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Research note

A three media transfer system for direct somatic embryogenesis from leaves of *Senecio x hybridus* Hyl. (Asteraceae)

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Abstract

Bisected leaves were cultured on semi-solid Murashige & Skoog medium amended with 13.5 μM 2,4-D and 4.5 μM BA for 7–14 days, transferred to 0.5% activated charcoal medium (without growth regulators) for three days, then transferred to MS basal medium. Control explants remained on initiation medium for comparison to transferred explants. Twenty-eight days after initiation of cultures, explants exposed to 11–14 days on induction medium yielded the largest number and most developmentally advanced embryos. Mature, white somatic embryos from transferred explants were visible after 35 days. Conversely, somatic embryos on control explants were not morphologically mature until 2–4 months. Mature embryos from transferred explants germinated without a resting stage, whereas embryos from control explants appeared quiescent. Histological examination confirmed embryo anatomy, however embryos, regardless of treatment, had abnormal cotyledons and regenerated plants had multiple stems.

Abbreviations: AC – activated charcoal, BA – 6-benzylaminopurine, CrAF III – chromium trioxide, acetic acid and formaldehyde, 2,4-D – 2,4-dichlorophenoxyacetic acid, IAA – indole-3-acetic acid, IBA – indole-3-butyric acid, MS – Murashige & Skoog medium, NAA – 1-naphthalenacetic acid

Senecio x hybridus Hyl. (florists' cineraria) is a daisy-like, flowering pot plant that exhibits a wide range of brilliant flower colors, including a strikingly beautiful blue. Because cineraria requires night temperatures of 13–18°C for growth and flowering, it is an inexpensive crop well-suited to the temperatures and light conditions of winter production, particularly in North America. Current commercial propagation is primarily by seeds that are heterozygous for flower color. The crop would be more valuable if less common blue flowering plants could be produced exclusively.

Clonal propagation by cutting or micropropagation by organogenesis does not provide a solution. Conventional propagation by cuttings is not economically feasible because only a limited number of cuttings can be made from a single plant. Furthermore, clones produced by this method decline in vigor after several propagation cycles (Barkley 1966). Micropropagation by organogenesis from tubular florets cultured on modified MS medium (Murashige & Skoog 1962) was not successful (Iizuka et al. 1973). In this report only 25% of the explants produced shoots and none of these shoots produced roots. Micro-

propagation from *in vitro*-grown seedling shoot tips and a two media transfer system was more recently reported (Cockrel et al. 1986). Shoot production was greatest on half-strength MS gerbera medium (Murashige et al. 1974) supplemented with IAA and kinetin, whereas rooting occurred most successfully on half-strength MS medium amended with NAA. Adventitious shoots have been produced from petiole segments using a three media transfer system (Gertsson 1988). Shoot initiation was most successful on modified MS medium containing IAA and BA. Shoot multiplication required a reduction of IAA and shoots were rooted on medium amended with IAA as the only growth regulator.

Micropropagation by adventitious shoot production or shoot tip culture is inefficient. Somatic embryogenesis offers the possibility of higher yields and efficient large-scale production, however there are usually complex requirements for embryo induction, development and maturation (Ammirato 1983). Plantlet production by somatic embryogenesis from cotyledons and first leaves using MS medium amended with 2,4-D and BA has been reported (Malueg et al. 1989). Embryogenesis was sparse and mature embryos were infrequently formed before 2–4 months after the initiation of cultures. The development of somatic embryos was arrested in the globular stage when the cultures remained on medium that contained growth regulators. In this study, we report a method to reduce the culture time and increase the rate of somatic embryogenesis from leaves of cineraria using a three media transfer system.

'Hansa' cineraria seeds (Park Seed Co., Greenwood, SC.) were sown in a 28 cm × 53 cm plastic flat containing vermiculite, covered with translucent plastic wrap and incubated at 21°C. Seedlings were grown on a laboratory bench with a 16 h photoperiod with 50 $\mu mol\, m^{-2}s^{-1}$ cool white fluorescent light.

The first true leaves were removed from seedlings 14 days post seedling emergence and surface sterilized in batches. Surface sterilization was accomplished by first immersing intact leaves in 70% ethanol for 30 sec and then rinsing with sterile water three times. Leaves were then treated with 10% (v/v) Clorox® (0.525% NaOCl) supplemented with 0.1% Triton X-100® for 10 min and rinsed three rinses with sterile water. Solutions were gently agitated using a magnetic stirrer. Leaves were bisected longitudinally along the midvein and transferred as a unit with abaxial surfaces in contact with the initiation medium contained in 60 mm petri dishes. Explants were cultured on initiation medium consisting of MS basal salts (Sigma, St. Louis MO, #6899) amended with 87.6 mM sucrose, 13.5 μM 2,4-D, 4.5 μM BA, and 0.8% (w/v) Phytagar® (Gibco Laboratories, Grand Island, NY) adjusted to pH 5.8 before sterilization in an autoclave at 0.1 MPa for 15 minutes. After 7–14 days, 8 leaf explants were transferred daily to activated charcoal (AC) medium for three days. The AC medium consisted of MS medium described above excluding 2,4-D, BA and including 0.5% (w/v) AC (Charcoal-Activated, U.S.P. #890–1134, Grand Island Biological Co., NY). A final transfer to MS basal medium with 87.6 mM sucrose completed the transfer system. Control explants remained on initiation medium for the entire culture period. All cultures were incubated in the dark at 22°C.

Evaluation for somatic embryogenesis began 28 days after initial culture. Somatic embryos were removed from the explant tissue after 38 days for embryo conversion to plantlet or histological examination. Somatic embryos for germination were placed on MS medium without growth regulators and incubated in the light (16-h photoperiod, $25\, \mu mol\, m^{-2}s^{-1}$) at 22°C. Plantlets, approximately 5 mm tall, were placed into GA7 boxes (Magenta Corp., Chicago, IL) on fresh basal MS medium amended with sucrose. When approximately 2 cm high, plants were acclimatized to laboratory conditions by placing them in a moist Jiffy-7 Peat Pellet® (Jiffy Products of America, Inc., Chicago, IL) inside clear plastic bags. Bags were opened for increasingly longer intervals over a two week period. When acclimatized, plantlets were transplanted to Fafard potting medium. (Mix No. 2, Conrad Fafard, Inc. Agawam, MA).

Globular, cordate, torpedo and mature somatic embryos were fixed in CrAF III (Sass 1958), dehydrated in a graded ethanol/TBA series and embedded in Paraplast+® (Sherwood Medical, St. Louis, MO). Ten μm sections were cut on a rotary microtome and stained in saf-

ranin O, crystal violet and fast green FCF (Johansen 1940). Imbibed zygotic embryos from mature seeds were processed in the same manner for comparison to somatic embryos.

All explants began to curl 4 days after culture initiation and a crystalline-like gloss could be seen after 6 days. At 13 days, pale yellow globular embryos, formed directly from the explants, were visible. At 28 days, control explants (no transfer from initiation medium) and transferred explants exposed to 7–10 days of induction medium developed mostly globular embryos, whereas transferrred explants exposed to 11–14 days of induction medium developed many more globular and advanced stage somatic embryos (Figs 1–2, Table 1). Somatic embryos did not develop from explants transferred after 10 days on induction medium, probably because the leaves were smaller than other explants used in this study.

The somatic embryo ontogeny noted above is very similar to that described by Dubois et al. (1991) in Cichorium, which is also member of Asteraceae. In both species, somatic embryos followed a similar developmental timetable and formed directly from leaf tissue exposed to auxin and cytokinin amended induction medium.

Mature, white somatic embryos (Fig. 3) were formed in 35 days from transferred explants and germinated without a resting stage. Roots often developed before shoots and conversion to plantlets was rapid. Experiments were assessed at 28 days post culture initiation, because our goal was to reduce the somatic embryo production time. However, control explants were held in a dark incubator without transfer to fresh medium for 3 months for observation. The few somatic embryos that developed on control explants were not morphologically mature until three months after culture initiation. These embryos seemed quiescent (in a resting state without root or shoot development). Most somatic embryos from both transferred and control explants exhibited abnormal cotyledonary development and multiple shoots.

Histological examination of mature somatic embryos confirmed embryo anatomy (Fig. 4). A vascular connection to the parental tissue was not evident, both shoot and root meristems were well-organized and a closed vascular system was apparent. However, somatic embryos were larger and contained more advanced vascular tissue than zygotic embryos that were imbibed for 24 h before fixation.

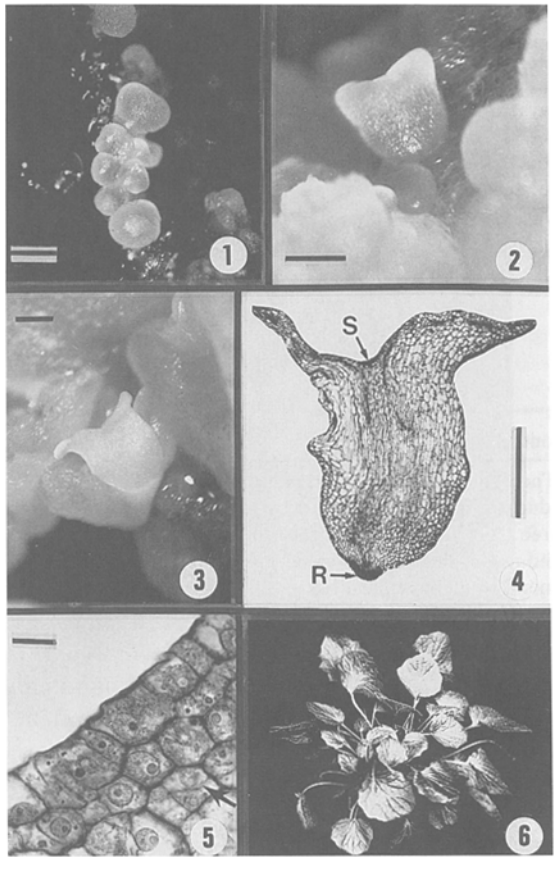

Fig. 1. Early stage yellow globular somatic embryos, 16 days post culture initiation, 12 days exposure to induction medium. Bar = 0.5 mm.

Fig. 2. Heart-shaped somatic embryo, 18 days post culture initiation, 11 days exposure to induction medium. Bar = 0.5 mm.

Fig. 3. Mature white somatic embryo showing malformed cotyledons, 34 days post culture initiation, 11 days exposure to induction medium. Bar = 0.5 mm.

Fig. 4. Longitudinal section of mature somatic embryo showing bipolar organization, cotyledon and provascular development, well organized root apex (R), and shoot apex (S), 37 days post culture initiation, 11 days exposure to induction medium. Bar = 0.5 mm.

Fig. 5. Enlargement of shoot meristem shown in Fig. 4. Small meristematic cells containing large nuclei with prominent nucleoi, small vacuoles, recent cell divisions (arrow), and densely staining cytoplasm. Bar = 25 μm.

Fig. 6. Plant with multiple stems, uncharacteristic of cineraria, derived from a somatic embryo.

Table 1. Effect of three media transfer system[1] on developmental stages and numbers of somatic embryos from leaves of *Senecio* × *hybridus* after 28 days.

Days on induction medium[2]	Developmental stages of embryos[3]			Mean number of somatic embryos per treatment[3]
	Globular	Cordate	Torpedo	
7	1.3	0	0	1.3
8	1.9	0	0	1.9
9	1.9	0.8	0	2.7
10	0	0	0	0
11	7.5	0.8	0	8.3
12	9.4	0.4	0.7	10.5
13	8.8	0.6	0.7	10.1
14	11.3	0.3	0.5	12.1
Control[4]	1.3	0	0	1.3

[1] Three media transfer system consisted of varying days on induction medium, followed by transfer to AC medium for three days and a final transfer to MS basal medium.
[2] induction medium contained growth regulators.
[3] mean of eight replications.
[4] not transferred from induction medium.

Shoot (Fig. 5) and root meristems of somatic embryos from transferred explants contained densely staining cells with small vacuoles, large nuclei and evidence of recent cell divisions, which are characteristic of intensely active meristematic regions. Conversely, embryos from control explants, after remaining on the initial induction medium for three months without transfer, had meristems that appeared to be quiescent (without recent cell divisions). Differences in anatomy/physiology may be caused by the gradual dehydration (decreased water potential) or increased osmotic potential of the control explant culture environment verses the hydrated conditions of the transferred explant environment. Gradual dehydration might have induced a quiescent state (inactive meristems) in these somatic embryos, thus preventing rapid germination and plantlet conversion. Perhaps the advantage of this observation might be the possibility of inducing a quiescent resting phase via gradual dehydration of the somatic embryo that can be reversed by hydration (Gray & Purohit 1991), while recognizing that other phenomena inside the culture vessel, such as possible endogenous hormone production, may be adding to or causing this effect. Additional studies are needed to investigate this observation.

The majority of regenerated plants, regardless of treatment, did not have a single basal stem, a normal characteristic of cineraria (Fig. 6). The regenerated plants flowered normally following typical protocols for floral induction (Larsen 1985). Plants were bushy and flowers smaller and more numerous because of the culture-induced multiple stem habit. Flower color and patterns were conserved. These variations might be valuable for the improvement of cineraria, as has been suggested for *Eustoma grandiflorum* (Griesbach et al. 1988), if commercial acceptance of the altered plant is strong.

Somatic embryogenesis is often influenced by exogenous plant growth regulators and nutrient medium selection (Ammirato 1987). Early research indicated that auxin or auxin-like compounds in the induction medium were necessary for somatic embryogenesis initiation, whereas the absence of auxins promoted maturation (Steward et al. 1967). The auxin used in these experiments was 2,4-D, which has been suspected as being responsible for inhibition of somatic embryo development and lack of high frequency conversion in other species (Kohlenbach 1981; Buchheim et al. 1989). Cytokinins have been found useful in fostering somatic embryo maturation (Fujimura & Komamine 1975), and cotyledon development (Ammirato & Steward 1971). The use of 2,4-D and BA for initiation of somatic embryogenesis has been reported for other species of Asteraceae (Heirwegh et al. 1985; May & Trigiano 1991). However, in this study, development beyond the globular stage was inhibited by remaining on medium containing these plant growth regulators.

Sequential multi-media transfer systems are often necessary to promote induction, maturation and conversion of somatic embryos and activated charcoal is often used in primary and/or maturation media for the absorption of inhibitory compounds (Ammirato 1983). AC is thought to absorb many substances, including phenol-like compounds, 5-hydroxymethylfurfural (a degradation product of autoclaving sucrose), benzoic acids and considerable amounts of auxins and cytokinins. Ebert and Taylor (1990) reported that the inclusion of 2.5 g l^{-1} AC in 0.6% semi-solid agar medium with 2×10^{-5} M 2,4-D absorbed 96% of the auxin in 1 day and

98% of the auxin in 10 days. Inclusion of AC in the maturation medium has proven advantageous for somatic embryo induction and maturation in several species (Buchheim et al. 1989; Litz & Conover 1980; Tisserat & De Mason 1980), but deleterious to others (Smith & Krikorian 1990). The use of 0.5% AC for three days appeared to lessen the inhibitory effects of the plant growth regulators used in the induction medium on somatic embryo maturation.

This study demonstrated that the three media transfer system successfully increased somatic embryo yields while reducing the developmental time from 2–4 months to 5 weeks. This system offers promise for the future production of elite blue flowering cineraria, however refinements of plant growth regulators, AC concentrations, transfer timing and quiescence induction will be necessary to improve the efficiency of somatic embryo production in this species. Additionally, studies focusing on cineraria zygotic embryo ontogeny and fluctuating endogenous hormone levels would be especially advantageous.

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