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 n was done using API (Difco) standard procedures. mples for electron microscopy were processed following ods described by Nevilopoulos 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-
mycin, Erithromycin, Tobramycin and Dibekacin.

:elly half of the new and preexisting buds became
:ed 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
:nerated a callus mass (3-4 mm diameter). In some
:condary callus, more pigmented and organogenetic,
n the primary callus. Subcultured isolated calli
:ly bleached and died.

30 days in culture, 40 - 45% of the viable explants
:ncia sp. produced a highly pigmented (red) compact
allus formation took place in the cut and apical
:he explant. The reculture of callus-producing
vas successful. Regeneration (4-5 thalli per callus)
n all the callus thalliclones (thallus regenerated
:) showed a high phototrophism. Differential thalli-
vth allowed the joining 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 eviden- 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 ıophyta 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 ıome 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 ilaria could be made, the question wether the endoıus bacteria [illegible] 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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