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Assessing Competence for Adventitious Shoot Formation in Hypocotyl-Explant Cultures from *Catharanthus roseus* Cultivars

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Hypocotyl explants from 22 cultivars of *Catharanthus roseus* were cultured on various shoot-inducing media to assess their competence for adventitious shoot formation. The Murashige and Skoog (MS) media had been supplemented with 14 μM zeatin and 2.5 μM indole-3-butyric acid (IBA), 4.5 μM BA and 0.5 μM α-naphthaleneacetic acid (NAA), or 14 μM thidiazuron and 2.5 μM IBA. After eight weeks, the explants from 'Cooler Raspberry Red' showed the greatest frequency of adventitious shoot formation, followed by 'Cooler Orchid' and 'Cooler Treated'. The highest frequency (86.7%) for 'Cooler Raspberry Red' was attained on the medium enhanced with 14 μM zeatin and 2.5 μM NAA. Excised adventitious shoots were then readily rooted on a half-strength MS basal medium. Afterward, the regenerated plantlets were transferred to potting soil and grown to maturity in a greenhouse.

Keywords: Apocynaceae, *Catharanthus roseus*, indole alkakoids, Madagascar periwinkle, organogenesis

Catharanthus roseus (Madagascar periwinkle) is a tropical and subtropical plant in the family Apocynaceae. This species has become important because it produces valuable alkaloid compounds, such as vinblastine and vincristine, which are used for treating blood cancer (Lounasmaa and Galambos, 1989). These compounds result from the coupling of two different monomeric indole alkaloids, vindoline and catharanthine (Endo et al., 1988; Misawa et al., 1988; Fujita et al., 1990). The former is present at relatively high concentrations in the plant, whereas natural levels of catharanthine are much lower. However, in cultured cells or hairy roots, vindoline is not produced, but catharanthine is found at a relatively high concentration (Constabel et al., 1982; Lounasmaa and Galambos, 1989; Jung et al., 1992a, 1992b). Therefore, it would be useful to produce those dimers by coupling vindoline from cultivated plants with catharanthine from cell or hairy root cultures (Fujita et al., 1990).

Yun et al. (1992) have succeeded in enhancing productivity of the tropane alkaloid scopolamine in *Atropa belladonna*. This was done by introducing cDNA for the gene from *Hyoscyamus niger* that encodes hyoscyamine-6-hydroxylase via *Agrobacterium*-mediated transformation. That enzyme catalyzes the conversion of hyoscyamine to scopolamine, which is rate-limited in *A. belladonna*. Their results suggest that the productivity of vinblastine or vincristine in *C. roseus* plants may be elevated by manipulating the expression level of the gene for any possible rate-limiting enzyme during the biosynthetic process of catharanthine. To do so, however, a genetic transformation system for this species is prerequisite. Hairy root cultures can be produced using *Agrobacterium* (Brillanceau et. al., 1989; Goddijn et al., 1995) and transgenic cell lines of *C. roseus* (van der Fits and Memelink, 1997; Hilliou et al., 1999). Although transgenic plants of *C. roseus* (via *Agrobacterium*) have been reported, no evidence of genetic transformation at the molecular level is available (Zarate et al., 1999). This shortcoming could be relieved by the development of a high-frequency plant regeneration system. Leaf explant-derived calli have been regenerated via organogenesis in *C. roseus* (Constabel et al., 1982). However, the frequency of plant regeneration has historically been too low for practical genetic transformation.

We previously established an alternative regeneration system based on somatic embryogenesis from *Catharanthus* (Kim et al., 1994). Anther-derived embryogenic calli yielded cell suspension cultures with a high competence for regeneration. However, in a preliminary

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experiment, we failed to regenerate somatic embryos into plantlets that arose from the *Agrobacterium*-mediated-transformed suspension cultures. Therefore, the objective of the current study was to develop a high-frequency regeneration system via adventitious shoot formation for subsequent use in genetic transformation. To do so, hypocotyl explants from 22 cultivars of *C. roseus* were cultured to determine which had the highest levels of competence.

MATERIALS AND METHODS

Plant Materials

Seeds of 22 cultivars of *Catharanthus roseus* (L.) G. Don (Table 1) were purchased from AustraHort Pty (Bomaderry, Australia), Ball Seed (West Chicago, IL, USA), Sakata Seed (Yokohama, Japan), and Takii Seed (Kyoto, Japan). The seeds were surface-sterilized, first in 70% (v/v) ethanol for 30 s, then for 20 min with occasional agitation in a 0.4% (v/v) sodium hypochlorite solution. They were then rinsed four times with sterile distilled water. Afterward, the seeds were placed on a half-strength Murashige and Skoog (1962) MS basal medium for germination. Two approximately 0.7-cm-long hypocotyl explants were excised consecutively from each 10-d-old seedling, immediately below the cotyledonary node, taking care to avoid the shoot apical meristem.

Culturing Media and Conditions

The basal medium for all experiments consisted of MS inorganic salts, 100 mgL^{-1} myo-inositol, 0.4 mgL^{-1} thiamine HCl, 3% (w/v) sucrose, and 4 gL^{-1} Gelrite (pH adjusted to 5.8 before autoclaving). Twenty-five mL of the medium was dispensed into each plastic Petri dish (87 × 15 mm). Unless mentioned otherwise, all cultures were maintained in the light (~3 W m^{-2} from cool-white fluorescent lamps) at 25°C, under a 16-h photoperiod.

Induction of Adventitious Shoots

To induce adventitious shoots, the hypocotyl explants were placed on MS media supplemented with one of the following combinations: 1) 14 µM zeatin and 2.5 µM indole-3-butyric acid (IBA); 2) 4.5 µM 6-benzy-

Table 1. Frequency (%) of adventitious shoot formation and number of adventitious shoots per explant in hypocotyl cultures of 22 *C. roseus* cultivars[a].

Cultivar	ZI	BN	TI	Mean frequency
Cascade Appleblossom	3.3	3.3	3.3	3.3 b[b]
Cooler Apricot	0.0	0.0	0.0	0.0 b
Cooler Coconut	3.3	3.3	0.0	2.2 b
Cooler Grape	0.0	0.0	3.3	1.1 b
Cooler Icy Pink	3.3	0.0	0.0	1.1 b
Cooler Mix Improved	0.0	3.3	0.0	1.1 b
Cooler Orchid	6.7	10.0	3.3	6.7 ab
Cooler Peppermint	0.0	3.3	3.3	2.2 b
Cooler Pink	3.3	3.3	3.3	3.3 b
Cooler Red	3.3	3.3	0.0	2.2 b
Cooler Rose	3.3	0.0	6.7	3.3 b
Cooler Raspberry Red	23.3	13.3	0.0	12.2 a
Cooler Strawberry Red	0.0	0.3	0.0	0.1 b
Cooler Treated	6.7	0.0	6.7	4.5 ab
Equaton Grape	6.7	0.0	0.0	2.2 b
Little Bright Eye	0.0	0.0	0.0	0.0 b
Little Linda	3.3	6.7	0.0	3.3 b
Pacifica Deep Orchid	0.0	0.0	0.0	0.0 b
Pacifica Red	0.0	0.0	0.0	0.0 b
Stardust Mix	6.7	0.0	0.0	2.2 b
Stardust Orchid	0.0	0.0	0.0	0.0 b
Stardust Pink	0.0	0.0	0.0	0.0 b

[a]Data were collected after eight weeks of culture. Each treatment consisted of 10 explants per dish with three replicates. MS medium supplemented with 14 µM zeatin and 2.5 µM IBA (ZI); 4.5 µM BA and 0.5 µM NAA (BN); or 14 µM thidiazuron and 2.5 µM IBA (TI) was used.

[b]Values followed by the same letter within the column are not significantly different at the 5% probability level.

ladenine (BA) and 0.5 μM α-naphthaleneacetic acid (NAA); or 3) 14 μM thidiazuron and 2.5 μM IBA. (The selection of plant growth regulators and their concentrations was based on the results of preliminary experiments and on logical deduction.) Each treatment consisted of 10 explants per dish, with three replicates. In addition, hypocotyl explants from 'Cooler Raspberry Red' (which had previously exhibited one of the highest frequencies of adventitious shoot formation) were cultured on an MS medium supplemented with zeatin or BA (2, 4, or 14 μM) in combination with IBA or NAA (0.5 or 2.5 μM). After eight weeks, competence for adventitious shoot formation was determined according to the number of explants producing such shoots. Data were subjected to ANOVA; mean frequencies of adventitious shoot formation by cultivar were separated by LSD at $P = 0.05$. The adventitious shoots that formed on the explants were excised with a scalpel and transferred to a rooting medium (half-strength MS basal medium). Regenerated plantlets were acclimated, transplanted to potting soil, then maintained in a greenhouse.

In parallel experiments of the 22 cultivars, cotyledonary explants from 10-d-old seedlings as well as leaf explants from four- to eight-week-old plants were maintained in 250-mL bottles containing a half-strength MS basal medium. Culturing conditions were as described above.

RESULTS AND DISCUSSION

After two weeks of culture, most of the hypocotyl explants had formed calli at the cut edges (Fig. 1A). By four weeks, a few explants had produced adventitious buds, which appeared to arise from those hypocotyl-derived calli (Fig. 1B). Adventitious shoots were elongated after five to eight weeks of culture (Fig. 1, C and D). After eight weeks, explants of 'Cooler Raspberry Red' that had been cultured on 14 μM zeatin and 2.5 μM IBA had the greatest frequency of adventitious shoot formation, with a mean shoot number of 0.57 per explant (Table 1). Cultivars 'Cooler Orchid' and 'Cooler Treated' performed almost as well. Our overall results suggest that competence for adventitious shoot formation is genotype/cultivar-dependent, an observation similar to that reported with *Gentiana* (Hosokawa et al., 1996) and delphinium (Hosokawa et al., 2001).

Supplementing the MS medium with 14 μM zeatin and 2.5 μM NAA increased the frequency of adventitious shoot formation to 86.7% in 'Cooler Raspberry Red'. That combination also produced the highest mean number of adventitious shoots per explant (Table 2).

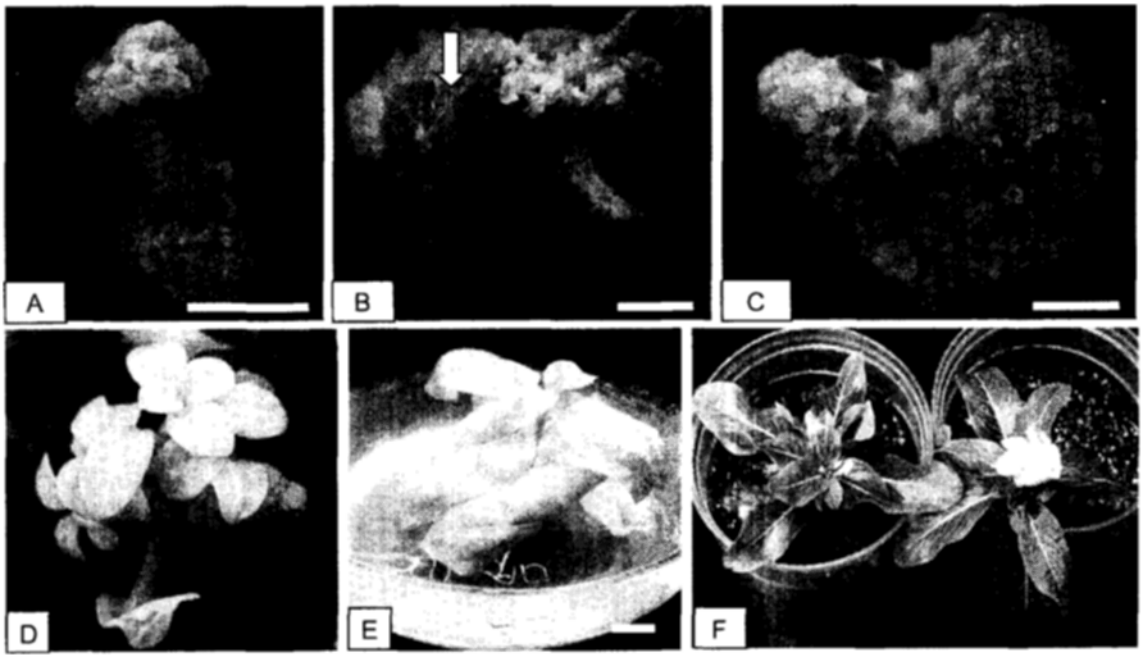

Figure 1. Adventitious shoot formation and plant regeneration in tissue cultures of *C. roseus*. **A,** Hypocotyl explants after two weeks of culture; **B,** Adventitious bud formation (at arrow) on hypocotyl-derived callus after four weeks of culture; **C and D,** Adventitious shoots formed on hypocotyl explant; **E,** Plantlet developed from adventitious shoot; **F,** Regenerated plant with flowers. Bars indicate 5 mm (**A, B, C, D**) or 10 mm (**E**).

Table 2. Frequency (%) of adventitious shoot formation per explant in hypocotyl cultures of *C. roseus* cv. Cooler Raspberry Red[a].

Plant growth regulator (μM)				Number of explants with adventitious shoots
Zeatin	BA	IBA	NAA	
2	–	0.5	–	3.3 d[b]
4	–	0.5	–	0.0 d
14	–	2.5	–	23.3 c
–	2	–	0.5	20.0 cd
–	4	–	0.5	10.0 cd
–	14	–	2.5	65.0 b
2	–	–	0.5	26.7 c
4	–	–	0.5	20.0 cd
14	–	–	2.5	86.7 a

[a]Data were collected after eight weeks of culture. Each treatment consisted of 10 explants per dish with three replicates.
[b]Values followed by the same letter within a column are not significantly different at the 5% probability level.

Zeatin plus NAA was more effective than either the combination of BA and NAA or zeatin plus IBA at the highest concentrations. Adventitious shoots were readily rooted (100% success) in the rooting medium (Fig. 1E), and the regenerated plantlets were grown to maturity in a greenhouse (Fig. 1F).

In the second parallel experiments, only the cotyledonary explants from 'Cooler Raspberry Red' formed adventitious shoots (frequency of 3.3%) when cultured with 14 μM thidiazuron and 2.5 μM IBA. This further confirmed that 'Cooler Raspberry Red' is one of the most competent cultivars. In contrast, none of the leaf explants produced adventitious shoots, thereby indicating that hypocotyl explants have a greater competence than either cotyledonary or leaf explants. Other explant sources, including the petiole and anther filament from 'Cooler Raspberry Red', are currently being investigated. Various combinations of cytokinins and auxins are being assessed to determine which cultivars and explant types yield adventitious shoots at frequencies adequate for use in routine genetic transformation of *C. roseus*.

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