Tissue Culture of Brown Seaweeds

H.J. LAWLOR, M.A. BOROWITZKA and J.A. McCOMB

School of Biological and Environmental Sciences, Murdoch University, Murdoch, WA 6150

The harvesting of macrophyte algae (seaweeds) is well established and on a world-wide scale more than 180,000 tonnes dry weight of algae such as the phaeophytes *Laminaria*, *Undaria*, *Sargassum* and *Macrocystis*, the red algae *Eucheuma*, *Gracilaria* and *Porphyra*, and the green algae *Ulva*, *Monostroma* and *Caulerpa* are harvested annually. Much of this algal biomass comes from farmed rather than wild species. The red and brown algae are the source of the phycocolloids agar, alginate, agarose and carrageenan which are of fundamental importance to the development of biotechnology; i.e. for the culture of micro-organisms (agar), for the separation of biomolecules (alginate and agarose) and for the production of food products (agar, carrageenan, alginate).

The successful large-scale cultivation of these algae requires, amongst other things, the ability to select fast growing and disease resistant strains which produce large quantities of the desired phycocolloid. To this purpose classical plant breeding programs are being carried out, however these are slow and the production of superior cultivars takes much time and effort (Van der Meer 1988). In recent years there has therefore been much interest in developing protoplast and tissue culture systems which would allow more rapid selection and propagation of suitable cell lines, the possibility of producing hybrids by cell fusion and new strains by genetic engineering (Polne-Fuller and Gibor 1987b, Le Gall et al. 1990).

Work in our laboratory has concentrated mainly on the brown algal genera *Ecklonia* and *Cystophora*. *Ecklonia* was chosen because it is easily obtained and is a potential source of alginate, and *Cystophora*, a genus endemic to Australia and New Zealand, because previous studies indicated that this genus appears to be a good source of tocopherols and tocotrienols (Gregson et al. 1977, Kazlauskas et al. 1981, unpubl. results). The tocopherols are of interest and also provide a convenient model for the study of the production of secondary metabolites in algal tissue culture. In this paper we describe some of our findings on the tissue culture of these species and on their tocopherol content.

Materials and methods

Plants of *Ecklonia radiata* (C. Ag.) J. Ag. (Laminariales, Phaeophyceae) were collected from shallow reefs off the coast of Perth, WA and immediately transported on ice to the laboratory. In the laboratory the plants were cleaned of visible epiphytes and up to 6 cm long sections were cut from the stipe, holdfast and lamina with a sterile scalpel. The pieces were then sterilized by shaking them for 30 sec. in 70% (v/v) ethanol, followed by 30 min. in sterile deionized water. They were then rinsed in two changes of sterile seawater and the cut surfaces of each piece were trimmed and discarded. Explants were prepared by either cutting small square pieces from the blade, or by taking small, 5 mm diameter, disk-shaped cores from the stipe.

The explants were placed on agar containing modified Murashige and Skoog medium (Lawlor et al. 1988). Other media were also examined, but this medium consistently gave the best growth.

Cystophora (Fucales, Phaeophyceae) species were collected from several sites as shown in Table 1. For tissue culture the plants were treated in the same way as *Ecklonia* except that no cores were prepared; instead sections of the secondary axis were used.

Table 1. Collection sites of the algae used for tocopherol assays.

	Malua Bay, NSW	Burri Point, NSW	Point Lonsdale, Vic.	Hamelin Bay, WA
Cystophora brownii				*
C. expansa	*	*		*
C. monilifera	*			*
C. moniliformis	*	*	*	
C. pectinata				*
C. racemosa				*
C. retorta			*	*
C. retroflexa		*		
C. siliquosa			*	*
C. subfarcinata			*	
C. torulosa			*	
Acrocarpia sp.				*
Caulocystis sp.	*	*	*	

Tocopherols were extracted by a modification of the methods of Muller-Mulot (1976) and Foss et al. (1984). Algal tissue (10 g) was macerated and extracted 2 times in 50 mL acetone plus 2 mL freshly prepared 12% (w/v) ascorbic acid as antioxidant.

Extraction was carried out in the dark in a N_2 atmosphere at 4°C overnight. The two extracts were combined and after removal of any precipitate they were dried by rotary evaporation under vacuum. After drying the extract was redissolved in acetone and stored at –20°C under N_2 until assay. Special care had to be taken to remove all water from the extract and this was achieved by freezing the acetone extract containing the tocopherols at –20°C and decanting off the non-frozen acetone containing the tocopherols and then removing any remaining water and the acetone by rotary evaporation. Before chromatography the samples were further purified by redissolving the sample in hexane and applying it to a silica Sep-Pak (Millipore) cartridge. The cartridge was then washed with 5 mL isopropyl ether and the tocopherol eluted with acetone.

Identification and quantification of the tocopherols was carried out using a Waters HPLC with a Waters 4 μm Novapak C18 reverse phase Radial Pak cartridge in a Waters Z module and a variable wavelength detector at 290 nm. The eluting solvent was methanol:water (97:3, v/v) at a flow rate of 1.5 mL/min and a column pressure of 600 p.s.i. Peaks were identified and calibrated using tocopherol standards (Eisai, Japan).

Results

Several forms of growth were observed from axenic explants of *Ecklonia radiata*. The most common form were short filaments of unpigmented or light-yellow cells which grew from the medullary and cortical cells of the cut surface of the explant (Figure 1b). These developed into an intermeshed and non-friable callus after about 2 weeks (Figure 1a). Electron microscopic examination of these cells showed them to have some plastids and perforated cell plates, similar to those in the medullary hyphae

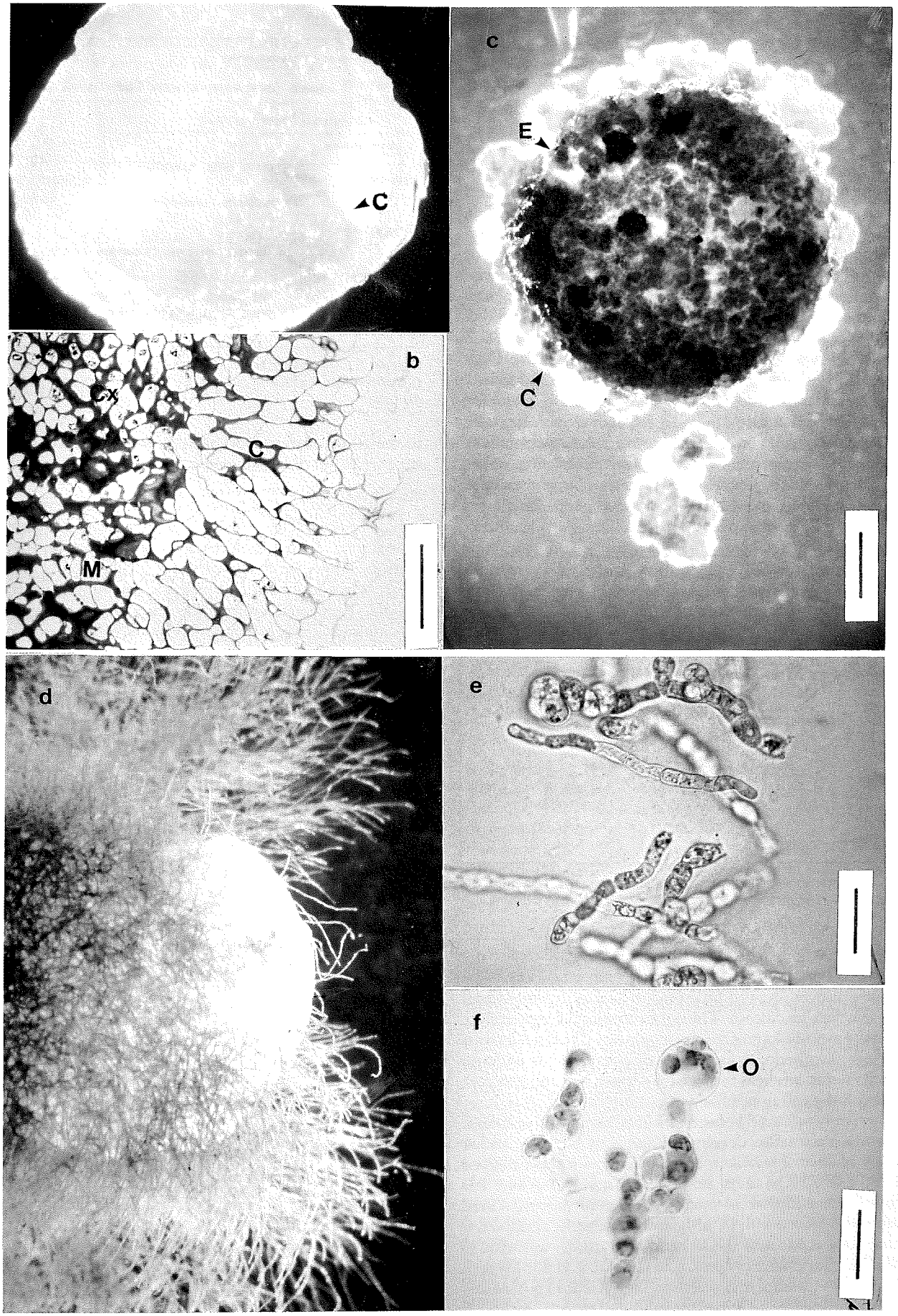

Figure 1. Light micrographs of the different cell morphologies observed in tissue culture of *Ecklonia radiata* (a-d,f) and *Cystophora retorta* (e). (a) Unpigmented/pale yellow callus growth on 17 day old stipe explant; (b) Section through the explant medulla and cortex and associated callus of same piece as shown in Figure 1a; (c) Pigmented callus growth on explant 9 weeks after appearance of pigmented centres; (d) Pigmented filamentous growth 9 weeks after appearance of pigmented centres; (e) Pigmented filaments; (f) Pigmented filamentous growth with a terminal oogonium-like cell. Symbols used: C = callus; Cx = cortex; E = explant; O = oogonium-like cells. (Scales–a,c,d, = 1 cm; b,e = 100 μm; f = 50 μm).

of Laminariales suggesting that they are derived from the filamentous hyphal cells of the parent tissue. These cells grew best in the dark and their growth was inhibited at even low photon flux density. Growth generally ceased after about 10 weeks.

The second type of growth observed in *E. radiata* originated as small centres of highly pigmented cells which developed within the explant after several weeks to months. These centres gave rise to callus cells (Figure 1c), filamentous cells (Figure 1d), or both. This callus was more pigmented and friable in comparison to the pale-yellow callus which developed soon after explant excision. Ultrastructural studies showed that the pigmented cells contained plastids and large amounts of osmiophilic granules throughout the cell. It is likely that these are composed of phenolic compounds (Ragan and Glombitza 1986, Klemm and Hallam 1987). Pigmented cultures could be transferred through many subcultures over several years. Most of the transferred cultures grew as masses of branched filaments which resembled gametophytes of *E. radiata*, but antheridia could not be easily identified although oogonia-like cells were common (Figure 1f). In media containing little agar, structures resembling young sporophytes developed in a few cases from the filamentous cells. The types of cells observed and their development is illustrated diagrammatically in Figure 2.

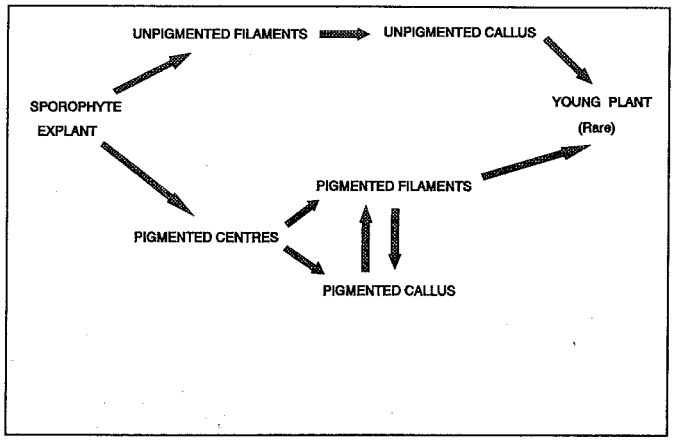

Figure 2. Diagrammatic representation of the cell types produced from explants of *Ecklonia radiata* in tissue culture.

The origin of the explant influenced the amount of callus produced. The stipe explants gave rise to the most callus and the holdfast explants also produced significant amounts. The blade explants, however, showed little growth and all growth ceased after 2–3 weeks in culture. The concentration of agar greatly affected the morphology of the tissue cultures. Thus, media with 2–4% agar favoured the formation of a hard callus with no visible filaments, whereas media with a lower agar content gave rise to both the callus and filaments.

Growth only occurred between 15–25°C with a pH optimum of 7.5 and a photon flux density of 50 μEinsteins/m^2.sec and at a salinity of 75% of seawater. Best callus growth was observed with 280 g/L KNO_3; urea, ammonium and glycine were less effective. The addition of auxins stimulated growth. Good growth was achieved with 1.0 μM napthalene acetic acid (NAA) and naphoxy acetic acid (NOA) and best growth was with 10 μM 2,4-dichlorophenoxy acetic acid (2,4-D). Cytokinins such as kinetin, N^6-(2-isopentyl) adenine, benzyl amino purine and zeatin had little effect on growth or inhibited growth. Although a range of organic supplements to the media were tested (i.e. glucose, fructose, mannitol, sucrose, sorbitol, glycerol, aspartic acid, acetate) none of these supported growth in the dark, nor stimulated growth in the light (Lawlor et al. 1989). The addition of 1% (w/v) Polyclar AT, a polyphenol binding compound,

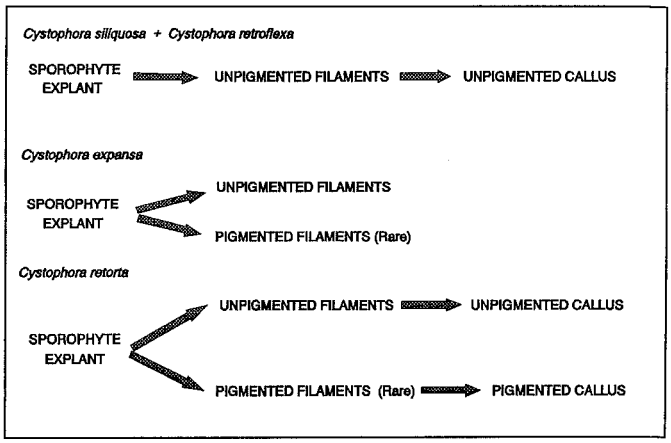

Figure 3. Diagrammatic representation of the cell types produced by explants of several *Cystophora* species in tissue culture.

increased the duration of callus growth, especially in uncored stipe tissue but did not increase the rate of growth (Lawlor et al. 1988).

The different *Cystophora* species produced a range of cell types in tissue culture (Figure 3). Unpigmented filaments grew within 4 weeks on the cut surfaces of explants from all the species cultured. However, only explants of *C. siliquosa*, *C. retorta* and *C. retroflexa* formed a pale-yellow callus similar to that of *E. radiata*. Pigmented growth was only rarely observed in *C. expansa* and *C. retorta* (Figure 3). Out of 98 axenic explants of *C. retorta* only one gave rise to pigmented filaments and callus after 10 months in culture (Figure 1e). Similarly, 2 explants of *C. expansa* out of a total of 109 gave rise to pigmented filaments, but no callus, after 5 months. One explant of *C. moniliformis* developed a shoot directly from the explant tissue without passing through the callus stage. Unlike *E. radiata*, no structures which resembled reproductive tissue nor sporophyte-like thalli were observed in *Cystophora*.

This may relate to the fact that *Cystophora* does not have a life history which includes a microscopic filamentous gametophyte stage.

Tocopherol assays

There was wide variation in the total tocopherol content and in the ratio of the constituent isomers between the different species examined. The highest tocopherol content was measured in *C. expansa*, *C. monilifera*, *C. retroflexa* and *C. subfarcinata* (Table 2). All of these species contained a mixture of the α-, β/γ- and δ-isomers. *Cystophora moniliformis* and *C. pectinata* had a lower tocopherol content and this consisted mainly of α-tocopherol with only 15% β/γ-tocopherol and a trace of δ-tocopherol. All the other species examined had less tocopherol. *Ecklonia radiata* contained far less tocopherol then *Cystophora*, *Caulocystis* and *Acrocarpia*, and only α-tocopherol was found (Table 2). The slightly yellow cultured tissue of *C. siliquosa* contained 246.50 ± 9.50 μg α-tocopherol/g dry weight as well as traces of the other tocopherols. This compares well with the tocopherol content of freshly collected plants.

Discussion

The results reported here show that tissue culture of brown macrophyte algae is possible. The two major types of growth (pigmented and unpigmented) in brown algal tissue cultures has also been observed by Polne-Fuller (1987a,b), Yan (1984), Notoya (1988) and others. The development of unpigmented callus-like growth in *E. radiata* resembles the response to wounding described by Moss (1961) for *Fucus vesiculosus*. This suggestion is

Table 2. Tocopherol content of several species of brown algae (All values are the mean of 5 plants and are expressed as μg tocopherol/g dry weight).

Species	Tocopherol isomers			
	α	β/γ	δ	Total
Cystophora expansa	550.47	183.80	222.07	956.34
C. moilifera	598.27	208.00	265.45	971.68
C. retorta	320.67	154.59	322.80	798.16
C. retroflexa	657.21	149.83	283.51	1130.56
C. subfarcinata	620.80	143.65	361.86	1126.30
C. torulosa	168.24	24.84	30.71	223.77
C. brownii	289.79	52.52	5.56	347.98
C. monilifera	439.46	80.41	7.07	526.14
C. pectinata	408.72	89.67	0.00	498.39
C. racemosa	221.09	73.88	0.00	294.97
C. siliquosa	394.06	131.98	108.14	634.17
Acrocarpia sp.	30.00	452.95	462.95	945.91
Caulocystis sp.	0.00	130.70	654.32	783.70
Ecklonia radiata	25.27	0.00	0.00	25.27

further supported by the observation that growth ceases after the wound is effectively sealed. This wounding response is probably under hormonal control and is stimulated by the presence of auxins (Lawlor et al. 1988).

The development of pigmented cells, on the other hand, is possibly a response to nutrient depletion within the explant since they take several weeks to develop when the remainder of the explant is bleached. The pigmented cell type in *E. radiata* may represent a form of reproduction, where the cells from the sporophytic explant tissue give rise to a gametophytic growth type. Fries (1980) and Lee (1985) have also described the appearance of both male and female gametophytic-like growth from aged tissue cultures of *Fucus spiralis* and *Laminaria saccharina*. The pigmented filaments of *Cystophora*, however, cannot be gametophytes since this genus does not have a filamentous gametophyte stage in its life history.

Measurement of the relative DNA content of the nuclei of the pigmented tissue culture of *E. radiata* by staining the DNA with DAPI and measuring the relative fluorescence showed that 22% of the nuclei were haploid, 44% diploid and 25% tetraploid with the remainder having a higher ploidy (Lawlor, unpubl. results). These results provide no information on the possible origin of the cultured cells, but they are of potential interest to future genetic studies of this alga.

Fries (1980) and Lee (1985) also describe the regeneration of small sporophytic plants from pigmented filamentous tissue cultures of *Fucus* and *Laminaria*. In our study and those of others such regeneration only occurred in liquid or soft medium. For example, Notoya and Aruga (1990) found that in *E. cava* only explants of the blade produced pigmented filamentous callus which survived for long periods and which developed into blade-like plantlets in liquid medium. Polne-Fuller and Gibor (1987b) also regenerated *Sargassum* plantlets from callus in liquid medium. On the other hand, regeneration of sporophytes from nonpigmented cultured cells has been reported only rarely (Polne-Fuller and Gibor 1987a).

The major problem facing algal tissue culture at this stage is the slow growth rate of most cultures and the difficulty in regenerating the alga from these cultures. These problems reflect our inadequate understanding of the growth requirements of these plants and of hormonal regulation of growth and development. Although cytokinins and auxins have been reported to occur naturally in many phaeophycean algae (e.g. Mooney and Van Staden 1986, Sanderson et al. 1987) and they have been shown to affect growth and differentiation in whole cultures (e.g. Borowczak et al. 1977, Yamanaka et al. 1990) the data on their effects on tissue cultures are conflicting and more detailed studies on whole plants and tissue cultures are required. Despite these difficulties it is clear that algal tissue culture has the potential to benefit the commercial culture of macroalgae in the same way that tissue culture has benefited the horticultural industry.

Tissue culture may also be a means for producing specific secondary metabolites such as the tocopherol reported in this paper. The tocopherol content of several of the *Cystophora* species reported here is higher than that found in most other algal species and higher plants (Jensen 1969, Janisowska and Pennock 1976, Tani 1989) although it is less (on a dry weight basis) than that reported in some microalgae (Aaronson et al. 1977, Ruggeri et al. 1985). The tissue cultures of *C. siliquosa* contained a similar amount of tocopherol as the intact plant and this indicates that it is possible to use tissue culture as a potential means of producing tocopherol. The potential advantage of tissue cultures is that the growth conditions can be more easily manipulated and controlled. However, better growth rates have to be achieved before this process can have any commercial application.

In conclusion, the results presented here show that algal tissue culture is a potentially valuable tool for the study of algal physiology and for the propagation of commercially important algal cultivars. Algal tissue culture also has the potential for the production of valuable secondary metabolites. ■

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