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Note

Development of embryogenic dogwood cultures and the regeneration of plants

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Abstract Somatic embryogenesis in flowering dogwood (*Cornus florida*) has been achieved, but not the regeneration of plants with active shoot growth. To improve plant regeneration, eight media treatments were tested for induction of somatic embryogenesis from immature zygotic embryos. Somatic embryogenesis was obtained on three media containing the plant growth regulators (PGRs) 0.1 mgl^{-1} picloram, 2.0 mgl^{-1} 2,4-dichlorophenoxyacetic acid, or 0.1 mgl^{-1} indole-3-butyric acid (IBA). Somatic embryogenesis was also induced on Woody Plant Medium without PGRs. Overall, 92% of the somatic embryos examined converted into plants with active root and shoot growth. This is the first report of somatic embryo-derived plants of *C. florida* that have active shoot growth and that could be transferred to soil. Embryogenic suspensions were established from IBA-treated cultures that could serve as a target for *C. florida* bioengineering.

Key words: *Cornus florida*, indole-3-butyric acid, plant growth regulator, regeneration, somatic embryogenesis.

Flowering dogwood (*Cornus florida* L.) is a small tree native to eastern North America that is used widely as an ornamental species in the United States and, increasingly, in Asia and Europe (McLemore 1990; Orton 1993). It is valued as a landscaping plant for its showy floral bracts in the spring and foliage color in the fall. *C. florida* is susceptible to powdery mildew and anthracnose pathogens, but there is variation in disease severity among cultivars (Hagan et al. 1998; Li et al. 2009). The genome of *C. florida* has been sequenced (Leebens-Mack et al. 2017), providing information that could be useful for improving horticultural traits like disease resistance. The lack of a genetic transformation system for *C. florida*, however, limits how genomic sequence information can be applied to cultivar development.

We are investigating somatic embryogenesis as a potential platform for *C. florida* transformation and gene editing. Trigiano et al. (1989) reported the induction of direct somatic embryos and embryogenic callus from immature zygotic embryos of *C. florida* exposed to the plant growth regulators (PGRs) 2,4-D and kinetin. In that study, plantlets were regenerated from somatic embryos, but they did not develop past the first true leaf stage. To improve plant regeneration, we examined the induction of somatic embryogenesis with 2,4-D and alternative PGRs picloram and IBA. We report here the development of embryogenic cultures that generated (1) actively growing *C. florida* plants and (2) suspension cultures that can be used for *C. florida* bioengineering.

Dogwood fruit were obtained from an open-pollinated tree located in Athens, Georgia (USA). Fruit were collected 14–17 weeks post-anthesis, a timeframe when immature zygotic embryos are responsive to somatic embryogenesis induction (Wilde and Gegogeine, unpublished). Following the procedures of Trigiano et al. (1989), the fruit were surface-sterilized, seeds were excised, and the immature zygotic embryos were isolated. Eight media treatments were prepared that consisted of Woody Plant Medium (WPM; Lloyd and McCown 1981) basal salts supplemented with 30 gl^{-1} sucrose, 3 g l^{-1} Phytagel (Sigma-Aldrich), and one of the following growth regulators: 2,4-D (2.0, 4.0, or 6.0 mgl^{-1}), IBA (0.1 mgl^{-1}), IBA (0.1 mgl^{-1})+6-benzylaminopurine (1.0 mgl^{-1}), picloram (0.1 or 1.0 mgl^{-1}), or no growth regulators. Based on the number of immature zygotic embryos available, 20 explants were cultured per treatment. Zygotic embryos were cultured in the dark at 25°C and the cultures were transferred to fresh media every four weeks. After five months, all cultures were transferred to petri plates containing PGR-free WPM and maintained in the dark (25°C) for somatic embryo development. The frequencies of somatic embryogenesis induction by the different PGR treatments were compared by a 2-sample test for equality of proportions with continuity correction using the R

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; PGR, plant growth regulator; WPM, Woody Plant Medium.

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Table 1. Embryogenic line induction. Explants were exposed to media treatments for 5 months and then transferred to PGR-free medium. All explants remained viable and axenic during the experiment. Organogenic explants produced shoots directly.

Treatment	PGR ($mg\,l^{-1}$)	Zygotic embryo explants (no.)	Embryogenic explants (no.)	Embryogenic explants (%)	Organogenic explants (no.)
A	none	20	6	30	0
B	IBA (0.1)	20	4	20	0
C	IBA (0.1)+BAP (1.0)	20	0	0	2
D	picloram (0.1)	20	1	5	0
E	picloram (1.0)	20	0	0	0
F	2,4-D (2.0)	20	1	5	0
G	2,4-D (4.0)	20	0	0	0
H	2,4-D (6.0)	20	0	0	0

statistical package, R-3.5.1.tar.gz (2018-07-02).

To establish suspension cultures, somatic embryos and embryogenic calli induced with $0.1\,mg\,l^{-1}$ IBA were transferred to 30 ml of liquid WPM+$0.1\,mg\,l^{-1}$ IBA in 125 ml flasks. Suspension cultures were maintained in the dark at 25°C on a rotary shaker (100 rpm) and subcultured every 4 weeks by introducing approximately 1 g of culture into fresh liquid medium. The embryogenic potential was determined by counting all somatic embryos produced 90 days after transfer of suspension culture to petri plates containing PGR-free WPM.

To examine plant regeneration, 26 mature, well-formed somatic embryos (e.g., Figure 1F) were selected from embryogenic cultures induced with $0.1\,mg\,l^{-1}$ IBA, $0.1\,mg\,l^{-1}$ picloram, or no PGRs and transferred to Magenta GA-7 boxes (Sigma-Aldrich) containing PGR-free WPM medium supplemented with activated charcoal ($0.25\,mg\,l^{-1}$). Plant development took place at 25°C under a 16 h photoperiod ($30\,\mu mol\,m^{-2}\,s^{-1}$). After three months, the number of somatic embryos that developed into plantlets with active root and shoot growth was determined. Plantlets were transferred to a 1:1 mix of vermiculite and Fafard 2B (Sun Gro) in 3-inch pots, covered with a Magenta box to maintain high humidity, and gradually acclimated to ambient conditions.

As shown in Table 1, somatic embryogenesis was induced from *C. florida* zygotic embryos cultured on media containing IBA ($0.1\,mg\,l^{-1}$), picloram ($0.1\,mg\,l^{-1}$), 2,4-D ($2.0\,mg\,l^{-1}$), or no PGRs. No statistical difference in the induction frequency of these treatments was observed (Table 2), due to the number of explants available for the media survey. Embryogenic cultures from 12 full-sib zygotic embryos were obtained for further study. Exposure of explants to higher levels of 2,4-D or picloram resulted in nonembryogenic callus. Treatment with $1.0\,mg\,l^{-1}$ BAP in addition to $0.1\,mg\,l^{-1}$ IBA induced shoot organogenesis directly from the explants.

Most somatic embryos from the PGR-free treatment originated directly from the surface of zygotic embryos (Figure 1A), although an indirect origin from embryogenic callus could not always be excluded (Figure 1B). In the presence of appropriate IBA, picloram, or 2,4-D levels, embryogenic callus was induced that produced somatic embryos when transferred to PGR-free medium (Figure 1C–E). In addition to somatic embryos with typical embryo morphology (e.g., Figure 1F), somatic embryos with additional cotyledons or fused cotyledons (e.g., Figure 1C) developed infrequently.

Table 2. Comparison of the induction frequency of somatic embryogenic (SE) cultures by media treatments. Treatments A–H as described in Table 1.

Comparison of SE frequency	X^{2a}	P[b]
A vs. B	0.13	0.7150
A vs. D, F	2.77	0.0960
A vs. C, E, G, H	4.90	0.0268*
B vs. D, F	0.91	0.3390
B vs. C, E, G, H	2.50	0.1138
D, F vs. C, E, G, H	0.00	1.0000

[a]Chi-square value with continuity correction. [b]X^2 probability with 1 degree of freedom. *Significant at $\alpha=0.05$

The majority of the somatic embryos examined (92%) germinated and converted into plants when transferred to PGR-free charcoal medium. Conversion was observed in somatic embryos originating from induction with $0.1\,mg\,l^{-1}$ IBA (9 plants) or $0.1\,mg\,l^{-1}$ picloram (2 plants), as well as no PGRs (13 plants). After the development of shoots with at least two nodes in culture, regenerated plants were transferred to soil (Figure 1I), where they continued to grow ex vitro. Somatic embryos of cultures induced with $2\,mg\,l^{-1}$ 2,4-D rarely developed beyond the globlular stage and did not produce plants.

The introduction of an IBA-induced somatic embryo culture into liquid WPM with $0.1\,mg\,l^{-1}$ IBA resulted in development of an embryogenic suspension culture (Figure 1G). The transfer of 1.2 g of suspension culture to semi-solid, PGR-free WPM produced 1,726 somatic embryos after three months (Figure 1H). Size-fractionation of suspension cultures is being examined to enrich for proembryogenic masses as a target for genetic transformation (e.g., Dhekney et al. 2008; Wilde et al. 1992).

In summary, this investigation of *C. florida* somatic

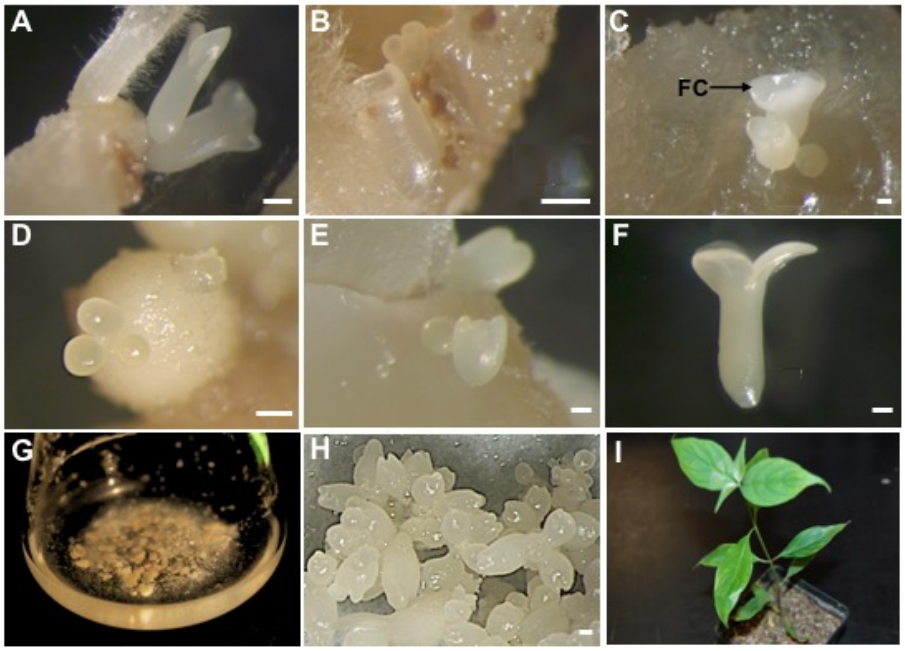

Figure 1. Somatic embryogenesis induction and plant regeneration. Direct somatic embryogenesis from zygotic embryo on PGR-free medium (A). Somatic embryos and callus from zygotic embryo on PGR-free medium (B). Embryogenic cultures induced with 0.1 mg l^{-1} picloram (C), 2.0 mg l^{-1} 2,4-D (D), or 0.1 mg l^{-1} IBA (E). Mature somatic embryo from IBA-induced culture (F). Embryogenic suspension culture in liquid WPM with 0.1 mg l^{-1} IBA (G). Somatic embryo development from suspension culture transferred to PGR-free solid medium (H). Plant regenerated from embryogenic culture induced with IBA (I). FC=somatic embryo with fused cotyledons. Scale bar=250 μm

embryogenesis has resulted in (1) the induction of embryogenic cultures with IBA, picloram, and 2,4-D, as well as with no PGRs, (2) the regeneration of plants with active shoot growth, and (3) embryogenic suspension cultures for transformation experiments. The recovery of embryogenic cultures on PGR-free medium was not anticipated, as explant tissues generally require PGRs to induce developmental reprogramming. A review of somatic embryogenesis literature found that in 94% of the studies, induction was carried out with either an auxin, a cytokinin, or a combination of the two PGRs (Gaj, 2004). In a few cases, abiotic stress has been found to induce somatic embryogenesis in the absence of PGRs (Ikeda-Iwai et al. 2003; Kamada et al. 1993, 1994; Kiyosue et al. 1989, 1990). It is possible that somatic embryogenesis in *C. florida* was induced by stress in the absence of PGRs, although the mechanism remains to be determined.

Somatic embryogenesis was induced from *C. florida* explants by media with 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} picloram, or 2 mg l^{-1} 2,4-D. These PGR treatments resulted in embryogenic calli that produced somatic embryos when transferred to PGR-free medium (Figure 1C–E). Overall, 92% of the somatic embryos transferred to germination medium converted into plantlets with active root and shoot growth. The improvement in conversion over previous *C. florida* research (Trigiano et al. 1989) may be due to the types of auxin used for the induction of somatic embryogenesis. In some plant species, 2,4-D has been found to have a carry-over effect on somatic embryo development, potentially due to a disruption in auxin polar transport (Jiménez 2005). The most effective PGR treatment in our pilot study was 0.1 mg l^{-1} IBA, a weak auxin that induced embryogenic callus from which plants could be regenerated. This is the first report of somatic embryo-derived plantlets of *C. florida* that had active shoot growth and that could be transferred to soil. The development of embryogenic suspension cultures and the regeneration of actively growing plants are significant steps towards a bioengineering platform for *C. florida*.

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