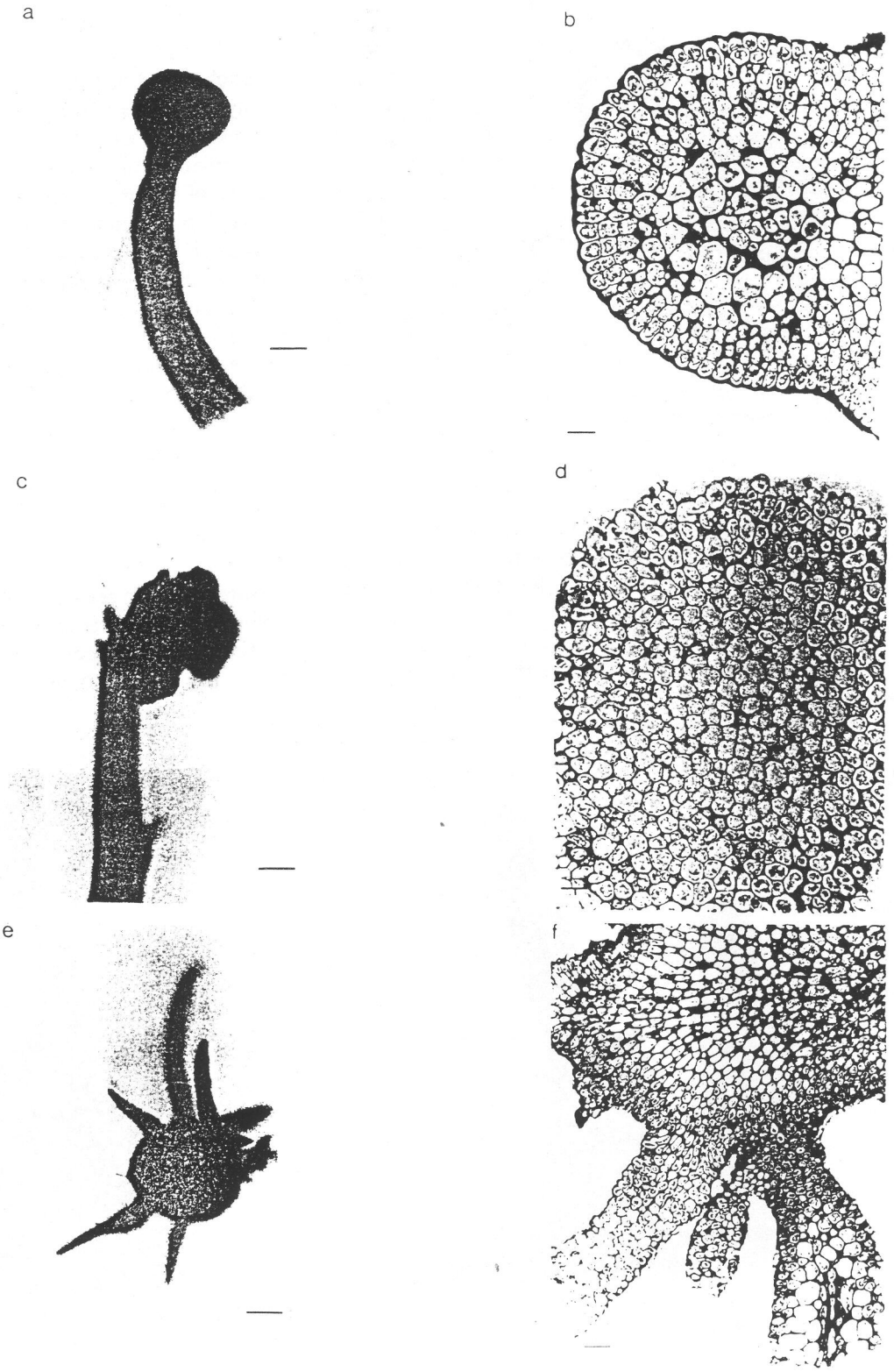
Tissues for light microscopic examination were fixed in 2.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (Pedersén *et al.*, 1980), embedded in Araldite resin, sectioned (1 μm) and stained with Toluidine blue (1%).

Results

Apices of plants grown in both turbulent systems disorganized into callus after the first week. The frequencies of callus development were significantly higher in 'air-turbulent' cultures than in the other treatments (Table 1). Only the apical parts of the plants transformed into calli. No callus formation was detected in the 'stationary' culture after the complete experimental period.

The development of callus structures from the apex of *Solieria filiformis* is shown in Fig. 1. Nor-

Fig. 1. Sequential disorganization of the apex of *Solieria filiformis* and its further reorganization (a) Initial callus development after one week in culture (b) Cross section of callus. (c) Mass of disorganized cells. (d) Transverse section of calli after two weeks. (e) Proliferation of branches from the callus after three weeks in culture. (f) Transverse section of branch developed from the callus. Scale bar = 0.5 mm for figures a, c, e. Scale bar = 8.5 μm for figures b, d, f.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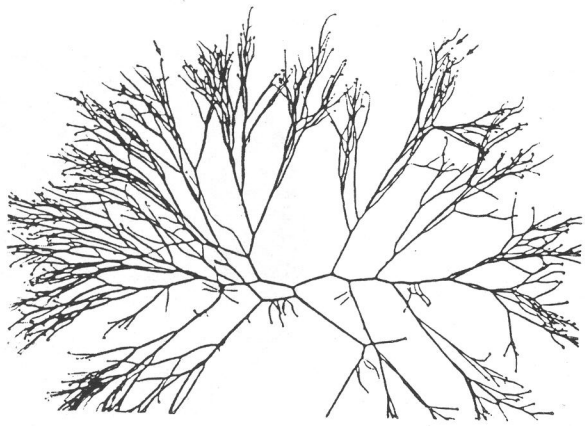

Fig. 2. Solieria filiformis after two weeks in air-turbulent culture showing the callus formation at the apices.

mal acute to long acuminate apex exhibited a progressive deepening in pigmentation and initiated the proliferation of a mass of highly differentiated and disorganized cells (callus) (Fig. 1a, b). After two weeks in culture the callus becomes more evident (Fig. 1c, d). After the third week in culture, the wider calli, between 1–2 mm in diameter, started to proliferate radially arranged branches and to form spherical balls (Fig. 1e, f). Only the apices of the plants in turbulent cultures formed these calli (Fig. 2).

Probably due to the turbulence or to fragmentation of the main axis, the organogenetic calli started to detach from the mother plant after four weeks. Free-floating calli continued the development of the branches forming spherical balls of up to 3 cm in diameter at the end of the experimental period.

Regeneration of branches from accidentally broken apices took place from a thin layer of cells (Fig. 3a, b), but no callus masses were obtained from these areas.

Discussion

Disorganization seems to be a consequence of the turbulence generated in the cultures, as other potentially inductive factors (*i.e.* irradiance, temperature, pH and nutrients) where identical in the

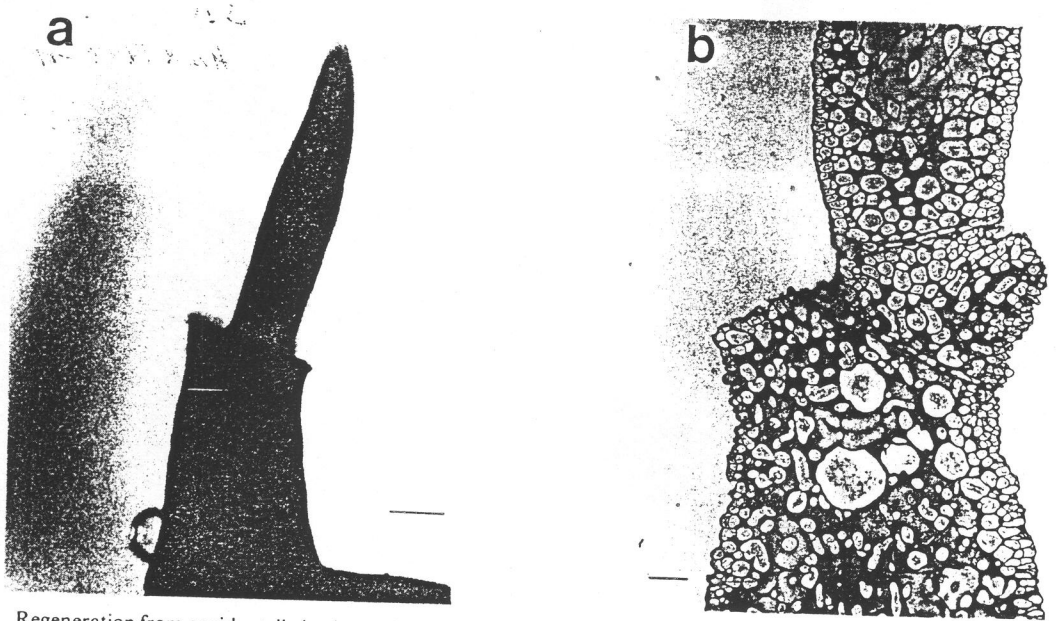

Fig. 3. Regeneration from accidentally broken apices. (a) Young bud regenerated from a broken area, scale bar = 0.5 mm (b) Longitudinal section of the regeneration surface, scale bar = 17.5 μm.

three tanks. Callus formation might be a response to abrasion of the apices against the tank walls whilst rolling around.

The higher frequencies of callus formation in 'air-turbulent' cultures might be explained by the higher intensity of shear forces or the frequency of contact stimuli against the tank walls, as the plants in this culture were more frequently overturned, despite the same rpm of circular motion as 'water-turbulent' cultures.

Norton & Mathieson (1983) suggested that loose-lying plants are modified versions of attached forms of the same species, and that these morphological changes could be a result of repeated tip damage. This response might be similar to those causing the adventituous formation of secondary attachment from cut surfaces and intact cortex of the apical region of *Solieria filiformis* described by Shintani (1988) and Perrone & Cecere (1991). As the contact stimuli in our turbulent cultures are not followed by continuous adherence to the substrate (but the opposite), the neogrowths develop and remain growing as callus.

The lack of directional guidance and polarity may influence the morphogenetic flexibility of *S. filiformis* apices. Although the polarity gradients in the thallus of seaweeds are well marked they are easily disrupted if the plant is unattached and frequently overturned (Buggeln, 1981).

Callus formation in *Solieria filiformis* seems to be a consequence of its high cellular plasticity and induced by the intensity and frequency of contact stimuli in turbulent cultures. The histology and behaviour of the calli (structure, induction, pigmentation, starch content) are quite similar to *in vitro* cultured calli of *Laurencia sp* and *Grateloupia doryphora* (Montagne) Howe (Garcia-Reina *et al.*, 1988; Robaina *et al.*, 1990).

Acknowledgements

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Ornithine cycle in *Nostoc* PCC 73102. Arginase, OCT and arginine deiminase, and the effects of addition of external arginine, ornithine, or citrulline

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Abstract. Arginase, ornithine carbamoyl transferase (OCT) and arginine deiminase activities were found in cell-free extracts of *Nostoc* PCC 73102, a free-living cyanobacterium originally isolated from the cycad *Macrozamia*. Addition of either arginine, ornithine or citrulline to the growth medium induced significant changes in their in vitro activities. Moreover, growth in darkness, compared to in light, induced higher in vitro activities. The in vitro activities of arginase and arginine deiminase, two catabolic enzymes primarily involved in the breakdown of arginine, increased substantially by a combination of growth in darkness and addition of either arginine, or ornithine, to the growth medium. The most significant effects on the in vitro OCT activities where observed in cells grown with the addition of ornithine. Cells grown in darkness exhibited about 6% of the in vivo nitrogenase activity observed in cells grown in light. However, addition of external carbon (glucose and fructose) to cells grown in darkness resulted in in vivo nitrogenase activity levels similar to, or even higher than, cells grown in light. Growth with high in vivo nitrogenase activity or in darkness with the addition of external carbon, resulted in repressed levels of in vitro arginase and arginine deiminase activities. It is suggested that nitrogen starvation induces a mobilization of the stored nitrogen, internal release of the amino compound arginine, and an induction of two catabolic enzymes arginase and arginine deiminase. A similar and even more pronunced induction can be observed by addition of external arginine to the growth medium

Key words: Arginase – Arginine deiminase – Cyanobacterium – Nitrogenase activity – *Nostoc* – Ornithine carbamoyl transferase

Many cyanobacteria are able to carry out biological nitrogen fixation, an energy requering process in which atmospheric N_2 is fixed into ammonia (Postgate 1987; Sprent and Sprent 1990; Fay 1992; Lindblad and Guerrero 1993). The ammonia is then incorporated into amino compounds, primarily through the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT) (Stewart et al. 1975; Guerrero and Lara 1987; Ohmori and Ohmori 1990; Sprent and Sprent 1990). Excess of nitrogen might be stored as cyanophycin, a copolymer of the amino compounds aspartate and arginine in 1 : 1 ratio (Simon 1976; Allen 1988). Arginine is formed by combining ornithine and carbamoyl phosphate (CP) into citrulline by ornithine carbamoyl transferase (OCT) (Fig. 1), before the citrulline is further converted to argininosuccinate and arginine by argininosuccinate synthetase and argininosuccinate lyase, respectively (Fig. 1) (Holm-Hansen and Brown 1963; Hood et al. 1969; Lehninger 1982; Carr 1983; Schubert and Boland 1990; Ohmori and Ohmori 1990). CP is synthesized from NH_4^+ or glutamine, CO_2/HCO_3^- and ATP by carbamoyl phosphate synthetase (CPS) (Fig. 1). Earlier studies showed the presence of both CPS and OCT in the filamentous heterocystous cyanobacterium *Nostoc* PCC 73102 (Lindblad 1989, 1992; Jansson et al. 1993). Immunolocalization and transmission electron microscopy demonstrated a cellular localization to both the nitrogen fixing heterocysts and the photosynthetic vegetative cells (Lindblad 1989, 1992). Native-polyacrylamide gel electrophoresis in combination with in situ activity stain revealed an in vitro active OCT with a molecular mass of approximately 80 kDa (Lindblad 1992). Moreover, addition of external arginine to the growth medium induced not only a higher in vitro OCT activity but also a second in vitro active OCT with a molecular mass of about 118 kDa (Jansson et al. 1993).

Cyanophycin is metabolized into the two amino compounds arginine and aspartate (Gupta and Carr 1981a, b; Carr 1983; Mackerras et al. 1990a, b). In the filamentous non heterocystous cyanobacterium *Oscillatoria chalybea* this catabolism has been suggested to be via an arginine-mediated induction (Bednarz and Schmid 1991). Arginase and arginine deiminase are the two enzymes primarely involved in this catabolism. Arginase hydrolyzes arginine to ornithine and urea, while arginine deiminase catalyzes the direct conversion of arginine into

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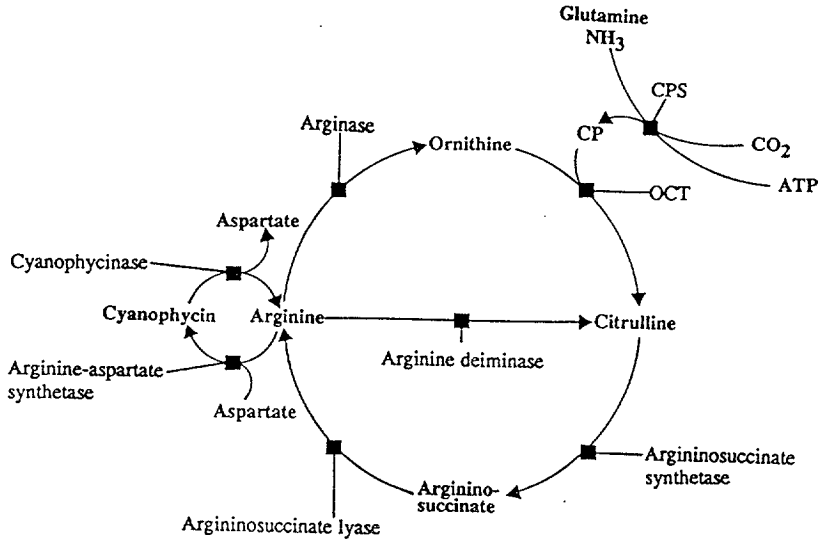

Fig. 1. Schematic presentation of the ornithine cycle in cyanobacteria capable of forming cyanophycin granules. *CP*, Carbamoyl phosphate; *CPS*, carbamoyl phosphate synthetase; *OCT*, ornithine carbamoyl transferase

citrulline (Fig. 1) (Hood and Carr 1971; Weathers et al. 1978; Carr 1983; Lehninger 1982; Schubert and Boland 1990).

The present work continues our characterization of the ornithine cycle in the cyanobacterium *Nostoc* PCC 73102. We demonstrate that certain amino compounds of the cycle are important in regulating the key enzymes arginase, OCT and arginine deiminase.

Material and methods

Organism and growth condition

Nostoc sp. strain PCC 73102 [American Type Culture Collection (ATCC) 29133], a free-living filamentous heterocystous cyanobacterium originally isolated from the cycad *Macrozamia*, was obtained from the Pasteur Culture Collection (PCC), Paris, France (Rippka et al. 1979). *Nostoc* PCC 73102, the reference strain for the genus *Nostoc* in the PCC classification, is a facultative photoheterotroph able to use glucose, fructose and/or ribose as carbon source(s) (Rippka et al. 1979). Axenic cultures were maintained in $BG11_0$-medium (Stanier et al. 1971), at 25 °C, using magnetic stirrers to continuously homogenize the cell suspensions. The experimental cells were transferred to, final volume, 30 ml $BG11_0$-N [$BG11_0$-medium + 10 mM Hepes-NaOH (pH 7.5)] in 100 ml E-flasks and placed shaking in light [Thorn Polylux 4000, and Osram Warmton Warm White (400–700 nm), providing 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the surface of the flasks] or in darkness, without or with the addition of external amino compounds (5 mM of ornithine, citrulline or arginine) and/or carbon (60 mM glucose, 60 mM fructose, or a combination of 30 mM glucose and 30 mM fructose). Growth in darkness was achieved by wrapping the E-flasks with aluminium foil. Cells were analyzed for in vivo nitrogenase activity (below) before harvested (centrifugation, 3500 × *g*), and kept in liquid nitrogen until being used in the in vitro enzyme activity measurements (below).

Nitrogenase activity

In vivo nitrogenase (EC 1.7.99.2) activity was measured as acetylene dependent ethylene production (Lindblad and Bergman 1986; Lindblad 1992; Lindblad and Guerrero 1993), and expressed per chlorophyll *a* and time. The chlorophyll *a* content was calculated according to Harborne (1973), extracting the cells in 80% (v/v) acetone for 6 to 12 h in darkness before the absorbances at 663 and 645 nm were measured using a Hitachi U-2000 (Tokyo, Japan) spectrophotometer.

In vitro ornithine carbamoyl transferase (OCT) activity

OCT (EC 2.1.3.3) was measured according to a modified method of Boyde and Rahmatullah (1980), based on the colorimetric detection of citrulline using diacetyl monoxime (DAMO, systematic name: 2,3-butanedoine monoxime; see Jansson et al. 1993). The background level (= control) used was a sample without the addition of carbamoyl phosphate. Major modifications of the originally described method (Boyde and Rahmatullah 1980) included: (i) a decrease in relative amount of chromogenic reagent, and (ii) an increase in the incubation time, both in order to increase the sensitivity in detecting the amino compound citrulline.

In vitro arginase and arginine deiminase activity

Cells were broken by sonication (MSE, Ultrasonic Power Unit # 12–63; London, UK) in 10 mM potassium-phosphate buffer (pH 8.0), centrifuged (14.000 × *g*, 4 °C, 1 h) before the supernatants were used in the in vitro assays. The reaction mixture contained 200 mM EPPS [N-(2-hydroxyethyl) piperazine-N'-(3-propanesulfonic acid), pH 8.5], 60 mM L-arginine, and 3 mM $MnCl_2$ in a final volume of 600 µl. The reaction was carried out for 30 min at 30 °C. Then the mixture was deproteinized, and the reaction stopped, by addition of 20 µl of 2 N H_2SO_4, centrifuged for 5 min and the supernatants collected. A sample without the addition of arginine was used as a control (= background level). The acidified reaction mixture was used to determine in vitro arginase (EC 3.5.3.1) and in vitro arginine deiminase (EC 3.5.3.6) activities, respectively.

In vitro arginase activity was measured according to a modified method of Archibald (1944), based on the colorimetric detection of urea using α-isonitrosophenone (INP); 500 µl of the acidified mixture was combined with 536 µl of phosphate sulphuric acid solution (concentrated H_3PO_4, H_2SO_4 and dH_2O in a ratio of 3 : 1 : 1) and 43 µl of INP solution (4 g of INP in 100 ml of 100% ethanol). The samples were boiled at 100 °C for one hour, the tubes allowed to cool down to room temperature (approximately 15 min) before the absorbance at 540 nm was measured. The in vitro arginase activities were then calculated using a standard curve, linear between 0 and 1 µmol of urea. Major modifications of the previously described method (Archibald 1944) included determinations of: (i)

the centrifugation time required for obtaining cell-free extracts, and (ii) the optimal arginine concentration in the reaction mixture.

The in vitro arginine deiminase activity was determined by colorimetric detection of citrulline, combining 100 µl of the acidified reaction mixture with DAMO as described above for the in vitro OCT activity (see also Jansson et al. 1993).

Total amount of protein in the cell-free extracts were calculated using the method described by Peterson (1983), and bovine serum albumin to standardize the assay procedure.

Results

Optimizing the methods for measuring in vitro ornithine carbamoyl transferase (OCT) and arginase activities in Nostoc PCC 73102

By modifying the original method of Boyde and Rahmatullah (1980) it was possible to measure in vitro OCT activities in cell-free extracts of the cyanobacterium *Nostoc* PCC 73102. Highest sensitivity was achieved by decreasing the volume of the chromogenic reagent from 3 ml to 600 µl and mix it with 200 µl of the sample (70 µl cell-free extract, 30 µl reaction mixture, and 100 µl TCA). Moreover, maximum colour development occurred after 30 min of boiling in a water bath at 100 °C, and both shorter or longer boiling times diminished the intensity of the colour. Similarly, several modifications were required to increase the sensitivity of the published method (Archibald 1944) for measuring in vitro arginase activity: (i) a centrifugation time of the sonicated sample for at least 1 h at 14.000 × *g*, and (ii) the optimal colour development occurred at an arginine concentration of 60 mM during the incubation.

Effect of external amino compounds on in vitro arginase, OCT and arginine deiminase activities

Generally, darkness induced higher in vitro activities compared to cells grown in the light (Table 1). The in vitro arginase activity increased substantially in cells grown with the addition of either arginine or ornithine to the growth medium. The highest activity was observed in cells grown in darkness with the addition of arginine followed by cells grown in darkness with the addition of ornithine, 71 × and 23 × increase compared to cells grown in the light, respectively (Table 1). Addition of citrulline to the growth medium had only minor stimulatory effects on the in vitro arginase activities. Interestingly, very similar effects were observed when assaying in vitro arginine deiminase activities. Again, the highest activity was observed in cells grown in darkness with the addition of arginine followed by cells grown in darkness with the addition of ornithine (Table 1). Darkness with the addition of ornithine induced the highest in vitro OCT activity followed by darkness with the addition of arginine, 12 × and 7 × the activities observed in cells grown in the light, respectively (Table 1). Addition of citrulline had either no (darkness) or negative (light) effect on the in vitro OCT activities.

In vitro nitrogenase activities

Cells grown in darkness exhibited about 6% of the in vivo nitrogenase activity observed in cells grown in light [18.1 (± 2.0) and 1.1 (± 0.3) nmol $C_2H_4 \cdot mg^{-1}$ (Chl*a*) $\cdot h^{-1}$ (means ± SD, $n = 4$), respectively]. However, addition of external carbon (glucose and/or fructose) greatly stimulated the in vivo nitrogenase activity in darkness (Fig. 2a). When a combination of glucose and fructose was added to cells grown in darkness the levels of in vivo nitrogenase activities reached those obtained for cells grown in the light. This capacity of external carbon to support high in vivo nitrogenase activity in cells grown in darkness remained even after keeping the cells for up to 12 days in darkness before the external carbon was added (Fig. 2b).

Effect of external carbon (glucose and fructose) on in vitro arginase, OCT and arginine deiminase activities

Growth in darkness, i.e. cells having only low in vivo nitrogenase activity (Fig. 2), induced higher in vitro

Table 1. In vitro arginase, ornithine carbamoyl transferase (OCT) and arginine deiminase activities in *Nostoc* PCC 73102. The cells were grown in light or darkness without or with the addition of either external arginine, ornithine or citrulline in the growth medium for 4 days before being analyzed for in vitro enzyme activities. Means ± SD ($n = 3$)

Growth condition[a]	Arginase		OCT		Arginine deiminase	
	nmol product formed · µg^{-1} (prot) · min^{-1}					
Light	69 ± 5	(100)[b]	64 ± 6[d]	(100)[b]	158 ± 2	(100)[b]
Light + Arg[c]	533 ± 127	(772)	136 ± 23[d]	(212)	1009 ± 53	(639)
Light + Orn[c]	797 ± 257	(1155)	196 ± 56	(306)	1576 ± 144	(997)
Light + Cit[c]	131 ± 3	(190)	26 ± 21	(41)	242 ± 8	(153)
Darkness	198 ± 4	(287)	140 ± 9[d]	(219)	490 ± 7	(310)
Darkness + Arg	4913 ± 92	(7120)	450 ± 19[d]	(703)	5913 ± 18	(3742)
Darkness + Orn	1578 ± 18	(2287)	780 ± 15	(1219)	1862 ± 13	(1178)
Darkness + Cit	270 ± 167	(391)	122 ± 3	(191)	318 ± 9	(201)

[a] Addition of arginine, ornithine or citrulline to light-grown cells reduced cell growth, measured as chlorophyll *a* content, with 6, 15, or 20%, respectively. However, similar additions to cells grown in darkness resulted in a 45, 15, or 16% increase, respectively

[b] % activity compared to cells grown in light without the addition of external arginine, ornithine or citrulline to the growth medium

[c] Arg, Arginine; Orn, Ornithine; Cit, Citrulline

[d] Data from Jansson et al. 1993

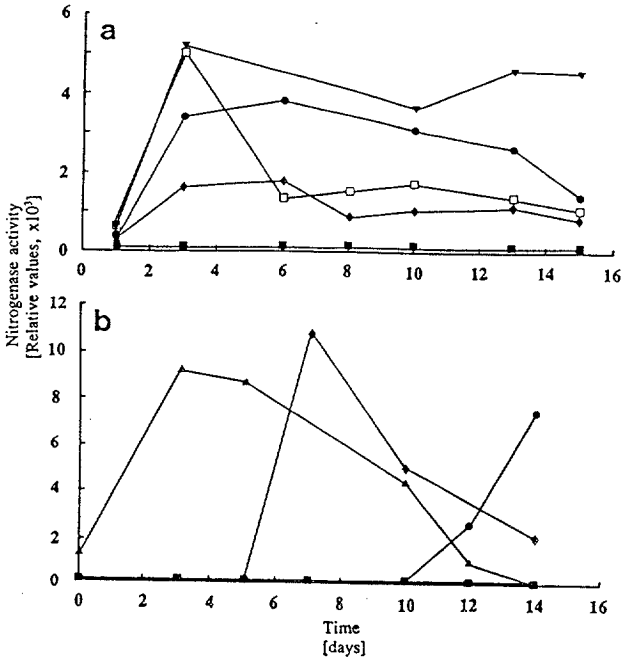

Fig. 2a, b. In vivo nitrogenase activity (acetylene reduction) by the free-living cyanobacterium *Nostoc* PCC 73102. *(a)* The cells were grown in light (□), or darkness without (■; = control, standardized to 100%) or with the addition(s) of external carbon [60 mM glucose (♦), 60 mM fructose (●), a combination of 30 mM glucose and 30 mM fructose (▼) to the growth medium. *(b) Nostoc* PCC 73102 grown in darkness without (■; = control, standardized to 100%) or with the addition of external carbon (a combination of 30 mM glucose and 30 mM fructose) to the growth medium. The external carbon sources were added after 0 (▲), 5 (♦), and 10 (●) days of incubation in darkness. Means [SD did not exceed ± 5% (n = 4)]

arginase activities (Fig. 3a). Cells grown in either light or in darkness with the addition of external carbon, i.e. cells having high in vivo nitrogenase activities (Fig. 2), showed decreased levels of in vitro arginase activities (Fig. 3a). Both light and darkness induced higher in vitro OCT activities, with the highest activities observed in cells grown in darkness without addition of external carbon (Fig. 3b). Interestingly, very similar effects were observed for in vitro arginine deiminase (Fig. 3c) as for arginase (Fig. 3a) activities.

Discussion

Arginase, ornithine carbamoyl transferase (OCT) and arginine deiminase were found to be present in cell-free extract of the filamentous heterocystous cyanobacterium *Nostoc* PCC 73102. Addition of either arginine, ornithine, or citrulline to the growth medium induced, with only one exception, higher in vitro arginase, OCT, and arginine deiminase activities. Not only the substrates for the respective enzymes induced higher in vitro activities, but also addition of eg external ornithine induced considerably higher in vitro arginase and arginine deiminase activities. The cells were grown for four days before respective enzymes were measured. During this growth period, external ornithine is rapidly converted to arginine/cyanophycin and induces higher in vitro arginase and arginine deiminase activities (Table 1). However, external citrulline might not be metabolized as quickly as ornithine and will therefore not induce any significant changes in the in vitro arginase, OCT, or arginine deiminase activities (Table 1). These results sharply contrast with those reported for *Anabaena variabilis* (Hood and Carr 1971) and *Aphanocapsa* PCC 6308 (Weathers et al. 1978), in which almost no alterations in the in vitro enzyme activities where observed when the cells were grown supplemented with arginine. However, in *Oscillatoria chalybea* the presence of arginine, ornithine or citrulline in the growth medium induced higher in vitro arginase activities (Bednarz and Schmid 1991).

Nostoc PCC 73102 is a facultative photoheterotroph (Rippka et al. 1979), with the capacity of high in vivo nitrogenase activity in darkness (Lindblad 1992). Nitrogen

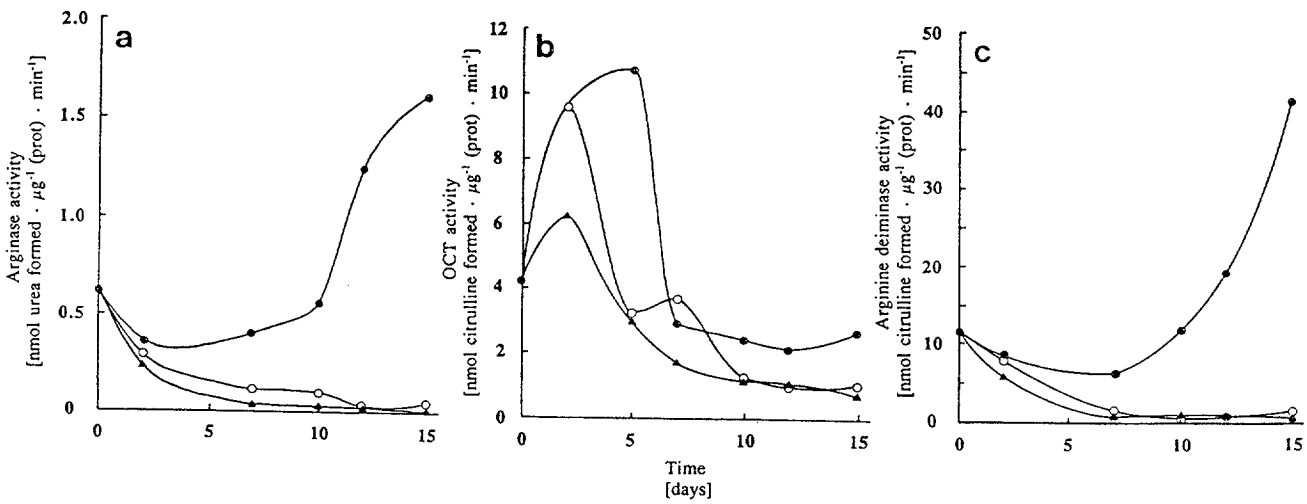

Fig. 3a–c. In vitro arginase *(a)*, ornithine carbamoyl transferase (OCT) *(b)*, and arginine deiminase *(c)* activities in *Nostoc* PCC 73102. The cells were grown in light (○), or darkness without (●) external carbon, or in darkness with (▲) the addition of external carbon (30 mM glucose and 30 mM fructose), before being analyzed for in vitro enzyme activities. Means [SD did not exceed ± 6% (n = 3)]

might be stored as cyanophycin either in the polar nodules of the heterocysts (Lindblad 1992) or in the cyanophycin granules present in the vegetative cells (not shown). Cells grown in light, or in darkness with the addition of external carbon, showed high in vivo nitrogenase activities (Fig. 2; Lindblad 1992) and decreased levels of both in vitro arginase and arginine deiminase activities (Fig. 3). However, growth in darkness resulted in low in vivo nitrogenase activity (Fig. 2) and increased in vitro arginase and arginine deiminase activities (Fig. 3). External carbon, added as a combination of glucose and fructose to cells growing in darkness, stimulated the in vivo nitrogenase activity and repressed the induction of the catabolic enzymes arginase and arginine deiminase. The external carbohydrates may serve as substrates stimulating the synthesis of both ATP through oxidative phosphorylation and NADPH by glycolysis (Bottomley and Stewart 1977; Sprent and Sprent 1990; Steinberg and Meeks 1991; Haselkorn and Buikema 1992), both required for nitrogenase activity in a cell-free system. In light, cyclic photophosphorylation (PSI) in the heterocysts may provide ATP to carry out biological nitrogen fixation (Tel-Or and Stewart 1977; Carr 1983; Sprent and Sprent 1990; Haselkorn and Buikema 1992).

An earlier study (Lindblad 1989) demonstrated that addition or either arginine, ornithine or citrulline to light grown cells *Nostoc* PCC 73102 resulted in decreased levels of in vivo nitrogenase activities. These amino compounds supplied the cells with a nitrogen source, thus reducing the demand(s) for energy requiring biological nitrogen fixation. However, in darkness, the ATP, produced mainly by oxidative phosphorylation (Bottomley and Stewart 1977; Tel-Or and Stewart 1977; Houchins 1985; Haselkorn and Buikema 1992), is not enough to support high levels of nitrogenase activity. The available nitrogen becomes insufficient to cover the metabolic demands of the cells. Consequently, the internal nitrogen storage compound, cyanophycin, might be mobilized, and, by induction of the catabolic enzymes arginase and arginine deiminase, arginine will be converted to a more available form of nitrogen. In *Anabaena cylindrica* cyanophycin has both a dynamic role in the nitrogen metabolism as well as a storage function (Mackerras et al. 1990a). Moreover, in cells grown in a dark/light cycle, the synthesis of cyanophycin continued in darkness, implying a function as a temporary storage that accumulates in the light for later amino compound synthesis in darkness (Mackerras et al. 1990a).

Addition of external arginine to the growth medium induced higher in vitro OCT activities. Moreover, as described earlier (Jansson et al. 1993), in addition to the previously described 80 kDa enzyme (Lindblad 1992), a second in vitro active OCT with a molecular mass of approximately 118 kDa appeared when the cells were grown with the addition of external arginine (Jansson et al. 1993).

In conclusion, arginase, OCT and arginine deiminase are present in cell-free extracts of the filamentous heterocystous cyanobacterium *Nostoc* PCC 73102. Their in vitro activities are siginificantly regulated by the amino compounds arginine, ornithine and citrulline.

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Marine Biology

Enzymes from marine phycophages that degrade cell walls of seaweeds

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Abstract. Agarase, cellulase and alginate lyase activities from crude extracts of *Aplysia dactylomela* Rang, *Haliotis coccinea canariensis* Nordsieck, *Littorina striata* King et Broderip and *Diadema antillarum* Phillipi were measured in vitro to compare digestive efficiencies against several components of complex seaweed cell walls. Commercial abalone acetone powder (AAP, an extract from *Haliotis* sp.; Sigma, Ref. A-7514) and purified (Sigma, Ref. A-6306) and non-purified agarases from *Pseudomonas atlantica* were used with the same objective. Optimum conditions for agarase and cellulase activities were 40 °C and pH 6.0. For alginate lyase, optimum temperature and pH were species-dependent. Highest reducing sugar release was shown by crude extracts from *A. dactylomela*. These crude extracts displayed high agarase activity compared with bacterial agarases at 40 °C, and were significantly higher at 25 °C. AAP and crude extracts from *L. striata* and *D. antillarum* exhibited high specific activities on all the substrates. Cold extract from *Gracilaria* spp. was the best substrate with which to measure agarase activity.

Introduction

Protoplast and somatic hybridization have been suggested as promising biotechnological tools for applied and basic studies in seaweed physiology, biochemistry and genetics (Cheney et al. 1986, Saga et al. 1986, Smith and Bidwell 1989, Butler et al. 1990, Davison and Polne-Fuller 1990, Jordan et al. 1991), but this potential is still constrained in agarophytes (and in other anatomically complex seaweeds) by the absence of highly efficient enzymes which degrade their cell walls.

Cellulose is the most common skeletal polymer in most macroalgae (Kreger 1962). Agar [a linear sulphated galactan composed of two regularly repeated galactose units alternatively linked by $\beta(1-4)$ and $\alpha(1-3)$ linkages] and carrageenan [highly sulphated galactans composed of D-galactose alternatively linked by $\beta(1-4)$ and $\alpha(1-3)$ linkages] in Rhodophyta, and algin [a polysaccharide made up of two uronic acids, β-1,4-D-mannuronic and α-1,4-L-guluronic acids] in Phaeophyta, are the most common matrix components of seaweeds (Kloareg and Quatrano 1988).

Inefficient digestion of the cell walls of anatomically complex brown and red seaweeds is not only due to their inherent complexity, but also to changes in cell-wall constitution due to age, life history, physiological status and culture conditions (Kloareg and Quatrano 1988, Björk et al. 1990). The complex and wide range of structural, matrix and storage carbohydrates found in seaweeds can be degraded by several marine herbivores and microorganisms (Galli and Giese 1959, Lewis 1964, Nisizawa et al. 1968, Benítez and Macaranas 1979, Torzilli and Andrykovitch 1980, Liu et al. 1984, Morrice et al. 1984, Onishi et al. 1985, Preston et al. 1985, Suzuki et al. 1986, Boyen et al. 1990). Broad-spectrum enzymatic systems freshly isolated from microorganisms (Polne-Fuller and Gibor 1987a, Smith and Bidwell 1989, Le Gall et al. 1990) or from the digestive tract of phycophages (Zhu 1983, Polne-Fuller et al. 1984, Saga and Sakai 1984, Chen 1987, Tokuda and Kawashima 1988, Butler et al. 1989) have been described as effective for the isolation of seaweed protoplasts, but the results have shown variable success.

There are very few data on enzymes from marine herbivores that degrade cell walls of seaweeds, and no quantitative studies of the agarolytic activity of extracts from marine molluscs have been reported. Knowledge of the specific polysaccharidase activity is a previous basic step to obtaining sustained high protoplast yields and plating efficiency (Boyen et al. 1990). The aim of the present study was the quantitative screening of enzymes from phycophages and bacteria that degrade seaweed cell walls, and the comparison of specific enzymatic activities at temperatures and pHs appropriate for the recovery of viable seaweed protoplasts.

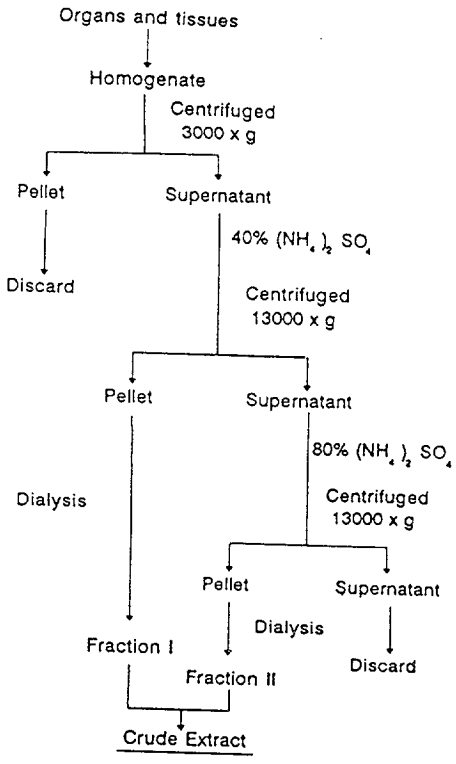

Fig. 1. Schematic of extraction procedure for preparing crude extracts from phycophages

Materials and methods

Crude extracts from the molluscs *Aplysia dactylomela* Rang, *Haliotis coccinea canariensis* Nordsieck, *Littorina striata* King et Broderip and the echinoderm *Diadema antillarum* Phillipi were prepared within 24 h of collection.

Preparation of crude extracts

Salivary glands, oesophagus and the digestive gland of *Aplysia dactylomela*, the digestive tract and hepatopancreas of *Haliotis coccinea canariensis*, the gut of *Diadema antillarum* and the whole body of *Littorina striata* (after shell removal), were used as enzymatic sources. Temperatures between 0 and 4 °C and 0.1 *M* phosphate buffer at pH 6.0 or 7.2 were used for all operations (except where indicated otherwise).

The pH of the oesophagus and digestive gland (separately) from *Aplysia dactylomela* and the gut from *Diadema antillarum* were measured immediately after dissection.

The extraction procedure is shown in Fig. 1. Glands and organs were homogenized and extracted with buffer supplemented with EDTA Na_2 (2.0 m*M*) in a mortar under liquid nitrogen. Homogenates were squeezed through a 170 µm nylon mesh and centrifuged at 3000 ×*g* for 30 min.

A saturated ammonium sulphate solution was added to the supernatants to 40% saturation. Suspensions were equilibrated for several hours and then centrifuged at 13 000 ×*g* for 20 min. Pellets were resuspended in buffer (Fraction I), and saturated ammonium sulphate was added to the new supernatants to 80% saturation. After equilibration and centrifugation at 13 000 ×*g* for 20 min, pellets were collected and dissolved in buffer (Fraction II). Fractions I and II were dialyzed against buffer overnight. Crude extracts consisted of the combined dialyzed Fractions I and II.

Purified agarase (PA) (EC 3.2.1.81, 1000 U ml^{-1} buffer; unit definition: 1 unit will produce 1.0 µg of reducing sugar, measured as D-galactose, from agar per min at 40 °C and pH 6.0) from *Pseudomonas atlantica* (Sigma, Ref. A-6306) and abalone acetone powder (AAP, a commercially available crude extract prepared from *Haliotis* sp. entrails; Sigma, Ref. A-7514) dissolved in buffer were used for a comparative experiment. After several hours equilibration, abalone acetone powder solution (1% w/v) was centrifuged at 3000 ×*g* for 30 min and dialyzed overnight against buffer.

A non-purified agarase (NPA), from a *Pseudomonas atlantica* culture (kindly provided by Boehringer Mannheim), containing 110 U ml^{-1} of agarase [unit definition: 1 unit of agarase will produce 1.0 µg/min of reducing sugars from low melting-point agarose, measured as D-galactose by the ferric-cyanide assay (Kidby and Davidson 1973) at 45 °C and pH 6.8] was also used for comparison of agarolytic activities.

All the enzymatic solutions were stored at −20 °C until use. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Substrates

Cold extract from *Gracilaria* spp. (kindly supplied by Professor M. Pedersén, University of Uppsala, Sweden), "natural" agar (dry powder without any additive, as extracted from *Gelidium* sp., kindly supplied by R. Armisén, Hispanagar S.A., Spain) and low melting-point agarose (Sigma, Ref. A-4018) were used as substrates to measure agarase activity. The extraction procedure of cold extract (Lahaye et al. 1986) is based on the differential solubility of agar polymers in ethanol and water at different temperatures. The agar content in the cold extract (extracted in distilled water at 22 °C) is equivalent to the agar content of the young tissue (Lahaye et al. 1986).

Carboxymethyl cellulose (CMC) and sodium alginate (Sigma, Ref. C-8758 and A-2158, respectively) were used for cellulase (EC 3.2.1.4) and alginate lyase (EC 4.2.2.3) activities, respectively.

Quantification of enzymatic activities

The assay system consisted of 900 µl of 0.3% (w/v) substrate solution dissolved in buffer and 100 µl crude extract. Mixtures were incubated for 4 h at 25 and 40 °C. Samples were removed at different intervals of time and reactions were stopped by boiling mixtures for 3 min in a water bath. After centrifugation (3000 ×*g*, 5 min) supernatants were used for the analysis of reducing sugars (RS). RS were determined spectrophotometrically by the method of Nelson (1944) as modified by Somogyi (1952), using galactose, glucose and galacturonic acid as standards for agarase, cellulase and alginate lyase activities, respectively. Results were corrected by subtraction of the corresponding blank.

Specific enzymatic activities were expressed as µg of reducing sugars produced per mg of protein per hour. Results are the means of 3 to 5 different determinations (5 observations each). The variability of each experiment never exceeded 10% of the mean.

Results

The pH in the oesophagus and digestive gland of *Aplysia dactylomela* was (mean ± SD) 5.58 ± 0.42 and 5.85 ± 0.19, respectively ($n = 8$), and in the gut of *Diadema antillarum* 6.92 ± 0.18 ($n = 4$). pH in the tract and hepatopancreas of *Haliotis coccinea canariensis* and in the whole body of *Littorina striata* was difficult to measure because of their small size, but the pH of the homogenates varied between 5.5 and 6.0.

Table 1. *Aplysia dactylomela, Haliotis coccinea canariensis, Littorina striata* and *Diadema antillarum*. Specific enzymatic activities of digestive enzymes at different conditions of temperature (25 and 40 °C) and pH (6.0 and 7.2). RS: reducing sugars, CX: cold extract, CMC: carboxymethyl cellulose, Alg: sodium alginate, –: not tested

Extract	pH	Specific activity (μg RS mg protein^{-1} h^{-1})								Protein conc, mean ± SD (mg ml^{-1})
		CX		Agar		CMC		Alg		
		25 °C	40 °C	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C	
A. dactylomela	6.0	63.4	76.2	19.4	47.0	73.9	74.1	14.2	45.1	6.2 ± 1.6
	7.2	38.6	58.1	19.8	46.2	62.6	77.9	18.0	52.2	(n = 10)
Abalone acetone powder	6.0	74.7	95.8	41.0	15.3	92.9	116.8	88.5	33.4	1.9 ± 0.4
	7.2	47.4	64.6	12.8	24.4	76.8	80.0	140.0	181.0	(n = 9)
H. coccinea canariensis	6.0	8.9	–	–	–	49.1	–	–	–	6.7 ± 1.2
	7.2	6.9	–	–	–	39.6	–	–	–	(n = 4)
L. striata	6.0	55.2	13.5	74.7	19.3	122.6	108.7	60.1	65.3	1.2 ± 0.3
	7.2	43.9	21.2	51.0	14.3	89.5	122.9	50.6	112.1	(n = 7)
D. antillarum	6.0	42.0	85.1	89.1	57.0	64.8	73.5	28.8	14.8	1.9 ± 0.5
	7.2	77.8	106.8	79.2	74.1	70.6	68.8	28.2	6.7	(n = 4)

Table 2. *Aplysia dactylomela* and *Pseudomonas atlantica*. Agarase activity of crude extract from *A. dactylomela*, and purified (PA) and non-purified (NPA) agarases from *P. atlantica*. CX: cold extract; –: not tested

Extract	pH	Agarase activity (μg RS h^{-1})					
		CX		Agar		Agarose	
		25 °C	40 °C	25 °C	40 °C	25 °C	40 °C
A. dactylomela (crude)	6.0	393.1	472.4	120.3	291.4	118.0	261.8
	7.2	239.3	360.2	122.7	286.4	–	–
Agarase (PA)	6.0	275.4	384.5	73.0	213.8	87.8	118.0
	7.2	234.9	370.5	82.0	180.0	39.7	84.0
Agarase (NPA)	6.0	119.5	138.5	37.0	90.0	33.3	77.0
	7.2	111.9	54.9	76.0	109.0	67.5	51.1

Specific enzymatic activities and protein concentration of crude extracts of *Aplysia dactylomela*, abalone acetone powder, *Haliotis coccinea canariensis, Littorina striata* and *Diadema antillarum* are given in Table 1. Total protein concentration was 3- to 5-fold higher in *A. dactylomela* and *H. coccinea canariensis* than in the abalone acetone powder, *L. striata* and *D. antillarum* extracts. These differences strongly affected the results for specific enzymatic activity on a protein concentration basis.

All the enzymatic systems degraded the experimental substrates, although clear differences were observed in substrate-specificity and as a function of temperature and pH. Optimal temperature and pH for agarase and cellulase activity were 40 °C and pH 6.0 for all the extracts, except for cellulase activity in *Littorina striata* (Table 1).

Highest alginate lyase activities were generally obtained at 40 °C and pH 7.2, with some exceptions; AAP showed very high activities at 25 °C, although the highest was observed at 40 °C (pH 7.2), *Diadema antillarum* crude extracts displayed highest activity at 25 °C, and *Littorina striata* at 40 °C and pH 7.2 (Table 1).

Agarolytic activities from *Aplysia dactylomela* and both purified and non-purified bacterial sources, expressed as μg RS h^{-1}, are shown in Table 2. Optimum temperature and pH for bacterial agarases appeared to be the same as for crude extracts from phycophages (40 °C and 6.0). The agarolytic activity of *A. dactylomela* crude extracts was significantly higher against cold extract, agar and agarose, and varied as a function of temperature and pH (Table 2).

Fig. 2 shows the hydrolytic evolution of some extracts on different substrates. The extract from *Aplysia dactylomela* had the highest RS-release rate on all the substrates except sodium alginate, and displayed significant differences between activities on cold extract and agar (Fig. 2A). No significant increases in RS were observed after 4 h of incubation.

Discussion

This is the first report which describes agarase activity from phycophages. Our results revealed similar optimum values for temperature and pH as those described for bacterial agarases, i.e., temperatures around 40 to 41 °C and pH between 5.0 and 7.2 (Yaphe 1966; Duckworth

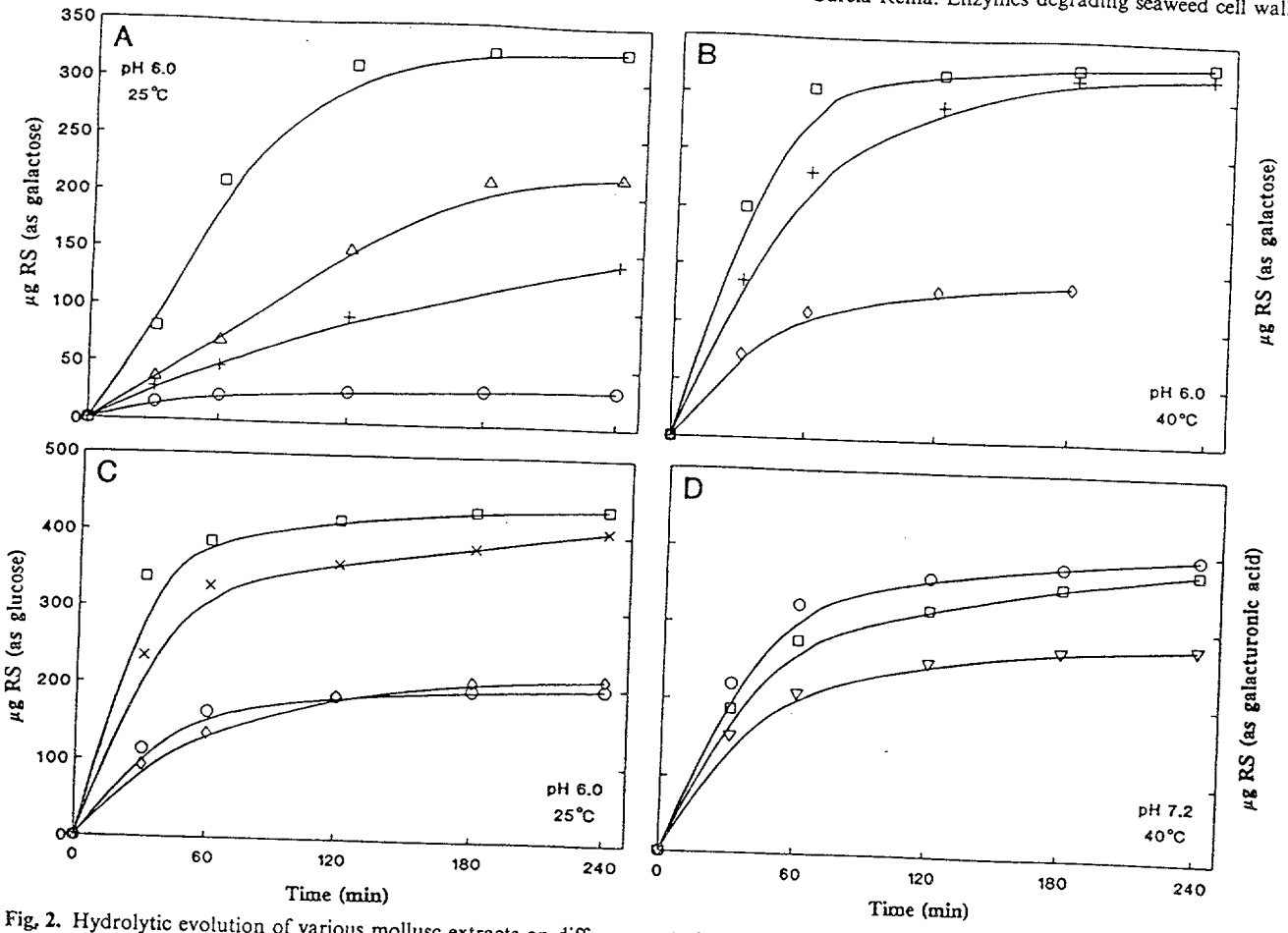

Fig. 2. Hydrolytic evolution of various mollusc extracts on different substrates. (A) On cold extract (□: *Aplysia dactylomela*; △: abalone acetone powder, AAP) and on natural agar (+: *A. dactylomela*; ○: AAP); (B) on natural agar (□: *A. dactylomela*; +: purified agarase; ◇: *Diadema antillarum*); (C) on carboxymethyl cellulose (□: *A. dactylomela*; ×: *Haliotis coccinea canariensis*; ○: AAP; ◇: *D. antillarum*); (D) On sodium alginate (○: AAP; □: *A. dactylomela*; ▽: *Littorina striata*). Incubation conditions are given inside each graph. RS: reducing sugars

and Turvey 1969, Van der Meulen 1975, Morrice et al. 1983). The agarolytic activities of crude extracts from *Aplysia dactylomela* (combined with high cellulase activity) appeared to be optimum at optimum conditions for protoplast isolation, 25 °C and pH 6.0, and were notably higher than activities of bacterial agarases. As expected, optimum pH conditions were similar to these measured in the digestive system, a fact common to all the enzymatic activities and phycophages.

The reducing power is hindered at temperatures <35 °C when agar and agarose are used as substrates, since these substrates do not form homogeneous solutions (Van der Meulen 1975). However, activities on cold extract (which does not form gels at any concentration and is easily dissolved in aqueous solution), natural agar and low melting-point agarose at concentrations up to 0.3%, can be measured at lower temperatures. Compared with natural agar and agarose (Tables 1 and 2), cold extract enabled the highest resolution of agarase activity measurements at a broad range of temperatures.

The results for cellulase activity are in accordance with those obtained for other species of molluscs (Yokoe and Yasumasu 1964, Hylleberg Kristensen 1972, Brock et al. 1986, Suzuki et al. 1986, Boyen et al. 1990), as are the optimum ranges of temperatures (24 to 35 °C) and pH (6.0 to 7.0) (Horiuchi and Lane 1965, Brock et al. 1986).

Boyen et al. (1990) found similar in vitro levels of activity between a commerical cellulase and crude and purified fractions of *Aplysia depilans* and *Haliotis tuberculata* using microcrystalline cellulose as substrate. The negligible cellulase activity of the crude extracts used for the isolation of protoplasts compared to commercial cellulase could be due to the use of an inappropriate substrate. To measure digestive cellulases, the most convenient substrate is carboxymethyl cellulose (CMC) which is easier to degrade than other cellulose derivatives (Hylleberg Kristensen 1972). However, the ability to digest CMC does not prove that an organism can digest the types of cellulose present in its natural diet (Friesen 1980). In our case, CMC was extensively digested by all the crude extracts, including these prepared from *Diadema antillarum*, although previous results (Lewis 1964) did not show cellulase activity in extracts from this sea urchin using filter paper as substrate.

Similar data on alginate lyase activities from *Aplysia, Haliotis* and *Littorina* species had been reported previously (Franssen and Jeuniaux 1965, Nakada and Sweeny 1967, Elyakova and Favorov 1974, Boyen et al. 1990), but not for *Diadema antillarum*, although Eppley and Lasker (1959) described degradation of sodium alginate solutions by other species of sea urchins.

Differences in agarase, cellulase and alginate lyase activities of crude extracts from *Aplysia dactylomela, Haliotis coccinea canariensis, Littorina striata*, and *Diadema antillarum*, are probably related to factors such as dietary preferences and availability of seaweeds. The highest alginate lyase activity was shown by the acetone powder from abalone (*Haliotis* sp.), and could be related to the preparation of extracts from individuals fed specific diets (i.e., brown seaweeds such as *Macrocystis pyrifera*) for long periods.

The acetone powder method for the extraction of abalone entrails was very effective, as reported previously by Polne-Fuller (1987). Substances which could be potentially harmful to plant tissues during the process of protoplast isolation (Cocking 1972, Berliner 1981) are removed from crude extracts by partial purification with ammonium sulphate and desalting. Nevertheless, new steps in the process of purification could be aimed at elucidating the behaviour and the biochemical properties of the different enzymes found in these phycophages.

Extracts from *Aplysia dactylomela*, commercial abalone acetone powder, *Haliotis coccinea canariensis, Littorina striata* and *Diadema antillarum* are highly efficient in digesting the complex carbohydrates in seaweed cell walls, at a temperature (25 °C) and pH (6.0) optimal for the recovery of high protoplast yields and plating efficiencies of complex seaweeds (Polne-Fuller and Gibor 1987b, Butler et al. 1990).

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GENETIC VARIABILITY OF *GELIDIUM CANARIENSIS* (RHODOPHYTA) DETERMINED BY ISOZYME ELECTROPHORESIS[1]

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ABSTRACT

The populations of Gelidium canariensis *(Grunow) Seoane-Camba from the Canary Islands were analyzed for genetic variability by isozyme electrophoresis in 1989 and 1990. Each population was divided into sporophytic and gametophytic subpopulations. Twenty-three to 27 putative alleles corresponding to 22 gene loci were analyzed. Seventeen loci were monomorphic in all six subpopulations, and five were polymorphic in at least one subpopulation. Significant deviations from Hardy-Weinberg equilibrium were found. The amount of genetic variability (percentage of polymorphic loci, mean number of alleles per locus, and average gene diversity) of haploid subpopulations was lower than that of diploid subpopulations. No correlation between genetic distance and geographical distance was found. Low genetic differentiation between sporophytic and gametophytic subpopulations of the same locality was observed in two populations. The low genetic diversity and genetic differentiation suggest that the genetic structure of the populations of* G. canariensis *from the Canary Islands is due to a combination of founder effects and the predominance of asexual reproduction. Initial differences in gene frequencies may have persisted because of insufficient time to reach a higher level of differentiation.*

Key index words: Canary Islands; electrophoresis; founder effect; Gelidium canariensis; *genetic variability; isozymes; Rhodophyta*

Quantitative estimates of genetic differentiation and variability have been greatly facilitated by isozyme electrophoresis. In red seaweeds, isozyme electrophoresis has been extensively applied in systematic studies (Mallery and Richardson 1971, 1972, Fujiura et al. 1978, Lindstrom and South 1989, Lindstrom and Cole 1990), but its application for analyzing genetic variability in populations has been very limited (Cheney 1985, Sosa and Garcia-Reina 1992). In four species of *Eucheuma*, Cheney and Babbel (1978) found similar values in the proportion of polymorphic loci, ranging from 0.25 for *E. acanthocladum* (= *Meristiella gelidium* (J. Agardh) Cheney and Gabrielson) to 0.36 for *E. isiforme* (C. Agardh) J. Agardh. Sosa and Garcia-Reina (1992) reported higher genetic variability for diploid subpopulations with an average proportion of polymorphic loci of 0.212) than gametophytic subpopulations (with an average of 0.060) for three locations of *Gelidium arbuscula* Bory from the Canary Islands. The proportion of polymorphic loci in Japanese populations of the haploid laver *Porphyra yezoensis* Ueda ranged from 0.000 for cultured populations (Miura et al. 1979) to 0.583 for wild populations (Fujio et al. 1987).

The application of isozyme electrophoresis to compare interspecific and/or intraspecific genetic differentiation has been very limited for marine algae compared to other organisms (Innes 1984). Cheney and Babbel (1978) reported high genetic differentiation in populations of *Eucheuma nudum* (= *E. isiforme* var. *denudatum* Cheney) from Florida populations, suggesting that a combination of selective forces and/or genetic drift coupled with genotypic differences originally present were the cause for the differentiation. High genetic differentiation was also found among Japanese populations of *Porphyra yezoensis*, with the genetic differentiation among gametophytic populations higher than that of other diploid organisms (Miura et al. 1979, Fujio et al. 1985, 1987). Similar results showing a higher genetic differentiation among gametophytic subpopulations than among sporophytic subpopulations were reported by Sosa and Garcia-Reina (1992) for three locations of *Gelidium arbuscula* from the Canary Islands.

The objective of the present study was to compare the levels of genetic variability of gametophytes and sporophytes of *Gelidium canariensis* and to examine the population structure and differentiation in this species from the Canary Islands by isozyme electrophoresis.

MATERIALS AND METHODS

Three populations of *Gelidium canariensis* (Grunow) Seoane-Camba from different sites in the Canary Islands were sampled during 1989 and 1990 (Fig. 1). Individuals from each population were collected at least twice per year and separated into sporophytic and gametophytic subpopulations by their reproductive structures. Thalli between 0.3 and 0.5 g fresh weight were used for the analysis. Collection, extraction, and electrophoresis were conducted following methods reported previously (Sosa and Garcia-Reina 1992). Eight enzymatic systems were studied by starch gel electrophoresis: alkaline phosphatase (ALP), esterase (EST), NADH-diaphorase (DIA), malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), phosphoglucose mutase (PGM), superoxide dismutase (SOD), and phycoerythrin (PE).

Genotype frequencies were inferred directly from isozyme phenotypes. A tentative interpretation of banding patterns was possible through identification of haploid (gametophytic) and diploid (tetrasporophytic) plants (Cheney and Babbel 1978, Sosa and Garcia-Reina 1992).

For each locus and allele, the R_f value (distance of migration of band/distance of migration for bromophenol blue) was calculated. The

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frequencies using Levene's formula for small samples (Levene 1949). The Chi-square test was used to estimate the deviations of genotype frequencies from the Hardy-Weinberg equilibrium.

The proportion of polymorphic loci, mean number of alleles per locus, and average gene diversity were calculated to quantify the amount of genetic variability for each subpopulation. Genetic identity and genetic distance values (Nei 1972) were computed for pairwise comparisons of subpopulations. Genetic differentiation between subpopulations was estimated using the coefficient of differentiation (G_{ST}) (Soltis and Soltis 1988):

$$G_{ST} = \frac{S^2}{p(1-p)},$$

where S^2 is the variance of gene frequencies among subpopulations and p is the average gene frequencies over all subpopulations compared. G_{ST} can vary from 0 (= populations with same allele frequencies at locus in question) to 1 (= populations fixed for different alleles).

RESULTS

A total of 27 alleles from 22 loci were inferred from the electromorph pattern. All bands migrated anodally (Fig. 2). Each locus containing an alternate allele was considered polymorphic. Seventeen loci (PE-1, PE-2, DIA-1, DIA-2, DIA-4, EST-1, EST-3, GDH, MDH-2, PGI-1, PGI-2, PGM, SOD-1, SOD-2, SOD-3, SOD-4, and SOD-5) were monomorphic for the same allele in all populations (Fig. 2). Only five loci (ALP-1, DIA-3, EST-2, MDH-1, and PGI-3) showed polymorphism in at least one subpopulation. All polymorphic loci were diallelic (Fig. 2). Additional bands were observed in gels stained for MDH, PGI, and EST, but due to their uninterpretable variation, they were omitted from this study.

Variation at the MDH-1 and PGI-3 conformed to a diallele, dimeric enzyme interpretation with three bands at heterozygous individuals. ALP-1, DIA-3, and EST-2 conformed to a diallele, monomeric enzyme interpretation, with two bands for heterozygotic individuals (Fig. 2)

When gene frequencies for each subpopulation were compared (Table 1), PGI-3 showed the greatest amount of variability (all subpopulations showed polymorphism), while ALP-1 showed polymorphism in only one subpopulation (Agaete sporophyte).

The proportion of polymorphic loci ranged from 0.182 for the combined diploid subpopulations to 0.091 for the combined gametophytic subpopulations. The average number of alleles per locus was 1.14 for all subpopulations, and gene diversity ranged from 0.050 for sporophytic subpopulations to 0.031 for gametophytic subpopulations (Table 2). For each population, the diploid subpopulation (tetrasporophytes) displayed higher variability than the haploid subpopulation, except for the Agaete population.

Important differences for polymorphic loci were observed when the observed and expected numbers of each genotype for each locality were compared (Table 3). Although EST-2bb was the only genotype expected in the Pto. Cruz population, EST-2ab was one of the most predominant genotypes observed. Similarly, clear differences can be observed at DIA-3

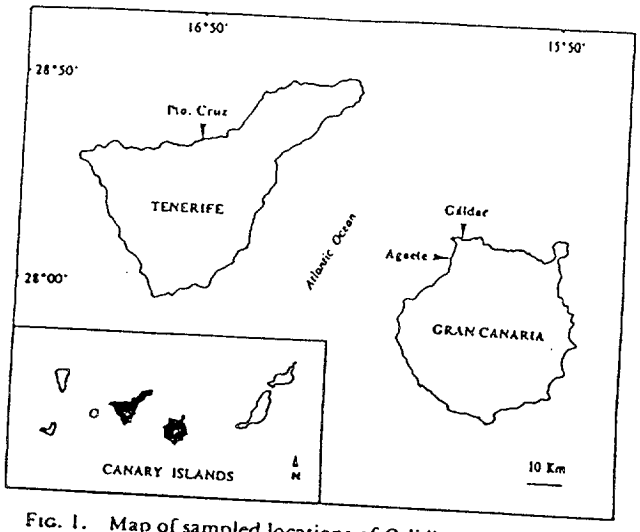

FIG. 1. Map of sampled locations of *Gelidium canariensis* from the Canary Islands.

and PGI-3 from Gáldar and DIA-3 from Pto. Cruz (Table 3). Although only three loci could be statistically analyzed by Chi-square test (Table 4), differences were significant at the 0.001% level.

Using Nei's genetic identity, the average genetic distance between all subpopulations (within *Gelidium canariensis*) was 0.017 (Table 5). Subpopulations from the same location (independent of their ploidy) displayed the least genetic distance, except for the Gáldar location, where sporophytic and gametophytic subpopulations showed the highest genetic distance. No relation was found between the genetic and geographic distance. Agaete and Pto. Cruz populations at a distance of more than 100 km and located on different islands showed less genetic distance than Agaete and Gáldar populations (separated by only 30 km and on the same shore of the same island; Table 5, Fig. 1).

The coefficient of genetic differentiation within each location was relatively low, except for the Gáldar population (Table 6).

DISCUSSION

Gelidium canariensis presents a low level of genetic variability and deviations from the Hardy-Weinberg equilibrium. The average number of alleles per locus (A) and the average proportion of polymorphic loci (P) were 1.18 and 0.182, respectively, for sporophytic subpopulations and 1.10 and 0.091, respectively, for gametophytic subpopulations (Table 2). These levels of genetic variation are lower than those described for other seaweeds (see Sosa and García-Reina 1992) and may be the consequence of a combination between a predominance of asexual reproduction and a founder effect (variation in gene frequency due to the founding of a population by a small number of individuals). Theoretically, *Gelidium canariensis*, like other species of *Gelidium*, has a

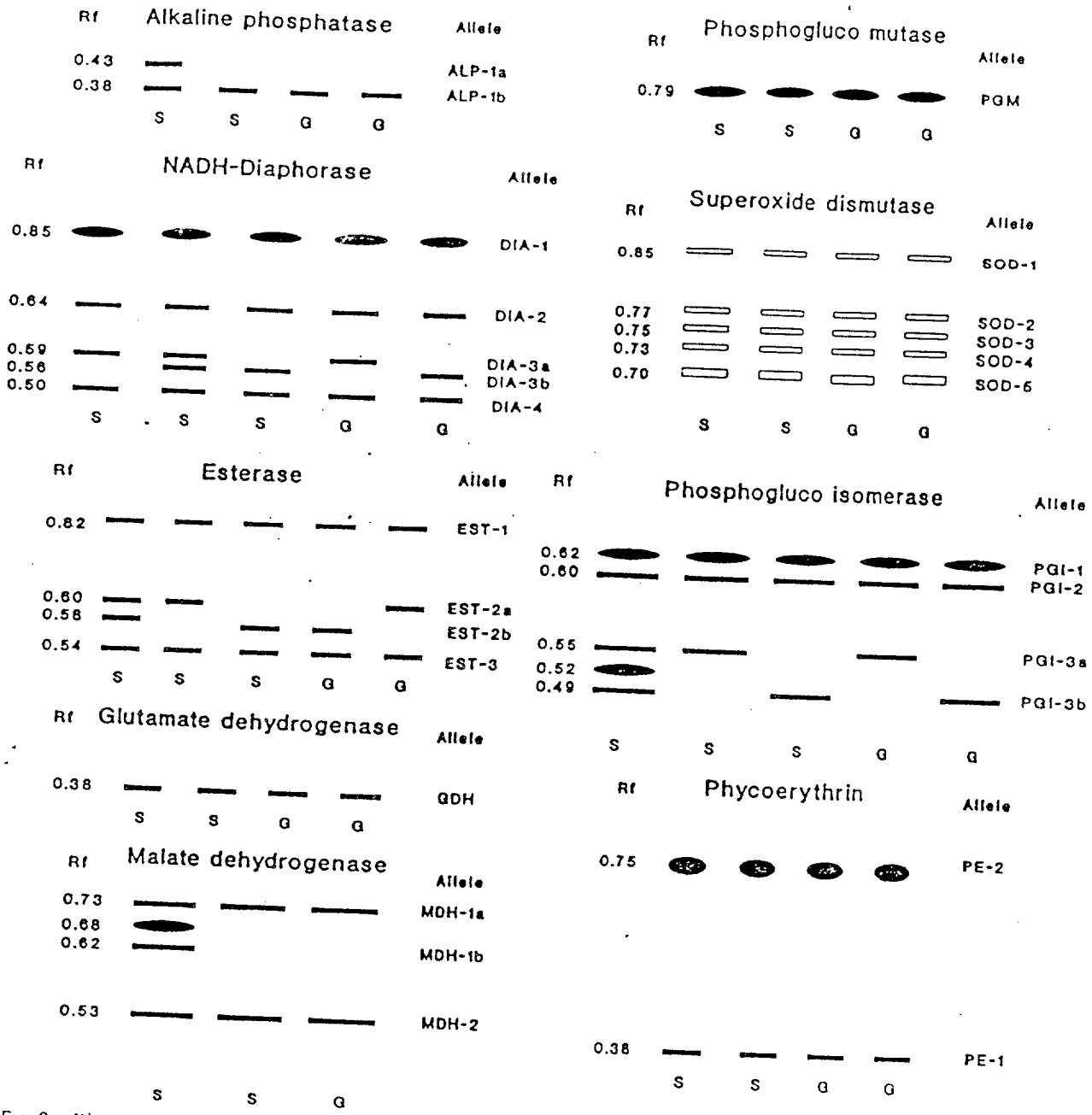

FIG. 2. Diagrammatic representation of total zymograms found in sporophytes (S) and gametophytes (G) of *Gelidium canariensis* from the Canary Islands. R_f = relative mobility. All bands migrated anodally. Direction of migration from bottom to top.

Polysiphonia-type life history with isomorphic generations. It is a slow-growing perennial, regenerating new individuals vegetatively from the established holdfast (unpubl. observ.). This life history is indirectly confirmed by the occurrences of fertile tetrasporophytic and cystocarpic fronds. These circumstances, combined with the endemic character of this species (Scoane-Camba 1979, Gil-Rodríguez and Afonso-Carrillo 1980) and their locations (volcanic islands), suggest that a founder effect may be responsible for the low levels of genetic variation observed in these populations. Asexual reproduction can result in reduced genetic variability in a population by magnifying the effects of random drift or founder effects (Black and Johnson 1979, Innes and Yarish 1984, Sosa and García-Reina 1992). A founder effect, combined with a predominant vegetative propagation of [illegible]

TABLE 1. *Gene frequencies of polymorphic loci detected in gametophytic (G) and sporophytic (S) subpopulations from three locations of* Gelidium canariensis *in the Canary Islands. Numbers in parentheses indicate number of individuals analyzed.*

	Population					
	Gáldar		Agaete		Pto. Cruz	
Allele	S	G	S	G	S	G
ALP-1a	0.00	0.00	0.03	0.00	0.00	0.00
ALP-1b	1.00	1.00	0.97	1.00	1.00	1.00
	(32)	(11)	(37)	(18)	(50)	(12)
DIA-3a	0.23	0.00	0.22	0.33	0.24	0.00
DIA-3b	0.77	1.00	0.78	0.67	0.76	1.00
	(33)	(11)	(37)	(18)	(42)	(12)
EST-2a	0.03	0.20	0.53	0.75	0.25	0.00
EST-2b	0.97	0.80	0.47	0.25	0.75	1.00
	(32)	(15)	(36)	(16)	(32)	(12)
MDH-1a	1.00	1.00	0.98	1.00	0.90	1.00
MDH-1b	0.00	0.00	0.02	0.00	0.10	0.00
	(40)	(18)	(23)	(17)	(39)	(11)
PGI-3a	0.32	0.89	0.08	0.22	0.37	0.25
PGI-3b	0.68	0.11	0.92	0.78	0.63	0.75
	(44)	(18)	(37)	(18)	(43)	(12)

TABLE 3. *Observed (O) and Hardy-Weinberg equilibrium expected (E) genotypes for each polymorphic locus of* Gelidium canariensis *at three locations analyzed.*

	Population					
	Gáldar		Agaete		Pto. Cruz	
Genotype	O	E	O	E	O	E
ALP-1aa	0	0	0	0	0	0
ALP-1bb	32	32	35	37	50	50
ALP-1ab	0	0	2	0	0	0
DIA-3aa	0	0	4	3.9	3	0
DIA-3bb	18	33	25	16.5	25	42
DIA-3ab	15	0	8	16.6	14	0
EST-2aa	0	1.2	10	20.2	2	0
EST-2bb	30	20.4	8	2.1	18	32
EST-2ab	2	10.4	18	13.7	12	0
MDH-1aa	40	40	22	23	31	39
MDH-1bb	0	0	0	0	0	0
MDH-1ab	0	0	1	0	8	0
PGI-3aa	14	34.8	0	1.7	11	2.6
PGI-3bb	30	0.5	31	22.4	22	24.1
PGI-3ab	0	9.8	6	5.5	10	20.3

sidered the reason for the low genetic variability detected in *G. canariensis*. A founder effect has been also hypothesized for the lack of genetic variation in other seaweeds (e.g. *Codium fragile* (Sur.) Hariot subsp. *tomentosoides* (van Goor) from Long Island populations (Malinowsky 1974) and *Gelidium arbuscula* populations from the Canary Islands (Sosa and Garcia-Reina 1992). Although the lower genetic variation of gametophytes could be the result of selection against the gametophytes (Maggs and Rico 1991), we suggest that an increased founder effect, due to a lower number of gametophytes during the colonizing process, would magnify the fixation of alleles, explaining the lower genetic variation of gametophytes. Initial differences in gametophytic and sporophytic subpopulations can persist and be carried beyond the site of their establishment by vegetative means; therefore, the actual population structure of *G. canariensis* would be the consequence of the colonization. As discussed by Systma and Schaal (1985), the loss of alleles rather than reduction in heterozygosity can be a primary result following the large population size reductions implicit in founder events. This interpretation suggests that there has not been sufficient time for selection to act upon the loci. The high genetic identities among subpopulations (Table 6) support these conclusions.

The deviations from the Hardy-Weinberg equilibrium in all three populations indicate that these populations are not randomly mating. A predominance of asexual reproduction in *Gelidium canariensis* would explain the departures from the Hardy-Weinberg equilibrium (Tables 3, 4). The departure from the Hardy-Weinberg equilibrium has been described in most of the species of seaweeds analyzed. Large deviations from expected genotypes with fixed heterozygous loci have been described in populations of *Codium fragile* (Malinowsky 1974). Cheney and Babbel (1978) described heterozygote deficiencies and Hardy-Weinberg deviations in two ACPH loci from two populations of *Eucheuma isiforme* var. *denudatum*, which exhibited a propensity for vegetative reproduction. No segregation was observed for heterozygous phenotypes in *Enteromorpha linza* (L.) J. Ag. from Long Island, with high deviations of observed from expected genotypes (Innes and Yarish 1984). Sosa and Garcia-Reina (1992) reported important deviations from the Hardy-Weinberg

TABLE 2. *Number of alleles (NA), average number of alleles per locus (A), proportion of polymorphic loci (P), and average gene diversity (H) detected in sporophytic (S) and gametophytic (G) subpopulations of* Gelidium canariensis *from three locations in the Canary Islands.*

Population		NA	A	P	H
Gáldar	S	25	1.14	0.136	0.039
	G	24	1.10	0.091	0.023
Agaete	S	27	1.23	0.227	0.049
	G	25	1.14	0.136	0.053
Pto. Cruz	S	26	1.18	0.182	0.063
	G	23	1.05	0.045	0.011
$\bar{X}$ S		26	1.18	0.182	0.052
$\bar{X}$ G		22	1.10	0.091	0.028
$\bar{X}$ S + G		24	1.14	0.136	0.039

TABLE 4. *Chi square test for the Hardy-Weinberg genotypic proportions of polymorphic loci in* Gelidium canariensis *populations from the Canary Islands. All comparisons with [illegible] degrees of freedom. [illegible] was possible due to degenerate cells.*

TABLE 5. *Nei's genetic distance (above diagonal) and genetic identity (below diagonal) (Nei 1972) between sporophytic (S) and gametophytic (G) subpopulations of* Gelidium canariensis *from three locations in the Canary Islands. $\bar{D} \pm SD = 0.016 \pm 0.011$ between gametophytic and sporophytic subpopulations. $\bar{D} \pm SD = 0.017 \pm 0.012$ among all populations of* G. canariensis. *$\bar{D} \pm SD = 0.009 \pm 0.004$ between sporophytic subpopulations. $\bar{D} \pm SD = 0.031 \pm 0.008$ between gametophytic subpopulations.*

	Gáldar (S)	Gáldar (G)	Agaete (S)	Agaete (G)	Pto. Cruz (S)	Pto. Cruz (G)
Gáldar (S)	·	0.019	0.015	0.026	0.003	0.003
Gáldar (G)	0.981		0.039	0.041	0.016	0.021
Agaete (S)	0.985	0.962		0.004	0.008	0.017
Agaete (G)	0.974	0.960	0.996		0.014	0.032
Pto. Cruz (S)	0.997	0.984	0.992	0.987		0.006
Pto. Cruz (G)	0.997	0.980	0.983	0.970	0.994	·

TABLE 6. *Coefficient of gene differentiation (G_{ST}) at polymorphic loci between sporophytes and gametophytes of the same location, between all gametophyte subpopulations, and between all sporophyte subpopulations of the three populations of* Gelidium canariensis *from the Canary Islands. — indicates no test is possible because of the invariable locus for compared subpopulations.*

Locus	Gáldar	Agaete	Pto. Cruz	Sporophytes	Gameto-phytes
ALP-1	—	0.033	—	0.020	—
DIA-3	0.132	0.018	0.142	0.000	0.247
EST-2	0.074	0.052	0.138	0.212	0.465
MDH-1	—	0.000	0.043	0.049	—
PGI-3	0.350	0.039	0.016	0.084	0.385
Average	0.231	0.034	0.073	0.073	0.366

equilibrium for most of the polymorphic loci in three populations of *Gelidium arbuscula* and suggested that a predominance of asexual reproduction in the populations was the reason for the deviations. Similar results have been described in other marine organisms (Black and Johnson 1979, Hebert and Crease 1980).

In the three populations analyzed here, the number of mature gametophytic individuals detected was always lower than the number of mature sporophytes (S:G = 5:1). Sporophyte dominance has been described in other species of *Gelidium* (Seoane-Camba 1965, 1969, Guzmán del Proó and de la Campa de Guzmán 1969, Carter 1985, Robertson et al. 1985, Akatsuka 1986, Gorostiaga 1990, Maggs and Rico 1991, Salinas 1991, Sosa and Garcia-Reina 1992) and indicates that the alternation of generations is not as important as are other means of regeneration. With high levels of perennation, as in *Gelidium canariensis*, alternation of generations will occur at a lower level. The capacity for vegetative propagation and differentiation of rhizoidal filaments from frond apical segments when in contact with the substrate is an important aspect in reproduction of *Gelidium* (Akatsuka 1986, Gorostiaga 1990, Santelices 1990, Juanes and Borja 1991). On the other hand, it appears that most of the new plants regenerated tend to be found in close proximity to the primary holdfasts. Such a situation may lead to the clumping of offspring next to their parents, resulting in effective inbreeding and causing deviations from the Hardy-Weinberg equilibrium. Similar results have been reported for *E. isiforme* var. *denudatum* populations (Cheney and Babbel 1978).

A high genetic identity between sporophytes and gametophytes from the same population (independent of the ploidy) was found. Similar results were described for *Gelidium arbuscula* from Canary Islands populations (Sosa and García-Reina 1992), but in contrast to results obtained previously with *G. arbuscula*, the coefficients of gene differentiation between gametophytes and sporophytes in Agaete and Pto. Cruz locations were low for *G. canariensis* (G_{ST} = 0.034 and 0.075, respectively; Table 6). Only the Gáldar population showed a relatively high coefficient of differentiation (G_{ST} = 0.231; Table 6). Since no differences in ecological factors such as light intensity, water motion, and temperature regimes have been observed among the three locations, it is difficult to explain the reason for higher differences of gene differentiation in the Gáldar population. Although we cannot exclude a different selective pressure acting on this population (e.g. at the microhabitat level; Innes 1987, 1988), or the existence of genetic flow between gametophytes and sporophytes within each of the Agaete and Pto. Cruz populations, we suggest that the initial differences in sporophyte and gametophyte gene frequencies from the colonization step have persisted beyond the site of their establishment due to a predominance of vegetative reproductive means and/or low incident of selective forces. The small genetic differentiation among all sporophytic subpopulations (G_{ST} = 0.073) and the high differentiation among all gametophytic subpopulations (G_{ST} = 0.366) may be attributable to higher levels of interpopulation gene flow in diploid individuals (higher migration capacity) and a lower gene flow between gametophytes (lower capacity of haploid individuals to migrate). The lack of genetic divergence among all sporophytic subpopulations may indicate that selective pressures on these loci have been weak and/or that insufficient generations have elapsed for selection to produce genetic differences. Similar results have been described in higher plants with adaptive radiation in the Hawaiian islands. Helenurm and Ganders (1985) reported the absence of genetic differentiation in *Bidens* taxa, considering that island-hopping results in only a slight differentiation among populations, with not enough time to change allele frequencies at isozyme loci.

Higher gene differentiation among haploid subpopulations (G_{ST} = 0.334) than diploid subpopulations (G_{ST} = 0.147) was also found in populations of *Gelidium arbuscula* (Sosa and García-Reina 1992). High genetic differentiation was reported in wild populations of the haploid laver *Porphyra* (G_{ST} = 0.472, 0.623, and 0.125; Miura et al.

Fujio et al. 1985, 1987, respectively). High genetic differentiation has been also described in *Chondrus crispus* populations (Cheney and Mathieson 1979). Innes (1987, 1988) reported that the genetic structure in *Enteromorpha linza* populations is dominated by factors operating on a microgeographic scale, with higher genetic differentiation among populations separated by a few meters than differentiation among populations separated by several kilometers.

The existence of similar levels of genetic variability and genetic structure in *Gelidium arbuscula* (Sosa and Garcia-Reina 1992) and *G. canariensis* (present study) suggests that populations of both species have originated by similar procedures and have followed a similar pattern of evolution. Both *Gelidium* species grow in the same localities, with no apparent evidence of unequal field distribution. Both species have 20 common isozyme loci, and it could be that range-wide similarity in selection pressure for both species has occurred. These conclusions are based on the relatively high average genetic identity between both species (I = 0.822). Similar values of genetic identity were described by Cheney and Babbel (1978).

A more detailed study including ecological, genetic, and physiological differences between gametophytes and sporophytes is necessary to understand the population structure and evolution of isomorphic species.

Finally, a note about our interpretations of the zymograms: Although our interpretations can only be considered hypotheses because direct evidence from progeny studies could not be obtained, we believe they are reasonable. Special mention will be given to the SOD banding pattern. This enzyme showed five bands for all analyzed individuals, independent of ploidy and population (Fig. 2). SOD, also known as indophenol oxidase or tetrazolium oxidase, has been described as both a monomeric (Cheney and Babbel 1978) and dimeric enzyme (Innes and Yarish 1984) in seaweeds and either dimeric or tetrameric in higher plants (Gottlieb 1981). Because equal SOD banding patterns were found in all gametophyte and sporophyte populations analyzed (Fig. 2), we considered the SOD zymogram to be a product of five invariable loci. Although only one or two SOD loci have been described for seaweeds (Cheney and Babbel 1978, Innes and Yarish 1984), a greater number of SOD isozyme loci has been described for higher plants. Four loci were reported for maize (Goodman and Stuber 1983) and six to eight for soybean (Kiang and Gorman 1983). On the other hand, because tetrazolium chloride is not a natural compound, an unknown number of nonspecific oxidative enzymes may be included in the SOD category. Some peroxidase enzymes [illegible] have SOD activity (Kiang and Gorman 1983, [illegible] could indicate that some SOD bands in *canariensis* correspond to peroxidase activ[illegible]

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Subcellular immuno-localization, amino acid composition and partial amino acid sequences of α-1,4-glucan phosphorylase of *Gracilaria* spp (Rhodophyta)

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Yu, S., Gómez-Pinchetti, J.-L., Ek, B., Garcia-Reina, G. and Pedersén, M. 1993. Subcellular immuno-localization, amino acid composition and partial amino acid sequences of α-1,4-glucan phosphorylase of *Gracilaria* spp (Rhodophyta). – Physiol. Plant. 89: 11–20.

Antibodies have been raised against an α-1,4-glucan phosphorylase (EC 2.4.1.1) purified from the red alga *Gracilaria chilensis*. Localization of α-1,4-glucan phosphorylase in thin sections of *G. chilensis* and *G. tenuistipitata* was performed using immuno-gold labelling and transmission electron microscopy. The enzyme was localized in the cytosol and around the cytosolic starch granules of the algal cells. The labelling was not associated with the chloroplast or the cell wall. Amino acid composition of the red algal phosphorylase was quite similar to that of potato tuber and rabbit muscle phosphorylases. Partial amino acid sequences showed 48, 54 and 65% homology with the rabbit, potato and *Escherichia coli* enzymes, respectively.

Key words – Gracilariales, α-1,4-glucan phosphorylase, immuno-gold localization, Rhodophyta, starch metabolism.

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Introduction

α-1,4-Glucan phosphorylase catalyzes the interconversion between α-1,4-glucan and glucose 1-phosphate. Its activity has been detected in nearly all organisms that use starch or glycogen as food storage (Steup 1988). The regulatory properties and subcellular localization may, however, vary from organism to organism (Yu 1992). Animal cells accumulate glycogen in the cytosol and their phosphorylase is located in the cytosol. In higher plant cells, starch accumulates in chloroplasts and amyloplasts and phosphorylase has been localized to both cytosol and plastids (Schneider et al. 1981, Mathews and van Holde 1990, van Berkel et al. 1991). The occurrence of cytosolic phosphorylase in higher plants has been an enigma, as no suitable substrate is known at present.

The red seaweeds of the order Gracilariales are agarophytes. More than 80% of their cell space can be filled up with starch granules (Yu 1992), implying that the metabolism of starch is one of the main metabolic activities in this group of organisms. α-1,4-Glucan phosphorylase is one of the major enzymes contributing to the control of starch metabolism (Steup 1988). Compared to other groups of organisms, there is a general lack of knowledge on the subcellular localization of α-glucan metabolizing enzymes in red algae. This is due to the fact that none of these enzymes has been purified earlier to make subcellular immuno-localization possible. Furthermore, the general knowledge of starch metabolism gained from studies on higher plants differs from that of red algae. Red algal chloroplasts do not contain any starch granules. Like glycogen in animal cells, starch granules accumulate only in the cytosol of red algae (Pueschel 1990). Only one form of phosphorylase has been detected

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in 11 species of red algae investigated (Yu 1992). Based on the cytosolic location of starch and the single form of phosphorylase, the red algal phosphorylase is likely to be present in the cytosol. The current communication presents the amino acid composition of the red algal phosphorylase, partial amino acid sequences, and the subcellular immuno-localization in two red seaweeds, *Gracilaria chilensis* and *G. tenuistipitata* (Gracilariales).

Materials and methods

Algal materials and cultivation

Gracilaria chilensis Bird, McLachlan et Oliveira (formerly called *Gracilaria sordida, G. secundata*) was collected from Auckland harbour (New Zealand) and *Gracilaria tenuistipitata* Zhang et Xia was collected from Hainan island (China). Unialgal cultures of the agarophytes were grown in enriched and filtered seawater (0.2 μm pore-size filter) in a cylinder system at 22±1°C in our laboratory as described by Lignell (1988). The salinity of the culture medium was 33‰ for *G. chilensis* and 17‰ for *G. tenuistipitata*. The cultures were illuminated continuously with an array of 36 W fluorescent tubes (Thorn, Polylux 4 000, UK) that delivered a photon irradiance of 110 μmol m^{-2} s^{-1}.

Purification of α-1,4-glucan phosphorylase and production of antibodies

α-1,4-Glucan phosphorylase was purified from *Gracilaria chilensis* by affinity chromatography on starch-Sepharose 6B (Yu and Pedersén 1991b). Further purification of phosphorylase I was achieved by FPLC on a Superose 12 column (1×30 cm, Pharmacia, Uppsala, Sweden) using 50 m*M* citrate-NaOH (pH 6.5), 0.2 *M* NaCl as elution buffer. A phosphorylase sample was concentrated to 100 μl by using Centricon-3 (Amicon, Danvers, MA, USA) and then loaded on the column. The elution rate was 0.15 ml min^{-1} and the fraction size was 0.5 ml. Absorbance was monitored at 280 nm. The enzyme obtained by this purification procedure had a specific activity of 50 μmol min^{-1} mg^{-1} protein with a recovery of 90%. Purified phosphorylase I (0.45 mg) was dissolved in 0.45 ml 0.85% NaCl and 0.1% NaN_3 and used for immunization of rabbits [carried out by DAKO A/S, Glostrup, Denmark according to the procedure detailed by Harboe and Ingild (1983)]. The first and second bleeding gave a titer of 1:16 and 1:64, respectively, as tested by double immuno-diffusion. The antiserum with a titer of 1:64 was used throughout this study.

Immuno-gold labelling

Growing tips of the algal thalli of *Gracilaria chilensis* and *G. tenuistipitata* were excised and fixed in excess of glutaraldehyde (2.5%, w/v) in 50 m*M* sodium cacodylate (pH 7.2) containing 1% (w/v) caffeine and 0.25 *M* sucrose for 2 h at 4°C, before being embedded in LR white (TAAB, UK). Ultra-thin sections were cut with a LKB Ultrotome (LKB, Sweden) using a diamond knife and placed on gold grids (300 mesh, ca 120 lines cm^{-2}, Brandsma, Hilversum, The Netherlands) covered with carbon-coated Formvar film.

The thin sections were first incubated with the rabbit antiserum to α-1,4-glucan phosphorylase (1:300 dilution) followed by incubation with goat anti-rabbit IgG conjugated with 10 nm colloidal gold particles (1:20 dilution) as described by Lindblad and Sellstedt (1991). After air-drying overnight, the sections were post-stained with lead citrate and uranyl acetate, and then exposed to OsO_4 vapor at room temperature for 1 h, before being viewed on a Philips CM 10 transmission electron microscope. For ultrastructural studies, the algal material was fixed as described earlier (Ekman et al. 1989). The fine structure of *Gracilaria* spp was difficult to preserve because of the thick cell wall which prevented good penetration. We found that a fixation time of one month with the addition of 1% caffeine improved preservation. The fine structure of immuno-gold labelled sections was enhanced by post-staining and exposure to OsO_4 vapor.

Electrophoresis and immuno-blotting

SDS-gel electrophoresis and immuno-blotting were performed with a PhastSystem™ (Pharmacia, Uppsala, Sweden) and the semi dry electrophoretic transfer unit PhastTansfer™ according to the manufacturer's instructions. Proteins from gels after SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes. The membranes were then incubated with the rabbit antiserum to the algal phosphorylase (1:100) overnight at room temperature, followed by incubation with secondary antibodies (goat-anti rabbit IgG) conjugated to horseradish peroxidase (1:1000). Phosphorylase was visualized using 4-chloro-1-naphthol and hydrogen peroxide as substrates for the peroxidase (Lindblad and Sellstedt 1991).

Amino acid composition

Amino acid analysis was performed on a LKB Model 4151 amino acid analyzer using norleucine as an internal standard. Phosphorylase purified from *G. chilensis* (9.9 μg salt-free sample) was used. The analysis was performed in the Central Amino Acid Analysis Laboratory at Uppsala University, Sweden.

Partial amino acid sequences

The red algal phosphorylase purified from *G. chilensis* (72 μg) was dissolved in 60 μl 6 *M* guanidine chloride. To 20 μl of this sample were added 40 μl 0.2 *M* Tris-HCl (pH 8.2) and 1 μl proteinase I from *Achromobacter lyticus* (specific for Lys-X, Wako Pure Chemical Industries LTD, Osaka, Japan). Digestion was carried out at

37°C overnight. Reduction of the polypeptides produced was performed at 56°C for 15 min in the presence of 0.1% 2-mercaptoethanol. The polypeptides were further derivatized with 0.3% of 4-vinylpyridine in the dark for 30 min at room temperature and then separated on HPLC with a linear gradient (conditions: Solution A, H_2O: TFA [100: 0.1, v/v]; Solution B, acetonitrile: H_2O: TFA [90: 10: 0.1, v/v/v]). The polypeptide peaks were monitored at 214 nm, collected, and stored at –20°C. Four polypeptide peaks (designated as Fragment 1, 2, 3 and 4) were chosen for sequencing. A gas phase sequencer (model 470A, Applied Biosystems, Foster City, USA) equipped with an on-line HPLC detection system was used for the sequence analysis.

Other methods

Activity of α-1,4-glucan phosphorylase was measured as described earlier (Yu and Pedersén 1991a). Ouchterlony double immuno-diffusion was performed as described by Zhang et al. (1984). The antibodies were diluted with 0.9% NaCl. Bacto-agar (1.5%, w/v) was prepared in barbitone buffer (pH 8.3) with a ionic strength of 50 m*M*. The diffusion was performed at 37°C.

Results and discussion

Specificity of the rabbit antiserum to α-1,4-glucan phosphorylase

When proteins in the whole cell extracts of *Gracilaria chilensis* and *G. tenuistipitata* were separated by SDS-PAGE, and then transferred to a nitrocellulose membrane, one protein band corresponding to the algal phosphorylase in migration was identified by the anti-phosphorylase serum (Fig. 1). In control experiments, the phosphorylase band was not observed when the antiserum was replaced with preimmune serum or the secondary antibodies were omitted. No phosphorylase band was identified by the antiserum when the cell-free extract had been passed through a starch-Sepharose column, which specifically removes phosphorylase (Yu and Pedersén 1991b). The phosphorylase antibodies did not identify other proteins in the cell free extract, such as biliproteins, which are the major soluble proteins of red algae. The antibodies did, however, cross-react with the molecular mass marker rabbit phosphorylase *b* (Fig. 1). This rabbit muscle phophorylase has the same relative molecular mass (97 400) as the algal phosphorylase (Fig. 1, Yu and Pedersén 1991b). In double immuno-diffusion experiments, purified algal α-1,4-glucan phosphorylase gave a single white precipitin band when tested against the immune serum in a dilution of 1:64.

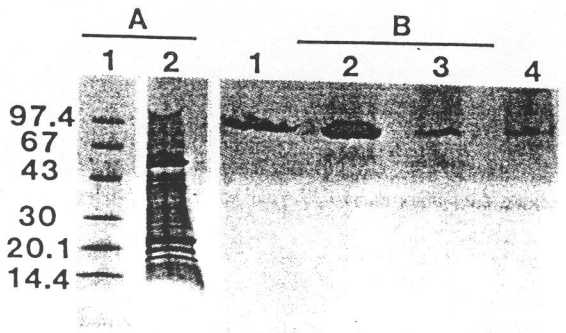


Fig. 1. SDS-PAGE (A) and immuno-blotting (B) of α-1,4-glucan phosphorylase. A: Lane 1, molecular mass markers (relative molecular mass × 10^{-3}) of rabbit phosphorylase *b* (97.4), albumin (67), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20.1) and α-lactalbumin (14.4). Lane 2, cell-free extract of *Gracilaria chilensis*. B: Lane 1, molecular mass markers as in Lane 1 of A, Lane 2, phosphorylase purified from *G. chilensis*, Lane 3 and 4, cell-free extracts of *G. chilensis* and *G. tenuistipitata*, respectively.

The cell structure of *Gracilaria tenuistipitata* and *G. chilensis*

The ultrastructure of *G. tenuistipitata* is characterized by a very thick cell wall. The chloroplasts, with unstacked, evenly spaced thylakoids, lie adjacent to the cell wall (Fig. 2). Starch granules with different shape and size are found free in the cytosol or in intimate contact with the chloroplasts. The cell wall material, which is mainly agar polymers, accounts for more than 30% of algal dry weight. The abundance of starch granules varies with the nitrogen status of the alga, being increased under nitrogen-starvation (Yu 1992). The basic features of the ultrastructures of *G. chilensis* is the same as those of *G. tenuistipitata*.

Subcellular immuno-localization of α-1,4-glucan phosphorylase

α-1,4-Glucan phosphorylase was localized in the cytosol, around starch granules, and to a lesser extent, in the immediate vicinity of the chloroplast outer membranes of the algal cells in both *G. chilensis* and *G. tenuistipitata* (Figs 3 and 4A). In the cytosol, the labelling was more or less concentrated to some electron dense areas of unknown composition. There were a few gold particles in the chloroplasts and in the cell wall areas (Figs 3 and 4 A). This may be due to non-specific labelling as such a level of labelling was also noticed using preimmune serum (Fig. 4B).

The observation that the red algal phosphorylase is localized to the cytosol is consistent with the fact that in red algae, starch granules are deposited in the cytosol (Pueschel 1990; Figs 2, 3 and 4), and with the fact, that the red algal phosphorylase resembles more the cytosolic phosphorylase of potato than the plastidic one in both kinetic properties, and molecular mass (Mori et al. 1991, Yu and Pedersén 1991a). It is also in accordance with the fact that there is only one form of phosphorylase in red algae (Yu 1992). Yu and Pedersén (1991b) have reported

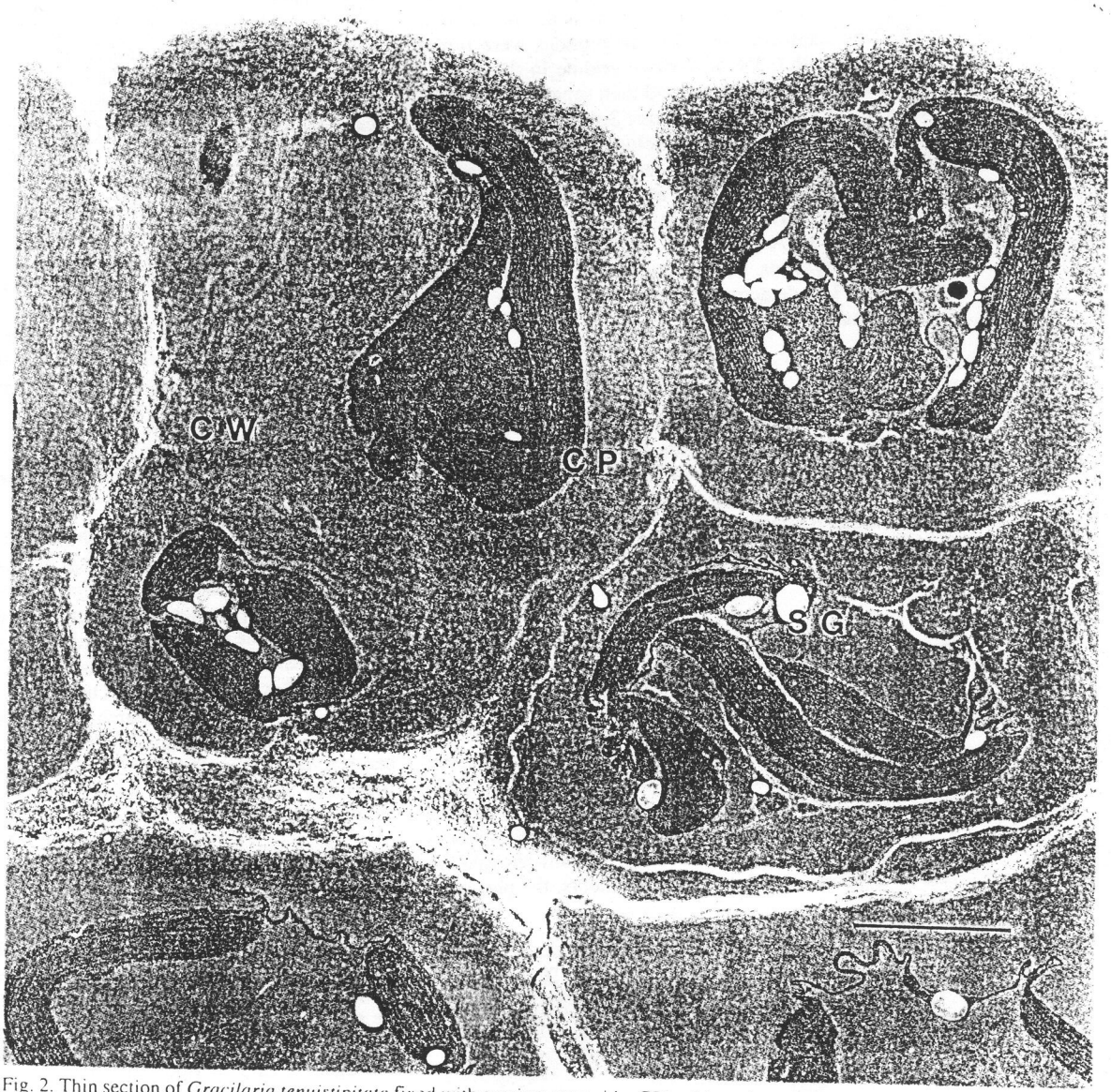


Fig. 2. Thin section of *Gracilaria tenuistipitata* fixed with osmium tetroxide. CP = chloroplast, CW = cell wall, SG = starch granules. ×8165, bar = 3 μm.

the occurrence of two forms of phosphorylase in *Gracilaria chilensis* during purification, namely, phosphorylase I and II. Further studies showed that they are simply different oligomer forms of the same subunit (Yu 1992) and in this study, the antiserum to phosphorylase I cross reacted with phosphorylase II in both double immuno-diffusion and immuno-blotting (figure not shown). The immuno-gold labelling technique has earlier been used for subcellular localization of Rubisco, phycoerytherin and α-1,4-glucan lyase in red algae. In none of these cases were any gold particles found associated with the cytosolic starch granules (Ekman et al. 1989, McKay and Gibbs 1990, Yu and Pedersén 1993). Using the antiserum to the red algal phosphorylase and the immuno-gold labelling technique, phosphorylase was detected in the pyrenoid of the green alga *Enteromorpha intestinalis* but not around the starch granules (S. Yu and M. Pedersen unpublished results).

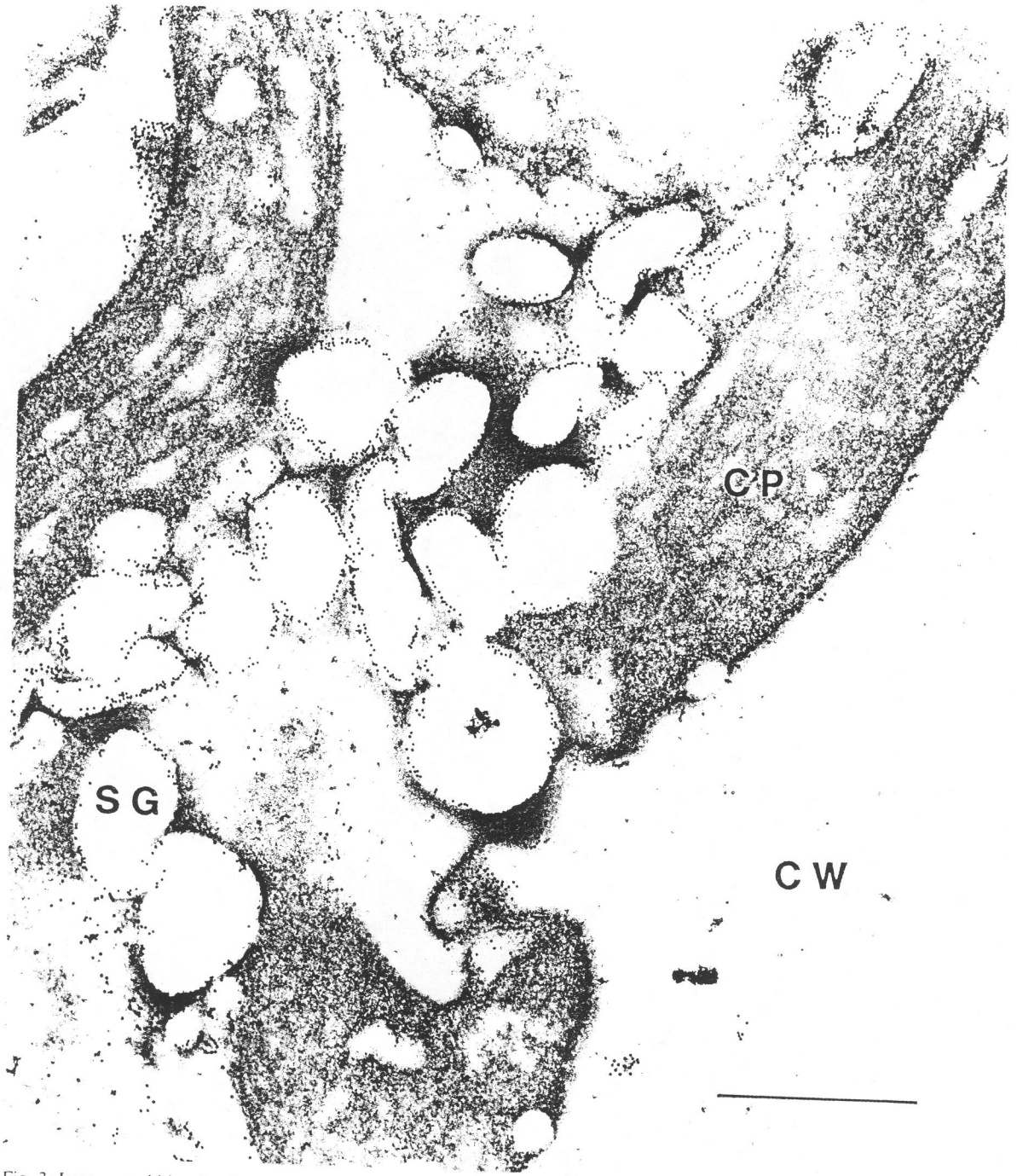
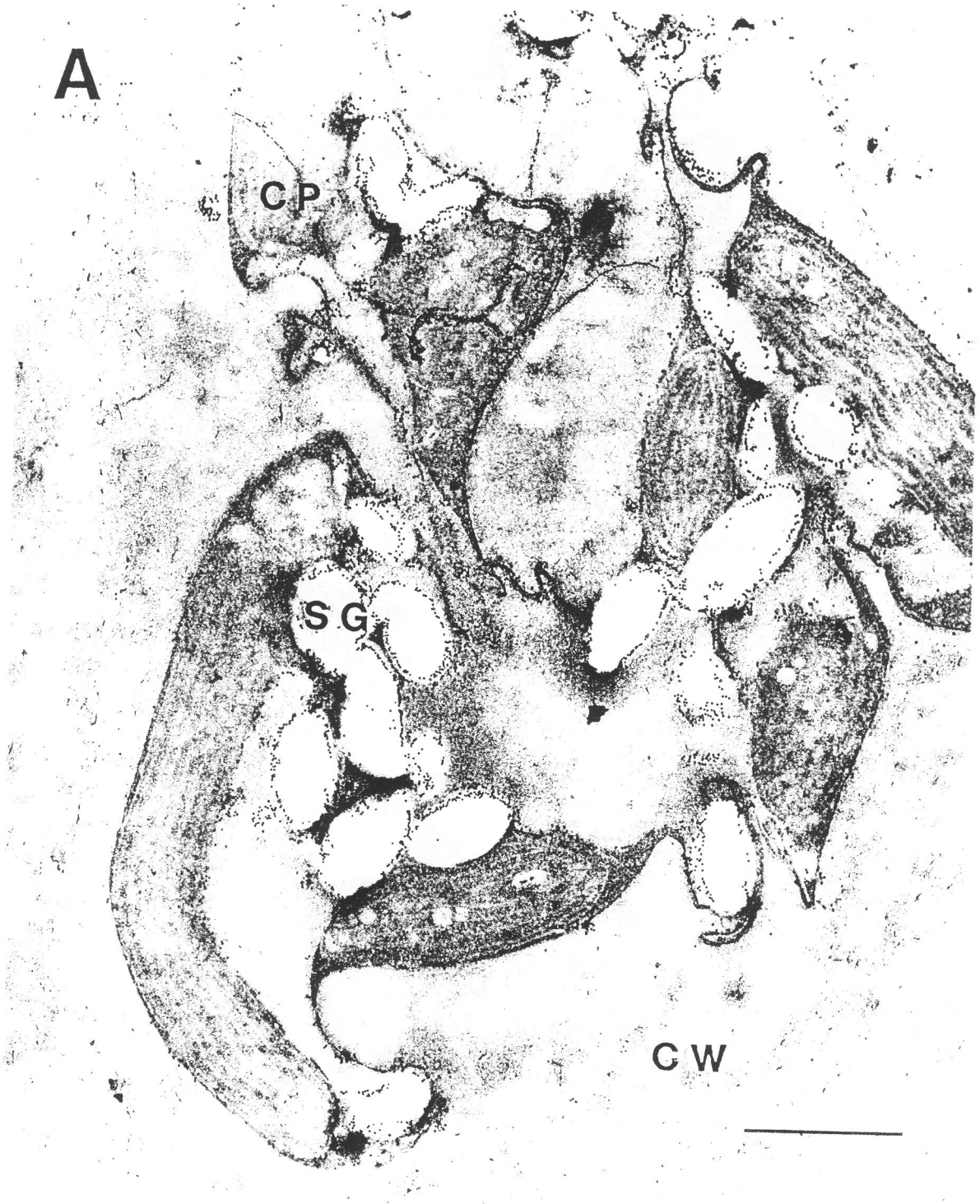


Fig. 3. Immuno-gold localization of α-1,4-glucan phosphorylase in the cell of *Gracilaria tenuistipitata*. The thin section was first fixed with glutaraldehyde, and subsequently incubated with rabbit antiserum to α-1,4-glucan phosphorylase and goat-anti rabbit IgG conjugated with 10 nm colloidal gold particles. The codes of the symbols are the same as in Fig. 2. ×34 500, bar = 1 μm.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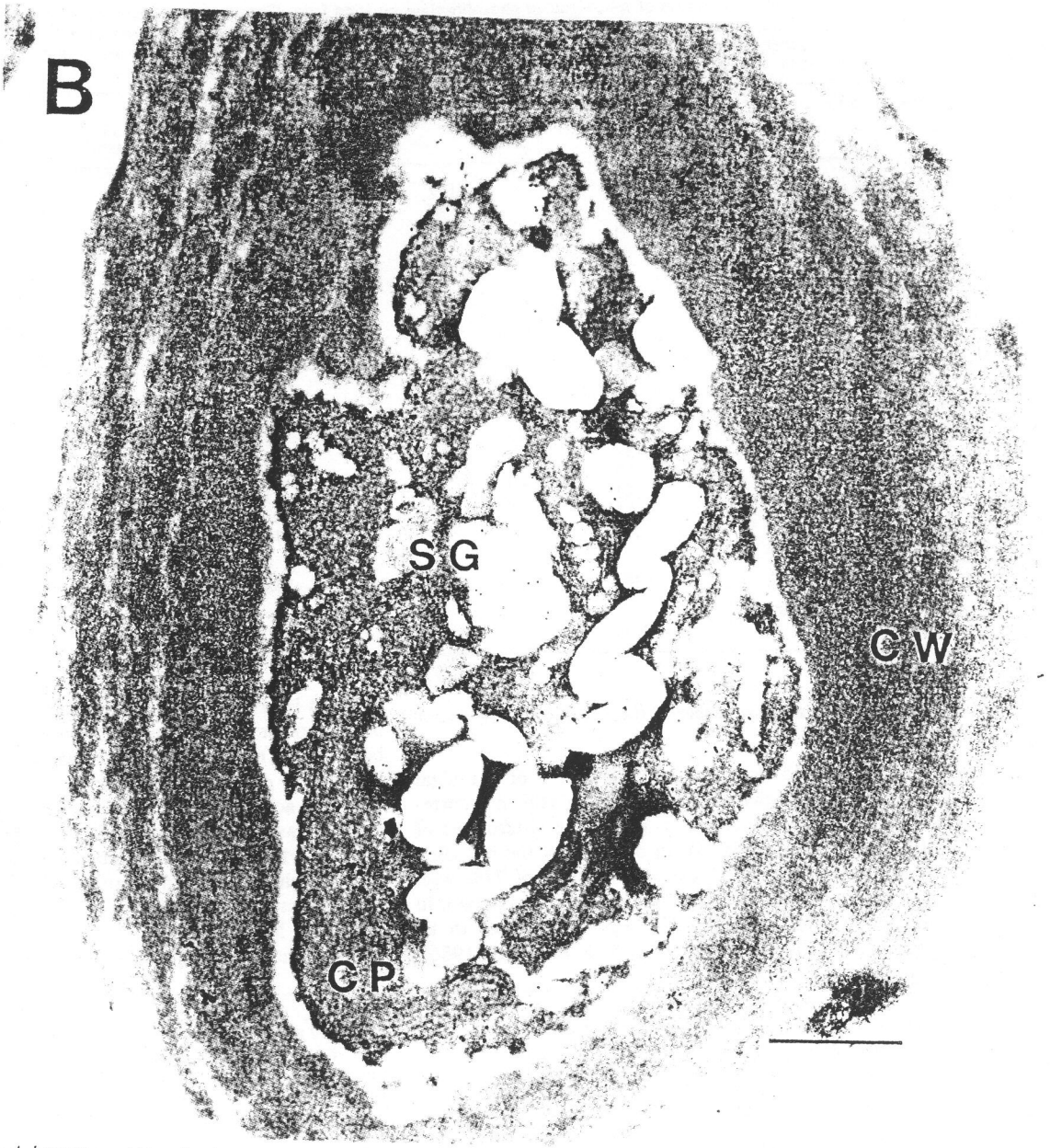

Fig. 4. Immuno-gold localization of α-1,4-glucan phosphorylase in the cell of *Gracilaria chilensis*. A: The thin section was treated as in Fig. 3. B: The thin section was treated as in Fig. 3 except that the antiserum was replaced with preimmune serum. The codes of the symbols are the same as in Fig. 2. bar = 1 μm.

The amino acid composition of the red algal α-1,4-glucan phosphorylase

Results of the amino acid analysis of the red algal phosphorylase are given in Tab. 1 along with values for phosphorylases of *E. coli*, potato tuber cytosol and plastid, and rabbit muscle (Titani et al. 1977, Nakano and Fukui 1986, Mori et al. 1991). Except for a slightly higher content of Tyr, Met and Ser residues, the red algal phosphorylase has a similar amino acid composition to phosphorylases of other organisms, such as a low content of Cys residues (Tab. 1).

Tab. 1. Amino acid composition of α-1,4-glucan phosphorylase purified from *Gracilaria chilensis*. Results are given as number of residues per subunit, assuming a subunit mol mass of 97 400. For comparison, the amino acid compositions of phosphorylases from *E. coli*, potato tuber cytosol and plastid, and rabbit muscle are included (Titani et al. 1977, Nakano and Fukui 1986, Choi et al. 1989, Mori et al. 1991). ND, not determined.

Amino acid	*G. chilensis*	*E. coli*	Potato tuber		Rabbit muscle
			Cytosol	Plastid	
Asp/Asn (D/N)	97	102	93	96	96
Thr (T)	47	34	42	49	35
Ser (S)	61	46	53	54	29
Glu/Gln (E/Q)	95	89	91	114	95
Pro (P)	35	26	38	36	36
Gly (G)	58	48	51	56	48
Ala (A)	62	54	66	73	63
Cys (C)	7	5	7	10	9
Val (V)	64	55	51	61	62
Met (M)	26	18	19	15	21
Ile (I)	56	49	46	62	49
Leu (L)	70	83	80	72	79
Tyr (Y)	41	35	33	34	36
Phe (F)	31	29	35	42	38
His (H)	19	24	21	17	22
Lys (K)	43	34	51	68	48
Arg (R)	55	45	43	40	63
Trp (W)	ND	14	18	17	12
Total amino acid residues	ND	790	838	916	841
Relative molecular mass of subunit	97400	90147	95382	103916	97412

Partial amino acid sequences of the red algal α-1,4-glucan phosphorylase

The amino acid sequence of Fragment 1 of the algal phosphorylase showed homology with a sequence corresponding to amino acids 163–185 in the N-terminus of the rabbit enzyme (Fig. 5). In this region, the red algal phosphorylase exhibited a residue identity of 77% with that of *E. coli*, and 47% with that of rabbit and potato. In the rabbit phosphorylase, this region is known to be involved in subunit interaction (Nakano and Fukui 1986). Such an interaction is vital since only the dimer but not the monomer shows enzyme activity. A high homology between the red algal phosphorylase and the phosphorylases of the other organisms are also found for Fragment 2 (amino acids 279–289 of the rabbit enzyme) and Fragment 3 (amino acids 641–661; Fig. 5). In the rabbit phosphorylase, these two regions are parts of the active sites for phosphate binding and for binding of the catalytic cofactor pyridoxal 5-phosphate, respectively (Fletterick and Madsen 1980).

Fragment 4 of the algal phosphorylase showed homol-

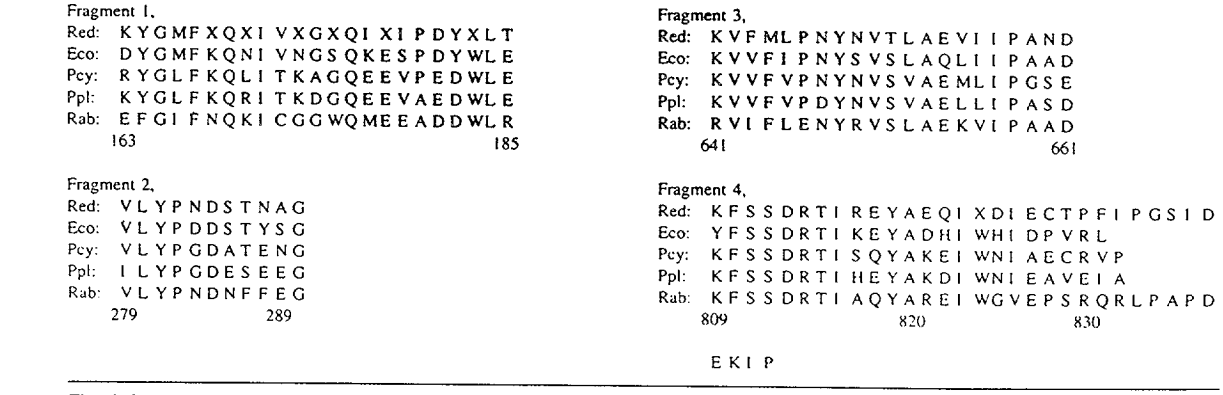

Fig. 5. Sequence comparison between the red algal phosphorylase (Red) and the phosphorylases of *E. coli* (Eco), potato tuber cytosol (Pcy) and plastid (Ppl), and rabbit muscle (Rab). Identical residues are in boldface. The numbering is according to the rabbit enzyme (Titani et al. 1977). The amino acid sequence data of the other phosphorylases are from Titani et al. (1977), Nakano and Fukui (1986), Yu et al. (1988) and Mori et al. (1991).

ogy with an amino acid sequence in the C-terminus (amino acids 809–830 of the rabbit enzyme) of the phosphorylase of *E. coli*, potato and rabbit muscle (Fig. 5). More than 40% residue identity was found when these sequences were aligned for maximal homology. It is noteworthy that the first 12 residues in this fragment (positions 809–820) are almost completely identical with those of the other phosphorylases. The high conservation of this region suggests that it is important for the function of the phosphorylase, though a specific role remains to be assigned. The region beyond amino acid 820 of Fragment 4 shows much less homology among the organisms. It has been suggested that this region does not play a major role in enzyme function (Choi et al. 1989).

Taking these partial sequence data together, the red algal phosphorylase is more similar to the phosphorylase of *E. coli* (65%) than to that of potato cytosol (53%), potato plastid (54%) and rabbit muscle (48%). This is interesting as red algae have been suggested to be the oldest extant groups of eukaryotic organisms.

α-1,4-Glucan phosphorylase is known as an evolutionarily well conserved protein. This has been shown by high similarities in amino acid sequences of the entire phosphorylase protein from phylogenetically diverse organisms (Palm et al. 1985, Nakano and Fukui 1986, Mori et al. 1991). The results presented in this study agree with this conclusion. Besides the sequence homology, the antibodies raised against the red algal phosphorylase exhibited cross-reactivity with phosphorylases of rabbit muscle (Fig. 1), the green alga *Enteromorpha intestinalis* and the cyanobacteria *Spirulina platensis* and *S. maxima*, as revealed by immuno-blotting (S. Yu, M. Pedersén, A. Martel and P. Lindblad, unpublished results).

Similar to other cytosolic phosphorylases, the red algal phosphorylase shows a high affinity towards the substrates amylopectin and glycogen and has a molecular mass similar to those of the potato cytosolic and rabbit phosphorylase (Tab. 1, Yu and Pedersén 1991a, b). By contrast, the potato plastidic phosphorylase shows low affinity towards amylopectin and glycogen and has a higher molecular mass (Tab. 1), characters typical for plastid phosphorylase (Steup 1988). These characters are due to a large mid-chain insertion of 78 residues in the potato plastid enzyme (Nakano and Fukui 1986, Mori et al. 1986).

It is assumed that phosphorylase evolved by diversification from a primordial ancestor molecule similar to the cytosolic form in plants (Nakano and Fukui 1986). Thus in animals, this ancestral phosphorylase acquired allosteric regulatory properties. The plastid phosphorylase of plants evolved by the inclusion of a 78-residue mid-chain insertion and a transit peptide needed for the transport of the enzyme from the cytosol to the plastid (Brisson et al. 1989, Lin et al. 1991). In this sense, the cytosolic phosphorylase of higher plants may be an evolutionary remnant without apparent physiological function, as the machinery for starch metabolism has been moved to the plastid. In red algae, however, the physiological function is obvious, since both the phosphorylase and starch are found in the cytosol.

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Physiological comparison between gametophytes and tetrasporophytes of *Gelidium canariensis* (Gelidiaceae: Rhodophyta).

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Key words: Calorific content, enzyme activity, gametophytes, *Gelidium canariensis*, photosynthesis, respiration, tetrasporophytes

Abstract

The physiological performances of tetrasporophytes and gametophytes of *Gelidium canariensis* (Grunow) Seoane-Camba were compared to estimate whether the field predominance of tetrasporophytes is due to lower fitness of gametophytes. No significant differences between tetrasporophytes and gametophytes were detected for calorific content, protein and pigment concentrations, NADH-Diaphorase, alkaline phosphatase and glucose-6-phosphate dehydrogenase activities and photosynthesis and respiration at 15, 20 and 25 °C, and pH 6.5, 8.2 and 9.2. Our results indicate that these physiological characteristics are not responsible for the scarcity of gametophytes in the field populations of *G. canariensis*.

Abbreviations: ALP = Alkaline phosphatase; G6PDH = glucose-6-phosphate dehydrogenase; DIA = NADH-Diaphorase; TRIS = Tris[hydroxymethyl]-aminomethane; PVPP = Polyvinylpolypyrrolidone.

Introduction

Gelidium canariensis (Grunow) Seoane-Camba, has a *Polysiphonia* type life history, with isomorphic generations, regenerating vegetatively new individuals from the established holdfast. As with other *Gelidium* species (Akatsuka, 1986; Santelices, 1990; Salinas, 1991) the populations of *G. canariensis* show a predominance of tetrasporophytes (S:G = 5:1) (Sosa *et al.*, results not shown). Unequal field distribution of tetrasporophytes and gametophytes of isomorphic algae has been explained in several ways, such as physiological differences between phases, differential mortality of one phase over the other or apomeiosis (Santelices, 1990; May, 1986).

Diploidy has been reported as a genetic advantage in several organisms (Benson, 1983) including seaweeds (Allender, 1977; Akatsuka, 1986). Akatsuka (1986) has suggested a higher fitness of *Gelidium* tetrasporophytes due to the higher potential for vegetative propagation of their prostrate axes in comparison to gametophytes. However, no evidence for higher fitness of diploidy have been found in other seaweeds (Hannach & Santelices, 1985; Littler *et al.*, 1987; Luxoro & Santelices, 1989).

The objective of the present study was to estimate whether the unequal distribution of the phases of *G. canariensis* are related to differences in physiological capabilities.

Material and methods

Gametophytic (bearing cystocarps) and tetrasporophytic individuals of *Gelidium canariensis* were collected during January and April 1992 over a 1 m^2 area throughout the intertidal north coast of the island of Gran Canaria (Canary Islands, Spain). Tetrasporophytes and gametophytes were cultivated under similar greenhouse conditions (daily temperature fluctuation from 19 to 23 °C) in 500 l continuously areated tanks at three seawater exchange rates per day, and maximum irradiance of 1100 to 1300 $\mu E\ m^{-2}\ s^{-1}$ (similar to the natural conditions where the plants were collected). Individuals were used for analyses within 7 days of cultivation.

Photosynthesis and respiration rates

Epiphyte-free apical fragments (20 to 28 mg of fresh weigh) were incubated in 2 ml of buffered seawater in a Clark-type electrode fitted with a measuring chamber (Hansatech Instruments LTD., Norlfolk, UK). A slide projector (Selecta, Germany) was used as light source. Irradiance levels were measured with a radiometer (LI-1000) using a spherical quantum sensor (LI-193SA). Between three to five replicates of each life-history phase were analyzed at different temperatures (15, 20 and 25 °C), and pH (6.5, 30 mmol MOPS; 8.2 and 9.2, 30 mmol TRIS). Photosynthesis versus irradiance curves were measured over a sequentially increasing range of photon flux density from 50 to 600 $\mu E\ m^{-2}\ s^{-1}$. Apical fragments were equilibrated for at least 30 min before each measurement. In order to minimize errors, gametophytes and tetrasporophytes were analyzed alternately and within 30 minutes for each set of temperature and pH conditions.

Pigments and protein analysis

Eight individuals of each phase were assayed to analyze the concentration of proteins and pigments. Chlorophylls were extracted following the methods described by Jensen (1978) and biliproteins according to Beer & Eshel (1985). Soluble protein was analyzed as described by Lowry *et al.* (1951).

Enzyme activity

One gram sample was ground to a fine powder with a mortar and pestle in liquid nitrogen. Two milliliters of extraction buffer (50 mM Tris-HCl, pH 7.4, 0.2 mM L-ascorbic acid, 1 mM Na_2 EDTA, 3% (w/v) PVPP, 0.1% 2-Mercaptoethanol) were added, and the resulting mixture was centrifuged for 20 minutes at 3500 rpm. The supernatant was loaded onto a Sephadex G-25 column (Pharmacia), previously equilibrated with Tris-HCl 50 mmol pH 8.0 or 7.4. Alkaline phosphatase (ALP), glucose-6-phosphate dehydrogenase (G6PDH) and NADH-Diaphorase (DIA) enzyme reactions were measured spectrophotometrically following the methods described in the Worthington Manual (1972), modified at 25 °C. Enzyme activity is reported in units defined as the increase (ALKPH, G6PDH) or decrease (DIA) in absorbance units per minute under the assay conditions. Four individuals of each phase were analyzed for each enzyme.

Calorific content

Two grams of air dried samples were milled to a fine powder and analyzed in a (Parr Model 1241) calorimeter. Eight individuals of each phase were assayed.

Statistical analysis

The data were analyzed by one-way ANOVA to test for a differential significant response between tetrasporophytes and gametophytes for each temperature and pH. All statistical comparisons of photosynthesis were conducted with the values at the highest irradiance (600 $\mu E\ m^{-2}\ s^{-1}$). A two-way ANOVA was performed to analyze the level of significance of the interaction between the rate of respiration and life-history phases with the temperature.

Results

No differences were found between tetrasporophytes and gametophytes for pigments (phycoerythrin, phycocyanin and chlorophyll), total protein concentrations, calorific content and enzymatic activity (Table 1).

Table 1. Compositional features of gametophytes and tetrasporophytes of *G. canariensis* (means ± SD are given), N = number of individuals of each life-history phase tested.

Component	N	Tetrasporophytes	Gametophytes
Protein concentration (mg g fresh weight^{-1})	9	2.00 ± 0.002	2.04 ± 0.003
Phycoerythrin (mg g fresh weight^{-1})	8	0.355 ± 0.03	0.328 ± 0.04
Phycocyanin (mg g fresh weight^{-1})	8	0.129 ± 0.008	0.116 ± 0.007
Chlorophyll (mg g fresh weight^{-1})	8	0.028 ± 0.005	0.027 ± 0.004
Calorific content (cal^{-1} g dry weight^{-1})	8	3.661 ± 51.1	3.763 ± 21.1
Enzyme activity (units mg prot^{-1})			
ALKPH	4	4.3 ± 0.5	4.2 ± 0.3
DIA	4	72.3 ± 12.3	88.7 ± 21.1
G6PDH	4	69.0 ± 25.0	63.3 ± 8.0

The rate of photosynthetic oxygen evolution in both phases of *Gelidium canariensis* was dependent on pH and temperature. Photosynthetic rates increased with the temperature and decreasing pH (Figs 1 and 2). Significant differences (at 600 μE m^{-2} s^{-1}), were detected among the three pH's tested ($P<0.05$, ANOVA), with the highest photosynthesis rates for both phases at pH 6.5 (Fig. 1). Photosynthesis was undetectable at irradiances below 200 μE m^{-2} s^{-1} and pH 9.2 in both phases. No significant differences ($P>0.05$, ANOVA) in O_2 evolution were detected between tetrasporophytes and gametophytes at either pH 8.2 and 9.2. Gametophytes showed lower significant ($P<0.05$) photosynthesis rate at pH 6.5 (Fig. 1).

Figure 2 shows the apparent photosynthetic rate of tetrasporophytes and gametophytes at pH 8.2 and 15, 20 and 25 °C. Gametophytes showed a significantly ($P<0.05$, ANOVA) higher photosynthesis rate at 25 °C, but no significant differences ($P>0.05$, ANOVA) were detected between gametophytes and tetrasporophytes at either 15 and 20 °C. Significant differences were found between 15 and 25 °C ($P<0.05$, ANOVA) and between 20 and 25 °C ($P<0.05$, ANOVA), but not ($P>0.05$, ANOVA) between 15 and 20 °C (Fig. 2).

Figure 3 shows respiration rates at 20 °C and pH 6.5, 8.2 and 9.2. A slightly lower respiration rate was detected in tetrasporophytes at the three pH's, but it was non-statistically significant ($P>0.05$, ANOVA) at any pH. The lowest respiration rate of both phases was recorded at pH 6.5 (Fig. 3).

Figure 4 shows the respiration rate of both phases at pH 8.2 and 15, 20 and 25 °C. Gametophytes showed a significantly ($P<0.05$, ANOVA) higher rate of respiration at 25 °C. No significant ($P>0.05$, ANOVA) differences were detected at either 15 and 20 °C. No significant interaction ($P>0.05$, two-way ANOVA) was found between temperature and life-history phases.

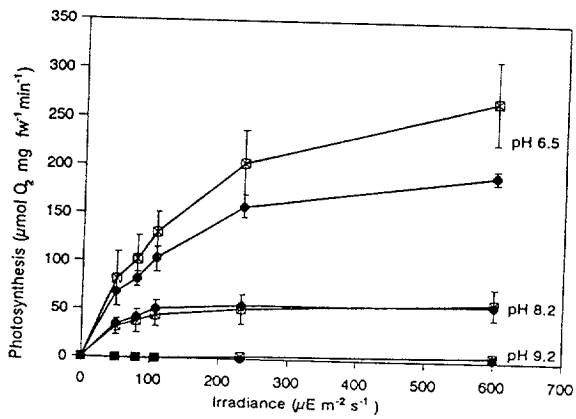

Fig. 1. Photosynthesis-light curves of tetrasporophytes (⊠) and gametophytes (●) of *Gelidium canariensis* at 20 °C (pH 6.5, 8.2 and 9.2). Results are expressed in µmol of oxygen produced per mg of fresh weight per minute. SE bars do not appear when they are smaller than the symbol.

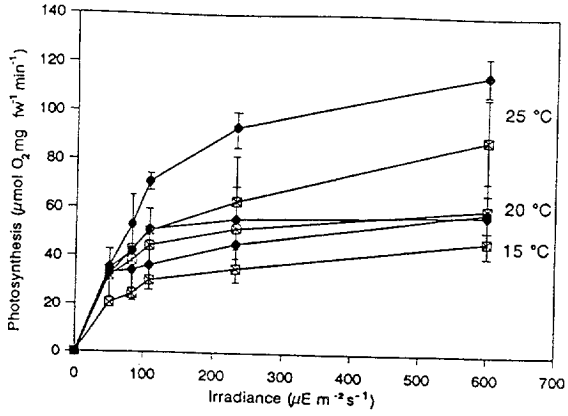

Fig. 2. Photosynthesis-light curves from tetrasporophytes (☒) and gametophytes (●) of *Gelidium canariensis* at pH 8.2 (Temperatures 15, 20 and 25 °C). Results are expressed in µmol of oxygen produced per mg of fresh weight per minute. SE bars do not appear when they are smaller than the symbol.

Discussion

Comparative studies between tetrasporophytes and gametophytes of *Gelidium* have been conducted on agar composition (showing no differences, Matsuhiro & Urzúa, 1988; 1990), but to our knowledge this is the first report comparing physiological characteristics.

The absence of significant differences between phases for calorific content (with similar values as other red seaweeds, Littler *et al.*, 1987), protein and biliprotein concentration, enzymatic activity, photosynthesis and respiration under natural conditions (20 °C, pH 8.2; coastal seawater temperatures surrounding Gran Canaria ranges from 17 °C in March to 25 °C in September, the average temperature and pH being around 20 °C and 8.2 respectively), provided evidence that neither ploidy nor these physiological characteristics are involved in the unequal field distribution of phases.

Although the higher rate of photosynthesis at 25 °C (Fig. 2) suggests a better adaptation of gametophytes to higher temperatures, its concomitant increase in respiration at this temperature (Fig. 4) obligates to evaluate the photosynthesis-respiration ratio to conclude for a higher fitness of gametophytes.

Optimal temperature for several *Gelidium* species have been reported in the range of 20 to 25 °C (Fralick *et al.*, 1990; Torres *et al.*, 1991), but our data indicates significantly superior photosynthetic performances at 25 °C for both life-history phases of *Gelidium canariensis*.

The clear differences in the photosynthetic rate of both phases between pH 6.5 and 9.2 must be

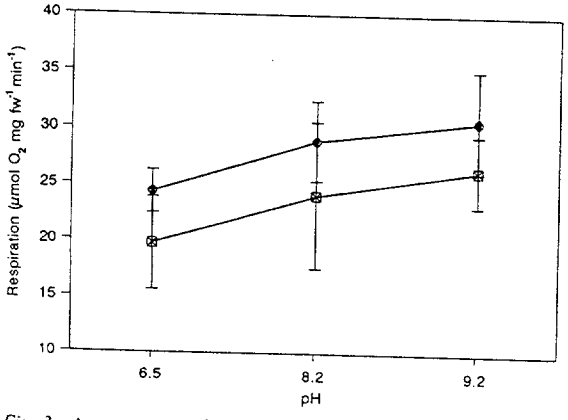

Fig. 3. Apparent respiration at different pH and 20 °C. All values are means of 3 measurements. Tetrasporophytes (☒); Gametophytes (●). Results are expressed in µmol of oxygen produced per mg of fresh weight per minute. SE bars do not appear when they are smaller than the symbol.

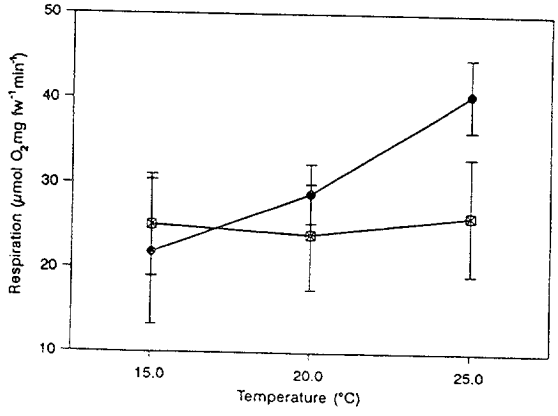

Fig. 4. Apparent respiration at different temperatures and pH 8.2. All values are means of 3 measurements. Tetrasporophytes (☒); Gametophytes (●). Results are expressed in µmol of oxygen produced per mg of fresh weight per minute. SE bars do not appear when they are smaller than the symbol.

due to differences in CO_2 (and HCO_3) availability at those pH's.

The lack of significant differences in the parameters investigated suggests that other factors must be involved in the unequal distribution of gametophytes and tetrasporophytes of *Gelidium canariensis*, supporting the stochastic model of May (1986).

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Plant Cell Reports

Factors affecting protoplast yield of the carrageenophyte *Solieria filiformis* (Gigartinales, Rhodophyta)

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Summary. The effect of age, pH of the culture medium, pre-treatment of tissues, enzymes sources and enzymatic adaptability of phycophages fed with a monospecific diet were analyzed on the protoplast yields of the red seaweed *Solieria filiformis* (Kützing) Gabrielson. New apices from fast growing plants showed the highest protoplast yields. The protoplast yield decreased when the pH of the culture medium increased from 6.0 to 9.0. Crude extracts from the abalone *Haliotis coccinea canariensis* Nordsieck, fed with *Solieria filiformis* thalli for three months in combination with cellulysin, released the highest number of viable cells and protoplasts. Yields ranged from 1.0 to 8.5 x 10^6 protoplasts per gram of fresh weight.

Key words: Protoplast - *Solieria filiformis* - Enzymes - *Haliotis coccinea canariensis* - Culture

Abbreviations: AAP= abalone acetone powder; Bis-Tris= Bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; CMC= carboxymethyl cellulose; EDTA= ethylenediaminetetraacetic acid; EGTA= ethylene glycol-*bis*(ß-aminoethyl ether) NNN'N'-tetraacetic acid; FDA= fluorescein diacetate; FW= fresh weight; Hepes= N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Tris= Tris(hydroxymethyl)aminomethane

Introduction

The inefficient digestion of the cell walls of anatomically complex brown and red seaweeds is usually due not only to changes in cell wall constitution (and consequently protoplast yield) in relation to age, life story, physiological status and culture conditions (Kloareg and Quatrano 1988; Björk et al. 1990), as in higher plants (Evans and Bravo 1984; Eriksson 1985), but also to the absence of highly efficient, cell wall degrading enzymes.

Commercially available enzymes (mostly purified from fungi) have been found to be effective for the preparation and regeneration of higher plant protoplasts (Eriksson 1985), but when used for complex seaweeds, a high number of failures have been reported (see review by Butler et al. 1990). A great variety of marine herbivores and microorganisms have been found to degrade the complex and wide range of structural and matrix carbohydrates found in seaweeds (Benítez and Macaranas 1979; Zhu 1983; Liu et al. 1984; Onishi et al. 1985; Polne-Fuller 1987; Aoki et al. 1990; Boyen et al. 1990; Potin et al. 1991). However, results on protoplast yields and plating efficiencies from these enzymatic sources have shown variable success (Saga and Sakai 1984; Tokuda and Kawashima 1988; Smith and Bidwell 1989; Le Gall et al. 1990). Problems with brown algae, such as *Laminaria* and *Macrocystis* species, appear to have been resolved since combinations of defined specific alginate lyases from marine molluscs (*Haliotis tuberculata*) and bacteria (*Pseudomonas alginovora*) have provided reproducible protocols (Butler et al. 1989; Kloareg et al. 1989; Benet and Kloareg 1991).

Work done so far in the division Rhodophyta almost exclusively concerns the genera *Porphyra* (Polne-Fuller and Gibor 1984, 1990; Chen 1987; Waaland et al. 1990) and *Gracilaria* (Cheney et al. 1986; Björk et al. 1990). However, recent reports have demonstrated success with new species such as *Chondrus crispus* (Smith and Bidwell 1989; Le Gall et al. 1990), *Gigartina corymbifera* (Gross 1990) and most recently *Palmaria palmata* (Liu et al. 1992). Except for protoplasts obtained from *Gracilaria* spp., cell wall degradations were successfully carried out with combinations of commercial cellulase and crude extracts prepared from marine invertebrates or bacteria. Although most of these protoplasts appeared to be viable, photosynthetically active, or developed new cell walls, complete regeneration has been only described in *Porphyra* species (Araki et al. 1987; Polne-Fuller and Gibor 1990; Waaland et al. 1990).

Neushul (1984) pointed out the possibility that marine herbivores fed with specific diets could alter specific enzymatic activities and, as a consequence, use of their digestive enzymes should improve protoplast yields. It

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appeared that the algal diet which the animals were fed for a month did not significantly affect their enzymatic activity, and new experiments were not developed further.

In the present study we describe a reproducible method to isolate large amounts of viable cells and protoplasts from the carrageenophyte *Solieria filiformis* using a commercial cellulase and various crude extracts prepared from *Haliotis coccinea canariensis*, wild and fed on a diet of this alga. Factors affecting growth, age and several pre-treatments of tissues were analyzed in order to obtain the best conditions for protoplast production.

Materials and methods

Sources and cultivation of plant material. Apices of *Solieria filiformis* (no longer than 1 cm in length) for enzymatic digestion were taken from healthy plants from four different systems:

1) "Wild apices": from wild plants taken from an unattached population at 3-4 m depth (Fig. 1A) on the east coast of Gran Canaria, Canary Islands, Spain. Plants were cleaned thoroughly and kept in 20 L tanks with UV-filtered running seawater 1-3 h before the experiments. New material was collected daily from the sea during the experimental period.

2) "Cylinder apices": taken from plants cultivated for 1 week in plexiglas cylinders, as described by Lignell et al. (1987).

3) "Ball apices": taken from young branches of balls ("Ball phenotype", Fig. 1B) developed after 2 months of culture in 300 L tanks.

4) Apices from plants cultured for 1 week in four different controlled pH values (6.0, 7.0, 8.0 and 9.0), in 20 L tanks. pH was adjusted throughout the day with diluted HCl and NaOH.

In the continuously aerated cultures (i.e., 2, 3 and 4), seawater (at a salinity of 3.7%) was enriched with minerals according to Provasoli (1968), except vitamins, and changed once a week. Maximum irradiance inside the greenhouse was 1,300 μmol m^{-2} s^{-1}. Growth rate was estimated daily as percent increase in FW per day, after mild centrifugation (2,000 rpm, 10 s) to remove surface water.

Mature fragments from thalli and branches were assayed to compare the effectiveness of degradation between tissues of different ages.

Sources of enzymes. Crude extracts were prepared from the endemic species of abalone, *Haliotis coccinea canariensis* (Haliotidae) (average shell length 3.0 cm). Crude extracts from wild individuals (H_w) were prepared within 12 h of collection and stored at - 20°C until used.

Crude extracts from diet-controlled specimens (H_f) were prepared from abalone fed three times a week with whole plants of *Solieria filiformis* as their only source of food for 3 months. Animals were maintained in fibreglass tanks with running, UV-filtered seawater at 19 ± 2°C.

Carrageenases were extracted from cultures of the marine bacterium *Pseudomonas carrageenovora* grown on both kappa- (κ-) and iota- (ι-) carrageenans, following the methods described by Knutsen (1991). Carrageenases were prepared after bacteria were grown in 1 L flasks containing 250 mL medium (Knutsen 1991) and placed on a rotary shaker (100 rpm) at 22°C for 48 h.

AAP (Ref. A-7514, Sigma, St Louis, USA), cellulysin (cellulase from *Trichoderma viride*, Ref. 219466, Calbiochem, San Diego, USA) cellulase (from *Trichoderma viride*, A-2274, Sigma), pectolyase (from *Aspergillus japonicus*, P-3026, Sigma) and agarase (from *Pseudomonas atlantica*, A-6306, Sigma) were also used singly or in combination (see Table 1).

Preparation of crude extracts. Crude extracts were prepared and characterized as previously described (Gómez-Pinchetti and García-Reina, 1993), with some modifications. The digestive tract and hepatopancreas from *Haliotis coccinea canariensis* were ground under liquid nitrogen after removal from the animals. The extraction was carried out in 0.1 M phosphate buffer (pH 6.0) supplemented with EDTA Na_2 (2.0 mM; 5.0 mL g tissue^{-1}). Homogenates were squeezed through a 170 μm nylon mesh and centrifuged at 27,000 g for 30 min. Ammonium sulphate was then added to the supernatant to 80% saturation. After several hours equilibration, the suspension was centrifuged at 37,000 g for 20 min. Pellets were resuspended in buffer and passed through a pre-packed Sephadex G-25 column (PD-10, Pharmacia LKB, Uppsala, Sweden) equilibrated with 20 mM Bis-Tris buffer (pH 6.0) dissolved in seawater for desalting and buffer exchange.

AAP was dissolved in buffer, equilibrated for several hours at 0-4 °C, centrifuged at 27,000 g for 15 min and passed through a PD-10 column.

For bacterial carrageenases, cells were collected by centrifugation at 27,000 g for 30 min and the supernatant used for enzyme preparation. Ammonium sulphate was added to the solution to 90% saturation and the following procedure carried out as described above. All the extracts were stored at - 20°C until use.

Quantification of enzymatic activities. κ- and ι- carrageenases (EC 3.2.1.83 and EC 3.2.1.-, respectively) and cellulase (EC 3.2.1.4) enzymatic activities of crude extracts were measured at 25°C and pH 6.0 and 7.2 on κ- and ι- carrageenans (kindly supplied by R. Armisén, Hispanagar S.A.) and CMC (Sigma, Ref. C-8758). The reaction mixture consisted of 900 μL of 0.3% (w/v) substrate dissolved in buffer and 100 μL crude extract. Reducing sugars were measured spectrophotometrically by the method of Nelson (1944) and Somogyi (1952). One unit (U) of enzyme activity is defined as the amount of enzyme which produce an increase in A_{510nm} min^{-1} of 0.1 in the reducing-sugar assay.

Plant pre-treatments

Treatment with ultrasound. "Wild apices" in sterile seawater were treated in an ultrasonic bath (50 Hz, Ultrasonic cleaner, Penta, Izasa S.A., Spain) for 1 to 3 min prior to pre-plasmolysis, (n=5).

Pre-plasmolysis. All algal material was chopped into millimeter pieces using a razor blade and then rinsed five times in wash buffer (20 mM Hepes in seawater, 0.2 M mannitol, pH 7.0) before incubation for 30 min in seawater supplemented with 20 mM Hepes and 0.8 M mannitol, pH 7.0, (n=35).

Metal chelators. EDTA and EGTA were used separately at concentrations from 20 to 40 mM in the pre-plasmolysis medium to check the effect of metal chelators on the protoplast yield of "ball" and "cylinder" apices, (n=10).

The effect of the different pre-treatments was compared with chopped apices directly incubated in the enzymatic solution (control).

Protoplast isolation. The enzymes mixtures used for the degradation procedure are shown in Table 1. Enzymatic solutions, supplemented with 0.4 M mannitol, were stirred for 30 min at 0-4°C, centrifuged at 27,000 g for 10 min, pH adjusted to 6.0 and filtered through 0.8 and 0.2 μm Minisart filters (Sartorius, Göttingen, Germany).

One gram of chopped apices was incubated in 10 mL enzyme solution in a 90 mm diameter Petri dish at 20°C under constant shaking (80 rpm) in the dark. After digestion, cell wall debris and non-digested material were removed by filtration through a 45 μm nylon mesh and protoplast yields estimated with a hemocytometer on an inverted microscope (IMT-2 Olympus, Hamburg, Germany). The suspension was rinsed with wash buffer and centrifuged in a swing out rotor at 100 g for 10 min. The pellet was resuspended in buffer and protoplasts were washed twice by centrifugation at 100 g for 5 min. Protoplasts were maintained in 5 cm plastic Petri dishes with Provasoli enriched seawater (Provasoli 1968) supplied with 0.2 M mannitol. Osmotic sensitivity of protoplasts was checked by immersion in distilled water. Cell viability was checked by the ability to accumulate FDA (Sigma, final concentration of 35 μg ml^{-1}) (Larkin 1976). The absence and regeneration of cell walls were followed by staining with Calcofluor White (0.01% w/v) (Galbraith 1981).

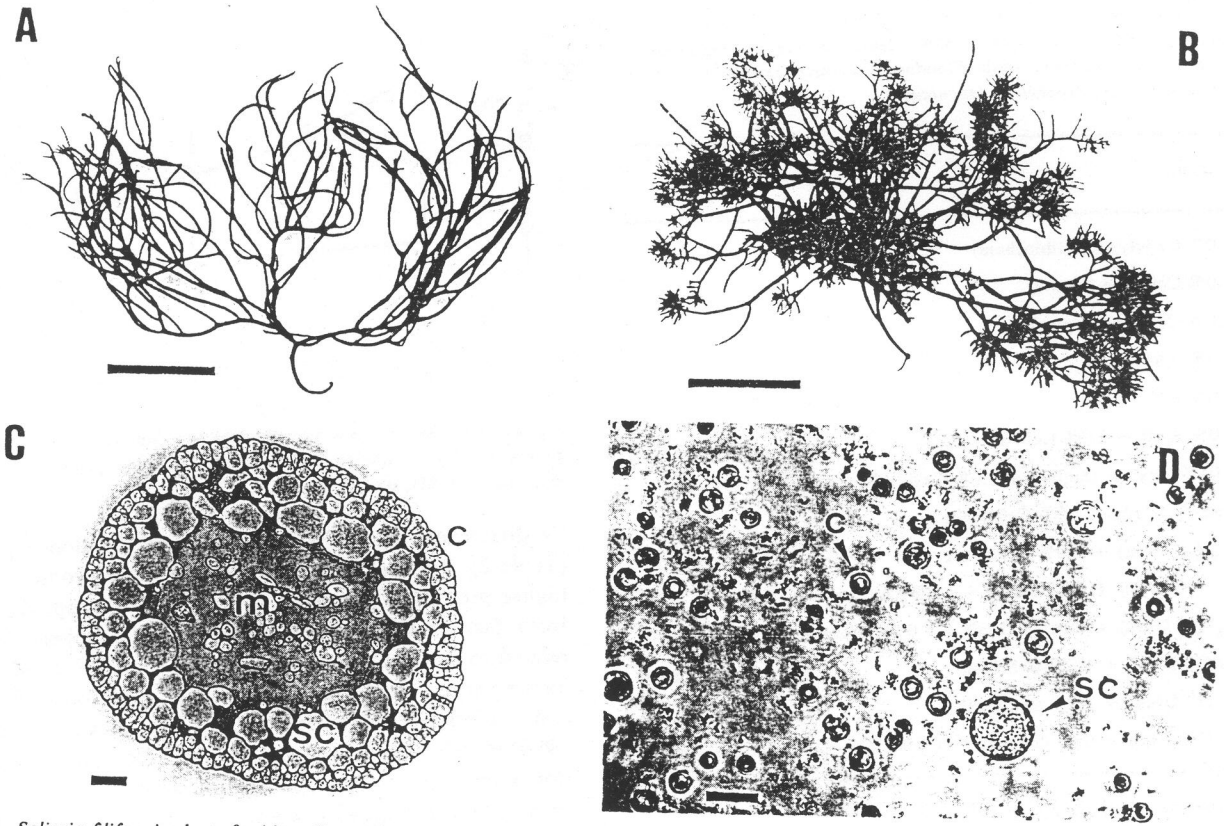

Fig. 1. *Solieria filiformis* plants freshly collected from the sea (A) and after 2 months in tank culture showing ball morphology (B) Scale bars= 2 cm. (C) Transverse section showing cell distribution; (c) cortical, (sc) sub-cortical and (m) medullary cells. Scale bar= 10 μm. (D) Freshly collected protoplasts produced from cortical (c) and sub-cortical cells (sc). Scale bar= 25 μm.

Results and discussion

Isolation of protoplasts

High numbers of cells and protoplasts were observed after 2 h, although maximum protoplasts yields (1.0 to 8.5 x 10^6 protoplasts per g FW) were obtained after 12 h of incubation in the enzymatic solution. Protoplasts from the sub-cortex and medulla (20-25 μm) and mostly from cortical cells (10-15 μm in diameter)(Fig. 1C) were released (Fig. 1D). Viable protoplasts, approximately 70-80%, appeared bright yellow-green when stained with FDA. After 2 days, 10-15% of the protoplasts showed cell wall regeneration and formation of cell clusters were observed.

The best protoplast yields were obtained with combinations of cellulysin and crude extracts from *Haliotis coccinea canariensis* fed with *Solieria filiformis* (H_f) (Table 1). No yield enhancement was observed when either bacterial carrageenases from *Pseudomonas carrageenovora*, or pectolyase, were added to the enzyme solution containing H_f crude extract, although an increase was observed when added to the H_w (Table 1). Cellulase played an important role in cell wall digestion. Increases in cellulysin concentration in the enzyme solution from 1.0 to 3.0 % ... rated protoplast production (none at 1%, to 10^4 at 3% after 2 h of incubation) without affecting cell viability. AAP, alone or in combination with cellulases, only degraded apices but no cells or protoplasts were observed (Table 1).

The increased efficiencies of H_f (and H_w), in comparison to AAP (Table 1), could be directly related to the diet of these phycophages. AAP is obtained from cultivated *Haliotis* sp. fed with brown seaweeds (mostly *Macrocystis pyrifera*), which might explain its high alginate lyase activity (40 U ml^{-1} at pH 7.2 and 25°C; Gómez-Pinchetti and García-Reina, 1993). Differences in carrageenase and alginate lyase activities between the different crude extracts (AAP, H_w and H_f) were observed. While alginate lyase was higher in AAP (40 U ml^{-1}) but not detected in H_w and H_f, carrageenase activity was not detected in AAP, but 10 in H_w and 30 U ml^{-1} in H_f. These results are in accordance to those described by van Weel (1961) who previously obtained higher production of carbohydrases (amylase and saccharase) when the mollusc *Helix fulica* was fed, during eight ... starch. Our data ... phycophages

Table 1. Effect of enzyme composition on protoplast yield (protoplasts g^{-1} FW) from "wild apices" of *Solieria filiformis*. -= no effect or slight tissue degradation. Values represent at least three repeated experiments. Carr = carrageenase from *Pseudomonas carrageenovora*; Pect = pectolyase from *Aspergillus japonicus*.

Enzymes	Yield
3.0% Cellulysin (Calbiochem)	-
3.0% Cellulase (Sigma)	-
6.0% Cellulase (Sigma)	-
3.0% AAP	-
6.0% AAP	-
2.0% AAP + 2.0% Cellulase	-
0.01% Agarase (Sigma) + 2.0% Cellulysin	$<10^4$
Carr (3 U/ml) + 1.0% Cellulysin	$<10^4$
H_w (10 U/ml) + 1.0% Cellulysin	$4.3 \pm 1.7 \times 10^4$
H_w (10 U/ml) + 0.25% Pect + 1.0% Cellulysin	$7.5 \pm 0.5 \times 10^4$
H_w (10 U/ml) + Carr (3 U/ml) + 1.0% Cellulysin	$8.7 \pm 1.0 \times 10^5$
H_f (30 U/ml) + 1.0% Cellulysin	$6.2 \pm 1.5 \times 10^6$
H_f (30 U/ml) + 3.0% Cellulysin	$7.3 \pm 1.2 \times 10^6$
H_f (30 U/ml) + Carr (3 U/ml) + 1.0% Cellulysin	$6.4 \pm 0.7 \times 10^6$

Sources of plant material

All the pieces used for the experiments showed the same anatomy and cell distribution as observed in Fig. 1C. Although alterations were produced in the morphology of plants cultured for more than 2 months in tanks ("ball phenotype", Fig. 1B) changes in the growth pattern of the "ball" apices were not observed.

Large differences in the release and viability of protoplasts were found depending on the donor tissue. "Wild" and "cylinder" apices showed the highest protoplast yields ($6.0\text{-}8.5 \times 10^6$ protoplast g^{-1} FW). In the few cases where protoplasts were obtained from mature thalli, yields were no higher than 10^4. Similar results have been described by Gross (1990) and LeGall et al. (1990) in other carrageenophytes. Yields of protoplasts from "ball apices" (Fig. 1B), were even lower. The differences in protoplast yield between "cylinder" and "ball" apices might be explained

Table 2. Protoplast yields of *Solieria filiformis* in relation to growth rates (% per day) and source of plant material.

Plant material	Growth rate	Yield
Ball apices	<1 %	$<10^4$
Cylinder apices	10-14 %	$8.0\text{-}8.5 \times 10^6$
Wild apices	-	$6.0\text{-}8.0 \times 10^6$

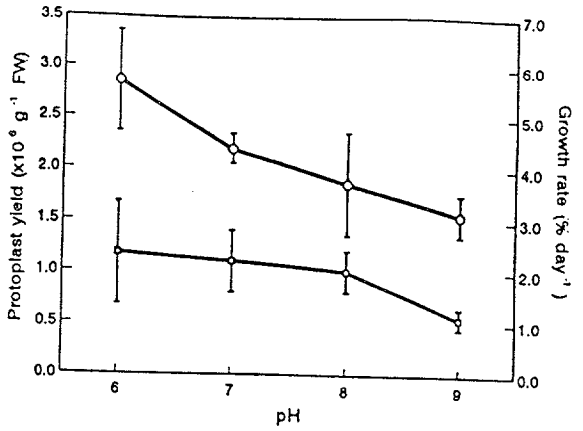

Fig. 2. Protoplast yield and growth rates as a function of the pH of the culture medium in *Solieria filiformis*. ○: Protoplast yield; □: Growth rate. (mean ± SD; n=5).

by differences in the growth rates of the donor plants (Table 2). Björk et al. (1990) described the production of higher protoplast numbers from *Gracilaria* spp. derived from fast growing plants. These results appear to be related to the fact that growth rate and culture conditions have a very strong influence on the composition of the cell walls (Lignell and Pedersén 1989; Ekman and Pedersén 1990) thereby requiring less complex enzymatic degradation during protoplast production. Intermediate growth rates obtained in the pH-experiment (Fig. 2) gave intermediate protoplast yields. An explanation of the similar yields observed with "wild" and "cylinder" grown apices might be related to a fast growth rate (not quantified) of wild plants during the period of the experiments. *Solieria filiformis* shows a marked seasonal growth and specimens were collected during the period of most growth activity.

Effect of the pH of the culture medium

The pH of the culture medium directly affected the protoplast yield (Fig. 2). Correlation between protoplast yield and growth rate (Pearson correlation coefficient, $\alpha = 0.86$) indicates a relationship between the pH of the culture medium and both parameters. Previous results have shown an increase of 25% in photosynthetic oxygen evolution of *Solieria filiformis* at pH 6.5 compared to measurements taken at pH 8.2 (Gómez-Pinchetti et al. 1992) which might explain the influence of pH on the growth rate and, as a consequence, the protoplast yield. However, the influence of the pH of the culture medium on the resistance to enzymatic digestion of the cell wall might also be another factor to be considered.

Effect of plant pre-treatments

Pre-plasmolysis. Pre-plasmolysis slightly improved protoplast yield (1-2%) and more so viability (90% of the cells appeared viable as detected by FDA staining). Viability of protoplasts obtained from tissues not pre-plasmolyzed was

less than 70% and cells did not look as uniform as those obtained from pre-plasmolyzed tissues. Similar beneficial effects of pre-plasmolysis have been shown in other red (*Gracilaria*, Björk 1992), brown (*Laminaria*, Butler et al. 1989) and green (*Ulva*, Björk et al. 1992) seaweeds. This has been generally described as a useful step to ensure protoplast stability during cell wall digestion (Butler et al. 1990) and to prevent the assimilation of toxic substances (Butler et al. 1989) present in enzymatic preparations (Tribe 1955; Berliner 1981; Fitzsimons and Weyers 1985). Pre-plasmolysis could have reduced the possible assimilation of substances such as proteases or ribonucleases, found in crude extracts, which might be detrimental to protoplast survival at certain pH and temperature conditions.

Treatment with ultrasound plus pre-plasmolysis. Protoplast yields were increased by approximately 20% (with respect to the control) when apices were incubated in an ultrasonic bath for no more than 2 min before pre-plasmolysis. Longer periods produced softening of tissues but also damaged cells, decreasing their viability. Neushul (1984) also reported the increase of effectiveness of the enzymatic treatment on *Sargassum* spp. when sonication was carried out prior to digestion. This kind of physical treatment of tissues can help to desintegrate the structure of cell walls facilitating the action of enzymes.

Pre-plasmolysis plus metal chelators. Addition of metal chelators such as EDTA or EGTA to the pre-plasmolysis solution had no significant effect on protoplast yield in *Solieria*. Although EGTA improved protoplast yield from *Laminaria* by removing calcium from the matrix polysaccharides (Butler et al. 1989), it seems unable to affect the matrix polysaccharides from carragenophytes. Similar results have been reported in *Chondrus crispus* with either EDTA or EGTA, although a 50% enhancement was obtained when Kryptofix 222 (a potassium chelator) was used as a chelating agent in the pre-incubation solution (Le Gall et al. 1990). Specificity of chelators in relation to the type of polysaccharide to be degraded seems to strongly affect the effectiveness of this pre-treatment.

This report indicates the possibility to expand protoplast techniques to difficult seaweeds species for digestion, inducing "tailor-made", species-specific, enzymes through specific dietary control of phycophages, and by an appropiate manipulation of pre-culture factors.

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Plant Cell Reports

Plant regeneration from protoplasts of peppermint (*Mentha piperita* L.)

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Summary. Enzymatically isolated leaf-derived protoplasts of peppermint (*Mentha piperita* L.) were cultured in modified B5 medium containing 1 mg/l NAA, 0.4 mg/l BA, 0.5% sucrose, 0.5 M mannitol and 0.1% Gelrite (first medium). After 30 d culture at 25°C in the dark, protoplasts formed colonies consisting of about 100 cells. Gelrite medium blocks were transferred into liquid medium to promote further growth. Colonies of 0.5 mm transferred to 0.2% Gelrite solidified medium (same components as first medium) formed green calli (1-2 mm) under incubation in the light. Green calli transferred to differentiation medium (B5, 0.1 mg/l NAA, 5 mg/l BA, 2% sucrose, 0.2 M mannitol, 0.2% Gelrite) developed shoot buds after 3-4 weeks. Whole plants were recovered following rooting of shoots in B5 medium without hormones.

Abbreviations: BA, 6-benzylaminopurine; NAA, α-naphthaleneacetic acid; KIN, kinetin; ZEA, zeatin; CPW, cell and protoplast wash solution; B5, Gamborg et al. (1968) mineral elements; MS, Murashige and Skoog (1962) mineral elements

Introduction

Peppermint (*Mentha piperita* L.) is a perennial herb of the *Labiate*, and contains essential oil that has been used as ingredient of medicine (Sanyal and Varma 1969; Herrmann and Kucera 1967) and spices since ancient times. At present, peppermint oil is widely used as a material for toothpaste, shampoo, chewing gum, etc. (Landing 1969; Hendrickson 1976). Some studies on cell culture of *Mentha* plants for the purpose of production of monoterpenes or fragrance components (Lin and Staba 1961; Wang and Staba 1963; Aviv and Galun 1978; Aviv et al. 1981; Karasawa and Shimizu 1980), and *in vitro* micropropagation (Rech and Pires 1986; Repcáková et al. 1986) have been reported.

Since *M. piperita* is a triploid (2n=72) plant and is sterile, its breeding by common crossing methods is impossible unless its ploidy is doubled for fertility (Sato et al. 1989). Recently, genetic manipulations that utilize protoplast culture are enhancing plant improvement efforts. To use such techniques as cell fusion, gene transfer, and somaclonal variation, techniques for plant regeneration from protoplasts have been established in many plants (Roest and Gilissen 1989). However, protoplast culture of *Mentha* species has not been reported.

We have been studying the tissue culture of *Mentha* plants, for the purpose of breeding useful mint with improved flavor, by protoplast manipulations such as cell fusion and protoclonal variation. This paper describes techniques for the isolation, culture, and plant regeneration of leaf-derived protoplasts of peppermint.

Materials and methods

Plant growth and pretreatment of leaf. Axillary buds of peppermint (*Mentha piperita* L. cv. Blackmint) excised from plants maintained in a greenhouse were disinfected with 70% ethanol (1 min) and 1% sodium hypochlorite (15 min), and washed 3 times with sterile distilled water. After surface sterilization, axillary buds were transplanted to 100 ml of B5 medium (Gamborg et al. 1968) containing 2% sucrose and 0.2% Gelrite (SCOTT LABORATORIES, INC.,) (pH 6.0) in 300 ml jars and cultured at 25°C, under the light (3,000 lux, 16 h photoperiod). Shoot cultures were maintained at subculture intervals of about one month. Leaves grown to 2-2.5 cm length from the 2nd to 4th nodes of each shoot were harvested. Approximately 0.8 g of leaves from 4 shoots were sliced into 1-2 mm strips by a scalpel, transferred into 7 ml of CPW salts solution (Xu et al. 1981) (0.5M mannitol, pH 6.0) in 60 mm plastic petri dishes, and shaken gently at 70 rpm for 4 h.

Protoplast isolation and purification. The pretreated leaves were transferred into each of the enzyme solutions (20 ml, filter sterilized) listed in Table 1, and incubated at 25°C for 12 h without shaking. Then, protoplasts were released by shaking (70 rpm) for 30 min. The enzymatically isolated protoplasts were filtered through Miracloth (Hoechst Co. Ltd.) and pelleted by centrifugation (100 x *g*, 2 min). Pelleted

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Ornithine Cycle in *Nostoc* PCC 73102: Stimulation of In Vitro Ornithine Carbamoyl Transferase Activity by Addition of Arginine

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Abstract. Cells of *Nostoc* PCC 73102, a free-living cyanobacterium originally isolated from the cycad *Macrozamia*, were cultured under different conditions and examined for the presence of *in vitro* active ornithine carbamoyl transferase (OCT). Cells grown in darkness showed a significant increase of *in vitro* OCT activity compared with the activity when grown in light. Addition of external arginine in the growth medium increased *in vitro* OCT activity both in light and in darkness. Moreover, the highest *in vitro* OCT activity was observed in cells grown in darkness and with the addition of external arginine, a sevenfold increase compared with cells grown in light. Native-PAGE in combination with on gel OCT activity stain demonstrated that external arginine induced the presence of two *in vitro* active OCT. In addition to the previously described 80 kDa OCT [Physiol Plant 84:275–282, 1992], a second *in vitro* active enzyme with a molecular weight of approximately 118 kDa appeared. Western immunoblots, with native cell-free extracts and antibodies directed either against native or denatured OCT purified from *Pisum sativum*, confirmed that both enzymes were OCT. Moreover, with a denatured cell-free extract only one polypeptide, with a molecular weight of about 40 kDa, was recognized, indicating that the second *in vitro* active OCT might be a trimer with three identical subunits.

Cyanobacteria can establish symbioses with a number of higher plants [15]. In the *Macrozamia-Nostoc* symbiosis, glutamine and citrulline have been identified as the main N-solutes transported in the xylem from the coralloid root to the rest of the cycad. $^{15}N_2$ experiments demonstrated that both amino compounds were involved in the transfer of fixed nitrogen [13]. Citrulline is synthesized from NH_4^+ or glutamine, CO_2, and ornithine with the two enzymes carbamoyl phosphate synthetase (CPS) and ornithine carbamoyl transferase (OCT, also named ornithine transcarbamoylase; EC 2.1.3.3) [9, 16]. Earlier studies have demonstrated that CPS and OCT are present in cell-free extracts of several filamentous heterocystous cyanobacteria [1, 3, 5, 7, 11]. In light-grown, nitrogen-fixing *Nostoc* PCC 73102, a free-living filamentous cyanobacterium originally isolated from *Macrozamia*, both CPS [10] and OCT [11] have been found to be present in both the nitrogen-fixing heterocysts and in the photosynthetic vegetative cells. Moreover, OCT was shown to be an *in vitro* active enzyme with a molecular weight of approximately 80 kDa and, based on SDS-PAGE/Western immunoblots, suggested to consist of two identical subunits with molecular weights of approximately 38 kDa [11].

OCT can have both an anabolic and a catabolic function. Anabolic OCT, catalyzing the formation of citrulline, is usually a trimeric molecule where the identical subunits have a molecular mass of 35–39 kDa [8, 19]. Catabolic OCT, producing carbamoylphosphate and ornithine from citrulline and inorganic phosphate, is, with only few exceptions, larger than the anabolic OCT and composed of six, eight, nine, or more identical subunits [8, 19]. In bacteria, catabolic OCT can be induced by including arginine in the growth medium [6, 8]. However, no catabolic enzyme could be induced by arginine in the three microalgae *Chlorella autotrophica, Chlorella saccharophila*, and *Dunaliella tertiolecta* [6]. These three microalgae all have arginine deiminase activity, where arginine is converted directly to citrulline and ammonium [6].

In the present work we continue our characterization of the citrulline synthesis in the free-living cyanobacterium *Nostoc* PCC 73102. We demon-

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strate that it is possible to induce higher OCT activity as well as a second *in vitro* active OCT by including arginine in the growth medium.

Materials and Methods

Organism and culture condition. Cells of *Nostoc* PCC 73102 (ATCC 29133), a free-living cyanobacterium originally isolated from the cycad *Macrozamia*, were grown in $BG11_0$-medium [18], as described [11]. Experimental cells were grown in $BG11_0$-HEPES ($BG11_0$ containing 10 mM HEPES-NaOH, pH 7.5) without or with the addition of 5 mM arginine. Cells of *Nostoc* PCC 73102 were also grown in $BG11_0$-HEPES with different pH: (i) $BG11_0$-HEPES pH 6.0, (ii) $BG11_0$-HEPES pH 7.0, (iii) $BG11_0$-HEPES pH 8.0, and (iv) $BG11_0$-HEPES pH 9.0.

***In vitro* ornithine carbamoyl transferase (OCT) activity.** OCT (EC 2.1.3.3.) was measured according to a modified method of Boyde and Rahmatulla [2], based on the colorimetric detection of citrulline with diacetyl monoxime (DAMO, systematic name: 2,3-butanedione monoxime). Cells were harvested (3000 *g*, 10 min), broken by sonication (MSE, Ultrasonic Power unit, 12-63, UK) in buffer [200 mM Tris-HCl (pH 8.5) containing 2 mM dithiothreitol] and centrifuged (14,000 *g*, 30 min, 4°C) before the supernatants were used in the assays. The reaction mixture contained 200 mM HEPES-NaOH (pH 8.5), 10 mM L-ornithine, 10 mM carbamoylphosphate-dilithium salt (CP) in a final volume of 0.1 ml. The reaction was carried out at 37°C for 15 min. The mixture was deproteinized and the enzyme reaction stopped by addition of 100 µl of 10% trichloroacetic acid; the mixture was then centrifuged for 5 min and the supernatant collected. A sample without the addition of CP was used as background level (control). The acidified reaction mixture was combined with 400 µl acid-ferric solution (1 *N* H_3PO_4, 9 *N* H_2SO_4, and 5 mM ferric ammonium sulfate) and 200 µl DAMO solution [50 mM DAMO and 20 mM antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin) added just before use]. The samples were mixed vigorously in a Vortex mixer (KEBO-Lab REAX 2000, Stockholm, Sweden), boiled at 100°C for 30 min, allowed to cool to room temperature before the absorbance was measured at 464 nm with a Hitachi U-2000 spectrophotometer (Tokyo, Japan). The *in vitro* OCT activities were then calculated by use of a standard curve, linear between 0 and 0.25 µmol of citrulline. Total amounts of protein in the cell-free extracts were calculated according to [14] with bovine serum albumin to standardize the assay procedure.

Native-PAGE and *in vitro* staining for OCT activity. The cyanobacterial cells were harvested by centrifugation (2500 *g*, 10 min) and resuspended in OCT-lysis buffer [10 mM HEPES-NaOH, 0.5% (w/v) Triton X-100, 2 mM dithiothreitol (pH 7.4)]. The cell suspension was sonicated (see above) on ice and centrifuged (13,000 *g*, 4°C, 10 min). By using Pharmacia PhastSystem (Pharmacia Biotechnology Ltd., Uppsala, Sweden) native-PAGE (Polyacrylamide gel electrophoresis) was performed on 10–15% gradient gels [11]. Two identical gels were run simultaneously; one stained for total protein profiles with Coomassie blue, and the second one stained for OCT activity by use of the method of Farkas et al. [4], outlined briefly below. The gel is incubated in a reaction mixture containing the substrates for OCT [5 mM ornithine and 15 mM carbamoylphosphate in 270 mM triethanolamine (pH 7.7)] for 10 min at 37°C. Through the activity of OCT, citrulline and inorganic phosphate are produced. The phosphate reacts with a 1 : 1 mixture of 1% nitric acid and 40 mM ammonium molybdate, and phosphomolybdic acid is formed. In the last step phosphomolybdic acid is reduced with 10% ascorbic acid, and the site(s) of the *in vitro* active OCT will appear as a blue band/precipitate. The sizes of the *in vitro* active OCT were calculated with high-molecular-weight markers (Pharmacia) as standards.

Table 1. *In vitro* ornithine carbamoyl transferase (OCT) activity in *Nostoc* PCC 73102. The cyanobacterial cells were grown for 4 days in light or darkness, without or with the addition of external arginine (5 mM) in the growth medium before being assayed for the presence of *in vitro* active OCT

Growth condition	nmol citrulline formed · µg protein^{-1} · min^{-1}	
Light	64 ± 6[a]	(100)[b]
Light + arginine	136 ± 23	(212)
Darkness	140 ± 9	(219)
Darkness + arginine	450 ± 19	(703)

[a] Means ± SE (n = 3).
[b] % activity compared with cells grown in light without the addition of external arginine in the growth medium.

Documentation of OCT activity stain. The on gel visualized OCT activities were viewed on a TV-screen (DAGE-MTI HR1000, USA)/saved on a diskette, by use of a Videocamera (DAGE-MTI CCD72, USA) and a Quantel image processor (Quantel, UK). Recalled images were photographed (Kodak TMAX 100) with a Polaroid FreezeFrame Video Recorder.

SDS-PAGE. The native cell-free extracts were denaturated by adding solubilization buffer [Tris-HCl (pH 8.0), containing 1 mM EDTA, 5% β-mercaptoethanol, 2.5% SDS, and 0.5% Triton X-100)] in a 1 : 1 ratio and were then boiled for 5 min. SDS-PAGE was performed on 10–15% gradient gels by use of Pharmacia PhastSystem [12].

Western immunoblot. The separated cyanobacterial proteins were transferred onto a nitrocellulose membrane before incubation with antibodies directed against either the native enzyme (1 : 400 dilution) or the denatured OCT (1 : 250 dilution) purified from *Pisum sativum* (rabbit-anti-OCT-antiserum) [17]. The recognition of the antibodies with the antigen(s) was visualized with goat-anti-rabbit IgG antibodies conjugated with horseradish peroxidase and chloro-1-naphthol as substrate [11, 12]. The results were documented with a Videocamera, a Quantel image processor, and a Polaroid FreezeFrame Video Recorder as described above.

Results and Discussion

By using a modified colorimetric method it was possible to measure *in vitro* OCT activity in the cyanobacterium *Nostoc* PCC 73102. Cells grown in light showed a value of 64 ± 6 (means ± SE, $n = 3$) nmol citrulline formed · µg protein^{-1} · min^{-1} (Table 1). However, by excluding light for 4 days, the specific

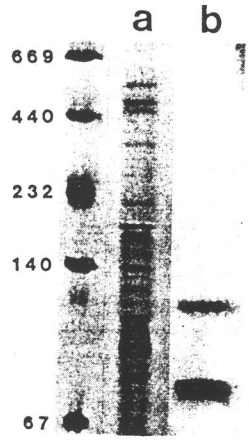

Fig. 1. Nitrogen-fixing *Nostoc* PCC 73102 contains two *in vitro* active OCTs when grown in darkness and with the addition of external arginine in the growth medium. Native-PAGE stained for total protein with Coomassie blue (a), and activity stain of *in vitro* active OCT (b). The two OCTs have molecular weights of approximately 80 and 118 kDa, respectively. High-molecular-weight markers (in kDa) are shown to the left.

in vitro OCT activity doubled. A similar increase was observed when arginine was added to the light-grown cells. Moreover, a combination of darkness and addition of external arginine induced a sevenfold stimulation of the *in vitro* OCT activity (Table 1). Interestingly, when native-PAGE and on gel OCT activity stain were used, two *in vitro* OCTs were present in cells grown with the addition of external arginine in the growth medium. In addition to the previously described 80 kDa enzyme [11], a second, *in vitro* active OCT of approximately 118 kDa appeared (Fig. 1). The 118-kDa enzyme was detectable after 26 h of induction (darkness + arginine) but reached maximal *in vitro* on gel activity after 70 h. Since the induction of the second *in vitro* active OCT was more pronounced in cells grown in darkness, these were chosen for further experiments.

Aspartate carbamoyl transferase (ACT, EC 2.1.3.2) is another enzyme generating inorganic phosphorus from carbamoylphosphate that theoretically could give a positive response with the method used. By replacing ornithine with aspartic acid in the reaction mixture, we examined the presence of *in vitro* active ACT in the cell-free extracts. No activity could be observed, demonstrating that the method used [4] is specific for OCT and that the second enzyme is OCT.

In the bacterium *Aeromonas formicans* OCT is a nonamer under acidic conditions (pH 6.0), but a trimer under more basic pH (8.0) [19]. By growing

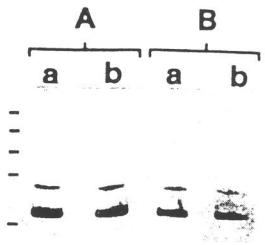

Fig. 2. Western immunoblotting of native cell-free extracts of *Nostoc* PCC 73102 grown in darkness and with the addition of external arginine in the growth medium. Before being visualized, with 4-chloro-1-naphthol as substrate, the cell-free extracts were electroblotted onto nitrocellulose membranes and incubated either with polyclonad rabbit-anti-native OCT antiserum (A) or rabbit-anti-denatured OCT antiserum (B), followed by goat-anti-rabbit IgG conjugated to horseradish peroxidase. (a) and (b) represent two individual cyanobacterial cultures. The positions of high-molecular-weight markers (see Fig. 1) are indicated to the left.

Nostoc PCC 73102 in darkness and in media with different pHs (6, 7, 8, or 9), the direct influence of pH on the presence of *in vitro* active OCT was evaluated. Under all pHs tested, only one *in vitro* active OCT, with a molecular weight of about 80 kDa, could be observed. Thus, the presence of the 118 kDa *in vitro* active enzyme is not an effect of low/high pH, but a response to the amino acid added. In several bacteria a catabolic OCT has been reported to be induced by external addition of arginine [8, 19]. Under the same conditions a second, larger OCT was induced in *Nostoc* PCC 73102. However, compared with other bacterial OCTs [8, 19], the cyanobacterial enzymes are, in general, smaller proteins (Fig. 1) [1, 11].

Native-PAGE/Western immunoblots and antibodies directed against either native or denatured OCT purified from *Pisum sativum* demonstrated recognitions against both the 80- and the 118-kDa enzymes (Fig. 2). This clearly demonstrates that both *in vitro* active enzymes are OCT, and that they share homology on subunit composition. Moreover, with a denatured cell-free extract and SDS-PAGE/Western immunoblots, only one single band, with a molecular weight of approximately 40 kDa, appeared (not shown). The 80-kDa enzyme was earlier suggested to be a dimer with two identical subunits [11], and the present data suggest that the 118-kDa enzyme might be a trimer with three identical subunits.

Nostoc PCC 73102 grown in darkness exhibits no significant nitrogenase activity [11]. Instead, nitrogen might be made available from the storage compound cyanophycin, a multipeptide consisting of arginine and aspartic acid in a 1 : 1 ratio [3]. The

released arginine may be converted either to citrulline directly by arginine deiminase, or to ornithine followed by citrulline by arginase and OCT, respectively. Addition of arginine, externally or by the catabolism of cyanophycin, will induce higher *in vitro* OCT activity (Table 1). Moreover, external arginine also induces a second, larger *in vitro* active OCT (Figs. 1, 2). The physiological function, anabolic/catabolic role, of this second *in vitro* active OCT requires further experiments.

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Dark induction of nitrate reductase in the halophilic alga *Dunaliella salina*

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Abstract. The effect of nitrogen starvation on the NO_3^--dependent induction of nitrate reductase (NR) and nitrite reductases (NIR) has been investigated in the halophilic alga *Dunaliella salina*. When *D. salina* cells previously grown in a medium with NH_4^+ as the only nitrogen source (NH_4^+-cells) were transferred into NO_3^- medium, NR was induced in the light. In contrast, when cells previously grown in N-free medium were transferred into a medium containing NO_3^-, NR was induced in light or in darkness. Nitrate-dependent NR induction, in darkness, in *D. salina* cells previously grown at a photon flux density of 500 $\mu mol \cdot m^{-2} s^{-1}$ was observed after 4 h preculture in N-free medium, whilst in cells grown at 100 $\mu mol \cdot m^{-2} s^{-1}$ NR induction was observed after 7–8 h. An inhibitor of mRNA synthesis (6-methylpurine) did not inhibit NO_3^--induced NR synthesis when the cells, previously grown in NH_4^+ medium, were transferred into NO_3^- medium (at time 0 h) after 4-h-N starvation. However, when 6-methylpurine was added simultaneously with the transfer of the cells from NH_4^+ to NO_3^- medium (at time 0 h), NO_3^- induced NR synthesis was completely inhibited. The activity of NIR decreased in N-starved cells and the addition of NO_3^- to those cells greatly stimulated NIR activity in the light. The ability to induce NR in darkness was observed when glutamine synthetase activity reached its maximal level during N starvation. Although cells grown in NO_3^- medium exhibited high NR activity, only 0.33% of the total NR was found in intact chloroplasts. We suggest that the ability, to induce NR in darkness is dependent on the level of N starvation, and that NR in *D. salina* is located in the cytosol. Light seems to play an indirect regulatory role on NO_3^- uptake and NR induction due to the expression of NR and NO_3^--transporter mRNAs.

Key words: *Dunaliella* – Nitrate reductase (dark induction, intracellular location) - Nitrite reductase

Abbreviations: GS = glutamine synthetase; NR = nitrate reductase; NIR = nitrite reductase

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Introduction

Microalgae assimilate nitrate by two closely linked steps: (i) the uptake of nitrate from the medium into the cell and (ii) intracellular reduction of NO_3^- to NH_4^+, catalyzed by nitrate reductase (NR) and nitrite reductase (NIR) (Guerrero et al. 1981; Syrett 1981; Solomonson and Barber 1990). Ammonium is incorporated into carbon skeletons via the glutamine synthetase/glutamate synthetase (GS/GOGAT) cycle (Guerrero et al. 1981).

However, the mechanism of NO_3^- transport into cells, the regulation of NR activity and the intracellular location of NR in algal cells are not yet fully understood. Several models of nitrate transport and regulation of NR activity in algae and higher plants have been described (Syrett 1981; Florencio and Vega 1982; Tischner et al. 1989; Solomonson and Barber 1990; Becker et al. 1992; Mohr et al. 1992; Campbell 1992). According to Guerrero et al. (1981) and Solomonson and Barber (1990), NR activity can be regulated at the transcription and translation level, and at the level of enzyme synthesis and degradation.

Light, the presence of NO_3^- and CO_2 promote NR synthesis (Herrera et al. 1972; Azuara and Aparicio 1983; Larsson et al. 1985; Ramazanov et al. 1988; Mohr et al. 1992). In contrast, darkness or the presence of NH_4^+ represses NR and NIR synthesis (Herrera et al. 1972; Guerrero et al. 1981; Florencio and Vega 1982; Franco et al. 1987, 1988; Mohr et al. 1992). However, Dixon and Syrett (1988) have reported NO_3^- uptake by an N-starved alga *Amphidium spp.* in darkness. In addition, several researchers have described an increase in respiration after the addition of NO_3^- to N-limited cultures of *Chlorella, Selenastrum* and *Dunaliella* (Syrett 1956; Turpin et al. 1988; Weger and Turpin 1989; Vanlerberghe et al. 1992). According to Turpin et al. (1988), mitochondrial respiration is involved in dark NO_3^- assimilation in the N-limited green algae *D. tertiolecta* and *Selenastrum minutum*. However, those experiments measured neither the level of NR activity nor the rate of NO_3^- uptake in N-limited and N-sufficient cells in the dark.

The intracellular location of NR in algae is still controversial. Lopez-Ruiz et al. (1985) demonstrated the localization of NR in the pyrenoids of several unicellular green algae by immunocytochemical methods. Similar results were reported by Okabe and Okada (1990) for the marine alga *Bryopsis maxima*. In contrast, Fischer and Klein (1988) demonstrated that NR in *Chlamydomonas reinhardtii* is located in the cytosol.

The work reported in this paper demonstrates that the ability to induce NR in darkness is dependent on the level of N starvation and that NR is located in the cytosol in the halophilic unicellular alga *D. salina*. We suggest that light plays an indirect regulatory role in NO_3^- uptake and NR induction via the induction of NR mRNA.

Material and methods

Algal material and culture conditions. Dunaliella salina strain Gran Canaria was isolated from a brine saltworks (5.5 M total salt concentration) on the island of Gran Canaria, Canary Islands, Spain. The cells were grown in 1-L glass bottles at 28° C, under continuous white light [100 μmol · m^{-2} · s^{-1} photosynthetically active radiation (PAR)] supplied by fluorescent lamps (Philips, Eindhoven, The Netherlands). The different culture media (according to Ramazanov and Cardenas 1992) contained in the N-free medium 2 M NaCl, 2 mM $MgSO_4$, 1.9 mM $MgCl_2$, 0.01 mM $Ca(NO_3)_2$, 4 mM K_2HPO_4. The NO_3^- and NH_4^+ media were obtained by the respective additions of 4 mM KNO_3 and 4 mM $ClNH_4$ to the N-free medium. These media were buffered at pH 7.5 with 20 mM Tris-HCl and sparged with a CO_2:air mixture (5:95, v/v).

Cells were transferred from N-free medium to NO_3^- medium by addition of 4 mM KNO_3 to the M-free medium. Cells were transferred from NH_4^+ medium to NO_3^- medium after washing the cells three times with N-free medium and centrifuging at 500·*g* for 10 min.

Enzyme determination. Nitrate reductase (EC 1.6.6.2) and NIR (EC 1.7.7.1) and activities were measured according to Fischer and Klein (1988). Transferase activity of glutamine synthetase (GS; EC 6.3.1.2) was determined colorimetrically by measuring the glutamylhydroxamate formed at 500 nm (Shaphiro and Stadman 1970). Phosphoenolpyruvate carboxylase activity was measured according to Maeba et al. (1969).

Chloroplast isolation. Exponentially growing cells (1 l) were harvested by centrifugation at 500 · *g* for 10 min at room temperature. Cells were washed with 25 mM Hepes-KOH (pH 7.5) supplemented with 2 M NaCl and the pellet resuspended in 30 ml of ice-cold disruption buffer containing 50 mM Hepes-KOH (pH 7.2), 300 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, and 1% bovine serum albumin (BSA). The algal suspension (2 ml) was withdrawn for marker-enzyme assays. Cells were disrupted according to the procedure of Mason et al. (1991). The lysate was removed at a flow rate of about 0.5 ml · s^{-1}.

After centrifugation of the homogenate at 1000 · *g* for 30 s, the pellet was resuspended in the disruption buffer and was layered onto a 70% and 45% discontinuous Percoll gradient (Percoll in 50 mM Hepes-KOH, pH 8.0, 300 mM sorbitol, 1 mM $Na_4P_2O_7$, 5 mM EDTA, 1 mM $MgCl_2$, and 1% BSA; Goldschmidt-Clermont et al. 1989). Centrifugation was carried, out using 15-ml Corex tubes in a swing-out rotor (JS-13.1; Beckman, Palo Alto, Cal., USA) at 6000 · *g* and 4° C for 20 min. After centrifugation, the chloroplast fraction was collected from the 45–70% interface and diluted four fold with cell-disruption buffer. Chloroplasts were pelleted by allowing the rotor to accelerate to 1000 · *g* and then stopping the run. The pellets were resuspended in 5 ml of appropriate buffer to measure enzyme activities.

Intactness assays. Chloroplast intactness was assayed by following the Hill reaction with ferricyanide as described by Lilley et al. (1975). Oxygen evolution was measured with a Clark-type oxygen electrode (Hansatech Instruments, Kings Lynn, Norfolk, UK). Intact chloroplasts and osmotically shocked chloroplasts, equivalent to 15 μg chlorophyll, were transferred to an assay medium (disruption buffer), to which 1000 U · ml^{-1} catalase, 2.5 mM NH_4Cl, and 2.5 mM $K_3Fe(CN)_6$ were added. Measurements were made under saturating (500 μmol photons · m^{-2} · s^{-1}) light conditions.

Respiration assays. The rate of respiratory oxygen consumption was measured in 1-ml algal samples with a Clark-type oxygen electrode (Hansatech).

Analytical measurements. The protein concentration was estimated according to Bradford (1976). The concentration of NO_2^- was estimated according to Snell and Snell (1949). Chlorophyll was extracted with absolute ethanol and quantified using the absorption coefficient given by Wintermans and De Mots (1965).

Inhibitors. An aliquot of 5 mM of 6-methylpurine (Sigma, St. Louis, Mo., USA) or 1 μg · ml^{-1} of cycloheximide (Sigma) was used as an inhibitor of mRNA or protein synthesis, respectively.

All experiments were repeated three to five times with similar results, and representative results are shown.

Results

Induction of NR in D. salina. It has been reported that the uptake and assimilation of NO_3^- by photoautotrophically grown algae is a strictly light-dependent process, and that darkness or the presence of NH_4^+ repress NO_3^- uptake and NR synthesis (Herrera et al. 1972; Guerrero

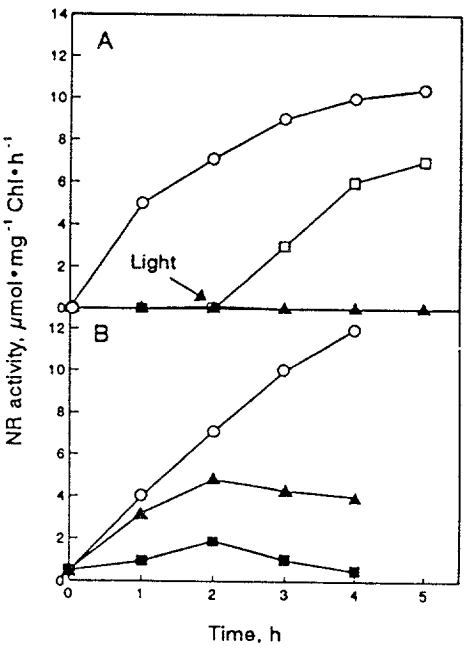

Fig. 1A, B. Effect of growth conditions on NR activities in *Dunaliella salina* precultured in NH_4^+ medium (A) or N-free medium (B). The precultured algae were transferred into NO_3^- medium at time zero, and NR activities were measured in light (○ ●), dark (▲ ▲), light + cycloheximide (□ ■) and dark + cycloheximide (■ ■) at the times indicated. Chlorophyll concentrations were 100 μg · ml^{-1} and the photon flux density 500 μmol · m^{-2} · s^{-1}

Table 1. Effect of N starvation at different iradiances on NO_3^--induced NR activity in *Dunaliella salina* in darkness. Algae previously grown in NH_4^+ medium were transferred into N-free medium at two different photon flux densities, 100 and 500 μmol · m^{-2} · s^{-1}. After various times of N starvation in the light, an aliquot of 4 mM KNO_3 was added to the cells in the dark and NR activity was measured 1 h after addition of KNO_3

Hours of N starvation in light	NR activity in darkness (μmol · mg Chl^{-1} · h^{-1})	
	100 μmol · m^{-2} · s^{-1}	500 μmol · m^{-2} · s^{-1}
0	0	0
2	0	0
4	0	2.2 ± 0.4
6	1.1 ± 0.4	4.7 ± 0.6
8	4.9 ± 1.1	6.9 ± 0.7

et al. 1981; Florencio and Vega 1982; Franco et al. 1987, 1988). Transfer of *D. salina* cells from NH_4^+ to NO_3^- medium increased NR activity in the light, but not in darkness (Fig. 1A). These results are in accordance with strictly light-dependent mechanisms of NR induction (Guerrero et al. 1981; Florencio and Vega 1982). Induction of NR synthesis by transferring cells from NH_4^+ to NO_3^- medium has been described in algae of different taxonomic groups and is a commonly observed phenomenon (Guerrero et al. 1981; Larsson et al. 1985).

A similar increase in NR activity was observed with the addition of NO_3^- to *D. salina* cells grown in N-free medium in light but, in contrast to cells transferred from NH_4^+ to NO_3^- medium, a pronounced increase in NR was also observed in darkness (Fig. 1B). This dark-induced NR activity was inhibited by cycloheximide, providing evidence for de-novo synthesis of NR at the translation level. Light has a strong influence on NO_3^- uptake and the induction of NR in algae (Guerrero et al. 1981; Azuara and Aparicio 1983; Solomonson and Barber 1990) and higher plants (Mohr et al. 1992; Becker et al. 1992). It has been demonstrated that light treatment given before addition of nitrate strongly stimulates NO_3^--induced NR synthesis in darkness in higher plants (Schuster et al. 1987; Mohr et al. 1992).

Table 1 shows the effect on NO_3^--induced NR synthesis in darkness of preculturing *D. salina* cells in N-free medium at different irradiances (in cells previously grown in NH_4^+ medium). In cells which were transferred into N-free medium at 100 μmol · m^{-2} · s^{-1}, the NO_3^--induced NR synthesis in darkness was observed after 8 h of N starvation, while in cells precultured in N-free medium at 500 μmol · m^{-2} · s^{-1}, NO_3^--induced NR synthesis was observed after 4 h. Although NO_3^- uptake was not measured, the increase in NR activity was observed only after the addition of NO_3^-. These results confirm the capacity of *D. salina* cells to take up NO_3^- in darkness and agree with the results described by Dixon and Syrett (1988) for *Amphidium spp.* Thus, our results indicate (as has been shown in higher plants, Cheng et al. 1992) that NO_3^- uptake and NR induction are not strictly light-dependent phenomena and that light plays an indirect role in NR induction.

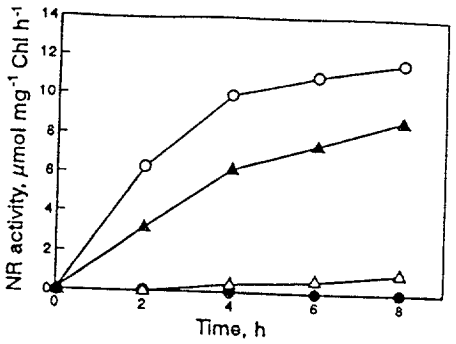

Fig. 2. Effect of 6-methylpurine on NO_3^--induced NR synthesis in *Dunaliella salina* in the light at a photon flux density of 500 μmol · m^{-2} · s^{-1}. NH_4^+-grown cells transferred directly in NO_3^- medium plus 6-methylpurine at time 0 h (△-△); NH_4^+-grown cells transferred into NO_3^- medium plus 6-methylpurine after 4-h preculture in N-free medium (▲-▲); controls, NH_4^+-grown cells transferred to NO_3^- medium (○-○) and to N-free medium (●-●)

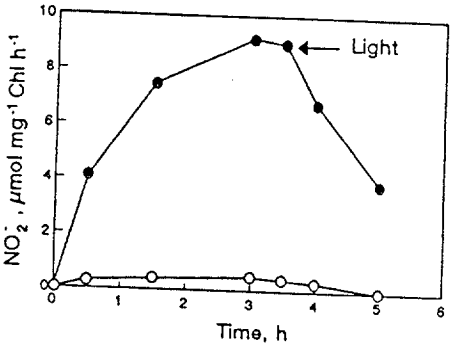

Fig. 3. Effect of NO_3^- on excretion of NO_2^- by *Dunaliella salina* grown in N-free medium. Algae precultured for 24 h in N-free medium were transferred into 5 mM NO_3^- medium in the light (○-○) and in darkness (●-●). The *arrow* shows the time of transfer of algal cells from dark to light. Chlorophyll concentration was 100 μmol · ml^{-1} and the photon flux density 500 μmol · m^{-2} · s^{-1}

Effect of 6-methylpurine on the induction of NR. Wolfner et al. (1975) demonstrated that in yeast, N starvation induced the expression of several genes involved in amino-acid synthesis. In addition, 6-methylpurine, an inhibitor of mRNA synthesis, did not inhibit NO_3^--induced NR synthesis in N-starved *Chlamydomonas reinhardtii* cells (Franco et al. 1988). In our case, the effect of 6-methylpurine on NO_3^--induced NR synthesis was dependent on the time of its addition to cells (Fig. 2). When 6-methylpurine was added simultaneously with the transfer of cells into NO_3^- medium (at time 0 h), NO_3^--dependent NR synthesis was completely inhibited. However, 6-methylpurine did not prevent the NO_3^--induced NR synthesis when added after 4 h culture in N-free medium.

Excretion of NO_2^-, and NR activity in N-starved cells. Figure 3 shows the effect of NO_3^- on NO_2^- excretion in N-starved *D. salina* cells in light and darkness. As can be seen from our results, N-starved cells excreted NO_2^- into the medium when it was supplemented with NO_3^-. The rate of NO_2^- excretion was substantially higher in dark-

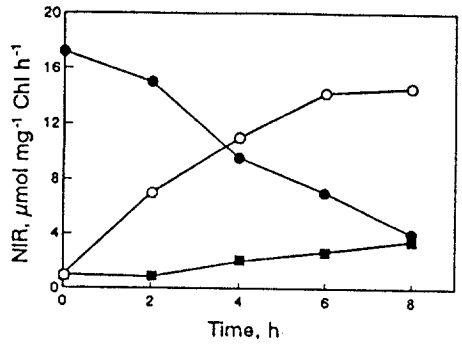

Fig. 4. Effect of NO_3^- and nitrogen starvation on NIR activity in *Dunaliella salina*. Cells grown in NO_3^- medium were transferred (at 0 h) to N-free medium in light (●-●). N-starved cells after NO_3^- addition (at 0 h) in darkness (■-■). N-starved cells after NO_3^- addition (at 0 h) in light (O-O). Photon flux density = 500 µmol · m^{-2} · s^{-1}

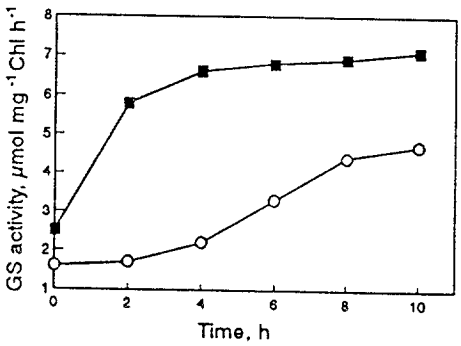

Fig. 5. Effect of photon flux density and N starvation on GS activity in *Dunaliella salina*. Cells were grown in NH_4^+ medium at 100 µmol · m^{-2} · s^{-2} for 24 h and were then transferred at 0 h into N-free medium at 100 µmol · m^{-2} · s^{-1} (O-O) and 500 µmol · m^{-2} · s^{-1} (■-■)

ness than in light. The excretion of NO_2^- decreased rapidly after the dark-grown cells were transferred into the light.

Figure 4 shows the effect of N starvation on NIR activity in *D. salina* cells. The NIR activity decreased during N starvation but the addition of NO_3^- greatly stimulated NIR activity in the light. Our results clearly show that NO_2^- excretion in N-starved *D. salina* is due to low NIR activity in those cells.

Glutamine synthetase activity in N-starved D. salina. Figure 5 shows the effect of N starvation on the total GS activity at two different irradiances. Transfer of cells from NH_4^+ medium to N-free medium was accompained by an increase in GS activity. An increase of GS activity during N starvation has been reported by several authors and is a commonly observed phenomenon in algae (Tischner and Hüttermann 1980; Sumar et al. 1984). However, GS activity increased more rapidly in N-starved cells that were grown at 500 µmol · m^{-2} · s^{-1} than in those grown at 100 µmol · m^{-2} · s^{-1}. The maximal level of GS activity was observed after 4 h at the high irradiance, while in cells grown at low irradiance the maximal GS activity was reached after 7–8 h. It is important to note that the

Table 2. Distribution of enzyme activities in whole cells and intact chloroplasts of *Dunaliella salina* grown in NO_3^- medium

Enzyme	Activity (µmol · mg^{-1} Chl · h^{-1})		Activity in chloroplast (%)
	Whole cells	Intact chloroplast	
Nitrate reductase	6.0 ± 0.1	0.02	0.33
Phosphoendpyruvate carboxylase	25.0 ± 3.9	0.23 ± 0.05	1.2

ability to induce NR synthesis in darkness was observed after 4 or 6 h in light-grown N-starved cells (Table 1) at the same time as the GS activity reached its maximum level.

Localization of NR in D. salina. The distribution of enzyme activities in whole cells and chloroplasts of *D. salina* isolated from NO_3^- medium is shown in Table 2. Chloroplast intactness, as estimated according to Lilley et al. (1975), was 85 ± 5%. The phosphoenolpyruvate carboxylase activity in the chloroplasts was about 1.2% of its total activity in the cell homogenate, which is a good indicator of the low cytosolic contamination of isolated chloroplasts. Although cells grown in NO_3^- medium exhibited relatively high NR activity, only 0.33% of total NR was found in intact chloroplasts. We suggest that this NR activity in the chloroplast fraction is due to cytosolic contamination. Our results show that NR in the *D. salina* cells is located in the cytosol, as reported for *Chlamydomonas reinhardtii* by Fischer and Klein (1988). Tischner et al. (1989) suggested the existence of a plasma-membrane-bound NR involved in NO_3^- uptake in *Chlorella sorokiniana*. However, this plasmalemma-bound NR represents only 0.8% of the total NR activity and has been suggested to be involved in NO_3^- transport into the cell.

Discussion

The ability to induce NR in darkness is strongly dependent on previous growth conditions. When precultured in N-free medium, *D. salina* cells had the ability to induce NR in darkness (Fig. 1, Table 1). In contrast, NH_4^+-grown cells induced NR only in light, which is typical also for many other algae (Guerrero et al. 1981; Larsson et al. 1985). Dixon and Syrett (1988) reported that N-starved cells of *Amphidium spp.* assimilated NO_3^- in darkness. It has been shown that NO_3^- and light act independently of each other in controlling the appearance of NR in higher plants (Rajasekhar and Mohr 1986). A possible explanation for the different capacities for NR induction in darkness in N-starved and N-sufficient *D. salina* cells could be that NO_3^- permease and NR mRNA are induced in both light and darkness in N-starved cells, while in NH_4^+-grown cells NR mRNA is induced only in light. It is well established that ammonium acts as a repressor for NR induction (Franco et al. 1987, 1988). Thus, our

results indicate that the mechanism of NO_3^- uptake and NR induction is more than just strictly light dependent, and must be regulated indirectly through the induction of NR mRNA and NO_3^--transporter mRNA. The effects of two inhibitors: cycloheximide and 6-methylpurine on NO_3^- induced NR synthesis in N-starved cells were completely different. Cycloheximide inhibited NO_3^--induced NR synthesis in darkness (Fig. 1), while 6-methylpurine did not (Fig. 2). It is probable that, during N starvation in *D. salina*, cells accumulate mRNA for NR and mRNA for NO_3^--transporter protein, and that NO_3^- plays a triggering role in NR induction. When 6-methylpurine was added to N-starved cells possessing NR mRNA, inhibition of NR synthesis was not observed (Fig. 2). Nitrogen starvation greatly stimulates GS activity and suppresses NIR activity. The ability to induce NR in darkness was observed when GS activity reached maximal levels during N starvation. When cells were precultured in N-free medium, at high photon flux density ($500\ \mu mol \cdot m^{-2} \cdot s^{-1}$) the maximal level of GS activity was observed after 4 h, giving those cells the ability to induce NR in darkness. Probably, the ability to induce NR in darkness is dependent on the level of N starvation, and light plays an important role in the induction of NR mRNA. It has been proposed that mitochondrial respiration is involved in dark NO_3^- assimilation in N-limited *D. tertiolecta* and *Selenastrum minutum* cultures (Weger and Turpin 1989; Venderberghe et al. 1992). Addition of NO_3^- to N-limited algae was found to greatly stimulate dark respiration (Turpin et al. 1988). Nitrogen-limited algal cells show a dramatic activation of respiratory carbon flow which supports amino-acid synthesis during assimilation of nitrate (Weger and Turpin 1989). In our case, the rate of respiration was similar in both NH_4^+- and N-starved cells, and either the addition of NO_3^- to N-starved cells did not affect the rate of respiration or this stimulation was not appreciable (data not shown). In spite of this, preculturing *D. salina* cells for 4 h in N-free medium markedly changed their physiological characteristics and was sufficient to induce NR in darkness (Fig. 1, Table 1). In contrast, when NH_4^+-cultured cells were transferred to NO_3^- medium in darkness, NR induction was not observed (Fig. 1). In spite of the fact that glutamine has been shown to decrease NR activity in spinach cells (Shiraishi et al. 1992), its intracellular level is low and changes little in algal cells (Syrett and Peplinska 1988). Therefore, we suggest that the contradictory result shown for *Dunaliella* is due to the low NIR activity in N-starved cells (Fig. 4), as NO_2^- was not assimilated by those cells but excreted into the medium (Fig. 3). The addition of NO_3^- substantially stimulated the NIR activity in light (Fig. 4). Similar results have been described for N-starved *Chlamydomonas reinhardtii* (Galvan et al. 1992). Therefore, in darkness the rate of NO_2^- assimilation in N-starved cells should be very low due to low NIR activity. We assume that NIR activity and the rate of NO_2^- assimilation in N-limited *S. minutum* (Turpin et al. 1988) were higher than in our N-starved *D. salina* cells. Nitrogen assimilation requires a high concentration of energy donors (ATP, NADPH and NADH) for nitrate transport, NR and NIR activities, and carbon skeletons for NH_4^+ fixation. In addition, Miyachi and Miyachi (1985) demonstrated that NH_4^+ induces starch degradation in algae.

Localization of NR in D. salina. López-Ruiz et al. (1985) reported that NR was located in the pyrenoid of *D. salina* cells. They used antiserum prepared against NR of the green alga *Monoraphidium braunii* to localize this enzyme. Our results clearly show that NR in *D. salina* cells is located in the cytosol (Table 2). This contradictory result may be due to fact that the NR antisera used in the immunocytochemical investigations by Lopez-Ruiz et al. (1985) were able to recognize another chloroplastic enzyme, i.e. glyceraldehyde-3-phosphate dehydrogenase (Gowri and Campbell 1989). After raising a polyclonal antibody against maize leaf NR, Gowri and Campbell (1989) selected a clone containing chloroplast glyceraldehyde-3-phosphate dehydrogenase cDNA, which further prompted a re-examination of immunocytochemical results concerning the location of NR. Recently, Okabe and Okada (1990) reported that the native, starchless pyrenoids purified from *Bryopsis maxima* showed NADH-NR activity. However, the extent of contamination of the isolated pyrenoid fraction by the cytosolic enzymes was not presented in this work.

An intrapyrenoid location of NR would require at least two permeases for NO_3^- uptake: the first one in the plasmalemma and the second in the chloroplast envelope. A plasmalemma-bound NR has been reported in algae (Tischner et al. 1989) and higher plants (Ward et al. 1988) but, at present, there is no evidence for a chloroplastic envelope-bound NR permease. Additionally, analysis of S_{35}-labeled NO_3^--induced protein shows that at least four polypeptides are induced in *D. salina* cells transferred from NH_4^+ medium to NO_3^- medium (data not shown). However, further studies are needed to identify and localize all of the NO_3^--induced proteins in order to elucidate the mechanism of NR induction and the functional role of light in NO_3^- uptake.

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Photosynthetic performance of healthy and virus-infected *Feldmannia irregularis* and *F. simplex* (Phaeophyceae)

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The filamentous brown algae *Feldmannia simplex* and *F. irregularis* are attacked by aquatic viruses, which propagate in deformed sporangia of the host plants. In order to evaluate a possible detrimental effect of the pathogen, photosynthesis–irradiance response curves and pigment concentrations of healthy and infected plants were compared. Photosynthetic performance of infected plants was significantly reduced and associated with a decrease in chlorophyll *a* and *c* content. In *Feldmannia irregularis*, which had a relatively low photosynthetic capacity, the virus infection produced a more severe effect than in *F. simplex*.

Key words: *Feldmannia*, pathology, Phaeophyceae, photosynthesis, virus infection.

Introduction

Filamentous brown algae of the order Ectocarpales have frequently been found to contain virus-like particles (Van Etten *et al.*, 1991). Beginning in 1988 (Müller *et al.*, 1990), virus-infected and healthy field specimens of *Ectocarpus siliculosus* (Dillwyn) Lyngbye were isolated and grown in well-defined laboratory cultures. *In vitro* infection experiments with *Ectocarpus siliculosus*, *Feldmannia simplex* (P. et H. Crouan) Hamel and *Feldmannia irregularis* (Kützing) Hamel established the pathogenic character of the viruses affecting these hosts (Müller & Frenzer, 1993). The genome of the *Ectocarpus* virus consists of double-stranded DNA (Lanka *et al.*, 1993). Infection sites are restricted to the unicellular motile zoids of the hosts, and experimental evidence points to an intimate association between virus and host genomes (Müller, 1991). During the development of a plant from an infected spore or gamete the virus genome is transmitted via mitosis to every cell of the host. However, virus symptoms do not appear until an adult plant begins to reproduce. Instead of functional zoidangia, homologous structures are produced, which are diverted to the formation of virus particles.

Parallel treatment of many healthy and infected host cultures under identical growth conditions revealed significant differences in their vitality. In both *Feldmannia* species, infected plants tend to grow more slowly than their healthy counterparts. In contrast, many infected gametophytes of *Ectocarpus siliculosus* show the same or even superior growth performance compared with uninfected parallel cultures. Since the reproductive potential of infected host plants is suppressed, differences in the vitality of the somatic thalli are a crucial factor for epidemiological dynamics in field populations.

The reduction of metabolic rates, including photosynthesis, as a result of virus infection in higher plants is well documented (Matthews, 1991), and some reports exist for cyanobacteria (Misra *et al.*, 1982) and eukaryotic phytoplankters (Suttle *et al.*, 1990). In macroalgae there is a general lack of knowledge of the effect of viral infection on host metabolism.

In this study we evaluated whether reduced growth in virus-infected plants of *Feldmannia* is associated with reduced photosynthetic performance caused by the virus genome in the host cells.

Materials and methods

Plant material

All cultures used in this study were unialgal and clonal. *Feldmannia simplex* was isolated from the west coast of Ireland (Müller & Stache, 1992). The following clones were used: (1) Flex 12 n, healthy, normal plant with pluri- and unilocular sporangia; (2) Flex 12 n-v, same isolate, experimentally infected with *F. simplex* virus, showing full virus expression; (3) Flex 12 n-v-n, normal, symptomless and fully fertile subisolate of Flex 12 n-v. *Feldmannia irregularis* was isolated from the Canary Islands (Müller & Frenzer, 1993). The following clones were used: (1) Fir 28 n from *Codium fragile* (Suringar) Hariot, Fuerteventura Island, healthy, normal plant with plurilocular zoidangia; (2) Fir 03 v, sterile isolate from *Cystoseira humilis* (Schousboe) Kützing, Hierro Island, with full virus expression (lateral vesicles forming virus particles).

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Cultures were grown in glass dishes with 50 ml culture medium (PES; natural seawater enriched according to Starr & Zeikus, 1987) at 12°C and illuminated with white fluorescent lamps (Osram daylight L36W/10) at 10 μmol photons $m^{-2} s^{-1}$ for 14 h per day. After transport from Konstanz to Las Palmas, 2 days before the experiments, the cultures were subjected to continuous white fluorescent light at a photon irradiance of 5·5 μmol $m^{-2} s^{-1}$ at 15°C. Nutrient, temperature and irradiance histories were the same for all clones used in the photosynthesis experiments.

Photosynthetic measurements

Photosynthetic oxygen evolution was measured at 15°C in a water-jacketed chamber, using a Clark-type oxygen electrode and a computer interface control circuit (Model CB1/IF-1; Hansatech, Norfolk, UK). Light was provided by a computer-controlled set of two red-light-emitting diode arrays (LH-7, 660 nm; Hansatech); light intensities provided by these light-emitting diodes were measured with a cosine-corrected sensor (Model LI-192SA; Li-Cor).

Samples of the algae (2–5 mg fresh weight) were placed into a water chamber which was modified to avoid entanglement of the filaments by the stirrer. Incubation water was replaced with 2·5 ml of fresh enriched seawater medium before introduction of new samples to avoid depletion of nutrients. After 5 min equilibration in darkness, the algal filaments were exposed to a series of 12 programmed incremental photon flux densities (0 to $\approx$ 800 μmol $m^{-2} s^{-1}$ photosynthetically active radiation). Data were continuously monitored for oxygen evolution rate calculation in real time using a microcomputer program (LeafDisc, Hansatech). Total exposure time at each photon flux density was 1·5 min, and rates were determined over the last 50 s.

Four to seven replicates of each clone were measured to determine independently each point on the photosynthesis–irradiance (P–I) curves. Measurements were done alternately between healthy and infected samples of the same species to minimise errors. The maximum rate of photosynthesis (P_{max}) was calculated as the average of values at saturation irradiance. Slopes of the P–I curves (α) were calculated as the average of values (four to seven replicates) derived by linear regression based on the linear portion (the first five points) of each curve. This parameter was used to measure the efficiency of photosynthesis since it is a function of photochemical reactions (Dunstand, 1973). Dark respiration (R_d) was taken as the intercept of this regression. The compensation point (I_c) was estimated from $I_c = R_d/\alpha$.

Chlorophyll determination

Chlorophylls were extracted from the algal tissue (50–100 mg fresh weight) with absolute methanol for 3 h at room temperature following the method of Duncan & Harrison (1982). In some cases the methanol treatment was repeated if the colour of the tissue indicated that the extraction was not complete. Chlorophyll *a* and *c* concentrations were calculated from absorbance at 668 and 635 nm (both corrected for scattering by subtracting absorbance at 700 nm) using the equations of Jeffrey & Haxo (1968).

Electron microscopy

Cultured *F. irregularis* was fixed for 2 h on ice with a solution of 30 g dm^{-3} glutaraldehyde in $\frac{1}{4}$ strength seawater which contained 2·5 g dm^{-3} caffeine and 0·05 M sodium cacodylate at pH 7·7. Washing in $\frac{1}{2}$ strength seawater with 5 g dm^{-3} caffeine and 0·1 M sodium cacodylate was followed by post-fixation in 10 g dm^{-3} osmium tetroxide. The material was washed in distilled water, dehydrated stepwise in acetone and embedded in Spurr's resin. Sections were stained with 20 g dm^{-3} aqueous uranyl acetate followed by 5 g dm^{-3} lead citrate in 0·1 N sodium hydroxide. Observations were made on a Zeiss EM9S instrument.

Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA), to evaluate the differences between healthy and virus-infected plants. Statistical pairwise comparisons were performed with values for photosynthesis maxima, dark respiration and the initial slope of each curve, and for chlorophyll content. Because the compensation point (I_c) is derived from R_d and α, which were compared statistically, comparisons between I_c were omitted. Individual values are presented for reference only.

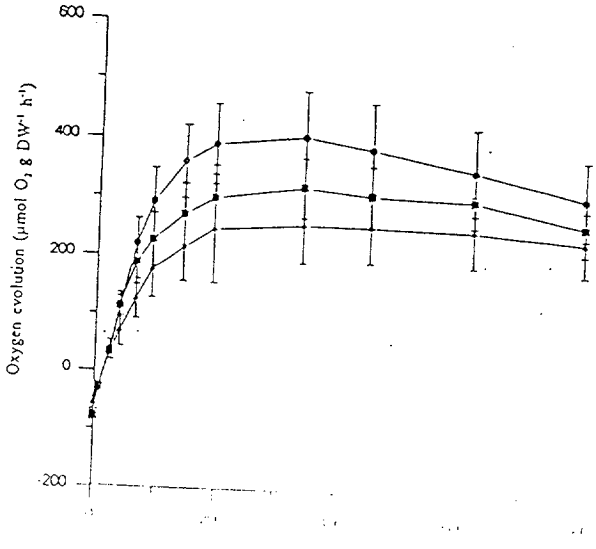

FIG. 1. Photosynthesis–irradiance curves for *Feldmannia simplex* (Crouan) Hamel, Flex 12 n, healthy plants, (n = 6); squares, Flex 12 v, virus-infected symptomless plants, (n = 4); triangles, Flex 12 v, virus-infected plants showing symptoms, (n = 6). Vertical bars represent SE of mean. PFD, photon flux density; DW, dry weight

Table 1. Photosynthesis–irradiance data for healthy and virus-infected clones of two *Feldmannia* species

Clone	P_{max}	R_d	α	I_c
Feldmannia simplex				
Flex 12 n (normal)	321·5 (194·7)	−72·0 (21·6)	4·536 (1·44)	16·15 (3·06)
Flex 12 n-v-n (virus-infected symptomless)	300·9 (111·6)	−68·4 (18·0)	4·104 (1·08)	16·84 (2·7)
Flex 12 n-v (with virus symptoms)	248·4 (126·0)	−49·32 (11·88)	2·808 (1·08)	19·48 (5·25)
Feldmannia irregularis				
Firr 28 n (normal)	250·2*** (51·12)	−49·32 (8·64)	3·06** (0·54)	16·38 (2·61)
Firr 63 v (with virus symptoms)	78·48*** (30·60)	−50·76 (36·00)	1·404** (0·72)	33·72 (7·07)

P_{max}, net photosynthesis at saturation irradiance (μmol O_2 g^{-1} dry weight h^{-1}); R_d, dark respiration (μmol O_2 g^{-1} dry weight h^{-1}); α, slope of the curve (μmol O_2 g^{-1} dry weight h^{-1} (μmol m^{-2} s^{-1}))$^{-1}$; I_c, compensation point (μmol m^{-2} s^{-1}). SD in brackets.
**$p < 0.01$; ***$p < 0.001$ (ANOVA).

Results

Feldmannia simplex

The healthy clone Flex 12 n showed a non-significant decrease in photosynthesis at irradiances higher than 350 μmol m^{-2} s^{-1}, while the symptomless Flex 12 n-v-n showed a slight decrease at 620 μmol m^{-2} s^{-1} (Fig. 1). No decrease was observed for Flex 12 n-v, the clone with virus symptoms. All the estimated parameters (R_d, P_{max}, α) for the P–I curves were lower for the Flex 12 n-v clone than for healthy or asymptomatic clones, although in pairwise comparisons the differences in these parameters were not significant (Table 1). Compensation points (I_c) differed little between the infected asymptomatic clone and the clone which displayed symptoms (Table 1). However, the chlorophyll *a* content was significantly higher ($p < 0.01$) in Flex 12 n than in Flex 12 n-v (Tables 2, 3). Chlorophyll content decreased relative to the healthy clone in the clone with apparent symptoms (Table 2). No significant differences were detected in chlorophyll *c* content and the ratios of chlorophyll *c* to *a* between all the clones (Tables 2, 3).

Feldmannia irregularis

No sign of photoinhibition was detected in either of the *F. irregularis* clones, even at the highest irradiance tested. There was a reduction in the photosynthetic rate in the clone with virus symptoms, Firr 63 v, when compared with the healthy one, Firr 28 n (Fig. 2). The difference in P_{max} observed in healthy and virus-infected *F. irregularis* was highly significant ($p < 0.001$). The initial slope of the curve was significantly lower ($p < 0.01$) (Table 1). There were no significant differences in dark respiration between clones. This species was photosynthetically less efficient than *F. simplex*.

Pigment content differed significantly between the two clones for both chlorophylls *a* and *c* ($p < 0.01$; Tables 2, 3). The chlorophyll *c* to chlorophyll *a* ratio was higher in Firr 28 n (0·47) than in Firr 63 v (0·37).

Infected *Feldmannia irregularis* plants showed severe deterioration of chloroplast structure in those cells where virus particles are formed, as can be seen in Fig. 3.

Discussion

The physiological effects of viral infection on the two *Feldmannia* species studied here included a decreased photosynthetic performance associated with a decrease in photosynthetic pigments, and a slight decrease in respiratory rates. Similar pathological reactions have been described in infected higher plants where photosynthetic activity in infected plants was impaired by changes in chloroplast structure, by reduced content of photosynthetic pigments, or by reduced content of specific proteins associated with photosystem II (van Kooten *et al.*, 1990).

Table 2. Pigment content of the experimental healthy and virus-infected clones ($n = 3$; SD indicated)

Clone	Chl*a*	Chl*c*	Chl*c*/Chl*a*
Feldmannia simplex			
Flex 12 n (normal)	332 ± 15	[illegible]	
Flex 12 n-v-n (virus-infected symptomless)	272 ± 60	[illegible]	
Flex 12 n-v (with virus symptoms)	193 ± 26		
Feldmannia irregularis			
Firr 28 n (normal)	398 ± 21		0·47 ± 0·02
Firr 63 v (with virus symptoms)	303 ± 13		0·37 ± 0·04

Chlorophyll concentration given as μg g^{-1} fresh weight. Chl*a* chlorophyll *a*; Chl*c*, chlorophyll *c*

Table 3. The level of significance of comparison of the content of chlorophyll *c* (Chl*c*), below diagonal, and chlorophyll *a* (Chl*a*), above diagonal, between different healthy and infected clones of *Feldmannia* species following one-way ANOVA

	F. simplex				*F. irregularis*	
Chl*a* / Chl*c*	Flex 12 n	Flex 12 n-v-n	Flex12 n-v	Chl*a* / Chl*c*	Firr 28 n	Firr 63 v
Flex 12 n		NS	**	Firr 28 n		**
Flex 12 n-v-n	NS		NS	Firr 63 v	**	
Flex 12 n-v	NS	NS				

See Table 2 for infection status of clones.
** $p < 0{\cdot}01$; NS, Not significant, $p > 0{\cdot}05$.

Photosynthetic rate was reduced in both virus-infected *Feldmannia irregularis* and *Feldmannia simplex*. The greater decrease in photosynthesis of infected *F. irregularis* plants (Fig. 1) may reflect a more virulent character of this particular virus. The difference in the initial photosynthesis–irradiance slope, α, between healthy and infected clones may be attributed to the reduction in pigment content that resulted in large changes in the light absorption properties of the thalli, and is in accordance with the interpretation of α in previous studies of photosynthesis in relation to pigment content (Ramus, 1981). The reduction in the amount of chlorophylls could have resulted from an inhibition of chloroplast development and chlorophyll biosynthesis, or from the destruction of chloroplasts in the virus-forming areas of the plant (Fig. 3).

For a long period, plant pathology has been concerned primarily with the physiology and molecular biology of pathogens and too little with the pathogenic effects on the host. In this study we describe the effects of virus infection on the fitness of a multicellular algal host, measured by its photosynthetic performance. Our results demonstrate that chloroplast disruption (Fig. 3) and reduction in chlorophyll content (Table 2) are consequences of virus infections in seaweeds, confirming their pathological character. The stunted growth of virus-infected plants might be a consequence of the decreased photosynthetic capacity caused by the infection. Although the differences in photosynthetic performance of the host plants examined in this study can be correlated with the presence of virus infections, the biochemical interactions between virus and host, and their consequences, remain to be established.

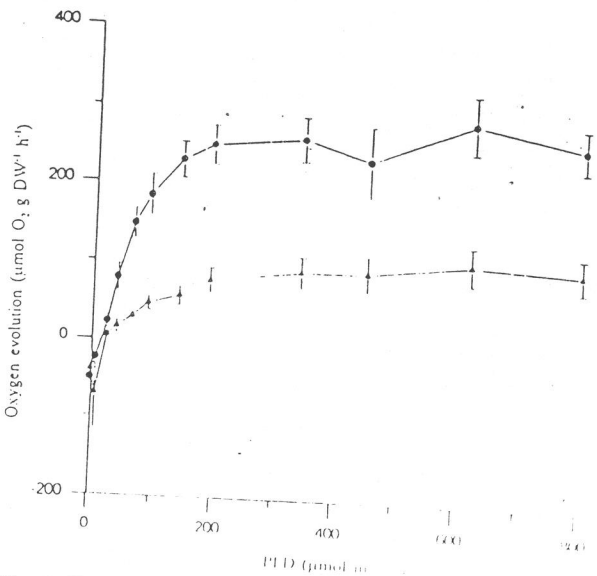

Fig. 2. Photosynthesis–irradiance curves for *Feldmannia irregularis* clones. Circles, Firr 28 n, healthy plants (o——o); triangles, Firr 63 v, virus-infected plants showing symptoms (▲——▲). Vertical bars represent SE of mean. PFD, photon flux density; DW, dry weight.

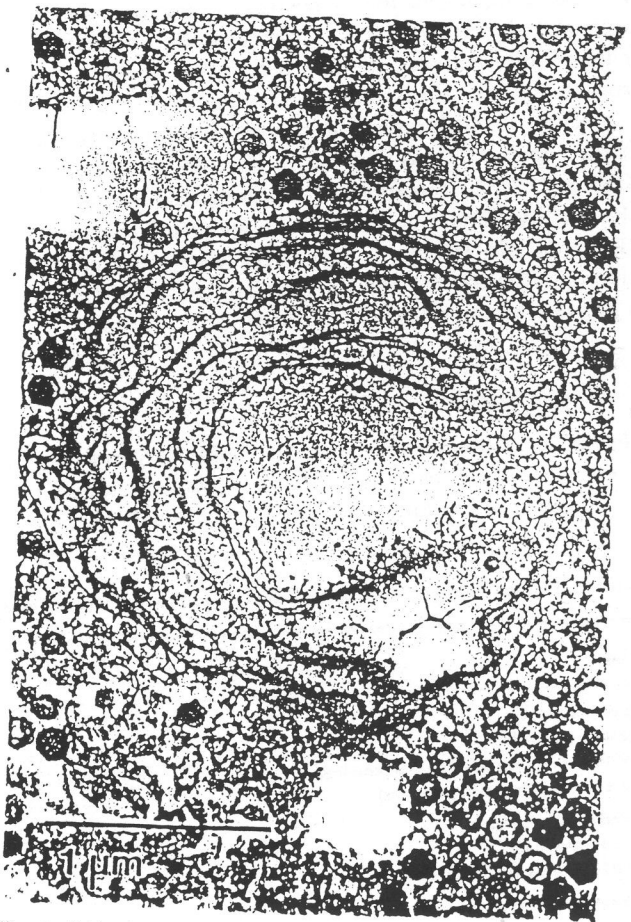

Fig. 3. *Feldmannia irregularis*. Cross section of a lateral vesicle of an infected plant, showing a rudimentary chloroplast, together with partly and fully assembled virus particles.

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$$1 = (1/z - 1)^{-1/B_1}\frac{C_1}{IC_{50_1}}[1 + (PI_2C_2)^{BP_2}]^{-1}$$

$$+ (1/z - 1)^{-1/B_2}\frac{C_2}{IC_{50_2}}[1 + (PI_1C_1)^{BP_1}]^{-1}$$

where $z = (a - y)/(a - d)$ is the normalized effect. In the equation for z, y is the measured p24 level in natural units, a is the level in the absence of drug, and d is the level at indefinitely high drug concentrations. The variable y defines a surface over C_1 and C_2, which represent the concentrations of the two drugs. IC_{50_1} and IC_{50_2} are the 50% inhibitory concentrations of the two drugs used separately; B_1 and B_2 are the corresponding 50%-effect slopes; BP_1 and BP_2 are slope parameters for the potentiating effects; PI_1 and PI_2 are potentiation indices for drug 1 acting on drug 2, and drug 2 acting on drug 1, respectively. For the present data, it sufficed to set $BP_1 = BP_2 = 1$ and $PI_1 = 0$. Weights for the fitting procedure were determined from the error structure of the data set itself with a Gaussian kernel windowing technique based on estimated responses.

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Isolation of Virus Capable of Lysing the Brown Tide Microalga, *Aureococcus anophagefferens*

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Viruses have been hypothesized to control blooms of *Aureococcus anophagefferens* gen. et sp. nov. (Chrysophyceae), a marine phytoplankton that since 1985 has caused devastating summer blooms called "brown tide." By means of ultrafiltration methods, viruses specific to this alga were isolated from both the Great South Bay and Peconic Bay systems of Long Island, New York, during the summer bloom period of 1992. Cell lysis of healthy algal cultures was demonstrated, as well as continuing reinfection with serial transfers of cultures. Electron microscope surveys yielded images of phage-like virus particles with tails that could attach to *A. anophagefferens* cells within minutes of exposure. The isolation and cultivation of this virus highlights the need for further study of viral infection of eukaryotic algae and the potential for a better understanding of algal bloom control by viral infection.

Brown tide blooms were first documented in 1985, in Narragansett Bay, Rhode Island, in Barnegat Bay, New Jersey, and in the Peconic Bay and Great South Bay systems of Long Island, New York. These bays experienced practically simultaneous algal blooms of the eukaryotic microalga, *Aureococcus anophagefferens* gen. et sp. nov. (Chrysophyceae) (*1*). The blooms colored the water a deep, golden-brown (*2*), drastically reduced light through the water column, and caused widespread death of eelgrass, *Zostera marina* (*3*). The bloom also devastated populations of *Argopecten irradians irradians* (bay scallop) and *Mytilus edulis* (blue mussel) by apparently causing starvation and total loss of larval recruitment (*4*, *5*). This resulted in severe monetary losses to local shellfishermen, especially the bay scallop industry in Peconic Bay, New York (*4*).

Massive "brown tide" blooms reappeared in Long Island bays in 1986 and, as in 1985, remained throughout the summer. Since then these blooms have recurred sporadically in isolated Long Island embayments, but have never returned to Narragansett Bay (*6*, *7*). Major environmental factors that have been found to contribute to these blooms include elevated salinities from drought conditions (*2*), elevation of organic compounds or micronutrients in bay waters from runoff (*7*, *8*), reduced grazing (*7*), and restricted circulation of bay waters (*9*).

Some cells observed during the original 1985 transmission electron microscope (TEM) survey from Rhode Island bloom water contained virus-like particles (VLPs) 130 to 150 nm in diameter, and the existence of a virus specific for *A. anophagefferens* was hypothesized to explain sudden decreases in algal populations (*1*). We therefore decided to isolate and cultivate these viruses.

At two Long Island bloom sites [West Neck Bay (WNB) in the Peconic Bay system and Blue Point (BP) in Great South Bay], a seawater sample of 20 liters was collected at the beginning of the bloom, on 6 July 1992 at WNB and on 7 July 1992 at BP. Sampling was repeated on 14 July 1992 at WNB. Each sample was filtered through a 0.2-μm Gelman Sciences capsule filter. The sample was then concentrated according to the methods of Suttle *et al.* (*10*), except that the lower filtration cutoff was 10,000 molecular weight (MW) instead of 30,000 MW to obtain a 200-ml concentrate that could contain potentially infective viruses. The ultrafiltrate (<10,000 MW) was also collected. Concentrate and ultrafiltrate were filter-sterilized through a 0.2-μm Nuclepore filter, transferred into sterile flasks, and stored at 5°C in the dark.

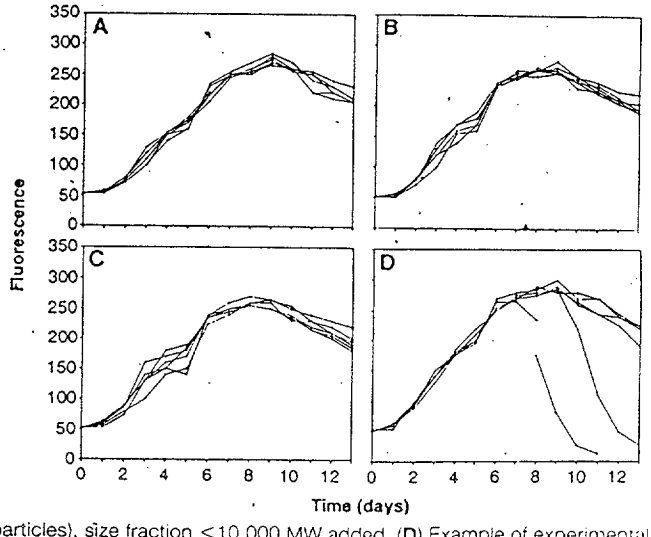

Fig. 1. Example of growth curves of *A. anophagefferens* in culture, testing for potential infectivity by viral concentrate from WNB 7/14/92 seawater. In each treatment, symbols (●) represent six replicate tubes, each containing 6 ml [some symbols overlap in (A), (B), (C), and (D)]. Treatment and control inocula were added on day 0 to healthy cultures. (**A**) Control, no virus added. (**B**) Control, 240 μl (4% of 6 ml) of microwaved (with inactivated viruses) concentrate added. (**C**) Control, 240 μl (4%) of ultrafiltrate (without viral particles), size fraction <10,000 MW added. (**D**) Example of experimental treatment, 360 μl (6%) of viral fraction concentrate added, with two infected replicates. Broken line represents inoculum extracted for serial transfer to healthy culture. Other experimental concentrations not shown were 120 μl (2%) and 240 μl (4%).

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Aureococcus anophagefferens was grown in Aquil media (*11*) to a cell concentration of approximately 4.5×10^5 cells per milliliter as determined by hemacytometer counts. The culture was divided among six replicate test tubes (6-ml volume) for each treatment. Treatments consisted of additions of various volumes of concentrate (viral fraction) and controls, to test for adverse effects from causes other than viral infection (Fig. 1).

Growth of *A. anophagefferens* in the tubes was monitored as in vivo fluorescence yield of cell chlorophyll a through the use of a Turner Designs fluorometer (Mountain View, California). When fluorescence reflected a sudden decrease of growth in experimental tubes relative to other replicates or to the control groups, a viral infection was suspected. Microscopic observations of infected cultures revealed only cellular debris and the absence of intact cells, indicating lysing of cells. Samples from lysed cultures were filter-sterilized through a 0.22-μm Millipore-GV filter, inoculated into fresh cultures of *A. anophagefferens*, and monitored for growth. Serial transfers of lysate were performed 10 times to amplify viruses that specifically attack *A. anophagefferens*.

There was a quantitative progression of infection in terms of percent viral concentrate from WNB 7/14/92. Two replicates of six showed infection in the 6% concentrate treatment (Fig. 1), only one replicate showed infection from the 4% concentrate, and no infection was observed from the 2% concentrate (*12*). Serial transfers from cultures that showed signs of lysis into healthy cultures consistently resulted in 100% reinfection (Fig. 2).

It was also possible to transfer the infection to large culture volumes of 600 ml with inoculum of 50 ml of lysate. Within 4 days after inoculation, the infected culture demonstrated a total loss of color, whereas there was continued growth in control cultures that had no inoculum added. Microscopic surveys again indicated cell lysis in the infected culture (*12*).

Samples from earlier bloom dates (WNB 7/6/92 and BP 7/7/92) showed infection in all concentrations (2 to 6%) and replicates of experimental treatments, suggesting that viral infectivity was greater at the peak of the bloom and demonstrating that viral activity was present in both the Great South Bay and Peconic Bay systems (*12*).

To evaluate whether isolated virus was DNA-based, we filtered samples through a 0.22-μm Millipore-GV filter and stained them with DNA-specific dye, 4-6-midino-2-phenylindole (DAPI), at a c tion of 0.1 ml of DAPI:1 ml of sample (*14*). Virus particles appeared as pinpoint blue under epifluorescence microscopy, dicating that the isolated virus conta DNA.

In electron microscope surveys of un fected cultures, *A. anophagefferens* appea as a small, round cell about 2.5 μm diameter, similar to previous studies (*1* Both uninfected and infected cultures w examined by transmission electron micr copy of uranyl acetate–stained samples three separate occasions. In infected but r uninfected cultures, virus particles we found that resemble phages with a polyg nal head approximately 50 to 70 nm (n 25) in diameter and attached tail, 80 to 1(nm ($n = 25$; Fig. 3A). Culture exposed f only 10 min to viral inoculum showed tai of the virus particles attached to the ce with the heads pointed away (Fig. 3B Culture exposed for approximately 45 mi to viral inoculum showed no intact c and some virus particles. The staining pro cedure resulted in clumping of cellular ma terial, so that quantitative enumeration c viral particles was not possible.

The potential for other species to be come infected by this virus was evaluated Inocula of 360 μl of lysed culture of A *anophagefferens* were filtered through 0.22 μm Millipore-GV filters and added to six replicate tubes for each unialgal species in exponential growth phase: *Thalassiosira weissflogii*, *Thalassiosira pseudonana*–clone 3H, *Nannochloris* sp., *Isochrysis* sp., and *A. anophagefferens*. Growth was monitored by in vivo fluorescence of cell chlorophyll a every 24 hours for a 2-week period. Of the species tested, *A. anophagefferens* was the only species in which cultures were lysed, indicating that the virus lysing *A. anophagefferens* would not infect the other species (*12*).

Only recently have viruses been described as having the potential to be significant factors in marine ecosystem dynamics (*16*). Virus-induced lysis was first established as a cause of mortality in microbial communities and is now being established as a significant factor in algal population control and as a contributor to dissolved organic levels in the marine system (*17*). Viral infections or the presence of VLPs have been identified in nine algal classes (*18*); however, most reports are incidental results of observations made during performance of general ultrastructural surveys of algal populations, and the virus was not isolated for cultivation and further study (*19*). Little is known about the significance of marine algal viruses in terms of their diversity, effectiveness as pathogens, and possible host ranges (*20*).

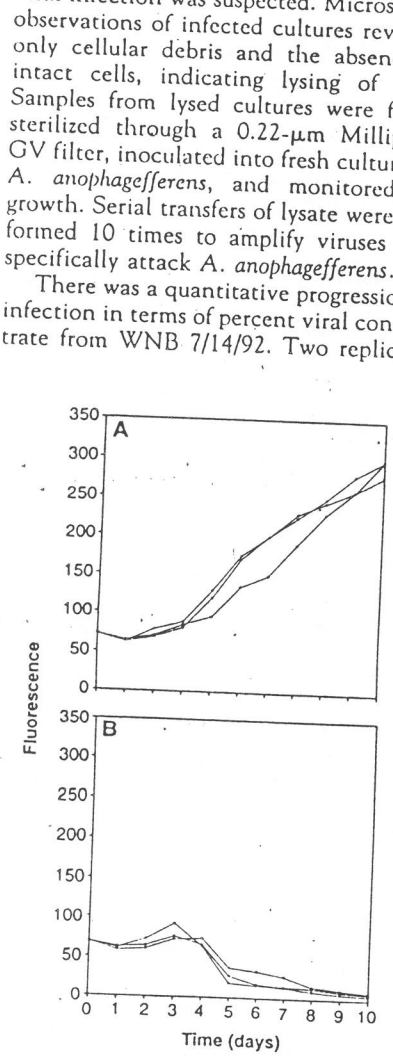

Fig. 2. Representative growth curves of cultures of *A. anophagefferens* after serial transfer of lysed cultures. Symbols (●) represent three replicate tubes, each containing 6 ml. Inocula were added on day 0 to healthy cultures. (**A**) Control, no lysate added. (**B**) Addition of 360 μl of inoculum from infected culture lysate from replicate in Fig. 1D.

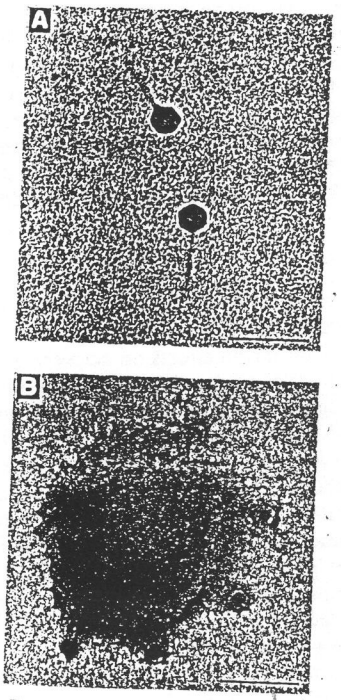

Fig. 3. Phage-like virus found in lysate and attached to *A. anophagefferens*. Samples were stained with 1% uranyl acetate and observed with TEM. (**A**) Example of free virus from lysed algal culture. Heads, 50 to 70 nm in diameter; tails, 80 to 100 nm in length (original magnification, ×60,000). Scale bar, 100 nm. (**B**) *Aureococcus anophagefferens* (cell size not representative because shrinkage occurred during staining) exposed to virus for 10 min (original magnification, ×40,000). Scale bar, 200 nm.

Although the phage-like appearance of the virus was surprising, marine viruses are highly diverse, and morphologies traditionally characteristic of phages cannot be assumed to infect only prokaryotes (*20*, *21*). Our experimental work indicates that the observed virus is the lysing agent of *A. anophagefferens*.

For *A. anophagefferens*, this study has established that viral control is an important consideration to be evaluated relative to the recurrence of blooms and their sudden dissipation. Viral lysis of *A. anophagefferens* during bloom periods may also release cellular products into the marine environment. This release would increase the concentration of dissolved and particulate organic matter (*22*) and possibly dimethylsulfide and acrylic acid (*23*–*26*), chemical compounds that might be associated with the chronic toxicity of bivalve starvation.

Another consideration in the population dynamics of *A. anophagefferens* is the inactivation of virus by environmental factors, thus allowing for blooms. For example, high concentrations of iron promote "brown tide" blooms in Long Island bays by stimulating growth of *A. anophagefferens* (*8*). Solid clay minerals and silts, iron oxides, and metal coagulants temporarily bind free virus particles (*27*–*29*), possibly precipitating and removing the potential for viral infection from the water column and effectively removing viral control on population densities.

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p53 Status and the Efficacy of Cancer Therapy in Vivo

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The therapeutic responsiveness of genetically defined tumors expressing or devoid of the *p53* tumor suppressor gene was compared in immunocompromised mice. Tumors expressing the *p53* gene contained a high proportion of apoptotic cells and typically regressed after treatment with gamma radiation or adriamycin. In contrast, *p53*-deficient tumors treated with the same regimens continued to enlarge and contained few apoptotic cells. Acquired mutations in *p53* were associated with both treatment resistance and relapse in *p53*-expressing tumors. These results establish that defects in apoptosis, here caused by the inactivation of *p53*, can produce treatment-resistant tumors and suggest that *p53* status may be an important determinant of tumor response to therapy.

Although the tumor-specific action of most anticancer agents has been attributed to their debilitating effects on actively proliferating cells, an increasing body of evidence suggests that anticancer agents instead induce apoptosis [reviewed in (*1*, *2*)]. This view has profound implications for understanding the therapeutic response of human tumors. First, events subsequent to the interaction between anticancer agents and their primary intracellular targets may have a substantial effect on tumor response. Second, factors that increase the propensity for apoptosis may determine the therapeutic index whereby anticancer agents selectively destroy tumor cells. Finally, because apoptosis requires a genetic program, mutations in apoptotic pathways could produce drug-resistant tumors.

Studies suggest that the *p53* tumor suppressor gene is an essential component of the apoptotic program induced by anticancer agents in oncogenically transformed cells (*3*, *4*). To determine whether *p53* influences tumor responsiveness in vivo, we developed a transplantable fibrosarcoma model in which tumors differed primarily in their *p53* status. Embryonic fibroblasts transformed by coexpression of the adenovirus early region 1A (E1A) and activated *ras* oncogenes form tumors when subcutaneously injected into nude mice regardless of their *p53* status, but $p53^{+/+}$ cells are highly sensitive to apoptosis in vitro (*5*). In agreement with earlier studies, oncogenically transformed $p53^{+/+}$ cells formed fewer tumors and with a longer latency than $p53^{-/-}$ cells. The $p53^{-/-}$ cells produced tumors at all injected sites with an average latency of 8 ± 4 days, whereas the $p53^{+/+}$ cells produced tumors at 82 ± 24% ($P = 0.11$, t test) of sites injected, with an average latency of 18 ± 7 days ($P < 0.03$). After reaching a palpable size, tumors derived from both $p53^{+/+}$ and $p53^{-/-}$ cells grew at similar rates.

The *p53* status had a dramatic effect on tumor response to gamma irradiation. Most

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Optimization of yield and biofiltering efficiencies of *Ulva rigida* C. Ag. cultivated with *Sparus aurata* L. waste waters*

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SUMMARY: *Ulva rigida* C. Ag. was cultivated in 750 l tanks at different densities and flow rates with waste waters from Sparus aurata cultivation systems, to estimate the conditions for optimizing biofiltering efficiency and yield. To maximize both characteristics: i) seaweed density should be adjusted to 2.7 g FW l^{-1} (1.2 kg m^{-2}) and ii) NH_4^+-nitrogen flow should be (at least) 150 mmol m^{-2} d^{-1}. Such conditions will yield 27 g DW m^{-2} d^{-1} with uptake efficiencies of 76 % of the daily input in our system at 12 volumes d^{-1} (110 mmol m^{-2} d^{-1}) at seaweed : fish biomass ratio of 1:4. NH_4^+ removal efficiencies would probably be increased if fish were feed as early as possible in the morning to provide maximum NH_4^+ concentrations during the light period, as uptake efficiencies are higher than in the dark.

Keywords: Ulva rigida, Sparus aurata, biofiltering, ammonia uptake, aquaculture

Abbreviations: ANE= Apparent Nitrogen Efficiency; RNE= Real Nitrogen Efficiency; PFD= *p*hoton *f*lux *d*ensity; DW= *d*ry *w*eight; FW= *f*resh *w*eight

RESUMEN: OPTIMIZACIÓN DEL RENDIMIENTO Y DE LA EFICIENCIA DE BIOFILTRACIÓN DE *ULVA RIGIDA* C. AG. CULTIVADA EN AGUAS EFLUENTES DE CULTIVO DE *SPARUS AURATA* L.– *Ulva rigida* C. Ag. fue cultivada en tanques de 750 l a diferentes densidades y tasas de renovación con efluentes de sistemas de cultivo del pez *Sparus aurata* L. con la finalidad de determinar las condiciones que permitieran optimizar tanto las eficiencias de biofiltración como la producción de algas. Para optimizar ambas características: i) la densidad de algas debería ajustarse a 2.7 g FW l^{-1} (1.2 kg m^{-2}) y ii) los flujos de amonio deberían ser de al menos 150 mmol m^{-2} d^{-1}. Bajo tales condiciones se podrían obtener producciones de 27 g PS m^{-2} d^{-1} con eficiencias de incorporación de amonio del 76% del amonio que entra al sistema a tasas de renovación de 12 vol d^{-1} (110 mmol m^{-2} d^{-1}) a una relación algas:peces de 1:4. La eficacia de incorporación de amonio podría incrementarse adelantando tanto como fuese posible el horario de comida de los peces para obtener las máximas concentraciones de amonio durante el día, coincidiendo con el periodo de máxima capacidad de incorporación del mismo por las algas.

Palabras clave: *Ulva rigida, Sparus aurata*, biofiltración, incorporación de amonio, acuicultura.

INTRODUCTION

Waste waters resulting from intensive fish cultivation systems contain large amounts of dissolved metabolites, mostly ammonia, generated from excretory products and bacterial ammonification of organic matter (KROM *et al.*, 1985a; PORTER *et al.*, 1987; KROM and VAN RIJN, 1989). Ammonia can increase to toxic levels, up to 450 µM, in fishponds with low or intermediate water exchange rates (PORTER *et al.*, 1987; KROM *et al.*, 1989), producing

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hypereutrophic conditions due to the fertilizing effects on marine phytoplankton (KROM *et al.*, 1985a; 1985b; NEORI *et al.*, 1989). From the point of view of minimum environmental impact, recycling or recirculation systems of aquaculture would be ideal, as there would be only reduced discharges into open water bodies. The development of such systems requires the removal of solid compounds and dissolved metabolites contained in outflowing water. Solids can be easily removed by filtration or other mechanical processes but removal of dissolved metabolites requires more complex and expensive processes.

The use of seaweeds as biofilters to remove dissolved nitrogen from fish pond effluent has been reported previously (DE BOER and RYTHER, 1977; FRALICK, 1979). Species of the genus *Gracilaria* and *Ulva* have been tested, showing high growth rates and high ammonia removal efficiencies (HARLIN *et al.*, 1979).

Ulva species not only show a higher N-removal capacity than *Gracilaria* but also a higher resistance to epiphytes. Ammonia removal efficiencies of up to 85%, independently of light or temperature fluctuations, were obtained with *Ulva lactuca* L. cultivated with effluents of the marine fish *Sparus aurata* at water exchange rates of 8 volumes per day and NH_4^+ concentrations of 10-20 µmol l^{-1} h^{-1} (VANDERMEULEN and GORDIN, 1990). Sustained *Ulva* yields over 30 g DW m^{-2} d^{-1} have been reported by several authors (VANDERMEULEN and GORDIN, 1990; NEORI *et al.*, 1991).

Seaweed stocking densities, water exchange (NH_4^+-nitrogen flow through tanks) and fish:seaweed biomass rates are key factors affecting growth rate, yield and biofiltering efficiency. Those variables are usually not clearly stated in the literature. Different optimum densities for maximum yield in tank cultures have been given for different species (*Ulva* = 0.8 kg FW m^{-2}, LAPOINTE and TENORE, 1981; *Gracilaria*= 2-3 kg FW m^{-2}, LAPOINTE and RYTHER, 1978). However, higher stocking densities than those given for maximal yield in *Ulva* have been recently reported for maximal ammonium uptake and tissue nitrogen content (NEORI *et al.*, 1991).

The present study was conducted to determine the interaction between NH_4^+ flow, seaweed density and seaweed:fish biomass ratio in a co-culture system, to maximize biofiltering efficiencies and seaweed yield.

MATERIAL AND METHODS

Plant material and culture conditions

Plants of *Ulva rigida* collected from the east coast of the island of Gran Canaria (Canary Islands, Spain) were precultivated for one month under continuous wastewater flow. Necrotic or epiphytized plants were removed during this period. Preculture and experiments were conducted under greenhouse conditions in 750 l (1.75 m^2) semicircular tanks 80 cm deep. Algae were grown suspended in the water column with the aid of air diffusers located at the bottom of the tank.

Fishpond effluent was pumped from a 1 m^3 reservoir tank (washout of sediments every two weeks) connected to the output of six 1 m^3 tanks with approximately 12 kg of *Sparus aurata* in each. The water exchange rate was 8-10 vol d^{-1} per seaweed tank. Water passed through seaweed tanks was directly released to the sea.

Noon average PFD during experiments was 710 ± 80 µmol m^{-2} s^{-1}, with a daily water temperature fluctuation of 25.6 ºC (at noon) to 19.9 ºC (during the night). Differences in temperature between tanks did not exceed 0.5 ºC in each experimental set.

Experimental design

Three sets of experiments (1, 2 and 3, Table 1) each in triplicate were conducted during a two week period to estimate the effects of: i) stocking densities (1, 2 and 4 g FW^{-1} l^{-1}) and ii) wastewater exchange rates (4, 8 and 12 vol d^{-1}) on: i) NH_4^+-removal efficiency (N-efficiency) and ii) seaweed yield. Two control experiments were carried out simultaneously to estimate i) yield in tanks under continuous flow of seawater with weekly pulse-feeding (expt. 4) and ii) NH_4^+ losses of aerated culture tanks (expt. 5). In experiment 4, water supply was turned off once a week and tanks were supplied with ammonium chloride and sodium orthophosphate to a final concentration of 2.0 mM NH_4^+ and 0.15 mM PO_4^{3-} (following the concentrations used in tank cultures of *Ulva lactuca* by DEBUSK *et al.*, 1986). After 24 h, the water supply was turned on and adjusted to a flow rate of 8 vol d^{-1}.

Inflow and outflow of NH_4^+ were measured as follows: i) every three hours (24 h period) the day after algae were re-stocked to their initial density and, ii) at 21:00 daily. PFD, temperature and pH values were taken simultaneously.

TABLE I.- Effect of stocking density and NH_4^+ flow on ammonia removal efficiencies, growth rate and yield of *Ulva rigida*.

Exp. nº	Exch. rate (d^{-1})	Density g FW l^{-1}	NH_4^+ inflow (mmol m^{-2} d^{-1}) ± SE			NH_4^+ outflow (mmol m^{-2} d^{-1})	NUR	NUE (%)			Growth rate (%d^{-1})	Yield g DW m^{-2} d^{-1} ±SE
			total	L*	D*	±SE	±SE	total	L*[1]	D*[1]		
1	4	1	48.2±3.4	19.3±1.4	28.9±2.0	7.2±0.3	40.9±2.8	85.0	92.9	76.0	8.7	10.5±0.7
	4	2	48.2±3.4	19.3±1.4	28.9±2.0	4.2±0.3	43.4±3.2	90.4	97.2	85.9	6.2	13.8±0.4
	4	4	51.4±2.0	20.6±0.8	30.8±1.3	3.4±0.3	47.9±1.9	93.3	98.6	90.2	3.0	11.9±1.0
2	8	1	98.3±4.1	40.0±1.7	58.3±2.5	29.8±2.4	68.7±2.4	69.6	78.4	58.5	13.2	19.2±1.7
	8	2	107.4±3.0	43.0±1.2	64.6±1.9	20.9±1.4	86.5±2.2	80.5	85.0	63.7	10.4	27.0±0.4
	8	4	107.4±3.0	43.0±1.2	64.6±1.9	13.4±0.7	93.9±2.5	87.4	96.5	82.3	5.0	21.2±0.8
3	12	1	154.6±3.9	68.5±1.6	86.1±2.3	70.5±1.5	85.0±2.1	55.0	63.2	40.0	14.0	21.0±0.6
	12	2	154.6±3.9	68.5±1.6	86.1±2.3	44.7±1.5	110.2±2.8	71.3	79.7	55.6	11.0	29.2±0.8
	12	4	157.7±2.6	69.8±0.9	88.8±1.5	37.5±1.0	120.7±2.3	76.6	92.3	67.9	5.6	24.1±0.5
4	8	1									12.6	17.9±0.4
	8	2									9.6	24.3±0.5
	8	4									5.4	23.6±0.5
5	8	0	107.4±3.0			106.1±2.2						

L*= light period; D*= dark period; [1]= % of the total NH_4^+ (100%) which enters the system during the respective periods.

Harvesting and growth rate determination

Ulva rigida plants were harvested weekly, spinned at 2,800 rpm in a domestic clothes centrifuge (Miele WZ268) for 30 s and re-stocked to the initial density. Specific growth rates (μ) were calculated according to the equation ($\mu = 100\ Ln(W_t/W_o)/t$) by D'ELIA and DEBOER (1978), where W_o= initial fresh weight; W_t= final fresh weight; and t= time (days). A sample of 10 g was removed and dried at 80 ºC to constant weight to obtain the wet weight/dry weight ratios. Yields were calculated according to the equation ($Y = [(N_t-N_0)/t * (DW/FW)] / A$) by DEBOER and RYTHER (1977), where N_t= initial fresh weight, N_0= final fresh weight and A= area in m^2.

Analytical measurements

Ammonia analysis was carried out using a Flow Injector Analyzer (FIAstar 5010 Analyzer, Tecator, Sweden), through the gas diffusion method (RONNESTAD and KNUTSEN, 1991). PFD was measured with a LI-COR LI-1000 Datalogger using a spherical quantum sensor to estimate the decrese of PFD into the tanks, at half depth (40 cm) and at the bottom (80 cm). A plane quantum sensor was used to measure the irradiation just above the surface of the tanks. The pH and temperature values were measured with a HI 9025 microcomputer pH meter (Hanna Instruments).

Nitrogen uptake efficiencies (NUE) shows the average reduction (%) in ammonia concentration. Nitrogen uptake rate (NUR) shows the amount of ammonium removed per unit of time. The light period for the calculation of NUE, NUR and NUR per unit of biomass was from sunrise (7:30) to sunset (20:30).

RESULTS

Stocking density

According to the results of several authors for *Ulva* spp. (LAPOINTE and TENORE, 1981), maximum *Ulva rigida* yields were obtained at an initial stocking density of 2 g FW^{-1} l^{-1} (0.86 kg FW m^{-2}), with declining yields at both lower and higher stocking densities at any water exchange rate (Table 1). Densities of 1, 2 and 4 g FW l^{-1} reduced PFD by 58.3, 75.6 and 95.4% at half depth of the tank (40 cm) and by 62.2, 93.3 and 100% at the bottom of the tank (80 cm), respectively.

As observed from Fig. 1 B, increments in stocking densities can also be related to reductions in inorganic carbon concentrations or high pH, caused by photosynthetic carbon assimilation, specially in low water exchange rate culture tanks.

Growth rate and Yield

Both the growth rate and yield at 12 vol d^{-1} were twice those observed at 4 vol d^{-1} at all densities

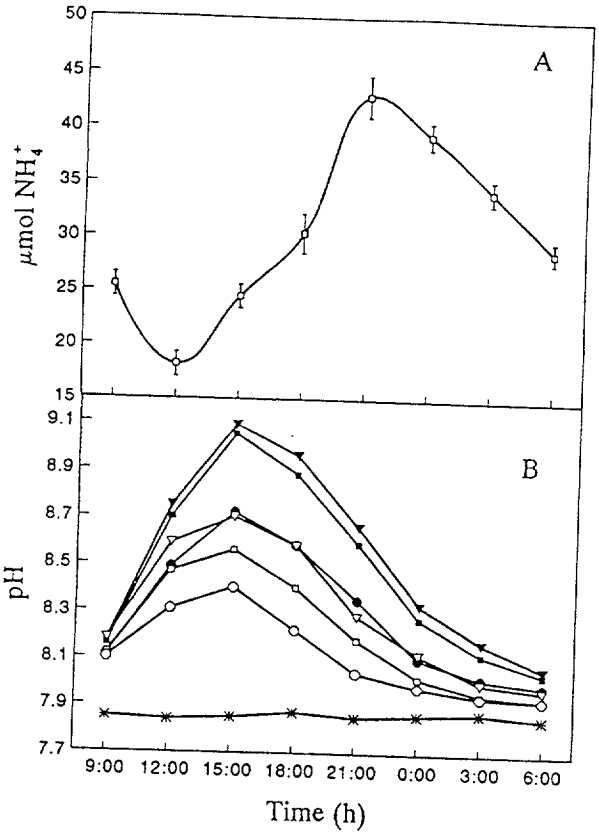

FIG. 1.-A: Daily ammonia concentration in the water of the fishpond effluent flowing into the tanks. Bars represent standard deviations (n=3). B: Daily pH evolution in: fish pond effluents (*) seaweed culture tanks at densities of 1 g l^{-1} (●), 2 g l^{-1} (■) and 4 g l^{-1} (▲) at 4 (closed symbols) and 12 (open symbols) daily water exchange rates.

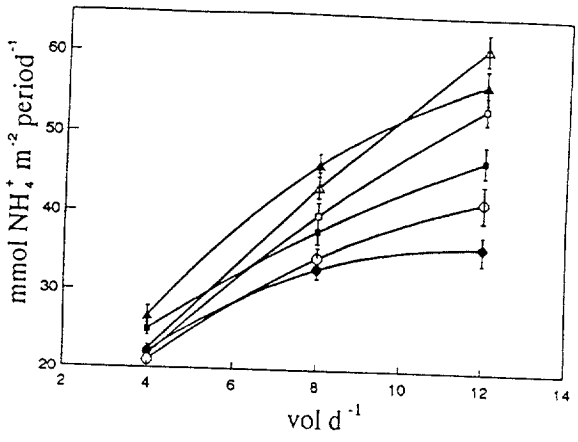

FIG. 2.- NUR during light (open symbols) and dark (closed symbols) periods at 4, 8 and 12 vol d^{-1} and densities of 1 g l^{-1} (●), 2 g l^{-1} (■)nd 4 g l^{-1} (▲). Bars represent standard deviation (n=3).

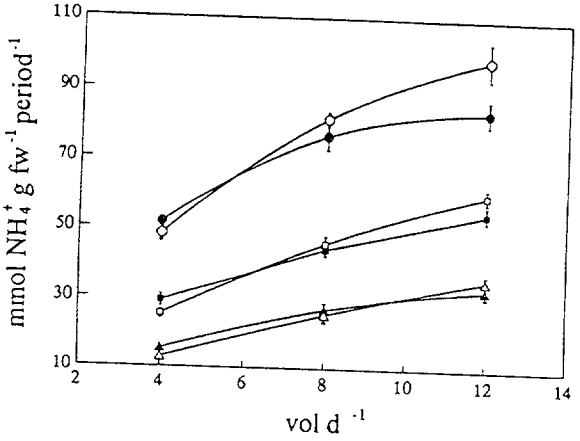

FIG. 3.- NUR per unit of biomass during light (open symbols) and dark (closed synbols) periods at 4, 8 and 12 vol d^{-1} and densities of 1 g l^{-1} (●), 2 g l^{-1} (■) and 4 g l^{-1} (▲). Bars represent standard deviation (n=3).

(Table 1). However, growth rate and yield at 8 and 12 vol d^{-1} were quite similar. Similar growth rates and yields were obtained between weekly-pulsed cultures with continuous flow of wastewater (Table 1). Disintegration of the plants and decrease in weight were observed in running through seawater tanks (non-fertilized cultures, data not shown).

Effect of water exchange rates

One of the main effects of the increase in water exchange rates were the increment in the amount of NH_4^+ and inorganic carbon provided to the plants per unit of time. Flow rates of 4 and 8 vol d^{-1} produced strong fluctuations in pH at densities of 2 and 4 g FW^{-1} l^{-1}, higher water exchange rates (12 vol d^{-1}) had a stabilizing effect on pH at all densities (Fig. 1B) and on temperature.

Ammonia levels in the fishpond effluent showed marked daily oscillations, with maximum concentrations during the "dark" period and minimum during the "light" period (Fig. 1A). Daily NH_4^+ flow was quite stable during the experimental period. NH_4^+ losses due to the system (by water recirculation and aeration of tanks without seaweed) were insignificant (less than 1.5%).

NUE were inversely related to water exchange rates and directly related with stocking density (Table 1). However NUR were directly related to

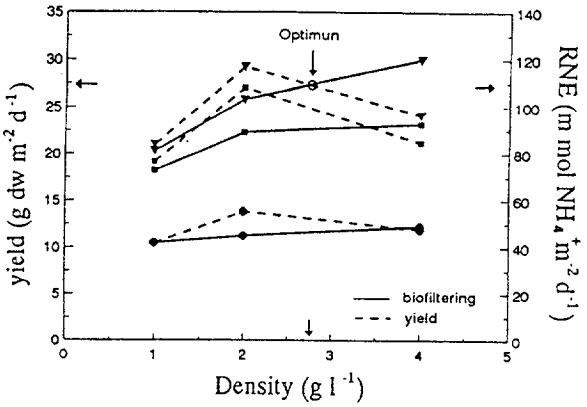

FIG. 4.- Yield (discontinuous line) and NUR (continuous line) at the three densities (1, 2 and 4 g l^{-1}) and water exchange rates of 4 (●), 8 (■) and 12 (▲) vol d^{-1}.

both flow rate and stocking densities (Table I). NUE during the light period were higher than during the dark period (Table 1). However, non significant differences in NUR ($p > 0.05$ ANOVA) were detected between light and dark periods at lowest water exchange rates (4 and 8 vol d^{-1}) (Fig.2). At higher ammonia flows (achieved by higher water exchange rates) NUR were higher during the light period at all densities ($p < 0.05$ ANOVA) (Fig.2).

NUR per unit of biomass was inversely related to density, both in dark and light periods (Fig. 3). Nitrogen uptake capacity by *Ulva rigida* plants in the light period seems to become saturated at NH_4^+ flow rates of 18.8 µmol NH_4^+ g FW^{-1} h^{-1}. However, during the dark period plants are nearly saturated at 11.8 µmol NH_4^+ g FW^{-1} h^{-1} (Fig.3). Increments in yield shows a very high correlation ($\alpha = 0.999$) with increments in NUR per unit of biomass per day.

DISCUSSION

Daily ammonia oscillations are due to the fish feeding period (between 9:00 and 14:00 h) which produce a peak of ammonia 7 hours later (Fig. 1A) due to fish excretion and microbial degradation of leftover food. Differences in the timing of peak ammonia levels reported by PORTER *et al.*, (1987) (4.5 hours after feeding) might be related to differences in fish age and feed.

The decrease in growth rate by the increase in density in our cultures is probably due to self-shading according to DUKE *et al.*, (1986); (1989a); LAPOINTE and TENORE, (1981), but may also depend on carbon limitation according to DEBUSK *et al.*, (1986).

Even though inorganic carbon concentrations in wastewater inflow have been increased by heterotrophic breakdown of dissolved organic substances (KROM and NEORI, 1989) and fish respiration (PIEDRAHITA, 1990) which results in a decrese of the pH, our results indicate that cultures over 2 g FW l^{-1} (0.86 kg m^{-2}) might be carbon limited (Fig. 1B). At 15:00 hours, the rise in pH, especially with lower exchange rate (4 vol d^{-1}) indicate that the buffering capacity of the carbonate system is decreasing, and photosynthesis causes the pH rise. According to VANDERMEULEN and GORDIN (1990), the higher pH values recorded under such conditions would indicate carbon limitation. Moreover, yield doubling (at the three densities), when doubling (8 vol d^{-1}) or tripling (12 vol d^{-1}) exchange rates (Table 1), fits well with similar increases in the amount of carbon provided to the plants per unit of time. The similar pH values of 1 g FW l^{-1} at 4 water exchanges per day and 4 g FW l^{-1} at 12 vol d^{-1} (Fig. 1) shows that photosynthetically assimilable carbon can be maintained at the same levels at four fold higher densities by a three fold increase in flow rate, which in turn produces almost 2.5 times more biomass and 3 times more NUR (Fig.3). However, NUE are lower than at 1 g FW l^{-1} and 4 vol d^{-1} (Table 1).

NUE are similar to those described by VANDERMEULEN and GORDIN (1990) and COHEN and NEORI (1991) for *Ulva lactuca*. The higher yields and NUR obtained by COHEN and NEORI (1991) at similar ammonia fluxes probably depends on the higher (almost double) irradiation in their experiments. Regarding the variable to maximize, efforts to maximize NUE and NUR values in biofiltering experiments resemble growth rate and yield values applied to production. Considerations based on only NUE will lead to wrong conclusions on its application as a biofilter (as growth rate on biomass production) (Figs. 2 and 3).

The maximum yields obtained at 2 g FW l^{-1} (0.86 kg FW m^{-2}) at any exchange rate, are in accordance with the optimum density to maximize production in *Ulva* described by LAPOINTE and TENORE (1981) and RYTHER *et al.*,(1984). The absence of correlation between yield and NUR might be explained by: i) a higher excretion of organic matter promoted by the stress (HELLEBUST, 1974) of highly dense cultures, or ii) a lower C:N ratio of the biomass at highly

dense cultures (as reported by DUKE *et al.*, 1989b; VANDERMEULEN and GORDIN, 1990; COHEN and NEORI, 1991). From these results, and in accordance with DUKE *et al.* (1986; 1989a) it seems evident that ammonium uptake by *Ulva* is less limited by light than growth. However, the conclusion is just the opposite when analysing the data per unit of biomass in light and dark periods (discussed below).

Contrary to results obtained by VANDERMEULEN and GORDIN (1990) (same NUE during day and night periods) our data shows differences in NUE during light and dark periods (Table 1). These apparent contradictory results might be explained by the low ammonia flow in the experiments of those authors. In agreement with the data reported by COHEN and NEORI (1991) with *Ulva lactuca*, differences in NUE during light and dark periods are shown at high ammonia fluxes.

Higher NUR during dark period at low water exchange rates (4 vol d^{-1}) (Fig. 2) might be explained by the lower NH_4^+ fluxes during the light period. Higher NUR in light at the highest water exchange rates (12 vol d^{-1}) at all densities (Fig. 2) can be explained by the increase in ammonia (achieved by higher water exchange rates) and inorganic carbon flows. Similarities of NUR (and NUR per unit of biomass) in the dark at 12 and 8 vol d^{-1} (Figs. 2 and 3) indicate that dark cultures are N-saturated at 8 exchange rates (107 mmol m^{-2} d^{-1}), but not light cultures at any density. From Fig. 3, it seems that N-saturating flow rate in light lies around 18.8 µmol NH_4^+ g FW^{-1} h^{-1}. As the N-saturating threshold flow per unit of biomass varies significatively between the three densities (higher uptakes in plants grown at lower densities = lower selfshading) (Fig. 3), clearly shows that light has a strong influence in N-efficiency of the plant, masked by opposite efficiencies of the system (Table 1).

HARLIN *et al.* (1979) used *Gracilaria* sp to remove the ammonium produced by the fish *Fundulus heteroclitus*, removing lightly more ammonium (30 - 54 mmol kg^{-1} FW d^{-1}) than was produced by an equal biomass of fish. Similar results were reported by HAGLUND and PEDERSEN (1993) in a co-culture system of *Gracilaria teniustipitata* Zhang et Xia with rainbow trout. Daily NH_4^+ uptake rates of 40 mmol kg^{-1} FW d^{-1} were obtained at a seaweed:fish biomass ratio of 1:1. Our results, indicate that at least two times higher (91.6 mmol kg^{-1} FW d^{-1}) uptake rate efficiencies than that of *Gracilaria teniustipitata* can be obtained at four times higher fish concentration.

To maximize biofiltering efficiency of the wastewater from *Sparus aurata* hatcheries and seaweed yield, the fish should be feed as early in the morning as possible (in order to provide maximum NH_4^+ concentrations during the light period), the density of seaweeds should be adjusted to 2.7 g FW l^{-1} (1.2 kg FW m^{-2}) and water exchange rates should be (at least) 12 vol d^{-1} (150 mmol m^{-2} d^{-1}). Such conditions (at a seaweed:fish biomass ratio of 1:4 in our experimental system), will yield 27 g DW m^{-2} d^{-1} and a daily ammonia uptake rate of 110 mmol m^{-2} d^{-1} (76 % of the daily input at 12 vol d^{-1}).

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Acid deoxyribonuclease activity in crude extracts from marine phycophages used for seaweed protoplast isolation*

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SUMMARY: Deoxyribonuclease activity (DNase) was studied in crude extracts prepared from the digestive tracts of the marine molluscs *Aplysia dactylomela* Rang, *Haliotis coccinea canariensis* Nordsieck, *Littorina striata* King et Broderip, the echinoderm *Diadema antillarum* Phillipi, and a commercially available abalone acetone powder (AAP; Sigma, Ref. A-7514) to evaluate their toxic effects when used for seaweed protoplast isolation. DNase was detected at pH 5.0, in presence of Mg^{2+}, in all the crude extracts except in those obtained from the digestive gland of *A. dactylomela*. At pH 6.0, activity was not detected. Maximal specific activities were measured in *L. striata* and *D. antillarum* crude extracts. AAP showed the lowest specific activity of all the assayed extracts. The pH in the different digestive organs of these phycophages ranged from 5.0 to 7.0, measured *in vivo*, providing new information about the optimum values at which digestion occurs and, together with results on DNase activities, those organs which should be used to degrade seaweed cell walls.

Key words: Phycophages, seaweed, protoplasts, DNase.

RESUMEN: ACTIVIDAD DESOXIRRIBONUCLEASA EN EXTRACTOS CRUDOS DE FICOFAGOS MARINOS EMPLEADOS PARA EL AISLAMIENTO DE PROTOPLASTOS DE MACROALGAS. — Actividad desoxirribonucleasa (DNasa) fue estudiada en extractos crudos, preparados de los sistemas digestivos, de los moluscos *Aplysia dactylomela* Rang, *Haliotis coccinea canariensis* Nordsieck, *Littorina striata* King et Broderip, el equinodermo *Diadema antillarum* Phillipi, y un extracto de *Haliotis* sp. en forma de polvos cetónicos, comercialmente disponible (abalone acetone powder, AAP; Sigma, Ref. A-7514), para evaluar sus efectos tóxicos al ser empleados en el aislamiento de protoplastos de macroalgas. Actividad DNasa fue detectada a pH 5.0, en presencia de Mg^{2+}, en todos los extractos crudos exceptuando aquellos obtenidos de la glándula digestiva de *A. dactylomela*. A pH 6.0, no fue detectada actividad DNasa en ninguno de los extractos. Las actividades específicas máximas fueron medidas en los extractos crudos de *L. striata* y *D. antillarum*. El AAP mostró la actividad específica más baja de todos los extractos ensayados. El pH en los diferentes órganos digestivos de estos ficófagos, medido *en vivo*, varió de 5.0 a 7.0, aportando nueva información sobre los valores óptimos de pH en los que la digestión tiene lugar y, junto a los resultados sobre la actividad DNasa, aquellos que deben ser usados para la digestión de la pared celular en macroalgas.

Palabras clave: Ficófagos, macroalgas, protoplastos, DNasa.

INTRODUCTION

The potential toxicity of proteases, lipases, peroxidases and deoxyribonucleases of crude extracts from the digestive tracts of a number of phycophages on the viability of plant protoplasts has been commented upon by several authors (see TRIBE, 1955; SCHENK and HILDEBRANDT, 1969; BERLINER, 1981; COCKING, 1972; FITZSIMONS and WEYERS, 1985; BUTLER *et al.*, 1990). However, few attempts have been made on the quantification and characterization of such potentially toxic enzymes.

Most of the crude extracts used for seaweed pro-

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toplast isolation are extracted from herbivorous marine molluscs and echinoderms such as *Aplysia*, *Haliotis*, *Littorina* or *Diadema* species (SAGA and SAKAI, 1984; CHEN, 1987; BUTLER *et al.*, 1989; BOYEN *et al.* 1990; GÓMEZ-PINCHETTI and GARCÍA-REINA, 1993). Low protease activity has been detected in crude extracts of *Aplysia depilans* and *Haliotis tuberculata* (BOYEN *et al.*, 1990). Lipases and proteases have been measured in other marine molluscs and fishes (CLIFFORD *et al.*, 1982; FERAL, 1989; TEO and SABAPATHY, 1990). Deoxyribonucleases in the marine environment have been studied only in some fishes and invertebrates (ASHE *et al.*, 1965; RASSKAZOV *et al.*, 1975; DOMINGO *et al.*, 1986; CHOU and LIAO, 1990; STRÆTKVERN *et al.*, 1990) including a few molluscs (GEORGATSOS and ANTONOGLOU, 1963), but none of which are used for seaweed cell wall degradation and protoplast isolation. These tissues of marine origin have revealed at least two groups of DNA-depolymerizing enzymes with quite different properties, namely acid and alkaline DNases (GEORGATSOS and ANTONOGLOU, 1963; RASSKAZOV *et al.*, 1975).

In this study, we report on the acid DNase activity of crude extracts from the most common phycophages used for the isolation of seaweed protoplasts.

MATERIAL AND METHODS

Source of enzymes

Crude extracts from *Aplysia dactylomela*, *Haliotis coccinea canariensis*, *Littorina striata* and *Diadema antillarum* were prepared within 24 h of collection. Abalone acetone powder (AAP; Sigma, St. Louis, USA, Ref. A-7514), a crude extract prepared from *Haliotis* sp. entrails was also used as a source of enzymes.

Preparation of crude extracts

The oesophagus and the digestive gland (separately) from *Aplysia dactylomela*, the digestive tract and hepatopancreas from *Haliotis coccinea canariensis*, the gut from *Diadema antillarum* and the whole body from *Littorina striata* (after shell removal), were used to prepare the crude extracts. Temperatures between 0 and 4 °C and 0.1 M phosphate buffer (pH 6.0) were used to perform all operations.

The pH from the oesophagus and the digestive gland of *Aplysia dactylomela*, the hepatopancreas of *Haliotis coccinea canariensis*, the homogenate of *Littorina striata* and the gut of *Diadema antillarum* were measured with a microelectrode (PHR-146, Lazar Res. Lab., CA, USA) immediately after dissection.

Crude extracts were prepared, as previously described by GÓMEZ-PINCHETTI and GARCÍA-REINA (1993), with some modifications. Glands and organs were homogenized and extracted with buffer, supplemented with EDTA Na_2 (2.0 mM), in a mortar under liquid nitrogen. The extraction was carried out in 0.1 M phosphate buffer (pH 6.0) supplemented with EDTA Na_2 (2.0 mM; 5.0 ml g tissue^{-1}). Homogenates were squeezed through a 170 μm nylon mesh and centrifuged at 27.000 x g for 30 min. Ammonium sulphate was then added to the supernatant to 80 % saturation. After several hours equilibration, the suspension was centrifuged at 37.000 × g for 20 min. Pellets were resuspended in buffer and passed through a pre-packed Sephadex G-25 column (PD-10, Pharmacia LKB, Uppsala, Sweden) equilibrated with 20 mM Bis-tris buffer (pH 6.0) dissolved in seawater, 1.0 M sodium acetate buffer (pH 5.0), or 0.1 M phosphate buffer (pH 6.0) for desalting and buffer exchange.

AAP was dissolved in buffer (2.0 % w/v), equilibrated for several hours between 0 and 4 °C, centrifuged at 27.000 × g for 15 min and passed through a Sephadex G-25 column.

Protein concentration was determined by the method of LOWRY *et al.* (1951) using bovine serum albumin as the standard.

Quantification of DNase activity

A DNA (from calf thymus; Ref. D-3664, Sigma) solution (0.004 %, final concentration) dissolved in distilled water and supplemented with the addition of 1.0 M sodium acetate buffer (0.01 %, pH 5.0) and 0.1 M $MgSO_4$ (0.005 %) was used as substrate. Activity was obtained by measuring the maximun linear rate at A_{260nm} in a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan) of a mixture containing 2.5 ml of substrate equilibrated at 25 °C and 0.5 ml of the crude extract.

Values were corrected to the standard activity of DNase I [EC 3.1.21.1; 2,000 Units per vial, one unit will produce a ΔA_{260nm} of 0.001 per min per ml at pH 5.0 at 25 °C using DNA as the substrate in presence of Mg^{2+} (4.2 mM); Ref. D-4263, Sigma].

RESULTS AND DISCUSSION

The pH of the different digestive systems ranged between 5.0 and 7.0 (Table I), coincident with the

TABLE 1. — The pH, after dissection, of the different digestive systems used as enzymatic sources, and the DNase activity of crude extracts at two different pH values [5.0 (1.0 M sodium acetate) and 6.0 (20 mM Bis-Tris in seawater or 0.1 M phosphate)]. T = 25 °C. AAP: Abalone acetone powder; –: no activity detected; nm: not measured.

Crude extract	pH (mean ± sd) (n = 5)	Prot. Conc. mg ml^{-1}	DNase activity Units ml^{-1}		Specific Activity Units mg^{-1} prot
			pH 5.0	pH 6.0	
Aplysia dactylomela					
Oesophagus	5.58 ± 0.42	6.2 ± 1.6	423.8	–	68.35
Digestive gland	5.85 ± 0.19	7.6 ± 0.6	–	–	–
Haliotis coccinea					
Hepatopancreas	6.56 ± 0.15	6.7 ± 1.2	257.9	–	38.49
Littorina striata					
Homogenate	5.75 ± 0.25	1.2 ± 0.3	134.1	–	111.75
Diadema antillarum					
Gut	6.92 ± 0.18	1.9 ± 0.5	219.5	–	115.53
Abalone Acetone Powder	nm	1.9 ± 0.4	35.6	–	18.70

optimum values for *in vitro* activities of agarase, cellulase, alginate lyase and other polysaccharidases from the digestive systems of those and other marine herbivores (ONISHI *et al.*, 1985; GÓMEZ-PINCHETTI and GARCÍA-REINA, 1993).

DNase activities of the different crude extracts are shown in Table 1. All the crude extracts degraded DNA in the presence of Mg^{2+} at pH 5.0, except those prepared from the digestive gland of *Aplysia dactylomela*. At pH 6.0 no DNase activity was detected in any of the different crude extracts regardless buffer (20 mM Bis-tris in seawater or 0.1 M phosphate). The significantly higher DNase specific activities from *Littorina striata* and *Diadema antillarum*, compared to these obtained with other crude extracts, might be explained by enzymatic contamination from other organs. In *L. striata,* DNase activity was measured from the whole body of the animal. In *D. antillarum*, although the gut was easily distinguishable, it was very difficult to excise the digestive system without contact from other fluids and organs. The abalone acetone powder showed the lowest specific activity of all the assayed crude extracts. This variability might be due to the utilization of the acetone precipitation method for the preparation of this crude extract from *Haliotis* sp. instead of ammonium sulfate precipitation used for the rest of crude extracts assayed.

Adjustment of the pH of the enzymatic solution to values between 6.0 and 7.0, depending on the phycophage and the seaweed to be digested, would avoid the action of DNases and be closely related to the optimum conditions in which natural digestion occurs in these phycophages and thus maximum activity of the polysaccharidases. pH values higher than 8.0, optimal for the action of alkaline DNases (GEORGATSOS and ANTONOGLOU, 1963; RASSKAZOV *et al.*, 1975), would be too high for the optimal action of cell wall degrading enzymes. The addition of inhibitors (i.e., potassium dextran sulphate) to the enzymatic solutions to prevent the effects of ribonucleases, employed by some authors (FUJIMURA *et al.*, 1989; WAALAND *et al.*, 1990; REDDY and FUJITA, 1991), would be unnecessary if the pH of the solution is adjusted to approximately 6.0. New steps in specific enzyme partial purification would be desirable to avoid toxic effects (see SCHENK and HILDEBRANDT, 1969; BUTLER *et al.*, 1990), however, the risk of loosing the degrading effects of other additional enzymes present in the crude extracts would be considerable. These data give us new information to improve seaweed protoplast production, cell viability and thallus regeneration.

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Low-CO_2-inducible protein synthesis in the green alga *Dunaliella tertiolecta*

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Abstract. In the green marine alga *Dunaliella tertiolecta*, a CO_2-concentrating mechanism is induced when the cells are grown under low-CO_2 conditions (0.03% CO_2). To identify proteins induced under low-CO_2 conditions the cells were labelled with $^{35}SO_4^{2-}$, and seven polypeptides with molecular weights of 45, 47, 49, 55, 60, 68 and 100 kDa were detected. The induction of these polypeptides was observed when cells grown in high CO_2 (5% CO_2 in air) were switched to low CO_2, but only while the cultures were growing in light. Immunoblot analysis of total cell protein against pea chloroplastic carbonic anhydrase polyclonal antibodies showed immunoreactive 30-kDa bands in both high- and low-CO_2-grown cells and an aditional 49-kDa band exclusively in low-CO_2-grown cells. The 30-kDa protein was shown to be located in the chloroplast. Western blot analysis of the plasma-membrane fraction against corn plasma-membrane ATPase polyclonal antibodies showed 60-kDa bands in both high- and low-CO_2 cell types as well as an immunoreactive 100-kDa band occurring only in low-CO_2-grown cells. These results suggest that there are two distinct forms of both carbonic anhydrase and plasma-membrane ATPase, and that one form of each of them can be regulated by the CO_2 concentration.

Key words: ATPase – Carbonic anhydrase – CO_2-concentrating mechanism – *Dunaliella* – Protein synthesis – Photosynthesis

Introduction

In algae, the assimilation of dissolved inorganic carbon ($CO_2 + HCO_3^-$) (DIC) from the environment is affected by the CO_2-concentrating mechanism (CCM), a transport system that enhances the delivery of CO_2 to ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Badger et al. 1980; Zenvirth and Kaplan 1981; Raven and Lucas 1985; Aizawa and Miyachi 1986; Badger 1987; Samuelsson et al. 1990; Moroney and Mason 1991; Spalding et al. 1991). The CCM appears to be present in most algal groups, although it has not yet been biochemically characterized.

A CCM is induced in the halotolerant marine unicellular alga *Dunaliella tertiolecta* when it is grown under low-CO_2 conditions (Aizawa and Miyachi 1984, 1986). This increased affinity for CO_2 facilitates both the active transport of DIC into the cell and the active assimilation of internal carbon. *Dunaliella* acclimates to low CO_2 conditions with an apparent photosynthetic *Km* (CO_2) of < 1–5 μM (Aizawa and Miyachi 1984; Ramazanov and Cardenas 1992).

The enzyme carbonic anhydrase (CA) has been found to play an important role in the CCM and in CO_2 assimilation in microalgae (Spalding et al. 1983, 1991; Aizawa and Miyachi 1984, 1986; Coleman and Grossman 1984; Moroney and Mason 1991; Sültemeyer et al. 1990, 1993; Ramazanov and Cardenas 1992). Clarification of the actual role played by CA in the regulation of carbon assimilation and in the CCM is complicated due to the existence of several forms of CA (Husic et al. 1989; Fukuzawa et al. 1990; Rawat and Moroney 1991; Ramazanov and Cardenas 1992). These CA isoforms differ not only in their intracellular location, but also in the nature of the dependence of their activity upon the cultivation conditions, CO_2 concentration in particular (Pronina et al. 1981; Husic et al. 1989; Fukuzawa et al. 1990; Ramazanov and Cardenas 1992; Sültemeyer et al. 1993). In *D. tertiolecta* and *D. salina*, CA has been reported to be located on the cell surface and to increase the affinity for CO_2 during photosynthesis (Aizawa and Miyachi 1984; Booth and Beardall 1991; Goyal et al. 1992; Gómez-Pinchetti et al. 1992; Ramazanov and Cardenas 1992).

However, CA activity alone cannot fully explain the internal inorganic-carbon accumulation that takes place

Abbreviations: CA = carbonic anhydrase; DIC = dissolved inorganic carbon ($CO_2 + HCO_3^-$); CCM = CO_2-concentrating mechanism; low CO_2 = air containing 0.03% CO_2; high CO_2 = air supplemented with 5% CO_2 (v/v)

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under conditions of low external DIC nor the organization of the fully implemented CCM in algae (Gehl et al. 1990; Martínez et al. 1992). At the same time that *Chlamydomonas reinhardtii* cells induce the CCM and increase their affinity for external inorganic carbon, at least five polypeptides are induced (Coleman and Grossman 1984; Manuel and Moroney 1988; Spalding and Jeffrey 1989; Spalding et al. 1991). These proteins include a 37-kDa periplasmic CA and four other proteins with molecular weights of 21, 36, 42 and 44 kDa (Coleman and Grossman 1984; Manuel and Moroney 1988; Geraghty et al. 1990; Spalding and Jeffrey 1989; Spalding et al. 1991; Ramazanov et al. 1993).

We have little information about low-CO_2-inducible protein synthesis in *D. tertiolecta*. Thielmann et al. (1992) reported that in *D. tertiolecta* the 45- and 47-kDa polypeptides induced by low CO_2 are constituents of the chloroplast envelope and that they appear to be required to activate the HCO_3^--transporting pump in these cells. However, the exact functional role of these polypeptides in the CCM remains unclear.

The requirement of a plasma-membrane ATPase for active DIC transport in algae has been suggested (Raven and Lucas 1985; Badger 1987; Thielmann et al. 1990; Rotatore et al. 1992; Karlsson et al. 1994). Several authors propose that the low-CO_2-induced vanadate sensitivity in photosynthesis may be caused by the appearance of a vanadate-sensitive ATPase involved in HCO_3^- transport (Raven and Lucas 1985; Thielmann et al. 1990; Karlsson et al. 1994). The mechanism by which such an ATPase may be involved in DIC transport needs more investigation. To date, none of the low-CO_2-inducible proteins in algae has been identified as a HCO_3^- transport protein or as an ATPase. Moreover, it has been suggested that in *Dunaliella* CO_2 is the general form of inorganic carbon transported across the plasma membrane and that bicarbonate enters the cell mainly by an "indirect" mechanism after dehydration to CO_2 (Aizawa and Miyachi 1986; Booth and Beardall 1991; Ramazanov and Cárdenas 1992).

In this work, experiments with *D. tertiolecta* cells were carried out to identify proteins that are preferentially synthesized under low-CO_2 conditions.

Materials and methods

Algal culture conditions. Dunaliella tertiolecta wild type, strain 999, was obtained from the UTEX Culture Collection (Texas, USA). Cells were first grown synchronously by using a 12 h light/12 h dark cycle for 3 d at 26 ± 1°C. After this period, cultures with a chlorophyll (Chl) concentration of 3–4 μg·ml^{-1} were switched to continuous illumination with white light (300 μmol quanta·m^{-2}·s^{-1}) supplied by fluorescent lamps. Under these conditions, the culture starts non-synchronous growth. Thus, non-synchronous cultures have been used for the experiments. The culture concentration of 3–4 μg Chl·ml^{-1} was maintained by daily dilution with fresh medium. The culture medium contained 0.5 M NaCl, 4 mM KNO_3, 2 mM $MgSO_4$, 1.9 mM $MgCl_2$, 0.01 mM $Ca(NO_3)_2$, 4 mM K_2HPO_4, and the micronutrient solution as described by Jiménez del Río et al. (1994). This medium was buffered at pH 7.5 with 20 mM Tris-HCl. Algae were grown in 0.5-L glass bottles (5 cm in diameter) sparged with either a CO_2:air mixture (5:95, v/v; high-CO_2 cells) or with air (0.03% CO_2, low-CO_2 cells). The gas flow was kept at a high rate (between 1500–2000 mL·min^{-1}) to favour an equilibrium concentration of DIC (Karlsson et al. 1994).

Photosynthesis assays. Photosynthesis was measured in 2-ml algal samples with a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK). Algae were centrifuged at 5000·g for 5 min and the pellet (3–4 μg Chl·ml^{-1}) resuspended in 2 ml of a 20 mM Hepes-KOH buffer supplemented with 0.5 M NaCl (pH 7.3) and transferred to the electrode chamber. Cells were allowed to consume the inorganic carbon of the buffer and the intracellular pool of DIC until no net photosynthesis was observed. Bicarbonate was added when net O_2 evolution had leveled off. The irradiance was 500 μmol quanta·m^{-2}·s^{-1}.

Carbonic anhydrase activity. The CA activity was determined in cells washed once with 10 mM Na-phosphate (pH 7.5), supplemented with 0.5 M NaCl (as an osmotic component). After centrifugation at 5000·g for 5 min, pellets were resuspended in ice-cold CA buffer [30 mM N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid) (EPPS), 1 mM EDTA, 0.1 mM dithiothreitol (DTT) and 1 mM $MgSO_4$, pH 8.2] to a Chl concentration of 10 μg·ml^{-1}. The CA activity was measured in cells disrupted in CA buffer as described previously (Ramazanov and Cárdenas 1992).

Activity of CA was also measured in fractions eluted from agarose affinity columns. Cell homogenates were centrifuged at 15000·g for 5 min, and supernatants subjected to 35% and 70% ammonium sulfate fractionation. The 70% ammonium sulfate precipitate was dialyzed against 2 L of 100 mM NaCl, 1 mM EDTA, 20 mM sodium phosphate (pH 6.8). The dialysate was centrifuged at 15000·g for 10 min and the supernatant was loaded on a *p*-aminomethylbenzene sulfonamide-agarose affinity column (Pharmacia, Uppsala, Sweden). The column was washed with 25 mM Tris, 22 mM Na_2SO_4 (pH 8.2), followed by 25 mM Tris, 300 mM $NaClO_4$ (pH 8.7). Elution of the protein fractions was monitored with a UV-detector. Following elution of nonspecifically associated proteins, the column was washed with 100 mM sodium acetate, 500 mM $NaClO_4$, pH 5.6 (Rawat and Moroney 1991), and eluted protein fractions that showed CA activity were subjected to Western blot protein analysis.

Labelling cells with $^{35}SO_4^{2-}$. Protein labelling by $^{35}SO_4^{2-}$ was performed according to Spalding and Jeffrey (1989). High-CO_2-grown cells were switched to minimal media containing 1/10 $MgSO_4$ concentration for 2 d. Cells were harvested by centrifugation at 5000·g for 5 min, the pellet washed twice with growth media lacking sulfate, then centrifuged again. The pellet was resuspended in growth media without sulfate to a Chl concentration of 5 μg·ml^{-1} and divided among seven 200-ml glass tubes (40 mm in diameter). Tubes were bubbled with air or with air supplemented with 5% CO_2. Then 455 kBq of carrier-free $H_2{}^{35}SO_4$ (370 TBq·mmol^{-1}) was added to the cultures. After 5 h incubation with $^{35}SO_4^{2-}$, cells were harvested by centrifugation at 5000·g for 5 min and the pellet was washed twice with 30 ml 30 mM Hepes-KOH (pH 7.5) supplemented with 0.5 M NaCl, and centrifuged again. The pellet was resuspended in 30 mM Hepes-KOH (pH 7.5) buffer for SDS-PAGE. To compare different treatments, samples were loaded to equal counts (250000 cpm per lane). The amount of radioactivity incorporated into the algal cells was determined by taking aliquots of cells in buffer and counting the sample using a Beckman LS 1801 liquid scintillation counter.

Plasma-membrane isolation. Plasma membranes were isolated according to the method of Larsson (1985). Cells were broken as described previously by Ramazanov and Cárdenas (1992) and the crude homogenate was centrifuged at 1500·g for 30 min to remove chloroplasts and cell debris. The supernatant was collected and centrifuged for 1 h at 28000·g to pellet microsomal membranes. The pellet was resuspended in 10 ml of Solution 1 (330 mM sucrose, 60 mM NaCl, 10 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 5 mM potassium phosphate, pH 7.8; Larsson 1985). This solution was overlaid with 50 g of Solution 2 to

give a final concentration of 6.5% (w/w) dextran T-500, 6.5% (w/w) polyethylene glycol (PEG) 3350, 330 mM sucrose, 2–5 mM KCl, 10 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF and 5 mM potassium phosphate (pH 7.8) in order to facilitate biphase partition (Widell 1987). The suspension was inverted several times and centrifuged for 5 min at 1500·*g* to separate the phases. Membranes were then separated by washing each of the two phases two additional times. The final upper phase (U_3) was diluted fivefold with Solution 1 and pelleted by centrifugation at 80000·*g* for 1 h. The final pellet was resuspended in Solution 2 and used for Western blot protein analysis.

Chloroplast isolation. Chloroplast isolation is described in detail by Jiménez del Río et al. (1994). Briefly, 1 L of exponentially grown high-CO_2- and low-CO_2-acclimated cells were harvested by centrifugation at 500·*g* for 10 min at room temperature. Cells were washed with 25 mM Hepes-KOH (pH 7.5) supplemented with 0.5 M NaCl and the pellet resuspended in 30 ml of ice-cold disruption buffer containing 50 mM Hepes-KOH (pH 7.2), 300 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, and 1% bovine serum albumin (BSA). Cells were disrupted according to the procedure of Ramazanov and Cardenas (1992).

Analytical measurements. Protein concentration was estimated according to Bradford (1976). Chlorophyll was extracted with absolute ethanol and quantified using the absorption coefficient given by Winterman and De Mots (1965).

Other methods. Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed with 12% (w/v) acrylamide concentration and/or gradient gel from 10 to 20% acrylamide concentration (Laemmli 1970). Autoradiography was performed using Kodak X-OMAT film.

Pea chloroplast CA antibodies were obtained from Prof. John Coleman (University of Toronto, Canada), *Chlamydomonas reinhardtii* periplasmic CA antibodies were obtained from Dr. James V. Moroney (Louisiana State University, USA), and corn plasma membrane ATPase antibodies were provided by Prof. Leonard T. Robert (University of California, Riverside, USA). The immunoblot assay was performed according to the protocol from Bio-Rad Laboratories (Bio-Rad, Richmond, Cal., USA) except that 5% non-fat dry milk was used to block the nitrocellulose. Goat anti-rabbit IgG(H + L) horseradish peroxidase conjugate and HRP Color development reagent were purchased from Bio-Rad Laboratories.

Results

Photosynthesis of D. tertiolecta. Table 1 shows differences in $K_{0.5}(CO_2)$ values for high- and low-CO_2-grown *D. tertiolecta*. Cells grown under low-CO_2 conditions showed increased affinity for inorganic carbon and required 10-fold less CO_2 to attain half-maximal photosynthetic rates than cells grown at high CO_2 levels. All experiments presented here were monitored by this method to verify that the CCM had actually been induced in healthy cells under low-CO_2 growth conditions.

Labelling cells with $^{35}SO_4^{2-}$. Identification of proteins that were preferentially synthesized under low-CO_2 conditions (Fig. 1) showed that at least seven polypeptides with molecular weights of approx. 45, 47, 49, 55, 60, 68 and 100 were induced by low CO_2. These polypeptides were induced under low-CO_2 conditions, but only in light, not in darkness. Three polypeptides with molecular masses of 45, 47 and 68 kDa appeared in large amounts in low-CO_2-grown cells (Fig. 1A). On the other hand, a 20-kDa protein is repressed in low-CO_2-adapting cells (Fig. 1A, compere lane 1 and 2). The 55-, 68- and 100-kDa proteins were observed in the low-speed pellet obtained after centrifugation of the low-CO_2-grown cell homogenate at 5000·*g* for 10 min, while a strong 60-kDa band appeared in the low-speed supernatant (Fig. 1B).

Carbonic anhydrase activity. Table 1 shows CA activity of cells under the two growth conditions. There was a signif-

Table 1. Photosynthesis and CA activity in *D. tertiolecta* grown under high- and low-CO_2 conditions. Data are means ± SD

Growth conditions	$K_{0.5}(CO_2)$[a] (μM)	CA[b] (units · mg Chl^{-1})
5% CO_2	40 ± 5	30 ± 10
0.03% CO_2	3 ± 1	390 ± 20

[a] $K_{0.5}(CO_2)$ is the half-maximal rate of photosynthesis. The rate of photosynthesis was measured in 20 mM Hepes-KOH supplemented with 0.5 M NaCl (pH 7.3) and an irradiance 500 μmol quanta·m^{-2}·s^{-1}.

[b] *D. tertiolecta* cells grown under high CO_2 concentrations were switched to low CO_2 for 6 h and the total CA activity in cell homogenates was measured

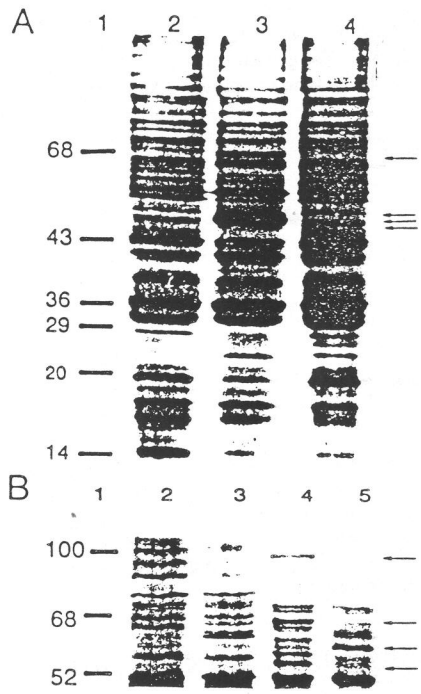

Fig. 1A, B. Autoradiographic analysis of ^{35}S-labeled protein produced by *D. tertiolecta* cells. The labeled cell extracts were subjected to gradient 10–20% SDS-PAGE analysis. A *Lane 1*, marker enzymes; *lane 2*, high-CO_2-grown cells; *lane 3*, low-CO_2 cells in light; *lane 4*, cells were switched to low CO_2 in darkness. B *Lane 1*, marker enzymes; *lanes 2, 3*, high-CO_2-grown cells; *lanes 4, 5*, low-CO_2-grown cells; *lanes 2, 4*, low-speed pellets that were obtained after centrifugation of cell homogenates at 5000·*g* for 10 min; *lanes 3, 5*, low-speed supernatants that were obtained after centrifugation of cell homogenates at 5000·*g* for 10 min. The *arrows* indicate the position of the low-CO_2-inducible proteins

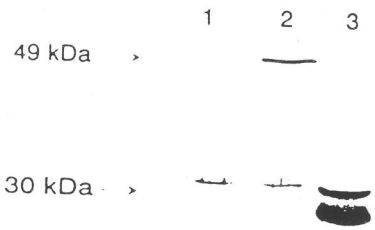

Fig. 2. Immunoblot protein analysis of whole-cell homogenates probed with antibodies raised against pea chloroplast CA. *Lane 1*, high-CO_2-grown cells; *lane 2*, low-CO_2-grown cells; *lane 3*, pea extract used as a positive control. Each lane contained 100 µg protein

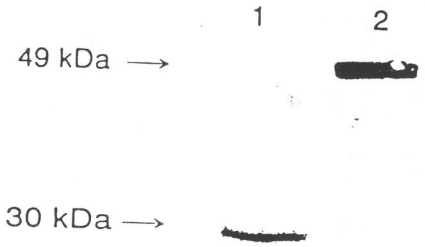

Fig. 3. Immunoblot protein analysis of lysed-chloroplast proteins and the soluble protein fraction eluted from a sulfonamide-agarose affinity column probed with pea chloroplast CA antiserum. *Lane 1*, lysed chloroplasts from low-CO_2-grown cells; *lane 2*, the soluble protein fraction eluted from a sulfonamide-agarose affinity column. Each lane contained 100 µg of protein

icant increase in CA activity in low-CO_2-grown *D. tertiolecta* cells.

Western blot protein analysis. Figure 2 shows protein immunoblot analysis using antibodies raised against pea chloroplastic CA. A band of 30 kDa appears in cells grown under both conditions, and a 49-kDa band specifically appears only in low-CO_2 cells.

Immunoblot analysis of the soluble protein fraction eluted from a sulfonamide-agarose affinity column and of lysed chloroplasts isolated from low-CO_2-cells showed that pea CA antibodies immunoreacted with a 49-kDa protein from the former and a 30-kDa protein from the latter (Fig. 3).

For the further localization of the 30-kDa protein, chloroplasts and plasma membrane were isolated from both high- and low-CO_2-grown cells and probed with antibodies raised against pea chloroplast CA. Figure 4 shows that the 30-kDa protein is located in the chloroplasts and not in plasma membranes. Similar results were obtained when spinach chloroplast CA antibodies were used (data not shown). An immunoblot of the *D. tertiolecta* proteins probed with antibodies raised purified *Chlamydomonas reinhardtii* periplasmic CA antibodies did not show cross-reactivity with cell homogenates from either high- or low-CO_2-grown cells (Fig. 5).

Figure 6 shows an immunoblot analysis of cell plasma membranes from both types of cell probed with antibodies raised against corn plasma membrane ATPase. The ATPase antibodies reacted with the 60-kDa protein in plasma membranes isolated from both cell types and with the 100-kDa protein that appears only in low-CO_2-grown cells.

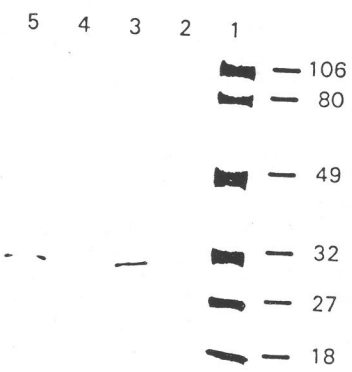

Fig. 4. Immunoblot protein analysis of isolated plasma membranes and lysed chloroplast proteins probed with pea chloroplast CA antiserum. *Lane 1*, marker enzymes; *lane 2*, plasma membranes from high-CO_2-grown cells; *lane 3*, lysed chloroplasts from high-CO_2-grown cells; *lane 4*, plasma membranes from low-CO_2-grown cells; *lane 5*, lysed chloroplasts from low-CO_2-grown cells. Each lane contained 100 µg of protein

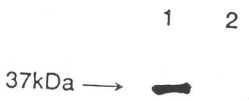

Fig. 5. Immunoblot protein analysis of *D. tertiolecta* cell homogenate probed with antibodies raised against *Chlamydomonas reinhardtii* periplasmic CA. *Lane 1*, low-CO_2-grown *C. reinhardtii* cells used as a positive control; *lane 2*, low-CO_2-grown *D. tertiolecta* cells

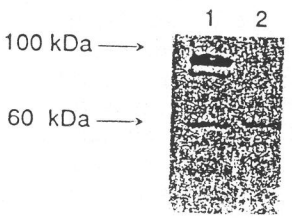

Fig. 6. Inmunoblot protein analysis of a plasma-membrane fraction from *D. tertiolecta* probed with antibodies raised against plasma-membrane ATPase. *Lane 1*, low-CO_2-grown cells; *lane 2*, high-CO_2-grown cells. Each lane contained 100 µg of protein

Discussion

The high affinity for DIC shown by *D. tertiolecta* cells grown in low CO_2 indicates that these cells induce the CCM. Since the appearance of the low-CO_2-inducible

proteins correlates with induction of the CCM (Coleman and Grossman 1984; Manuel and Moroney 1988; Spalding and Jeffrey 1989; Spalding et al. 1991), these polypeptides have been suggested as candidates for involvement in the CCM. Our results show that at least seven polypeptides with molecular weights of 45, 47, 49, 55, 60, 68 and 100 kDa are induced by low CO_2 (Fig. 1). Of these, three polypeptides with molecular masses of 45, 47 and 68 kDa appear in large amounts in low-CO_2 cells. The 45-kDa and the 47-kDa polypeptides have been specifically localized to the chloroplast envelope of *D. tertiolecta* cells grown under low-CO_2 conditions by Thielmann et al. (1992).

In many microalgae the CCM induced by low CO_2 involves both an extracellular and an intracellular CA (Spalding et al. 1983, 1991; Aizawa and Miyachi 1984, 1986; Badger 1987; Sültemeyer et al. 1990, 1993; Moroney and Mason 1991; Ramazanov and Cardenas 1992). Recently, the presence of an extracellular and an intracellular CA were also demonstrated in *D. tertiolecta* cells (Dionisio-Sese and Miyachi 1992; Goyal et al. 1992). In addition, Aizawa and Miyachi (1984, 1986) demonstrated that low-CO_2-grown *D. tertiolecta* have higher CA activity on their surfaces than within.

Our results (Table 1) also show an increase in CA activity in low-CO_2-grown cells, coincident with the appearence of a 49-kDa band which reacts with pea chloroplast CA antibody (Fig. 2). A 49-kDa protein in low-CO_2 *D. tertiolecta* is also shown to be a soluble protein which binds to the sulfonamide/agarose affinity column, and which is the sole immunoreactant among the soluble proteins eluted from the column (Fig. 3). This 49-kDa soluble protein possibly accounts for the rise in surface (or extracellular) CA activity even though the cross-reactivity is against a chloroplast membrane (or intracellular) CA. Clarification of this point is of special interest in order to understand (i) the roles of the different isoforms of CA in the biochemical mechanisms underlying adaptation of the photosynthesizing cells from high to low CO_2 concentrations, and (ii) the functional role of the cell organelles in this process.

The anti-CA reactive 30-kDa band appears to be a chloroplast protein and is present in both high- and low CO_2-grown cells (Fig. 3, 4). This protein does not appear in the soluble protein fraction which binds to the sulfonamide/agarose affinity column, but presumably passes freely through it, being bound to other membrane components. Sültemeyer et al. (1993) reported a similar characteristic for the chloroplastic CA of *Chlamydomonas reinhardtii*. When blotted against antibodies to the 37-kDa periplasmic (extracellular) CA of *C. reinhardtii* (Rawat and Moroney 1991) however, the chloroplast (intracellular) 30-kDa protein of *D. tertiolecta* did not cross-react (Fig. 5). This finding contradicts the results of Goyal et al. (1992) who reported that antiserum prepared against the 37-kDa polypeptide of periplasmic CA from *C. reinhardtii* did immunoreact with the 30-kDa polypeptide of *D. tertiolecta*, (without reference to the cellular localization of their 30-kDa protein). A possible explanation is that antibodies raised in different hosts were used in different experiments. Nevertheless, the immunoreactivity of the pea chloroplast CA antibodies with *D. tertiolecta* proteins provides evidence worthy of further exploration.

It is generally accepted that green algae are capable of inducing an ATPase-dependent HCO_3^- transport mechanism (Raven and Lucas 1985; Badger 1987; Thielmann et al. 1990; Karlsson et al. 1994). This mechanism is induced by limited CO_2 concentrations (Thielmann et al. 1990) and seems to be required for the transport of HCO_3^- across plasma membranes and the inner chloroplast membrane (Beardall 1981; Beardall and Raven 1981; Raven and Lucas 1985; Goyal and Tolbert 1989; Thielmann et al.1992). The requirement of a plasma-membrane ATPase for DIC transport in algae has been demonstrated in cells growing under low CO_2 concentrations (Thielmann et al. 1990; Karlsson et al. 1994), although the mechanism by which this ATPase is involved in DIC transport is still unknown. A specific protein for the transport of bicarbonate has not yet been identified, and there is some controversy about the localization of the putative transporter (Marcus et al. 1984; Goyal and Tolbert 1989; Thielmann et al. 1990). Pick (1992) proposed that the ATPase in plasma membranes and chloroplast envelopes is probably not a bicarbonate pump for the following reasons: (i) adaptation to low CO_2 does not increase the vanadate-sensitive ATPase activity in the plasma membrane, (ii) the ATPase activity in plasma membranes is not affected by bicarbonate, and (iii) ATP-dependent bicarbonate transport in plasma-membrane vesicles could not be demonstrated. In the same study, Pick reported that *Dunaliella* species contain plasma-membrane ATPases with molecular weights of about 60 and 100 kDa. Our results show that a 100-kDa protein is present among the $^{35}SO_4^{2-}$-labelled low-CO_2-inducible proteins (Fig. 6), that both a 100-kDa protein and a 60-kDa protein are immunoreact with antibodies raised against purified plasma-membrane ATPase of higher plants, and that both are located in the plasma-membrane fraction. This evidence suggests that the constitutive 60-kDa and the low-CO_2-inducible 100-kDa proteins in *D. tertiolecta* cells are the same plasma-membrane ATPases that have been characterized by Pick (1992). The 60-kDa protein could be the constitutive plasma-membrane ATPase whose activity is changed neither by low-CO_2 adaptation (CCM), nor by varying bicarbonate concentration. The 100-kDa inducible protein, localized in the plasma membrane, also immunoreacts with corn plasma-membrane ATPase antibodies. This protein may be responsible for the vanadate inhibition of photosynthetic HCO_3^- transport which is seen only in algae grown under low-CO_2 conditions (Thielmann et al. 1990; Karlsson et al. 1994).

In conclusion, our results suggest that both CA and the plasma-membrane ATPase each exist in two forms in *D. tertiolecta*, that one of each pair is controlled by low CO_2 in the culture medium, and that the 49-kDa and 100-kDa labelled polypeptides from low-CO_2-grown cells represent these inducible enzymes.

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antibodies raised against the 37-kDa periplasmic carbonic anhydrase of CO_2 *Chlamydomonas reinhardtii*, and Prof. Leonard T. Robert for a gift of corn plasma-membrane 100-kDa ATPase antibodies. We thank Dr. Jeanine Olsen (University of Groningen, the Netherlands) for style comments. This work was supported by the Instituto Tecnológico de Canarias (Spain).

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Photosynthetic production of hydrogen peroxide by *Ulva rigida* C. Ag. (Chlorophyta)

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Abstract. Production of hydrogen peroxide has been found in *Ulva rigida* (Chlorophyta). The formation of H_2O_2 was light dependent with a production of 1.2 μmol·g $FW^{-1}·h^{-1}$ in sea water (pH 8.2) at an irradiance of 700 μmol photons $m^{-2}·s^{-1}$. The excretion was also pH dependent: in pH 6.5 the production was not detectable (< 5 nmol·g $FW^{-1}·h^{-1}$) but at pH 9.0 the production was 5.0 μmol·g $FW^{-1}·h^{-1}$. The production of H_2O_2 was totally inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The ability of *U. rigida* growing in tanks (750 l) under a natural light regime to excrete H_2O_2 was checked and found to be seven times higher at 08.00 hours than other times of the day. The H_2O_2 concentration in the cultivation tank (density: 2 g $FW·l^{-1}$) reached the highest value (3 μM) at 11.00 hours. Photosynthesis was not influenced by H_2O_2 formation. The H_2O_2 is suggested to come from the Mehler reaction (pseudocyclic photophosphorylation). With an oxygen evolution of 120 mmol·g $FW^{-1}·h^{-1}$ at pH 8.2 and 90 mmol·g $FW^{-1}·h^{-1}$ at pH 9.0, 0.5% and 2.7% of the electrons were used for extracellular H_2O_2 production. The H_2O_2 production is sufficiently high to be of physiological and ecological significance, and is suggested to be a part of the defence against epi- and endophytes.

Key words: Chemical defence – Hydrogen peroxide – Mehler reaction – Photosynthesis – *Ulva*

Introduction

Oxygen-evolving photosynthetic organisms are exposed to increased concentrations of oxygen during photosynthesis. This is especially true for seaweeds with carbon-concentrating mechanisms, where oxygen pressures can be up to five times air equilibrium (Raven et al. 1994). Oxygen per se is not particularly reactive but it is potentially very toxic to organisms because partial reduction of oxygen causes the formation of active oxygen species, such as superoxide radicals ($O_2·^-$) and hydrogen peroxide (Fridovich 1978). It can also cause the formation of the very reactive hydroxyl radical (OH·), which will react with lipids, proteins and nucleic acids (Asada and Takahashi 1987). The production of active oxygen is usually considered detrimental to the organism, but H_2O_2 is also used in biosynthesis, in for example peroxidase activity, and in plant defence, as a part of the hypersensitive response (e.g. Apostol et al. 1989).

Production of H_2O_2 in plants can be divided into two types: photosynthetic and non-photosynthetic. Photosynthetic H_2O_2 production is mainly due to the Mehler reaction (pseudocyclic photophosphorylation) and photorespiration, here used in the restricted sense as the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Asada and Takahashi 1987). In the Mehler reaction, oxygen is used as electron acceptor, mostly in photosystem I, during photosynthesis forming $O_2·^-$ (Mehler 1951; Badger 1985). The radical $O_2·^-$ is disproportionated to H_2O_2 and O_2, spontaneously or with the aid of a superoxide dismutase (SOD). Hydrogen peroxide is formed in photorespiration when glycolate is converted into glyoxylate with the aid of an oxygenase (Asada and Takahashi 1987). Non-photosynthetic production of H_2O_2 can occur by, for example, incomplete reduction of oxygen in mitochondria and oxidase reactions, forming $O_2·^-$ and H_2O_2 (Fridovich 1986).

Excretion of H_2O_2 has been shown in several cyanobacteria, *Chlamydomonas sp.* and *Chlorogonium* (Chlorophyta), *Hymenomonas cartarae* (Prymnesiophyceae), *Euglena gracilis* and in *Pleurochrysis cartarae* (Chrysophyta) (Patterson and Myers 1973; Stevens et al. 1973; Palenik et al. 1987; Zepp et al. 1987; Palenik and Morel 1991; Ishikawa et al. 1993). Formation of H_2O_2 has also been shown from spinach chloroplasts (Steiger and Beck 1981). Uptake of oxygen, due to use of O_2 as the

Abbreviations: ACL = artificial, continuous light; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethylurea; GNL = greenhouse; LDC = Luminol-dependent chemiluminescence; SOD = superoxide dismutase

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electron acceptor in photosynthesis, has been shown in, for example, cyanobacteria (Miller et al. 1988), green microalgae (Sültemeyer et al. 1993) and higher plants (Marsho et al. 1979; Furbank et al. 1983). The photoreduction of O_2 took place even when the Calvin cycle was inhibited, showing that the activity is not due to the oxygenase activity of Rubisco (Radmer and Kok 1976; Miller et al. 1988).

Biological production of H_2O_2 has also been suggested as a way of manufacturing H_2O_2, as well as converting solar energy to chemical energy (Navarro et al. 1987). High, short-term production of H_2O_2 has been achieved by adding auto-oxidizable redox mediators, such as methyl viologen to cyanobacteria and chloroplasts (Morales and de la Rosa 1989; Morales et al. 1992).

The aim of this study was to examine the production of H_2O_2 by *Ulva rigida*.

Materials and methods

Plant material and culture conditions. Ulva rigida C. Ag. was collected from Taliarte, on the east coast of Gran Canaria in August 1993. Plants were cultivated at the Institute of Applied Algology/ITC, Canary Islands, Spain. Two cultivation methods were used:
(i) Under greenhouse conditions (GNL = greenhouse, natural light), free floating with continuous supply of air from the bottom of round-bottomed fiberglass tanks (750 l). Sunrise was at 07.00 hours and sunset at 20.30 hours; maximum irradiance reached 800 µmol photons·m^{-2}·s^{-1}. The initial density was 2 g FW·l^{-1} and the water exchange rate was 5 l·min^{-1}.
(ii) In 5 l tanks in a thermostatted room (25°C) with continuous light (ACL = artificial continuous light) of 50 µmol photons·m^{-2}·s^{-1} supplied by cool-white fluorescent lamps. The initial density was 2 g FW·l^{-1} and the water-exchange rate was 1 l·min^{-1}.

All cultures were pulse-fed with ammonium nitrate and phosphate 2–4 d before experiments.

Measurement of H_2O_2. Hydrogen peroxide was determined by measuring luminol-dependent chemiluminescence (LDC) following a modification of the method used by Glazener et al. (1991). Luminol solution was prepared by dissolving 8.8 mg luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma, St. Louis, Mo., USA) in 1 ml 1 M NaOH and adding 9 ml 0.4 M pH 7.0 Mops (3-[N-morpholino]propanesulfonic acid; Sigma) giving a final pH of 7.6. Horseradish peroxidase (Sigma) was dissolved in phosphate buffer (0.10 M pH 7.0) to a concentration of 11 µkat·ml^{-1}. Peroxidase (16 µl) and luminol solution (16 µl) were mixed and added to 1.0 ml sample. This gave a measuring pH of 7.3 ± 0.15. The LDC was measured using an LKB 1250 luminometer (LKB, Bromma, Sweden) and a flatbed recorder. A standard curve for H_2O_2 was made by diluting 30% H_2O_2 (Merck, Darmstadt, Germany) to approx. 20 mM, mixing 500 µl of diluted H_2O_2 with 500 µl acetate buffer (0.1 M pH 4), 500 µl 0.5 M KI and 20 µl saturated $(NH_4)_6Mo_7O_{24}$·4 H_2O, and further diluting to 10 ml (Pamatmat 1990). The amount of I_3^- released was measured spectrophotometrically after 10 min using an absorption coefficient of 22 900·M^{-1} at 353 nm (Alexander 1962). The detection limit was 50 nM.

Production of H_2O_2. Artificial, continuous light cultures. The experiments on H_2O_2 production were performed with 0.50 g FW of *U. rigida* in 50 ml sea water. To study the nature of the H_2O_2 production, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea dissolved in 96% ethanol) was added to the sea water to a final concentration of 400 µM. Hydrogen peroxide production at different pH values was measured in unbuffered sea water at 700 µmol photons·m^{-2}·s^{-1}. Adjustments to the pH were made with HCl or NaOH. In all the experiments, the sample was continuously stirred with a magnetic stirrer that was not in mechanical contact with the seaweed. The sea water used was initially devoid of H_2O_2.

Greenhouse, natural light cultures. U. rigida (3.0 g) was incubated in 300 ml sea water (pH 8.2) and the LDC was measured after 50 min. Measurements were performed every hour for 24 h and repeated over several days. The results are given per hour assuming that the production is linear. The H_2O_2 concentration in the tank where *U. rigida* was growing in the greenhouse was measured and also in an identical tank without seaweed.

Photosynthesis measurements. Photosynthesis was measured at 25°C with a Clark-type oxygen electrode (Hansatech, Kings Lynn, Norfolk, UK) incubating 15 mg of *U. rigida* in 2 ml medium. Light was supplied by a slide projector and measured by a Li-Cor Quantum Photometer (LI1000) with a quantum sensor (LI-193SA; Li-Cor, Lincoln, Neb., USA). Oxygen evolution at different pH values was measured in unbuffered sea water at 700 µmol photons·m^{-2}·s^{-1} with a minimum of four samples at each pH. Adjustments to pH were made with HCl or NaOH. The possible effect of H_2O_2 production on photosynthesis by GNL plants was analysed by measuring the photosynthesis in 700 µmol photons·m^{-2}·s^{-1} and at ambient (greenhouse) irradiance during a 24-h period.

Catalase (100 U·ml^{-1}; US Biochemical Company; Cleveland, Ohio, USA) was added to the reaction chamber of the oxygen electrode to study potentially harmful effects of H_2O_2 on photosynthesis on ACL-grown material. One unit of catalase is defined as the amount that decomposes µ 1 mol·min^{-1} at a concentration of H_2O_2 of 10 mM in pH 7 and 25°C.

Results

Artificial, continuous light cultures. Ulva rigida produced H_2O_2 in the light at a rate of 1.2 µmol·g FW^{-1}·h^{-1} at pH 8.2. Production of H_2O_2 was not detectable at pH 6.5, and at pH 9.0 it reached 5 µmol·g FW^{-1}·h^{-1} (Fig. 1). The rate of production in normal sea water was constant for at least 3 h (not shown). The LDC was inhibited by catalase (100 U·ml^{-1}), darkness and 400 µM DCMU (data not shown).

Photosynthetic oxygen evolution was 0.26 ± 0.04, 0.12 ± 0.03 and 0.09 ± 0.01 mmol O_2·g FW^{-1}·h^{-1} at

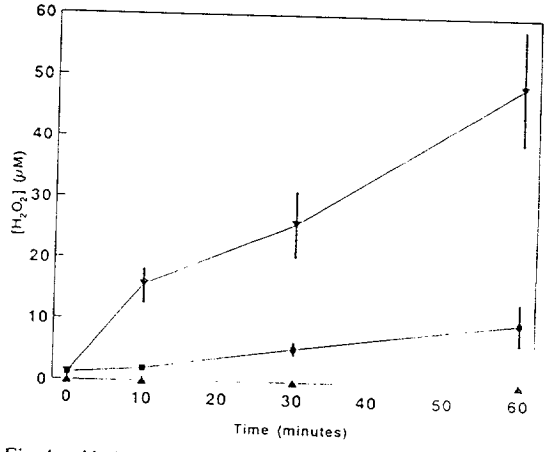

Fig. 1. pH dependence of H_2O_2 production by *Ulva rigida* (ACL cultures) in light (700 µmol photons·m^{-2}·s^{-1}). Experiments were performed with 0.50 g FW in 50 ml sea water at different pH values: 6.5 (▲), 8.2 (■) and 9.0 (▼). Shown are means ± SE (n = 3)

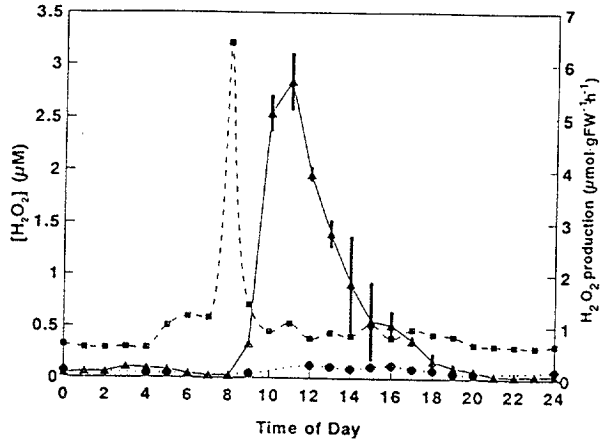

Fig. 2. Ability of *Ulva rigida* to produce H_2O_2 at different times of day. Production of H_2O_2 was measured in greenhouse material (GNL cultures) that was collected hourly and measured immediately after collection. In experiments, 3.0 g of alga was incubated in 300 ml sea water at 700 μmol photons·m^{-2}·s^{-1} and the production of H_2O_2 after 50 min was measured and expressed as production per hour, assuming that production was linear (■). The mean of two experiments (performed over 2 d) is shown; the difference between the experiments did not exceed 20%. Also shown is the H_2O_2 concentration in the cultivation tank (▲) and in a control tank without seaweed (●). Data are means ±SD of two experiments each with triplicate measurements

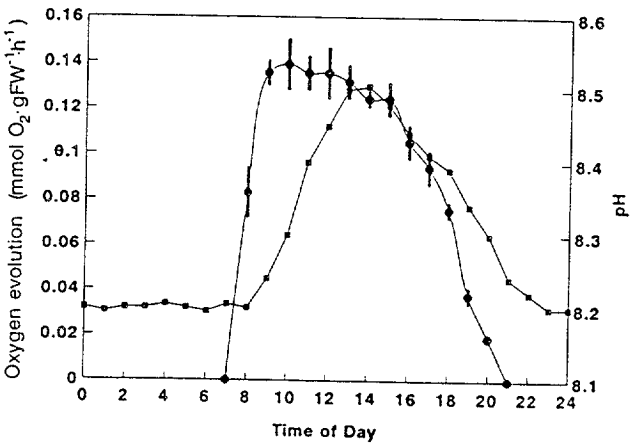

Fig. 3. Photosynthesis of greenhouse-grown *Ulva rigida* (GNL cultures), in ambient light (●), and pH in the tank (■). Photosynthesis is shown as mean ±SE ($n=3$)

pH 6.5, 8.2 and 9.0 respectively. Addition of catalase at pH 9.0 had no effect on oxygen evolution. The measurements for photosynthesis at different pH values are similar to those found by Björk et al. (1992) for *U. rigida*.

Greenhouse, natural light cultures. The ability to excrete H_2O_2 differed with the time of day. During most of the day and night, H_2O_2 production was approximately 0.8 μmol·g FW^{-1}·h^{-1}. There was, however, a drastic increase in the ability to excrete H_2O_2 at 08.00 hours to 6.3 μmol g FW^{-1}·h^{-1} (Fig. 2). The H_2O_2 concentrations in the *U. rigida* tank and a control tank without algae are

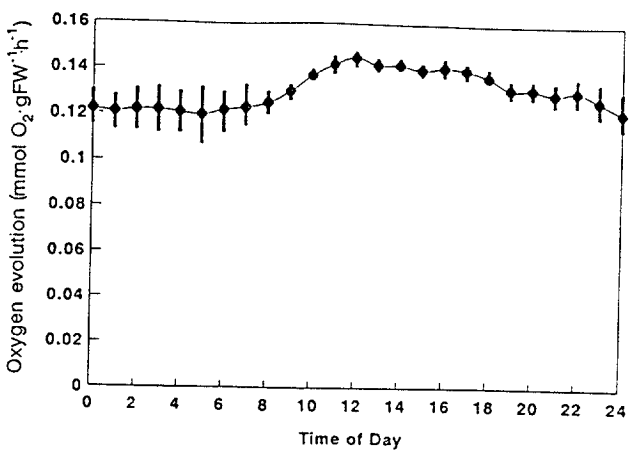

Fig. 4. Photosynthesis of greenhouse-grown *Ulva rigida* (GNL cultures) at 700 μmol photons·m^{-2}·s^{-1} at different times of day. In the experiments, 15 mg of alga was incubated in 2.0 ml sea water and the oxygen evolution was measured. Shown are means ±SE ($n=3$)

shown in Fig. 2. The highest concentrations of H_2O_2 were measured at 11.00 hours; thereafter H_2O_2 levels decreased. A low amount of H_2O_2 was formed in the control tank without seaweed, probably due to photochemical reactions. Similar data were recorded over a period of 10 d. However, the maximum amount of H_2O_2 produced differed between days and the time of the highest production varied by up to 1 h. The photosynthesis in ambient light changed with the irradiance (Fig. 3), the highest photosynthesis occurring at highest irradiances. The pH increased to 8.5 in the tank between 08.00 hours and 14.00 hours and then started to decrease to 8.2 again (Fig. 3). The photosynthesis in constant light (700 μmol photons·m^{-2}·s^{-1}) did not change during the day (Fig. 4).

Discussion

The disappearance of the LDC after addition of catalase shows that H_2O_2 has been formed in the experiments. It does not, however, prove that H_2O_2 is produced by *U. rigida* directly. Production of $O_2^{\cdot-}$ would produce similar response in the presence of sufficient amounts of SOD. To our knowledge, photosynthetic production of H_2O_2 has not been reported previously in seaweed.

The inhibition by DCMU and the light requirement for H_2O_2 production shows its photosynthetic nature. The probable source is the Mehler reaction, since glycolate dehydrogenase but not glycolate oxidase was found in *Ulva conglobata* (Suzuki et al. 1991). In many other green macroalgae a dehydrogenase is used in the conversion between glycolate and glyoxalate in stead of an oxidase, with the exception of *Nitella sp.* (Charophyta; Frederick et al. 1973; Tolbert 1976), and hence no H_2O_2 is produced during photorespiration. Björk et al. (1993) did not find any detectable photorespiration in *U. rigida* at pH 8.2 even at low total inorganic carbon, measured as the difference in oxygen evolution between 2% and 20%

O_2. This supports the hypothesis that H_2O_2 production in *U. rigida* is mainly derived from the Mehler reaction.

The Mehler reaction is a way, albeit ineffective, of producing ATP without simultaneous formation of NADPH (Raven and Beardall 1981). If ascorbate together with ascorbate peroxidase is used in scavenging of H_2O_2, the regeneration of ascorbate will cause NADPH-dependent ATP production (Foyer and Lelandais 1993). The ATP requirement for carbon-concentrating mechanisms in *Chlamydomonas reinhardtii* (Sültemeyer et al. 1993), *Synechoccus* (Miller et al. 1991) and in mesophyll chloroplasts of C_4 plants (Furbank et al. 1983) has been shown to be partly supplied by or dependent of the Mehler reaction. *Ulva* spp. have several different carbon-concentrating mechanisms, including extra- and intracellular carbonic anhydrase and anion transporters in order to increase intracellular concentrations of CO_2 (Björk et al. 1992, 1993; Drechsler et al. 1993). The increased H_2O_2 production with increased pH might thus be explained as an increased ATP need for carbon-concentrating mechanisms since the amount of available CO_2 will decrease with increasing pH. This is also consistent with the low production of H_2O_2 at pH 6.5, when the CO_2 concentration is high. An increased Mehler reaction with increased pH has also been found in *Chlamydomonas* (Sültemeyer et al. 1993). Reduced photosynthesis due to CO_2 limitation can also increase the amount of Mehler reaction when limited amounts of ribulose-1,5-bisphosphate (RuBP) are available for Rubisco. The Mehler reaction can then work as a sink for energy, causing a reduction of excited chlorophyll molecules (Raven and Beardall 1981). At pH 9.0, CO_2 concentrations and photosynthesis are lower and there is thus a higher need for energy dissipation. An increased amount of superoxide radicals is also formed by the Mehler reaction in chloroplasts when the ratio of NADPH/$NADP^+$ to O_2 is high (Steiger and Beck 1981; Scandalios 1993); this will occur when the amount of CO_2 is limiting for photosynthesis. The Mehler reaction has also been suggested to enable cyclic electron transport by controlling the redox state of photosynthetic electron carriers (Steiger and Beck 1981).

If the H_2O_2 comes from O_2, two electrons are needed for each H_2O_2 formed.

$$2e^- + 2O_2 + 2H^+ \rightarrow H_2O_2 + O_2$$

Four electrons are needed for each O_2 evolved. This means that more than 2.7% of the electrons at pH 9.0 and more than 0.4% of the electrons at pH 8.2 are used to produce H_2O_2.

The pattern found in the 24-h-study might be explained by dynamic pools of H_2O_2-scavenging enzymes. When the sun starts to shine in the morning, the production of H_2O_2 starts. Light might also induce production of H_2O_2-scavenging enzymes. Both synthesis and degradation of catalase has been shown to increase with light intensity (Hertwig et al. 1992). The high H_2O_2 production seen at 08.00 hours might thus be caused by low H_2O_2-scavenging activity. The broad peak of high H_2O_2 concentration compared to the short period of maximal H_2O_2 production in the cultivation tank (Fig. 2) can be explained by the higher pH found later in the day which will cause increased production of H_2O_2. The time difference between the highest production of H_2O_2 and increased content in the tank probably reflects the probability that the increased production is due to the increased pH of the cultivation medium rather than the higher ability to produce H_2O_2 (in pH 8.2) seen at 08.00 hours. The constant photosynthesis in constant light during the time of high H_2O_2 production shows, somewhat surprisingly, that the production of H_2O_2 does not seem to be detrimental to the photosynthesis of the alga (Fig. 4). This is also suggested by the absence of increased photosynthesis after addition of catalase.

A possible explanation for the limited breakdown of H_2O_2 in *U. rigida* might be that seaweed, in contrast to land plants, can scavenge H_2O_2 by diffusion. The H_2O_2 will diffuse readily through biological membranes out in the sea water (Takahashi and Asada 1983) but, because of its low vapour pressure, it will not easily evaporate from leaves. Hydrogen peroxide concentrations of 70 nmol·g FW^{-1} have been found in cucumber leaves (Patterson et al. 1984). This is about twice the concentrations achieved after 180 min in pH 8.2 by *U. rigida*, assuming similar concentrations of H_2O_2 in the media and inside the cells. The excretion of H_2O_2 from *Anacystis nidulans* (Patterson and Myers 1973) has been suggested to be due to the high K_m value of catalase for H_2O_2. This will cause leakage of H_2O_2 (Miyake et al. 1991). Frederick et al. (1973) pointed out that unicellular green algae with glycolate dehydrogenase that did not produce H_2O_2 in the conversion of glycolate had catalase activities of about 10% of that in higher plants.

The ecological consequences of H_2O_2 production are not clear but H_2O_2 has been used to control "red spot injury" caused by *Alteromonas* sp. in commercial cultivations of the brown alga *Laminaria japonica* (Ezura et al. 1990). Hydrogen peroxide has also been shown to inhibit fungal development; partial and total inhibition of germination in three species of pathogenic fungi was seen at 6.5 μM H_2O_2 and 26 μM, respectively (Peng and Kuc 1992). The acute toxicity of *Chatonella antigua* (Chromophyta) to fish in fish farms is thought to be caused partly by the excretion of hydrogen peroxide and superoxide into sea water (Shimada et al. 1991). It has also been shown that addition of 10 μM H_2O_2 decreased photosynthesis by 50% in chloroplasts of spinach, when H_2O_2 scavenging was suppressed by cyanide. Addition of catalase to the chloroplasts increased photosynthesis two- to sixfold (Kaiser 1976). Hydrogen peroxide also inhibits a number of metabolic enzymes, for example fructose bisphosphatase, ribulose phosphate kinase, NADP glyceraldehyd-3-fosfatase, Rubisco, CuZnSOD and FeSOD (Kaiser 1979; Badger et al. 1980; Asada et al. 1975). Increased amounts of H_2O_2 will also cause increased production of volatile halogenated compounds, for example $CHBr_3$, from the red macroalga *Meristiella gelidium* (Collén et al. 1994). The direct toxicity of H_2O_2 will increase in the presence of some metal ions, for example Fe^{2+} and Cu^+, and extremely reactive hydroxyl radicals will form. If O_2^- is produced, metal ions will act as catalysts in the production of hydroxyl radicals:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$$

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

Addition of 100 μM H_2O_2 caused a decrease in growth of *Euglena gracilis* between 5% and 45% depending on the internal concentrations of iron, with the lower value in iron-depleted cells (Radtke et al. 1992). All the above-mentioned reports indicate that the concentrations of H_2O_2 achieved by *U. rigida* can have ecological importance in the struggle against pathogens, epiphytes and endophytes, especially in tidal pools and similar areas with low water exchange where the pH increases as a consequence of carbon uptake. The concentration of H_2O_2 on the surface of the algae and in the cell wall is also probably much higher than the concentration measured in the sea water surrounding the algae. The short burst of H_2O_2 measured at 08.00 hours could be less detrimental for the organism than a continuous H_2O_2 production; but still cause protection. The advantage of using H_2O_2 as a chemical defence is probably the low cost, it will not deplete the seaweed of nutrients like nitrogen or carbon. The only direct cost is in the form of energy (electrons). The disadvantage is the general toxicity of H_2O_2. Hydrogen peroxide and other species of active oxygen could be important, but neglected, ecological variables.

Despite the fact that scavenging of H_2O_2 is generally considered to be very efficient in the chloroplast, because of the action of ascorbate and ascorbate peroxidases (Foyer and Lelandais 1993), *U. rigida* has a relatively high production of photosynthetic H_2O_2. This makes it an interesting species for further investigations on oxidative stress and photosynthesis.

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Effect of nitrogen supply on photosynthesis and carbonic anhydrase activity in the green seaweed *Ulva rigida* (Chlorophyta)

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Abstract Photosynthesis rate and carbonic anhydrase (CA) activity have been studied in the green seaweed *Ulva rigida* C. Agardh (Chlorophyta) grown in seawater (SW) and SW supplemented with 40 μM NH_4Cl (N–SW). Higher growth and maximal O_2 evolution rates were observed in N–SW- than in SW-grown seaweeds. Western blot analysis of the total homogenates probed with antibodies raised against small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) showed crossreaction with a 15 kdalton polypeptide in both SW- and N–SW-grown plants, although the band was more intense in N–SW-grown plants. Carbonic anhydrase activities in the total homogenate and in the soluble protein fraction were higher in N–SW-grown plants. Although the pellets from both plants showed a considerable CA activity, the activity of CA in the thylakoid membranes was undetectable. The low nitrogen concentration is a major environmental factor that affects the level of RuBisCO and CA, and therefore CO_2 assimilation in *U. rigida*.

Introduction

At pH 8.2, the concentration of HCO_3^- in SW is $\simeq 2.2$ μM, while dissolved CO_2 is $\simeq 12$ μM (Skirrow 1975). In aquatic photosynthetic organisms, the assimilation of dissolved inorganic carbon (CO_2 + HCO_3^-) (DIC) from the environment is affected by a CO_2-concentrating mechanism (CCM), which is a transport system that enhances the delivery of CO_2 to RuBisCO (Badger et al. 1980; Spalding and Portis 1985; Aizawa and Miyachi 1986). A CCM was found in *Ulva rigida* and other aquatic plants grown under natural environmental conditions (Drechler and Beer 1991; Björk et al. 1992, 1993). Apparently all seaweeds can use CO_2, which diffuses readily across the cell membranes (Raven and Lucas 1985; Smith and Bidwell 1987), but mechanisms for uptake of HCO_3^- are not yet clear. Since RuBisCO uses CO_2 as a substrate, HCO_3^- must be dehydrated to CO_2. In many seaweeds the CCM involves the enzyme carbonic anhydrase, which significantly increases the interconversion of CO_2 and HCO_3^- (Aizawa and Miyachi 1986; Björk et al. 1992, 1993; Haglund et al. 1992; Sültemeyer et al. 1993). Clarification of the actual role of carbonic anhydrase (CA) in CCM is complicated by the fact that several forms of CA have been found in seaweeds (Bjork et al. 1992; Haglund et al. 1992; Sültemeyer et al. 1993). Recently, the presence of an extracellular and an intracellular form of CA has been suggested in *U. rigida* (Björk et al. 1992, 1993). The inhibition of CA activity in algae leads to increased levels of photorespiration, which indicates that CA might be involved in the regulation of carbon metabolism through the photorespiratory glycolate pathway (Nilsen and Johnsen 1982; Ramazanov and Cárdenas 1992, 1994).

The concentration of inorganic nitrogen (NO_3^-, NO_2^- and NH_4^+) in Atlantic seawater is < 3 μM (Parson and Harrison 1983). Limitation of algal growth by nitrogen availability results in a decrease in N:C ratios, chlorophyll and protein concentrations and a lower fluorescence and photosynthetic yield (Lapointe and Duke 1984; Osborne and Geider 1986; Falkowski et al. 1989; Turpin 1991). Beardall et al. (1982) demonstrated that in *Chlorella emersonii* nitrogen limitation caused induction of the CCM, irrespective of the CO_2 concentration used for growth. Therefore, induction of the CCM in marine algae could be due to N-limitation (or starvation) and not only due to CO_2 limitation.

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In this work we studied the effect of ammonium availability on growth rate, photosynthesis, pigments, CA activity, RuBisCO concentration and NH_4^+ excretion in the green seaweed *Ulva rigida*.

Materials and methods

Plant material and culture conditions

Ulva rigida C. Agardh collected from the east coast of Gran Canaria (Canary Islands, Spain) was cultivated in 750-litre tanks under greenhouse conditions with a continuous flow of natural seawater (SW plants)- or nitrogen (40 μM NH_4^+)-supplemented seawater (N–SW plants) at an exchange rate of 8 vol d^{-1}. Inorganic nitrogen concentration in SW was undetectable. Plants (2 g fresh wt l^{-1}) were suspended in the water column by bubbling air from the bottom of the tanks. Mean photon flux density (PFD) was 710 $\pm$ 30 µmol m^{-2} s^{-1} at 14:00 hrs, with a daily fluctuation of water temperature from 20 to 25° C. Specific growth rates (μ) were calculated according to the equation ($\mu = 100 \ln (W_t/W_0)/t$) by D'Elia and Deboer (1978), where W_0 = initial fresh weight (FW); W_t = final fresh weight; and t = time (days).

Oxygen evolution

Photosynthetic O_2 evolution was measured with a Clark-type electrode fitted with a measuring chamber (Hansatech Instruments LTD., UK) thermostated at 20° C. Plant fragments of 5 mg FW were transferred to the chamber filled with seawater at pH 8.2. Sequential experiments were performed with the same piece of alga, but changing the incubation medium (seawater). A slide projector (Reflecta, Germany) was used as light source. Different values of illumination were achieved by placing the light source at different distances from the chamber. Light measurements were carried out with a radiometer LI-1000 data logger using a spherical quantum sensor LI-193SA (LI-COR, Nebraska, USA).

Enzyme assay

Carbonic anhydrase (EC 4.2.1.1.) activity in cell fractions was determined by the method described by Stemler (1993). Unique to this method is the use of $^{14}HCO_3^-$ and a reaction vessel with a gas-permeable membrane (Stemler 1993). A 10 g sample of algal thallus was homogenized in liquid nitrogen, extracted with a buffer containing 50 mM MES [2-(*N*-morpholino) ethanesulphonic acid], pH 6.5, and 5 mM EDTA. The crude homogenate thus obtained was centrifuged at 20 000 xg and 4° C for 1 h. CA activities were measured in the total homogenate, in the liquid phase, and in the pellets. The assay procedure was as follows: the glass filters at the bottom of the scintillation vials were wetted with 20 µl 0.1 M NaOH that acted as a $^{14}CO_2$ trap. The glass reaction-vessels were inserted into the scintillation vials. The membrane was then in direct contact with the NaOH-wetted glass filter. The fractions were resuspended in 50 mM MES, pH 6.5, and after an incubation period of 60 s the reaction was started by injection of 100 µl of a bicarbonate solution that contained 0.5 µCi $Na^{14}HCO_3$. The final concentration of $^{14}HCO_3^-$ was 28 μM and the final reaction volume was 300 µl (Stemler 1993; Moubarak-Milad and Stemler 1994). The reaction was stopped after 5 s by removing the reaction vessel from the scintilation vial.The radioactivity was then measured in a scintillation counter. Since samples are identical in their quenching characteristics, counts per min (cpm) values gave directly relative enzyme activity (Stemler 1993).

Isolation of thylakoid membranes

Isolation of thylakoid membranes particles from *Ulva rigida* was performed following the procedures described by Ramazanov et al. (1993). A 10 g sample of algal thallus was homogenized in liquid nitrogen and extracted with a buffer containing 50 mM MES, pH 6.5, and 5 mM EDTA. Crude homogenates were centrifuged at 50 000 xg and 4° C for 1 h and pellets were resuspended in 15 ml of 0.6 M sucrose in 10 mM Tris, pH 7.8, 1 mM EDTA, and incubated for 10 min at 0° C. The suspension was vigorously homogenized with a Potter-Elvehjem homogenizer. The homogenates were then adjusted to 1.3 M sucrose by dropwise addition of 1.8 M sucrose in 10 mM Tris, 1 mM EDTA, pH 7.8. On the top of the crude homogenate, 1.2 and 0.3 M of sucrose solutions were loaded in Tris-EDTA, pH 7.8, and were centrifuged at 75 000 xg for 5 h. After the flotation step, the green band of thylakoids was collected, washed twice with 50 mM MES buffer, pH 6.5, concentrated by centrifugation, and resuspended in 50 mM MES buffer, pH 6.5.

Other methods

SDS PAGE (sodium dodecyl-sulphate polyacramide-gel electrophoresis) of the total homogenate was performed with 12% (w/v) acrylamide concentration and/or a gradient gel (10 to 20% acrylamide concentration). The immunoblot assay was performed according to the Bio-Rad Laboratories protocol, except that 5% non-fat dry milk was used to block the nitrocellulose. Goat anti-rabbit IgG(H + L), horseradish peroxidase conjugate, and HRP (horseradish peroxide) colour development reagent were purchased from Bio-Rad Laboratories. Antibodies against spinach small-subunit RuBisCO were kindly provided by Professor S. Bartlett (Louisiana State University, USA).

Analytical measurements

Protein concentration was estimated according to Bradford (1976). Chlorophyll *a* was extracted with absolute ethanol and the concentration was determined by using the absorption coefficients given by Wintermans and De Mots (1965).

Ammonium excretion

Ten grams of *Ulva rigida*, previously cultivated in SW and N–SW, were washed with SW, transferred to a 1-litre Erlenmeyer flask containing SW, and maintained at 25° C and a light intensity of 200 µmol m^{-2} s^{-1} provided by white fluorescent lamps. N–SW plants maintained under the same conditions but in darkness were used as controls. Ammonium concentration in the assay medium medium was measured every 2 h according to the gas-diffusion method described by Ronnestad and Knutsen (1991), using a flow injection analyzer (FIA-Star 5010 Analizer, Tecator, Sweden)

Inhibitors

Ethoxyzolamide (EZ) (Sigma, St. Louis, Missouri, USA) was dissolved in 50 mM NaOH to a concentration of 100 mM. The pH of the solution was adjusted to 8.0 using HCl. A final concentration of 100 μM in the assay medium was used to inhibit CA activity. 1 mM L-methionine-D,L-sulfoximine (MSX) (Sigma, St. Louis, Missouri, USA) was used to inhibit glutamine synthetase (GS) activity

Table 1 *Ulva rigida*. Growth characteristics and CA activity in seaweed grown in natural seawater (*SW*) and in seawater supplemented with nitrogen (*N–SW*) and in presence of ethoxyzolamide (*EZ*). Protein concentration in each sample was 4 mg ml^{-1}. CA activity in blank (buffer without sample) was 1.0 ± 0.2 cpm × 10^3 (*FW* fresh wt)

Growth conditions	Growth rate (% d^{-1})	Chlorphyll conc (mg g^{-1} FW)	CA activity (cpm × 10^3)			
			Homogenate	Soluble proteins	Pellet	Thylakoids
SW	6.5 ± 1.2	0.83 ± 0.04	8.0 ± 1.0	4.0 ± 1.0	3.0 ± 0.1	1.0 ± 0.3
SW + 100 μ*M* EZ			1.0 ± 0.1	0.7 ± 0.2	0.9 ± 0.1	0.9 ± 0.1
N–SW	14.2 ± 2.7	1.16 ± 0.06	14.0 ± 1.0	10.0 ± 0.8	3.0 ± 1.0	1.0 ± 0.2
N–SW + 100 μ*M* EZ			1.0 ± 0.3	0.9 ± 0.4	0.8 ± 0.1	0.9 ± 0.3

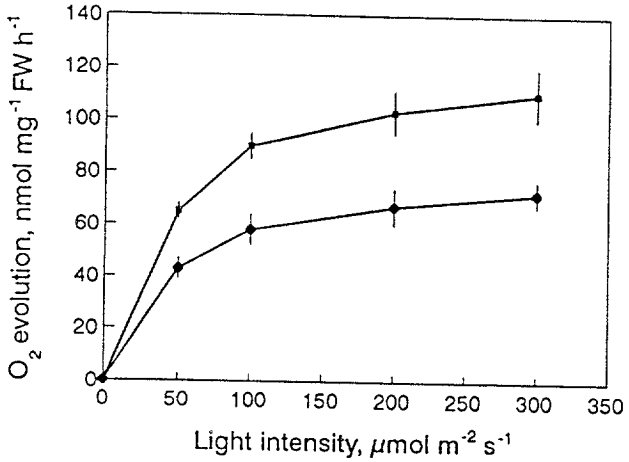

Fig. 1 *Ulva rigida*. Light-dependent photosynthetic oxygen evolution of seaweed in natural seawater (●) and in seawater supplemented with nitrogen (■) (*Bars* ± SD, *n* = 6; *FW* fresh wt)

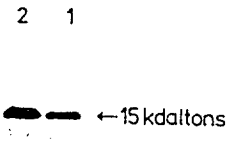

Fig. 2 *Ulva rigida*. Immunoblot protein analysis of total homogenates probed with antibodies raised against small subunits of Rubisco (*Lane 1* crude extract from SW-grown seaweed; *Lane 2* crude extract from N–SW grown-seaweed). Each lane contains 100 μg protein

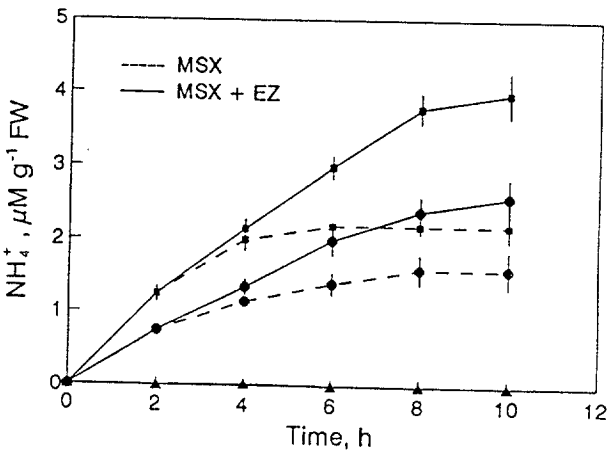

Fig. 3 *Ulva rigida*. Effect of L-methionine-sulphoximine (*MSX*) and ethoxyzolamide (*EZ*) on ammonium excretion in SW (●)- and N–SW (■)-grown seaweed (▲ ammonium excretion without inhibitors; *bars* ± SD, *n* = 6) Light intensity was 200 μmol m^{-2} s^{-1}

Results

Ulva rigida grown in seawater supplemented with nitrogen displayed a growth rate of 14.2 ± 2.7%, while the growth rate in SW was only 6.5 ± 1.2% increment d^{-1} of fresh wt (Table 1).

Photosynthetic O_2 evolution as a function of light intensity in *Ulva rigida* at pH 8.2 exhibited saturation at 200 μmol photons m^{-2} s^{-1} in both SW and N–SW-grown plants, although the maximal rate of photosynthesis was higher in the N–SW-grown plants (Fig. 1).

Immunoblots of total proteins probed with antibodies raised against small subunits of RuBisCO showed crossreaction with a 15 kdalton protein from both SW- and N–SW-grown seaweeds, although the band was more intense in N–SW-grown plants (Fig. 2).

Total CA activity in the homogenate and in the soluble protein fraction was higher in N–SW-grown plants, while CA activity in the pellets was similar in both SW- and N–SW-grown plants (Table 1). CA activity associated with thylakoid membranes was undetectable, whereas 100 μ*M* of EZ completely inhibited CA activity in the other fractions. These results indicate that CA in *Ulva rigida* is not a thylakoid membrane-bound enzyme.

Fig. 3 shows the effect of L-methionine-D,L-sulfoximine (MSX) and EZ on the rate of ammonium excretion in *Ulva rigida*. Ammonium excretion was observed only in the presence of MSX in light, being stimulated by the inhibition of CA by EZ. The NH_4^+ excretion rate was higher in seaweeds previously grown in N SW. When incubated with EZ, the ammonium excretion rate was 50% higher in N SW- than in SW-grown plants.

Discussion

The concentration of nitrogen is one of the factors that most frequently limits productivity of macroalgae in natural seawater (Ryther and Dunstan 1971; Hanisak 1979 a, b; Smith 1984). Our results demonstrate that NH_4^+ availability in the medium controls the level of RuBisCO and CA activity in *Ulva rigida*. Several authors have suggested that the level of DIC available for photosynthesis in SW is sufficient to allow maximum rates of photosynthesis of seaweeds at very low PFD but, under low hydrodynamic fluxes and higher PFD levels that sometimes occur under natural conditions, the concentration of DIC can be a limiting factor (Holbrook et al. 1988; Levavasseur et al. 1991; Raven 1991). However, growth and photosynthesis rates, at the same pH and DIC concentration in seawater, were enhanced in N–SW (Fig. 1). The degree of N limitation influences the net photosynthetic rates (Li and Goldman 1981; Osborne and Geider 1986), probably due to a reduction of the amount of RuBisCO (Lapointe and Duke 1984; Duke et al. 1986; Beardall et al. 1991; García-Sánchez et al. 1993) and pigment concentrations (Turpin 1991; García-Sánchez et al. 1993; Vergara and Niell 1993). The results of the western-blot protein analysis showed that the concentration of RuBisCO in *U. rigida* is dependent on the availability of NH_4^+ in the growth media (Fig. 2), and its content decreased when plants grew in N-limited conditions (Fig 2). We suggest that the higher O_2-evolution rate in N–SW-grown *U. rigida* is due to relatively higher RuBisCO concentration (Fig. 2) and CA activity (Table 1). The relative increase in CA activity with the increase in RuBisCO content is consistent with the optimization model of protein budget between RuBisCO and CA developed by Cowan (1986). Majeau and Coleman (1994) demonstrated a coordinated expression of RuBisCO and CA genes in the pea, and suggested that a link between RuBisCO and CA expression may be required for the maintenance of a CA:RuBisCO ratio that is optimal for efficient photosynthetic carbon assimilation. Since the substrate for RuBisCO is CO_2, HCO_3^- must be dehydrated to CO_2, and therefore CA is the most likely enzyme for this conversion (Aizawa and Miyachi 1986; Kuchitsu et al. 1991; Badger and Price 1994). Pronina and Semenenko (1990) speculated that thylakoid membranes in the green alga *Chlorella vulgaris* are freely permeable to the HCO_3^- ion, and that the intrathylakoid membrane-bound CA catalyzes the dehydration of HCO_3^- to CO_2. However, the existence of a HCO_3^- transport mechanism into the intrathylakoidal space has not yet been demonstrated. Both soluble and insoluble fractions showed a considerable CA activity, but CA activity in the thylakoid membranes was undetectable (Table 1). Therefore, it can be concluded that in *U. rigida* CA is located in the soluble and membrane fractions, but neither of these is associated with thylakoid membranes. Our data with respect to the location of CA contradict the results of Pronina and Semenenko. This could be due to possible contamination of the thylakoid membranes by other cell membranes in the latter study. The exact location and functional role of the different forms of CA in *U. rigida* is not yet clear, and the variation in the intracellular location of CA in different organisms cannot be excluded.

Most marine algae assimilate CO_2 via the C_3 pathway with RuBisCO as a carboxylating enzyme (Kerby and Raven 1985). The C_3 pathway of CO_2 assimilation is competitively inhibited by high O_2 concentration, and a high intracellular O_2:CO_2 ratio is favourable for oxygenase activity and the photorespiratory glycolate pathway (Bowes and Ogren 1972; Lorimer 1981). However, many seaweeds have a suppressed rate of photorespiration due to the activity of the CCM (Beer and Israel 1986, 1990; Björk et al. 1993). In the presence of MSX, photorespiratory ammonium is excreted into the medium in *Ulva rigida* (Fig. 3), as has been described for *Chlamydomonas reinhardtii* cells by Peltier and Thibault (1983). The inhibition of CA activity leads to increased levels of ammonium excretion (Fig. 3), indicating that in algae the carbon flow through the photorespiratory glycolate pathway may be influenced by CA activity and the CCM.

Nitrogen availability determines not only the concentration of RuBisCO and therefore the photosynthetic performance in *Ulva rigida*, but also the concentration of CA, which plays an important role in the regulation of the CCM and carbon flow through the photorespiratory glycolate pathway in most aquatic plants.

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Natural Decoloration, Composition and Increase in Dietary Fibre Content of an Edible Marine Algae, *Ulva rigida* (Chlorophyta), Grown under Different Nitrogen Conditions

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Abstract: The colour, dietary fibre content and characteristics of the edible marine green algae, *Ulva rigida*, were investigated in relation with the nitrogen content of the algal culture medium. Colour brightness decreased and total nitrogen of the algae increased in nitrogen-enriched conditions. When nitrogen-enriched seaweeds were grown again in normal seawater for 10 days, colour brightness increased and total nitrogen concentration fell. Total dietary fibre content of the wild and nitrogen enriched algae were close (~38·0% DW) but increased markedly when the nitrogen-rich plants were cultured in normal seawater (54·4% after 10 days). Soluble fibre were xylorhamnoglucuronan sulphate (ulvan) with close molar compositions for all the seaweed samples. Insoluble polysaccharides were composed of glucose, xylose, uronic acid, rhamnose and sulphate which molar proportions varied for the different algal samples. The soluble fibre intrinsic viscosity and the water holding capacity of insoluble fibre were not markedly affected by the growth conditions. This report demonstrates that edible seaweed aquaculture provides an alternative or a complement to post-harvest transformations of seaweeds to modify organoleptic and nutritional characteristics.

Key words: Decoloration, dietary fibre, marine algae, *Ulva rigida*, aquaculture, nitrogen, ulvan, chemical composition, ^{13}C NMR spectroscopy, viscosity, water holding capacity.

INTRODUCTION

Interest in edible seaweeds as novel foods is increasing in Europe (Mabeau and Fleurence 1993) and among their nutritional benefits (see Darcy-Vrillon 1993) is their high dietary fibre contents (Lahaye 1991; Nishimune *et al* 1991). These fibres are food polysaccharides resistant to degradation by endogeneous digestive enzymes (Trowell 1974) and are involved through various mechanisms in the prevention of certain pathologies (digestive diseases, obesity, cancer heart diseases) (Southgate 1990). The nutritional properties of dietary fibres depend on their physico-chemical properties such as their solubility in water and their ability to be fermented by human colonic bacteria to short-chain fatty acids (SCFA) (Johnson 1990; Kritchevsky 1990; Southgate 1990). Among edible seaweeds two green macrophytes *Ulva lactuca* and *Enteromorpha compressa* contain 36·6 and 40·0% total dietary fibres on the algal dry weight basis, respectively (Lahaye and Jegou 1993).

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Soluble fibres (15·8 and 14·9% for *U lactuca* and *E compressa*, respectively) consist of low viscosity sulphate xylorhamnoglycuronans, referred to as ulvan, that were shown in some species to form gels in presence of boric acid and calcium (Haug 1976; Lahaye and Axelos 1993). The hydrophilic insoluble fibres (24·2 and 21·6% for *U lactuca* and *E compressa*, respectively) are enriched in glucans, alkali-insoluble xyloglucans (Lahaye *et al* 1994) β-1,4-glucuronan and ulvan (Jegou and Lahaye 1992; Ray and Lahaye 1995). For *Ulva*, only the insoluble fibres were partially fermented to SCFA and the resistance of ulvan to gut flora degradation was related to the chemical linkages between the constituent sugars (Bobin-Dubigeon *et al* 1993). Physico-chemical properties of dietary fibres such as, water solubility, viscosity, ion exchange capacities, hydration properties (water holding, water binding capacities, swelling) are closely related to their chemical composition and structure (Morris 1992). In the case of algae, this structure can vary according to several factors such as the growing conditions (Percival and McDowell 1981). Among them, the nitrogen content in the culture medium affects the biosynthesis of the algal cell wall polysaccharides but also of other compounds such as proteins and pigments (DeBoer 1981; Lobban *et al* 1985). As the deep colour of edible seaweeds is one of the factors that can limit their use as source of fibres by food industries, a natural means of decoloration would be beneficial. This paper reports on the effect of nitrogen concentration variations in the culture medium of *Ulva rigida* on its colour and dietary fibre content and characteristics.

MATERIALS AND METHODS

Materials

Ulva rigida C Ag. (Chlorophyta) was cultivated for a period of 2 months in 750 litre aerated tanks with running seawater enriched with ammonium at a concentration of 1·25 ($\pm$0·41) mg litre^{-1} (maximum light intensity 1500 μmol m^{-2} s^{-1}, water temperature 24 $\pm$ 2°C). After this period plants were maintained under the same conditions with normal seawater (without any nitrogen addition, natural nitrate, nitrite and ammonium concentrations $\leq$20, 0·5 and 3 μM, respectively; Parson and Harrison 1983) for a period of 2 weeks during August 1993. Samples were taken every 2 days and sun dried to constant weight.

Methods

Colour determination

Algal colour brightness (L^* following the Minolta method) was determined in duplicate on powders of particle size between 250 and 500 nm using a Uvikon 810P colorimeter (Kontron Instruments). Whatman microcrystalline cellulose was used as a white colour reference.

Dietary fibre

Soluble and insoluble dietary fibre contents were determined according to the AOAC method (Prosky *et al* 1988) as modified by Lahaye (1991). Percentage of soluble and insoluble dietary fibres were obtained by subtracting total residual proteins and ash contents from the respective yield of the liquid and solid fractions.

Chemical analysis

All chemical analysis were done at least in duplicate on moisture free samples. Proteins were measured as nitrogen by the Kjelhdal method (N $\times$ 6·25). Ash content was determined after 16 h incineration at 550°C followed by 2 h at 900°C. Neutral sugars were quantified after sulphuric acid hydrolysis by gas–liquid chromatography as described by Hoebler *et al* (1989). Uronic acids were quantified by the automated colorimetric method using *m*-phenylphenol (Thibault 1979). Sulphate was measured by high-performance liquid chromatography after HCl acid hydrolysis as described by Lahaye and Axelos (1993).

Nuclear magnetic resonance spectroscopy

^{13}C NMR spectra of 4% polysaccharide solution in deuterium oxide were recorded at 60°C on a Bruker ARX 400 spectrometer. Chemical shifts were calculated from dimethyl sulphoxide assigned to 39·4 ppm.

Physico-chemical characterisation

Water holding capacities were determined with water at 25°C on insoluble fibre fractions by capillarity using the Baumann apparatus (Baumann 1967). Viscosity of solutions of soluble fibres in 150 mM NaCl were measured at 37°C with an automatic viscosimeter (Viscomatic VCD Amtec, France). Intrinsic viscosities were derived from the Kramer and Huggins equations by extrapolation to infinite dilutions (Billmayer 1984).

RESULTS AND DISCUSSION

The particular organoleptic properties of edible algae can limit their consumption in the West. To improve the acceptance of these novel foods and to extend their use as ingredients for the food industries, chemical, physical and enzymatic methods of modification are now under study (Darcy-Vrillon 1993). As an alternative or in complement to these modifications is the culture of 'sea-vegetables' under controlled conditions that modify texture, colour, taste and/or nutritional values. The effect of nitrogen enrichment in the growing

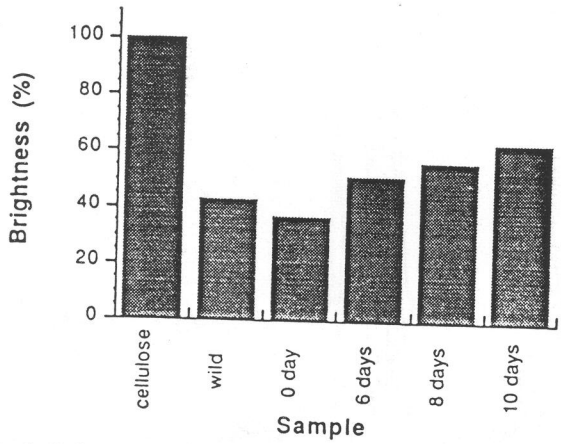

Fig 1. Brightness (% of reflected light) of wild *rigida* and samples grown 0, 6, 8 and 10 days in seawater after being cultured in a nitrogen-enriched environment.

medium on the pigmentation and dietary fibre content and characteristics of *Ulva rigida* was investigated. Deeply pigmented biomass was rapidly produced under nitrogen-enriched conditions (maximum production 38–40 g DW m^{-2} day^{-1}) which was then bleached by a subsequent culture in normal seawater for several days (Fig 1). The marked increase in nitrogen content of the algae (from 2·1 to 4·2% total N in wild and 0 day *U rigida* samples, respectively, Table 1) seen during the fast growth period was followed by the consumption and/or the loss of the nitrogen containing constituents (proteins, pigments, etc) when the seaweed was placed back in normal seawater (to reach 0·9% total N in the 10 days sample, Table 1). Nitrogen enrichment of the growing medium did not affect markedly the total dietary fibre contents (on the dry weight basis) of *U rigida* compared to that of the wild sample (39·0 and 38·0% for the 0 day and wild samples, respectively, Table 2) and the amounts obtained agreed with literature data (Lahaye 1991; Lahaye and Jegou 1993). However, fibre content was higher in nitrogen enriched samples cultured for 6 and 10 days in normal seawater (43·8 and 54·4%, respectively; Table 2). The crude fibre fractions from these algal samples also contained lower residual protein contents than the wild and nitrogen enriched *Ulva* (Table 2). This most likely reflected the low initial protein content in the plants rather than their greater protease sensitivity during fibre extraction. If the total dietary fibre content is expressed on ash and protein free dry algae (Table 1, assuming that all the N measured comes from proteins), they amount to 68·2,

TABLE 1

Ash and total nitrogen content of wild *Ulva rigida* and samples cultured for 0, 6 and 10 days in seawater after being grown in nitrogen-enriched seawater

Sample	*Ash (% dry weight)*	*Nitrogen (% dry weight)*
Wild	31·2	2·1
0 day	22·3	4·2
6 days	12·9	1·7
10 days	27·5	0·9

TABLE 2

Dietary fibres content and chemical composition of soluble (Sol) and insoluble (Ins) dietary fibers from wild *Ulva rigida* and samples cultured for 0, 6 and 10 days in seawater after being grown in nitrogen-enriched seawater

	Wild			*0 day*			*6 days*			*10 days*		
	Sol	*Ins*	*Total*	*Sol*	*Ins*	*Total*	*Sol*	*Ins*	*Total*	*Sol*	*Ins*	*Total*
Fibre content[a]	18·5 (1·1)	19·5 (1·3)	38·0	21·5 (0·5)	17·5 (1·0)	39·0	23·3 (0·9)	20·5 (0·5)	43·8	29·0 (0·3)	25·4 (0·8)	54·4
Uronic acids[b]	17·0	7·1		14·9	5·2		16·6	6·1		17·4	6·2	
Neutral sugars[b]	47·3	38·4		51·5	38·7		53·3	51·9		56·6	55·6	
Ash[b]	20·4	11·5		15·7	14·8		22·3	8·7		16·8	13·0	
Protein[b]	14·7	24·8		12·5	26·6		11·2	14·7		7·4	6·0	
Rhamnose[c]	26·9	10·8		25·1	9·3		25·6	8·7		26·3	8·7	
Xylose[c]	10·6	15·2		15·3	23·9		14·6	22·8		13·3	22·5	
Mannose[c]	2·5			3·1			2·2			2·3		
Galactose[c]	tr			0·9			0·3			0·7		
Glucose[c]	1·7	37·5		2·0	35·8		1·6	41·1		2·4	42·5	
Uronic acids[c]	21·5	14·0		17·3	10·2		18·3	9·2		18·2	8·9	
Sulphate[c]	36·7	22·4		36·4	20·8		37·3	18·3		36·8	17·4	

[a] Weight percentage of the algae weight (SD, $n = 3$).
[b] Weight percentage of the fraction dry weight ($n = 2$).
[c] molar percentage ($n = 2$).

75·9, 83·4, and 81·3% of the wild, 0, 6 and 10 days *U rigida* samples, respectively. Thus, an increase in polysaccharide content was observed for all the cultured algae and may not only be related to the loss and/or consumption of nitrogeneous compounds but also probably of other molecules such as reserve metabolites (starch, lipids, etc). Growing conditions also affected soluble to insoluble dietary fibre ratios (S/I). The higher proportion of soluble polysaccharides in nitrogen enriched *U rigida* (S/I = 1·23) than in the wild algae (S/I = 0·93) may be related to its high growth rate. Rapidly dividing algae produce new cell walls that may contain more matricial polysaccharides with lower self-associating abilities (gelling properties) as it is encountered in young actively growing brown and red algal tissues (Craigie *et al* 1984; Craigie 1990). A constant S/I = 1·14 was calculated for the 6 and 10 days samples. On a nutritional point of view, the amounts of *U rigida* soluble dietary fibres measured in this study represent maximum values as all of them will probably not be solubilised in the digestive tract. Indeed, the method used to measure them, although close to the generally accepted official methods (Prosky and De Vries 1992), is far from physiological conditions. Lower amounts of soluble fibres are generally obtained from edible seaweeds, fruits and vegetables when temperature, pH and ionic conditions are closer to those found in the digestive tract (Fleury and Lahaye 1991; Lahaye *et al* 1992; Monro 1993). For example, only about 56% of the *U lactuca* soluble fibres were extracted with these conditions (Lahaye and Jegou 1993). Fibres contents will also be affected by processing such as cooking (Monro 1993; Suzuki *et al* 1993).

The chemical composition of the dietary fibres (Table 2) were in agreement with the literature data (Jegou and Lahaye 1992; Lahaye and Jegou 1993). Polysaccharides in all the soluble fibres fractions were sulphated and essentially composed of rhamnose, glucuronic acid and xylose with low amounts of glucose and mannose. Trace quantities of galactose and of an unidentified monomethylated hexose were also detected. On a molar percentage basis, the content in sugars (except xylose) and sulphate of the different soluble fibres isolated from cultured algal samples remained close regardless of the time spent in normal seawater. Soluble fibres from wild *U rigida* contained slightly less xylose and more uronic acid than the cultured *U rigida* samples.

The ^{13}C NMR spectra of the water soluble polysaccharides from the wild sample and the *Ulva* grown 0 and 10 days in normal seawater after nitrogen enrichment showed well resolved signals demonstrating a regular structure for the polysaccharides (Fig 2). Characteristic signals for C6 of rhamnose and uronic acid were observed at 17·8 and 175·8 ppm, respectively. The attribution of the other resonances is under study. No major difference was seen between the spectra of ulvan from *U rigida* samples cultivated for 0 and 10 days in

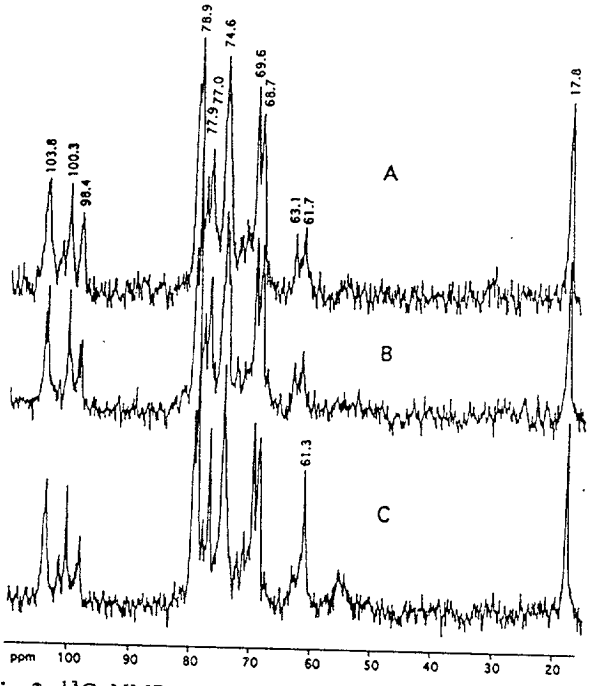

Fig 2. ^{13}C NMR spectra of water-soluble polysaccharides from *Ulva rigida* grown for (A) 0 and (B) 10 days in normal seawater after nitrogen enrichement and (C) from wild *U rigida*.

normal seawater after nitrogen enrichment (Fig 2(A) and 2(B)). This indicated that the polymers from the latter sample were probably those that were synthesised during the fast-growing period and it also suggested that no major structural modification occured once the polysaccharides were deposited in the cell wall. The spectrum of ulvan from the wild *U rigida* sample differed from these two spectra in the intensity of the signal at 77·0 ppm and by the presence of a new resonance at 61·3 ppm (Fig 2(C)). Thus, although the different culture conditions did not affect the sugar and sulphate contents of the soluble fibres, growth in the wild and under controlled conditions possibly affected their chemical structure (sugar sequence, sulphation pattern, etc).

Sulphated polysaccharides also comprised the insoluble fibres fractions from the wild sample and the cultured algae (Table 2). Major sugars were glucose, xylose, uronic acid, rhamnose and trace quantities of mannose and galactose. On a molar percentage basis, the insoluble fibres from the wild algal sample contained less xylose and more uronic acid, rhamnose and sulphate than the cultured samples. The sugar composition and sulphate content of the insoluble fibres from the cultured seaweeds were affected by the time spent in normal seawater after being removed from the nitrogen-enriched medium. The uronic acid and sulphate content decreased (from 10·2 to 8·9% and 20·8 to 17·4%, respectively, for the 0 and 10 days samples) whereas that of glucose increased to reach 42·5 mol% in the

10 days sample. The decrease in ulvan (rhamnose, uronic acid and sulphate) and the increase in xylose and glucose contents observed between the insoluble fibres of the wild and those of the different cultured *Ulva* samples most likely resulted from a better extraction of ulvans rather than a modification of the biosynthesis of the cell wall polymers. Proteins of intra- and/or extracellular origin may complex and thus insolubilise part of the ulvan during extraction or may belong to glycoproteins cross-linking ulvan with themselves or with the insoluble glucans/xyloglucans. The presence of glycoproteins in *U lactuca* has been proposed (Abdel-Fattah and Sary 1987).

Physico-chemical properties were not markedly affected by the particular growing conditions. The intrinsic viscosity of the soluble fibres were 169 ml g^{-1} for the wild and 0 day samples, 209 and 233 ml g^{-1} for the 6 and 10 days samples, respectively, and were close to published values (Lahaye and Jegou 1993). The slightly higher values for samples grown in normal seawater after nitrogen enrichment may indicate a small molecular weight increase of the polymers or a lower contribution of residual proteins to viscosity. The water holding capacities (WHC) of the insoluble fibres were 24·6, 25·6 and 26·1 g g^{-1} for the wild, 0 and 10 days samples, respectively. These values were within the range of those reported for other seaweeds (Fleury and Lahaye 1993) and were close to those of fruits and vegetables (Thibault *et al* 1992). The slight increase in the WHC of insoluble fibres from cultured *Ulva* can be related to several factors such as different polymers associations allowing for a greater number of pores and/or different chemical compositions since the fibres containing low residual protein contents had slightly higher WHC (WHC of 24·6 g g^{-1} with 24·8% proteins for wild *Ulva* and WHC of 26·1 g g^{-} with 6·0% proteins for the 10 days sample).

In conclusion, growth of *U rigida* in a nitrogen enriched environment followed by a cultivation in normal seawater allows the rapid generation of biomass that is then decolored and enriched in cell wall polysaccharides by a mechanism probably similar to that occuring in nature when algae are exposed to low nutrient levels and high light intensities (Mouradi-Givernaud *et al* 1993 and references therein). This report demonstrates that particular aquaculture conditions of edible seaweeds can be used to modify their organoleptic and nutritional characteristics.

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Effect of external CO_2 concentrations on protein synthesis in the green algae *Scenedesmus obliquus* (Turp.) Kütz and *Chlorella vulgaris* (Kosikov)

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Abstract. Unicellular algae grown under low-CO_2 conditions (0.03% CO_2) have developed a means of concentrating CO_2 at the site of ribulose-1,5-bisphosphate carboxylase/oxygenase. Cells with the CO_2-concentrating mechanism (CCM) acquire the ability to accumulate inorganic carbon to a level higher than that obtained by simple diffusion. To identify proteins which are involved in the organization of the CCM, cells of *Scenedesumus obliquus* and *Chlorella vulgaris* grown in high CO_2 (5% CO_2 in air) were transferred to low-CO_2 (0.03%) conditions in the presence of $^{35}SO_4^{2-}$ and, thereafter, polypeptides labeled with ^{35}S were detected. Under low-CO_2 conditions the inducton of 36-, 39-, 94- and 110- to 116-kDa polypeptides were particularly observed in *S. obliquus* and 16-, 19-, 27-, 36-, 38- and 45-kDa polypeptides were induced in *C. vulgaris*. Western blots with antibodies raised against 37-kDa subunits of the periplasmic carbonic anhydrase (CA) of *Chlamydomonas reinhardtii* showed immunoreactive bands with the 39-kDa polypeptide in the whole-cell homogenates from *S. obliquus* and with 36- and 38-kDa polypeptides in both high- and low-CO_2-grown cells of *C. vulgaris*. Anti-pea-chloroplast CA antibodies cross-reacted with a single polypeptide of 30 kDa in the whole-cell homogenates but not with thylakoid membranes. The CA activity was associated with soluble and membrane-bound fractions, except thylakoid membranes.

Key words: Carbon dioxide concentrating mechanism – Carbonic anhydrase – *Chlorella* – Photosynthesis – Protein synthesis – *Scenedesmus*

Introduction

The unicellular green algae *Scenedesmus* and *Chlorella*, like many other algae, can grow photoautotrophically at very low CO_2 concentrations due to the existence of a CO_2-concentrating mechanism (CCM; Beardall 1981; Beardall and Raven 1981; Shiraiwa and Miyachi 1985; Thielmann et al. 1990; Shiraiwa and Umino 1991; Shiraiwa et al. 1991; Tsuzuki and Miyachi 1991; Coleman 1992). Information about the organization of the CCM in *Scenedesmus* and in *Chlorella* cells is scarce compared with that in *Chlamydomonas reinhardtii* (Spalding et al. 1983, 1991; Coleman 1992) and blue-green algae (Kalpan et al. 1991; Badger and Price 1992). The appearance of the low-CO_2-inducible proteins in algae correlates with the induction of the CCM, and these polypeptides have therefore been suggested as candidates to be involved in this mechanism, mainly in *Chlamydomonas reinhardtii* (Coleman and Grossman 1984; Manuel and Moroney 1988; Spalding et al. 1991). However, there is little information about the low-CO_2-inducible protein synthesis in *S. obliquus* and *C. vulgaris* cells.

In many microalgae, extracellular and intracellular carbonic anhydrases (CAs) play an important role in the mechanism for pumping dissolved inorganic carbon (DIC) up from the medium into the site for CO_2 fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Aizawa and Miyachi 1986; Husic et al. 1989; Palmqvist et al. 1990; Rawat and Moroney 1991; Shiraiwa and Umino 1991; Shiraiwa et al. 1991; Sültemeyer et al. 1993; Badger and Price 1994). However, CA activity alone cannot fully explain the highly efficient acquisition of DIC at CO_2-limiting conditions in cells with the fully implemented CCM of *Scenedesmus* and *Chlorella* species (Gehl et al. 1990; Martinez et al. 1992); even in these algae external or internal forms of CA have been reported (Findenegg 1977; Semenenko et al. 1977; Pesheva et al. 1988; Coleman et al. 1991; Williams and Colman 1993). In *Chlamydomonas reinhardtii* cells with a single [illegible] CCM and an affinity for external inorganic [illegible] polypeptides were found [illegible] low-CO_2

Abbreviations: CA = carbonic anhydrase; CCM = CO_2-concentrating mechanism; DIC = dissolved inorganic carbon ($CO_2 + HCO_3^- + CO_3^{2-}$); Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase

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conditions (Coleman and Grossman 1984; Manuel and Moroney 1988; Spalding and Jeffrey 1989; Spalding et al. 1991). These proteins comprised periplasmic CA with a subunit of 37 kDa and four other proteins with relative molecular masses (M_rs) of 21, 36, 42 and 44 kDa (Coleman and Grossman 1984; Manuel and Moroney 1988; Spalding and Jeffrey 1989; Geraghty et al. 1990; Moroney and Mason 1991; Spalding et al. 1991; Ramazanov et al. 1993). Recently, low-CO_2-inducible proteins have been described in the halotolerant unicellular green alga *Dunaliella tertiolecta* (Ramazanov et al. 1994). However, we still have little information about the synthesis of low-CO_2-inducible proteins in *Scenedesmus* and *Chlorella* cells.

In this paper, low-CO_2-inducible proteins from *S. obliquus* and *C. vulgaris* were analyzed using techniques of labeling with $^{35}SO_4^{2-}$ and immunological methods. The CA activities were found to be associated with both soluble proteins and membrane-bound fractions, but not with thylakoid membranes.

Materials and methods

Algal strains and culture conditions. *Scenedesmus obliquus* (Turp.) Kürtz and *Chlorella vulgaris* (Kosikov) were obtained from the Russian Culture Collection (CALU) at the Moscow Institute of Plant Physiology (Semenenko et al. 1977) and *Chlamydomonas reinhardtii* 6145 wild type was a gift from Prof. E. Fernández (University of Córdoba, Spain). In a suspension culture, the cells were grown in minimal medium (Sueoka 1960) at 28°C under aeration with either a CO_2: air mixture (5:95 v/v) for high-CO_2-grown cells or with air (0.03% CO_2) for low-CO_2-grown cells. The algal suspension was illuminated with white light at a photon flux density of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Labeling of cells with $^{35}SO_4^{2-}$. Labeling of protein with $^{35}SO_4^{2-}$ was performed according to Manuel and Moroney (1988).

Western blot analysis of polypeptides. An SDS-PAGE analysis was performed with a gel composed of 12% (w/v) acrylamide and/or with a gradient gel composed of 10–20% acrylamide (Laemmli 1970). The immunoblot assay was performed according to the protocol from Bio-Rad Laboratories (Richmond, Calif., USA) except that 5% non-fat dry milk was used to block the nitrocellulose membrane. Polyclonal antibodies raised against the 37-kDa subunits of periplasmic CA of *Chlamydomonas reinhardtii* were provided by Dr. J. Moroney (Louisiana State University, USA). Polyclonal antibodies raised against large subunits of spinach Rubisco were a gift from Prof. S. Bartlett (Louisiana State University, USA) and polyclonal antibodies against pea chloroplast CA were provided by Prof. J. Coleman (University of Toronto, Canada). Goat anti-rabbit IgG(H + L) horseradish-peroxidase (HRP) conjugate and HRP color-development reagent were purchased from Bio-Rad Laboratories.

Carbonic anhydrase assay. Carbonic anhydrase (EC 4.2.1.1.) activity in cell fractions was determined by the method described previously Stemler (1993). Unique to this method is the use of $^{14}HCO_3^-$ and a reaction vessel with a gas-permeable membrane (Stemler 1993). Algae were harvested by centrifugation, washed twice with 50 mM 2-2-(N-morpholinio)ethane sulfonic acid–NaOH (Mes-NaOH) buffer (pH 6.5), and then broken by sonication. After centrifugation of the cell-free homogenates at 50000 · *g* for 2 h, the resultant supernatants and the pellets were used for CA assay. Thylakoid membranes isolated form *S. obliquus* and *C. vulgaris* by the method of Metz and Seibert (1984) were centrifuged at 100000 · *g* for 1 h and the resultant pellet was resuspended in 50 mM Mes-NaOH buffer (pH 6.5). After incubation for 60 s, the reaction was started by the injection of 100 µL of $Na^{14}HCO_3$ (0.5 µBq). The radioactivity of $^{14}CO_2$ which passed through the gas-permeable membrane from the reaction medium into the buffer in another part of the reaction vessel was used to calculate the activity of CA (Stemler 1993; Moubarak-Milad and Stemler 1994). Proteins were estimated by the method of Bradford (1976). Chlorophyll was extracted with absolute ethanol and the concentration determined using the absorption coefficients given by Wintermans and De Mots (1965).

Statistics. All values of CA activity measurements are means of ten independent experiments, and the standard error of the mean is indicated where appropriate.

Results

Analysis of ^{35}S*-labeled proteins.* Autoadiography of ^{35}S-labeled proteins from *S. obliquus* cells grown either with air or elevated CO_2 concentrations showed that at least four polypeptides with M_rs of approx. 36, 39, 94 and 116 kDa were induced under low CO_2 (Fig. 1). Two polypeptides ith M_rs of 35 and 37 kDa appeared in large amounts in low-CO_2-grown cells.

In *C. vulgaris* the induction of 16-, 19-, 27-, 36-, 38- and 45-kDa polypeptides was observed in low-CO_2 cells (Fig. 2).

Western blot analysis of proteins. Antibodies raised against a 37-kDa subunit of the low-CO_2-inducible periplasmic CA of *Chlamydomonas* cross-reacted with a 39-kDa polypeptide from *S. obliquus* in both high- and low-CO_2-grown cells (Fig. 3A). In low-CO_2-grown cells of *Chlamydomonas* used as a positive control, the anti-periplasmic CA antibodies cross-reacted with two polypeptides

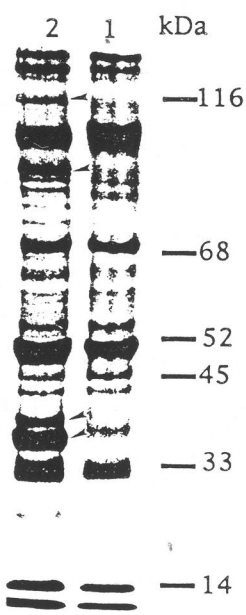

Fig. 1. Autoradiographic analysis of $^{35}SO_4^{2-}$-labeled proteins of *S. obliquus* cells. Labeled cell extracts were subjected to gradient 10–20% SDS-PAGE analysis. *Lane 1*, high-CO_2-grown cells; *lane 2*, low-CO_2-adapted cells; *Numbers*: molecular-weight (kDa) markers. *Arrowheads* (*lane 2*) indicate polypeptides apparently induced in the cells by limiting CO_2

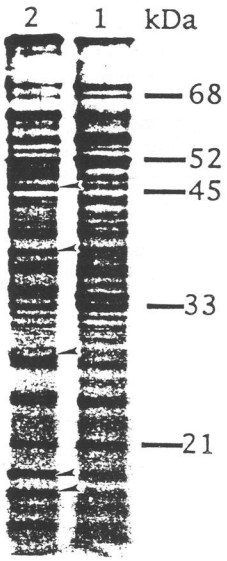

Fig. 2. Autoradiograhic analysis of $^{35}SO_4^{2-}$-labeled proteins of *C. vulgaris* cells. Labeled cells extracts were subjected to gradient 10–20% SDS-PAGE analysis. *Lane 1*, high-CO_2-cells; *lane 2*, low-CO_2-adapted cells; *Numbers*: molecular-weight (kDa) markers. *Arrowheads* (*lane 2*) indicate polypeptides apparently induced in the cells by limiting CO_2

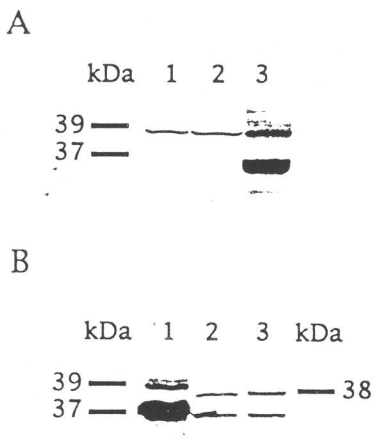

Fig. 3A, B. Immunoblot analysis of the total proteins from *S. obliquus* and *C. vulgaris* cells probed with antibodies raised against a 37-kDa subunit of periplasmic CA of *C. reinhardtii*. A Extracts of *S. obliquus* cells were subjected to gradient 10–20% SDS-PAGE analysis. *Lane 1*, high-CO_2 cells; *lane 2*, low-CO_2 cells; *lane 3*, low-CO_2-grown *C. reinhardtii* cells used as a positive control. Each lane contained 100 µg of protein. B Extracts of *C. vulgaris* cells were subjected to gradient 10–20% SDS-PAGE analysis. *Lane 1*, low-CO_2-grown *C. reinhardtii* cells used as a positive control; *lane 2*, high-CO_2-cells; *lane 3*, low-CO_2 cells. Each lane contained 100 µg of protein

of 37 and 39 kDa, although the 37-kDa band was much stronger.

In the crude cell homogenates from *Chlorella* the periplasmic CA antibodies of *Chlamydomonas* immunoreacted with 36- and 38-kDa polypeptides from both high- and low-CO_2-grown cells (Fig. 3B). However, the cross-reactivity with the 38-kDa band was stronger in low-CO_2-grown cells than in high-CO_2-grown cells.

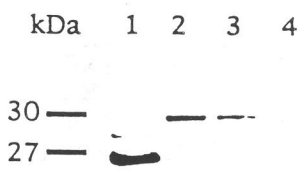

Fig. 4. Immunoblots of whole-cell homogenates and thylakoid membranes from *S. obliquus* cells probed with antibodies to pea CA., *lane 1*, crude homogenate from pea used as a positive control; *lane 2*, high-CO_2-grown cells; *lane 3*, low-CO_2-grown cells; *lane 4*, thylakoid membranes from low-CO_2-grown *S. obliquus* cells. Each lane contained 100 µg protein

Fig. 5. Immunoblots of whole-cell homogenates and thylakoid membranes probed with antibodies to the large subunits of Rubisco. *Lane 1*, crude homogenate from low-CO_2-grown *S. obliquus* cells; *lane 2*, thylakoid membranes from *S. obliquus*; *lane 3*, thylakoid membranes from low-CO_2-adapted *C. vulgaris* cells. Each lane contained 100 µg protein

Immunoblot analysis with anti-pea-chloroplast CA antibodies showed that a 30-kDa band was immunoreactive in crude cell homogenates from both high- and low-CO_2-grown *S. obliquus* cells, but not in the thylakoid membrane fraction (Fig. 4). Similar results were obtained in *Chlorella* cells (data not shown). These results suggest that CA is not a thylakoid membrane-bound protein in *S. obliquus* and *C. vulgaris* cells and/or that this form of CA has different immunological characteristics.

Immunoblot analysis also showed that no cross-reactivity with antibodies raised against the large subunits of Rubisco was observed in the thylakoid membrane proteins from either *S. obliquus* or *C. vulgaris* cells, indicating that the thylakoid membranes were free of soluble proteins (Fig. 5).

Carbonic anhydrase activities. There was considerable CA activity (expressed as cpm) in algal cells grown under both conditions, although activity was higher in the low-CO_2-grown cells (Table 1). *Chlorella* cells showed higher CA activity than *Scenedesmus* cells. Addition of 100 µM ethoxyzolamide, a known inhibitor of CA, inhibited CA activity in both crude homogenates and cell fractions.

Table 1. Carbonic anhydrase activity in extracts of *S. obliquus* and *C. vulgaris*, in the presence and absence of the CA inhibior ethoxyzolamide (EZ) (for details, see *Materials and methods*). Protein concentration in each sample was 1 mg·ml^{-1}. Data are means ± SE

Fraction	CA activity (cpm × 10^3)			
	S. obliquus		*C. vulgaris*	
	High CO_2	Low CO_2	High CO_2	Low CO_2
Homogenate	8 ± 2.0	19 ± 1.0	18 ± 1.0	59 ± 2.5
+ 100 μM EZ	2 ± 0.5	10 ± 0.7	3 ± 1.0	4 ± 0.5
Soluble proteins	5 ± 1.3	10 ± 1.5	7 ± 1.3	23 ± 1.1
+ 100 μM EZ	2 ± 0.5	2 ± 1.1	3 ± 0.5	4 ± 1.2
Pellets	4 ± 0.9	10 ± 1.2	10 ± 1.1	39 ± 1.1
+ 100 μM EZ	2 ± 1.1	2 ± 0.6	2 ± 0.5	3 ± 0.3
Thylakoids	2 ± 0.4	2 ± 0.2	2 ± 0.2	2 ± 1.0
Buffer (no sample)	2 ± 0.1	2 ± 0.3	2 ± 0.5	2 ± 0.3

Values in the thylakoid membranes were similar to those in the Mes buffer used as a background control (Table 1).

Discussion

In algae, the acquisition of DIC from the environment is facilitated by the CCM, which is a transport system that enhances the delivery of CO_2 to Rubisco (Badger et al. 1980; Aizawa and Miyachi 1986; Spalding et al. 1991; Sültemeyer et al. 1993). The appearance of low-CO_2-inducible proteins has been shown to be correlated with the induction of the CCM and therefore these polypeptides have been suggested to be involved in the CCM (Coleman and Grossman 1984; Manuel and Moroney 1988; Spalding and Jeffrey 1989; Spalding et al. 1991). It is generally accepted that CA, which catalyzes the equilibration reaction between CO_2 and HCO_3^-, plays an important role in the mechanism of DIC transport from the medium to Rubisco (Spalding et al. 1983; Aizawa and Miyachi 1986; Badger and Price 1992, 1994; Sültemeyer et al. 1993). However, clarification of the role of CA in the regulation of carbon nutrition and the CCM in algal cells is complicated by the fact that several isoforms of CA have been found (Husic et al. 1989; Badger and Price 1992, 1994; Ramazanov and Cárdenas 1992, 1994; Sültemeyer et al. 1993. These isoforms of CA differ not only in their intracellular location, but also in the dependence of their activity upon the cultivation conditions, CO_2 concentration in particular (Semenenko et al. 1977; Pronina et al. 1981; Fukuzawa et al. 1990; Rawat and Moroney 1991; Suzuki et al. 1994). The activity of these CAs, however, cannot fully explain the whole activity of the CCM (Martínez et al. 1992). In low-CO_2-adapted *S. obliquus* and *C. vulgaris* cells which increased their photosynthetic affinity for external DIC by inducing the CCM, at least four to five low-CO_2-specific polypeptides were induced (Figs. 1, 2). Polypeptides of M_r 36, 39, 94 and 116 kDa in *S. obliquus* (Fig. 1) and 16, 19, 27, 36, 38 and 45 kDa in *C. vulgaris* (Fig. 2) were induced under low CO_2.

At present we have little information about the actual role of the low-CO_2-inducible proteins in the CCM of *Scenedesmus* and *Chlorella* cells. For further characterization of the proteins Western-blot protein analysis was used. Antibodies raised against the 37-kDa subunit of the periplasmic CA of *Chlamydomonas reinhardtii* showed cross-reactivity with a 39-kDa polypeptide in *S. obliquus* (Fig. 3A). However, the 39-kDa band was not specific to low-CO_2-grown cells and was observed in high-CO_2-grown cells also.

Recently, Fukuzawa et al. (1990) and Rawat and Moroney (1991) isolated and purified a new 39-kDa isoenzyme of CA from high-CO_2-grown *Chlamydomonas reinhardtii* cells. It has been shown tha the *CAH1* gene codes for a low-CO_2-inducible periplasmic CA with subunits of 37 kDa (Fujiwara et al. 1990), while the *CAH2* gene codes for a second periplasmic CA of M_r 39 kDa. The isoenzyme of CA encoded in the *CAH2* gene is expressed under high- CO_2 conditions, but not under low CO_2. Findenegg (1977) and Semenenko et al. (1977) had already reported considerable CA activity in both high- and low-CO_2-grown *Scenedesmus* cells, although the activity induced under low CO_2 was significantly higher than that under high CO_2, as also shown in Table 1. Results of Western-blot protein analysis in our study suggest that the expression of a 39-kDa protein, which showed weak cross-reactivity against the antibody of *Chlamydomonas* CA was not affected by the ambient CO_2 concentration given during growth Fig. 3A).

According to the immunoblot analysis of the crude cell homogenates from *C. vulgaris* the anti-*Chlamydomonas* periplasmic CA (37 kDa) antibody immunoreacted with a 36-kDa polypeptide which was present equally in both high- and low-CO_2-grown cells. On the other hand, cross-reactivity of a 38-kDa polypeptide was increased by a decrease in ambient CO_2 concentration (Fig. 3B). Recently, Coleman et al. (1991), by using antibodies raised against the periplasmic CA of *Chlamydomonas*, identified two isoforms of CA (M_rs 36 and 38 kDa) in *Chlorella saccharaphila* cells and only one isoform of CA (M_r 38 kDa) in *Chlorella ellipsoidea*. Since this strain of *C. ellipsoidea* has no extracellular CA, it has been suggested that the 38-kDa protein may be an intracellular CA. Both *C. vulgaris* (Pronina and Semenenko 1990) and *C. saccharaphila* (Coleman et al. 1991) exhibit extracellular CA activity even under high- CO_2 conditions. Therefore, those 36- and 38-kDa polypeptides, suggested to be a cytosolic CA (Coleman et al. 1991), were shown to be immunologically identical in these *Chlorella* species.

The activity of CA was considerabe in the whole-cell membrane fraction, but undetectable in the thylakoid membrane fraction in *Scenedesmus* and *Chlorella* cells (Table 1). Immunoblots probed with antibodies raised against the large subunits of Rubisco showed no cross-reactivity in the thylakoid membranes, although a strong signal was observed in the total-protein fraction (Fig. 5). These results suggest that the thylakoid membrane fraction was not contaminated with soluble proteins. A 30-kDa band, which was reactive with the anti-pea-chloroplast CA antibodies was detected in whole-cell

homogenates, but not in the thylakoid membranes from either high- or low-CO_2-grown cells of *Scenedesmus* (Fig. 4). Recently, Husic and Marcus (1994) demonstrated that a CA-directed photoaffinity reagent, ^{125}I-labeled *p*-aminomethyl-benzenesulfon-amide, specifically bound with a 30-kDa polypeptide in *Chlamydomonas reinhardtii*. In *Dunaliella tertiolecta*, antibody raised against the 37-kDa polypeptide of *Chlamydomonas* was immunoreactive with a polypeptide of 30 kDa from air-adapted cells (Goyal et al. 1992). Ramazanov et al. (1994) also found a 30-kDa polypeptide in the chloroplasts which is cross-reactive with anti-pea-chloroplastic CA antibody. These findings were supported by Husic and Marcus (1994) who suggested that a 30-kDa polypeptide is the intracellular (probably chloroplastic) CA. The immunoreactivity of the 30-kDa polypeptide in *Scenedesmus* (Fig. 4) with the anti-pea-chloroplast CA antibodies provides evidence worthy of further exploration.

Several isoforms of CA have been reported in algae. None of them, including those found by us, has been shown to be a thylakoid membrane-bound protein, with one exception, reported by Pronina and Semenenko (1990) in the same *Chlorella* species as used in our experiments. However, the results of Pronina and Semenenko (1990) can be explained by the possible contamination of the thylakoid membranes with other cell fractions.

Results of ^{35}S-labeling analyses have shown that most of the low-CO_2-inducible polypeptides, except the 36-kDa polypeptide, are different in *Scenedesmus* (Fig. 1), *Chlorella* (Fig. 2) and *Chlamydomonas* (Spalding et al. 1991). At this time it is not clear whether all of these low-CO_2-inducible polypeptides are involved in the CCM, or not. Analysis of ^{35}S-labeled proteins in *C. vulgaris* cells has shown that the 45-kDa polypeptide is induced either under N-limitation conditions (data not shown) or under low-CO_2 conditions (Fig. 2). Some of the polypeptides induced under low CO_2 may be stress proteins which are also induced under a high ratio of O_2/CO_2 and are not essentially involved in the CCM. The actual roles of these proteins in photosynthesizing cells are worthy of further exploration.

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Seasonal agar yield and quality in *Gelidium canariensis* (Grunow) Seoane-Camba (Gelidiales, Rhodophyta) from Gran Canaria, Spain

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Abstract

The seasonal effects on yield and gel properties of *Gelidium canariensis* agar were investigated at two intertidal populations at the northern coast of Gran Canaria. Physical and rheological properties were measured in 1.5% w/v solutions after treatment with alkali. No significant differences were found on agar characteristics between the two sites studied. The highest yields were obtained during summer with a maximum in June (27.8%) and minimum during late autumn and winter (18–18.6%). Overall quality was highest in winter (November-January), when gel strength peaked above 850 g cm^{-2}. The results showed an agar of industrial quality.

Introduction

Gelidium and *Pterocladia* species are usually regarded as yielding the best quality agar and the highest prices. The former genera constitute about 47.3% of the world's annual use for agar extraction, estimated to be about 6683 t (Armisén, 1994). Species of *Gelidium* are extremely important because their extract best meets the requirements of gel strength and temperature hysteresis for bacteriological agar, along with that from *Pterocladia* which is available in only small amounts (Armisén & Galatas, 1987).

Most resources of *Gelidium* are obtained from Spain, Portugal, North Africa and Korea with the Spanish harvest accounting for 20% of the world total *Gelidium* harvest (McHugh, 1991). Estimated agar production in Spain is around 890 t y^{-1} representing approximately 65% of European production (Indergaard & Ostgaard, 1991). Although the main agar source is *G. sesquipedale* (Clemente) Thuret, there are other less known species that could be used by local industries. *G. canariensis* (Grunow) Seoane-Camba is an agarophyte restricted to the northen coasts of some of the Canary Islands. There have been no published studies on the agar content and quality of this seaweed.

According to Santelices (1988), a comprehensive management model based on seasonal changes in the rheological and physical properties of agar should precede intensive harvesting and exploitation of agarophytes. It is well known that agar composition and content in agarophytes depends on the season of the year; these changes are usually associated with variations in water temperature, light intensity, photoperiod and geography (Santelices, 1988). Seasonal changes in the yield and quality of agar in Gelidiales have been reported by several authors (Carter & Anderson, 1986; Onraët & Robertson, 1987; García, 1988; Mouradi-Givernaud *et al.*, 1992), but differences in the results exist because different methods used for agar extraction. Also, Armisén (1994) has noted the need to consider the level of 'pure seaweed' within a normal harvest in order to determine realistic yields.

The present study was undertaken to ascertain whether seasonal variation occurs in the yield and rheological and physical properties of the agar obtained from *G. canariensis* growing at two populations in the northern coast of Gran Canaria and to evaluate whether

this seaweed could be of importance to the agar industry.

Materials and methods

Plant collection

Gelidium canariensis plants were collected monthly at Bocabarranco (28°09'N, 15°40'W) and Agaete (28°06' N, 15°43' W) from August 1991 to July 1992 at an exposed northern rocky shore of Gran Canaria Island. Seawater temperature was recorded *in situ* during the monthly sampling. Plants were collected during low tide, transported to the laboratory, washed thoroughly with tap water, centrifuged to remove excess water, weighed and oven dried (60–70 °C) overnight. Samples were stored in sealed plastic bags until agar extraction. To determine levels of pure seaweed, subsamples of fresh material were weighed, cleaned and reweighed. Three samples (1 g each) of fresh pure seaweed were oven dried for 24 h at 60 °C to estimate monthly changes of percent dry weight.

Agar extraction

Seaweeds were exposed to a 0.5% solution of Na_2CO_3 at 85–90 °C for 30 min prior to extraction and washed with running tap water for 10 min. Agar was extracted ($n = 3$) with distilled water at a pH between 6.0–6.5 and autoclaved at 120 °C for 2 h. The extract was ground with a commercial blender and heated at 90 °C with diatomaceous earth for 30 min and pressured filtered. The filtrate was allowed to gel at room temperature, frozen overnight and thawed. Finally the agar was oven dried for 24 h at 60 °C, cooled and weighed to calculate percent agar.

Gel properties

The dry agar was ground in a Tecator mill and reconstituted into 1.5% w/v solutions to measure gel strength, melting and gelling temperature ($n = 3$). Gel strength was measured after gelling overnight at room temperature by measuring the load ($g\,cm^{-2}$) that causes a cylindrical plunger (1 cm^2 cross section) to break a standard gel in 20 s (Armisén & Galatas, 1987). Gelling temperature was obtained by the addition of 10 ml hot agar solution into a test tube (2.3 cm diameter, 6 cm height). A glass bead (5 mm diameter) was placed in the test tube. The tube was tilted up and down in a water bath (room temperature) until the glass bead ceased moving. The gel temperature in the tube was immediately measured introducing a precision thermometer (0.1 °C divisions). Melting temperature of the gel in a test tube (2.3 cm diameter, 16.5 cm height) was measured by placing a lead bead (9 mm diameter) on the gel surface. The test tube was clamped in a water-bath and the temperature raised from 50 to 100 °C; the melting point was recorded with a precision thermometer when the bead sank into the solution.

Statistical analysis

The data were tested for normality (Kolmogorov-Smirnov) and homogeneity of variance (Bartlett test) using a statistical software package. Simple linear correlation analysis were use to correlate data. Statistical significant differences in yield, gel strength and dry weight between months and localities were tested using a multiple analysis of variance (MANOVA) followed by a least significant difference test.

Results

The *Gelidium canariensis* population at Bocabarranco was more homogeneous than the population located at the northwest. In Agaete, the plants were shorter (5–10 cm) with obvious epiphyte cover throughout the year with a mean value of 13.9% fresh weight corresponding to epiphytes, when compared with Bocabarranco plants (8.86% fresh weight).

The dry weight oscillated around 30% depending on the season, with a peak in September for Agaete (52%) and in October for Bocabarranco (65%). Agar yield and percent dry weight were generally inversely related (Fig. 1). Sea water temperature ranged between 17.7 to 23.5 °C and was correlated positively with dry weight values ($p \leq 0.05$, $r = 0.57$ and $r = 0.72$, Agaete and Bocabarranco respectively).

Agar yield from *G. canariensis* fluctuated seasonally in both localities from 16.7% to 32.6% with a mean value of 23.1% (Fig. 1). The mean agar yield values in Agaete were slightly higher (23.4%) than in Bocabarranco (22.7%). The highest yields were obtained from plants harvested during summer with a maximum value in June for Agaete (32.6%) and in August for Bocabarranco (30.2%). Agar content was minimal at both sites during November and January-February with values of 18.2%–17% in Bocabarranco, and 16.6–16.8% in Agaete

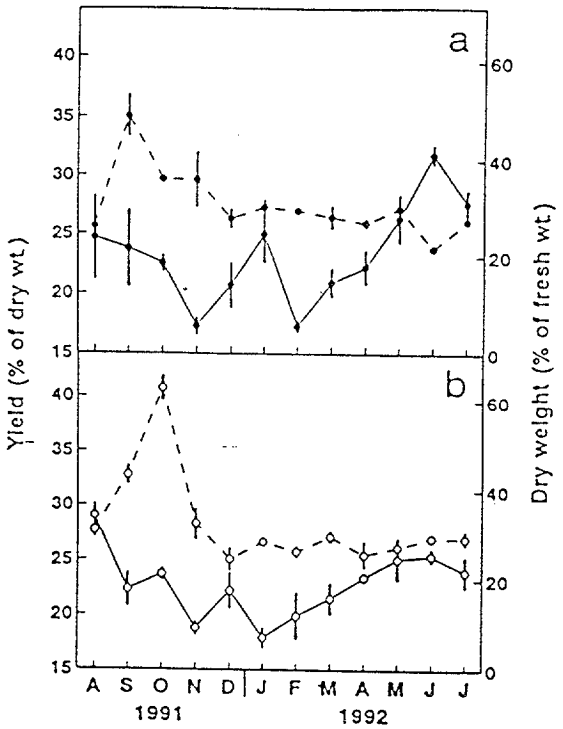

Fig. 1. Seasonal variation of the agar content (–) and dry weight (- -) of *Gelidium canariensis* in a. Agaete (●) and b. Bocabarranco (○).

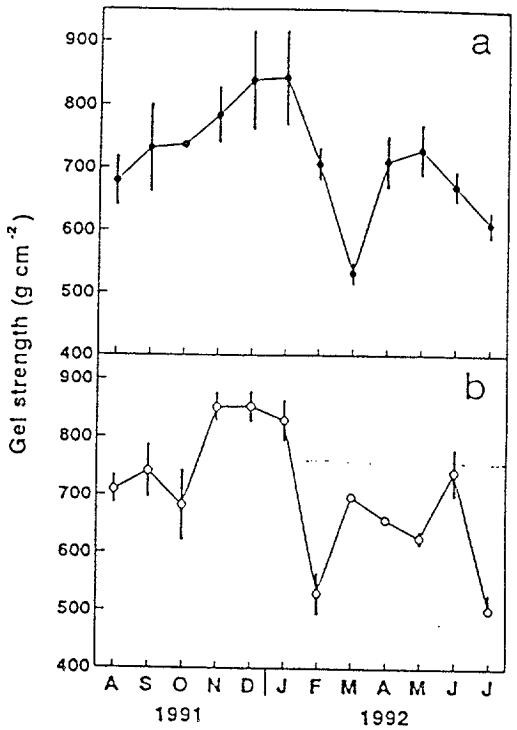

Fig. 2. Seasonal variation of the agar gel strength from *Gelidium Canariensis* in a. Agaete (●) and in b. Bocabarranco (○).

. Gel strength varied from 513 g cm^{-2} to 903 g cm^{-2} for Agaete and between 470 to 873 g cm^{-2} for Bocabarranco (Fig. 2). In both localities the higher gel strength occurred in November, December and January coinciding with the lowest yield values ($p < 0.05$, $r = -0.25$). Minimum gel strength values were found during March (513 g cm^{-2}) and July (588 g cm^{-2}) in Agaete, while in Bocabarranco were recorded during February (492 g cm^{-2}) and July (502 g cm^{-2}).

Gelling temperature ranged from 35.7 °C to 39.3 °C with a mean value of 37.5 °C for Agaete and from 34.6 °C to 37.5 °C with a mean of 36.4 °C for Bocabarranco, while melting temperature ranged from 85.1 °C to 93.7 °C with a mean of 89.2 °C for Agaete and 85.5 °C to 92.1 °C with a mean of 89.5 °C for Bocabarranco. There was a positive correlation between gel strength and melting temperature in Agaete ($p < 0.05$, $r = 0.39$) and in Bocabarranco ($p < 0.05$, $r = 0.44$). Multiple analysis of variance showed a significant difference in the variables studied (gel strength, yield, dry weight) both seasonally ($p < 0.0001$) or between localities ($p < 0.005$), confirming the annual variation in the agar characteristics. However, when the locality was used as the main effect for the same variables, the only significant difference between Agaete and Bocabarranco was for the dry weight ($p < 0.005$).

Discussion

There were substantial seasonal changes in agar characteristics of *G. canariensis* at Gran Canaria, but no significant differences in the agar characteristics between the two populations. Dry weight was found to differ between localities probably due to the plants from Agaete being smaller than those from Bocabarranco. Betancort and González (1991) found that *G. canariensis* was larger in the Bocabarranco population (size classes 10–15 cm and >15 cm) than the Agaete population where there were no individuals >15 cm and with the medium size class (5–10 cm) predominating.

High agar yield occurred during late spring and summer, with a minimum during winter similar to *G. sesquipedale* (García, 1988) Mouradi-Givernaud

et al. (1992) also reported high yield during summer for *G. latifolium*, but they found another maximum in November. The highest agar yields may be related to the development of the thermocline and the lowest nutrient concentrations at that season of the year. An increase in agar content with nutrient deficiency has been also found by Carter and Anderson (1986).

The inverse relationship between agar yield and dry weight suggests that agar synthesis occurs at the expense of biomass production (Fig. 1). Similar results have been found by Mouradi-Givernaud *et al.* (1992) for *Gelidium latifolium*. Increased biomass was also related to an increased in sea water temperature based on dry weight changes. Sosa *et al.* (1993) reported superior photosynthetic performance at 25 °C for *G. canariensis*.

Seasonal changes in gel strength of *G. canariensis* agar are comparable with those obtained for *Onikusa pristoides* (Onraët & Robertson, 1987) and for *Gelidium latifolium* (Mouradi-Givernaud *et al.*, 1992). In contrast, there have been reports of high values in gel strength for summer and low ones for winter from *G. sesquipedale* (Establier, 1964). The relationship between gel strength and melting temperature has been known for many years, and both measurements are usually considered when agar quality is of interest (Selby & Wynne, 1973). Higher melting temperatures and higher gel strength have been related with decreasing amounts of sulfate in agar (Yaphe & Duckworth, 1972). The sulfate content in agar from *G. canariensis* was inversely correlated with gel strength in both localities (data not shown).

Gel properties of *G. sesquipedale* are higher than those obtained for *G. canariensis*: gel strength and agar yields ranged between 920 to 1500 g cm^{-2} and 29–35%, respectively (Establier, 1964; García, 1988) while in *G. canariensis* they were 700 g cm^{-2} and 23%. Thus, *G. canariensis* is a potential a source for bacteriological agar. The best period for harvest to obtain high gel strength occurs in the winter months, but studies on biomass are needed before an optimum harvesting programme can be recommended.

Acknowledgments

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UNDERWATER LIGHT AND ALGAL PHOTOBIOLOGY, *F.L. FIGUEROA, C. JIMÉNEZ, J.L. PÉREZ-LLORÉNS and F.X. NIELL (eds.)*

The presence of ribulose 1,5-biphosphate carboxylase/oxigenase is required for the induction of the 37 KDa periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii**

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SUMMARY: The effects of DCMU, acetate and temperature on the induction of the 37 kDa periplasmic carbonic anhydrase (37 kDa pCA) in *Chlamydomonas reinhardtii* wild type and in a temperature sensitive mutant strain, designated as *68-4PP*, were studied. Transfer of cells from high CO_2 (5%, v/v) to low CO_2 (0.03%) provoked induction of the 37 kDa pCA and this induction was repressed by DCMU, an inhibitor of photosynthesis. However, in the presence of acetate, DCMU did not prevent the induction of the 37 kDa pCA. For further identification of the signal of the CA induction, the effect of temperature on the induction of the 37 kDa pCA was studied in the wild type and in the temperature sensitive mutant strain. When grown at the permissive temperature (25°C) the mutant had a reduced level of Rubisco. Immunoblot analysis of the total cell homogenates against the 37 kDa pCA of *C. reinhardtii* showed a cross-reaction with a 37 kDa polypeptide from low CO_2 grown wild type and mutant cells, although the level of the signal was lower in mutant cells. At the nonpermissive temperature (35°C), the level of Rubisco was not detectable by Western blot analysis and the cells required acetate for growth. When cells were switched from high CO_2 plus acetate to low CO_2 plus acetate media, the induction of the 37 kDa pCA was observed only in the wild type cells, but not in the mutant. We suggest that the presence of Rubisco is required for the induction of the 37 kDa pCA in *C. reinhardtii*.

Key words: carbonic anhydrase, CO_2 concentrating mechanism, photosynthesis, Rubisco.

Abbreviations: CA, carbonic anhydrase; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DIC, dissolved inorganic carbon (CO_2 + HCO_3^-); RuBP, ribulose 1,5-biphosphate; Rubisco, ribulose 1,5-biphosphate carboxylase/oxygenase; CCM, carbon concentrating mechanism.

INTRODUCTION

The low concentration of inorganic carbon is an important environmental factor limiting the photosynthetic productivity of aquatic plants. Upon adaptation to low DIC conditions, unicellular microalgae induce a CCM (Badger *et al.*, 1980; Aizawa and Miyachi, 1986; Spalding *et al.*, 1991).

The majority of investigators adhere to the opinion that an important role in the CCM is played by enzymatic reactions involving CA (Aizawa and Miyachi, 1986; Palmqvist *et al.*, 1990; Sültemeyer *et al.*, 1993). Carbonic anhydrase catalyses the hydration/dehydration reaction between CO_2 and HCO_3^- and the activity of CA increases during adap-

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tation of algae to conditions of carbon dioxide limitation (Coleman and Grossman, 1984; Aizawa and Miyachi, 1986; Bailly and Coleman, 1988; Palmqvist *et al.*, 1990; Sültemeyer *et al*, 1993). At least 5 other polypeptides with molecular weights of 21, 36, 37 and 42-45 kDa that are either absent or present in low amounts in cells grown on high CO_2 concentrations are induced during adaptation of *C. reinhardtii* cells to low CO_2 conditions (Spalding *et al*, 1991; Manuel and Moroney, 1988). So far, only one of these polypeptides, a 35-37 kDa polypeptide, has been identified as a subunit of periplasmic CA (Toguri *et al.*, 1986; Fukuzawa *et al*, 1990). However, none of the low CO_2 induced polypeptides has been linked to a specific function in the CCM (Spalding *et al*, 1991).

Apparently, low CO_2 concentration is not the factor directly involved in the induction of the CCM and CA synthesis (Spalding and Ogren, 1982; Spalding *et al.*, 1983 a, b, c; Ramazanov and Semenenko, 1984, 1986; Umino and Shiraiwa, 1991). Thus, induction of CA synthesis in *Chlorella* is triggered not only by lowering the CO_2 concentration from 2% to 0.03%, but also by increasing the O_2/CO_2 ratio in high CO_2 grown cells (Ramazanov and Semenenko, 1984, 1986). It has been suggested that the signal for induction of the CO_2 concentrating mechanism in *Anabaena variabilis* is phosphoglycolate (Marcus *et al.*, 1983). Umino and Shiraiwa (1991) also suggest that the photorespiratory nitrogen cycle or some aspect of N-metabolism may be related to the regulation of CA induction in *Chlorella regularis*. Several authors reported that DCMU, a specific inhibitor of photosynthesis, represses the induction of CA (Ramazanov and Semenenko,1988; Dionisio-Sese *et al.*, 1990). Photosynthesis-deficient mutants have reduced CA and apparently lack the capacity for active accumulation of inorganic carbon (Spalding and Ogren, 1982). Therefore it has been proposed that photosynthesis is required for the induction of CCM and CA (Spalding and Ogren, 1982; Ramazanov and Semenenko, 1988; Dionisio-Sese *et al*, 1990).

In this work, we have studied the effect of photosynthesis inhibitors and acetate on the induction of the CCM and low CO_2 inducible proteins in *C. reinhardtii* wild type and in a temperature sensitive mutant strain, designated as *68-4PP* (Chen *et al.*, 1990) with the aim to identify the signal for the induction of CCM. Results suggest that the function of Rubisco is essential for the induction of CA and CCM in *C. reinhardtii*.

MATERIAL AND METHODS

Algal strains and culture conditions.

Chlamydomonas reinhardtii wild type cells strain 6145c (Dr. R. Sager, Sidney Farber Center, New York, USA) and the temperature sensitive mutant of *C. reinhardtii* (kindly supplied by Dr. Robert Spreitzer, Lincoln, Nebraska, USA) were grown at 25°C under continuous illumination (200 $\mu mol\ m^{-2}s^{-1}$) with white fluorescent lamps in the minimal medium of Sueoka *et al.* (1967) and/or minimal media supplemented with acetate. Fresh cultures were inoculated with algae axenically grown on agar slants, supplied with 5% CO_2-air mixture and/or with air in 1 L glass bottles and kept in the logarithmic phase of growth by daily dilution with fresh medium.

Labelling cells with $^{35}SO_4^{-2}$.

Protein labelling by $^{35}SO_4^{-2}$ was performed according to Manuel and Moroney (1988). Cells, previously grown on minimal media at 25°C, were centrifuged and resuspended to minimal media with 1/10 $MgSO_4$ concentration for two days, aerated with 5% CO_2. After two days of cultivation, cells were harvested by centrifugation at 5000 g for 5 minutes and the pellet was washed twice with growth media lacking sulfate and resuspended in growth media without sulfate to a chlorophyll concentration of 3-4 $\mu g\ ml^{-1}$. Cells were bubbled with air or air supplemented with 5% CO_2. Then, 15 µCi of carrier-free $H_2{}^{35}SO_4$ (1000 C_i/mmol) were added to the cultures. After incubation for 6h with $^{35}SO_4^{-2}$, cells were harvested by centrifugation at 5000 g for 5 minutes and the pellet was washed twice with 30 mL of 30 mM Hepes-KOH, pH 7.5 and centrifuged again. The pellet was resuspended in 30 mM Hepes-KOH buffer (pH 7.5) used for SDS-polyacrilamide gel electrophoresis. To compare different treatments, samples were loaded to equal counts (250000 count min^{-1} per line). Autoradiography was performed using Kodak X-OMAT film. The amount of radioactivity incorporated into the algal cells was determined by taking aliquots of cells in buffer and counting the sample using a Beckman LS 1801 liquid scintillation counter.

Isolation of carbonic anhydrase.

Wild type and mutant cells grown in the acetate media at 35°C were harvested and sonicated.

Resulting homogenates were centrifuged at 15000 g for 5 min and the supernatant was subjected to ammonium sulfate fractionations of 35 and 70%. The 70% ammonium sulfate precipitate was dialyzed and centrifuged at 15000 g for 10 minutes and the supernatant was loaded on a p-aminomethylbenzene sulfonamide-agarose affinity column (Pharmacia, Uppsala, Sweden). Elution of the isolated CA was performed as described previously (Rawat and Moroney, 1991).

SDS-PAGE and Western blot analysis.

SDS-PAGE was performed with 12% (w/v) acrylamide concentration and/or gradient gel from 10 to 20% acrylamide concentration (Laemmli, 1970). The immunoblot assay was performed according to the protocol from Bio-Rad Laboratories except that 5% non-fat dry milk was used to block the nitrocellulose. Goat anti-rabbit IgG(H+L) Horseradish Peroxidase conjugate and HRP Color development reagent were purchased from Bio-Rad Laboratories.

Polyclonal antibodies against large subunits of Rubisco were provided by Prof. Sue Bartlett (Louisiana State University, USA) and polyclonal antibodies raised against the purified 37 kDa periplasmic CA of *C. reinhardtii* were kindly provided by Dr. James V. Moroney (Louisiana State University, USA).

Analytical measurements.

Protein concentration was estimated according to Bradford (1976). Chlorophylls were extracted with absolute ethanol and quantified using the absorption coefficient given by Wintermans and De Mots (1965).

RESULTS AND DISCUSSION

Effect of DCMU and acetate on the induction of the 37 kDa periplasmic carbonic anhydrase.

Immunoblot analysis of the total cell homogenates probed with antibodies raised against the periplasmic CA antibodies showed cross-reaction with a 37 kDa polypeptide from low CO_2 grown cells, which indicates that under these conditions the cells induced CA (Fig. 1, lane 1). The induction of the 37 kDa periplasmic CA by lowering CO_2 concentration is a common phenomenon and has been described previously by other authors (Coleman and Grossman, 1984; Husic *et al.*, 1989; Dionisio-Sese *et al.*, 1990; Fukuzawa *et al.*, 1990; Husic and Marcus, 1994; Ramazanov *et al.*, 1994). Synthesis *de novo* of the 37 kDa periplasmic CA has been shown to be correlated with the induction of the CO_2-concentrating mechanism (Badger and Price, 1992; Coleman, 1992; Ramazanov *et al.*, 1994). In the presence of DCMU, a specific inhibitor of photosynthesis, the induction of the 37 kDa pCA under low CO_2 was repressed (Fig. 1, lane 2). Inhibition of the CA induction by DCMU was first shown for *Chlorella vulgaris* (Ramazanov *et al.*, 1988) and then confirmed in *C. reinhardtii* (Dionisio-Sese *et al.*, 1990). The analysis of the CA induction in a photosynthesis-deficient mutant of *C. reinhardtii* and inhibition of the CA induction by DCMU suggest that photosynthesis and specifically the function of photosystem II are required for the induction of CA (Spalding and Ogren, 1982; Ramazanov and Semenenko, 1988; Dionisio-Sese *et al*, 1990). Surprisingly, algae grown in the media supplemented with acetate and DCMU induced the 37 kDa pCA (Fig.1, lane 3). These results clearly indicate that the process of photosynthesis is indirectly involved in the induction of CA.

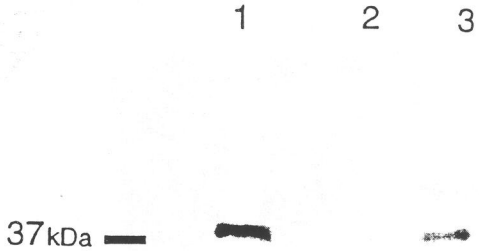

FIG. 1. – Immunoblot of the total homogenates from wild type of *C. reinhardtii* cells probed with antibodies raised against the 37 kDa periplasmic carbonic anhydrase of *C. reinhardtii*. Lane 1, low CO_2 cells; lane 2, low CO_2 cells plus 50 µM DCMU; lane 3, cells grown in acetate media switched to low CO_2 in the presence of 50 µM DCMU. Each lane contains 100 µg of protein.

Effect of temperature on the accumulation of Rubisco in a wild type and in a temperature sensitive mutant of *C. reinhardtii*

It has been recently reported that glycolaldehyde, a powerful inhibitor of triose phosphate to RuBP

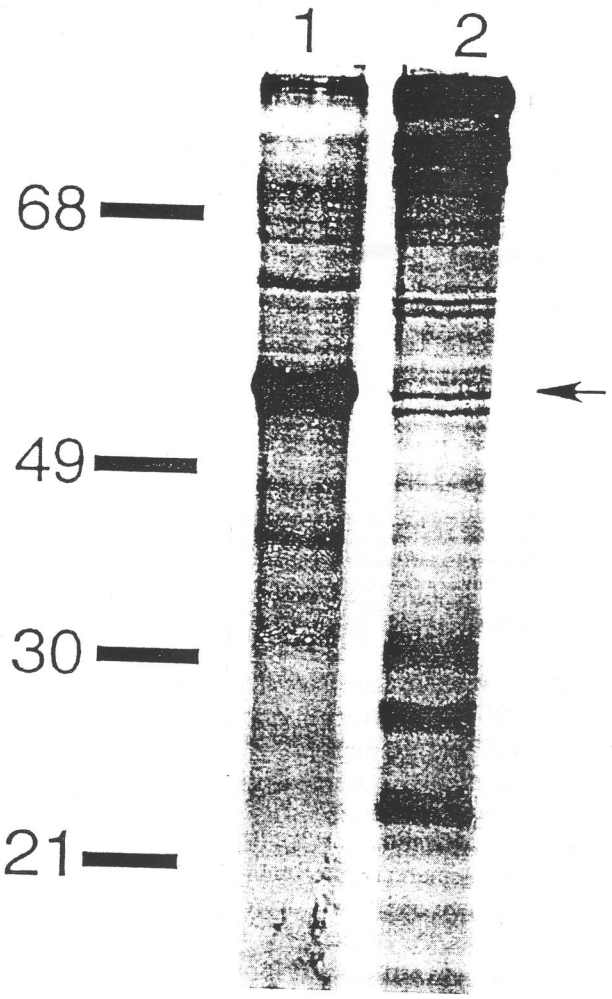

FIG. 2. – Autoradiography of ^{35}S-labelled protein analysis of high CO_2 grown *C. reinhardtii* wild type and the mutant cells at 25°C and 200 µmol m^{-2} s^{-1}. The labelled cell extracts were subjected to gradient 10-20% SDS-PAGE analysis. Lane 1, wild type; lane 2, temperature sensitive mutant cells. The arrow shows the presence of the 52 kDa polypeptide: the large subunits of Rubisco.

conversion, completely inhibited the induction of CA in *C. reinhardtii* (Ramazanov and Cárdenas, 1992). RuBP is the substrate for both the carboxylation and oxygenation reaction of Rubisco and consequently, glycolaldehyde seriously can interfere with the glycolate pathway during adaptation to low CO_2 conditions. Umino and Shiraiwa (1991) and Ramazanov and Cárdenas (1992) hypothesized that the induction of CA under low CO_2 conditions in *Chlorella* cells is reversibly controlled by metabolites and also regulated by the operation of the glycolate pathway and the photorespiratory nitrogen cycle. To test this hypothesis, we studied the effect of temperature on the 37 kDa pCA induction in a temperature sensitive mutant of *C. reinhardtii* (Chen *et al.*, 1990). It has been shown that the mutant cells grown at the permissive temperature (25°C) had a reduced level of Rubisco (Chen *et al*, 1990). Figure 2 shows $^{35}SO_4^{-2}$ labelling of proteins in wild type and in mutant cells under 25°C and high CO_2 conditions. Our results clearly indicate a strong labelling of a 52 kDa polypeptide, (the large subunits of Rubisco) in the wild type of *C. reinhardtii* cells, but not in the mutant. This is in agreement with results that have been previously reported by Chen *et al.* (1990).

At the nonpermissive temperature (35°C) the level of Rubisco in mutant cells was not detectable by Western blot analysis and the cells required acetate for growth (Fig. 3). At 35°C the wild type cells accumulated a large amount of Rubisco in minimal media.

Effect of temperature on the induction of the 37 kDa periplasmic carbonic anhydrase.

The fractions eluted from p-aminomethylbenzene sulfonamide-agarose affinity column with CA activity were subjected to Western blot protein analysis. Analysis of these fractions against the 37 kDa pCA antibodies showed a strong immunosignal in low CO_2 adapted wild type cells despite the fact that growth medium was supplemented with acetate. This is in agreement with our previous results (Ramazanov *et al*, 1994). However, the induction the 37 kDa pCA was not observed in the mutant cells.

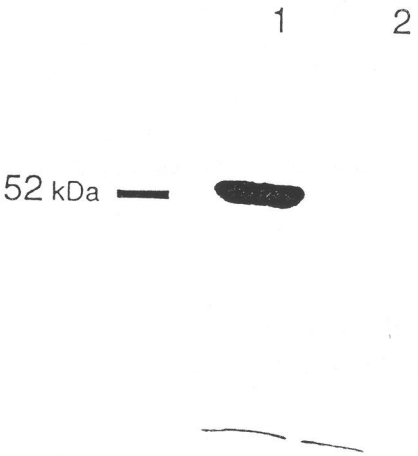

FIG. 3. – Immunoblot of the total soluble proteins from the wild type and temperature sensitive mutant of *C. reinhardtii* cells probed with antibodies raised against spinach large subunits of Rubisco. Cells were grown at 35°C in acetate media. Each lane contains 100 µg of protein.

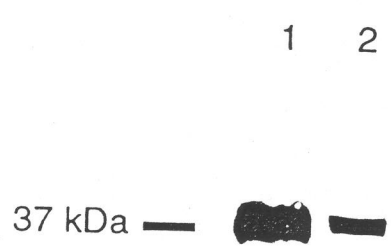

FIG. 4. – Immunoblot analysis of the soluble proteins fraction eluted from sulfonamide-agarose affinity column and probed with antibodies raised against the 37 kDa periplasmic carbonic anhydrase of *C. reinhardtii* . Lane 1, low CO_2 adapted wild type cells grown in acetate media at 35°C; lane 2, low CO_2 adapted mutant cells grown in acetate media at 25° C ; lane 3, low CO_2 adapted mutant cells grown in acetate media at 35°C . Each lane contains 100 µg of eluted protein.

CONCLUSION

The above results clearly indicate that the presence of Rubisco is essential for the induction of the 37 kDa pCA in *C. reinhardtii* and this signal is probably related with the function of Rubisco. It may be assumed that one metabolite of the photorespiratory glycolate pathway plays the role of an effector in CA induction. This metabolite can act as a signal in the cytoplasm pointing to an "underloading" of carboxylation centers of Rubisco. The photosynthetic cell synthesizes CA in response to and depending on this signal. However, further investigations are needed to clarify the detailed organization of this regulatory mechanism.

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UNDERWATER LIGHT AND ALGAL PHOTOBIOLOGY, F.L. FIGUEROA, C. JIMÉNEZ, J.L. PÉREZ-LLORÉNS and F.X. NIELL (eds.)

The ultrastructure and polypeptide composition of the pyrenoid from *Dunaliella tertiolecta**

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SUMMARY: The pyrenoid is a prominent proteinaceous structure found in the stroma of the chloroplast in unicellular and multicellular algae, and contains the majority of the cellular ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco), a key enzyme in the Calvin-Benson cycle of photosynthetic CO_2 assimilation. Pyrenoids from the marine unicellular algae *Dunaliella tertiolecta* were isolated after the treatment of the chloroplast with mercurium chloride. Transmission and scanning electron microscopy showed that tubule-like structures enter into the intrapyrenoidal space. SDS-PAGE of the pyrenoids showed that most of the components consisted of the large (55 kDa) and small subunits (15 kDa) of Rubisco, and several minor polypeptides (52 and 18 kDa). The possible role of the pyrenoid in CO_2 assimilation is discussed.

Key words: CO_2-concentrating mechanism, *Dunaliella tertiolecta*, polypeptide composition, pyrenoid.

INTRODUCTION

The pyrenoid is a prominent proteinaceous structure found in the chloroplast of algal cells, sometimes surrounded by a starch sheath and transversed by thylakoid lamellae (Gibbs, 1962; Griffiths, 1980; Miyachi *et al.*, 1986; Kuchitsu *et al.*, 1988a,b, 1991; Ramazanov *et al.*, 1994a). The pyrenoid is a specialized region of the chloroplast of algae, an electron dense inclusion body structure, containing the majority of the cell's Rubisco, a key enzyme in the Calvin-Benson cycle of CO_2 assimilation. Although pyrenoid morphology has been investigated in diverse groups of algae, little is known about the physiological role of this structure. It has been proposed that the pyrenoid is a protein and nitrogen accumulating region and can be mobilized in different environmental stress conditions (Griffiths; 1980, McKay and Gibbs, 1991; Okada, 1992).

In this work we studied the ultrastructure and polypeptide composition of pyrenoids isolated from the marine unicellular alga *Dunaliella tertiolecta*.

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MATERIAL AND METHODS

Algal culture conditions

Cultures of *Dunaliella tertiolecta* (UTEX strain 999) were grown in the medium previously described by Jiménez del Río *et al.* (1994) at 25-27° C under continuous white light (300 µmol m^{-2} s^{-1}) illumination supplied by fluorescent lamps. The culture density during growth was maintained at a chlorophyll concentration of 4 µg ml^{-1}. Algae were grown in 0.5 l glass bottles (5 cm in diameter) aerated with 5% and/or 0.03% CO_2 in air.

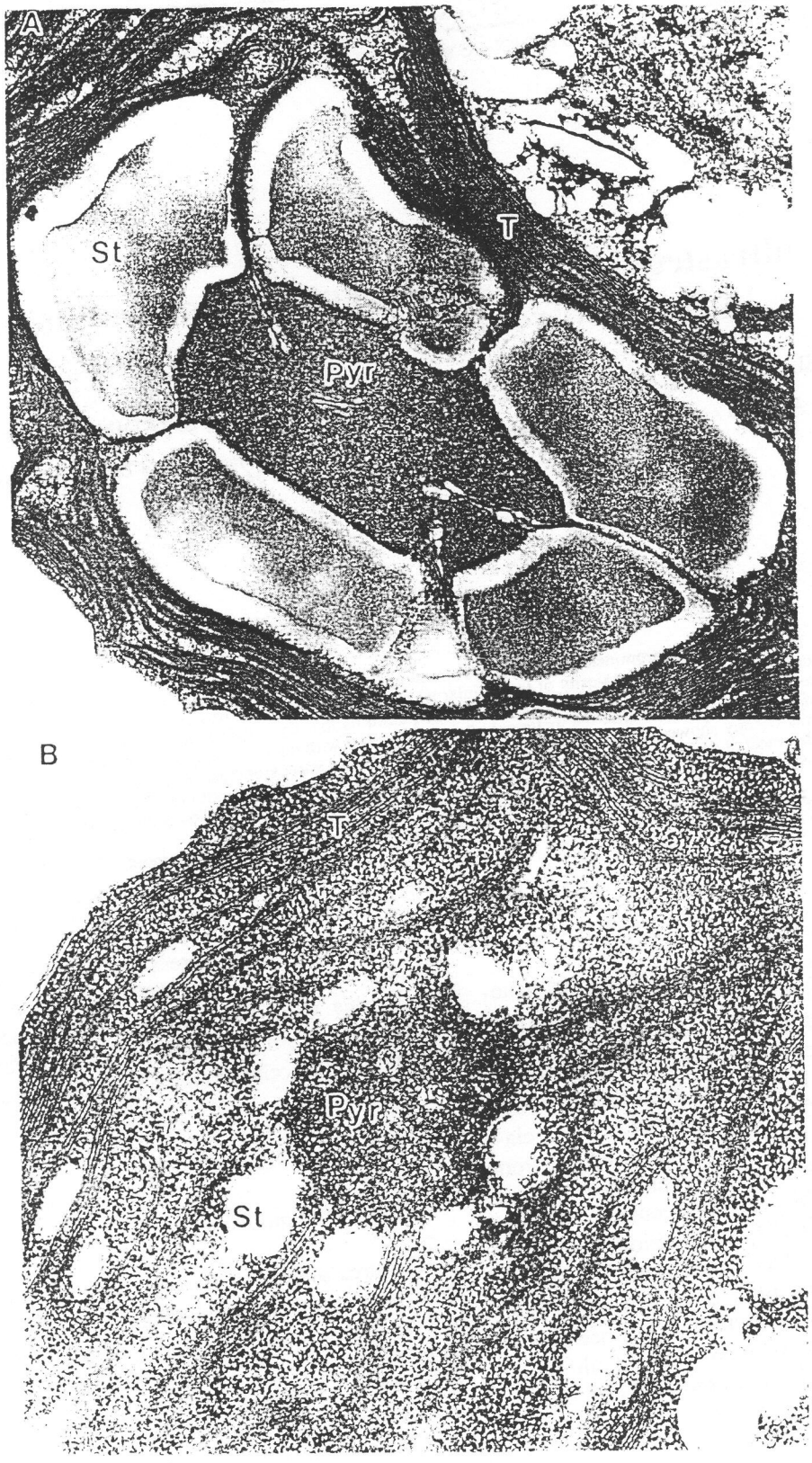

FIG. 1. Transmission electron micrograph of *Dactylosphaera* [illegible] (B) grown in high CO_2 showing the pyrenoid [illegible]

Pyrenoid isolation

Pyrenoid isolation was performed according to Kuchitsu *et al.* (1988a) after chloroplasts isolation (Jiménez del Río *et al.*, 1994).

Electron Microscopy

For transmission electron microscopy, whole cells were fixed as described by Ramazanov *et al.* (1994a).

For scanning electron microscopy, samples of isolated pyrenoids were collected on a 13 mm membrane filter with 0.2 µm pore size, sputter coated with 20 nm AuPd, and examined and photographed using a Cambridge S-260 scanning electron microscope.

Other methods

SDS-PAGE was performed with 12% (w/v) acrylamide concentration and/or gradient gel from 10 to 20% acrylamide concentration (Laemmli, 1970). The protein concentration was estimated according to Bradford (1976) using the BioRad protein assay. Chlorophyll was extracted with absolute ethanol and the concentration determined using the absorption coefficients given by Wintermans and de Mots (1965).

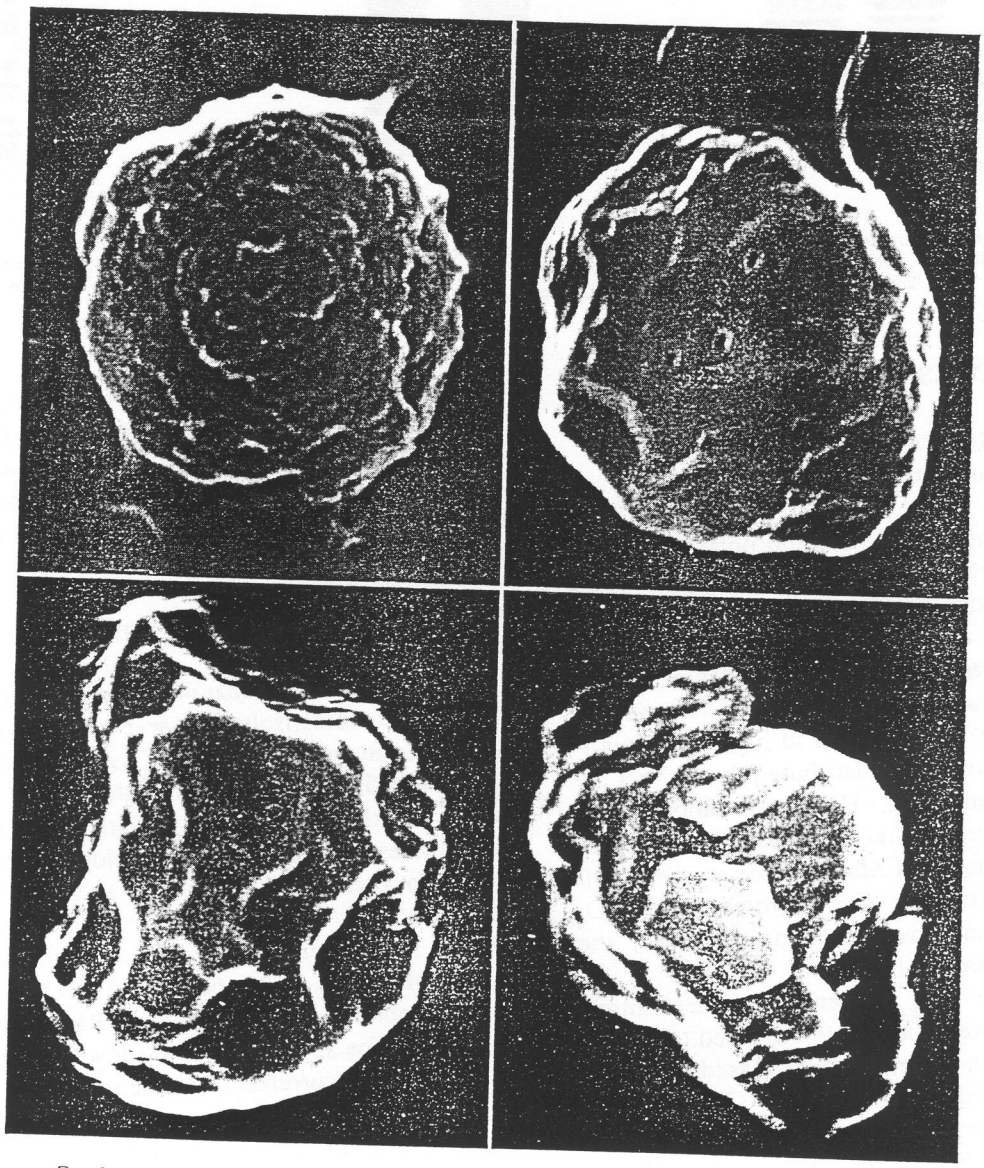

FIG. 2. – Scanning electron micrographs from isolated pyrenoids showing tubule-like structures.

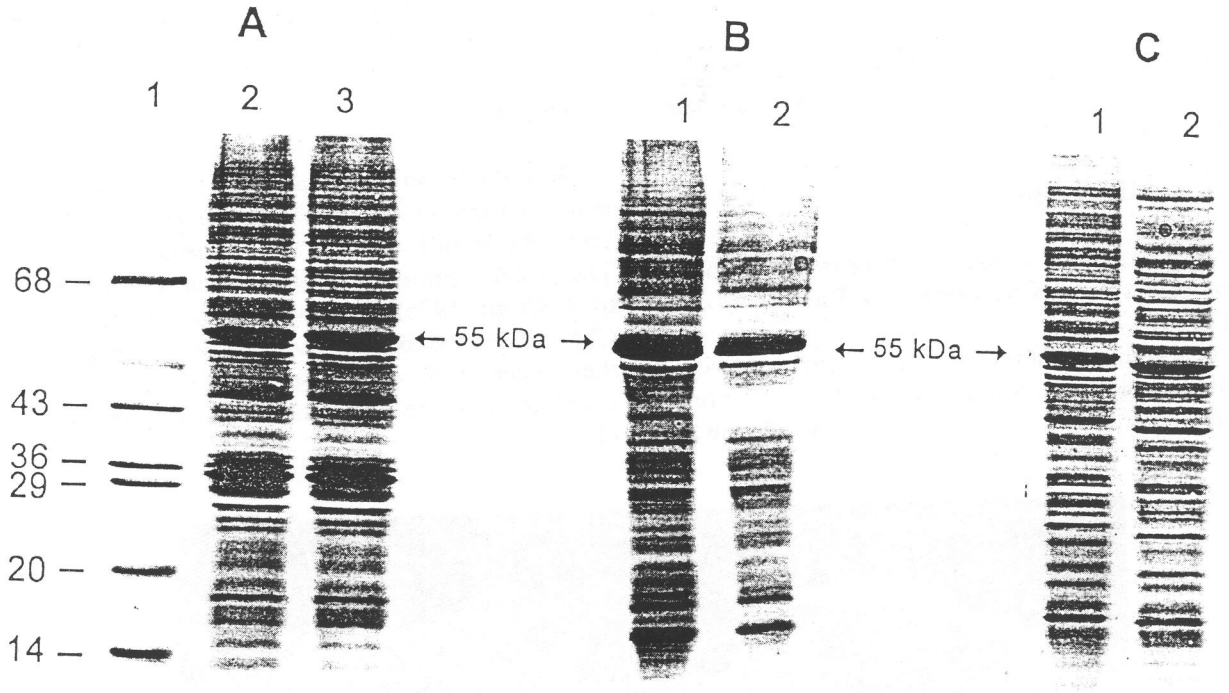

FIG. 3. – Protein pattern of pyrenoids from *Dunaliella tertiolecta*. A: Cell homogenates: lane 1= molecular weight markers; lane 2= high CO_2 cells; lane 3= low CO_2 cells; B: isolated pyrenoids: lane 1= high CO_2 cells; lane 2= low CO_2 cells; C: Cells soluble protein fraction: lane 1= high CO_2 cells; lane 2= low CO_2 cells. Each lane contained 100 µg of protein.

RESULTS AND DISCUSSION

The ultrastructure of the chloroplast of *Dunaliella tertiolecta*

The ultrastructure of the chloroplast and specially the area around the pyrenoid, strongly reflects the CO_2 conditions during growth (Miyachi *et al.*, 1986; Kuchitsu *et al.*, 1988a; Ramazanov *et al.*, 1994). In *D. tertiolecta*, cells fully adapted to low CO_2 have a sheath of starch around the pyrenoid (Fig. 1A), while cells adapted to high CO_2 conditions usually have starch distributed throughout the chloroplast stroma (Fig. 1B). Ramazanov *et al.* (1994a) demonstrated that the deposition of starch around the pyrenoid in *Chlamydomonas reinhardtii* occured after the transfer of cells grown at high CO_2 to low CO_2 conditions and followed closely the increase in apparent affinity for CO_2 due to the induction of the CO_2 concentrating mechanism. Thylakoid membranes are often observed to dissect pyrenoids in green algae, being decreased to tubule-like structures within the pyrenoid (Gibbs, 1962). Scanning electron micrographs of isolated pyrenoids of *Dunaliella tertiolecta* shows similar tubule-like structures (Fig 2). The actual role of these membranes in the structural organization of the pyrenoid is not clear yet. McKay and Gibbs (1991), suggested that a decreased PSII activity in intrapyrenoidal thylakoid membranes would provide a means to maintain a high CO_2/O_2 ratio in the intrapyrenoidal thylakoids.

Polypeptide composition of the pyrenoid

SDS-PAGE showed that the polypeptide composition of pyrenoids isolated from *Dunaliella tertiolecta* included the two subunits of Rubisco (the large subunit of 55 kDa molecular weight and the small one of 15 kDa) together with some other minor polypeptides (Fig. 3). The polypeptide pattern was similar in both high and low CO_2 grown cells. However, $^{35}SO_4^{-2}$ labelling of *D. tertiolecta* cells showed that seven polypeptides with molecular weights of 45, 47, 49, 55, 60, 68 and 100 kDa were induced under low CO_2 conditions (Ramazanov *et al.*, 1994b). The appearance of this low CO_2-inducible proteins was correlated with the induction of the CO_2-concentrating mechanism in *D. tertiolecta* (Ramazanov *et al.*, 1994b). Previously we demonstrated that pea chloroplast CA antibodies recognized a 30 kDa polypeptide [illegible] *D. tertiolecta*

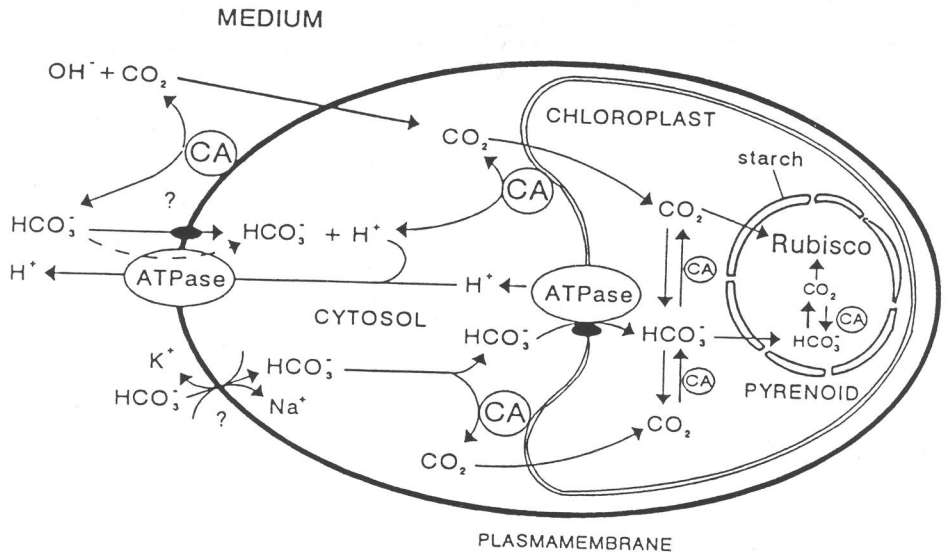

FIG. 4. – Theoretical scheme for inorganic carbon transport and assimilation in *Dunaliella tertiolecta*.

ta chloroplasts, but not with plasma membranes (Ramazanov *et al.*, 1994b), (ii) crossreacted with a 30 kDa polypeptide from isolated pyrenoids of *C. reinhardtii* and *D. tertiolecta* (Ramazanov, unpublished results). Dansylamide and acetazolamide (inhibitors of CA activity) were shown to specifically stain the pyrenoid region in *Chlamydomonas reinhardtii* strain C-9 (Kuchitsu et al., 1991). These results suggest that CA would be closely related to pyrenoid which may serve as the site where bicarbonate is accumulated by the alga and preferentially dehydrated to CO_2 (Fig. 4). This reaction would generate an elevated CO_2 concentration in the pyrenoid, the location of Rubisco. The starch sheath surrounding the pyrenoid might aid in carboxylation by slowing the diffusion of CO_2 out of the pyrenoid. The mechanism of algal adaptation to low CO_2 conditions represents a process complex fairly, including the induction of synthesis (or de-repression) of extracellular and intracellular CAs and ATPase (Ramazanov and Cárdenas, 1992; Ramazanov *et al.*, 1994b), and involving several cell compartments.

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UNDERWATER LIGHT AND ALGAL PHOTOBIOLOGY, *F.L. FIGUEROA, C. JIMÉNEZ, J.L. PÉREZ-LLORÉNS and F.X. NIELL (eds.)*

Photosynthesis and low CO_2 inducible protein synthesis in a newly isolated high CO_2-preferring mutant of *Chlamydomonas reinhardtii**

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SUMMARY: The effect of external CO_2 concentrations on the protein synthesis in *Chlamydomonas reinhardtii* wild-type is compared with that of a new high CO_2-preferring mutant, designated as *pyr*-45. Radiolabeled wild-type and *pyr*-45 cells exhibit up-regulation of two polypeptides (42-45 kDa) when adapted from high (5% CO_2 in air) to low CO_2 (0.03%), and wild-type induces three new ones (21, 36 37 kDa), but *pyr*-45 induces no new polypeptides. Total proteins from *pyr*-45 mutant cells do not crossreact with antibodies against the three low CO_2-inducible polypeptides of wild-type. The CO_2 requirement for half maximal rates of photosynthesis decreases when *pyr*-45 cells are switched from high to low CO_2, but not to the extent of wild-type cells. When exogenous carbonic anhydrase (CA) is added to these partially adapted cells, the CO_2 requirement is further reduced, but still not completely. The up-regulation of the 42-45 kDa polypeptides under low CO_2 growth conditions suggests these changes play a role in the adaptation of algal cells to limiting CO_2 concentrations in the environment and in the function of the CO_2 concentrating mechanism (CCM) in *Chlamydomonas reinhardtii*.

Key words: Adaptation, carbonic anhydrase, *Chlamydomonas*, CO_2-concentrating mechanism, mutant, photosynthesis, protein synthesis.

INTRODUCTION

The unicellular green alga *Chlamydomonas reinhardtii*, like many other algae, induces the CO_2-concentrating mechanism (CCM) when grown on limiting CO_2 concentrations (Badger *et al.*, 1980; Spalding *et al.*, 1983a,b,c; Moroney and Mason, 1991; Coleman, 1992). The nature of the CCM is not completely understood and several models have been proposed (Coleman, 1992; Ramazanov and Cárdenas, 1992). The clarification of the specific role played by carbonic anhydrase (CA) in regulation of the inorganic carbon nutrition and the CCM in cells is complicated by the fact that several isoforms of CA have been found in algae (Pronina *et al.*, 1981; Husic and Marcus, 1994; Fukuzawa *et al.*, 1990; Rawat and Moroney, 1991; Sultemeyer *et al.*, 1993). These isoforms of CA differ not only in intracellular location but also in their activity, which is dependent to varying degrees on the culture conditions, in particular CO_2 concentration (Pronina *et al.*, 1981; Fukuzawa *et al.*, 1990). At least 5 other polypeptides that are either absent or present in low

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amounts in cells grown on high CO_2 concentrations and induced during adaptation of *C. reinhardtii* cells to low CO_2 conditions (Manuel and Moroney, 1988; Spalding and Jeffrey, 1989; Spalding *et al.*, 1991). These proteins include two polypeptides of approximately 42-45 kDa, two membrane-associated proteins with molecular weights of 36 kDa (LIP-36) and 21 kDa (LIP-21) (Husic and Marcus, 1994; Manuel and Moroney, 1988; Spalding *et al.*, 1991; Geraghty *et al.*, 1990; Spalding and Jeffrey, 1989; Ramazanov *et al.*, 1993), and 37 kDa-soluble polypeptide, which has been identified as a subunit of periplasmic CA (Coleman *et al.*, 1984a,b; Fukuzawa *et al.*, 1990). LIP-36 has been shown to be specifically localized in the chloroplast envelope membranes isolated from low CO_2-grown *C. reinhardtii* cells (Ramazanov *et al.*, 1993). However, none of the low CO_2-induced polypeptides have been linked to a specific function in the CCM (Spalding *et al.*, 1991).

A number of *C. reinhardtii* mutants that lacks low CO_2-inducible proteins have been isolated and shown not to grow under low CO_2 conditions (Spalding *et al.*, 1983a,b,c, 1991; Moroney *et al.*, 1989). Among these mutants, *cia*-5, synthesizes none of the low CO_2-inducible proteins (Moroney *et al.*, 1989) nor low CO_2-inducible mRNA's (Spalding *et al.*, 1991) when placed in low CO_2 conditions. Manuel and Moroney (1988) reported that the high CO_2-requiring mutant strain, designated as *pmp*-1 (Spalding *et al.*, 1983b), lacks two low CO_2 inducible polypeptides of approximately 42-45 kDa. In addition, Spalding *et al.*, (1991) provided evidence that the induction of these 42-45 kDa polypeptides in the wild-type represents an up-regulation in the synthesis of the polypeptides rather than *de novo* induction of new polypeptides. According to Spalding *et al.*, (1991) the *pmp*-1 lacks the up-regulation, although the 42-45 kDa polypeptides are present in this strain (Spalding *et al.*, 1991). Isolation of the mutant strain that is defective in these 42-45 kDa and/or induces only these two polypedtides could add a significance meaning to our understanding of the actual role of these proteins (if any) in the CCM.

In this work the effect of CO_2 concentration on the protein synthesis of wild type *C. reinhardtii* and in a newly isolated high CO_2-preferring mutant strain *pyr*-45 is studied. Labelling wild-type cells with $^{35}SO_4^{-2}$ shows the induction of 21, 36, 37, and 42-45 kDa polypeptides, while mutant cells induced only two polypeptides of 42-45 kDa. We suggest that the up-regulation in the synthesis of the 42-45 kDa polypeptides under limiting CO_2 conditions might play a role in the adaptation to limiting CO_2 concentrations in the environment and in the function of the CCM in *C. reinhardtii*.

MATERIAL AND METHODS

Algal strains and culture conditions

The wild-type strain of *Chlamydomonas reinhardtii* 6145c is a gift from Prof. Emilio Fernández (University of Córdoba, Spain), and high CO_2-preferring mutant *pyr*-45 cells have been isolated via UV mutagenesis. Algae were grown in minimal medium (Sueoka, 1960) in a specially constructed glass bioreactor with plane-parallel walls (0.5 cm inside) illuminated with 400 $\mu mol\ m^{-2}s^{-1}$ and aerated with either a high CO_2:air mixture (5:95, v/v) or with low CO_2 (air containing 0.03% CO_2).

Mutant isolation

High CO_2-preferring mutants were isolated following UV mutagenesis. High CO_2-grown wild-type cells were exposed to UV light for different times and aliquots of the cell suspension were plated onto minimal medium. After 24 h dark exposure these plates were illuminated (200 $\mu mol\ m^{-2}s^{-1}$) for two weeks in a high CO_2 chamber. After two weeks colonies were picked and transferred to new plates with minimal medium and exposed to the same light intensity in the growth chamber aerated with 0.03% CO_2. Colonies that grew poorly in low CO_2 were picked up and used for further analysis (see also results).

Labelling cells with $^{35}SO_4^{-2}$

Protein labelling with $^{35}SO_4^{-2}$ was performed according to Manuel and Moroney (1988). Cells previously grown on minimal medium were centrifuged and resuspended in minimal medium with 1/10 $MgSO_4$ concentration, aerated with 5% CO_2. After two days of cultivation cells were harvested by centrifugation at 5000 g for 5 min. The pellet was washed twice with growth medium lacking sulfate and resuspended in growth medium without sulfate to a chlorophyll concentration of 3-4 $\mu g\ ml^{-1}$. Cells were bubbled with low or high CO_2 and 15 μCi of carrier-free $H_2{}^{35}SO_4$ (1000 Ci/mmol) was added to

the cultures. After incubation for 6 h with $^{35}SO_4^{-2}$ cells were harvested by centrifugation at 5000 g for 5 min and the pellet was washed 3 times with 30 ml of 30 mM Hepes-KOH, pH 7.5 and resuspended in the buffer. To compare different treatments, samples were loaded to equal counts (250,000 count min^{-1} per lane). Autoradiography was performed using Kodak X-OMAT film. The amount of radioactivity incorporated into the algal cells was determined by taking aliquots of cells in buffer and counting the sample using a Beckman LS 1801 liquid scintillation counter.

Photosynthesis assays

Photosynthesis of algal cells was measured in 1-ml samples with an oxygen electrode (Hansatech Ltd., Norfolk, England). Algae were centrifuged at 5,000 g for 5 min, resuspended in 1 ml of 25 mM Hepes-KOH (pH 7.3) to the Chl concentration of 10 µg, and transferred to the electrode chamber, where they were allowed to consume the dissolved inorganic carbon (DIC) of the buffer and intracellular pool until no net photosynthesis was observed. Bicarbonate was added when net oxygen evolution had levelled off.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed with 12% (w/v) acrylamide concentration and/or gradient gel from 10 to 20% acrylamide concentration (Laemmli, 1970). The immunoblot assay was performed according to the protocol from Bio-Rad Laboratories except that 5% non-fat dry milk was used to block the nitrocellulose. Goat anti-rabbit IgG(H+L) horseradish peroxidase conjugate and HRP color development reagent were purchased from Bio-Rad Laboratories.

Polyclonal antibodies raised against a 37 kDa periplasmic CA of *C. reinhardtii* were kindly provided by Dr. James V. Moroney (Louisiana State University, USA) and antibodies raised against low CO_2 inducible LIP-21 polypeptide were a gift from Prof. Martin Spalding (Iowa State University, USA). Protein concentration was estimated according to Bradford (1976). Chlorophylls were extracted with absolute ethanol and quantitated using the absorption coefficients given by Wintermans and de Mots (1965).

TABLE 1. – Half-maximal photosynthetic rate $K_{0.5}(CO_2)$ values of *C. reinhardtii* wild-type and *pyr*-45 cells grown under different CO_2 concentrations. Photosynthesis measured in 25 mM Hepes-KOH, pH 7.3 and light intensity 400 µmol $m^{-2}s^{-1}$.

Growth conditions	Wild type	*pyr*-45
	$K_{0.5}(CO_2)$ µM	
5% CO_2	40±5	40±5
0.03% CO_2	2±1	10±2
1 µg CA	2±1	6±1

RESULTS

Photosynthesis assay

When *C. reinhardtii* cells grown on high concentrations of CO_2 were switched to low CO_2 conditions the algal cells required 5-6 h to adapt to the limiting CO_2 conditions. During this transition the apparent affinity of the cells for CO_2 increased (Table 1). The concentration of CO_2 required for half maximal rates of photosynthesis [$K_{0.5}(CO_2)$] in high CO_2-grown cells is about 40 µM CO_2 in both strains, while the low CO_2-grown wild-type cells required about 2±1 µM and mutant cells about 10±1 µM CO_2 (Table 1). The high affinity for DIC shown by *C. reinhardtii* wild-type cells grown in low CO_2 clearly indicates that these cells induce the CCM. *Pyr*-45 also was able to adapt partly to limiting CO_2 concentrations in the environment. The addition of 1 µg of bovine CA to the cells decreased the [$K_{0.5}(CO_2)$] for photosynthesis from 10 to 6±1 µM of CO_2 in the low CO_2-grown *pyr*-45 cells but not in wild-type irrespective of the growth conditions, nor in high CO_2-grown *pyr*-45 cells.

Labelling with $^{35}SO_4^{-2}$

Figure 1 shows an autoradiogram of newly synthesized proteins from cells grown either with low or high CO_2, enabling identification of proteins that are preferentially synthesized under low CO_2 conditions. $^{35}SO_4^{-2}$ labelling shows that at least 5 polypeptides with molecular weights of approximately 21, 36, 37 and 42-45 kDa were induced by low CO_2 in wild-type, while mutant cells induced only two polypeptides of 42-45 kDa. A small amount of 42-45 kDa polypeptides were also present in high CO_2-grown cells. Therefore, the increased amount of these polypeptides in both the wild-type and mutant cells represent an up-regulation in the synthesis of the polypeptides rather than a *de novo* induction of new polypeptides.

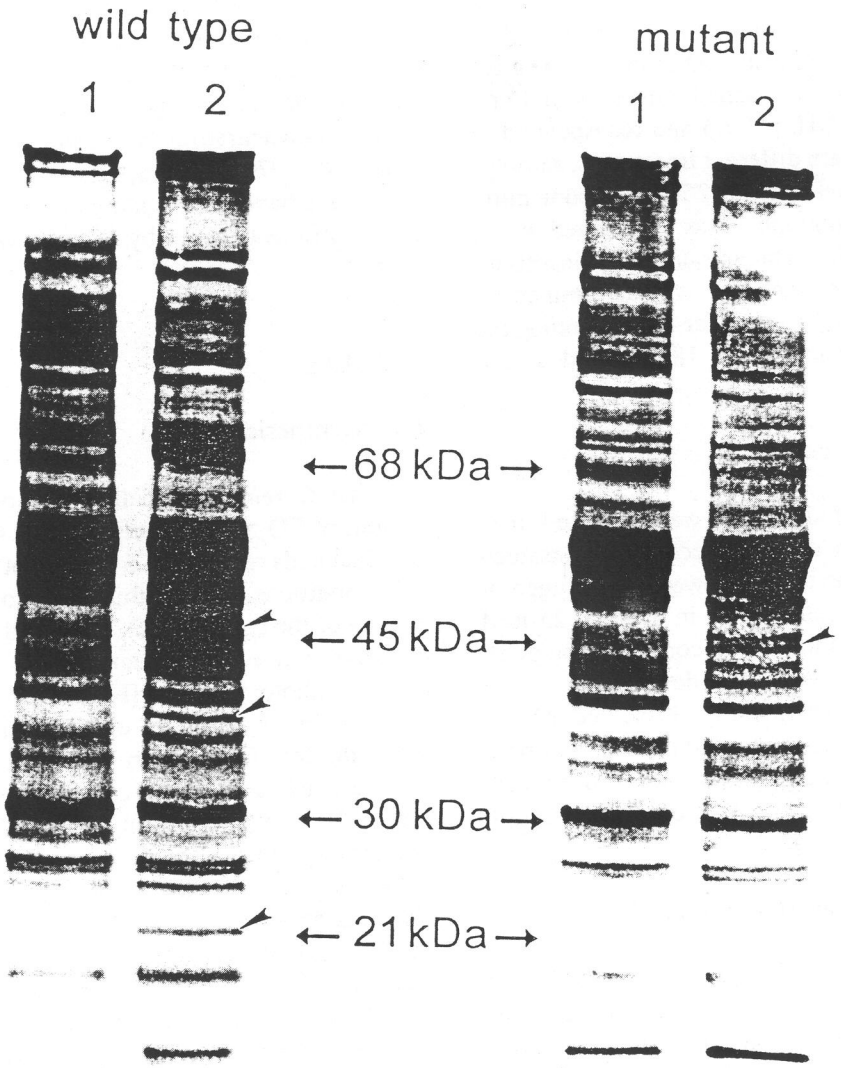

FIG. 1. – Autoradiography of ^{35}S-labelled protein analysis of *C. reinhardtii* wild-type and the *pyr*-45 cells. The labelled cell extracts were subjected to gradient 10-20% SDS-PAGE analysis. In lane 1, high CO_2-grown cells; lane 2, low CO_2-grown cells. The arrows indicate polypeptides which were preferentially labelled in wild-type and mutant cells on low CO_2.

Western blot protein analysis

Immunoblot analysis of wild-type total homogenates probed with antibodies raised against *C. reinhardtii* periplasmic CA showed reaction with a 37 kDa protein from low CO_2-grown cells only. Low CO_2 *pyr*-45 cells showed no such reaction (Fig. 2).

Immunoblot analysis of the total homogenates probed with antibodies raised against LIP-21 (Fig. 4) showed reaction with the 21 kDa polypeptide that appeared in low CO_2-grown wild type. No reaction was observed in *pyr*-45 total homogenates.

DISCUSSION

C. reinhardtii can grow photoautotrophically on very low levels of CO_2 due to the presence of a CCM. The CCM is inducible since only cells grown on low CO_2 exhibited a high apparent affinity for CO_2 (Badger *et al.*, 1980). The mechanism of the algal cells adaptation to low CO_2 conditions represents a process with a fairly complex organization and has not been characterized in detail. Several cell compartments are involved and inhibition of the activity (or its loss after mutations) of one or more enzymes leads to the malfunction of the mechanism

that governs adaptation of the photosynthesizing cell to conditions of CO_2 limitation, thus producing cells unable to adapt to low DIC conditions (Spalding *et al.*, 1983a,b,c; Badger and Price, 1992).

The appearance of low CO_2 inducible proteins is correlated with the induction of the CCM and these polypeptides have been suggested as participants in the mechanism or its induction (Manuel and Moroney, 1988; Spalding and Jeffrey, 1989; Geraghty *et al.*, 1990; Spalding *et al.*, 1991; Ramazanov *et al.*, 1994a,b). Simultananeosly with the increase of the affinity of *C. reinhardtii* cells for external inorganic carbon, at least five polypeptides are induced (Coleman and Grossman, 1984a,b; Manuel and Moroney, 1988; Geraghty *et al.*, 1980; Mason *et al.*, 1990; Moroney and Mason, 1991; Spalding *et al.*, 1991). Actually, $^{35}SO_4^{-2}$ labelling (Fig. 1) and our immunoblot protein analysis (Fig. 2 and Fig. 3) show the induction of a 21, 36, 37 and 42-45 kDa in wild-type cells under low CO_2 conditions. These same polypeptides in *C. reinhardtii* have been described previously by other authors (Coleman and Grossman, 1984a,b; Manuel and Moroney, 1988; Spalding and Jeffrey, 1989; Geraghty *et al.*, 1990; Mason *et al.*, 1990; Spalding *et al.*, 1991). In the mutant *pyr*-45 the induction of only two polypeptides of 42-45 kDa is observed under low CO_2 conditions (Fig. 1). These 42-45 polypeptides are also present in high CO_2-grown cells, althought in small amount. These results indicate that the induction of these polypeptides in the wild-type and in *pyr*-45 cells represent an up-regulation in the synthesis of the polypeptides rather than *de novo* induction of new polypeptides. This is in agreement with results previously described for wild-type by Spalding *et al.* (1991).

In many microalgae the CCM, which is induced by low CO_2, involves an extracellular and an intracellular CA (Spalding *et al.*, 1983a,b,c; Aizawa and Miyachi, 1986; Moroney and Mason, 1991; Fukuzawa *et al.*, 1990; Palmqvist *et al.*, 1990; Sultemeyer *et al.*, 1993; Ramazanov and Cárdenas, 1994). The mutant *cia*-5 has been shown to lack 37 kDa periplasmic CA as well as all the other low CO_2 inducible proteins. *Cia*-5 cells never show increased affinity for CO_2 even when they are grown on limiting CO_2 concentrations (Moroney *et al.*, 1989). It has been suggested that *cia*-5 mutant strain is defective in some factor which may either sense the CO_2 concentration or be responsible for the induction of the transcription of low CO_2-inducible genes (Moroney *et al.*, 1989; Spalding *et al.*, 1991). Our

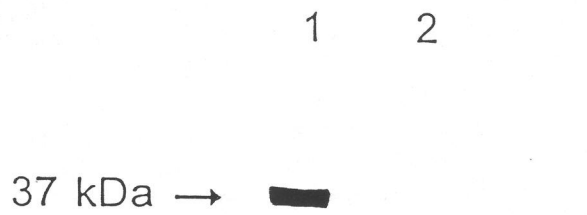

FIG. 2. – Immunoblot of the total homogenates from wild-type and mutant cells of *C. reinhardtii* probed with antibodies raised against the 37 kDa periplasmic carbonic anhydrase of *C. reinhardtii*. Lane 1, high CO_2 cells; lane 2, mutant cells. Each lane contained 100 µg of protein.

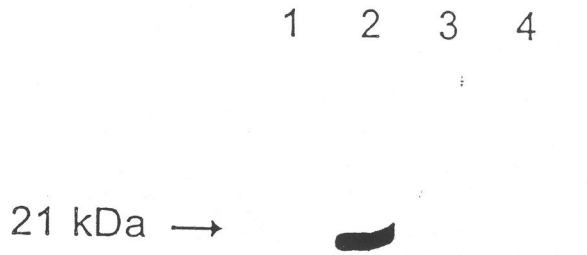

FIG. 3. – Immunoblot of the total cell homogenates from wild-type and the mutant cells probed with antibodies raised against LIP-21 kDa polypeptide. Lanes 1 and 2, wild-type cells; lane 1, high CO_2; lane 2, low CO_2; lanes 3 and 4, mutant cells; lane 3, high CO_2; lane 4, low CO_2. All lanes contained 100 µg of protein.

results show that, like *cia*-5 (Moroney *et al.*, 1989), *pyr*-45 induces neither LIP-36, LIP-21 nor the 37 kDa periplasmic CA proteins (Fig. 2). However, the *pyr*-45 mutant differs from *cia*-5, because the latter strain clearly senses the CO_2 conditions by increasing its affinity for DIC, although not to the level shown by the *C. reinhardtii* wild-type cells with induced CCM. Data for wild-type $K_{0.5}(CO_2)$ in our experiments (Table 1) were similar to those described for algae by others authors (Badger *et al.*, 1980; Spalding *et al.*, 1983a,b,c; Moroney *et al.*, 1989; Moroney and Mason, 1991). The addition of exogenous CA to low CO_2 *pyr*-45 cultures decreased the $K_{0.5}(CO_2)$ for photosynthesis from 10 to 6±1 µM

CO_2, still 3-fold greater than that of wild type. These results suggest that, in addition to the 37 periplasmic CA and 42-45 kDa proteins, the full functioning of the CCM requires other low CO_2-inducible proteins. Ramazanov *et al.* (1993) suggested that the 36 kDa polypeptide induced under low CO_2 conditions located in the chloroplast envelope may play an important functional role in the CCM. The actual role of all these polypeptides in this complex process still remains unclear.

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Ulva rigida (Ulvales, Chlorophyta) tank culture as biofilters for dissolved inorganic nitrogen from fishpond effluents

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Abstract

Ulva rigida was cultivated in 750 l tanks at different densities with direct and continuous inflow (at 2, 4, 8 and 12 volumes d^{-1}) of the effluents from a commercial marine fishpond (40 metric tonnes, Tm, of *Sparus aurata*, water exchange rate of 16 m^3 Tm^{-1}) in order to assess the maximum and optimum dissolved inorganic nitrogen (DIN) uptake rate and the annual stability of the '*Ulva* tank biofiltering system'. Maximum yields (40 g DW m^{-2} d^{-1}) were obtained at a density of 2.5 g FW l^{-1} and at a DIN inflow rate of 1.7 g DIN m^{-2} d^{-1}. Maximum DIN uptake rates were obtained during summer (2.2 g DIN m^{-2} d^{-1}), and minimum in winter (1.1 g DIN m^{-2} d^{-1}) with a yearly average DIN uptake rate of 1.77 g DIN m^{-2} d^{-1}. At yearly average DIN removal efficiency (2.0 g DIN m^{-2} d^{-1}, if winter period is excluded), 153 m^2 of *Ulva* tank surface would be needed to recover 100% of the DIN produced by 1 Tm of fish.

Abbreviations: DIN= dissolved inorganic nitrogen (NH_4^+ + NO_3^- + NO_2^-); FW= fresh weight; DW= dry weight; PFD= photon flux density; V= DIN uptake rate

Introduction

Animal aquaculture and mariculture are techniques of food production that like other food production techniques affect adversely the environment in one way or another (Council Planning Coor. Res. 1982; Ackefors & Enell 1990). The restrictions for fish culture in protected bays, fjörds and other coastal areas due to the increase in sedimentation, biochemical oxygen demand, nutrient loading, etc. inherent to highly intensive stocking and feeding, are well known. The development of clean mariculture practices is of great importance both for mariculture and for the coastal environment.

Wastewaters leaving a fishfarm contain large amounts of nitrogen excreted by fish into the water either as particulated or dissolved matter. According to Krom & Rijm (1989), 26% of the total nitrogen input (100%) is assimilated by fish, 46% is excreted as particulated matter, 13% as dissolved inorganic nitrogen and 10% is found in the detritus. Removal of dissolved nitrogen compounds can be performed by (i) microbial oxydation by means of the active sludge technique or biological beds, (ii) two step removal process, where wastewaters are use to growth bivalves and the residual nutrients dissolved into the water are removed by seaweeds (Fitt *et al.*, 1993; Shpigel *et al.*, 1993) and (iii) removed by direct absortion of dissolved N by seaweeds (Vandermeulen & Gordin, 1990; Cohen & Neori, 1991; Jiménez del Río *et al.*, 1994). The first technique requires long retention times. The use of bivalves and seaweed in a two step process seems to allow higher biofiltration rates of dissolved and particulate matter (Shpigel *et al.*, 1993) with shorter retention times, but is more unstable and sophisticated. The third technique is simplest and cheapest, allowing the biofiltration of higher water volumes in shorter time.

Most of the work on the use of seaweeds as biofilters for marine fishpond effluents is based on NH_4^+ biofiltration (Cohen & Neori 1991; Jiménez del Río *et al.*, 1994). Although NH_4^+ is only a fraction of the total inorganic nitrogen of fishfarm effluents (but toxic at the high concentrations reached under intensive fishpond cultures), the large amount of effluents leaving a commercial fishpond farm produce a considerable environmental impact. The use of *Ulva* spp as biofilters has been suggested as an efficient method to recover large amounts of dissolved inorganic nitrogen (Fralick *et al.*, 1979; Vandermeullen & Gordin, 1990). However, most of the work done so far is based on laboratory scale experiments (i.e. low NH_4^+ concentration and low water flow rates) involving relatively short time periods. The present study was conducted to determine the interaction, under full commercial/natural scale conditions, amoung the different factors which affect the '*Ulva* biofiltering system': NH_4^+ concentration, wastewater flow and seaweed density. The experiments were carried out during a one-year period in order to assess the annual stability of the system as both biofilter and seaweed biomass production unit.

Material and methods

Fish culture conditions and water quality

Fishpond effluents were pumped directly from a 2000 m^3 tank with approximately 40 Tm of *Sparus aurata*. The water exchange rate in the fish tank was approximately 16 m^3 Tm^{-1} fish h^{-1} (8 vol d^{-1}). The fish were fed daily with a pelleted diet (50% proteins, 44% lipids and 6% carbohydrates) at a rate of 1–5% (5–25 kg) of their body weight depending on their body size, between 9:00 to 18:00 hours. Mean concentrations of NH_4^+, NO_3^- + NO_2^- and DIN during the experimental period varied between 0.9–1.2, 0.09–0.13 and 1.0–1.33 mg l^{-1} respectively, with daily fluctuations between 0.5–1.7, 0.06–0.15 and 0.56–1.85 mg l^{-1} for NH_4^+, NO_3^- + NO_2^- and DIN respectively. The pH of the effluents varied daily between 7.1 to 8.0 and the temperature range was 17.5 to 23.6 °C. From November 1993 fish were selectively culled according to body size and effluents were taken from an identical tank when density decreased below 16 kg fish m^{-3} (January 1994), in order to maintain similar DIN concentrations in the fish tank effluents.

Table 1. Average PFD (μmol m^{-2} s^{-1}) and water temperatures during day (T_d, at 12.00) and night (T_n, at 22.00) during the experimental period (at 8 vol d^{-1} turnover).

	April	July	October	January
noon PFD	1550 ± 71	1975 ± 98	1787 ± 78	1336 ± 82
Water T_d. (°C)	23.0 ± 0.5	24.2 ± 1	23.8 ± 0.2	22.2 ± 1
Water T_n. (°C)	19.1 ± 0.3	20.0 ± 0.7	19.3 ± 0.4	18.6 ± 0.4

Plant material and culture conditions

Ulva rigida C. Ag. was collected from the south-east coast of the island of Gran Canaria. Plants were preculitvated under continuous wastewater flow during one month (April 1993) prior to the start of the experiments. Preculture and culture experiments were performed in 750 l (1.75 m^2) semicircular bottom tanks 80 cm deep. Algae were suspended in the water column with the aid of air diffusers located at the bottom of the tank. Necrotic or epiphytized plants were removed during this period.

Noon average PFD and water temperature during experimental period are shown in Table 1.

Experimental design

The optimum density for maximum yield was determined by culturing under different stocking densities (1.5, 2, 2.5, 3, 4 and 6 g l^{-1}) at flow rates exceeding the rate of nitrogen removal (12 vol d^{-1}) during one month (April-1993).

Once the optimum density (for maximum yields under the experimental conditions) was established (2.5 g FW l^{-1}), DIN uptake experiments were performed during one year period (May 1993- April 1994) to evaluate the effect of DIN flow (2, 4, 8 and 12 vol d^{-1}) on DIN-removal efficiency (V), N content of the biomass (% DW) and seasonal fluctuation on biofiltering efficiencies and yield. Simultaneously, one control experiment to estimate DIN losses of an aerated tank was carried out in a tank without seaweeds.

Water sampling for DIN determination at the inlet and outlet of each experimental tank was conducted every three hours (24 h period) once a week (the day after plants were re-stocked to their initial density).

Harvesting and growth rate

Ulva rigida was harvested weekly, centrifugated at 2800 × rpm in a domestic clothes centrifuge (Miele

Table 2. Seasonal variation in growth rate and yield of *Ulva rigida* at four different fishpond wastewater exchange rates.

Period	Water exchange rate d^{-1}	DIN concentration (mg l^{-1} ±SD)* Inflow	Outflow	Growth rate (% d^{-1})	Yield (g DW m^{-2} d^{-1})
April	2	1.2 ± 0.02	0.12 ± 0.03	6.8 ± 0.8	21.4 ± 3.4
	4		0.26 ± 0.02	9.4 ± 0.4	30.3 ± 2.5
	8		0.71 ± 0.05	9.6 ± 0.6	32.2 ± 3.7
	12		0.84 ± 0.08	9.6 ± 0.4	33.7 ± 3.2
July	2	1.27 ± 0.02	0.04 ± 0.03	8.4 ± 0.5	28.6 ± 2.2
	4		0.17 ± 0.04	12.4 ± 0.9	39.3 ± 4.5
	8		0.66 ± 0.05	12.6 ± 1.0	40.2 ± 5.3
	12		0.85 ± 0.07	12.5 ± 0.9	40.0 ± 3.9
October	2	1.32 ± 0.03	0.10 ± 0.02	7.3 ± 0.3	28.9 ± 1.2
	4		0.30 ± 0.04	11.4 ± 0.5	34.5 ± 2.9
	8		0.79 ± 0.06	11.7 ± 0.6	35.9 ± 3.2
	12		0.95 ± 0.06	11.6 ± 0.4	35.7 ± 2.5
January	2	1.08 ± 0.03	0.16 ± 0.03	3.6 ± 0.3	12.2 ± 1.1
	4		0.54 ± 0.05	6.8 ± 0.8	17.5 ± 4.4
	8		0.77 ± 0.06	6.9 ± 0.4	17.8 ± 2.2
	12		0.87 ± 0.08	6.9 ± 0.3	17.9 ± 2.0

*= Mean daily DIN concentration in the effluents.

Table 3. Seasonal variation in DIN concentration, DIN removal efficiency (as% and as *V*) and nitrogen content of *Ulva* (% of DW), of *Ulva rigida* at four different fishpond wastewater exchange rates (n= 3).

Period	Water exchange rate d^{-1}	DIN concentration (mg l^{-1} ±SD)* Inflow	Outflow	DIN removal efficiency (%)	*V*	N in DW (%)
April	2	1.20 ± 0.02	0.12 ± 0.03	93.2 ± 4	0.98 ± 0.07	3.1 ± 0.10
	4		0.26 ± 0.02	78.5 ± 2	1.66 ± 0.11	3.9 ± 0.15
	8		0.71 ± 0.05	41.4 ± 3	1.73 ± 0.12	4.2 ± 0.20
	12		0.84 ± 0.08	29.7 ± 2	1.85 ± 0.10	4.3 ± 0.10
July	2	1.27 ± 0.02	0.04 ± 0.03	96.6 ± 5	1.08 ± 0.02	3.2 ± 0.05
	4		0.17 ± 0.04	86.1 ± 4	1.92 ± 0.06	4.1 ± 0.05
	8		0.66 ± 0.05	48.3 ± 3	2.15 ± 0.12	4.3 ± 0.10
	12		0.85 ± 0.07	33.9 ± 2	2.21 ± 0.13	4.5 ± 0.10
October	2	1.32 ± 0.03	0.10 ± 0.02	92.3 ± 4	1.06 ± 0.06	3.2 ± 0.10
	4		0.30 ± 0.04	77.5 ± 3	1.80 ± 0.06	3.9 ± 0.02
	8		0.79 ± 0.06	40.7 ± 3	1.86 ± 0.11	4.1 ± 0.15
	12		0.95 ± 0.06	28.6 ± 1	1.95 ± 0.09	4.5 ± 0.12
January	2	1.08 ± 0.03	0.16 ± 0.03	85.3 ± 3	0.80 ± 0.03	3.1 ± 0.05
	4		0.54 ± 0.05	50.1 ± 2	0.95 ± 0.08	3.8 ± 0.5
	8		0.77 ± 0.06	28.3 ± 2	1.06 ± 0.17	3.9 ± 0.08
	12		0.87 ± 0.08	19.0 ± 1	1.08 ± 0.19	4.1 ± 0.06

*= Mean daily DIN concentration in the effluents.

WZ268) for 30 s to eliminate the superficial water and re-stocked to the initial density. Specific growth rates (μ) were calculated according to the equation, μ = 100 $Ln(W_t/W_o)/t$ (D'Elia & DeBoer, 1978), where W_o= initial fresh weight; W_t= final fresh weight; and t= time (days). A sample of 10 g was removed and

dried at 90 °C to constant weight to obtain the wet weight/dry weight ratios. Yields (Y) were calculated according to the equation, $Y = [(N_t - N_0)/t \cdot (DW/FW)] / A$ (DeBoer & Ryther, 1977), where N_t= final fresh weight, N_0= initial fresh weight and A= area in m^2.

Analytical measurements

Dissolved inorganic nitrogen (NH_4^+, NO_3^- and NO_2^-) analyses were carried out using a Flow Injector Analyzer (FIAstar 5010 Analyzer, Tecator, Sweden). Nitrogen concentration in the tissue was determinated by digestion and Kjëlhdal neutralization (Tecator, Sweden). Photon flux density, PFD was measured with a LI-COR LI-1000 Datalogger using a plane quantum sensor. The pH was measured with a HI 9025 microcomputer pH meter (Hanna Instruments, Italy).

DIN uptake rate (V = g DIN m^{-2} d^{-1}) was calculated according to the formula $V = Q(S_i - S)/A/T$, where Q = flow rate, S_i= DIN inflow (g l^{-1}), S = DIN outflow (g l^{-1}), A = *Ulva* surface (m^2) and T = time.

Results

Environmental conditions

The maximum averages of noon PFD and water temperature were recorded during summer. Daily fluctuation in water temperature of the tanks was 3 to 5 °C between day and night at 8 vol d^{-1} (Table 1).

Effect of plant density on growth and yield

Maximum growth rates (13.8% d^{-1}) were recorded at the lowest density (1.5 g FW l^{-1}) (Figure 1). Yield reached a maximum (40 g DW m^{-2} d^{-1}) at a density of 2.5 g FW l^{-1}, decreasing at higher densities (6 g FW l^{-1}) to least than 10 g DW m^{-2} d^{-1} (Figure 1). Table 2 shows that increasing the exchange rate from 4 vol d^{-1} to 8 vol d^{-1} and 12 vol d^{-1} had no effect on increasing the growth rate and yield.

Effect DIN flow on V and V seasonal fluctuation

Although the DIN concentration in fishpond effluents entering the seaweed tanks exhibited strong daily fluctuations (Figure 2), average concentrations of DIN influxing were similar during the whole year (Table 3).

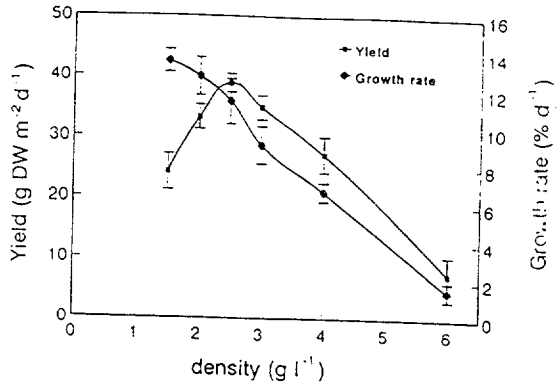

Figure 1. Effect of stocking density on growth rate and yield of *Ulva rigida* cultivated at a water exchange rate of 12 vol d^{-1}. Error bars are ± SD (n= 3).

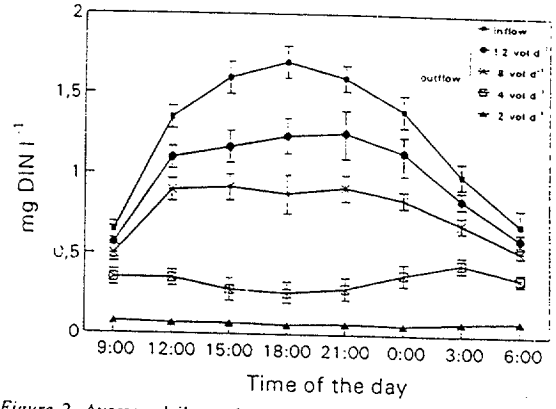

Figure 2. Average daily evolution of DIN concentration in water flowing into and out of the *Ulva rigida* tanks at four different flow rates (July 1993). Error bars are ± SD (n= 3).

Maximum DIN uptake rates (V= 2.2 g DIN m^{-2} d^{-1}) were recorded during summer, decreasing during winter to values below 1.1 g DIN m^{-2} d^{-1} (Table 3). V and yield were directly related regardless of season (Figure 4). Yield was only limited at DIN flow rates lower than 1.2 g DIN m^{-2} d^{-1} (equivalent to a flow rate of 2 vol d^{-1}) during all the year (Table 2).

At non-limiting DIN flow rates (higher than 4 vol d^{-1} equivalent to 2.4 g DIN m^{-2} d^{-1}) the yield exhibited seasonal fluctuation, with higher values during summer (40 g DW m^{-2} d^{-1}) and lowest during winter (17 g DW m^{-2} d^{-1}). Outflow concentrations of DIN correlated positively with water exchange rate (and to some extent with V), but negatively with the percentage of DIN removed ($p < 0.05$) (Table 3).

At high DIN fluxes (8 and 12 vol d^{-1}) V decreased markedly at night (Figure 2). V and N plant con-

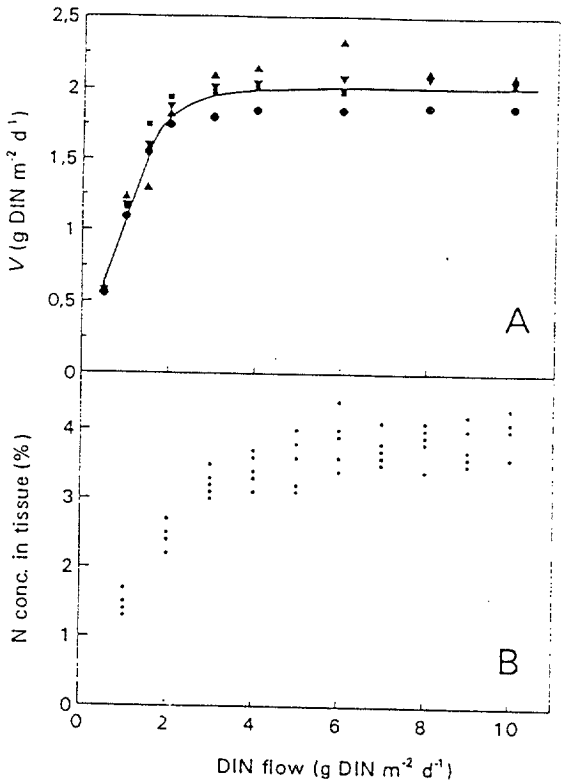

Figure 3. A: DIN-uptake rate by *Ulva rigida* as a function of DIN-flux (n= 5). B: Nitrogen concentration in *Ulva rigida* tissue (percent of DW) as a function of DIN-flux (n= 5). Both figures at a flow rate of 12 vol d^{-1}.

tent increased with DIN flow (Figure 3), following a Michaelis-Menten type kinetics. The *V*max (2.3 g DIN g^{-1} FW d^{-1}) and maximum N content (4.5%) were reached at a flow rate of 4 vol d^{-1} (2.4 g DIN m^{-2} d^{-1}) (Figure 3). No ammonium losses were detected in the aerated tanks without seaweeds (control).

Assuming that a commercial scale *Sparus aurata* tank has a water exchange rate of 16 m^3 water Tm^{-1} fish h^{-1} and an average daily DIN concentration in the effluents of 0.8 mg l^{-1}, a total amount of 307 g DIN Tm^{-1} fish will be produced daily. Considering an annual average *Ulva V* of 2.0 g DIN m^{-2} d^{-1} (excluding winter), a surface of 153 m^2 of *U. rigida* culture tanks will be needed to biofilter 100% of the DIN produced by this 1 Tm of *Sparus aurata*.

Discussion

The great potential of the genus *Ulva* to treat wastewaters has been demostrated both under laboratory and field conditions (Vandermeulen & Gordin, 1990; Cohen & Neori, 1991, Jiménez del Río *et al.*, 1994). *Ulva* has the ability to quickly absorb and metabolize nitrogen. This, together with its high growth rate, low epiphytism subsceptibility, high resistance to environmental stress conditions and wide distribution, make *Ulva* a perfect candidate for the development of wastewater biofiltering systems.

The values (2.5 g l^{-1}) obtained for the optimum density for maximun yield (Figure 1), are higher than the optimum values reported previously for *Ulva lactuca* (Ryther *et al.*, 1984; DeBusk *et al.*, 1986) and also for *Ulva rigida* C. Ag. in our own reports (2 g l^{-1}, Jiménez del Río *et al.*, 1994). This is probably due to the improvement in the design of the tanks (increasing the rpm's of the rolling biomass in the tank), which improves the light harvest of the system, and to the higher PFD (almost double that of our previous experiments). Stocking densities over the optimum (under not N-limiting conditions) did not increased yield, probably due to light limitation, according to the results of Duke *et al.* (1986, 1989) & Neori *et al.* (1991.)

Increments in growth rate and yield correlate with increments in DIN-flow through seaweed tanks. However both growth rate and yield showed saturation kinetics when correlated with DIN-flux (Table 2). Fluxes of DIN over 2.2 g DIN m^{-2} d^{-1} (approx. 4 vol d^{-1}) did not increased growth rate and yield. Low pH observed in fish pond effluents (produced by fish respiration) may provide the seaweeds with an extra source of inorganic carbon for photosynthesis. Similar growth rates and yield at different flow rates (Table 2) confirm this hypothesis.

The saturation kinetics of *V* and the N content of the tissue as a function of DIN-flux (Figure 3), as well as the decrease in growth rate and yield (Table 2), indicate that *Ulva rigida* is N-limited at DIN fluxes below 2.0 g DIN m^{-2} d^{-1} (4 vol d^{-1} in our system). Although *V* correlates with yield and N concentration in the tissue, there is a slight disproportion between the amount of nitrogen incorporated into *Ulva* biomass and that removed from the effluents (Tables 2, 3). The higher values obtained for *V* in comparison from the expected values (%N × yield, Table 3) might be due to the washout of fragments and spores.

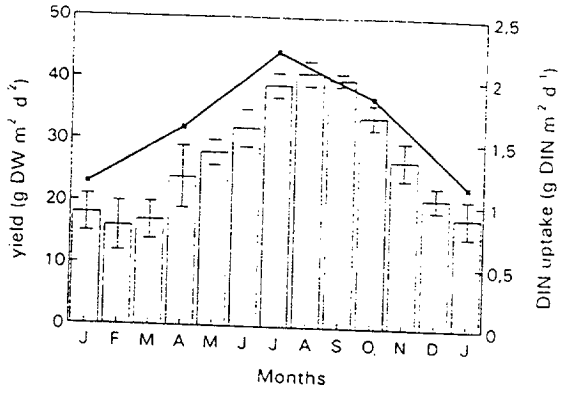

Figure 4. Average (± SD) annual yield (bars) and DIN-uptake rate of *Ulva rigida* cultivated at a density of 2.5 g l^{-1} and a water flow rate of 12 vol d^{-1}.

Maximum growth rates and yield under N-saturating conditions (DIN flow over 2.2 g DIN m^{-2} d^{-1}) were recorded during summer (Figure 4). These results are in accordance with the data reported by DeBusk *et al.* (1989) for *Ulva lactuca*. The seasonal pattern shown by *Ulva rigida* in the present study is similar to that of the *Ulva* populations in Gran Canaria which begin to spread in April and proliferate until November. Seasonal variation in biofiltering efficiency correlates with variations in seaweed yield.

The high DIN removal capacity of *Ulva rigida* as a biofilter unit (*V* annual average= 1,77 g DIN m^{-2} d^{-1}, Table 3), demonstrates the feasibility of the system under commercial scale conditions. The main limitations for the development of N-biofiltering system with *Ulva rigida* reside in the winter seasonal fluctuation, which reduces *V*, and increases dramatically the susceptibility of *Ulva rigida* to epi-endo infections (data not shown), and would thus require quite more land for a given biofiltering efficiency. However, this biofiltering system is a very promising proposition due to the low operation expenses and potential economic benefits from a biomass that needs to be valorized properly.

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The CO_2-concentrating mechanism in a starchless mutant of the green unicellular alga *Chlorella pyrenoidosa*

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Abstract The CO_2-concentrating mechanism (CCM) was induced in the green unicellular alga *Chlorella* when cells were transferred from high (5% CO_2) to low (0.03%) CO_2 concentrations. The induction of the CCM correlated with the formation of a starch sheath specifically around the pyrenoid in the chloroplast. With the aim of clarifying whether the starch sheath was involved in the operation of the CCM, we isolated and physiologically characterized a starchless mutant of *Chlorella pyrenoidosa*, designated as IAA-36. The mutant strain grew as vigorously as the wild type under high and low CO_2 concentrations, continuous light and a 12 h light/12 h dark photoperiod. The CO_2 requirement for half-maximal rates of photosynthesis [$K_{0.5}$ (CO_2)] decreased from 40 μM to 2–3 μM of CO_2 when both wild type and mutant were switched from high to low CO_2. The high affinity for inorganic carbon indicates that the IAA-36 mutant is able to induce a fully active CCM. Since the mutant does not have the pyrenoid starch sheath, we conclude that the sheath is not involved in the operation of the CCM in *Chlorella* cells.

Key words: *Chlorella* – CO_2-concentrating mechanism – Photosynthesis – Pyrenoid – Starch

Introduction

In contrast to most higher plants, unicellular algae have developed a means of concentrating CO_2 at the site of the ribulose-bisphosphate carboxylase/oxygenase (Rubisco) when grown under low CO_2 conditions (Badger et al. 1980; Spalding et al. 1991). The CO_2-concentrating mechanism (CCM) results in an increase in internal CO_2 concentration, which favours the carboxylation reaction of Rubisco over the competitive oxygenation reaction (Badger et al. 1980; Aizawa and Miyachi 1986). The nature of the CCM is not completely understood, and several models have been proposed (Badger et al. 1980; Moroney and Mason 1991; Spalding et al. 1991).

The discovery that the Rubisco protein is located in the pyrenoid (Kuchitsu et al. 1988; MacKay and Gibbs 1991) has raised the question of whether or not this cell structure is involved in the operation of the CCM (Ramazanov et al. 1994). In addition, the ultrastructure of the algal chloroplast, especially the area around the pyrenoid, undergoes rapid and dramatic changes in response to the CO_2 concentrations in the environment (Miyachi et al. 1986; Ramazanov et al. 1994). Several authors have reported that the sheath of starch surrounding the pyrenoid is formed rapidly in response to a decrease in CO_2 concentration in the environment (Miyachi et al. 1986; Kuchitsu et al. 1988). The formation of the pyrenoid starch sheath has been shown to be correlated with the induction of the CCM in the green unicellular alga *Chlamydomonas reinhardtii* (Ramazanov et al. 1994). Based on these correlative observations, it has been hypothesized that the starch sheath could act as the diffusion barrier to slow CO_2 efflux from the pyrenoid and therefore may play a role in the CCM (Badger et al. 1993; Ramazanov et al. 1994), although there is no direct evidence supporting this conclusion. In the present study, we demonstrate that a starchless mutant of *Chlorella pyrenoidosa* grows as vigorously as the wild type (WT) under both high and low CO_2 conditions and that it induces an active CCM. These results suggest that the pyrenoid starch sheath is not involved in the operation of the CCM.

Materials and methods

Algal strains and culture conditions. *Chlorella pyrenoidosa* WT strain 82T was obtained as a gift from Prof. E. S. Kuptzova (Institute of Plant Physiology, Moscow, Russia) and starchless mutants of *Chlorella* were isolated via UV-mutagenesis (Ball et al. 1991). Algae were grown in minimal medium (Sueoka 1960) in 1 l glass bottles illuminated with 500 μmol m[illegible] continuous light and on a 12 h light/12 h dark cycle [illegible] with either a high CO_2 [illegible] mixture (5–95, v/v) or with [illegible] CO_2 [illegible] mixture (0.03% CO_2

Abbreviations: CCM = CO_2 concentrating mechanism; high CO_2 = air supplemented with 5% CO_2 (v/v); low CO_2 = air containing ambient 0.03% CO_2 (v/v); WT = wild type

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Photosynthesis assays. Photosynthesis of algal cells was measured in 1-mL samples with an oxygen electrode (Hansatech, Norfolk, UK). Algae were centrifuged at 5000·*g* for 5 min, resuspended in 1 mL of 25 mM Hepes-KOH (pH 7.3) to a chlorophyll concentration of 10 $\mu g \cdot ml^{-1}$, and transferred to the electrode chamber, where they were allowed to consume the dissolved inorganic carbon ($CO_2 + HCO_3^-$) of the buffer and the intracellular pool until no net photosynthesis was observed. Different concentrations of bicarbonate were added when net oxygen evolution had leveled off.

Respiration assays. The respiration rate of algal cells was measured in darkness in 1-mL samples with an oxygen electrode (Hansatech) Algae were centrifuged at 5000·*g* for 5 min, and resuspended in 1 mL of 25 mM Hepes-KOH (pH 7.3) to a chlorophyll concentration of 10 $\mu g \cdot ml^{-1}$.

Electron microscopy For transmission electron microscopy, whole cells were fixed as described by Harris (1986).

Analytical measurements. Protein concentration was estimated according to Bradford (1976). Chlorophyll was extracted with absolute ethanol and quantified using the absorption coefficient given by Wintermans and De Mots (1965).

Results

Chlorella mutants isolation. After UV-mutagenesis of the WT *Chlorella*, over 40000 colonies were isolated on minimal medium. The colonies were then replica-plated onto nitrogen-free medium with and without acetate. Under nitrogen starvation, algae accumulate large amounts of starch and it also leads to a pronounced degradation of chlorophylls, thus enabling immediate colony staining by iodine vapor (Ball et al. 1991). Under these conditions, WT colonies were revealed by their typical dark-blue colour, while starchless mutants had a red-yellow colour. After iodine staining, five starchless mutants were selected, and one of them, designated as IAA-36, was chosen for further characterization.

Growth characteristics. To test the growth characteristics, both the WT and the starchless mutant were cultured under different growth conditions. Surprisingly, the starchless mutant of *Chlorella* grew as vigorously as the WT under high and low CO_2 concentrations (Fig. 1). No differences were observed in the growth rates when the WT and starchless mutant were cultured under 12 h light/12 h dark or under 6 h light/18 h dark photoperiods and high CO_2 concentration.

Photosynthesis. When high-CO_2-grown cells of both the WT and the IAA-36 mutant were switched to low CO_2 conditions, the algal cells required about 4–5 h to adapt to the limiting CO_2 conditions. During this transition time, the apparent photosynthetic affinity for CO_2 increased (Fig. 2, insert). *Chlorella* cells grown under high CO_2 required about 35–40 μM CO_2 for half-maximal rate of photosynthesis [$K_{0.5}(CO_2)$], while low-CO_2-grown cells required only about 2–3μM CO_2 for the same rate of photosynthesis (Fig. 2).

Dark respiration. The dark respiration rates were measured in the WT and mutant cells cultured under continuous light and in cells grown under a 12 h light/12 h dark photoperiod. Higher respiratory rates were observed in the cells grown under continuous light [60 μmol $O_2 \cdot (mg\ Ch)^{-1}$] compared with cultures grown under the light/dark regime [25 μmol $O_2 \cdot (mg\ Ch)^{-1}$], although the rates of respiration were similar in both strains (data not shown). Respiration of the cells grown under photoperiodic condition was measured at the end of dark period.

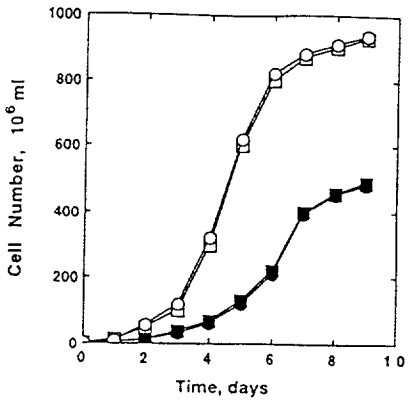

Fig. 1. Growth of *Chlorella pyrenoidosa* WT (O, ●) and the starchless mutant (□, ■) under high (O, □) and low (●, ■) CO_2 concentrat ions. Algae were cultured under continuous light conditions

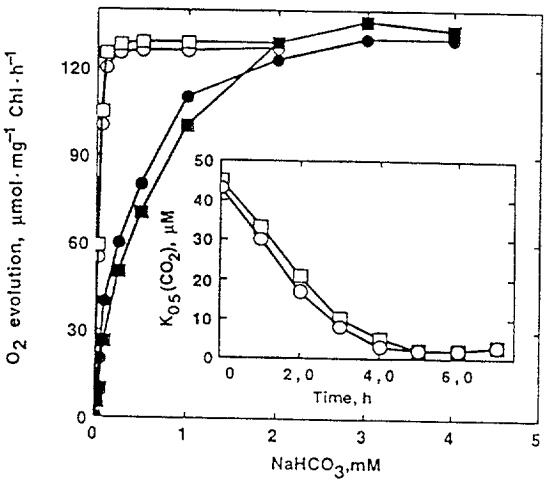

Fig. 2. The rate of photosynthesis versus inorganic carbon concentration for WT (O, ●) and mutant (□, ■) *C. pyrenoidosa* grown under high CO_2 (●, ■) or adapted to low CO_2 (O, □) conditions. The *insert* shows the kinetics of adaptation to low CO_2 and how the concentration of CO_2 required for half-maximal rates of photosynthesis [$K_{0.5}$ (CO_2)] decreases as the cells adapt to the low CO_2 conditions

Effect of CO_2 concentrations on ultrastructure. Chlorella WT cells grown under high CO_2 usually had starch granules evenly distributed throughout the chloroplast stroma (Fig. 3A), while low-CO_2-grown cells had a sheath of starch completely surrounding the pyrenoid (Fig. 3B) Neither high-(Fig. 3C) nor low-(Fig. 3D) CO_2-grown mutant cells accumulated starch granules

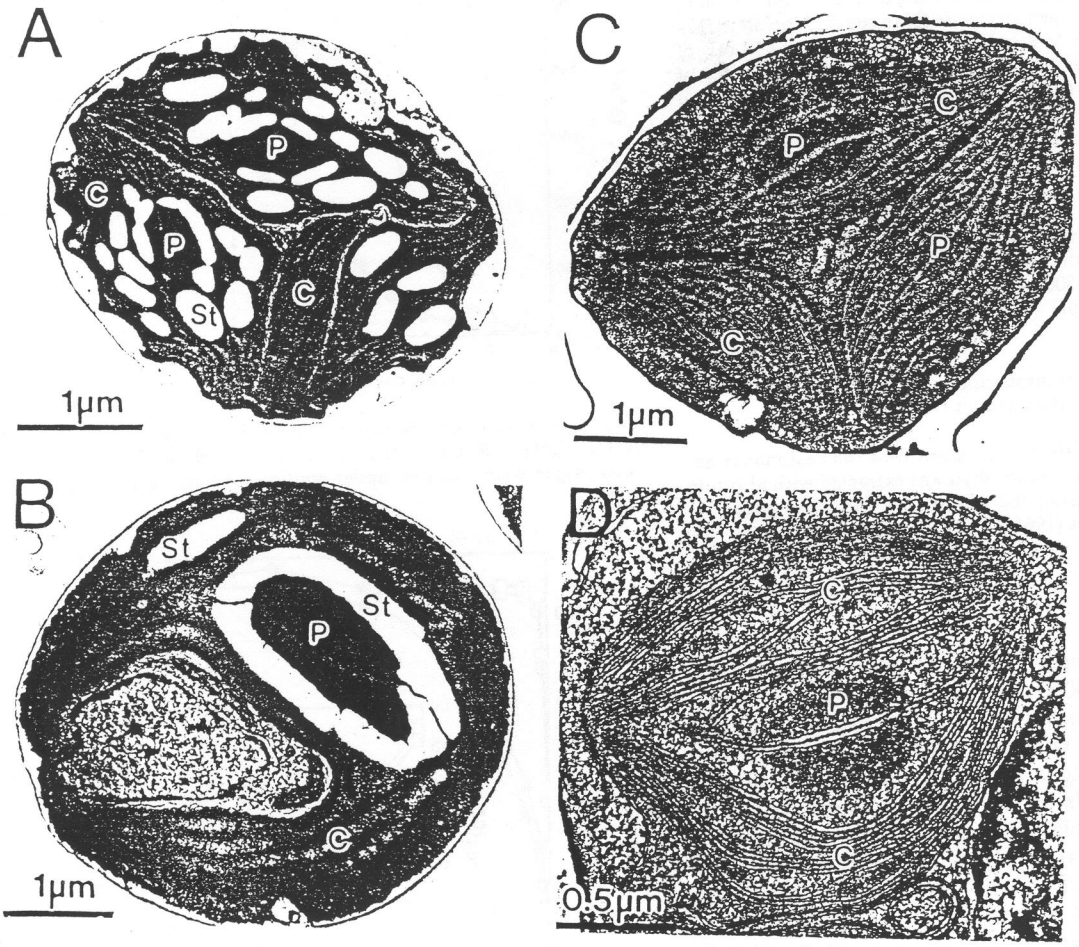

Fig. 3A–D. Electron micrographs of *C. pyrenoidosa* WT (A, B) and the IAA-36 mutant (C, D) grown in high CO_2 (A, C) or adapted to low CO_2 concentrations for 6 h (B, D). *P*, pyrenoid, *St*, starch; *C*, chloroplast. Algae were cultured under continuous light conditions

Discussion

In this work, we isolated and partially characterized a starchless mutant of *C. pyrenoidosa*. This allowed us to address several key questions concerned with (i) the CO_2 requirement of the starchless unicellular algal mutant, (ii) the mutant's ability to induce the CCM, and (iii) a presumed involvement of the pyrenoid starch sheath in the operation of the CCM.

The growth rate of the starchless mutant cells was tested both under high and low CO_2 conditions. In either case, IAA-36 cells showed the same growth rate as WT (Fig. 1). No detectable differences in growth rates were observed when the WT and mutant were subjected to light/dark photoperiods. Thus, the lack of starch in IAA-36 mutant cells did not affect the growth rates. Results obtained with this algal mutant are different from those reported for the starchless mutants of higher plants. It has been demonstrated that the growth rates of the starchless mutants of *Arabidopsis* (Lin et al. 1988) and *Nicotiana* (Huber and Hanson 1992) are restricted when compared with their WT under a shortened photoperiod. Possibly, starch plays a more important role in higher plants than in the unicellular algae. We did not determine the levels of photosynthetic metabolites that specifically accumulate in the IAA-36 cells, but evidently the pool of these metabolites in both the WT and mutant cells was high enough to support the growth and respiration rates under a shortened photoperiod.

Despite the fact that the starchless mutant of *Chlorella* formed no pyrenoid starch sheath under low CO_2 conditions (Fig. 3), this mutant did induce a fully active CCM, as was evident from the comparison of the $K_{0.5}(CO_2)$ for photosynthesis of the WT and IAA-36 cells (Fig. 2). This strongly suggests that the pyrenoid starch sheath is not involved in the operation of the CCM in *Chlorella* cells. The formation of a starch sheath and the induction of the CCM seem to represent two independent mechanisms regulated by CO_2 concentrations in the

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Low-temperature-induced β-carotene and fatty acid synthesis, and ultrastructural reorganization of the chloroplast in *Dunaliella salina* (Chlorophyta)

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The effect of suboptimal growth temperature on β-carotene and fatty acid biosynthesis, and on the ultrastructural reorganization of the chloroplast in the green unicellular alga *Dunaliella salina* has been studied. A decrease from the optimal temperature for growth (30°C) to suboptimal (18°C) temperatures induced β-carotene synthesis and increased lipid content in *D. salina* cells, thereby promoting the formation of lipid–carotene globules in the chloroplast periphery. The content of polyunsaturated fatty acids was higher in cells cultured at low temperature. Results suggest that the induction of carotenogenesis and accumulation of polyunsaturated fatty acids are mechanisms of acclimation to unfavourable environmental conditions for growth.

Key words: adaptation, β-carotene, *Dunaliella*, fatty acids, low temperature, ultrastructure

Introduction

The halotolerant green alga *Dunaliella salina* and other species of *Dunaliella* are able to accumulate large quantities of β-carotene (Semenenko & Abdullaev, 1980; Ben-Amotz & Avron, 1983, 1989; Borowitzka *et al.*, 1984). The extent of this accumulation is a direct function of the amount of light to which algae are exposed during a division cycle (Ben-Amotz *et al.*, 1982; Ben-Amotz & Avron, 1983). Maximal concentrations of β-carotene were found in *D. bardawil* and *D. salina* cells exposed to a high light intensity and high concentrations of NaCl (4–5·5 M) and grown under nitrogen-limiting conditions (Semenenko & Abdullaev, 1980; Ben-Amotz *et al.*, 1987; Lers *et al.*, 1990). The amount of β-carotene was also dependent on the spectral composition of the light. Blue light has been reported to greatly stimulate biosynthesis of β-carotene in *D. salina* (Semenenko & Abdullaev, 1980), whereas *D. bardawil* cells grown under continuous red light (> 645 nm) or high intensities of white light had increased β-carotene synthesis (Ben-Amotz & Avron, 1989). Differential steroisomeric composition of β,β-carotene in thylakoids and in pigment globules as a function of the light intensity and temperature has also been reported (Jiménez & Pick, 1994). The massive accumulation of β-carotene is concentrated in lipid–carotenoid globules located in the interthylakoid space of the chloroplasts (Vladimirova *et al.*, 1978; Ben-Amotz & Avron, 1983, 1989; Shaish *et al.*, 1991). Most of the information regarding the factors controlling β-carotene synthesis points to a synergistic effect of light intensity and nutrient imbalance on algal metabolic activity.

In the work presented here we have demonstrated that a simple change in culture temperature of *D. salina* from the optimal growth temperature (30°C) to a suboptimal temperature (18°C) increased the cellular content of β-carotene. The transfer of the cells to suboptimal temperature dramatically changed the fatty acid composition.

Materials and methods

Algal material and culture conditions

Dunaliella salina Teodoresco (Chlorophyceae, Dunaliellales) was isolated by Gómez-Pinchetti *et al.* (1992) from a hypersaline pond at the east coast of the island of Gran Canaria (Canary Islands, Spain). *D. salina* cells were grown at 30°C and 18°C under continuous illumination with white light at 450 μmol photon $m^{-2} s^{-1}$ (400–700 nm). The culture medium contained 2 M NaCl, 80 mM KNO_3, 200 mM $MgSO_4$, 4 mM K_2HPO_4, 0·001 mM $FeSO_4$, 0·1 mM EDTA, and 2 ml/l of a micronutrient solution (Jiménez del Río *et al.*, 1993). After preparation and sterilization, the medium was adjusted to pH 7·5 with 0·1 M NaOH. The algal suspensions were grown in 300 ml glass bottles and sparged with a CO_2/air mixture (5 : 95, v/v).

Analytical assays

The β-carotene concentration in *D. salina* cells was

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determined as described by Ramazanov *et al.* (1988). Lipid extraction and fatty acid analysis were performed according to methods described elsewhere (Petkov *et al.*, 1990). Culture growth was determined by direct count of cell numbers in a cell counter (Coulter-Counter Q-10).

Electron microscopy

For transmission electron microscopy, whole cells were fixed for 2 h at 4°C in 2·5% glutaraldehyde added to chloroplast isolation buffer containing 1% bovine serum albumin (BSA) or, in the case of whole cells, Hepes-KOH (pH 7·5) buffer. Samples were rinsed in the appropriate buffer, then postfixed overnight at 4°C in 1% osmium tetroxide. After a rinse in distilled water, the samples were dehydrated in a graded ethanol series, with 2% uranyl acetate added to the 80% step and held overnight at 4°C, to a final concentration of 100% ethanol. Samples were pelleted by centrifugation between each change. The samples were slowly infiltrated at 4°C with hard grade LR White epoxy resin, with seven changes over a period of several days, then polymerized for 24 h at 60°C. Thin sections were cut using a diamond knife mounted on a Sorvall Porter-Blum MT-2 Ultramicrotome, poststained with uranyl acetate and lead citrate, and examined and photographed on a Jeol JEM 100CX transmission electron microscope.

Results and discussion

Fig. 1 shows the growth rate and β-carotene content of *D. salina* cells grown at optimal (30°C) and suboptimal (18°C) growth temperatures. Optimal conditions for algal cell growth and for β-carotene synthesis were not the same, with β-carotene content at suboptimal growth temperature being significantly higher than at optimal growth temperature. The change in temperature from 30°C to 18°C was accompanied by a considerable decrease in cell growth (Fig. 1) and a decrease in the chlorophyll content in *D. salina* cells. This is in accordance with the hypothesis that metabolite synthesis is controlled by the uncoupling of cell functions and photosynthesis (Semenenko & Abdullaev, 1980; Ben-Amotz & Avron, 1989). Such an uncoupling opens up alternative pathways for transformation of the products of carbon assimilation into storage substances (polysaccharides, lipids, β-carotene, etc.), although the exact nature of this pathway is determined by the genetic properties of each strain.

The growth of *D. salina* cells under suboptimal temperature conditions dramatically changes the ultrastructural organization of the chloroplast. In *D. salina* cells grown under optimal temperature conditions, the chloroplast has a large pyrenoid which occupies the major part of the cell and does not contain carotene-containing lipid (Vladimirova *et al.*, 1978). However, in *D. salina* cells grown under suboptimal temperature the thylakoids occupy only a small part of the stroma and

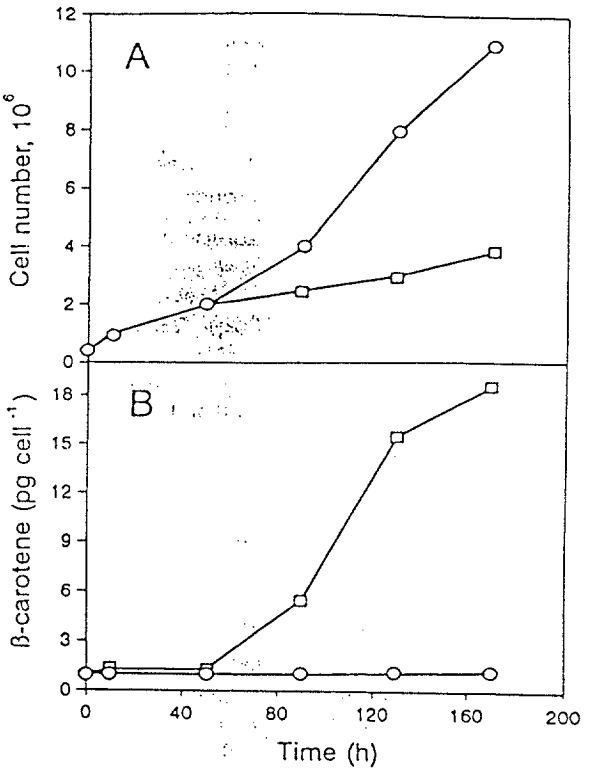

Fig. 1. Effect of suboptimal temperature on growth rate (*A*) and concentration of β-carotene (*B*) in *Dunaliella salina*. Algae previously grown at 30°C and 450 μmol m^{-2} s^{-1} were cultivated at 18°C at the same light intensity for 4 days. Growth rate and β-carotene concentration per cell are shown for 30°C (circles) and 18°C (squares).

there is an increase in the number of lipid–carotene globules in the periphery of the chloroplast. Similar effects have been observed in the chloroplast of *D. bardawil* by Ben-Amotz & Avron (1982) and by Lers *et al.* (1990), although in this case carotene-rich globules were induced by transferring algal cells from low to high light intensity at optimal temperatures for growth. The appearance of lipid globules in *D. salina* cells suggests the possibility that the induction of carotenogenesis is correlated to lipid accumulation under suboptimal temperature for growth. To test this, analyses were made of the total lipid and fatty acid composition in *D. salina* cells cultured under optimal and suboptimal temperatures. The cell transfer from optimal to suboptimal growth temperature conditions caused significant changes in the lipid content and in the fatty acid composition of lipids (Table 1). The content of fatty acids increased from 11% to 24·8% during carotenogenesis. The decrease in growth temperature from 30°C to 18°C caused significant changes in the fatty acid composition of lipids. It is notable that concentrations of palmitic and oleic acids increased significantly in cells cultured at 18°C. These results indicate that under unfavourable growth conditions, the cells accumulate storage lipids, a phenomenon observed in many algae from different taxa. These results agree with our pre-

Table 1. Effect of temperature on the fatty acid composition of *Dunaliella salina*

Fatty acid	Growth temperature	
	30°C	18°C
Myristic (14 : 0)	24·8	49·5
Palmitoleic (16 : 1n9)	8·1	5·1
Hexadecanoic (16 : 1n13)	3·3	2·1
Hexadecadienoic (16 : 2n6)	2·6	2·4
Hexadecatrienoic (16 : 3n3)	0·5	4·4
Hexadecatetraenoic (16 : 4)	20·6	39·0
Stearic (18 : 0)	1·9	3·2
Oleic (18 : 1)	9·1	44·2
γ-Linolenic (18 : 3n6)	1·1	7·2
α-Linolenic (18 : 3n3)	38·4	89·7
Octadecatetraenoic (18 : 4)	0·1	1·3
Total fatty acids	111·2	248·1

Values are $\mu g\ mg^{-1}$ dry mass.

vious data obtained with another strain of *D. salina* (Ramazanov *et al.*, 1988). The observed parallelism between accumulation of β-carotene and polyunsaturated fatty acids suggests that these compounds are involved in the mechanism governing the acclimation of cells at suboptimal temperatures. In *D. salina*, high levels of β-carotene imply resistance to high light intensity (Ben-Amotz *et al.*, 1982; Gómez-Pinchetti *et al.*, 1992). It has been suggested that the resistance to photoinhibition at low temperature demonstrated by *Chlamydomonas reinhardtii* cells is due to the increase in the resynthesis of photosystem II (Falk *et al.*, 1990).

Thus, suboptimal temperatures in combination with high light intensities are shown to be highly effective in enhancing carotenogenesis and can be used for the control of β-carotene biosynthesis in *D. salina*, although the molecular mechanisms of the β-carotene induction under the influence of suboptimal temperature for growth remain unclear.

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Seasonal changes in agar characteristics of two populations of *Pterocladia capillacea* in Gran Canaria, Spain

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Key words: Pterocladia capillacea, agar, seasonality

Abstract

Agar characteristics of *Pterocladia capillacea* were examined seasonally at two intertidal populations exposed to different wave energy on the northern rocky shore of Gran Canaria Island. Plants were collected monthly from August 1991 to July 1992. Agar yield, gel strength, melting and gelling temperature and chemical properties such as sulphate and pyruvate content were measured. Percent epiphytism was determined on both populations, together with the changes in biomass as dry weight. Specimens in the sheltered habitat were larger and more epiphytized than ones in the exposed area. There was a clear seasonal change in agar characteristics in both populations. Agar yields decreased in late spring and early summer, although in the sheltered habitat fluctuations were more erratic. Gel strength increased in winter, reaching a maximum in December-February. No significant differences were found in agar yield, gel strength or melting and gelling temperatures, but there was a difference between fish to dry weight ratio. The role of the exposure degree as a possible environmental factor responsible for this behavior is discussed. Agars of *Pterocladia capillacea* from Canary Islands show characteristics for industrial use.

Introduction

Agars are polysaccharides in the intercellular matrix of primarily two red algal families, Gracilariaceae and Gelidiaceae (Craigie, 1990). Agar provides structural support in response to water movements resulting in elasticity and rigidity of the alga. The structure of agar is basically composed of neutral and charged galactoses consisting of alternating molecules of D-galactose and 3,6 anhydro-L-galactose (Duckworth & Yaphe, 1971). Charged residues such as sulphate esters and pyruvate acetal play an important role in the physical and rheological properties of agar.

Pterocladia spp. are of considerable commercial significance as a source of the phycocolloid agar. They are the next important source of bacteriological agar and agarose to *Gelidium* (Armisén & Galatas, 1987). Both genera are exploited in the Azores and in New Zealand for the agar industry (McHugh, 1991). *Pterocladia* includes 10–12 tropical species having only two species common to temperate waters, *P. capillacea* (Gmelin) Bornet et Thuret and *P. lucida* (Turner) J. Agardh. The temperate species grow where there is strong water motion, which is considered to be the main factor affecting shore plant distribution (Santelices, 1988). Survival of this macroalga depends on its ability to withstand the hydrodynamic forces generated by breaking waves, an ability that may be related to both the morphology and size of the plant.

Various factors such as habitat, water temperature, light intensity and geography as well as biotic interactions such as epiphytism influence the relative proportions of seaweed constituents (Santelices, 1988). The effect of environmental conditions on agar composition is usually studied by following seasonal variations in quantity and quality. These variations may be regarded

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as biological alterations of the chemical and physical properties of the cell wall to meet environmental or physiological demands (Craigie & Wen, 1984). Several authors have reported these changes in other seaweed species, with only limited data for *Pterocladia capillacea* (Friedlander & Zelikovitch, 1984; Oliveira et al., 1995) (Table 1).

The influence of local or micro environmental factors on seasonal changes in phycocolloid yields and properties are often not known or inadequately documented (Craigie, 1990). Correlations have been found between species zonation, ecological distribution, and cell wall composition suggesting that matrix polysaccharides such as agar may be involved in mechanical regulations (Kloareg & Quatrano, 1988). For exmaple, in *Mastocarpus stellatus* and *Chondrus crispus* Dudgeon and Johnson (1992) found that differences in mechanical properties of the stipe may be reflected in differences of plant size which could be explained as different cell wall polysaccharides composition; however, they did not evaluate polysaccharides of these species.

Aim of this study was to present the seasonal differences in yield, rheological and physico-chemical properties of agar from *Pterocladia capillacea*.

Materials and methods

Study area

The Canary Islands are volcanic in origin with a rocky coastline consisting mostly of weathered basalt. The Islands are bathed by the relatively cold water of the Canary current flowing from NNE with surface temperatures at Gran Canaria between 18 and 23 °C. Salinity in oceanic waters around Canary Islands is stable at 37‰. Tidal range is moderate with a mean high tide level of 2 m and a mean low tide level of 0.8 m. In the northern rocky coast of the Canary Islands, *Pterocladia capillacea* is commonly found in the middle to low intertidal zone forming dense patches. Two localities exhibiting different wave exposure degree were sampled monthly. Quantitative measurements of water movement applicable to benthic situations are difficult to apply in wave beaten habitats; therefore the wave exposure was described in terms of prevailing currents, wind direction and coastal topography. Bocabarranco at the north (28°09′N, 15°40′W) is a sheltered zone protected from direct wave action by rock outcroppings while Agaete at the northwest (28°06′N, 15°43′W) is a more exposed boulder site directly impacted by the waves from the open coast.

Plant collection

Pterocladia capillacea plants were collected from August 1991 to July 1992 together with measurements of water temperature. Plants were cut above the holdfast during the lowest tides of each month. In the laboratory they were washed thoroughly with tap water to remove silt and sand. Wet weight was measured to the nearest 0.01 g after centrifugation in a commercial laundry centrifuge for 10 s to remove excess water. 100 g wet material was weighed ($n = 3$), oven dried overnight at 60–70 °C and reweighed. Samples were stored in sealed plastic bags until agar extraction.

To determine percent of epiphytism on *Pterocladia capillacea*, three sub-samples from the fresh material were weighed. Epiphytes were cleaned by brushing and scrapping *Pterocladia* fronds. The weight recorded after this procedure was taken as the value of pure seaweed (percent of agarophyte from original samples). Three samples (1 g each) of fresh pure seaweed were oven dried for 24 h at 60 °C to estimate monthly biomass changes in terms of dry weight.

Agar extraction

Dry seaweeds were exposed to a 0.5% solution of Na_2CO_3 at 85–90 °C for 30 min prior to extraction and washed with running tap water for 10 min. Agar was extracted ($n = 3$) with distilled water at pH between 6.0–6.5 and autoclaved at 120 °C for 2 h. The mixture was ground with a commercial blender and heated at 90 °C with diatomaceous earth for 30 min and finally pressure filtered (Armisén & Galatas, 1987). The filtrate was allowed to gel at room temperature, frozen overnight and thawed. Finally the agar was oven dried for 24 h at 60 °C, coooled and weighed to calculate percent agar yields.

Gel properties

Dry agar was ground in a Tecator mill and reconstituted into 1.5% w/v solutions to measure physico-chemical characteristics (gel strength, melting and gelling temperature, $n = 3$). Gel strength was measured after gelling overnight at room temperature by measuring the load (g cm^{-2}) causing the cylindrical plunger (1 cm^2 cross-section) to break a standard gel in 20 s (Armisén & Galatas, 1987).

Table 1. Pterocladia capillacea. Agar content physical properties and chemical characteristics found by other authors at different locations.

Locality	Agar content (%)	Gel strength (g cm^{-2})	Gelling T (°C)	Melting T (°C)	Sulphate (%)	Pyruvate (%)	Source
Barbados	15.3^a	--	--	--	3.7	0.65	Young et al. (1971)
Egypt	14.0–27.0^a	--	--	--	--	--	Rao & Bekheet (1976)
Brazil	36.5–37.0	--	--	--	--	--	Santos (1980)
Hawai	28.9–31.1	--	--	--	--	--	Santos (1980)
Florida	33.0–41.0^a	110–190	41.0–45.0^a	82.0–85.0^a	1.80–2.20	--	Cote & Hanisak (1986)
Israel	28.5^a	448^{a,b}	27.7	98	0.48	--	Friedlander & Lipkin (1982)
Israel	5.5–32.0^a	150–950^{a,b}	21.0–32.0^{a,b}	91.0–99.0^{a,b}	1.20–2.50^a	--	Friedlander & Zelikovitch (1984)
Venezuela	12.4–27.6	1313–1470	35.0–35.5	97	0.30–2.60	0.0	Lemus et al. (1991)
Brazil	15.0–34.0	--	--	--	--	--	Oliveira & Berchez (1993)
Brazil	5.5–32.2	--	--	--	1.00–4.60	--	Oliveira et al. (1995)
Spain	15.0–29.5^a	813–1428^a	34.6–36.3^a	88.9–95.5^a	2.12–3.43^a	0.14–0.47^a	This study (Agaete)
Spain	16.7–29.8^a	912–1354^a	34.4–36.9^a	86.9–94.9^a	1.98–3.10^a	0.12–0.50^a	This study (Bocabarranco)

[a] Native agar (without alkali treatment);
[b] 1% agar solutions.

Gelling temperature was obtained by the addition of 10 ml hot agar solution into a test tube (2.3 cm diameter, 6 cm height). A glass bead (5 mm diameter) was placed in the test tube. The tube was tilted up and down in a water bath at room temperature until the glass bead ceased moving. The gel temperature in the tube was immediately measured introducing a precision thermometer (0.1 °C divisions). Melting temperature of the gel in a test tube (2.3 cm diameter, 16.5 cm height) was measured by placing an iron bead (9 mm diameter) on the gel surface. The test tube was clamped in a water-bath and the temperature raised from 50 to 100 °C; melting point was recorded with a precision thermometer when the bead sank into the solution.

Agar substitutions

Percent sulphate was determined by hydrolyzing 1 g of agar powder (previously dried at 105 °C) in 10 ml HNO_3 in 100 ml Kjeldahl flasks that results in complete hydrolysis of the ester sulphate followed by quantitative precipitation with barium chloride of the liberated sulphate. The precipitates were collected on ash-free gravimetric filters, dried, ignited and weighed on a precision balance (0.0001 g). The weight of the obtained barium sulphate, multiplied by 0.4116, gave the equivalent of sulphate. Percent pyruvate was determined by hydrolyzing 0.5 g of agar powder in oxalic acid 0.02 M following the spectrophotometric method based on lactic dehydrogenase by Duckworth & Yaphe (1970).

Statistical analysis

The data were tested for normality (Kolmogorov-Smirnov) using a statistical software package (Stasoft). Data were subjected to the Bartlett's test for homogeneity of group variances and to Pearson's product moment correlation test to determine linear relationship between treatments. Spearman correlation was used when necessary. Multifactorial analyses of variance were applied to determine the effects of single treatments and degrees of interaction between different treatments on agar characteristics whenever data groups exhibited homogeneity. The single and combined effects of season and locality on agar properties for *Pterocladia capillacea* were determined as well through multifactorial analysis of variance. MANOVA comparison of means was done using Tukey's HSD test. All heterogeneous data groups were transformed by different methods including arcsin square root of x, log(x + 1) and ln(x + 1) in order to produce th ehomogeneity required for a multifactorial analysis of variance. Monthly mean differences for data groups that retained their heterogeneous character were tested using non-parametric Kruskal-Wallis one way analyses of variance.

Results

Pterocladia capillacea grows year round in the two study areas on the northern rocky shore of Gran

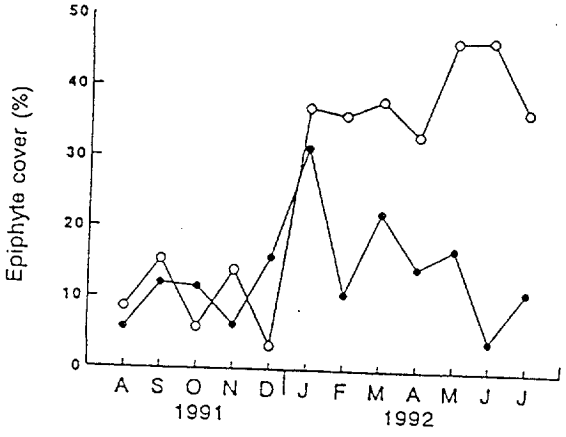

Figure 1. Epiphytic coverage on samples collected at the exposed Agaete site (•) and the sheltered Bocabarranco site (o) during the study period. Bars represents standard deviation.

Canaria. Specimens collected in Agaete were shorter, forming dense mats while in Bocabarranco were larger and quantitatively more epiphyted throughout the year. The mean value for epiphyte coverage was 26.6% of the fresh weight in Bocabarranco, whereas in Agaete only 13.6% of plant fresh weight was from epiphytes. In Bocabarranco the increase in epiphyte cover was more evident from late winter to the beginning of summer with the maximum value between May–June (46.8%) while in Agaete the maximum was recorded in January, 31.4% (Figure 1). The most common epiphyte was the crustose red alga *Lithothamnion* sp. although during the summer months *Ulva rigida* was most evident coinciding with an increase in water temperature.

Fluctuations in percent dry weight followed a seasonal pattern. In both localities the maximum values were found in early autumn, September in Agaete and October in Bocabarranco, and minimum in winter and spring (Figure 2). The mean value in Agaete (28.6%) was higher than in Bocabarranco (25.8%).

Sea water temperature ranged between 17.7 and 23.5 °C with maximum values recorded during late summer and early autumn (Figure 2). There was a positive correlation between sea water temperature and dry weight in Agaete ($r = 0.36$, $p < 0.05$) and in Bocabarranco ($r = 0.52$, $p < 0.05$). No significant correlation was found between sea water temperature and agar yield at Agaete ($r = 0.23$) or Bocabarranco ($r = 0.29$).

Agar content ranged from 15% to 29.5% in Agaete and from 16.7% to 29.8% in Bocabarranco (Figure 3a). Agar content was slightly higher in Agaete (22.9%)

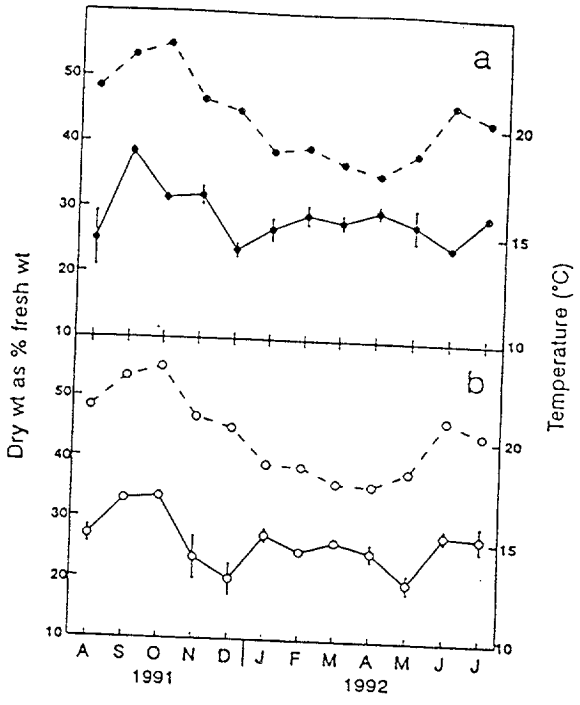

Figure 2. Dry weight fluctuations (—) in relation to seawater temperature (– –) in (a) Agaete and (b) Bocabarranco. Bars represents standard deviation.

than in Bocabarranco (21.4%). Overall, the highest yields were obtained from plants harvested during late summer and autumn, with a maximum value in August for Bacabarranco, whereas in Agaete the highest peak value was found in December. In both localities, agar yields declined in late spring and early summer (Figure 3a). There was a positive correlation between agar yield values in Agaete and Bocabarranco ($r = 0.35$, $p < 0.05$).

Gel strength ranged from 813 to 1428 g cm^{-2} for Agaete (mean, 1145 g cm^{-2}) and between 912 to 1354 g cm^{-2} (mean, 1170 g cm^{-2}) for Bocabarranco. In both localities it increased in winter, reaching a maximum in December–February (Figure 3b). Minimum values were found in August at Bocabarranco, and in November at Agaete. There was a positive correlation ($r = 0.53$, $p < 0.01$) in this variable between the sites.

In both localities, the sulphate content was highest in March 3.43% in Agaete and 3.11% in Bocabarranco (Figure 3c), and was positively correlated between both sites ($r = 0.59$, $p < 0.05$). There was no correlation between gel strength and sulphate content in either site (Table 2). Percent of pyruvic acid ranged from

Table 2. Correlation coefficient matrix of various chemical and physical properties of agar characteristics sampled in this study. Correlation coefficients greater than 0.36 are significant at the 95% level ($p < 0.05$).

Agaete/Bocabarranco	Gelling temperature (°C)	Melting temperature (°C)	Gel strength (g cm^{-2})	Sulphate content (%)
Melting temperature (°C)	0.36/0.55	--		
Gel strength (g cm^{-2})	-0.21/0.26	0.36/0.44	--	
Sulphate content (%)	-0.24/-0.32	-0.13/-0.42	0.18/-0.01	--
Agar content (%)	0.43/0.29	0.34/0.22	0.15/-0.37	-0.30/0.13

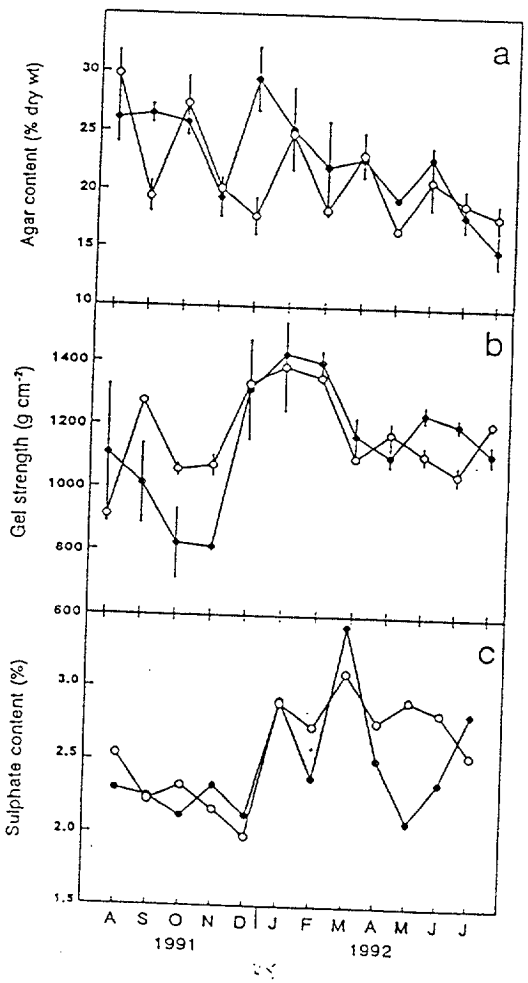

Figure 3. Seasonal variation of the agar characteristics at Agaete (•) and Bocabarranco (○). a. Agar yield expressed as percent of dry weight from pure seaweed. b. Gel strength of 1.5% agar solution. c. Sulphate content in agar samples. Bars represents standard deviation.

0.14% to 0.47% in Agaete and from 0.12% to 0.50% in Bocabarranco.

Mean gelling temperatures were 35.4 and 35.6 °C for Agaete and Bocabarranco respectively, while melting temperature was 91.9 and 91.6 °C, respectively (Table 3). There was a positive correlation for gelling temperature between Agaete and Bocabarranco ($r = 0.47$, $p < 0.01$) and for melting temperatures between both sites ($r = 0.69$, $p < 0.01$). Data showed a positive correlation between gel strength and melting temperature in Agaete and Bocabarranco (Table 2).

Gel strength data showed monthly significant differences when analyzed by the MANOVA test. However, no significant differences between Agaete and Bocabarranco were found for this variable. Due to the heterogeneity of variance data for agar content, dry weight, gelling and melting temperatures were analyzed using Kruskal Wallis one way analysis of variances. This showed monthly significant differences ($p < 0.01$), and a significant difference between localities for the dry weight ($p < 0.01$) (Table 4).

Discussion

Two main findings were obtained from this study. Agar characteristics changed seasonally in both *Pterocladia capillacea* populations, agar yield decreased in late spring and gel strength increased in winter. No evidence of influence of wave exposure degree on the agar characteristics was found, although differences between localities were found in plant size, dry weight and epiphyte cover.

The seasonal pattern of agar yield showed a more erratic fluctuation over the year in the sheltered intertidal population at Bocabarranco. Although few data exist in the literature for the agar in *Pterocladia capillacea* comparisons on agar yield values were similar

Table 3. Gelling and melting temperatures of agar at both localities.

Month	Agaete		Bocabarranco	
	Gelling T°C	Melting T°C	Gelling T°C	Melting T°C
Aug	36.0±0.5	92.6±1.0	35.7±0.6	94.2±0.3
Sep	36.3±0.2	89.2±1.1	34.7±0.3	93.7±0.6
Oct	35.9±0.6	93.5±0.5	36.6±0.6	92.9±0.8
Nov	35.4±0.5	93.9±0.7	36.4±0.4	92.7±0.7
Dec	35.4±0.2	93.2±0.8	36.1±0.0	93.8±0.6
Jan	35.6±0.3	95.5±0.3	36.9±0.8	93.7±0.2
Feb	35.6±0.1	94.6±1.0	36.5±0.3	94.9±0.1
Mar	34.6±0.1	88.9±0.0	34.4±0.3	88.7±0.5
Apr	35.1±0.3	89.2±0.0	35.1±0.0	89.8±0.8
May	35.4±0.5	90.2±0.0	34.8±0.3	89.4±0.3
Jun	35.3±0.2	91.1±0.6	35.0±0.0	86.9±0.4
Jul	34.9±0.1	89.5±0.4	34.7±0.7	88.3±0.3

to those obtained by others (Table 1). Minimum values were found in early summer coinciding with the lowest values found by other authors in the same months (Friedlander & Zelikovitch, 1984; Oliveira & Berchez, 1993; Oliveira et al., 1995). However, in this study agar yield evaluations were based on dry seaweed free of epiphytes that gives more reliable yield values.

Dry to wet weight ratio may reflect many physiological and biochemical processes within the alga, such as storage of high molecular weight reserves (Craigie, 1990). Although no significant correlation between dry weight and agar yield were found in both populations, the highest values of dry weight and agar yield occurs in the same season. The increase in biomass based on dry weight changes was related to an increase in seawater temperature. It appears that an increase in dry weight was stimulated in autumn by conditions of high temperature and high light intensity (see also Fralick & Andrade, 1981) for populations at the Azores. The mean dry weight values for *Pterocladia capillacea* in the Canary Islands were higher than those obtained by Friedlander & Lipkin (1982) in the Mediterranean (24.8%).

The difference between the two localities in dry weight values may be related to plant size. The morphological variations observed in *Pterocladia capillacea* between sheltered and exposed population can be explained by differences in water movement. Similar effects of wave exposure on morphology in *P. capillacea* have been observed in Brazil (Oliveira & Berchez, 1993) and morphological variations in *P. caeruelescens* in Hawaii (Santelices, 1978).

Epiphytism also seems to be affected by wave exposure. Lowest epiphytism was found during autumn associated with higher tides and increased water movement (Figure 1). In Agaete epiphytism showed a more homogeneous pattern throughout the year most probably due to higher exposure. In addition, epiphyte coverage may have affected dry weight values. According to Santelices (1988) epiphytes are particularly common on larger subtidal thalli, reducing the amount of available light and decreasing the agar yield. In this regard, Torres et al. (1991) showed a direct relationship between photosynthetic rates and carbon and nitrogen content in *Gelidium sesquipedale*, with a reduction in the C:N ratio under decreasing irradiance and leading to a decrease in cell-wall polysaccharides. Perhaps the small differences in agar yield between the sites in this study are due to differences in epiphyte cover.

Oliveira et al. (1995) found a higher agar content in *P. capillacea* plants inhabiting more turbulent waters in Brazil. In the present study there was no evidence for this, although the values of agar yield and gel strength were slightly higher in the more exposed intertidal population (Agaete). However, it is difficult to compare our study in Gran Canaria with that of Brazil because the factors involved in seasonal effects are likely to differ from one location to another.

Gel strength followed a similar seasonal pattern in both localities in this 12-month study. Friedlander & Zelikovitch (1984) showed a different gel strength pattern in a 7-month study, with a maximum in summer (950 g cm^{-2}). Our gel strength values are comparable with those obtained by Lemus et al. (1991), although no alkali treatment was performed. Thus, the gel strength

Table 4. Kruskall-Wallis ANOVA test for significance differences between the two localities in agar characteristics and dry weight.

Variable	n	Sum of ranks	H	p
Agar yield				
Agaete	32	1206.0	2.194	0.138
Bocabarranco	35	1072.0		
Dry weight				
Agaete	32	1237.0	7.057	<0.01
Bocabarranco	32	834.0		
Gelling temperature				
Agaete	33	1086.5	0.198	0.655
Bocabarranco	34	1191.5		
Melting temperature				
Agaete	31	1050.5	0.647	0.421
Bocabarranco	32	965.5		

values obtained here are higher than previous studies (Table 1).

There was no correlation between sulphate and gel strength as stated by Yaphe & Duckworth (1972). During alkali treatment the L-galactose 6-sulfate can be converted to 3,6-anhydro L-galactose, however the low concentration of Na_2CO_3 would not hydrolyze sulphate in this position and would not affect the chemical composition of native agar. Similar to our results, Mouradi-Givernaud et al. (1992) could not find any correlation between the sulphate content and gel strength in *Gelidium latifolium*.

Pyruvate content was low when compared to the other authors (Table 1), however, values should be taken with caution since absolute values for the pyruvate content of agar can only be quantified with the use of nuclear magnetic resonance spectroscopy (NMR).

Any correlation between gel strength and melting temperature can in part be explained by the methoxyl substitutions at various position in the agar molecule (Yaphe & Duckworth, 1972). High values of gelling and melting temperatures of the agar in *Pterocladia capillacea* corresponds with high gel hysteresis, thus gel strength is the most important parameter used to determine applications of agar.

In the genus *Pterocladia* gel strengths above 700 g cm^{-2} and low sulphate and pyruvate values (below 5% and 0.5% respectively) is critical for bacteriological uses (Armisén & Galatas, 1987). Our results show that agar from *Pterocladia capillacea* in Gran Canaria has commercial value.

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UNDERWATER LIGHT AND ALGAL PHOTOBIOLOGY, *F.L. FIGUEROA, C. JIMÉNEZ, J.L. PÉREZ-LLORÉNS and F.X. NIELL (eds.)*

Ultrastructural and biochemical adaptation of algal cells to limiting CO_2 concentrations*

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SUMMARY: The pyrenoid is a prominent proteinaceous structure found in the stroma of the chloroplast in unicellular eukaryotic algae, most multicellular algae, and some hornworts. The pyrenoid contains the enzyme ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) and is sometimes surrounded by a carbohydrate sheath. In *Chlamydomonas reinhardtii* the pyrenoid starch sheath is formed rapidly in response to a decrease in the CO_2 concentration in the environment. In this report we show that the unicellular green algae *Chlamydomonas acidophila* and *Chlorella spK* also form a starch sheath when adapted to low CO_2 conditions. We also report that in some high CO_2-requiring *C. reinhardtii* strains, pyrenoid starch sheath formation is partly inhibited or absent. The inhibition of the pyrenoid starch sheath is also seen when the carbonic anhydrase inhibitor ethoxyzolamide is added to cells placed in an environment low in CO_2. These observations support the idea that the ultrastructural reorganization of the pyrenoid starch sheath under low CO_2 conditions plays a role in the CO_2 concentrating mechanism in *C. reinhardtii* as well as in other eukaryotic algae.

Key words: CO_2, photsynthesis, pyrenoid, starch, *Chlamydomonas, Chlorella.*

INTRODUCTION

Since the discovery of the reductive photosynthetic carbon cycle by Calvin and his colleagues over forty years ago a number of variations on this basic metabolic pathway have been identified. In terrestrial plants, C_4 photosynthesis has evolved in response to decreasing atmospheric CO_2 levels while Crassulacean Acid Metabolism (CAM) is found in many plants growing in arid conditions. Like C_4 plants, aquatic organisms must be able to efficiently acquire C_i from the environment since the diffusion of CO_2 and HCO_3^- is thousands of times slower in aqueous solutions than in air. Most aquatic photosynthetic organisms have adapted to the aqueous environment by having some form of a CO_2 concentrating mechanism (CCM), a means of concentrating CO_2 at the site of Rubisco (Badger *et al.*, 1980). Cells with a CCM have the ability to accumulate inorganic carbon (CO_2 and HCO_3^-) to levels higher than can be obtained by simple diffusion. Algae with the CCM can grow on very low CO_2 concentrations, lower than that tolerated by plants with C_3-type photosynthesis.

The intracellular localization of ribulose bisphosphate carboxylase oxygenase (Rubisco) appears to be critical in the operation of C_3-photosynthesis and the CCM. In plants with C_3 photosynthesis, Rubisco is distributed throughout chloroplast stroma

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TABLE I. – The localization of Rubisco in plants using different photosynthetic pathways.

Photosynthesis Type	Type of Organism	CO_2 affinity	Rubisco location
C-3 photosynthesis	most land plants	low	chloroplast stroma-leaf mesophyll cells
C-4 photosynthesis	diverse land plants	high	chloroplast stroma-bundle sheath cells
CO_2 concentrating mechanism (CCM)	most algae and cyanobacteria	high	unicellular green algae-chloroplast pyrenoid cyanobacteria - carboxysomes

of the mesophyll cells of the leaf (Table 1). For plants with C_4 photosynthesis, Rubisco is still soluble in the chloroplast but now is localized only within the bundle sheath cells of the leaf. Finally in algae, Rubisco is packaged within the cell or chloroplast. In cyanobacteria Rubisco is localized in proteinaceous structures called carboxysomes. In eukaryotic algae Rubisco is localized in the pyrenoid. Recent research summarized below indicates that the carboxysome and the pyrenoid may be essential to the functioning of the CCM in algae.

In cyanobacteria Rubisco is localized within the carboxysome (Codd and Marsden, 1984). Kaplan and his colleagues (Friedberg *et al.*, 1989; Lieman-Hurwitz *et al.*, 1991) as well as Badger and his colleagues (Price *et al.*, 1993) used insertional mutagenesis to select for cyanobacterial mutants that have a defective CCM. The phenotype of these mutant strains is that they are unable to grow on low levels of CO_2. One class of mutant that they have identified are cells that have aberrant carboxysomes or have no carboxysomes at all. Mutants identified have point mutations or insertions in genes known as ccmL or just identified as an open reading frame (ORF). Recently English *et al.* (1994) have identified the gene encoding the carboxysome coat protein of the chemolithotroph *Thiobacillus neapolitanus* and this gene has significant homology to the ORFII identified in *Synechococcus* (Friedberg *et al.*, 1989). A second type of carboxysome mutant has an insertion within the Rubisco small subunit gene. This results in a cell that has very long rod-shaped carboxysomes and also requires high CO_2 for growth. The implication of these results is that the carboxysome must be intact for the CCM to operate.

In eukaryotic algae Rubisco is found in pyrenoids. The pyrenoid is a large protein complex found within the chloroplast sometimes surrounded by a sheath of carbohydrate such as starch, amylose or paramylon (Gibbs, 1962a, 1962b; Griffiths, 1980; Kuchitsu *et al.*, 1988a; Okada, 1992). In the past the most common physiological role suggested for the pyrenoid was that of a protein or carbohydrate storage body (Griffiths, 1980; McKay and Gibbs, 1991; Okada, 1992). However recent immunolocalization studies have indicated that most, if not all, of the Rubisco is localized in the pyrenoid (Lacoste-Royal and Gibbs, 1987; Osafune *et al.*, 1990; Kuchitsu *et al.*, 1991; McKay and Gibbs, 1991; Okada *et al.*, 1991), indicating that the pyrenoid is the active location of CO_2 fixation and not simply a storage structure.

Light microscopy studies have shown that cells adapted to low CO_2 conditions are more likely to have starch around the pyrenoid than cells adapted to high CO_2 conditions (Miyachi *et al.*, 1986; Kuchitsu *et al.*, 1988b; Kuchitsu *et al.*, 1991). In *Chlamydomonas reinhardtii*, as well as *Chlorella*, *Scenedesmus* and *Dunaliella* species, the CCM is inducible; only algal cells grown on air levels of CO_2 (0.03%) have an active CCM (Badger *et al.*, 1980; Aizawa and Miyachi, 1986). Recent work from our laboratory has shown that the formation of the starch sheath around the pyrenoid correlates well with the induction of the CCM in *Chlamydomonas* (Ramazanov *et al.*, 1994). This is further evidence that the pyrenoid may play an important role in the CCM in these eukaryotic algae, a role similar to that of the carboxysome in cyanobacteria. In this report we have looked at pyrenoid morphology in other unicellular green algae growing in an environment low in CO_2. In addition we report on the pyrenoid structure of some high CO_2-requiring mutants of *Chlamydomonas*.

MATERIAL AND METHODS

Algal culture and growth conditions

Chlamydomonas reinhardtii 137 wild type was obtained from Dr. R. K. Togasaki, Indiana University, Bloomington IN, USA and maintained in this laboratory. *Chlamydomonas acidophila* was

obtained from Prof. J. Kalina of the Czech culture collection. *Chlorella spK* was obtained from the Russian algal collection (Moscow). *C. reinhardtii* and *Chlorella spK* were grown in minimal media (Sueoka, 1960) while *C. acidophila* was grown in Murashige and Skoog media without sucrose (Murashige and Skoog, 1962) with the pH adjusted to 4.5. In liquid culture, the strains were inoculated at a cell density of 4 x 10^4 cells ml^{-1} and aerated with 5% CO_2 in air with continuously illumination at 100 µmol $m^{-2}s^{-1}$ of white light for 3 days. The cultures were then switched to air levels of CO_2, where indicated, and maintained at that level of CO_2 until they were harvested.

Electron Microscopy

For transmission electron microscopy two methods of fixation and embedding were used. In the first procedure, cells were fixed for 2 h at 4° C in 2.5% glutaraldehyde added to Hepes-KOH (pH 7.5) buffer. Samples were rinsed in the same buffer, then postfixed overnight at 4° C in 1% osmium tetroxide. After a rinse in distilled water, the samples were dehydrated in a graded series of ethanol, with 2% uranyl acetate added to the 80% step and held overnight at 4° C, to a final concentration of 100% ethanol. Samples were pelleted by centrifugation between each change. The samples were slowly infiltrated at 4° C with Spurr's resin (Spurr, 1969), with seven changes over a period of several days, then polymerized for 12 h at 60° C.

In the second procedure, cells were fixed in a mixture of 2% glutaraldehyde and 2% osmium tetroxide in half-strength growth medium for 15 min. The cells were then filtered and further fixed in the same fixative mixture in 0.05M cacodylate buffer (pH 7.1), for 20 min, rinsed for 5 min, stained *en bloc* with 0.5% uranyl acetate for 30 min, dehydrated in ethanol, then infiltrated and embedded in LR White medium resin. Thin sections were cut using a diamond knife mounted on a Sorvall Porter-Blum MT-2 or MT 5000 Ultramicrotome, poststained with uranyl acetate and or lead citrate, and examined and photographed on a JEOL JEM 100CX Transmission Electron Microscope.

Photosynthesis assays

The photosynthetic rate of algal cells was measured with an oxygen electrode (Rank Brothers, Cambridge, England). Algae were centrifuged at

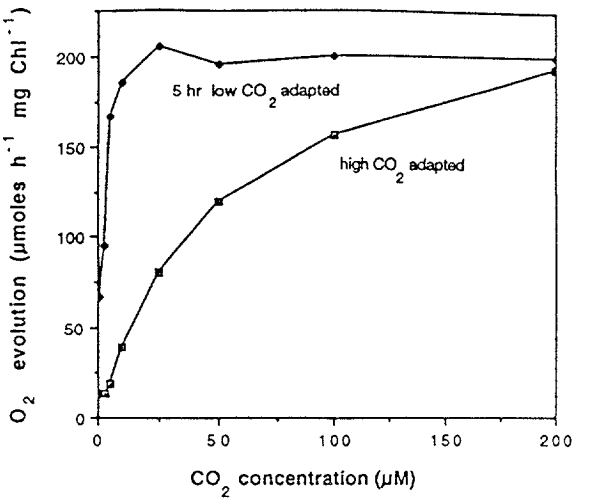


FIG. 1. – The rate of photosynthesis versus inorganic carbon concentration for wild-type *C. reinhardtii* cells grown under high CO_2 concentrations (○) or adapted to low CO_2 for five hours (•).

5,000 rpm for 5 min and the pelleted algae were resuspended at 25 µg Chl ml^{-1} in 4 ml of 25 mM Hepes-KOH (pH 7.3) and transferred to the electrode chamber, where they were allowed to consume the inorganic carbon of the buffer and intracellular pool of C_i until no net O_2 exchange was observed which took between 3 and 10 min. Bicarbonate at the indicated concentrations was added and the rate of O_2 evolution measured over the next 0.5-2 min. Chlorophyll concentrations were determined spectrophotometrically.

RESULTS

In unicellular green algae, the CCM is inducible. When these cells are grown on elevated CO_2 they do not induce the CCM and the cells' affinity for CO_2 remains low. Cells grown under these conditions require about 25 to 40 µM CO_2 for half maximal rates of CO_2 fixation (Fig. 1). However when these cells are placed under limiting CO_2 they induce the CCM and they now require about 1 to 3 µM CO_2 for half maximal rates of photosynthesis (Fig. 1). This inducibility has been seen in a large number of unicellular algae (Aizawa and Miyachi, 1986; Merret, 1991). The induction of the CCM requires about five hours. The pyrenoid starch sheath also is formed within this period of time. Figure 2 shows that *Chlamydomonas* cells that have adapted to low CO_2 have a complete starch sheath while those on elevated CO_2 have only the normal form of starch

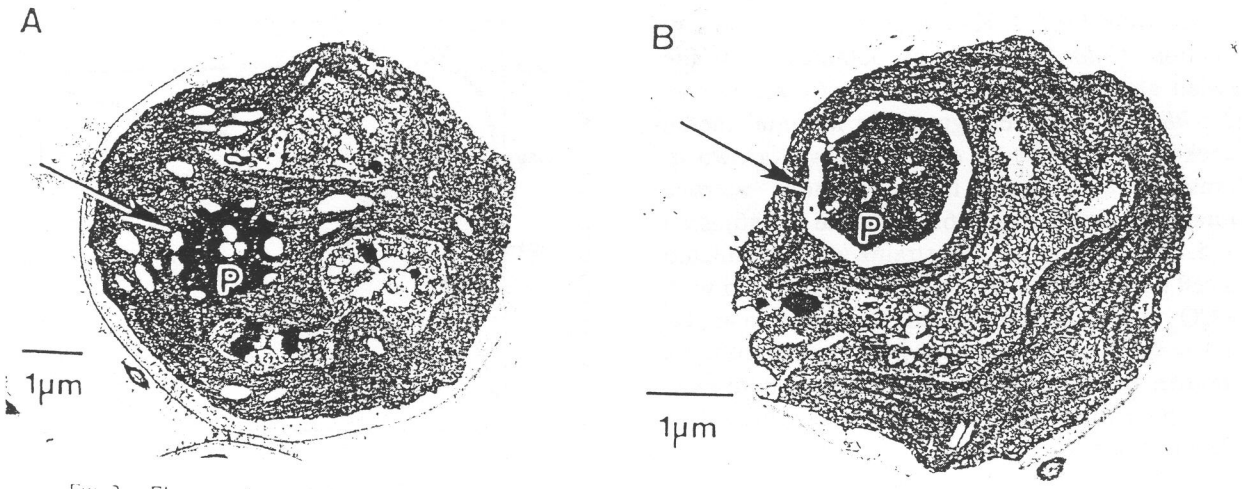


FIG. 2. – Electron micrographs of *C. reinhardtii* cells grown on elevated CO_2 (A) or adapted for five hours on low CO_2 (B).

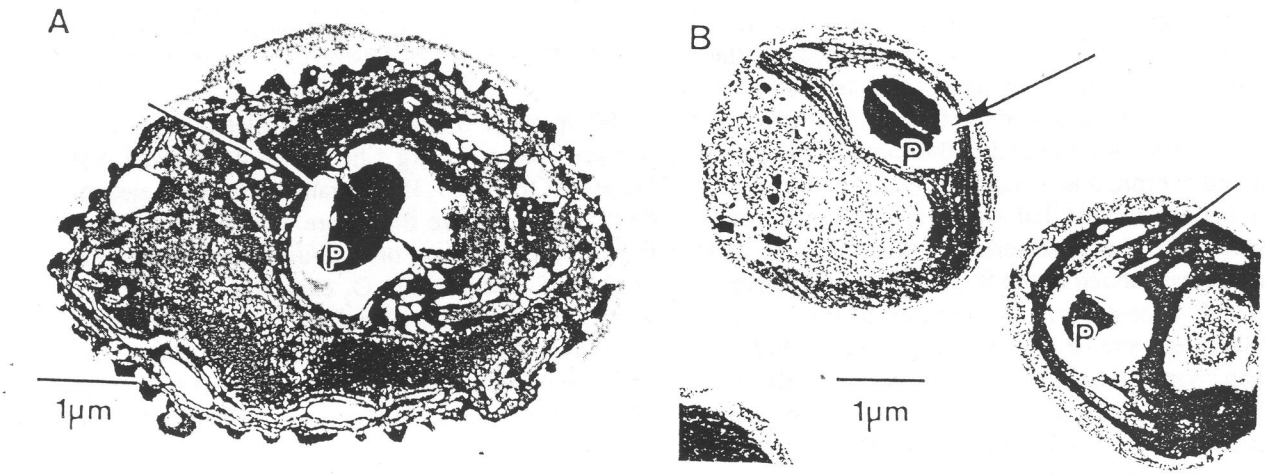


FIG. 3. – Electron micrographs of *Chlamydomonas acidophila* (A) and *Chlorella spK* (B) grown on low CO_2.

Recent work from this laboratory has shown that the biosynthesis and breakdown of the starch sheath coincides well with the induction and loss of the CCM in *C. reinhardtii* (Ramazanov *et al.*, 1994).

This thick starch sheath is also seen in other unicellular green algae when they are grown on limiting CO_2. *Chlamydomonas acidophila* is an acid tolerant alga which has a different type of pyrenoid than *C. reinhardtii* with fewer thylakoid membranes within the pyrenoid. The *Chlorella spK* has also been used in the study of CCM organization and carbonic anhydrase activity (Pronina *et al.*, 1981). These two algae both induce a CCM as does *C. reinhardtii*. In addition, both make the pyrenoid starch sheath when exposed to low CO_2 conditions (Fig. 3). These results support the contention that the pyrenoid starch sheath may be playing a [illegible] in a variety of green algae.

If the pyrenoid is playing a role in the CCM then one might expect that mutants that have a disrupted pyrenoid might also have a less efficient CCM. With this in mind we investigated the pyrenoid structure of some high CO_2 requiring *C. reinhardtii* mutants. In Table 2 we show the apparent CO_2 affinity of three of these high CO_2 requiring strains and in Figure 4 we show the pyrenoid morphology of these strains. Cia-5 is a strain that adapts poorly to low CO_2 conditions (Moroney *et al.*, 1989). When placed in a low CO_2 environment this strain has a lower affinity for CO_2 than wild-type cells and fails to make any of the low CO_2 inducible proteins normally seen in wild-type cells (Moroney *et al.*, [illegible]

[illegible] Figure 4

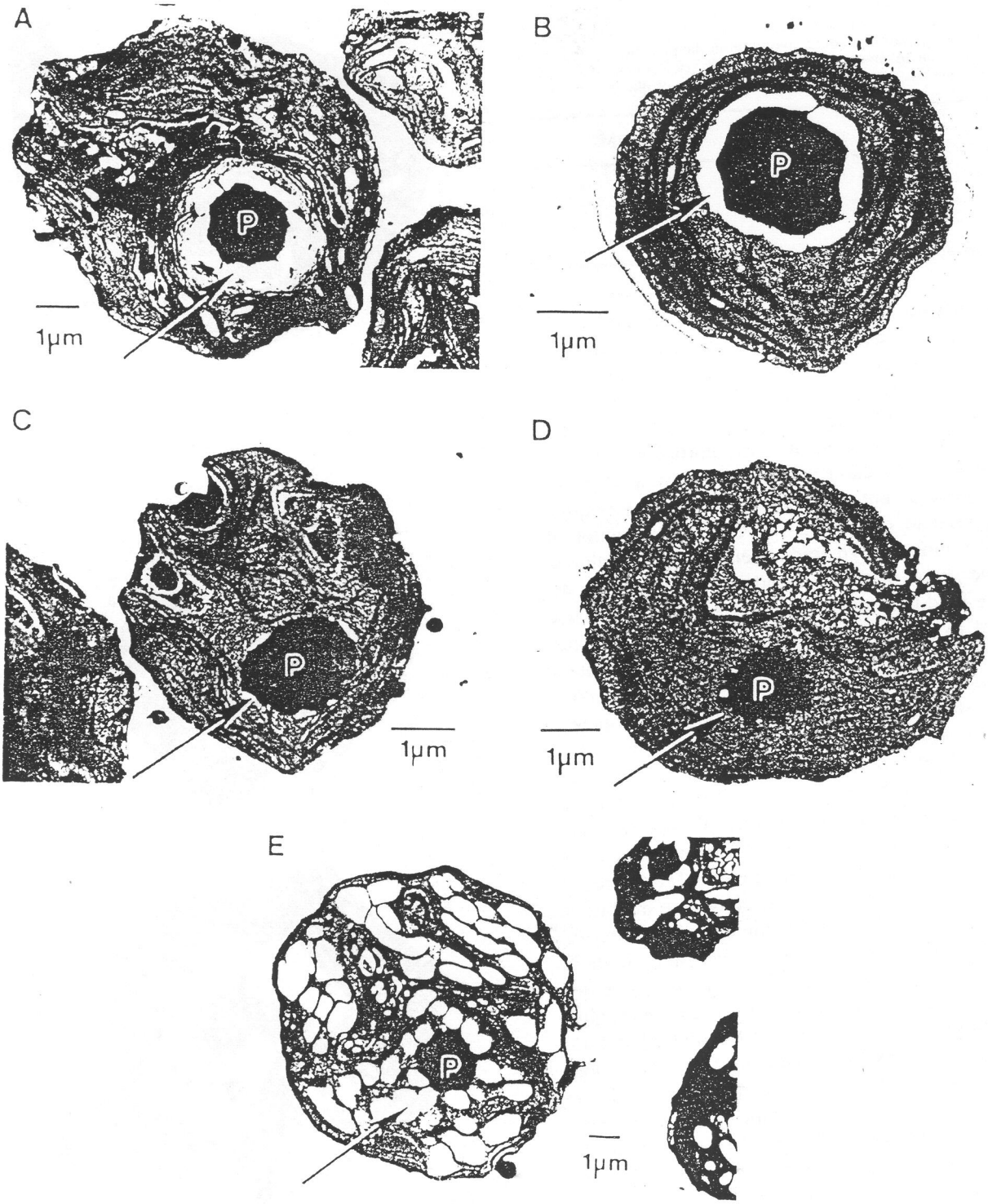


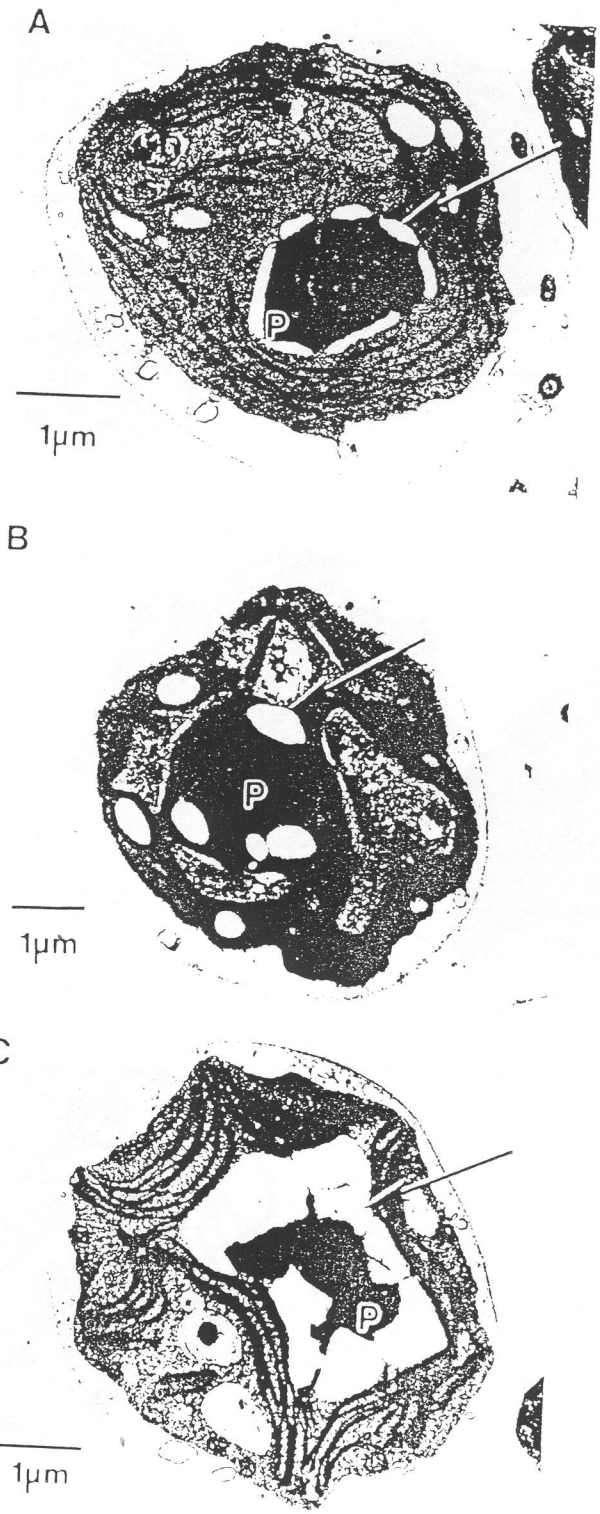
Fig. 5. The pyrenoid morphology wild type *C. reinhardtii* [illegible] 5g 0.055% CO_2 (A); acetazolamide 0.055% CO_2 (B) [illegible]

TABLE 2. – Apparent affinity for CO_2 of wild-type cells and the high CO_2 requiring mutants cia-5, cia-3 and ca-1. Cells were switched from a high CO_2 environment to one low in CO_2 for five hours prior to measuring their rates of O_2 evolution in the presence of differing concentrations of inorganic carbon. The $K_{0.5}(CO_2)$ is the CO_2 concentration which supports half-maximal rates of photosynthesis.

Strain	$K_{0.5}(CO_2)$ (μM)
wild-type	2.5
cia-5	17
cia-3	65
ca-1	52

1986; Husic and Marcus, 1994; Katzman *et al.*, 1994). This strain has a very poor affinity for CO_2 (Table 2) and fails to synthesize the pyrenoid starch sheath (Fig. 4). Ca-1 is a high CO_2 requiring mutant selected by Spalding *et al.* (1983) which is also thought to be deficient in the chloroplast carbonic anhydrase. Like cia-3 it has a very low affinity for CO_2 (Table 2) but its pyrenoid morphology is quite different than cia-3 (Fig. 4). Ca-1 makes a lot of starch around the pyrenoid under low CO_2 conditions but the shape of the pyrenoid is abnormal (Fig. 4). Unlike wild type cells one often observes irregularly shaped pyrenoids and starch that is deposited unevenly around the pyrenoid (Fig. 4). From these data it appears that some of the high CO_2-requiring *C. reinhardtii* strains have abnormal pyrenoid development similar to the carboxysome mutants of cyanobacteria.

Earlier genetic studies indicated that ca-1 and cia-3 were allelic (Moroney *et al.*, 1986). Since both of these strains were deficient in the chloroplast carbonic anhydrase, there might be a link between the chloroplast carbonic anhydrase and pyrenoid structure. To test this we added two carbonic anhydrase inhibitors, acetazolamide and ethoxyzolamide, to *C. reinhardtii* cells and looked at the pyrenoid morphology in these cells. Acetazolamide is a less permeant inhibitor thought to only inhibit the periplasmic carbonic anhydrase (Moroney *et al.*, 1985). This inhibitor had little affect on the affinity of the cells for CO_2 or pyrenoid structure (Fig. 5B). However cells treated with the membrane permeant inhibitor, ethoxyzolamide, failed to make a starch sheath (Fig. 5C). This result resembles the results obtained with cia-3 (Fig. 4B). One additional effect of ethoxyzolamide was observed in cells grown on elevated CO_2 (Fig. 5E). Cells treated with ethoxyzolamide made a lot of starch that was distributed throughout the chloroplast stroma. These results indicate that there might be a link between the chloroplast carbonic anhydr
deposition by these algae



DISCUSSION

The intracellular packaging of Rubisco is very common among photosynthetic organisms that possess a CCM. In cyanobacteria Rubisco is localized in carboxysomes while in eukaryotic unicellular algae it is localized to the pyrenoid. It is thought that the carboxysome and the pyrenoid are the sites where the bicarbonate accumulated by the cell is dehydrated to CO_2 causing a localized elevated CO_2 concentration. This mechanism would allow the Rubisco to take advantage of the higher CO_2 concentration increasing the carboxylation reaction while decreasing photorespiration (Badger and Price, 1994).

The evidence supporting this hypothesis is strong in cyanobacteria. First it is clear that these organisms take up bicarbonate from the medium (Miller *et al.*, 1990; Reinhold *et al.*, 1991). Recently, a carbonic anhydrase has been localized in or at the carboxysome (Price *et al.*, 1992). In addition, the introduction of a human carbonic anhydrase into the cytoplasm causes the dehydration of bicarbonate to occur in an inappropriate location which disables the CCM (Price and Badger, 1989). Finally a number of carboxysome mutants have been discovered (Friedberg *et al.*, 1989; Lieman-Hurwitz *et al.*, 1991; Price *et al.*, 1993), including a strain mutated in the carboxysome coat protein (English *et al.*, 1994). All of these strains exhibit the high CO_2 growth requirement.

In this communication we provide evidence that the pyrenoid is playing a similar role in the eukaryotic algae. This chloroplast structure is almost ubiquitous among eukaryotic unicellular algae (Bold and Wynne, 1985). In cyanobacteria there is an increase in the number of carboxysomes when cells are placed in a low CO_2 environment (Turpin *et al.*, 1984; McKay *et al.*, 1993). In *C. reinhardtii*, there is a rapid biosynthesis of a starch sheath when the cells are placed in low CO_2 (Ramazanov *et al.*, 1994 and Fig. 2). Other algae also make a starch sheath in response to low CO_2 growth conditions (Fig. 3 and Kuchitsu *et al.*, 1988). In addition, some of the high CO_2-requiring strains of *C. reinhardtii* contain abnormal pyrenoids (Table 2 and Fig. 4). These strains are reminiscent of the cyanobacterial carboxysome mutants in which disruption of the carboxysome structure leads to a high CO_2-requiring phenotype. It appears that the correct packaging of Rubisco might be an essential component of the operation of the CCM in algae.

The data presented in this paper also suggest that there might be a link between the chloroplast carbonic anhydrase and the functioning of the pyrenoid. In two cases, mutants with defects in the chloroplast carbonic anhydrase exhibit abnormalities in their pyrenoid structure. Ca-1 has pyrenoids that are not spherical while cia-3 fails to produce a pyrenoid starch sheath (Fig. 4).

Under low CO_2 conditions, the effects of ethoxyzolamide mimic the cia-3 mutants, namely a pyrenoid starch sheath is not produced. Under high CO_2 the chloroplast deposits a lot of starch throughout the stroma when ethoxyzolamide is present.

At this point it is unclear whether the altered pyrenoid morphology observed in the high CO_2 requiring mutants is directly due to the carbonic anhydrase defect or is due to an additional mutation. We are presently addressing this question by crossing cia-3 and ca-1 with wild-type cells to determine whether there is a linkage between the chloroplast carbonic anhydrase and the pyrenoid. In addition a number of researchers are working at purifying the chloroplast carbonic anhydrase to determine whether or not it is localized to the pyrenoid. While the data presented in this manuscript are consistent with the carbonic anhydrase being localized to the pyrenoid, this important point remains to be proven.

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