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Growth Regulator Effects on *in Vitro* Shoot Regeneration of *Crassula helmsii*[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]) and concentration (0-10 µM) on *in vitro* shoot regeneration of *Crassula helmsii* (T. Kirk) Cockayne (swamp stonecrop) from single-node explants were examined. The influence of either BA, 2iP, or zeatin (0-25 µM) in factorial combination with 0 or 1.0 µM α-naphthaleneacetic acid (NAA) on the capacity to form adventitious shoots *in vitro* from leaf blade and internode explants was also examined. Axillary shoot production from nodal explants was promoted in media supplemented with cytokinin. Maximum shoot regeneration from nodal explants occurred in liquid medium consisting of full-strength Murashige & Skoog mineral salts, 0.56 mM myo-inositol and 1.2 µM thiamine-HCL and 58.4 mM sucrose supplemented with 5.0 µM BA. Zeatin and 2iP were ineffective in promoting lateral branching from single node explants. Cultured internode explants produced adventitious shoots in the absence of exogenous growth regulators. Both leaf blade and internode explants exhibited rapid adventitious shoot development (ASD) when cultured on agar-solidified medium supplemented with a cytokinin and 1.0 µM NAA. These results suggest *in vitro* culture techniques may be used to rapidly screen aquatic plant growth potential.

Key words: aquatic plants, adventitious shoot development, cytokinins, growth potential.

INTRODUCTION

Most weedy aquatic angiosperms expand within their range primarily through effective vegetative reproduction (Cook 1987). Rapid colonization of water bodies is attributed, in part, to the high capacity of these plants to regenerate and grow from stem fragments and specialized hibernacula including tubers or turions (Sculthorpe 1967, Madsen *et al.* 1988, Sutton *et al.* 1992).

It is well documented that aquatic plant growth and development *in situ* is influenced by many abiotic and biotic factors acting in concert (Spencer and Bowes 1985). However, it is plant genotype which ultimately determines maximum

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proliferation *under nonlimiting growth conditions*. Therefore, it may be feasible to determine the relative growth potential of aquatic species by comparing their capacity for growth and regeneration under nonlimiting culture conditions of light and temperature as well as nutrient and carbon availability. Such conditions can be provided by using *in vitro* whole plant and tissue culture techniques. This approach involves the sterile culture of whole plants or tissues under nonlimiting conditions of nutrient, carbon, and growth regulator enrichment under controlled conditions of light and temperature.

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, Mohan Ram and Kakkar 1983, Kane and Albert 1989a). Studies in our laboratory suggest that aggressive species such as *Myriophyllum heterophyllum* and *M. aquaticum* possess inherently high capacities for rapid axillary branching and adventitious shoot production from isolated tissues cultured *in vitro* (Kane and Albert 1989b, Kane and Gilman 1991, Kane *et al.* 1991). Preliminary data suggest that a close relationship may exist between the cellular capacity for shoot regeneration and growth *in vitro* and growth potential *in situ*. Comparative studies of *in vitro* growth and regeneration of designated known weedy and putative nonweedy species could provide valuable baseline information with which to evaluate aquatic plant growth potential. However, the *in vitro* growth performance of diverse aquatic plant genera must be screened to verify this relationship.

Crassula helmsii (swamp stonecrop), a succulent perennial aquatic angiosperm native to Australasia, has become established in Europe and naturalized in over 140 sites in Britain (Dawson and Warman 1987). The rapid spread of this species has been attributed to its enormous potential to regenerate from small stem fragments (Dawson and Warman 1987). In the present study, we examined the influence of growth regulators on the regenerative capacity of swamp stonecrop in vitro from single-node, leaf blade and internode segments.

MATERIALS AND METHODS

Initial establishment of in vitro shoot cultures. Shoots of swamp stonecrop were kindly provided by Dr. F. H. Dawson, Freshwater Biological Association River Laboratory, Great Britain. Defoliated stem segments (consisting of two to three nodes) were rinsed for 30 min in tap water and then 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 transferred into 500-ml aluminum-foil-capped Erlenmeyer flasks containing 250 ml of sterile liquid basal medium (BM) consisting of half-strength Murashige and Skoog mineral salts (1962) supplemented with 0.56 mM myo-inositol, 1.2 μM thiamine-HCl and 58.4 mM sucrose. The medium was adjusted to pH 5.7 with 0.1 N KOH before autoclaving at 1.2 $kg \cdot cm^{-2}$ for 20 min at 121C. Stock cultures and experiments were maintained at 25 ± 2C under a 16-hr photoperiod provided by cool-white fluorescent tubes (Sylvania F96T12/CW) at a photosynthetic flux density of 38 $\mu mol \cdot s^{-1} \cdot m^{-2}$ as measured at culture level. Stock plant cultures were further increased by aseptically subculturing the branch shoot tips produced at 4-wk intervals (Figure 1A).

Cytokinin effects on regeneration from nodal explants. The effects of cytokinin enrichment on shoot regeneration from single node segments (explants) cultured in liquid BM were examined. Single node explants 5 to 7 mm long (Figure 1A) were transferred into 150- by 25-mm culture tubes containing 12 ml of liquid BM supplemented with 1-10 μM of either N^6-benzylaminopurine (BA), 2-isopentenyladenine (2iP), or 6-[4-hydroxy-3-methylbut-2-enyamino]purine (zeatin). A tube containing a single node explant represented the experimental unit. Each treatment was replicated nine times. Shoot number, length and node number were determined after 28 days in culture. Treatment effects were statistically analyzed using the General Linear Models (GLM) procedures developed by Statistical Analyses System (SAS 1985). Mean separation was determined using Tukey's (HSD) studentized range test ($\alpha = 0.05$). For brevity, only the optimal responses at the 5-μM cytokinin level are described.

Capacity for adventitious shoot formation from leaf and internode explants. The capacities of 3.0-mm-long leaf blade (Figure 1A) and 5- to 8-mm-long internode (Figure 1A) explants to form adventitious shoots *in vitro* were evaluated on a modified BM (MBM) supplemented with either BA, 2iP, or zeatin (0-25 μM) in factorial combination with 0 or 1.0 μM α-naphthaleneacetic acid (NAA). The modified BM components were the same as previously described except that 87.6 mM sucrose was substituted and the medium was solidified with 0.8% (w/v) TC® agar (JRH Biosciences, Lenexa, KS). Media were dispensed as 10-ml aliquots into 60- by 15-mm Falcon® #1007 polystyrene petri dishes (Becton Dickerson, Lincoln Park, NJ). Each replicate consisted of a petri dish inoculated with a leaf blade and internode explant placed horizontally on the surface of the medium. Treatments were replicated nine times. Treatment effects on adventitious shoot number were determined after 28 days in culture. For brevity, effects of medium supplementation with only 20 μM cytokinin and 1.0 μM NAA are described.

Histological sectioning. For histological observations of adventitious shoot development, internode explants were cul-

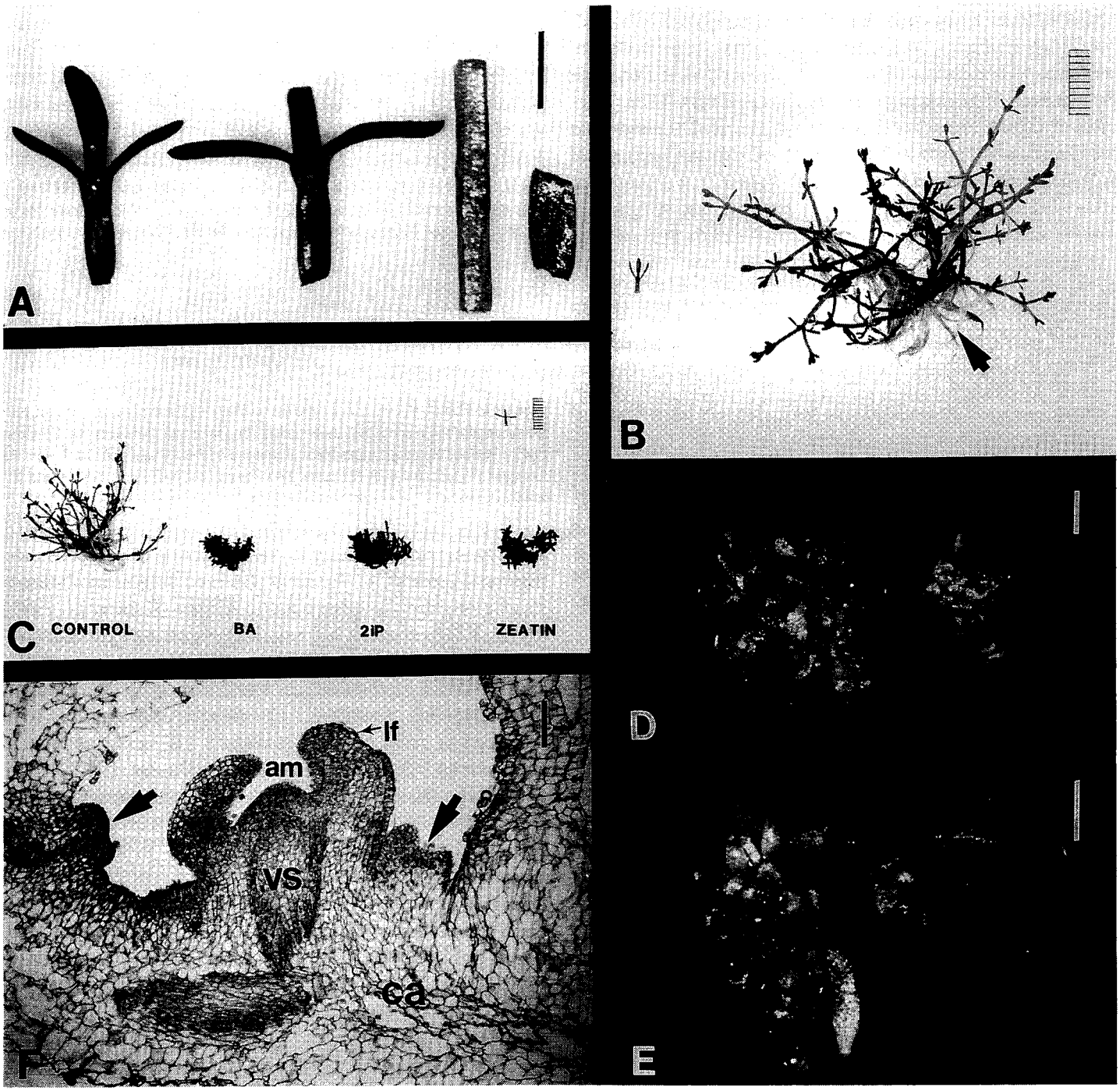

Figure 1A-F. Effect of explant and cytokinin type on *in vitro* shoot regeneration from shoot tip, single node, internode and leaf blade explants of *Crassula helmsii*. Figure 1A. Explant types (left to right): shoot tip, single node, internode and leaf blade. Scale bar = 3.0 mm. Figure 1B. Rooted (arrow) shoot mass produced from a single shoot tip (left) cultured in basal medium for 28 days. Scale bar = 10 mm. Figure 1C. Effect of cytokinin type (5 μM) on shoot regeneration from a nodal explant (upper right) cultured in liquid medium for 28 days. Control = basal medium without cytokinin; BA = N^6-benzylaminopurine; 2iP = 2-isopentenyladenine; zeatin = 6-[4-hydroxy-3-methylbut-2-enyamino]purine. Scale bar = 10 mm. Figure 1D. Multiple adventitious shoot formation from internode explant cultured on agar-solidified medium supplemented with 10 μM 2iP and 1.0 μM NAA for 28 days. Scale bar = 1.0 mm. Figure 1E. Adventitious shoot formation from leaf blade explant cultured on agar-solidified medium supplemented with 10 μM 2iP and 1.0 μM NAA for 28 days. Scale bar = 1.0 mm. Figure 1F. Multiple adventitious shoot development (arrows) from callus (ca) produced on internode explant after 28 days culture on agar-solidified medium supplemented with 10 μM 2iP and 1.0 μM NAA. Mature adventitious shoot bud consists of apical meristem (am) and leaf primordia (lf). Note provascular strand (vs) development. Scale bar = 100 μm.

tured on modified BM supplemented with 10 µM 2iP and 1.0 µM NAA for 28 days and then 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, St. Louis, MO). Embedded tissues were sectioned at 10 µm and stained with 0.05% toluidine blue (w/v) in citrate phosphate buffer (pH 6.0) for 25 sec (Sakai 1973).

RESULTS AND DISCUSSION

In outdoor experimental conditions, swamp stonecrop exhibits the capacity for rapid regeneration (1.4 to 2 shoots per node) from single node fragments (Dawson and Warman 1987). This regrowth potential is further accentuated *in vitro*. Both shoot tip and single node explants exhibit similar but extraordinarily high capacities to form densely branched and rooted shoot masses when cultured in liquid BM for 28 days in the absence of plant growth regulators (Figure 1B; 1C control). A shoot mass comprised of 62 shoots and a total of 127 rooted nodes and 254 lateral buds is regenerated from a single shoot tip in 28 days (Figure 1B and 2). The *in vitro* shoot regeneration rate of swamp stonecrop in basal medium is approximately five times greater than that observed for *M. aquaticum* (Kane *et al.* 1991).

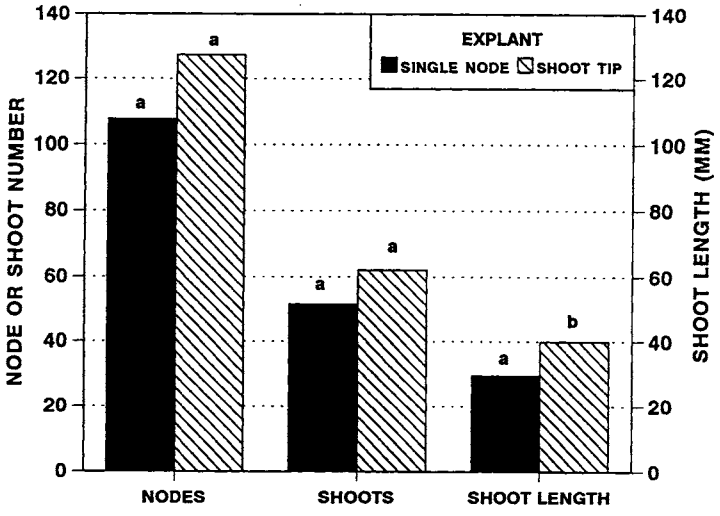

Figure 2. Comparative shoot regeneration from single node and shoot tip explants cultured in liquid basal medium for 28 days. Each histobar represents the mean response of 9 explants. For each response, histobars with the same letter are not significantly different; 5% level.

Over the concentration range used (0 to 10 µM) only the synthetic cytokinin BA promoted shoot regeneration and node number over that observed from single node explants cultured in BM only (5-µM treatment responses shown; Figure 3). Medium supplementation with cytokinins inhibited

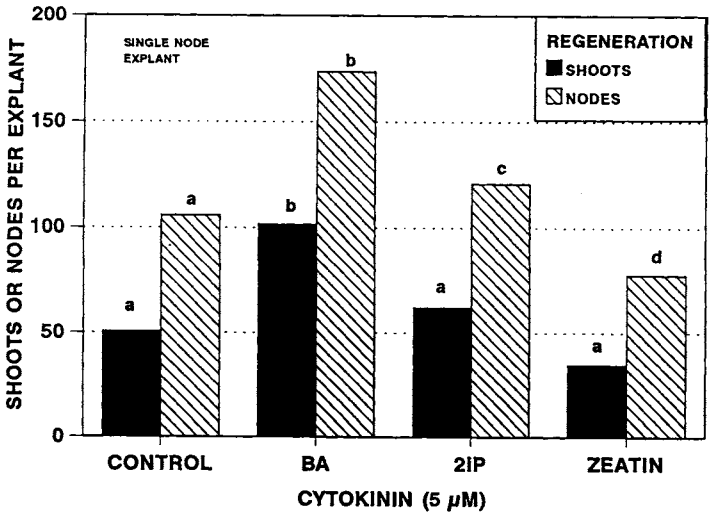

Figure 3. Effect of cytokinin type on axillary shoot and node regeneration from single node explants cultured in liquid basal medium for 28 days. Each histobar represents the mean response of 9 explants. For each specific response, histobars with the same letter are not significantly different; 5% level.

shoot elongation resulting in the production of compact shoot masses (Figure 1C). We have observed similar inhibition of shoot elongation in other aquatic species (Kane *et al.* 1991). The ineffectiveness of the naturally occurring cytokinins 2iP and zeatin to further promote shoot production suggests that axillary shoot production in swamp stonecrop is not limited by endogenous cytokinins. Conceivably, the multiple roots produced at most nodes (see Figure 1B) serve as sites of endogenous cytokinin biosynthesis (Davies 1987). This would explain the extremely highly branched growth habit of plants observed *in situ* (Dawson and Warman 1987). Conversely, the lack of multiple nodal roots in *Myriophyllum aquaticum* could account for the significant promotion of axillary shoot production we have observed following cytokinin supplementation (Kane *et al.* 1991). Cytokinin treatments only slightly enhance axillary shoot production in *Hydrilla verticillata* (Anderson 1985). However, in hydrilla, apical meristem and nodal explants exhibit dissimilar responses to both exogenous cytokinin type and level.

Numerous aquatic plants exhibit the capacity to regenerate through formation of adventitious shoots from fragmented tissues (Hagemann 1932; Sculthorpe 1967). In swamp stonecrop, both leaf blade and internode explants exhibited the capacity to produce adventive shoots. No ASD occurred on leaf blade explants cultured on MBM. In contrast, internode tissues exhibited the capacity (11% responsive explants) for ASD (mean: 1.5 shoots/explant) when cultured on agar-solidified MBM without cytokinin supplementation. Dissimilar regenerative capacities may be related to the difference in the initial size of the leaf blade and internode explants. Medium

supplementation with up to 20 μM cytokinin only slightly enhanced ASD on internode explants in the absence of NAA (data not shown). However, ASD from both leaf blade and internode explants was significantly promoted with increased cytokinin level in the presence of 1.0 μM NAA. Comparative maximum ASD responses for leaf blade and internode explants in the presence of 20 μM cytokinin and 1.0 μM NAA are depicted in Figure 4. The promotive effects of the three cytokinins on ASD were not significantly different. Rost and Paterson (1976) reported a similar requirement for medium supplementation with both cytokinin (2iP) and auxin for optimal ASD from leaf explants of the terrestrial species *Crassula argentea*.

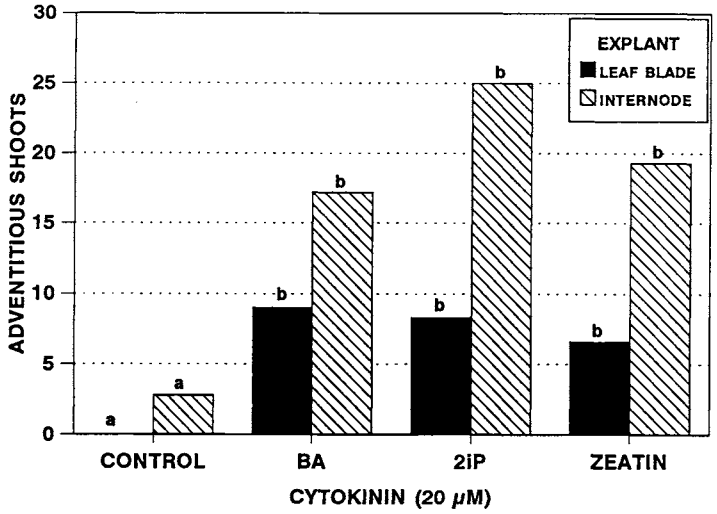

Figure 4. Effects of explant and cytokinin type on adventitious shoot development from leaf blade and internode explants cultured on agar-solidified media supplemented with 1.0 μM α-naphthaleneacetic acid (NAA). Each histobar represents the mean response of 9 explants. For a given explant type, histobars with the same letter are not significantly different; 5% level.

Multiple adventitious shoot meristems formed secondarily from callus which first developed on the basipetal cut surfaces of both explants types (Figure 1D-F). By day 28, multiple adventitious shoots covered both cut ends of the internode explants (Figure 1D). Members of the Crassulaceae are noted for their capacity for ASD from stem and leaf segments. The developmental pattern of ASD observed in *Crassula helmsii* is similar to that described for other *Crassula* species (Rost and Paterson 1976). Given that adventitious shoot regeneration from internode tissues arises *in vitro* on basal medium, the possibility arises that regrowth from fragmented stem segments without pre-existing buds probably occurs under field conditions as observed in other aquatic plants (Hagemann 1932, Sculthorpe 1967).

The regenerative capacity exhibited by *Crassula helmsii in situ* is clearly reflected in its growth *in vitro*. Our results suggest that a close correlation exists between regenerative capacity *in vitro* and the capacity for prolific shoot regeneration and growth *in situ* such as observed following the naturalization of *Crassula* in Britain (Dawson and Warman 1987). Although typically not considered weedy in its native range in Australia, *Crassula* has recently become obstructive in flowing water channels (L. Anderson pers. comm.). This implies that abiotic factors act in concert to modulate expression of the inherently high growth potential in this species.

Our results indicate that *in vitro* culture responses, particularly shoot regeneration and growth, may prove useful for screening aquatic plant growth potential in other species. However, care must be taken in making broad generalizations. The occurrence of minimal shoot regeneration from cultured hydrilla 2-node explants (Anderson 1985) suggests that shoot regeneration alone may not be a reliable indicator of weed potential in all species. Consequently, in addition to *in vitro* shoot regeneration, concurrent consideration of other biotic and abiotic parameters may be necessary for reliable assessment of overall weed potential.

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