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# Regenerative Capacity of *Myriophyllum aquaticum* Tissues Cultured *In Vitro*[1]

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## ABSTRACT

Effects of cytokinin type ($N^6$-benzylaminopurine [BA], 2-isopentenyladenine [2iP] or 6-[4-hydroxy-3-methylbut-2-enyamino]purine [zeatin]) on *in vitro* shoot regeneration from nodal segments of parrotfeather (*Myriophyllum aquaticum* Verdc.) were examined. The influence of either BA, 2iP, or zeatin at 0, 2.5, 5, 10, 2 or 40 $\mu$M in factorial combination with 0, 0.1, or 1 $\mu$M $\alpha$-naphthaleneacetic acid (NAA) on adventitious shoot formation from 1.0 cm long aerial stem internode segments was also examined. Axillary shoot production from single node segments was significantly promoted in media supplemented with cytokinin. Maximum shoot regeneration from nodal segments occurred in liquid medium supplemented with 5 $\mu$M zeatin. Internode segments exhibited the capacity for rapid adventitious shoot formation when cultured on basal medium without cytokinins. Adventitious shoot development (ADS) was inhibited in the presence of BA but was promoted by the naturally occurring cytokinins 2iP and zeatin. Maximum adventitious shoot formation occurred from internode explants cultured on basal medium supplemented with 40 $\mu$M 2iP. Medium supplementation with the synthetic auxin NAA antagonized promotion of ADS by 2iP and zeatin and promoted callus formation. Adventitious shoot meristems formed directly from the epidermal layer of the internode explants within 2 days after inoculation.


*Key words*: aquatic plants, tissue culture, adventitious shoot development, cytokinins.

## INTRODUCTION

Many aquatic plants have the capacity to rapidly colonize water bodies and become problematic. This phenomenon has been attributed, in part, to their diverse and effective means for rapid vegetative growth and regeneration (Sculthorpe 1967; Madsen et al. 1988; Smith and Barko 1990). Cook (1987) observed that, of the 12 most weedy aquatic plants, eleven species expand within their range through effective vegetative reproduction from propagules including shoot fragments and specialized hibernacula (tubers or turions). The remaining species, *Trapa natans* L., relies sorely on sexual reproduction for its spread. The effectiveness of vegetative reproduction for rapid dispersal is exemplified by the infestation of water bodies in the southern United States by the non-

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seed bearing dioecious (pistillate flowered) stain of hydrilla [*Hydrilla verticillata* (L.f.) Royle] (Langeland 1989).

Many aquatic macrophytes can regenerate rapidly from stem fragments processing a single axillary or modified bud (Sculthorpe 1967; Langeland and Sutton 1980; Spencer and Bowes 1985). In several taxonomically diverse aquatic plant genera, including *Utricularia, Rorippa, Ceratopteris, Hygrophila* and *Podostemum* production of adventitious plantlets on detached and/or injured leaves is also an effective method of regeneration *in situ* (Sculthorpe 1967; Muhlberg 1982). Hagemann (1932) reported that, within the Haloragaceae, detached leaves of Western milfoil (*Myriophyllum hippuroides* Nutt. ex Torr. & Gray) and mermaidweed (*Proserpinaca palustris* L.) produced adventitious shoots when maintained under moist conditions. It is not known, however, what role, if any, adventitious shoot formation from fragmented tissues may play in the dispersal of these Haloragaceae taxa.

Parrotfeather is a popular heterophyllic amphibious aquatic used in aquaria and water gardens. Parrotfeather is native to South America and has become naturalized in 29 states since its first documented introduction into the United States in the early 1890's. Parrotfeather, while widespread, is not considered to pose a widespread weed problem (Nelson and Couch 1985).

Only pistillate flowering plants of parrotfeather are known to occur in the United States (Sutton 1985). Thus, the spread of parrotfeather in its adventive range has been a function of its inherent capacity for rapid vegetative reproduction from plant fragments and human intervention (Sutton 1985). Detached parrotfeather leaves also exhibit the capacity for adventitious shoot formation when maintained under moist conditions (Hagemann 1932). Hence, the *in vitro* culture of parrotfeather may be a viable system for probing the physiological mechanisms regulating vegetative regeneration both from pre-existing meristems and adventitious shoot development in aquatic plants.

*In vitro* whole plant and tissue culture systems have proven useful to precisely study the physiological factors controlling development in certain aquatic plants (Mohan Ram and Kapoor 1976; Kane and Albert 1989a; 1989b). Recent studies in our laboratory suggest that *Myriophyllum* species, in general, may possess inherently high capacities for rapid axillary branching and adventitious shoot production from isolated tissues cultered *in vitro* (Kane and Albert 1989b; Kane and Gilman 1991). In the present study, we examined the influence of growth regulators and culture conditions on the *in vitro* regenerative capacity of parrotfeather from nodal and internodal stem tissues.

## MATERIALS AND METHODS

*Initial establishment of submerged and aerial shoot cultures in vitro.* Shoot of parrotfeather, originally collected from Piersol Lake, Shelby Co., Tenn., were kindly provided by Dr. Susan Aiken. Defoliated aerial stem segments (consisting of two to three nodes), collected from aerially grown greenhouse plants, were surface sterilized in aqueous 1.05% (w/v) NaClO containing 0.01% (v/v) Tween-20 for 12 min, followed by three 5-min rinses in sterile deionized water. Stem segments were then transferred into 500 ml aluminum foil capped Erlenmeyer flasks containing 250 ml sterile liquid basal medium. Surface sterilized stem segments were initially established in liquid basal medium to facilitate early detection of microbial contamination. The basal medium (BM) consisted of half-strength Murashige and Skoog mineral salts (1962) supplemented with 0.56 mM myo-inositol, 1.2 $\mu$M thiamine-HCl and 87.6 mM sucrose. The medium was adjusted to pH 5.7 with 0.1 N KOH before autoclaving at 1.2 kg cm$^{-2}$ for 20 min at 121 C. For this study, all stock cultures and experiments were maintained at 25 ± 2 C under a 16-hr photoperiod provided by cool-white fluorescent tubes (Sylvania F96T12/CW) at a photosynthetic flux density of 90 $\mu$mol·s$^{-1}$·m$^{-2}$ as measured at culture level. Stock liquid cultures were further increased by propagating lateral branches which developed from the initial stem segments after 4 weeks culture in liquid BM. Flasks were not shaken. Stock plants were subcultured at three week intervals. All transfers were performed aseptically in a laminar air flow transfer hood.

Stock aerial shoot cultures of parrotfeather were initially established by rooting single node stem cuttings (explants) from submerged stock cultures in 150 x 25 mm culture tubes containing 15 ml sterile BM solidified with 1.5% (w/v) TC™ agar (JRH Biosciences, Inc., Lenexa, KS). Single node (single whorl) cuttings obtained from the aerial shoots which developed were subcultured at three week intervals to increase to number of aerial shoot stock cultures used for subsequent experiments.

*Effect of nodal explant position on shoot regrowth.* Effect of node position on shoot regrowth capacity was examined by culturing the first, second, third, and fourth aerial nodal explant (Figure 1) immediately below the terminal leaf cluster into individual 150 x 25 mm culture tubes containing 15 ml sterile agar solidified BM. Nodal explants were placed into the medium with the basal cut end of the stem embedded up to the node. A culture tube inoculated with a single nodal explant represented the experimental unit. Each treatment was replicated 25 times. Shoot number, length and node number were determined after 14 days culture.

*Cytokinin effects on regeneration from nodal explants.* Cytokinin effects on shoot regeneration from aerial nodal explants cultured in liquid and agar solidified BM were examined. Single node explants were transferred into 150 x 25 mm culture tubes containing 15 ml liquid or agar-solidified (1.5% w/v TC™ agar) BM supplemented with 5 $\mu$M of either N$^6$-benzylaminopurine (BA), 2-isopentenyladenine (2iP), or 6-[4-hydroxy-3-methylbut-2-enyamino]purine (zeatin) for 14 days. Cytokinins were prepared as concentrated aqueous stock solutions and sterilized by Millipore filtration (pore size: 0.22 $\mu$m) before they were added to molten (40 C) autoclaved agar solidified or liquid BM. A culture tube containing a single node explant represented the experimental unit. Each treatment was replicated 25 times. Shoot number and length were determined after 14 days culture. Treatment effects were statistically analyzed using the General Linear Models (GLM) procedures developed by Statistical Analysis System (SAS 1985). Mean separation was determined using Tukey's (HSD) studentized range test ($\alpha$=0.05).

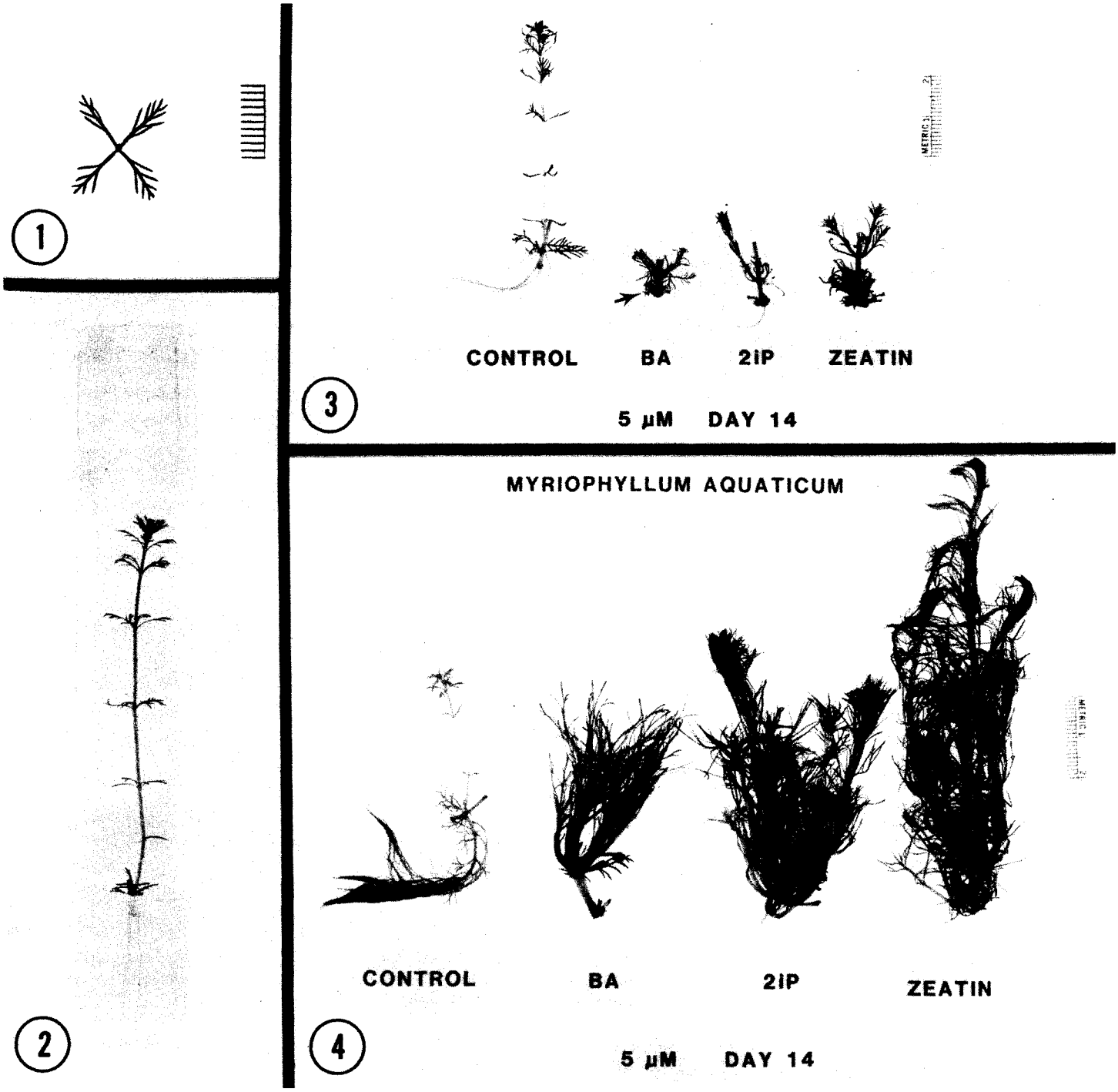



Figure 1-4. Effects of agar-solidified and liquid media and cytokinin type on shoot regeneration from nodal explants of *Myriophyllum aquaticum*. Figure 1. Aerial nodal explant consisting of a four-leaf whorl. Scale bar = 10 mm. Figure 2. Development of a single aerial shoot produced from a nodal explants after 14 days culture on agar-solidified basal medium. Figure 3. Effect of cytokinin type on shoot regeneration from a nodal explant cultured on agar-solidified medium for 14 days. Control = basal medium without cytokinin; BA = $N^6$-benzylaminopurine; 2iP = 2-isopentenyladenine; zeatin = 6-[4-hydroxy-3-methylbut-2-enyamino]purine. Note presence of adventitious shoots (arrow) at base nodal explant cultured in presence of BA. Scale bar = 20 mm. Figure 4. Effect of cytokinin type on shoot regeneration from a nodal explant cultured in liquid medium for 14 days. Scale bar = 20 mm.

*Plant growth regulator effects on adventitious shoot formation from internode explants.* Effects of factorial combination of either BA, 2iP, or zeatin (0, 2.5, 5, 10, 20, or 40 μM) with α-naphthaleneacetic acid (NAA) at 0, 0.1, or 1 μM on adventitious shoot formation from 1.0 cm long aerial stem internode segments were examined. Each experiment was comprised of 18 treatments consisting of all combinations of a specific cytokinin and NAA. Growth regulators were filter sterilized as described above and then added individually or in combination to molten autoclaved agar sup-

plemented BM. Media were dispensed as 10 ml aliquots into sterile 20 ml glass scintillation vials. Vials were loosely covered with polypropylene caps. Each replicate consisted of a vial inoculated with a single internode explant placed horizontally and in firm contact with surface of the medium. Treatments were replicated 10 times. Treatment effects on adventitious shoot number and length were determined after 28 days culture. Main treatment effects and interactions were statistically analyzed using GLM procedures.

*Light and scanning electron microscopy.* For histological observations, internode explants were cultured on agar-solidified BM with 5 $\mu$M 2iP. At two-day intervals until day 28, explants were fixed in formalin-acetic-alcohol (FAA) under vacuum, dehydrated through a graded ethanol-tertiary butyl alcohol series and embedded in Paraplast Plus™ (mp: 56 C, Monojet Scientific, Saint Louis, MO). Embedded internode explants were sectioned at 10 $\mu$m and stained with 0.05% toludine blue (w/v) in citrate phosphate buffer (pH 6.0) for 25 sec (Sakai 1973). For scanning electron microscopy (SEM), explants were fixed in FAA, dehydrated through an ethanol series, critical point dried, mounted on aluminum studs, and sputter coated with gold in an Eiko 1B-2 ion coater. Samples were then examined in an Hitachi S-450 scanning electron microscope at 20 kV and images were recorded photographically on Polaroid-type 55P/N film.

## RESULTS AND DISCUSSION

Node position had no significant effect on the capacity of single node explants to regenerate shoot or the number of nodes produced per regenerated shoot when cultured *in vitro* on agar solidified BM. Shoot regrowth occurred 100% at all node positions. Each parrotfeather nodal explant consisted of a four-leaf whorl with four axillary buds (Figure 1); but only a single shoot consisting of an average of six nodes usually developed from each nodal explant within 14 days (Figure 2). We feel that this supports other work that, in nature, a 5.0 mm parrotfeather stem fragment with a single node is capable of forming a new plant *in situ* (Jacot Guillarmod 1979). Single node hydrilla fragments are also capable of regrowth. However, the percentage of fragments exhibiting regrowth is positively correlated with number of nodes per fragment under both controlled culture and field conditions (Langeland and Sutton 1980). Spencer and Bowes (1985) observed a similar dependence on stem fragment node number for regrowth in cultured *Limnophila sessiliflora* (Vahl) Blume, *Hygrophila polysperma* Roxb. T. Anderson and hydrilla. Growth potential from notes at specific position along the stem was not examined in their studies. However, in *Hygrophila*, Cutter and Chiu (1975) have attributed the observed lag in lateral shoot development from cultured single nodal explants taken from the most apical nodes to be the result of initially smaller axillary buds at these nodes.

Cytokinin supplementation significantly promoted axillary shoot production from single node explants cultured in agar-solidified or liquid media (Figure 3 and 4). Cytokinin promoted shoot production was significantly greater in liquid than on agar-solidified media (compare Figure 3 and 4; Figure 5). The naturally occurring cytokinin zeatin was most effective in promoting shoot production from nodal explants in cultured in liquid medium (Figure 5). Axillary shoots produced in the presence of the synthetic cytokinin BA were moderately chlorotic. Adventitious shoot formation also occurred along the basal cut end of nodal explants on solid medium supplemented with 5 $\mu$M BA (Figure 3). Except in control treatments, shoot growth was significantly greater in liquid media (Figure 6). Compared to controls, shoot growth was significantly inhibited in both liquid and agar-solified media supplemented with cytokinin (Figure 6). Maximum shoot growth inhibition occurred in the presence of BA in liquid and solid media.

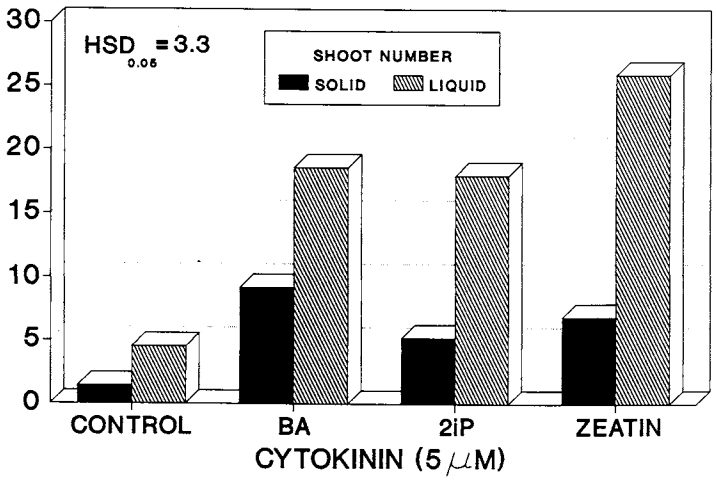



Figure 5. Effect of agar-solidified and liquid media and cytokinins $N^6$-benzylaminopurine (BA), 2-isopentenyladenine 2iP) and zeatin on axillary shoot production from nodal explants of *Myriophyllum aquaticum* after 14 days culture. Each histobar represents the mean response of 25 explants.

Internodal segments of parrotfeather exhibited a high capacity for rapid formation of adventitious shoot when cultured *in vitro*. Adventitious shoot development (ASD) [mean: 6 adventitious shoot buds/internode explant] and

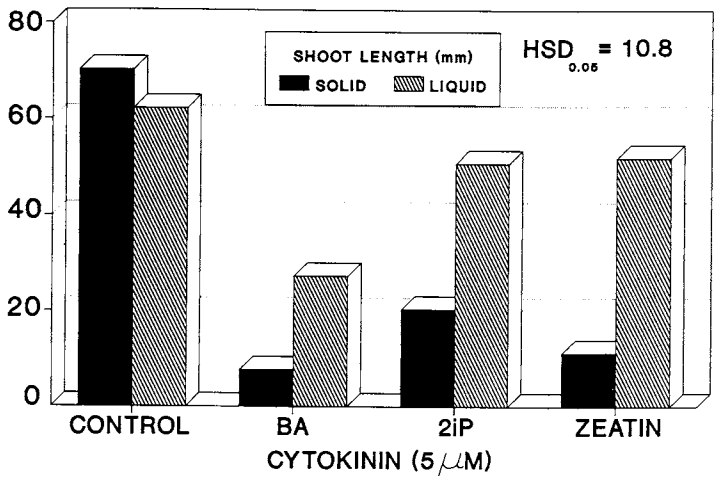



Figure 6. Effects of agar-solidified and liquid media and cytokinins $N^6$-benzylaminopurine (BA), 2-isopentenyladenine (2iP) and zeatin on axillary shoot length from nodal explants of *Myriophyllum aquaticum* after 14 days culture. Each histobar represents the mean response of 25 explants.

subsequent rooting occurred in 100% of the explants cultured on BM without growth regulator supplementation (see "control" Figure 16). This observation supports Hagemann's (1932) report that isolated non-nodal tissues of parrotfeather exhibit the capacity for regeneration through formation of adveititious shoots. Given the high capacity for *in vitro* ASD in parrotfeather, it is probable that this mode of regeneration probably occurs following stem fragmentation in nature.

Adventitious shoot development was significantly inhibited on BM supplemented with the synthetic cytokinin BA (Figure 7). All internode explants cultured on BM supplemented with greater than 10 $\mu$M BA died. There was a significant interaction among the various BA and NAA treatments. Medium supplementation with NAA mitigated the inhibitory and toxic effects of BA on ASD (Figure 7). However, addition of NAA along was inhibitory to ASD. *In vitro* adventitious shoot development on *Myriophyllum heterophyllum* Michx. (variable-leaf milfoil) leaf explants was inhibited by BA and this inhibition was similarly mitigated by addition of NAA (Kane and Albert 1989b).

In contrast to BA treatments, BM supplementation with the naturally occurring cytokinin 2iP alone significantly promoted ASD (Figure 8). Maximum ASD (mean: 28 adventitious shoots/internode) occurred on BM supplemented with 40 $\mu$M 2iP. Internode explants cultured on BM supplemented with greater than 10$\mu$M for 28 days produced masses for stubby shoots with short internodes (Figure 16). Medium supplementation with NAA significantly promoted the formation of callus on the internode explants (Figure 17) and inhibited 2iP promoted ASD (Figure 8). Adventitious shoot formation on internodal explants was also significantly promoted on BM supplemented with the naturally occurring cytokinin zeatin (Figure 9) and was similarly suppressed in the presence of NAA.

The promotive effect of cytokinins on axillary branching and adventitious shoot formation *in vitro* have been

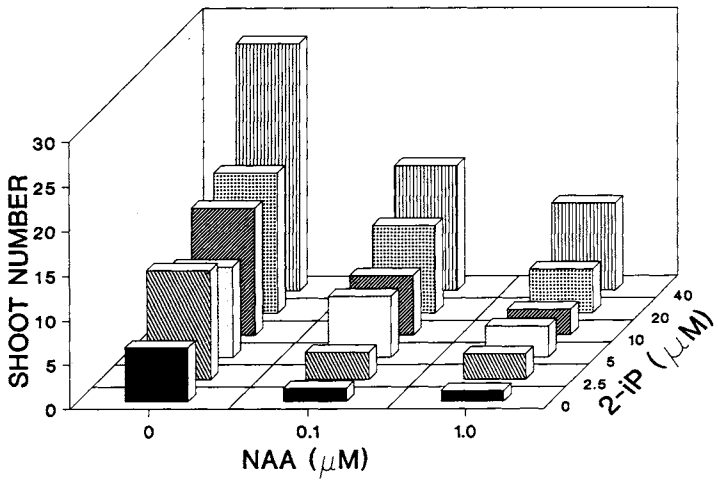



Figure 8. Effect of 2-isopentenyladenine (2iP) and $\alpha$-naphthaleneacetic acid (NAA) combinations on adventitious shoot development on internode explants cultured for 28 days. Each histobar represents the mean number of shoots for 10 explants.

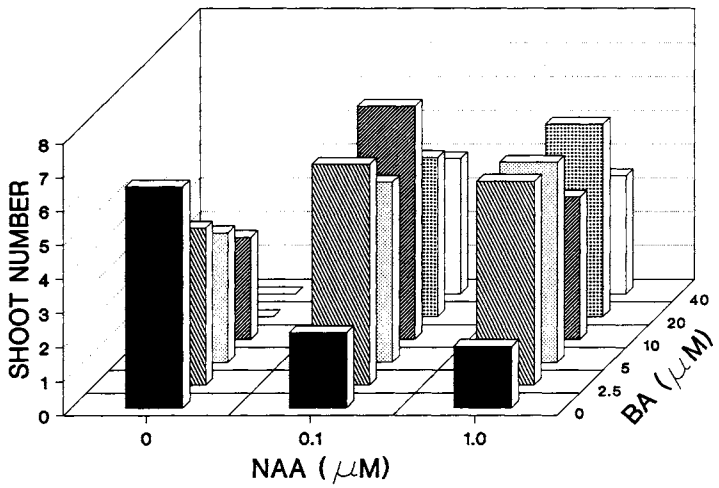



Figure 7. Effect of benzyladeine (BA) and $\alpha$-naphthaleneacetic acid (NAA) combinations on adventitious shoot development on internode explants cultured for 28 days. Each histobar represents the mean number of shoots for 10 explants.

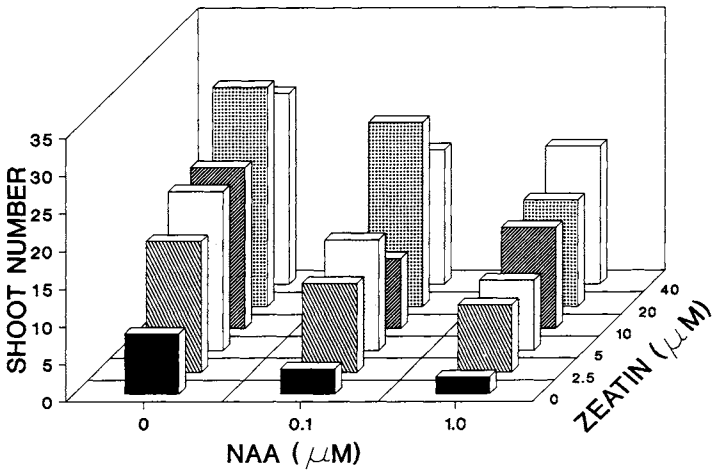
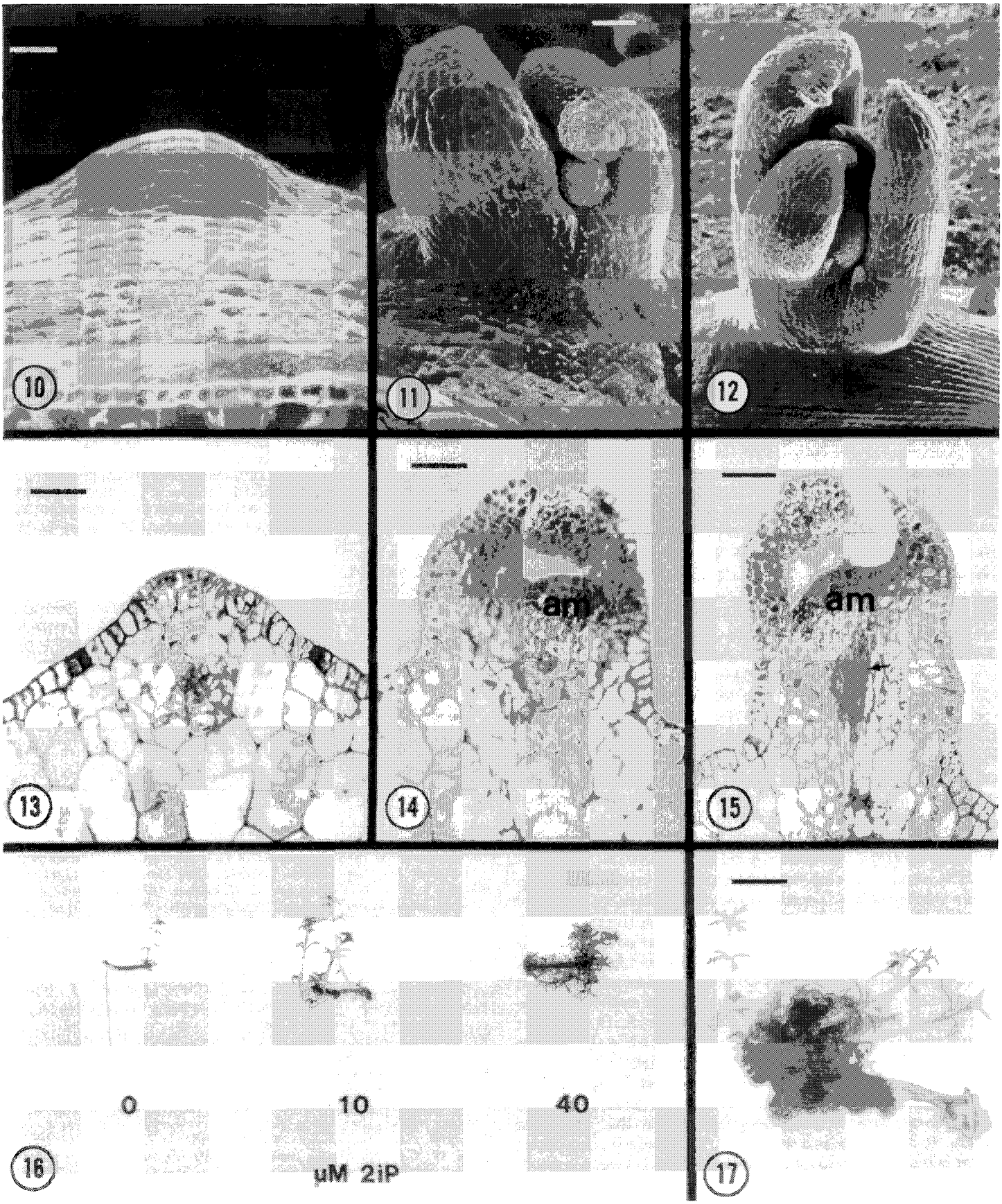



Figure 9. Effect of zeatin and $\alpha$-naphthaleneacetic acid (NAA) combinations on adventitious shoot development on internode explants cultured for 28 days. Each histobar represents the mean number of shoots for 10 explants.

Figure 10-15. Histological origin and development of adventitious shoots from internode explants of *Myriophyllum aquaticum* cultured *in vitro* in presence of 5 $\mu$M 2iP alone. Figure 10, 13. Dome shaped meristematic center initially formed within the epidermal layer after 2 day culture in the presence of 5 $\mu$M 2iP. Figures 11, 14. Adventitious shoot-bud with distinct apical meristem (am) and leaf primordia after 4 days culture. Figures 12, 15. Development of protruding bud consisting of apical meristem (am) flanked by numerous leaf primordia. Note provascular strand developmental (arrow). Figure 16. Effect of 2iP without $\alpha$-naphthaleneacetic acid (NAA) on adventitious shoot development on internode explants cultured for 28 days.

Figure 17. Induction of callus formation on internode explant in presence of 1 $\mu$M NAA and 20 $\mu$M 2iP after 28 days culture. In Figures 10, 11, 12 scale bars= 100 $\mu$m.. In Figures 13, 14, 15 scales bars= 25 $\mu$m. In Figures 16, 17; scale bars= 10 and 2.5 mm, respectively.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documented in many terrestrial plants (George and Sherrington 1984). However, there is little information on the role of cytokinins in controlling these processes in aquatic angiosperms. In *Myriophyllum aquaticum* and *M. heterophyllum* (Kane and Albert 1989b), the naturally occurring cytokinins 2iP and zeatin more effectively promoted axillary and adventitious shoot development and growth than did the synthetic cytokinin BA. The physiological basis for this is not known; but it may reflect the inability of *Myriophyllum* species to metabolize BA resulting in the endogenous buildup of supra-optimal levels of BA (Blakesley and Lenton 1987). We are not aware of any other studies which have specifically examined the efficacy of synthetic and naturally occurring cytokinins on *in vitro* regeneration in aquatic plants. Work indicating that the synthetic cytokinins kinetin and BA effectively promoted shoot regeneration *in vitro* in some aquatic genera including *Potamogeton* and *Cryptocoryne*, while having no effect on other aquatic species such as American lotus (*Nelumbo lutea* L.), suggests that distinct differences in the hormonal mechanisms controlling growth exist among different aquatic taxa (Ailstock 1986; Kane et al. 1990).

The histological origin and development of adventitious shoots from *M. aquaticum* internode explants was similar to that observed in *M. heterophyllum* leaf explants (Kane and Albert 1989b). Histological sectioning and scanning electron microscopy of internode segments cultured on BM supplemented with 5 $\mu$M 2iP revealed that, in *M. aquaticum*, adventitious shoots develop directly from the epidermal layer of the internode explants. By day 2, dome shaped meristematic centers consisting of small densely cytoplasmic cells developed following initial anticlinal and periclinal cell divisions within the epidermal layer and subsequent cell divisions from within the underlying cortical tissue (Figure 10, 13). Several leaf promordia developed from the margins of the protruding meristem by day 4 (Figure 11, 14). Following rapid cell division and elongation, by day 8 buds averaged 0.7 mm in length with each consisting of an apical meristem flanked by numerous leaf primordia (Figure 12). Provascular strand development was also evident within these buds (Figure 15). No secondary development of adventitious buds was observed on the primary adventitious shoots produced.

Differences in regenerative capacity *in vitro* exist between *Myriophyllum* species. Although *in vitro* initiation of ASD from internode tissues in parrotfeather is very rapid, the *total* regenerative capacity of this species is relatively less than we have observed in *Myriophyllum heterophyllum*. From a single exercise leaf explant of *M. heterophyllum*, rapid formation and subsequent axillary branching of primary adventitious shoots and secondary ASD on these shoots results in production of extremely dense shoot masses comprised of more than 2600 shoots following 28 days culture (Kane and Albert 1989b; Kane and Gilman 1991). *Myriophyllum heterophyllum* also exhibits aggressive growth *in situ* throughout its habitat range (Crow and Hellquist 1983). Further baseline studies with other aquatic species are needed to determine the relationship between growth potential *in situ* and regenerative capacity *in vitro*. Results of this study further indicate that *in vitro* culture techniques are useful for screening aquatic plant regenerative potential on the cellular and tissue levels.

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