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Micropropagation of a Cold Hardy Selection of *Cercis canadensis* L. Through Single-Node Culture[1]

Wenhao Dai[2], Victoria Jacques[3], Dale Herman[4], and Zong-Ming Cheng[5]
Department of Plant Sciences, North Dakota State University, Fargo, ND 58105

Abstract

A cold hardy eastern redbud (*Cercis canadensis* L., TS 0092) was propagated using single-node micropropagation. *In vitro* shoot cultures were initiated from rapidly growing shoot tips of a mature tree. The effects of basal media and plant growth regulators on proliferation were investigated. Maximum proliferation rate was obtained from all three tested media, Murashige and Skoog (MS), Woody Plant Medium (WPM), and Driver-Kuniyuki walnut (DKW), supplemented with 0.5 µM thidiazuron (TDZ). Shoots produced on WPM medium appeared stressed showing yellowish and thin leaves compared to those on MS and DKW media. Shoots were *in vitro* rooted in auxin-free half strength WPM medium after being pulsed for 1 to 16 days in half strength WPM medium with 12.5 to 100 µM indole-3-butyric acid (IBA). *In vitro* rooting rate was influenced by IBA concentration, pulsing time, and shoot sources (media in which shoots were produced). Shoots produced in DKW medium rooted best after being pulsed in 25 to 100 µM IBA for 8 days although root numbers were similar among treatments. Direct *ex vitro* rooting was achieved by quick-dipping the microshoots in 10 µM IBA or NAA solutions and inserting them in Jiffy Mix potting medium.

Index words: redbud, auxin pulsing, *ex vitro* rooting.

Species used in this study: *Cercis canadensis* L.

Significance to the Nursery Industry

To speed up the release of newly selected woody plants, a sufficient quantity of clonal plants is required for multiple field tests. Unfortunately, some woody species are very difficult to propagate by such traditional means as cuttings, grafting, and division. An eastern redbud tree hardy to USDA hardiness zone 3 was discovered in central South Dakota. This plant is difficult to propagate readily by cuttings and grafting. Lack of sufficient uniform plants is a bottleneck in evaluating and releasing this elite selection for future commercial propagation and use. This study demonstrated that 0.5 µM TDZ produced healthy microcuttings that could be *in vitro* rooted after being pulsed with 25 to 100 µM IBA for 8 days and *ex vitro* rooted after 10 µM NAA or IBA treatment in the soilless medium. The propagation method developed in this study can be used to mass propagate this selection and could be applied for propagation of other related redbud species or cultivars by commercial tissue culture laboratories.

Introduction

Eastern redbud (*Cercis canadensis* L.), a small flowering landscape tree, is popular because of its unique display of colorful flowers on leafless branches in the early spring. Native to the eastern United States, eastern redbud is adaptable to a wide range of soils and climate (2). Several cultivars with various flower colors have been introduced, but they are unable to tolerate the harsh winters in the northern Great Plains. Efforts have been made to select redbuds from cold hardy seed sources for many years by horticulturists, but with little progress. A redbud tree was discovered to bloom reliably in the Northern Plains based upon extended observations. Like other taxa in the genus *Cercis*, this selection is difficult to propagate clonally by cuttings.

Although several researchers have reported on tissue culture of the genus *Cercis* (1, 3, 4, 5, 9, 13, 14), data on micropropagation are still limited. Shoot tips have been used to propagate eastern redbud (*C. canadensis*) (1, 9, 14) and Chinese redbud (*C. yunnanensis*) (3). Embryonic or immature seedling tissues, such as cotyledonary nodes (4), somatic embryos (5), and immature embryos (13) have been used to *in vitro* propagate redbud species. Results from the previous research showed that effects of explants, media, and plant growth regulators on redbud proliferation and rooting appear to be genotype-dependent and controversial.

Tissue cultures have to be initiated with vegetative tissues to maintain the existing traits and to clonally propagate woody selections. In woody plant breeding, some traits have to be evaluated when plants are mature. Therefore, micropropagation must be initiated from mature vegetative tissues that are regarded as recalcitrant to *in vitro* culture. With only few reports of redbud micropropagation using shoot tips from mature plants (1, 9, 14), we conducted this study to develop an efficient *in vitro* propagation protocol for this elite hardy selection. The specific objectives in this study were to investigate factors affecting micropropagation, including shoot proliferation, rooting and acclimatization, of this eastern redbud selection.

Materials and Methods

Establishment of tissue culture. Newly flushed shoots [1 to 1.5 cm (0.4 to 0.6 in)] were collected from a mature eastern redbud selection (TS 0092) found by G. Morgenson, Lincoln-Oakes nursery, Bismarck, ND. The shoots were washed with running tap water for 1 hour and then surface disin-

[1]Received for publication August 17, 2004; in revised form January 3, 2005. This research was supported in part by McIntire-Stennis Project ND06212 and in part by the NDSU Development Foundation's Gordon A. Larson Agriculture Research Fund. We thank Drs. E.L. Deckard and D. Li for reviewing this manuscript and Dr. C. Huang and Koji Fujiwara for the statistical analysis.

[2]Assistant Professor, Department of Plant Sciences, e-mail: <wenhao.dai@ndsu.edu>. To whom reprint requests should be addressed.

[3]Research Specialist.

[4]Professor, Department of Plant Sciences, North Dakota State University, Fargo, ND 58105.

[5]Associate Professor, Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996.

fected in 70% ethanol for 30 seconds followed by immersion in 10% Clorox (0.6% sodium hypochlorite) containing 3 drops of liquid hand soap per 100 ml solution for 15 min. Shoots were rinsed three times in sterile distilled water and blotted dry with sterile paper towels, then inserted into Murashige and Skoog (MS) medium (10) supplemented with 5 µM BA in 100 ml baby food jars containing 25 ml medium. After 4 weeks initial culture, explants were transferred to three basal media, MS, Woody Plant Medium (WPM) (8), and Driver-Kuniyuki walnut (DKW) (6) containing 2.5 µM BA or a combination of 1.0 µM BA, 2.5 µM TDZ, and 1.0 µM IBA. All media above were supplemented with MS vitamins, 3% sucrose and 0.7% agar (Difco Co., Detroit, MI, #0140-01-0) and adjusted to pH 5.8 before autoclaving. Each treatment consisted of two replications (jars) with 4 shoot explants per jar. Shoot cultures were maintained at 25C (76F) under cool-white fluorescent light at approximately 36.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 16-hour photoperiod. All other experiments were performed under these conditions except otherwise noted.

Shoot proliferation. To optimize proliferation conditions, three basal media (WPM, MS, and DKW) and two cytokinins (BA and TDZ) at four concentrations (BA at 2.5, 5.0, 10.0, and 20 µM; TDZ at 0.1, 0.5, 1.0, and 2.0 µM) were tested. All basal media were supplemented with MS vitamins, 3% sucrose and 0.7% agar, and the pH was adjusted to 5.8 before autoclaving. Single nodes from *in vitro* plants were used for proliferation. Three jars with 4 nodes in each jar were used for each treatment. After culture for 3 weeks, the proliferation was evaluated by counting the node number of the new shoots based on the fact that each node can develop a new plant.

Rooting and acclimatization. Two rooting experiments were carried out to examine the effects of microcutting source, auxin concentration, and pulse time on rooting percentages. For *in vitro* rooting, microcuttings longer than 1.5 cm (0.6 in) were placed in half strength of WPM medium containing IBA at 0, 12.5, 25.0, 50.0, and 100 µM for root induction (pulsing). After being in auxin supplemented medium for 1, 8, and 16 days, microcuttings were then transferred to the same medium without auxin for root development. Each treatment in this pulse experiment consisted of three jars with four shoots per jar. After being in the auxin-free medium for three weeks, rooting percentages and number of roots per microcutting were recorded. Rooted plants were then transferred to flats filled with Jiffy Mix (Jiffy Mix. Shippegan, NB, Canada) and covered with clear plastic tops. After one week, the covers were gradually removed during the following one-week period. Surviving plants were potted into Sunshine Mix #1 (Fisons Western Corp., Vancouver, BC, Canada) and grown in the greenhouse.

For *ex vitro* rooting, the basal portion (approximately 3 to 5 mm) of microcuttings were quick-dipped (5 seconds) in 10 µM IBA or NAA, and then inserted into Jiffy Mix medium in plastic flats covered with clear plastic tops. Each treatment included at least 50 microcuttings. After three weeks, the covers were gradually removed during a one-week period. Rooting percentages were recorded, and the rooted plants were transferred to Sunshine Mix #1 and grown in the greenhouse. Both *in vitro* and *ex vitro* rooting experiments were replicated twice.

Shoot proliferation and *in vitro* rooting experiments were conducted as a completely randomized design (CRD) consisting of two replications of a factorial arrangement with a $3 \times 2 \times 4$ of basal medium, hormone type, and hormone concentration, respectively for shoot proliferation experiment and a $3 \times 5 \times 3$ of cutting source (CS), IBA concentration, and pulsing time, respectively for *in vitro* rooting experiment. *Ex vitro* rooting experiment was conducted as a CRD and replicated twice. Data were subject to analysis of variation and mean comparison using the GLM procedure of SAS software Version 9.1 (11).

Results and Discussion

In vitro establishment. When the initial shoot tips were placed into MS medium, the explants exuded large amounts of phenolic compounds, showing brownish to black color around the base of explants. Growth of the explants was stunted and leaves appeared nutrient deficient. Shoot culture establishment was affected by basal medium and cytokinins. After 4 weeks culture, all explants on all three media with 2.5 µM BA as the sole growth regulator died, whereas 75% of explants survived on the media with a combination of 1.0 µM BA, 2.5 µM TDZ, and 1.0 µM IBA. More than 80% of new growing shoots were vitrified, especially on MS medium. Reducing TDZ from 2.5 µM to 0.5 µM dramatically reduced shoot vitrification. Addition of 0.1% activated charcoal during the stabilization period significantly inhibited the growth of new shoots, especially on the DKW medium. Most cultures appeared to be stable after 6 subcultures with a 4-week interval in media with a combination of 1.0 µM BA, 2.5 µM TDZ, and 1.0 µM IBA.

Shoot proliferation. Shoot proliferation was significantly affected by basal medium and cytokinin (Table 1). Significant interactions in medium × cytokinin and cytokinin × (medium × cytokinin) were observed (Table 1). Overall, BA at 5 µM or TDZ at 0.5 µM induced higher proliferation rates for explants in all three media. Shoot proliferation was significantly lower on MS medium than on the other two media when high concentration of BA (> 5 µM) was used. Proliferation rates among explants in the three basal media were similar if 0.5 µM TDZ was in the medium. However, shoots produced on DKW medium had thick stems and dark green leaves, whereas shoots produced on WPM medium grew quickly with longer internodes and yellowish leaves. High TDZ concentrations (≥1.0 µM) inhibited redbud proliferation. Proliferation rates were also slightly inhibited for explants receiving high BA concentrations (≥10 µM) in WPM and DKW media.

Results in this study clearly showed that the basal medium affected the establishment and proliferation of redbud. Explants on WPM used in most studies on micropropagation of the genus *Cercis* (1, 9, 14) produced yellowish and thin shoots, resulted in lower rooting percentage and lower survival rate after acclimatization (discussed below). High quality shoots with thick stems and dark green leaves were produced on DKW medium. Because all media used in this study contained MS vitamins, the effect of medium on shoot proliferation most likely resulted from the difference in macronutrients and micronutrients in the respective medium. The overall low macronutrient concentration of WPM may be the cause of deficiency symptoms. DKW medium, richer in several nutrients, such as magnesium, manganese, zinc, and

Table 1. Effect of basal medium and cytokinins on shoot proliferation of *Cercis canadensis* L[z].

Cytokinin (µM)	Proliferation rate[y]			Analysis of variation			
	MS	DKW	WPM[x]	Source of variation	df	Mean square	F
BA 0	0.4j	1.2f–j	0.5j	Medium	2	1.8427	7.73**
BA 2.5	1.6e–i	1.4e–j	1.9c–g	Cytokinin	1	1.2298	5.16*
BA 5.0	1.9c–g	2.8a–c	3.1ab	Medium × cytokinin	2	0.9766	4.10*
BA 10.0	1.0g–j	1.7d–h	2.8a–d	Concentration × (Medium × cytokinin)	24	2.2460	9.43***
BA 20.0	0.5j	2.1b–f	2.3b–e				
TDZ 0.1	1.3e–j	1.2f–j	1.6e–i				
TDZ 0.5	3.7a	3.4a	3.6a				
TDZ 1.0	0.6ij	1.0g–j	0.8h–j				
TDZ 2.0	0.4j	0.7ij	0.5j				

[z]Different letters indicate significant differences ($p \leq 0.05$).

[y]The proliferation rate is expressed as the number of nodes produced from a single-node explant after 4 weeks *in vitro* culture.

[x]MS: Murashige and Skoog medium; DKW: Driver-Kuniyuki Walnut medium; WPM: Woody Plant Medium.

*, **, ***Significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

nickel, may be more suitable than the other media for *in vitro* shoot growth of redbud.

In this study, nodal explants produced single shoots with no shoot multiplication from preexisting buds. It is surprising that relatively high concentrations of BA and TDZ failed to stimulate lateral shoot multiplication. Unlike other commonly used micropropagation methods for redbud, such as meristem tip culture and plant regeneration, micropropagation through a single node has an advantage of producing true-to-genotype plants because no shoots will be produced from calli induced from the base of explants. Regeneration of shoots from callus is considered to be a major cause of somaclonal variation (7).

In vitro and ex vitro rooting. After being pulsed in IBA-containing half strength WPM medium for 1 to 16 days and transferred to auxin-free half strength WPM medium, microcuttings started producing roots in two weeks (Table 2). Microcuttings without IBA pulsing failed to root, indicating that auxin is required for *in vitro* rooting of this redbud selection. Cutting sources (CS) significantly affected *in vitro* root initiation. Cuttings from DKW and WPM media produced roots after 1-day IBA pulsing, whereas very few shoots developed from MS-produced cuttings. Pulsing time significantly affected rooting. The best rooting percentage resulted from 8-day pulsing for all cutting sources with several exceptions. No significant CS × IBA and CS × pulse interactions were determined. However, interactions of IBA × pulse and CS × IBA × pulse were significant (Table 2). In general, the lower the auxin concentration used, the longer the pulsing time needed. However, high IBA (>100 µM) and long pulsing (>8 days) resulted in inhibition of *in vitro* rooting of redbud. Root number was also affected by cutting sources, auxin, and pulsing time (Table 3). However, no significant interactions were identified from any combinations of CS, IBA concentration, and pulsing time. Microcuttings collected from DKW produced more roots (3.52 roots/cutting) than those from MS (2.27 roots/cutting) and WPM (1.85 roots/cutting) media. Overall, cuttings pulsed for 8 and more days produced more roots than those pulsed for 1 day. No significant difference in root number was found between 8-day and 16-day pulsing. DKW-generated cuttings rooted best

Table 2. Effect of cutting sources, auxin, and pulsing time on *in vitro* rooting percentage of *Cercis canadensis* L[z].

Cutting sources	Auxin (µM)	Rooting percentage, in days			Analysis of variation			
		1	8	16	Source of variation	df	Mean square	F
MS	IBA 0	0.0h	0.0h	0.0h	Cutting source (CS)	2	1392.57	4.53*
	IBA 12.5	0.0h	41.7d–g	66.7a–d	IBA	4	8664.55	28.21***
	IBA 25	0.0h	50.0c–f	33.4e–g	CS × IBA	8	373.08	1.21NS
	IBA 50	8.4h	33.4e–g	41.7d–g	Pulse	2	10360.81	33.73***
	IBA 100	0.0h	100.0a	50.0c–f	CS × pulse	4	413.91	1.35NS
DKW	IBA 0	0.0h	0.0h	0.0h	IBA × pulse	8	1045.40	3.4**
	IBA 12.5	33.3e–g	50.0c–f	25.0fg	CS × IBA × pulse	16	990.81	3.23**
	IBA 25	16.7gh	91.7a	11.0h				
	IBA 50	16.7gh	83.3ab	91.7a				
	IBA 100	41.7d–g	83.4ab	83.3ab				
WPM	IBA 0	0.0h	0.0h	0.0h				
	IBA 12.5	16.8gh	41.7d–g	58.3b–e				
	IBA 25	8.4h	41.7d–g	75.0a–c				
	IBA 50	25.0fg	66.7a–d	41.7d–g				
	IBA 100	58.4b–e	83.3ab	33.4e–g				

[z]Different letters indicate significant differences ($p \leq 0.05$).

*, **, ***Significant at $P \leq 0.05$, 0.01, and 0.001, respectively.

NSNonsignificant.

Table 3. Effect of cutting sources, auxin, and pulsing time on root number produced *in vitro* of *Cercis canadensis* L.[z].

Cutting sources	Auxin (µM)	Rooting number/cutting, in days			Analysis of variation			
		1	8	16	Source of variation	df	Mean square	F
MS	IBA 0	0k	0k	0k	Cutting source (CS)	2	14.4211	10.05***
	IBA 12.5	0k	3.84a–f	4.75a–c	IBA	4	23.8916	16.66***
	IBA 25	0k	2.88c–j	5.00ab	CS × IBA	8	2.5177	1.76NS
	IBA 50	0.5k	1.5h–k	2.88c–j	Pulse	2	27.2821	19.02***
	IBA 100	0k	3.0b–i	2.88c–j	CS × pulse	4	4.9264	3.43*
DKW	IBA 0	0k	0k	0k	IBA × pulse	8	2.0903	1.46NS
	IBA 12.5	1.75g–k	2.34e–k	4.25a–d	CS × IBA × pulse	16	1.5048	1.05NS
	IBA 25	2.5d–i	3.9a–f	3.65a–g				
	IBA 50	1.5h–k	4.2a–e	4.9ab				
	IBA 100	2.13f–k	5.59a	5.50a				
WPM	IBA 0	0k	0k	0k				
	IBA 12.5	0.75k	2.63c–j	1.1jk				
	IBA 25	1.0jk	3.13b–i	2.13f–k				
	IBA 50	1.75g–k	1.67h–k	2.34e–k				
	IBA 100	2.34e–k	1.9f–k	1.5h–k				

[z]Different letters indicate significant differences ($p \leq 0.05$).
*, **, ***Significant at $P \leq 0.05$, 0.01, and 0.001, respectively.
NSNonsignificant.

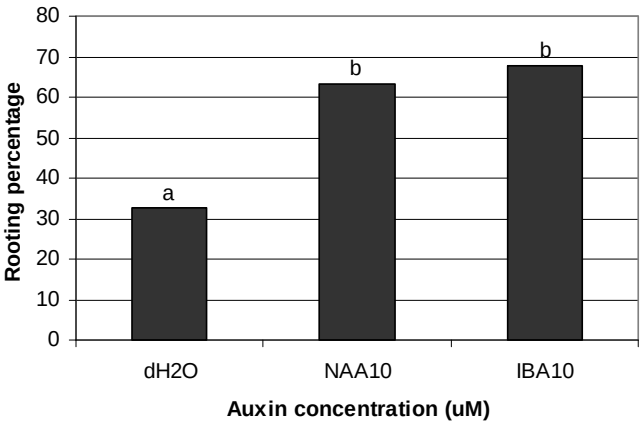

Fig. 1. Effect of auxin treatment on *ex vitro* rooting percentage of *Cercis canadensis* L. Different letters above the bars indicate significant differences ($p \leq 0.05$).

(highest rooting percentage) after being 8- or 16-day pulsed with 50 or 100 µM IBA. Morphologically, high IBA (>50 µM) and long pulsing time (>8 days) produced thick and short roots with less lateral roots, which may reduce chance of survival in the soils. In this study, we found that although high concentrations of auxin and long auxin pulsing time were unnecessary to induce more roots, treatment with auxin was required for *in vitro* rooting of this selection, which is different from results of Mackay et al. (9) in which the control (no auxin treatment) produced good quality roots with 60% of rooting percentage for Mexican redbud.

Auxin treatment significantly affected *ex vitro* root formation (Fig. 1). An average of 65.5% of the shoots rooted when quick-dipped in NAA or IBA solution, whereas only 32.8% of shoots developed roots when treated with water. The number of roots formed per shoot was similar among treatments with an average of 2.3 roots formed per cutting. A total of 378 microshoots rooted *ex vitro*. After one month of acclimatization, up to 85% of rooted plants survived in the greenhouse (Fig. 2).

In this research, we obtained good rooting results both *in vitro* and *ex vitro*. Using the *ex vitro* rooting coupled with

Fig. 2. Micropropagation of *Cercis canadensis* L. (A) *In vitro* shoot proliferation on DKW medium containing 0.5 µM TDZ; (B) Roots were induced *ex vitro* from microcuttings after quick-dipping in 10 µM IBA; (C) Hardened plants were potted and grown in the greenhouse.

acclimatization can significantly increase the quality of plants even though this procedure had a lower rooting rate. In general, *in vitro* rooting increases cost of final products about 30% compared to *ex vitro* rooting (12). Therefore, the direct *ex vitro* rooting method, with a higher rooting rate and shorter time by eliminating the *in vitro* rooting stage, was more practical and economical for redbud micropropagation.

The research results showed that this redbud selection can be efficiently micropropagated using MS or DKW media supplemented with 5 µM BA or 0.5µM TDZ for proliferation and direct *ex vitro* rooting method for rooting and acclimatization. This protocol should also be applicable to other redbud cultivars or new selections.

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