Botanica Marina
Vol. 34, pp. 261–264, 1991

A Rapid and Inexpensive Method for Surface Sterilisation of *Ecklonia radiata* (Phaeophyta) for Tissue Culture

H. J. Lawlor, M. A. Borowitzka and J. A. McComb[1]

School of Biological and Environmental Sciences, Murdoch University, Murdoch, Western Australia, 6150, Australia

(Accepted 23 February 1991)

Abstract

Sections of the thallus of the brown alga *Ecklonia radiata* (C. Ag.) J. Ag. were surface sterilised for tissue culture by dipping in 70% ethanol for 30 s, followed by sterile deionised water for 30 min. A high percentage of aseptic viable explants could be obtained using this treatment, and growth *in vitro* was initiated.

Introduction

Tissue culture provides a useful technique for both basic research on algae and possible commercial exploitation of valuable products. Control over the growth conditions of cultures is improved when they are axenic and unialgal. Epiphytes (*i.e.* algae, bacteria, etc) are a major source of contamination in tissue cultures of macrophytes and many techniques have been used to remove them. For example, dragging the plant material through sterile seawater solidified with agar (0.8%) eliminated epiphytes from *Chondrus crispus* Stackhouse (Chen and Taylor 1978). Manual brushing, mechanical shaking in a suspension of abrasives, or ultrasound treatment were found to be effective for cleaning some red and brown macroalgae (Gibor *et al.* 1981). However, these methods are laborious, and the long term effect of disruptive procedures on the macroalgal tissues is unknown.

Most methods reported for surface sterilisation of macroalgae have been based on those used for higher plants. A saturated solution of calcium hypochlorite has been used to sterilise tissue from *Laminaria digitata* (Hudson) Lamouroux and *L. hyperborea* (Gunn.) Foslie (30–60 s treatment) (Fries 1980) and *Fucus spiralis* L. (2–5 min treatment) (Fries 1977, 1980), while a combination of 1.5% KI solution (10 min) and 70% ethanol (incubation time not given) sterilised tissue of *Laminaria japonica* Areschoug and *Undaria pinnatifida* (Harvey) Suringar (Yan 1984). Iodine was also the active ingredient in a sterilisation method for red algae using Jodopax (Fries 1963). A wide range of germicidal agents have also been tested by Gibor *et al.* (1981) on several species of red and brown macroalgae. Best results were obtained from a 5 min incubation in 1% Betadine which contains the active ingredient iodide. Stipe explants of *Laminaria saccharina* (L.) Lamouroux have also been sterilised with 1% Betadine or 1% sodium hypochlorite for 1–12 min (Lee 1985). For *L. angustata* Kjellman, an alcohol dip followed by flaming and the use of a 25 mm core, parallel to the long axis of the stipe, yielded a high percentage of sterile explants (Saga and Sakai 1983).

Antibiotics have also been used to sterilise algal cells (Fries 1963, Gibor *et al.* 1980, Saga and Sakai 1982, Polne-Fuller and Gibor 1982, Bradley *et al.* 1986, Fisher and Gibor 1987, Bradley *et al.* 1988). Generally explants were first surface sterilised, using one of the above methods, and then incubated on media to which the antibiotics were added. However, antibiotics are not always a satisfactory sterilising agent (Chen and Taylor 1978, Saga and Sakai 1983).

This report describes the establishment of *Ecklonia radiata* in axenic culture using a quick and inexpensive method for surface sterilisation.

[1] Author and address for reprint requests

Material and Methods

Mature *Ecklonia radiata* (C. Ag.) J. Ag. plants were collected at a depth of 1 m on limestone reefs off the coast near Perth, Western Australia. Plants with the fewest epiphytes were selected and were transported in plastic bags filled with air to minimise damage. Plants were stored at ambient seawater temperature and were sterilised and cultured within five hours of collection. In the laboratory, visible epiphytes were removed with a sterile scalpel and sections up to 6 cm long were cut from the holdfast, stipe, and lamina. These pieces were treated (85 per 250 mL screwcap jar) by submerging and shaking in the following solutions for the range of times shown in Table I: – ethanol (70% v/v in deionised water); potassium iodide, analytical grade (1.5% w/v in seawater); and sterile deionised water. Sections were then rinsed in two changes of sterile seawater and the cut surfaces of each piece were trimmed and discarded. Explants were cut into cubes about 4 mm each side and placed on solid medium in 9 cm Petri dishes sealed with parafilm (5–10 explants per dish). From stipes of sufficient diameter, a core comprising medullary and cortical tissues was removed parallel to the long axis, using a flame-sterilised 5 mm steel corer. The ends of the core were trimmed and discarded, and the remaining core was cut into 2 mm high discs.

The culture medium used was modified PES medium solidified with 7 gL^{-1} agar (Lawlor *et al.* 1988). A seawater based bacterial nutrient broth was used to detect the presence of contaminants (Gibor *et al.* 1981). Cultures were kept at 25 °C and continuous light (50 $\mu mole^{-2}\ s^{-1}$). Sterility of the explants was determined by examination of the medium around and below the explant for bacterial and fungal growth, for at least 6 weeks.

Viability of the algal tissues was estimated after sterilisation, using hand sections stained with Evan's blue or neutral red (Millner *et al.* 1979). Fresh unsterilised tissue and tissue incubated for 10 min at 80 °C were used as the live and dead controls, respectively.

Results

Initially a solid sterility test medium based on seawater and bacterial nutrient broth (Gibor *et al.* 1981) was used to detect the presence of contaminants. However, for *Ecklonia radiata* this medium greatly reduced the induction and rate of callus growth. Since

Table I. Percentage of sterile explants of *E. radiata* following sterilisation and 6 weeks incubation on tissue culture medium. 100 explants were used per treatment. Viability was determined immediately after surface sterilisation and an asterisk indicates that treatment killed more than 20% of the tissue in all explants.

Treatment		Percentage sterile explants			
		Stipe		Holdfast	Lamina
(Time)		Not cored	Cored		
KI (1.5% w/v)					
10 s		0	0	0	0
15		0	0	0	0
30		0*	0*	0	0*
60		28*	31*	0*	25*
90		43*	40*	0*	25*
Ethanol (70% v/v)					
15 s		0	0	0	0
30		30	31	0	20
60		57	61	0	51*
90		63*	78*	10	60*
120		70*	100*	21	62*
Ethanol (70% v/v)	then KI (1.5% w/v)				
5 s	15 s	0	0	0	0
10	30	28*	30*	0*	25*
20	60	57*	80*	12*	58*
30	90	78*	100*	19*	80*
Ethanol (70% v/v)	then DI water				
30 s	10 min	60	64	0	61
30	20	69	71	10	65
30	30	75	85	20	81
30	60	82	85	20	80*
30	120	86*	100*	25*	85*
30	180	88*	100*	26	84*

any contamination was clearly visible on the solid tissue culture medium this step was eliminated.

Potassium iodide killed *Ecklonia radiata* tissue, whether used alone or following ethanol (Table I). Treatment for 30 to 60 s in ethanol resulted in live sterile explants. When the explants were treated with ethanol and then shaken in deionised water for 30 to 60 min the highest percentages of live sterile explants were obtained. Holdfasts gave the lowest percentage of sterile explants. Lamina tissues were killed during the 60 min freshwater treatment. The percentage of sterile stipe explants was increased by taking a core. When sterile explants were placed on PES medium, callus-like growth was apparent after 3 to 7 days (Lawlor *et al.* 1988). Cultures found to be clean after 6 weeks showed no contamination in later subcultures.

Discussion

Explants of *Ecklonia radiata* were successfully surface sterilised when treated for 30 s with 70% ethanol, followed by sterile deionised water. This may be due to the possibility that marine contaminants, especially marine bacteria, cannot survive in fresh water. Sterilisation periods for longer than one hour in deionised water damaged the algal cells. Although KI solution followed by ethanol has been used to sterilise *Laminaria japonica* and *Undaria pinnatifida* (Yan 1984); treatments sufficient to eliminate contamination in *E. radiata* killed the cells. Similarly, 70% ethanol alone, although a potent microbicide (Frobisher *et al.* 1974), also killed the algal cells.

Many factors influence the success of sterilisation of plants for tissue culture. The method of collection and transportation should not be underestimated; macroalgae which were damaged, allowed to become warm, or were stored before use, always had higher levels of contamination. Best results were obtained by using undamaged material, sterilised and cultured within five hours of collection.

The use of a scalpel to remove visible epiphytes is labour intensive, but effective for macroalgae such as *E. radiata*, causing little damage except to the outer layer of cells. Removal of a core of explant tissue is a further important step in the elimination of algal epiphytes, as well as surface bacterial and fungal contamination. In *E. radiata* this procedure is suited to the stipe and meristematic zone. As well as minimising contamination, this step eliminated the problem of leakage of phenolics from the epidermal and cortical cells into the culture medium, where these compounds may then interfere with growth (George and Sherrington 1984).

This paper provides an alternative approach for surface sterilisation of macroalgae. The method is now being used routinely for the tissue culture of *E. radiata*, and it has been applied successfully to species of *Sargassum* and *Cystophora*.

Acknowledgements

This study was supported by a grant from the Marine Sciences and Technology Grant Scheme.

References

Bradley, P. M., D. P. Cheney and N. Saga. 1986. A new one-step method for obtaining axenic cultures of algae. *Appl. Phyc. Forum 3*: 4–5.

Bradley, P. M., D. P. Cheney and N. Saga. 1988. One-step antibiotic disk method for obtaining axenic cultures of multicellular marine algae. *Plant Cell Tissue and Organ Culture 12*: 55–60.

Chen, L. C. M. and A. R. A. Taylor. 1978. Medullary tissue culture of the red alga *Chondrus crispus*. *Can. J. Bot. 56*: 883–886.

Fisher, D. D. and A. Gibor. 1987. Production of protoplasts from the brown alga *Sargassum muticum* (Yendo) Fensholt (Phaeophyta). *Phycologia 26*: 488–495.

Fries, L. 1963. On the cultivation of axenic red algae. *Physiol. Plant. 16*: 695–708.

Fries, L. 1977. Growth regulating effects of phenylacetic acid and p-hydroxyphenylacetic acid on *Fucus spiralis* L. (Phaeophyceae, Fucales) in axenic culture. *Phycologia 16*: 451–455.

Fries, L. 1980. Axenic tissue cultures for the sporophytes of *Laminaria digitata* and *Laminaria hyperborea* (Phaeophyta). *J. Phycol. 16*: 475–477.

Frobisher, M., R. D. Hinsdill, K. T. Crabtree and C. R. Goodheart. 1974. *Fundamentals of Microbiology*. Saunders, Philadelphia. pp. 850.

George, E. F. and P. D. Sherrington. 1984. *Plant Propagation by Tissue Culture*. Eastern Press, Reading. pp. 709.

Gibor, A., M. Polne-Fuller, M. Biniaminov and M. Neushul. 1981. Exploratory studies of vegetative propagation of marine algae; procedure for obtaining axenic tissues. *In*: (T. Levring, ed) *Proc. of Xth Internat. Seaweed Symposium*. Walter de Gruyter, Berlin. pp. 578–593.

Lawlor, H. J., J. A. McComb and M. A. Borowitzka. 1988. The development of filamentous and callus-like growth in axenic tissue cultures of *Ecklonia radiata* (Phaeophyta). *In*: (T. Stadler, J. Mollion, M. C. Verdus, Y. Karamanos, H. Horvan and D. Christian, eds) *Algal Biotechnology*. Elsevier Applied Science, London. pp. 139–150.

Lee, T. F. 1985. Aposporous gametophyte formation in stipe explants from *Laminaria saccharina* (Phaeophyta). *Bot. Mar. 28*: 179–185.

Millner, P. A., M. E. Callow and L. V. Evans, 1979. Preparation of protoplasts from the green alga *Enteromorpha intestinalis* (L.) Link. *Planta 147*: 174–177.

Polne-Fuller, M. and A. Gibor. 1984. Developmental studies in *Porphyra*. 1. Blade differentiation in *P. perforata* as expressed by morphology, enzymatic digestion and protoplast regeneration. *J. Phycol. 20*: 609–616.

Saga, N. and Y. Sakai. 1982. A new method for the culture of macroscopic algae, the one step selection method. *Jap. J. Phycol. 30*: 40–43.

Saga, N. and Y. Sakai. 1983. Axenic tissue culture and callus formation of the marine brown alga *Laminaria angustata*. *Bull. Jap. Soc. Sci. Fish. 49*: 1561–1563.

Yan, Z. M. 1984. Studies on tissue culture of *Laminaria japonica* and *Undaria pinnatifida*. *Hydrobiologica 115/116*: 314–316.