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In Vitro Propagation and Bioassay Systems for Evaluating Growth Regulator Effects on *Myriophyllum* Species[1]

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ABSTRACT

In vitro culture procedures were developed for efficient propagation of *Myriophyllum aquaticum* (Vell.) Verde, *M. heterophyllum* Michx., *M. pinnatum* (Walt.) BSP, *M. spicatum* L., and *M.* 'Frill' for use in growth regulator screening studies. Shoots formed rapidly and grew into dense multiple shoot masses in all species cultured on a shoot multiplication medium consisting of Murashige and Skoog mineral salts (MS) supplemented with 0.56 mM myo-inositol, 1.2 μM thiamine-HCL, 87.6 mM sucrose, and 30 μM *N*-(3-methyl-2-butenyl)*H*-purin-6-amine) (2iP) solidified with 0.8% (w/v) agar. Shoots of *M. heterophyllum* were stored *in vitro* for 14 months at 5 C without loss of regenerative capacity. Single rooted submerged shoots of each species were produced by subdividing the shoot masses and subculturing in liquid half-strength MS supplemented with 0.56 mM myo-inositol, 1.2 μM thiamine-HCL, and 87.6 mM sucrose for two 6-day culture cycles. *M. heterophyllum*, *M.* 'Frill', and *M. spicatum* exhibited interspecific differences in sensitivities to the growth retardant Cycocel. Potential applications of these culture protocols for further growth regulator screening are discussed.

Key words: tissue culture, aquatic plants, growth retardant, Cycocel.

INTRODUCTION

Bioassay techniques have been used to document allelopathy in aquatic plants and characterize allelochemicals for potential use in the management of aquatic plants (Szczepanski, 1977; Cheng and Reimer, 1989; Sutton and Portier, 1989). Application of aquatic plant bioassay techniques to monitor herbicide residue levels have also been proposed (Burkhart and Stross, 1990). Most studies, however, have relied upon bioassay techniques using terrestrial plant seed germination or duckweed (*Lemna* spp.) *in vitro* frond growth responses to evaluate the allelopathic potential of aquatic plant extracts (El-Ghazal and Reimer, 1986; Cheng and Riemer, 1989; Sutton and Portier, 1989). Elakovich and Wooten (1989) observed dissimilarities between sensitivities of both the lettuce and duckweed bioassay systems to aquatic plant extracts and questioned the appropriateness of using terrestrial target species such as lettuce. *Hydrilla verticillata*, an aquatic species, has been used under non-axenic conditions (Klaine, 1986; Sutton, 1986; Martin and Martin, 1986; 1988); but, there are no *in vitro* submerged aquatic plant culture systems designed for rapid standardized screening of allelochemicals or other growth regulator substances.

Mohan Ram and Kapoor (1976) described the potential benefits of using both tissue and whole plant *in vitro* culture to study the environmental and physiological factors controlling growth and development in aquatic plants. One limitation to using this approach has been the elaborate procedures often required to establish sterile cultures especially with oblilgate aquatic species (Wetzel and McGregor, 1968; Klaine and Ward, 1981; Madsen, 1985; Francko, 1986). However, with those aquatic species successfully established *in vitro*, growth regulator effects on both adventitious shoot regeneration (Kane and Sheehan, 1988; Kane and Albert, 1989a) and whole plant development (Mohan Ram and Kapoor, 1976; Mohan Ram and Rao; 1982; Ailstock, 1986; Kane et al., 1988; Kane and Albert, 1989b) have been precisely examined. Conceivably, similar culture systems could be modified for growth regulator screening. The objective of this study was to develop *in vitro* propagation procedures for providing a reliable and uniform source of *Myriophyllum* spp. plantlets for use in bioassay studies.

MATERIALS AND METHODS

An earlier report (Kane and Albert, 1989a), established that aerial-leaf explants of *Myriophyllum heterophyllum* could be readily surface sterilized and exhibited high capacity for adventitious shoot formation (organogenesis) when cultured *in vitro*. Consequently, initial emphasis was placed on developing a propagation system using this species. Aerial leaves excised from greenhouse-grown stock plants were rinsed in flowing tap water for 1 hr and surface sterilized in aqueous 1.05% (v/v) NaOCl containing 0.01% (v/v) Tween-20 for 12 min, followed by three 5-min rinses in sterile deionized water. Leaf explants were then transferred into individual 150 x 25 mm culture tubes containing 12 ml Shoot Multiplication Medium (SMM) consisting of full strength Murashige and Skoog mineral salts (Murashige and Skoog, 1962) plus 0.56 mM myo-inositol, 1.2 μM thiamine-HCL, 87.6 mM sucrose and 30 μM *N*-(3-methyl-2-butenyl)*H*-purin-6-amine) (2iP) solidified with 0.8% (w/v) TC Agar™ (Hazelton Research Products, Lenexa, KS). The medium was adjusted to ph 5.7 with 0.1

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N KOH before autoclaving at 1.2 kg · cm^{-2} for 20 min at 121 C. All cultures and bioassays were maintained at 25 ± 2 C under a 16-hr photoperiod provided by cool-white fluorescent tubes (Sylvania F96T12/CW) at 26 μmol · s^{-1} · m^{-2} as measured at culture level.

After 28 days initial culture, the compact mass of adventitious aerial shoots formed from each leaf explant was excised, subdivided and transferred onto fresh SMM to increase the number of stock cultures. To induce formation of single shoots of the submerged form of *M. heterophyllum*, shoot masses from four culture tubes were aseptically removed after 21 days culture, divided into fragments and transferred into a 500 ml aluminum foil capped Erlenmeyer flasks containing 250 ml sterile Liquid Basal Medium (LBM) consisting of half-strength Murashige and Skoog mineral salts supplemented with 0.56 mM myo-inositol, 1.2 μM thiamine-HCL, and 87.6 mM sucrose (pH 5.7). After 6 days liquid culture, 1.0 cm long shoot tips were harvested from the elongated shoots and subcultured in LBM for another 6 days. Apical shoot tips (1.0 cm long) obtained from the second 6-day subculture were used as inocula for the growth regulator evaluation experiment.

Submerged shoot cultures of *Myriophyllum aquaticum, M. pinnatum, M. spicatum*, and *M.* 'Frill'[3] were initially established *in vitro* in LBM from defoliated aerial stem segments (consisting of two to three nodes) using the aforementioned surface sterilization procedure. Clonal stocks were increased by further propagating lateral brnaches which developed from the initial stem segments after 4 weeks culture in LBM. The capacity of these species to produce high density multiople shoot masses was examined by culturing 1.0 cm long shoot tips on SMM in culture tubes for 28 days as described above.

The plant growth retardant Cycocel (2-chloroethyltrimethyl ammonium chloride) was selected to compare treatment effects on shoot growth of *M. heterophyllum, M. spicatum*, and *M.* 'Frill' in liquid culture. Apical shoot tip cuttings (1.0 cm long) obtained from *in vitro* liquid culture stocks were transferred into 100 ml sterile LBM in 250 ml Erlenmeyer flasks loosely capped with aluminum foil. A flask containing 3 shoot tips served as the experimental unit. Treatments were replicated 3 times using a completely randomized designed. Technical grade (a.i. 97%) Cycocel (American Cyanamid Company, Princeton, N.J.) was prepared as a 1.0 mg/ml aqueous stock solution and sterilized by Milipore filtration (pore size: 0.2 μm) before adding to sterile LBM. Cycocel treatment effects on shoot length and dry weight were evaluated for each species after 14 days static culture. Statistical significance ($\alpha = 0.05$) for differences in shoot growth and dry weight was calculated using analysis of variance followed by Dunnett's means separation test for comparison of treatments with a control (Steel and Torrie, 1960).

[3]*Myriophyllum* 'Frill' (also called 'Filigree Myrio') is a non-flowering cultivar propagated and sold by the aquarium plant trade. This cultivar is morphologically similar to *Myriophyllium aquaticum* but is of unknown origin.

RESULTS AND DISCUSSION

Adventitious shoots formed rapidly on surface sterilized leaf explants of *M. heterophyllum* cultured on SSM. As previously described (Kane and Albert, 1989a), the primary adventitious shoots formed directly from the epidermis within 3 to 4 days (Figure 1). Continued development of primary adventitious shoots, subsequent lateral branching of these shoots and secondary formation of adventitious shoots on primary shoots resulted in production of compact rootless shoot masses after 28 days. Kane and Albert (1989a) reported more than 2600 shoots can be produced *in vitro* from a single leaf explant within a 28 day culture period. Rapid shoot regeneration of *M. heterophyllum* was sustained when explants (ca 8.0 mm^2) from the original shoot masses were subcultured onto fresh SMM for 21 days. By day 21, these cultures consisted of a solid "tube" of densely compacted shoot tips (Figure 2). Shoot multiplication cultures maintained longer than 28 days at 25 C, without subculture, usually exhibited moderate chlorosis. However, the need for frequent subculture was eliminated by storing stock cultures on SMM in the dark at 5 C. *Myriophyllum meterophyllum* shoot cultures were stored for up to 14 months at 5 C without loss of regenerative capacity (Kane, unpublished). *Myriophyllum aquaticum, M. pinnatum, M. spicatum*, and *M.* 'Frill' also exhibited the capacity for high density shoot formation when shoot tips were transferred to SSM containing 30 μM 2iP. Thus, *Myriophyllum* species displayed inherently high rates of

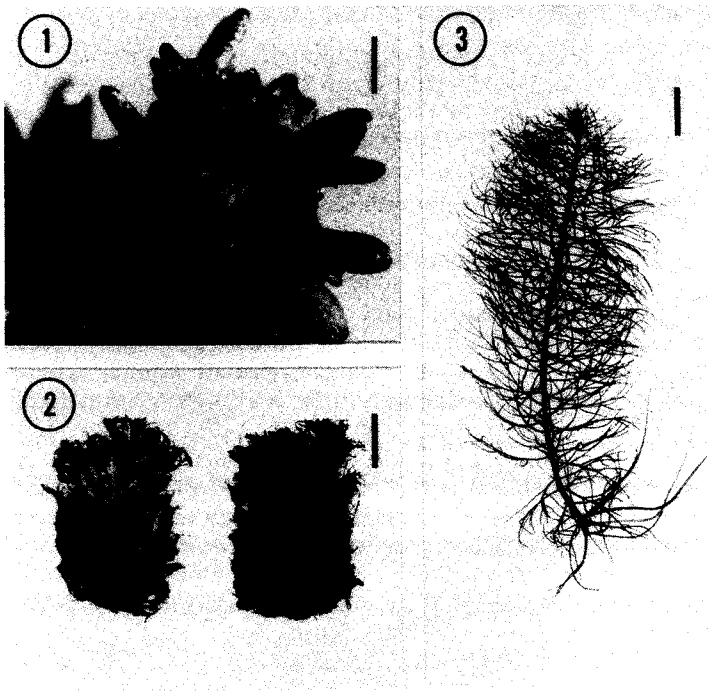

Figure 1-3. *In vitro* propagation of *Myriophyllum* species. Fig. 1. Early multiple adventitious shoot formation from a leaf explant of *Myriophyllum heterophyllum* cultured for 12 days on Shoot Multiplication Medium (SMM). Scale = 0.25 mm. Fig. 2. Longitudinal section through a typical *Myrophyllum heterophyllum* shoot mass produced following 21 days culture on SSM. **Left**: inner cut surface; **Right**; outer surface. Scale = 10 mm. Fig. 3. Typical development of a rooted *Myriophyllium heterophyllum* plant from a 1.0 cm shoot tip cultured in Liquid Basal Medium. Scale = 10 mm.

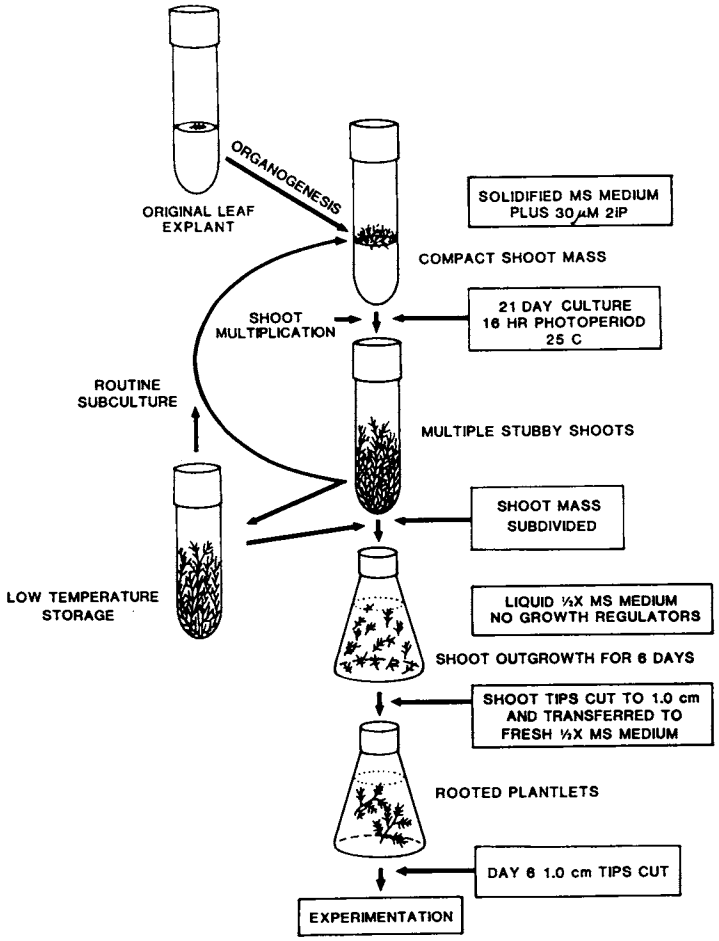

Figure 4. Protocol for *in vitro* propagation and storage of *Myriophyllum* species for use in bioassays and physiological/developmental studies.

shoot regeneration when cultured *in vitro* on a medium supplemented with the naturally occurring cytokinin 2iP.

Although convenient for high density shoot storage of *Myriophyllum* spp., the compact shoots produced on SSM were too small for direct use as propagules in Cycocel bioassays. Higher cytokinin levels promote *in vitro* shoot formation but inhibit shoot elongation and root development in many terrestrial species (Pierik, 1987). Consequently, rapid elongation of submerged shoots was achieved after shoot masses produced on SMM were divided and subcultured into LBM without the cytokinin 2iP for 6 days. Cytokinin-induced inhibition of rooting was eliminated by the two 6-day subcultures in LBM. This provided rooted plantlets from which 1.0 cm long excised shoot tips served as uniform propagules for the Cycocel bioassays (Figure 3). The generalized *Myriophyllum in vitro* propagation and storage procedure is depicted in Figure 4.

The three *Myriophyllum* species screened exhibited different sensitivities to Cycocel at the levels tested. *Myriophyllum heterophyllum* exhibited the greatest sensitivity to Cycocel. Both shoot elongation and total plant dry weight were significantly inhibited by 80 mg/L Cycocel, dry weight accumulation was not significantly inhibited at any of the levels tested (Figure 6). There was no significant Cycocel treatment effect on either shoot growth or dry weight ac-

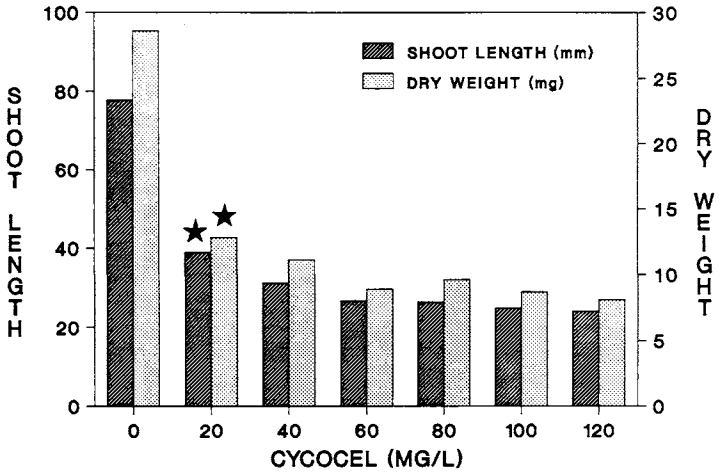

Figure 5. Effect of Cycocel on growth of 1.0 cm shoot tips of *Myriophyllum heterophyllum* cultured *in vitro* for 14 days. Values represent mean response of 9 shoot tips per treatment. Initial shoot tip dry weight = 2.9 ± 1.0 mg. The lowest concentration resulting in a response statistically less ($\propto$ 0.05) than the control is denoted by a star.

cumulation of *Myriophyllum* 'Frill' at the levels tested. Cycocel indirectly inhibits cell elongation and division through blockage of *ent*-kaurene synthetase A activity in the gibberellin biosynthetic pathway but its effectiveness is species specific (Sponsel, 1987). Differences among species in sensitivity to Cycocel were rapidly determined using the bioassay protocol developed in this study. This protocol could be used for screening more relevant allelochemic and synthetic growth regulators having potential use for aquatic plant management.

One important criterion for any standardized bioassay using aquatic plants is that the plants be available in sufficient supply year round with minimal culture and space requirements. The ability to both propagate *Myriophyllum* spp. *in vitro* as compact shoot masses and store them for

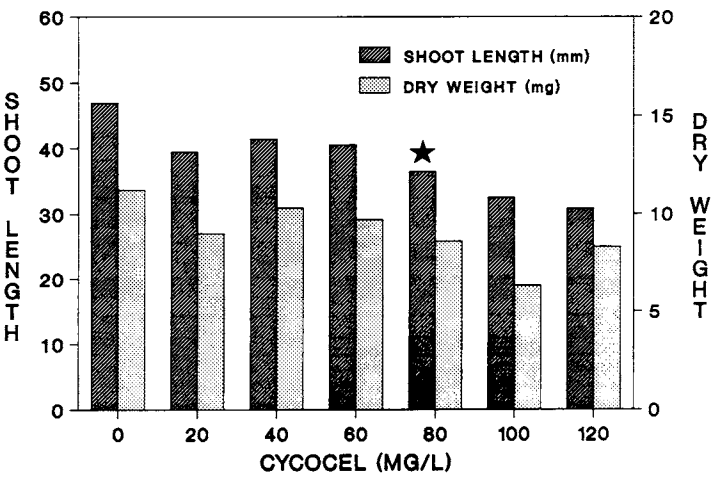

Figure 6. Effect of Cycocel on growth of 1.0 cm shoot tips of *Myriophyllum spicatum* cultured *in vitro* for 14 days. Values represent mean response of 9 shoot tips per treatment. Initial shoot tip dry weight = 0.9 ± 0.3 mg. The lowest concentration resulting in a response statistically less ($\propto$ 0.05) than the control is denoted by a star. Cycocel had no significant effect on dry weight accumulation.

extended periods before use meet this criterion. Presently, the duckweed bioassay, utilizing a floating aquatic, is the only *in vitro* culture system available. The availability of numerous submerged *Myriophyllum* species may now provide the means for more comprehensive *in vitro* growth regulator screening.

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