Somatic Embryogenesis and Plant Regeneration from Leaves of *Dendranthema grandiflora*

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Abstract. Somatic embryogenesis from leaf midrib explants of *Dendranthema grandiflora* Tzvelev. 'Iridon' cultured on modified Murashige and Skoog basal medium (MSB) containing 1.0 mg 2,4-D and 0.2 mg BA/liter was influenced by light and sucrose concentration. Somatic embryos formed directly from explants when cultured on medium containing 9% to 18% sucrose and incubated first in the dark for 28 days, followed by 10 days in light, and then returned to the dark for 14 days. Embryogenesis did not occur in continuous darkness and was drastically reduced when explants were incubated in light only. The most embryos were formed on medium containing either 12% or 15% sucrose; lower concentrations stimulated shoot and root development. Light also mediated embryogenesis from leaf explants of 'other cultivars. White-opaque or occasionally light-green cotyledon-stage somatic embryos germinated on MSB medium without growth regulators but containing 3% sucrose. Twelve of the 23 cultivars evaluated produced somatic embryos, but plants were recovered from only five. Regenerated plants were phenotypically similar to parent plants in growth habit, leaf morphology, and flower color. Chemical names used: *N*-(phenylmethyl)-1 *H*-purine-6-amine (BA); (2,4-dichlorophenoxy) acetic acid (2,4-D).

Chrysanthemum [*Dendranthema grandiflora* Tzvelev. (Anderson, 1987)], an important floricultural crop in the Asteraceae, is conventionally propagated vegetatively by stem cuttings. Chrysanthemums also have been successfully micropropagated by adventitious shoot formation from various tissues and callus cultures (Earle and Langhans, 1974; Hill, 1968; Iizuka et al., 1973; Khalid et al., 1989; Roest and Bokelmann, 1975). Tissue culture methods have provided a means to eliminate various pathogens (Ahmed and Andrea, 1987; Horst, 1990) and to induce and select desirable mutations (Broertjes et al., 1976; Dalsou and Short, 1987; DeJong and Custers, 1986; Huitema et al., 1987; Preil et al., 1983).

Somatic embryogenesis is another in vitro process used for regeneration and potentially offers a very efficient system for mass clonal production of horticultural crops (Ammirato and Styer, 1985; Lutz et al., 1984; Sharp et al., 1982). Although numerous species are reportedly capable of forming somatic embryos (Tisserat et al., 1979; Warm, 1988; Williams and Maheswaran, 1986), relatively few instances of somatic embryogenesis have been documented for members of the Asteraceae and, among these, none are strictly ornamental or floricultural species. These reports are limited to chicory and endive, *Cichorium* species (Guedira et al., 1989; Heirwegh et al., 1985; Vasil and Hildebrandt, 1966; Vasil et al., 1964), sunflower, *Helianthus annuus* L. (Finer, 1987; Patterson and Everett, 1985), *Brachycome lineariloba* (DC) Druce (Gould, 1978), and hawkweed, *Pterotheca falconeri* Hook. [syn. *Crepis sanceta* (L.) Babcock (Mehra and Mehra, 1971)]. Earle and Langhans (1974) noted that "embryoids" were formed in callus cultures of *Chrysanthemum morifolium* Ramat. (*D. grandiflora*), but did not present substantiating evidence.

Production of somatic embryos from leaf tissue and subse-

quent regeneration of plants is an attractive possibility for propagating cultivars of chrysanthemum. Additionally, recovery of chemical or radiation-induced mutations or genetically transformed plants should be possible using this technique since somatic embryos are generally believed to arise from single cells (Haccius, 1978). Therefore, the objectives of this investigation were to devise protocols to induce somatic embryogenesis from leaves of *D. grandiflora*, to regenerate plants, and to survey pot and garden cultivars for the ability to produce somatic embryos.

Materials and Methods

General. Cuttings of 23 cultivars of *D. grandiflora* were obtained from Yoder brothers, Barberton, Ohio (Table 3). 'Iridon' and 'Goldmine' were maintained vegetatively in a growth chamber with 16-hr days at 27C and 8-hr nights at 18C. During the day, plants received 150 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ provided by cool-white fluorescent and incandescent lamps. This and all other measurements of irradiance were recorded using a LI-COR-L170 light meter (LI-COR, Lincoln, Neb.). All other cultivars were maintained at $24 \pm 3\text{C}$ on light stands with similar irradiance and photoperiod. Stock plants were fertigated three times weekly with 200 mg N/liter using Peters 20N-10P-20K water-soluble fertilizer (W.R. Grace, Fogelsville, Pa.). Shoot tips were removed every 21 to 28 days to encourage branching and growth of new leaves.

Young leaves, 3 to 5 cm long, were surface sterilized for 5 min in 0.26% NaOCl and 0.1% (v/v) Triton X-100 with constant agitation, then rinsed three times in sterile distilled water. Four or five 3×8 mm midrib explants, excluding the distal and basal portions of the lamina, were excised and placed with the abaxial surface in contact with culture medium. The basal medium (MSB) consisted of Murashige and Skoog (1962) macro and micro salts, and (mg-liter⁻¹, 100 *myo*-inositol, 1.0 thiamine-HCl, 1.0 2,4-D, 0.2 BA and 7000 agar. Depending on the experiment, 3% to 18% sucrose was used to supplement MSB medium. The pH of all media was adjusted to 5.7 before sterilization for 20 min at 121C. Unless otherwise noted, 'Iridon' was used in all experiments and cultures were incubated at either $24 \pm 1\text{C}$ with a 16-hr photoperiod of 25 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ provided by cool-white fluorescent lamps or at $24 \pm 1\text{C}$ in darkness.

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Effects of light. Five milliliters of MSB medium supplemented with 12% sucrose (MSB-12) was dispensed into each well of twelve 12-well dishes. A single leaf explant was placed in each well. Treatments consisted of either dark, light, or 10 combinations of dark and light incubation periods (Table 1). One dish containing 12 samples was prepared for each treatment and the dishes arranged in a completely randomized design. The number of leaf explants that responded and the number of somatic embryos (globular through cotyledon-stage) per treatment were determined after 45 or 52 days, depending on the treatment. The experiment was repeated twice. Data were transformed using the square root of the counts plus one to stabilize variation and subjected to analysis of variance (ANOVA). Significant differences between means were determined using Waller-Duncan K-ratio *t* test ($K = 100$ and $P = 0.05$).

Effects of initial dark incubation period. Five milliliters of MSB-12 medium was poured into each well of four 12-well dishes and one leaf explant placed in each well. A treatment consisted of one dish (12 samples) and was initially incubated for either 7, 14, 21, or 28 days in darkness, then in light for 10 days and finally placed in darkness for the remainder of the culture period. Treatments were arranged in a completely randomized design. Both the number of explants forming embryos and the number of somatic embryos were recorded after 52 days. The experiment was repeated once. Data were transformed using the square root of counts plus one to stabilize variation and subjected to ANOVA. Significant differences between means were determined using Waller-Duncan K-ratio *t* test ($K = 100$ and $P = 0.05$).

Effects of sucrose concentration. Leaf explants from 'Iridon' and 'Goldmine' were used in a split-plot design with treatments nested within cultivars in this experiment. Treatments consisted of MSB medium supplemented with either 3%, 6%, 9%, 12%, 15%, or 18% sucrose. Media were dispensed into eight 12-well dishes (four dishes per cultivar), in randomized complete blocks within plots and replicated eight times. One leaf explant was

placed in each well. Cultures were initially incubated in darkness for 28 days, then in light for 10 days, and finally in darkness for 14 days. The number of somatic embryos formed after 52 days was recorded for each cultivar-treatment combination. Data were transformed using the square root of the counts plus one and subjected to ANOVA.

Evaluation of cultivars for somatic embryogenesis. Twenty-three cultivars were surveyed for the ability to produce somatic embryos from leaf midrib explants. MSB-12 medium, modified to contain 2,4-D at 0.5, 1.0, or 2.0 mg·liter⁻¹, was dispensed into three 12-well dishes in a randomized complete-block design. For each cultivar, 12 explants per treatment were incubated for 52 days either in light, in darkness, or in the 28/10/14-day dark/light/dark protocol described previously. The number of somatic embryos produced in each of the three incubation conditions, regardless of the 2,4-D concentration, was recorded after 52 days. A cultivar was considered to be embryogenic regardless of the morphological stages of the embryos observed.

Plant regeneration. Cotyledon-stage somatic embryos were removed from leaf explants and placed on MSB medium supplemented with 3% sucrose and without growth regulators. Embryos were cultured in the light until radicles emerged and shoots had a minimum of two leaves. Plantlets were transferred to sterilized potting soil in 0.45-liter pots, covered with GA-7 boxes (Magenta Corp., Chicago) to facilitate acclimation, and incubated at 24C with a 16-hr photoperiod of 70 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ of light provided by cool-white fluorescent lamps. Plants were pinched after 4 and 8 weeks and then transplanted to 2.6-liter pots. Flowering was initiated using a 10-hr photoperiod (150 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) and a 27/18C day/night cycle in a growth chamber.

Histology. Somatic embryos were immobilized in 1% (w/v) low melting point agarose before fixation using a modification of a method described by Hock (1974). Agarose blocks containing specimens and leaf explants were fixed in Craf III (Johansen, 1940), dehydrated in a graded ethanol-tertiary butanol

Table 1. Effect of light on somatic embryogenesis from leaf midrib explants of *Den-dranthema grandiflora* 'Iridon'.

Light/dark transfers and duration in days						Explants forming somatic embryos ^{x,v} (no.)	Somatic embryos per treatment ^{w,v}
First		Second		Third			
L ^z	D ^y	L	D	L	D		
	52					0.0 d	0.0 e
52						0.7 bcd	0.7 cde
14			10	21		0.0 d	0.0 e
21			10	14		0.3 cd	0.3 de
28			5	19		1.3 b	2.3 c
28			10	14		0.7 bcd	0.7 cde
28			15	9		0.0 d	0.0 e
	14	10			21	3.7 a	16.3 b
	21	10			14	3.0 a	15.0 b
	28	5			19	1.0 bc	2.0 cd
	28	10			14	3.3 a	29.0 a
	28	15			9	3.7 a	13.7 b

^zL = incubation under a 16-hr photoperiod.

^yD = incubation in continuous darkness.

^{*}Mean number (three replications) of explants that produced somatic embryos from a sample of 12 leaf explants.

^uMean number (three replications) of somatic embryos produced on a sample consisting of 12 leaf explants.

^vMeans within columns followed by the same letter are not significantly different according to Waller-Duncan K-ratio *t* test ($K = 100$, $P = 0.05$).

series, and embedded in Paraplast Plus (Sherwood Medical Industries, St. Louis, Mo.), Eight- to 10- μ m-thick sections were cut and stained with crystal violet, safranin, and fast green (Johansen, 1940).

Results

Effects of light. Leaf explants of 'Iridon' incubated in darkness only formed white, compact callus along the cut edges but did not initiate somatic embryos. Light-green, crystalline callus formed along the cut edges of explants cultured in the light for the 52-day period and, except for one explant, failed to differentiate somatic embryos (Table 1). Very few somatic embryos developed on explants initially cultured in the light. Some leaf sections exposed to any treatment that included an initial dark period directly produced somatic embryos. Significantly more embryos formed on explants cultured for 28 days in darkness, followed by 10 days in light, and then returned to darkness for 14 days than in any other treatment (Table 1). Evidence of somatic embryo initiation and development were not visible until the final dark incubation period.

Effects of the initial dark incubation period. The number of leaf explants of 'Iridon' that exhibited somatic embryogenesis and the number of somatic embryos produced was significantly influenced by the initial dark incubation period (Table 2). Very limited somatic embryogenesis was stimulated by dark incubation for 7 days. Explants that were incubated longer in darkness initiated substantially more direct somatic embryos, although the effect of 14, 21 and 28 days was similar.

Effects of sucrose concentration. Leaf sections of 'Iridon' cultured on medium containing either 3% or 6% sucrose produced only shoots and roots (organogenesis), whereas organogenesis and somatic embryogenesis occurred on medium containing 9% sucrose. Explants of 'Goldmine' did not exhibit organogenesis on 3% or 6% sucrose, but produced some somatic embryos with 9% sucrose. Somatic embryogenesis occurred on media containing 12% to 18% sucrose and statistically similar numbers of somatic embryos were initiated from explants of both cultivars on similar media. Fifteen percent sucrose in the medium supported the formation of the greatest number of somatic embryos for both cultivars (Fig. 1). Shoot and root development from explants of either cultivar did not occur on media containing >9% sucrose.

Evaluation of cultivars for somatic embryogenesis. Twelve of the 23 cultivars evaluated produced somatic embryos (Table

Fig. 1. Effect of sucrose concentration on somatic embryo formation from leaf midrib explants of *Dendranthema grandiflora* 'Iridon' and 'Goldmine'. There was no significant ($P = 0.05$) interaction between cultivar and sucrose concentration; therefore, data were combined and a single line represents the response of both cultivars. Vertical bars represent ± 1 SE of eight replications.

Table 3. Effect of light on induction of somatic embryos from midrib leaf explants of selected cultivars of *Dendranthema grandiflora*.

Production of somatic embryos by cultivars		Somatic embryos produced per incubation condition ² (no.)			Plants regenerated (no.)
		Dark ¹	Light ³	D-L-D ⁴	
Adorn	Capri	3	0	3	0
Ballarina	Champ	0	12	7	0
Boaldi	Debonair	0	2	1	0
Bronze Charm	Fortune	2	8	5	1
Circus	Goldmine	1	24	15	9
Coral Charm	Hekla	5	0	5	1
Encore	Iridon	0	5	17	3
Grenadine	Neoga	6	4	3	0
Legend	Patriot	1	0	0	0
Remarkable	Pink Arola	5	0	0	0
Salmon Charm	Rave	0	0	8	0
	Zest	0	0	12	0

¹Number of somatic embryos (globular- through cotyledon-stage) produced on 12 leaf explants.

²Fifty-two days of incubation in continuous darkness.

³Fifty-two days of incubation in light (16-hr photoperiod).

⁴Twenty-eight days of initial incubation in darkness, followed by 10 days in light and 14 days in darkness.

Table 2. Effect of initial dark incubation period on induction of somatic embryos from leaf midrib explants of *Dendranthema grandiflora* 'Iridon'.

Condition (days)			Explants forming somatic embryos ^{y,w}	Somatic embryos per treatment ^{x,w}
Dark	Light ^z	Dark		
7	10	35	0.5 a	0.5 a
14	10	28	6.0 b	19.0 b
21	10	21	7.0 b	17.5 b
28	10	14	5.5 b	27.0 b

¹Incubated under a 16-hr photoperiod.

²Mean number (two replications) of explants that produced somatic embryos from a sample consisting of 12 leaf explants.

³Mean number (two replications) of somatic embryos produced on a sample consisting of 12 leaf explants.

⁴Means within columns followed by the same letter are not significantly different according to Waller-Duncan K-ratio t test ($K = 100$, $P = 0.05$).

3). Explants capable of somatic embryogenesis formed embryos regardless of the concentration of 2,4-D in the medium; however, embryogenesis was mediated by incubation conditions. Two cultivars produced embryos only when incubated in darkness, 10 of the cultivars formed embryos when the explants were cultured with some light and three cultivars formed somatic embryos under all three irradiation conditions. The frequency of morphologically mature somatic embryos was greater from leaf explants that had been exposed to some light than those that had not received light during incubation. In some cases, these embryos were competent to regenerate plantlets. Development of embryos from explants incubated in darkness usually did not progress past the torpedo stage and, although capable of radicle formation, were incapable of whole-plant regeneration. The ability to form morphologically mature embryos was also cultivar-dependent under these experimental condi-

Fig. 2. Photomicrographs of somatic embryos of *Dendranthema grandiflora* 'Iridon'. (A) Early globular-stage somatic embryo with well-differentiated protoderm (P) that originated (O) from a cell layer subjacent to the adaxial leaf epidermis. Bar = 200 μ . (B) Cotyledon-stage somatic embryo (E) attached directly to the leaf explant (L) by a broad suspensor-like column of cells (S). Bar = 1 mm. (C) Group of somatic embryos that developed asynchronously on the surface of a leaf explant. Arrows indicate globular embryos. Bar = 3.5 mm. (D) A single morphologically mature somatic embryo. Bar = 2.0 mm. (E) Longitudinal section of the somatic embryo in Fig. 2D showing a dome-shaped shoot meristem (arrow) and procambial tissue (V). Bar = 1 mm. (F) A serial longitudinal section of the somatic embryo in Fig. 2D showing a root meristem (arrow). Bar = 1 mm.

tions. 'Fortune', 'Goldmine', 'Hekla', 'Iridon', and 'Zest' produced cotyledon-stage embryos, whereas, 'Capri', 'Champ', 'Debonair', 'Neoga Patriot', 'Pink Arola', and 'Rave' only formed globular through torpedo-stage embryos.

Plant regeneration. Radicles usually elongated within 5 days after embryos with well-developed cotyledons were placed on MSB medium supplemented with 3% sucrose and lacking growth regulators. Shoot development was delayed for 5 to 15 days after radicle emergence. Conversion rate of somatic embryos to plantlets ranged from 0% to 23%, depending on the cultivar. Plants from 'Fortune', 'Goldmine', 'Hekla', 'Iridon', and 'Zest'

were regenerated from somatic embryos. Regenerated plants appeared phenotypically normal for growth habit, leaf morphology, and flower color.

Embryo development. Somatic embryos presumably originated directly from single cells of either the epidermis or subjacent cell layers (Fig. 2A); indirect embryogenesis was not observed. Morphologically mature embryos were attached from the mid-hypocotyl region to the leaf explant by a broad column of cells and were typically reflexed parallel to the leaf surface (Fig. 2B). Embryo ontogeny was asynchronous; globular-through cotyledon-stage embryos were often observed on an individual

explant (Fig. 2C). White-opaque or occasionally light-green somatic embryos were initiated either as closely grouped individuals (Fig. 2C) or singly (Fig. 2D) from both adaxial and abaxial leaf surfaces. Histological examination of somatic embryos formed 52 days after culture initiation demonstrated that root meristems had formed in most embryos, but shoot apices were either incompletely formed or absent. However, in a few embryos both shoot (Fig. 2E) and root (Fig. 2F) meristems were present.

Discussion

Somatic embryogenesis from leaf explants of 'Iridon' was inhibited by continuous incubation in light, whereas other cultivars were able to initiate somatic embryos under all of the incubation conditions. Although many species can form embryos in light as well as in darkness (e.g., Gingas and Lineberger, 1989; Michler and Lineberger, 1987), promotion and inhibition of embryo formation by light are also well-documented. The greatest number of somatic embryos of red oak (*Quercus rubra* L.) was generated from explants incubated in light (Gingas and Lineberger, 1989), whereas high irradiance inhibited embryogenesis in cotyledon cultures of soybean, *Glycine max* (L.) Merr. (Lazzeri et al., 1987). Spectral band width may also influence embryogenesis and embryo ontogeny (Michler and Lineberger, 1987). The mechanisms underlying the interaction of light with either explanted tissue or cell cultures to influence embryogenesis have not been determined.

Most studies of somatic embryogenesis have only employed treatments that included continuous incubation under a specific daily photoperiod or in continuous darkness. Few studies have examined the effects of alternating exposures to dark and light incubation conditions on embryo initiation. Somatic embryogenesis in olive (*Olea europaea* L.) only occurred from zygotic embryos that were first cultured in darkness for 3 weeks and thereafter in light. Incubation only in light completely inhibited embryogenesis (Rugini, 1988). However, the effects of shorter dark culture periods or incubation in continuous darkness on embryogenesis in olive were not reported. A minimum of 14 days of incubation in darkness was required before leaf explants of 'Iridon' produced a significant number of embryos; a useful initial dark incubation period was 28 days although the optimum has not been established. Evaluation of other cultivars of chrysanthemum that formed embryos under these incubation conditions was beyond the scope of this study.

The concentration of sucrose in the basal medium regulated a change in developmental pathways from organogenesis (low sucrose) to somatic embryogenesis (high sucrose). Two percent to 3% sucrose is commonly used for optimum shoot development in chrysanthemum (Earle and Langhans, 1974; Hill, 1968; Khalid et al., 1989); organogenesis from 'Iridon' occurred on medium containing up to 9% sucrose. Shoot and root formation were completely inhibited by sucrose concentrations >9%. Adventitious shoot formation from callus cultures of tobacco (*Nicotiana tabacum* L.) was completely suppressed by 12% sucrose (Hammersley-Straw and Thorpe, 1988). Relatively high sugar concentrations (>9%) were required to support somatic embryogenesis from leaves of 'Iridon' and 'Goldmine'. Other plants, including sunflower (Finer, 1987), endive (Guedira et al., 1989), and corn (*Zea mays* L.) (Close and Ludeman, 1987; Lu et al., 1982) exhibited greater frequencies of somatic embryogenesis on medium containing elevated sucrose concentrations.

Expression of somatic embryogenesis from leaf explants of chrysanthemum on high-sucrose containing medium may be related to osmotic effects rather than to absolute nutritional re-

quirements. In other investigations, somatic embryogenesis could be maintained by substituting mannitol or sorbitol for all (Ammirato and Steward, 1971) or part (Close and Ludeman, 1987) of the sucrose in the medium. Furthermore, in vitro maturation of some zygotic embryos is promoted by high concentrations of either sucrose or other osmotically active sugar alcohols (Chandler and Beard, 1983; Finkelstein and Crouch, 1986; Narayanaswami and Norstog, 1964).

The ability to form somatic embryos from leaf explants was limited to about one-half of the cultivars evaluated. Somatic embryogenesis in some species has been demonstrated to be highly dependent on genotype (e.g., Hanning and Conger, 1986; Parrott et al., 1989; Trigiano et al., 1989). Alternatively, interactions between genotype and culture medium may affect the expression of somatic embryogenesis (Chen and Marowitch, 1987; Lu et al., 1982). Although embryogenesis appeared to be independent of 2,4-D concentration, only one sucrose concentration was used to evaluate most of the cultivars. Nonresponsive cultivars may be able to express embryogenesis if an appropriate osmotically adjusted culture medium is provided.

Our study demonstrated that direct somatic embryogenesis from midrib leaf explants of chrysanthemum was influenced by light, sucrose concentration in the medium, and genotype. Phenotypically normal plants were regenerated for several cultivars. The protocols described herein are not competitive with current large-scale propagation methods, but do have the potential for commercial exploitation. If the rate of somatic embryogenesis and conversion of embryos to plantlets can be improved, these methods may be useful for recovering unique genotypes generated by mutation or genetic engineering techniques.

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