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MICROPROPAGATION OF AN INTERSPECIFIC HYBRID DOGWOOD (*CORNUS* 'NCCH1')

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Abstract

A micropropagation protocol was developed for a novel hybrid dogwood, *Cornus* 'NCCH1' (an F_2 hybrid of *Cornus kousa* Buerger ex Miq. 'Miss Satomi' × *Cornus hongkongensis* Hemsl. 'Summer Passion'). Murashige and Skoog (MS) medium, woody plant medium (WPM), Driver and Kuniyuki Walnut (DKW) medium, Quoirin and Lepoivre (QL) medium, and Schenk and Hildebrandt (SH) medium, supplemented with 5 µM 6-benzylaminopurine (BAP), were evaluated as basal multiplication media. Benzylaminopurine, zeatin (Ztn), *meta*-Topolin (*m*T), or kinetin (Kin) at 0.625, 1.25, 2.5, 5, or 10 µM on WPM were investigated. Woody plant medium supplemented with 10 µM BAP provided the highest mean number of 4.27 axillary shoots (5-10 mm long) after 5 weeks of culture. The effect of supplementing multiplication media with indole-3-acetic acid (IAA) at 0, 0.1, 0.5, or 1.25 µM on shoot elongation was evaluated. The addition of IAA at 0.5 µM produced a 19% increase in mean shoot length per subsample over 5 weeks. The influence of media strength and sucrose concentration on rooting and *ex vitro* establishment was investigated. Quarter-strength WPM supplemented with 5 g l^{-1} sucrose produced the highest *ex vitro* establishment of 72.5%.

Key words: auxins, cytokinins, elongation, microshoots, multiplication rate, phenolics

INTRODUCTION

Dogwoods are valuable nursery and landscape crops that are grown throughout the world (Cappiello 2006). The North American species, *Cornus florida* L. and *Cornus nuttallii* Audubon, are generally susceptible to both dogwood anthracnose (*Discula destructive* Redlin) and powdery mildew (*Microsphaera pulchra* Cooke and Peck) (Cappiello 2006). Many Asian dogwoods including *Cornus kousa* F. Buerger ex Miq. have more resistance to these diseases (Ranney et al. 1995) and some species including *Cornus honkongensis* Hemsl. have persistent evergreen foliage. The dogwood breeding program at NC State University has recently developed hybrids between *C. kousa* and *C. hongkongensis* including *Cornus* 'NCCH1', an F_2 hybrid of *C. kousa* 'Miss Satomi' × *C. hongkongensis* 'Summer Passion'. This hybrid dogwood is a small tree or shrub, semi-evergreen (down to -10°C) with dense branching, red fall foliage, and excellent resistance to powdery mildew.

Asexual propagation of dogwood cultivars can be achieved from budding, softwood stem cuttings, or from *in vitro* propagation (Hadziabdic 2005). However, cultivars regenerated from cuttings frequently lack vigor (Hartmann et al. 2002). *In vitro* propagation may prove useful for maintaining vigorous juvenile tissue and for rapid multiplication rates of new elite cultivars. In addition, *in vitro* propagation protocols provide a platform for further cultivar improvements through ploidy manipulation, mutation treatments, and transgenic applications (Touchell et al. 2008).

In vitro shoot regeneration protocols have been reported for both North American and Asian dogwoods including *C. nuttallii* Audubon (Edson et al. 1994), *C. canadensis* L. (Feng et al. 2009), *C. florida* (Trigiano et al. 1989, De Klerk and Korban 1994, Kaveriappa et al. 1997, Wedge and Tainter 1997, Sharma et al. 2005), *C. mas* L. (Ďurkovič 2008), *C. officinalis* Torr. ex Dur. (Lu 1984, 1985, Xue et al. 2003), *C. capitata* Wall. (Ishimaru 1998), and *C. kousa* (Ishimaru et al. 1993, 1998, Hadziabdic 2005). Basal nuttrient compositions comprised of woody plant medium (WPM, Lloyd and McCowan 1980) have been used for the micropropagation of a diverse range of dogwoods, including *C. nuttallii* (Edson et al. 1994), *C. officinalis* (Xue et al. 2003), *C. mas* (Ďurkovič 2008), *C. florida* (Kaveriappa et al. 1997). However, several basal nuttrient compositions

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have been used for *C. kousa*. Ishimaru et al. (1993) successfully established callus cultures using Murashige and Skoog (MS) medium (Murashige and Skoog 1962). Further Ishimaru et al. (1998) established cultures of *C. kousa* var. *chinensis* Osborn, *C. kousa* 'Milky Way', and *C. capitata* 'Mountain Moon' on Broadleaf Tree (BW) medium (Chalupa 1984). Hadziabdic (2005) established cultures of *C. kousa* cultivars ('Little Beauty', 'Samaritan', 'Heart Throb', 'Rosabella', and 'Christian Prince') on WPM or half-strength BW basal nutrients and vitamins.

In most *in vitro* shoot regeneration studies on dogwoods 6-benzylaminopurine (BAP) at 2 to 8 µM has been used as an effective cytokinin. While BAP alone was sufficient for microshoot proliferation of *C. nuttallii* (Edson et al. 1994), *C. florida* (De Klerk and Korban 1994, Kaveriappa et al. 1997), and *C. kousa* (Hadziabdic 2005), low concentrations of auxin were necessary for efficient *in vitro* growth in other dogwood species. Ďurkovič (2008) reported microshoot regeneration using 3.1 µM BAP supplemented with 0.3 µM 1-naphthaleneacetic acid (NAA) for *C. mas* 'Macrocarpa'. Xue et al. (2003) reported proliferation of *C. officinalis* microshoots on media containing 0.5 µM BAP, 0.5 µM (6-[4-Hydrooxy-3-methil-but-2-enylamino]purine (zeatin, Ztn), and 0.5 µM NAA, and elongation on media containing 4.4 µM BAP, 0.5 µM Ztn, 0.5 µM NAA, and 2.3 µM gibberellic acid (GA_3).

In vitro rooting of microcuttings has been successfully achieved for dogwoods. For North American dogwoods, indole-3-butyric acid (IBA) has been the predominant auxin used for *in vitro* root formation (Edson et al. 1994, Feng et al. 2009). For *C. florida*, *in vitro* rooting was highest using 4.9 µM IBA over 4 weeks (Kaveriappa et al. 1997, Sharma et al 2005). In contrast, NAA or indole-3-acatic acid (IAA) have been more effective in inducing *in vitro* rooting in some Asian dogwoods. Ďurkovič (2008) reported an *in vitro* rooting medium of using 2.7 µM NAA for *C. mas* 'Macrocarpa'. For *C. kousa*, NAA or IAA at concentrations of 0-13.5 µM proved better for *in vitro* root production than IBA (Hadziabdic 2005).

Though several studies have been conducted on *in vitro* propagation of *C. kousa*, few studies have been conducted on evergreen dogwood species or their hybrids. Currently, no reports on the micropropagation of the evergreen *C. hongkongensis* exist. Therefore the objectives of this study were to evaluate a range of basal media compositions, cytokinins, and auxin to develop micropropagation protocols for a new interspecific hybrid *Cornus* 'NCCH1'.

METHODS AND MATERIALS

Plant Material

In vitro cultures of *Cornus* 'NCCH1' were initiated from apical and axillary bud explants. Explants were collected from containerized, greenhouse-maintained plants and rinsed under tap water for 4 h. Then, explants were surface-disinfected in a 20% (v/v) commercial bleach (6.15% NaOCl) / water solution containing two to three drops of Tween® 20 (Sigma-Aldrich Corporation, St. Louis, MO), per 100 ml. Explants were agitated periodically for 17 min followed by three 5-min rinses in sterile distilled water. Explants were cultured on axillary shoot induction medium consisting of WPM basal nutrients and vitamins supplemented with 5 µM BAP, 100 mg l^{-1} myo-inositol, 100 mg l^{-1} 2-(N-Morpholino) ethanesulfonic acid (MES) monohydrate, and 30 g l^{-1} sucrose. Medium was adjusted to a pH of 5.75 and solidified with 7.5 g l^{-1} agar. Regenerated axillary shoots were used as initial explants for all experiments (unless stated otherwise) and maintained by transferring to fresh regeneration medium (25 ml in 180-ml glass jars) every 4 to 6 weeks for 12 months and incubated under standard culture conditions [23 ± 2°C and a 16-h photoperiod of 30 µmol m^{-2} s^{-1} (400-700 nm) provided by cool-white fluorescent lamps].

Effect of the medium on axillary shoot proliferation

The effects of MS nutrients and vitamins, WPM nutrients and vitamins, Driver and Kuniyuki (DKW) nutrients and vitamins (Driver and Kuniyuki 1984), Quoirin and Lepoivre (QL) nutrients (Quoirin and Lepoivre 1977) and Gamborg B5 vitamins (Gamborg et al. 1968), and Schenk and Hildebrandt (SH) nutrients and vitamins (Schenk and Hildebrandt 1972) on axillary shoot proliferation of *Cornus* 'NCCH1' were examined. All media treatments were supplemented with 5 µM BAP, 100 mg l^{-1} myo-inositol, 100 mg l^{-1} MES, and 30 g l^{-1} sucrose. Media were adjusted to a pH of 5.75, solidified with 7.5 g l^{-1} agar, and dispensed at 25 ml into 180-ml glass jars. Each treatment consisted of ten replications with five axillary shoots (5-10 mm in length) for a total of 50 shoots. All jars were completely randomized and incubated under standard culture conditions as previously described. Two subcultures were performed at 5 week intervals over a total treatment duration of 10 weeks. Data were collected on number of shoots, mean shoot length, and multiplication rate (number of 5-10 mm microcuttings obtained per microshoot per 5 weeks). Phenolic discoloration was scored per replicate (data for explants were pooled) using the following scale for diameter of phenolic plume per explant: 0 = 0 mm, 1 = 1 to 2 mm, 2 = 2 to 3 mm, 3 = 3 to 4 mm, and 4 ≥ 4 mm. Data sets were subjected to analysis of variance (ANOVA) and means were separated using Tukeys's test (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Effect of cytokinins on axillary shoot proliferation

To improve shoot proliferation, the effects of WPM containing BAP, Ztn, *meta*-Topolin (*m*T), and Kin at

0.625, 1.25, 2.5, 5, or 10 µM were examined. All media were supplemented with 100 mg l^{-1} myo-inositol, 100 mg l^{-1} MES, and 30 g l^{-1} sucrose. Media were adjusted to a pH of 5.75, solidified with 7.5 g l^{-1} agar, and dispensed at 25 ml into 180-ml glass jars. Zeatin was added to cooled autoclave media prior to dispensing. The experiment consisted of 8 replicates with five axillary shoots (5-10 mm in length) for a total of 40 shoots per treatment. Jars were completely randomized under standard culture conditions (as described for shoot initiation). Two subcultures were performed at 5 week intervals for a total treatment duration of 10 weeks. Data were collected on number of shoots, mean shoot length, and multiplication rate (number of 5-10 mm shoots obtained per explant per 5 weeks). Total phenolics were scored per replicate as described for basal salts. Data were subjected to ANOVA and regression analysis (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Elongation

Axillary shoots elongation was examined by combining 0, 0.1, 0.5, or 1.25 µM IAA with WPM and 2 µM BAP. All media were supplemented with 100 mg l^{-1} myo-inositol, 100 mg l^{-1} MES, and 30 g l^{-1} sucrose. Media were adjusted to a pH of 5.75, solidified with 7.5 g l^{-1} agar, and dispensed at 25 ml into 180-ml glass jars. The experiment consisted of 10 replicates with five shoots (5-10 mm in length) for a total of 50 shoots per treatment. Jars were arranged in a completely randomized design under standard culture conditions (as described above under plant material). After 5 weeks, data were collected on number of shoots, mean shoot length, multiplication rate (number of 5-10 mm microcuttings obtained per subsample per 5 weeks), and total phenolics (as described for basal nutrients). Data were subjected to regression analysis (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Rooting and acclimatization

Media used for *in vitro* rooting consisted of half-strength or quarter-strength WPM nutrients with full strength WPM vitamins supplemented with 5, 10, 20, 40 or 80 g l^{-1} sucrose, 2.5 µM IAA, 0.1 g l^{-1} myo-inositol, and 0.1 g l^{-1} MES monohydrate. Media were solidified with 7.5 g l^{-1} agar and pH adjusted to 5.75. Unrooted shoots (10 to 15 mm in length), were subcultured on 25 ml of in 180 ml jars. Each treatment consisted of six replicates each containing five axillary shoots for a total of 30 shoots per treatment. Treatments were arranged in a completely randomized design. After 4 weeks, shoots were evaluated for *in vitro* rooting and then rinsed carefully with water to reduce transfer of sucrose to the soilless media. Shoots were inserted with one leafless node placed below the surface of the media (2 peat : 1 vermiculite, v : v) in 50-cell trays in a randomized block design and placed under intermittent mist (10-s duration at 10-min intervals). Data were collected on percentage of shoots rooted at 8 weeks *ex vitro*. Data were subjected to ANOVA procedures and regression analysis (Proc GLM, SAS Version 9.1; SAS Institute, Inc., 2002).

RESULTS AND DISCUSSION

Effect of medium on axillary shoot proliferation

Axillary shoots regeneration was achieved for all basal nutrient treatments (Table 1, Fig. 3A). For both subculture periods, basal nutrients had a significant effect on shoot number ($p < 0.01$), shoot length ($p < 0.05$), multiplication rate ($p < 0.01$), and phenolics ($p < 0.01$). During both subcultures, WPM supplemented with BAP typically produced the greatest number of shoots, the longest shoots, and the highest multiplication rate after 5 weeks (Table 1). The nutrient medium SH produced the smallest amount of phenolics (7.50 ± 0.93) after 5 weeks (Table 1).

Shoot number, length, and multiplication rate for most basal nutrient treatments remained stable over the two subcultures (Table 1). However, phenolics and oxidative browning was generally lower for all treatments in the second subculture. When phenolic compounds are oxidized, toxic quinones are produced that lead to oxidative damage to plant tissues (Dobranszki and Teixeira da Silva 2010). Phenolic-reducing compounds are frequently added to media to reduce phenolics; however, frequent subcultures may be an alternate method to reduce phenolics and soluble fractions of peroxidase and polyphenoloxidase without chemically altering plant metabolism (Baziz et al. 1994, El Hadrami 1995).

In the present study, WPM basal medium produced the best shoot growth. The production of shoots was significantly influenced by nitrogen levels in the basal media. Woody plant media containing lowest total nitrogen (12.5 mM) produced the most shoots (4.27 ± 0.34) after the second subculture (Fig. 1). Further, WPM had the least amount of variability in number of shoots, shoot length, and multiplication rate between the two subcultures (Table 1). Several former studies yielded similar results using WPM. De Klerk and Korban (1994), Kaveriappa et al. (1997), and Sharma et al. (2005) successfully regenerated microshoots of *C. florida* on WPM supplemented with BAP. *Cornus nuttallii* (Edson et al. 1994), *C. mas* 'Macrocarpa' (Ďurkovič 2008), *C. officinalis* (Xue et al. 2003), and cultivars of *C. kousa* (Hadziabdic 2005) were also successfully propagated on combinations of WPM and BAP.

Effect of cytokinins on axillary shoot proliferation

Two subcultures were performed to reduce possible lag effects from previous propagation media. Due to consistency between subcultures, data is presented for second subculture only (Fig. 2). There was a significant

Table 1. Effect of basal nutrients and vitamins on *in vitro* shoot proliferation and phenolic production of *Cornus* 'NCCH1'.

Media	Subculture 1			
	Shoot number	Shoot length (mm)	Multiplication rate	Phenolics
MS	1.54 ± 0.40 b	5.11 ± 0.71 b	1.56 ± 0.41 b	12.00 ± 0.99 a
WPM	4.21 ± 0.35 a	7.76 ± 0.63 a	4.21 ± 0.36 a	11.00 ± 0.87 ab
DKW	2.16 ± 0.35 b	8.33 ± 0.63 a	2.30 ± 0.36 b	8.00 ± 0.87 c
QL	2.34 ± 0.40 b	6.79 ± 0.71 ab	2.36 ± 0.41 b	8.86 ± 0.99 bc
SH	2.11 ± 0.38 b	6.71 ± 0.67 ab	2.11 ± 0.38 b	7.50 ± 0.93 c
Media	Subculture 2			
	Shoot number	Shoot length (mm)	Multiplication rate	Phenolics
MS	1.83 ± 0.36 b	5.79 ± 0.43 c	1.86 ± 0.38 d	3.00 ± 0.46 a
WPM	4.27 ± 0.34 a	8.58 ± 0.41 a	4.97 ± 0.36 a	0.56 ± 0.43 b
DKW	2.72 ± 0.36 b	6.73 ± 0.43 bc	2.80 ± 0.38 cd	2.00 ± 0.46 a
QL	3.88 ± 0.33 a	8.50 ± 0.39 a	4.06 ± 0.34 ab	0.50 ± 0.41 b
SH	3.76 ± 0.33 a	7.12 ± 0.39 b	3.76 ± 0.34 bc	0.50 ± 0.41 b

All media treatments were supplemented with BAP at 5 µM.
Means ± standard error followed by different letters within columns are significantly different, $p < 0.05$.
Multiplication rate defined as the number of 5 - 10 mm long microcuttings produced per subsample after five weeks.
Phenolics were scored per replicate (data for each subsamples pooled) using the following scale for diameter of phenolic plume per subsample: 0 = 0 mm, 1 = 1 to 2 mm, 2 = 2 to 3 mm, 3 = 3 to 4 mm, and 4 = 4+ mm.

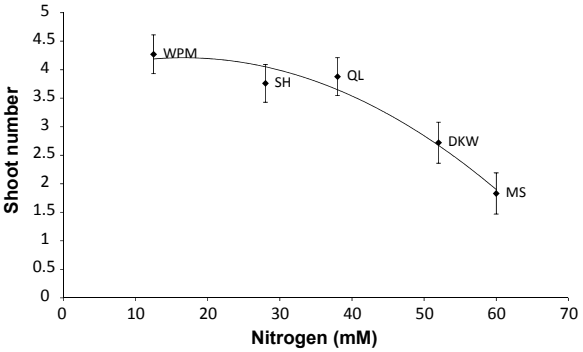

Fig. 1. Effect of basal media on axillary shoot production of *Cornus* 'NCCH1'. Symbols represent means ± standard error. Shoot number; $y = -0.0012x^2 + 0.041x + 3.8689$, $r^2 = 0.96$. Abbreviations: WPM (Lloyd and McCown woody plant medium), SH (Schenk and Hildebrandt medium), QL (Quoirin and Lepoivre medium), DKW (Driver and Kuniyuki walnut medium), and MS (Murashige and Skoog medium).

interaction between cytokinin type and concentration for number of axillary shoots, shoot length, multiplication rate, and phenolics ($p < 0.05$). After the second subculture, both number of shoots and multiplication rate exhibited a quadratic response to BAP concentration. Regression analysis predicted 9 µM BAP to provide the highest axillary shoot productivity with an estimated 4.28 shoots produced at a multiplication rate of 4.35 shoots per explant after 5 weeks (Figs. 2A and 2C). However, BAP also produced the highest phenolics (7.63 ± 0.73) of all the media treatments and exhibited many hyperhydrated shoots (data not shown). While not significant, BAP at 1.25 µM produced the longest shoots (8.98 mm ± 0.87) after 5 weeks (Fig. 2B). Similar responses were observed in Ztn, with number of shoots and multiplication rate exhibiting quadratic responses to Ztn. Regression analysis predicted 8 µM Ztn also produced high axillary shoot production with 3.19 shoots produced at a multiplication rate of 3.45 shoots per explant over 5 weeks (Figs. 2A and 2C). Both Kin and *m*T had a negative effect on shoot length while not influencing shoot production and multiplication rate (Fig. 2A,B,C).

Because of hyperhydration at higher concentrations, 5 µM BAP was considered the preferred cytokinin type and concentration. Similar concentrations of BAP were used in micropropagation of *C. nuttallii* (Edson et al. 1994), *C. canadense* (Feng et al. 2009), and *C. florida* (Kaveriappa et al. 1997, Sharma et al. 2005). The low cost and effectiveness of BAP have made it the most widely utilized cytokinin for micropropagation (Bairu et al. 2007). However, previous studies have demonstrated that BAP can accumulate in plant tissues, can be slowly released over time, and can be associated with hyperhydricity, heterogeneity of growth, or inhibition of *ex vitro* rooting in many species (Leshem and Sachs 1985, Leshem et al. 1988, Teramoto et al. 1993, Bairu et al. 2007).

Elongation

Axillary shoot production was achieved for all elongation treatments. Shoot length exhibited a quadratic response to IAA concentration ($p < 0.05$) (6.1 ± 0.3, 6.6 ± 0.5, 7.5 ± 0.5, and 5.9 ± 0.6 mm for 0, 0.1, 0.5, 1.25

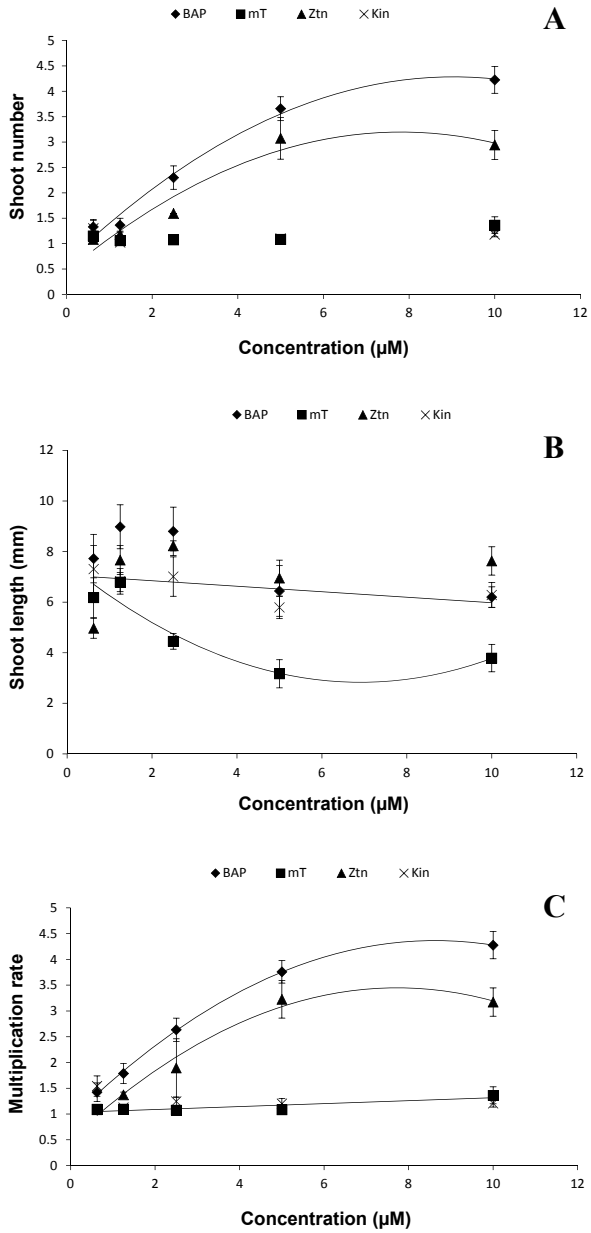

Fig. 2. Effect of cytokinin type and concentration on axillary shoot development of *Cornus* 'NCCH1'. Symbols represent means ± standard error. A) Mean number of shoots, B) Mean shoot length, C) Multiplication rate defined as the number of 10 mm long axillary shoot produced per explant after five weeks.

µM IAA respectively). A 19% increase in shoot length was achieved after 5 weeks on WPM supplemented with 5 µM BAP and 0.5 µM IAA compared to media without IAA. However, there was no significant effect on multiplication, suggesting IAA can have a positive influence on shoot length without compromising shoot number. In a previous study, IAA was also successfully utilized in shoot elongation of *C. officinalis* (Xue et al. 2003).

Auxins are associated with cell elongation in plant tissues, and can work in concert with cytokinins to enhance multiplication (Branca et al. 1991). Cytokinins such as BAP have been demonstrated to reduce apical dominance in plant meristems (Madhulatha et al. 2004). However, other studies have demonstrated that induced mutant plants that overproduce IAA exhibit increased apical dominance (Zhao et al. 2001). Therefore, auxins play a critical role in meristem biology, particularly in the apical meristem, and may interact with cytokinins to influence microshoot development (Vernoux et al. 2010). These combined effects in the present study may explain why an increase in shoot elongation was obtained without a reduction in multiplication rate.

Rooting and acclimatization

In vitro rooting was achieved, but remained low (Fig. 3B). *In vitro* roots were first observed 3 weeks after subculture. After 4 weeks only 20% of axillary shoots formed roots. All shoots (rooted and unrooted) were transferred *ex vitro*. After an additional 8 weeks *ex vitro*, there was a significant interaction between media strength and sucrose concentration for the percentage of rooted shoots ($p < 0.05$). Further, regression analysis showed for both half-strength and quarter-strength WPM the percentage of rooted shoots exhibited a negative quadratic response to sucrose concentration (Fig. 4). Highest rooting percentage (72.5 ± 10.1%) was achieved on quarter-strength WPM supplemented with 5 g l^{-1} sucrose and 2.5 µM IAA. Similarly, Hadzibdic (2005) found between 0.5 and 13.5 µM IAA effective in rooting different cultivars of *C. kousa.* Once established *ex vitro*, plantlets grew vigorous and normally (Fig. 3B,C).

The reduction in media nutrients strength and sucrose are often used to induce rooting and harden *in vitro* grown plants. For example, Pretto and Santarém (2000) achieved improved rooting for *Hypericum perforatum* using half-strength nutrients. Similarly, Sharma et al. (2011) also found improved rooting for *Jatropha curcas* with reduced sucrose and half-strength nutrients.

This research provides effective protocol for micropropagation of *Cornus* 'NCCH1'. This protocol will also provide a platform for further improvement of propagation protocols and cultivar development through ploidy manipulation, mutation treatments, and transgenic applications.

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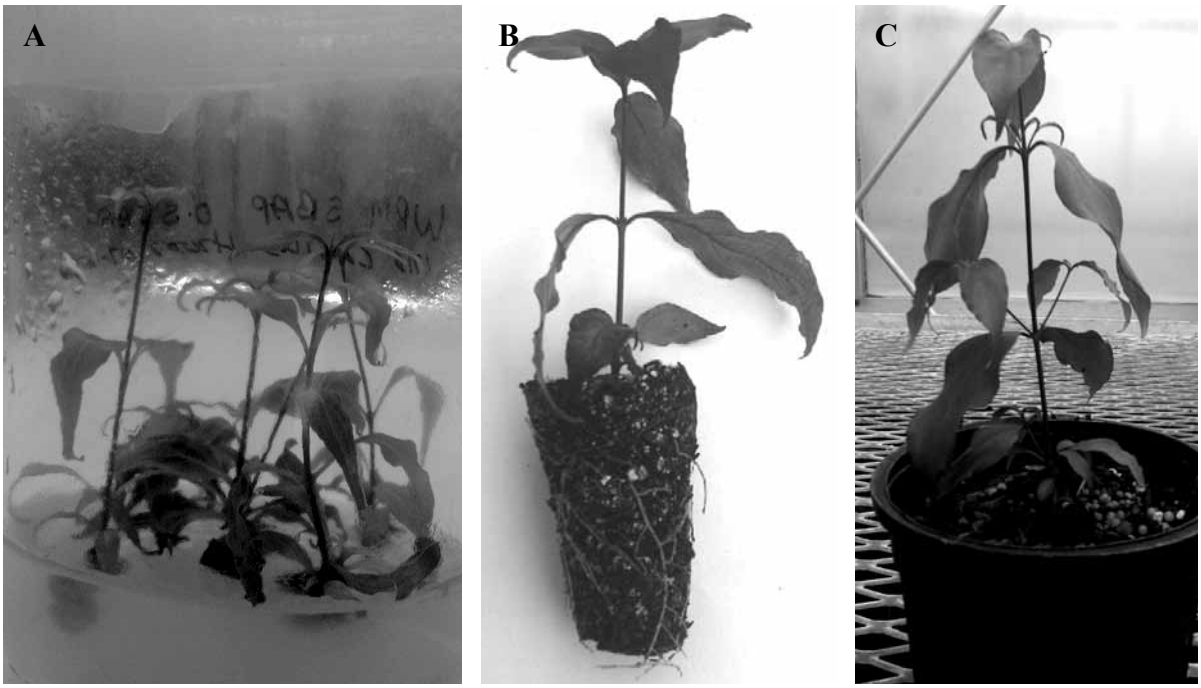

Fig. 3. *Cornus* 'NCCH1' A) *In vitro* propagated axillary shoots, B) Rooted shoot 8 weeks after being transferred to the greenhouse, C) *Cornus* 'NCCH1' 12 weeks after being transferred to the greenhouse.

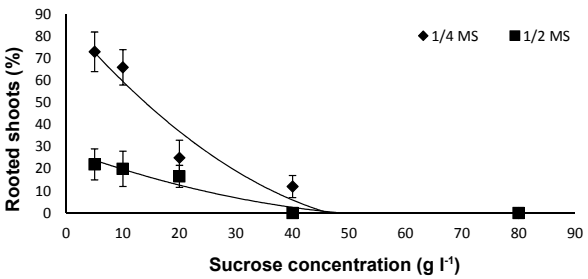

Fig. 4. Effect of media strength and sucrose concentrations on *ex vitro* root formation. Symbols represent means ± standard error.
Abbreviations: WPM (Lloyd and McCown woody plant medium). Lines represent trends fitted using quadratic regression analysis. For half-strength WPM, $y = 28.73 - 0.98x + 0.008x^2$, $r^2 = 0.97$. For quarter-strength WPM, $y = 85.69 - 2.96x + 0.024x^2$, $r^2 = 0.93$.

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