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In vitro culture of Heterzostera tasmanica and Zostera muelleri

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Introduction

The use of micro-propagation methods for the in vitro cultivation of plants is widespread and is used in forestry, horticulture and revegetation work. (Figure 1). The potential of tissue culture to enable the generation of large numbers of plantlets for revegetating denuded seagrass beds is a particularly attractive proposition. This is a relatively new field of study with only a limited number of published reports having been produced in the international literature and very few successful cases of culture and/or transfer to the external environment (eg the estuarine species Ruppia maritima L – Bird et al., 1994). A report by C.B. Burgess (1996) provides relevant background information and details the outcome of a recent US study into the potential of tissue culture for culturing sea grasses and estuarine plants. This study involved leading US scientists in this field, including Drs Kimon Bird (University of North Caroline) and Jack Gallagher (University of Delaware), and was funded by the National Oceanic and Atmospheric Administration and other bodies (grant NA 36RG00499).

The current project explored the potential for culturing two species indigenous to Western Port, Heterozostera tasmanica and Zostera muelleri, with the intention of defining conditions that may allow for the propagation of these true seagrasses under controlled conditions prior to successful transfer to the marine environment. The experimental program involved an initial trial of sea grass cultivation in pots containing natural substrata maintained in aerated sea water (sourced from Bass straight) in 30 - 40 litre plastic aquaria housed in temperature-controlled greenhouses under artificial illumination (21° ± 2º C; 14/10 h light/dark regime; light intensity of approx 95 mM m–1 lux provided by fluorescent lights of variable spectra). These initial experiments proved largely unsuccessful due to relatively rapid microbial/algal overgrowth leading to death of sea grass plants within 3 - 8 weeks of transplantation1. (Figure 2). It was noticed however that illumination of aquaria using Gro-Lux fluorescent tubes (commonly used in greenhouses to promote plant growth; red: blue ratio of 1.4) led to more rapid overgrowth of algae than did illumination by blue-enhanced fluorescent tubes (3 -4 weeks vs _ 8 weeks until algal overgrowth occurred). This area may merit further investigation in future experiments that are concerned with successful transfer of plants from an aseptic in vitro environment into sea water aquaria to enable successful acclimatisation prior to transplantation into Western Port.

1 Initial aquaria experiments were conducted by Mr Andrew Mains who was employed as a part – time research assistant on the project from April – July 2002. Plant material sourced with the assistance of Mr Jason Walker.

Initial aquaria experiments were followed by more intensive attempts2 to establish axenic, proliferating cultures in vitro that may be used as a source of material for future transplantation programs as summarised in Figure 3. As in all tissue culture programs involving new species, an iterative process was followed with advances being made on the basis of a series of incremental partial successes (summarised in Figure 4). The experimental program involved an initial assessment of various trial surface-sterilisation regimes to remove contaminating parasites and microorganisms without destroying plant viability. In vitro culture conditions were then established to simulate a summer external environment (14 hrs light/10 hrs dark per day; 22 ± 2 º C) and numerous growth media, containing variable combinations of plant (phyto) hormones and other additives commonly used to culture tissues of land plants in vitro. In principle, micro-propagation of higher plants can utilise either seeds or explants of whole plant material that have been surface-sterilised to remove endogenous bacteria and fungi (and algae in the case of aquatic plants). In this project, we focussed on the use of explant material sourced from sea grass meadows as it was not feasible to harvest seed in appreciable quantities.

Methods

Sea Grass Tissues and Surface Serilisation

Plants were sourced from Ricketts Point, Melbourne, and from Western Port. They were acclimatised in a temperature-controlled greenhouse (_21 ° C ; 14/10 h light/dark regime; light intensity of approx 95 mM m–1 lux; daylight-spectrum equivalent fluorescent tubes) in aerated sea water for 2-3 days before use. Extraneous plant matter was excised with a scalpel blade immediately prior to sterilisation, leaving essentially a 1-2 cm region intact that contained the shoot meristem portion of the growing plant.

Decontamination involving aquatic plants that have been published in the scientific literature to date are usually very tedious and the use of several antibiotics. In most cases, they are of limited effectiveness, particularly against the growth of fungi - the most difficult class of microbial contaminants that are encountered in plant tissue culture. In this project we examined the use of Plant Preservative Medium (PPM), a relatively new commercially available anti-microbial agent for plant tissue culture media which uses azide as its core antimicrobial agent. Successful attempts to attain asepsis involved a 60 second submersion of sea grass plant material in commercial bleach (Domestos) solutions varying in strength (5 - 0.25%), followed by submersion in PPM solution for various lengths of time (12 h, 6 h and 0.5 h). Culture media were either free of PPM or contained PPM at a low concentration of 0.025%.

Growth media and conditions.

Growth of sea grass tissues in three commonly used marine algal media (f, fE, PhK) or terrestrial plant tissue culture medium (M + S) was assessed over a period of several weeks. Agitated liquid solutions (rotary shaker, 60 rpm) and solid nutrient media (containing either 8 gl agar or 2 gl Phytagel [Sigma] as gelling agents) were trialled (Figure 5). Media were made up using autoclaved sea-water (Bass Straight) or autoclaved artificial sea-water (commercially available). Media were either not supplemented with sucrose or contained 20 gl-1 sucrose. Tissues were cultured under a lighting regime of 112 mM m–1 lux (Gro-Lux fluorescent tubes) for 14 hrs light/10 hrs dark per day at a constant temperature of 22 ± 1°C.

2 Axenic tissue culture experiments were conducted by Mr Jon Sumby who was employed as a part-time research assistant on the project from Aug – Nov 2002. Plant material was sourced with the assistance of Mr Jason Walker.

Phytohormones

Of the various classes of plant (phyto) hormones that are available, recent literature involving the in vitro culture of sea grasses has reported some successes following inclusion of members of the cytokinin group. In this project, the cytokinins 6 benzyl amino purine (BAP), kinetin and 2- isopentenyl adenine (2iP) were added (individually) at a final concentration of 17 µM (this concentration being similar to levels reported as being effective in other studies).

Additives

The use of soluble polyvinylpyrollidine (PVP) at 1% final concentration (used as an anti-phenolic/anti-browning agent in some plant tissue culture media) and 1% glutamic acid as a source of reduced Nitrogen were trialed.

Summary of Results

As anticipated, non-effective surface sterilisation of plant tissues led to extensive microbial growth in vitro leading to the death of sea grass tissues (Figure 6). Attempts to surface sterilise plants of Zostera muelleri showed them to be highly sensitive to the sterilisation/initial culturing process with tissues rapidly becoming brown and dying in both plate and liquid culture media. Rapid browning/ death of tissues of H. tasmanica also occurred rapidly in vitro when higher concentrations of bleach/PPM were used However a mild sterilisation regime involving submersion in a 0.25% bleach bath for 60 secs, followed by submersion in a 4% solution of PPM for

0.5 hr and then overnight transfer to sterile sea water (for recovery) was found to be relatively non-damaging to H. tasmanica plantlets whilst also allowing for acceptable levels of subsequent asepsis in media containing a low concentration of PPM (0.025%).

Growth measurements of leaf material after transfer of plantlets to in vitro conditions and culturing on various media provided evidence that, of all conditions tested, survival and aseptic growth of Heterozostera tasmanica plants in vitro occurred best in illuminated, agitated liquid M + S plant tissue culture medium supplemented with 2% sucrose and the cytokinin 6-BAP (Figures 7 - 11).

Conclusions and Future Directions

This study indicates that viable, aseptic tissues of Heterozostera tasmanica can be established in vitro using standard (commercially available) tissue culture medium (M + S; Murashige and Skoog 1962) supplemented with sucrose, cytokinin and a low level of an azide-based plant tissue culture antibiotic. Using these observations as a baseline, future studies could profitably be undertaken to examine effects of altering conditions and media further to enable stable, proliferating axenic tissue cultures of sea grass species to be established in the laboratory with the ultimate aim of successful transfer to the external environment (ie Western Port). Such future experiments would involve

- Further alterations in the nutritional regime (eg different higher plant tissue culture basal media (Gamborg et al., 1976) with various combinations of phytohormones, individually and in combination).
- Alterations in the physical environment (eg altered agitation rates, variations in dissolved oxygen/CO2 levels, variable temperature and lighting regimes).
- Examination of alternative sterilisation regimes to reduce toxicity to Zostera muelleri tissues whilst still facilitating asepsis after culture in vitro.

The availability of axenic, proliferating, long-term tissue cultures of Western Port sea grass species would also facilitate fundamental studies into the

nutritional requirements of these plants (eg as undertaken for Ruppia maritima, Thursby 1984) and possibly also studies to shed more light on their reproductive strategies. Furthermore, such cultures could, in conjunction with genotyping/DNA profiling activities, act as genetic reservoirs of particular ecotypes as well as being useful for interstate/ international exchange without concerns of disease transfer and/or depletion of natural germplasm stocks.

Bibliography

General

Burgess C. B. (1996) Wetland plants from test tubes – NCU-W -95 – 002 C3 (35 pages: copy available from John Hamill upon request)

This report was prepared by Dr Carla Burgess following a workshop held in 1995 at the University of North Carolina USA, funded by the NOAA's coastal ocean program and the Sea Grant College program. In addition to providing specific details concerned with the tissue culture of marine and coastal plants, it also contains useful general information on the history and state of the art of plan tissue culture.

Relevant tissue culture scientific literature

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