

# Micropropagation and Polyploid Induction of *Acer platanoides* ‘Crimson Sentry’<sup>1</sup>

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## Abstract

Protocols were developed for micropropagation and induction of autopolyploids in a fastigiate cultivar of Norway maple (*A. platanoides* L. ‘Crimson Sentry’). Murashige and Skoog (MS) medium, woody plant medium (WPM), and Quoirin and Lepoivre medium were supplemented with 2  $\mu$ M 6-benzylaminopurine (BA), *meta*-Topolin, 6-( $\gamma,\gamma$ -dimethylallylamino) purine, kinetin, or thidiazuron to evaluate microshoot proliferation. Murashige and Skoog medium with 2  $\mu$ M BA yielded the most microshoots (3.2) and longest microshoots (30.6 mm) per subsample after 5 weeks. The influence of BA concentration on proliferation was evaluated at 0, 2, 4, 8, or 16  $\mu$ M. Optimal multiplication rate was achieved at 2 or 4  $\mu$ M BA producing approximately 2.8 microcuttings per subsample after 5 weeks. To induce in vitro rooting, half-strength WPM was supplemented with 0, 5, 10, 20, 40, or 80  $\mu$ M indole-3-butyric acid (IBA). Optimal in vitro rooting (70%), number of roots (2.5), and root length (15 mm) per subsample were achieved with 10  $\mu$ M IBA after 8 weeks. To induce polyploidy, microcuttings were pretreated for 7 days on MS medium with 4  $\mu$ M BA alone or combined with 1  $\mu$ M IBA, indole-3-acetic acid (IAA), or 1-naphthaleneacetic acid prior to treatment in liquid MS medium containing 15  $\mu$ M oryzalin for 3 days. Homogenous tetraploids were only obtained from shoots pretreated with IAA. This research provides optimized protocols for micropropagation and autopolyploid induction of *A. platanoides* ‘Crimson Sentry’ and demonstrates the development of tetraploid lines for use in future improvement programs.

**Index words:** 6-benzylaminopurine, auxins, Norway maple, oryzalin, polyploidy.

**Species used in this study:** Norway maple ‘Crimson Sentry’ (*Acer platanoides* L.).

**Chemical used in this study:** BA (6-benzylaminopurine), IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), 2iP (6-( $\gamma,\gamma$ -dimethylallylamino) purine), kin (kinetin), *mT* (*meta*-Topolin 6-( $\gamma,\gamma$ -dimethylallylamino)), TDZ (Thidiazuron (1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea)).

## Significance to the Nursery Industry

Norway maple (*Acer platanoides* L.) is a valuable landscape tree known for its attractive foliage and architecture. After its introduction to North America by Bartram in 1756, Norway maple has played a major role in replacing the American elm following Dutch elm disease. Pest and disease resistance, and tolerance of poor soils have made Norway Maple a popular choice as a municipal street tree. However, Norway maple has become invasive in disturbed forests, along roadside edges, and within intact forests bordering ornamental plantings in the Northeast and Midwest United States. Inducing autopolyploids may improve ornamental features of Norway maple and provide an opportunity to develop triploid, seedless cultivars. In this study, in vitro regeneration protocols were developed for micropropagation and induction of polyploidy as a platform for future breeding for sterile cultivars and novel traits

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## Introduction

Norway maple (*Acer platanoides* L.) was introduced to North America by Bartram in 1756 (31) and has served as an important substitute for American elm (*Ulmus americana* L.) following the introduction of Dutch elm disease (45). Its attractive architecture, pest and disease resistance, and tolerance of poor soils and pollution have made it a successful municipal street tree (4). *Acer platanoides* has increased in popularity as new cultivars have become widely available for the urban landscape. Unfortunately, *A. platanoides* has become invasive in some areas and often naturalizes in areas bordering landscape plantings (16, 37, 38, 45).

Although *A. platanoides* can be propagated by seed, plants tend to display a diverse range of phenotypes. Therefore, elite cultivars with improved ornamental characteristics have typically been propagated asexually by budding onto seedling rootstocks (19). Micropropagation can provide an alternative means for rapid propagation of valuable ornamental cultivars, as well as providing a platform for improving ornamental traits. Studies on micropropagation of *A. platanoides* have focused on wild types (8, 19) or the popular cultivar ‘Crimson King’ (22, 23), with a single report on cultivar ‘Crimson Sentry’ (5). Initial studies on micropropagation of *A. platanoides* ‘Crimson Sentry’ utilized Murashige and Skoog (MS) medium (29) for microshoot regeneration (5). More recent studies employed Linsmaier and Skoog (LS) medium (20) and woody plant medium (WPM) (21) for *A. platanoides* ‘Crimson King’ (22, 23) and wild type *A. platanoides* (8, 19), respectively.

The cytokinin 6-benzylaminopurine (BA), used solely or in combination with other plant growth regulators, was the most commonly used cytokinin for in vitro propagation

of *A. platanoides*. Marks and Simpson (22) reported 1  $\mu\text{M}$  BA alone promoted microshoot formation in *A. platanoides* 'Crimson King'. Additional studies showed that microshoot proliferation could be increased in *A. platanoides* 'Crimson King' when medium containing BA was supplemented with low concentrations of thidiazuron (TDZ); however, significant callus formation was also observed (23). Lindén and Riikonen (19) demonstrated significant microshoot proliferation of apical and axillary buds of *A. platanoides* with 0.01 to 0.1  $\mu\text{M}$  of TDZ. Kinetin (Kin) has also proven effective for inducing microshoot regeneration in *A. platanoides* (8, 19). For *A. platanoides* 'Crimson Sentry', in vitro cultures were established using 5  $\mu\text{M}$  BA in combination with 0.5 to 5  $\mu\text{M}$  indole-3-butyric acid (IBA); however, exact proliferation rates were not provided (5).

Topolins are a group of natural aromatic cytokinins having a similar structure to BA, yet have not been associated with hyperhydricity, heterogeneity of growth, or inhibition of rooting (1, 2). However, effects of *meta*-Topolin (*mT*) on in vitro growth and development of *A. platanoides* have yet to be investigated. Toxicity of BA was the result of its stable nature within the plant, whereas *mT* and its metabolites were easily degradable, and accumulation of *mT* was prevented by its rapid translocation in plant tissues (1, 2). In addition, *mT*'s origin in *Populus* may indicate a greater activity in woody plants (2, 43), and therefore a potential usefulness for in vitro culture of *A. platanoides*.

In vitro rooting of microcuttings of *A. platanoides* usually consists of low salt medium supplemented with IBA (5, 23). Marks and Simpson (23) achieved rooting for cultivar 'Crimson King' when microcuttings were cultured for 7 days on half-strength modified LS medium containing 5  $\mu\text{M}$  IBA, followed plant growth regulator free by half-strength modified LS media. Similarly, Đurković (8) found microcuttings of *A. platanoides* successfully rooted on half-strength WPM supplemented with 5  $\mu\text{M}$  IBA.

In vitro regeneration protocols provide an excellent platform for inducing polyploidy. Induced polyploids may be useful for overcoming hybridization barriers, producing sterile cultivars, restoring fertility to wide hybrids, enhancing pest resistance and stress tolerance, enhancing vigor, or enlarging flowers, leaves, or fruit (36). Polyploidy is usually achieved by treating plant tissues with a spindle fiber inhibitor such as colchicine, oryzalin, or trifluralin (36). Colchicine [N-5,6,7,9-tetrahydro-1,2,3,10-tetra-methoxy-9-oxobenzo(a)heptalen-7-yl] acetamide is the most common mitotic inhibitor based on the ability to be autoclaved without affecting the capacity to promote polyploidy (7, 46). However, colchicine's affinity for animal microtubules makes it also toxic to humans (7, 27) while its weak affinity for plant tubulins necessitates higher doses to maintain effectiveness in plant tissues (7). In contrast, oryzalin (3,5-dinitro-N4,N4-dipropylsufanilamide) is a mitosis-inhibiting dinitroaniline herbicide with a high affinity for plant tubulin dimers (26). This affinity allows for effective use of oryzalin at low concentrations with reduced toxicity and incidences of mutation or abnormal growth (3, 7, 11, 26). Oryzalin has been used successfully to create polyploid lines of woody and semi-woody plants including *Rosa* L. (14), *Rhododendron* L. (10, 12, 44), *Chaenomeles* Lindl. (42), *Hypericum* L. (25), and *Berberis* L. (18).

Development of an efficient micropropagation protocol for *A. platanoides* 'Crimson Sentry' would provide a mechanism for rapid propagation of elite cultivars and provide a platform

for ploidy manipulation. Thus, the objectives of this research were to 1) develop in vitro protocols for propagation of the popular columnar cultivar *A. platanoides* 'Crimson Sentry', and 2) develop procedures for polyploid induction of *A. platanoides* 'Crimson Sentry' for future use in developing novel seedless cultivars.

## Methods and Materials

**Plant material and culture conditions.** Actively growing apical and axillary shoots were used to initiate in vitro cultures. Actively growing shoots were collected from a 3 year old field grown plants and rinsed under tap water for 4 h. Explants were surface-disinfested in 20% (v/v) bleach (6.15% NaOCl) solution containing two to three drops of Tween® 20 (Sigma-Aldrich Corporation, St. Louis, MO) for 17 min with periodic mixing followed by three 5-min rinses in sterile distilled water. Explants were cultured on regeneration medium consisting of MS basal salts and vitamins supplemented with 2  $\mu\text{M}$  BA, 100 mg·liter<sup>-1</sup> *myo*-Inositol, 100 mg·liter<sup>-1</sup> 2-(N-Morpholino) ethanesulfonic acid (MES) monohydrate, and 30 g·liter<sup>-1</sup> sucrose. Media were solidified with 7.5 g·liter<sup>-1</sup> agar (Phytotechnology Laboratories, Shawnee Mission, KS) and adjusted to pH of 5.75, and 25 mL was dispensed to 180-mL glass jars. Regenerated microshoots were maintained by transfer to fresh regeneration medium every 4 to 6 weeks and incubated under standard culture conditions [ $23 \pm 2^\circ\text{C}$  and a 16 h photoperiod of 75  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  provided by cool-white fluorescent lamps].

**Basal salts and cytokinins.** Effects of basal salts and cytokinins on microshoot growth and proliferation were examined. Media treatments included MS salts and vitamins, WPM salts and vitamins, and Quoirin and Lepoivre (35) salts (QL) and Gamborg vitamins (9) each supplemented with 2  $\mu\text{M}$  BA, TDZ, 2iP, *mT*, or Kin. All media were supplemented with 100 mg·liter<sup>-1</sup> *myo*-Inositol, 100 mg·liter<sup>-1</sup> MES, and 30 g·liter<sup>-1</sup> sucrose, solidified with 7.5 g·liter<sup>-1</sup> agar and adjusted to pH 5.75. Media (25 mL) was dispensed into 180-mL glass jars. Five microcuttings (10–20 mm long) were placed vertically in each jar. Six replicates of each media composition were incubated under standard culture conditions, as described previously, in a completely randomized design. After 5 weeks, data were recorded on the number of microshoots, microshoot length (of longest shoot), number of leaves, and number of nodes (of longest microshoot). Microshoot length was measured from the apical meristem to the base of the microshoot, callus not included. Data sets were subjected to analysis of variance (ANOVA) and means were separated using Fisher's least significant difference (LSD) (40).

**Cytokinin concentration.** To further improve microshoot proliferation of *A. platanoides* 'Crimson Sentry', a second study was conducted to optimize cytokinin concentration from the best resulting medium from the basal salt-cytokinin experiment. Media consisted of MS salts and vitamins with BA at 0, 2, 4, 8, or 16  $\mu\text{M}$ . All media were supplemented with 100 mg·liter<sup>-1</sup> *myo*-Inositol, 100 mg·liter<sup>-1</sup> MES, and 30 g·liter<sup>-1</sup> sucrose, solidified with 7.5 g·liter<sup>-1</sup> agar and adjusted to pH 5.75. Media (25 mL) was dispensed into 180-mL glass jars. The experiment consisted of seven replicates with five subsamples (20 mm microcuttings) per treatment. Jars were arranged in a completely randomized design under standard culture conditions (as described previously). After 5 weeks,

data were recorded on the number of microshoots, microshoot length (of longest shoot), number of leaves, and number of nodes (of longest shoot). Microshoot length was measured from the apical meristem to the base of the microshoot, callus not included. Data were subjected to regression analysis (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

**Rooting.** Media used for in vitro rooting consisted of half-strength WPM salts and vitamins supplemented with 0, 5, 10, 20, 40, or 80  $\mu\text{M}$  IBA, 30 g·liter<sup>-1</sup> sucrose, 100 mg·liter<sup>-1</sup> MES, and 100 mg·liter<sup>-1</sup> *myo*-Inositol. Media were solidified with 7.5 g·liter<sup>-1</sup> agar and adjusted to a pH of 5.75. Microcuttings 10 to 20 mm long were subcultured 25 mL of media in 180-mL jars. Each of the six treatments consisted of six replications with five subsamples each. All jars were completely randomized under standard culture conditions as described previously. Following 8 weeks of growth, microshoots were scored for in vitro rooting percentage, number of roots, and root length (longest root). Data were subjected to regression analyses (40).

**Polyploid induction.** A  $2 \times 3 \times 4$  complete factorial design was used to test effects of BA, oryzalin exposure time, and oryzalin concentration on polyploid induction of *A. platanoides* 'Crimson Sentry'. Liquid media (adjusted to pH 5.75) containing MS salts and vitamins, 100 mg·liter<sup>-1</sup> *myo*-Inositol, 100 mg·liter<sup>-1</sup> MES, and 30 g·liter<sup>-1</sup> sucrose were supplemented with 0 or 2  $\mu\text{M}$  BA and 0, 15, 30, or 45  $\mu\text{M}$  oryzalin. Microshoots 10–20 mm long were first cultured horizontally on regeneration medium in 90 mm diameter petri dishes for 7 days. A stock solution of 3 mM oryzalin was dissolved in 95% ethanol and added to autoclaved media in specified volumes to achieve the desired concentrations. A final concentration of 0.9% ethanol was added to all treatments, including controls. Microshoots were then transferred to 100 mL jars containing 25 mL liquid oryzalin medium and placed on a platform shaker in the dark at 23°C for 3, 5, or 7 d. Microshoots were then washed for 24 h in liquid regeneration medium to remove excess oryzalin and cultured on solidified regeneration medium. Each treatment consisted of six replicates with five subsamples (microshoots) each. All jars were completely randomized under standard culture conditions as described for microshoot initiation.

A second trial was established to evaluate the influence of auxin pretreatments on polyploidy induction of *A. platanoides* 'Crimson Sentry'. Microshoots (10 to 20 mm long) were cultured horizontally on pretreatment medium consisting of MS salts and vitamins, 100 mg·liter<sup>-1</sup> *myo*-Inositol, 100 mg·liter<sup>-1</sup> MES, 30 g·liter<sup>-1</sup> sucrose and pH 5.75. Pretreatment medium was supplemented with either 4  $\mu\text{M}$  BA, 4  $\mu\text{M}$  BA and 1  $\mu\text{M}$  IBA, 4  $\mu\text{M}$  BA and 1  $\mu\text{M}$  indole-3-acetic acid (IAA) or 4  $\mu\text{M}$  BA and 1  $\mu\text{M}$  1-naphthaleneacetic acid (NAA) for 7 days. Each treatment consisted of six replicates with five subsamples each. All jars were completely randomized under standard culture conditions as described for microshoot initiation. Microshoots were then transferred to jars containing liquid MS media supplemented with 15  $\mu\text{M}$  oryzalin (as described previously) for 3 days. Following treatment, microshoots were washed for 24 h in liquid regeneration medium to remove excess oryzalin and then cultured on solidified regeneration media.

After 8 weeks, data were recorded on percent survival and number of microshoots produced. Ploidy level was

determined via flow cytometry. For each sample, 1 to 2 expanded leaves were placed in a petri dish containing 400  $\mu\text{L}$  of nuclei extraction buffer (CyStain ultraviolet Precise P Nuclei Extraction Buffer®; Partec, Münster, Germany) and chopped finely with a razor blade until the sample was completely incorporated into the buffer. The resulting solution was passed through a Partec CellTrics™ disposable filter with a pore size of 50  $\mu\text{m}$  to remove debris. Then, 1.2 mL of a nucleic acid staining buffer (4', 6-diamidino-2-phenylindole; CyStain ultraviolet Precise P Staining Buffer®; Partec) was added to the filtered solution, and resulting stained nuclei were analyzed with a flow cytometer (Partec PA II; Partec). Ploidy level for each sample was determined by comparing peak position with that of a known diploid microshoot of *A. platanoides* 'Crimson Sentry'. Three microshoots (subsamples) were analyzed for each replicate.

## Results and Discussion

**Basal salts and cytokinins.** Microshoot regeneration was achieved for all treatments. There were significant interactions between basal salts and cytokinins that affected both microshoot length ( $P < 0.01$ ) and number of nodes ( $P < 0.05$ ) (Table 1). Murashige and Skoog medium with 2  $\mu\text{M}$  BA produced the longest mean microshoot length ( $30.6 \pm 1.4$  mm) and there was a general trend of BA producing longer microshoots among all medium types. The most nodes per microshoot were produced with MS medium and BA ( $3.6 \pm 0.2$ ), as well as QL medium and BA ( $3.5 \pm 0.2$ ). Microshoot number was influenced only by cytokinin ( $P < 0.01$ ). In general, BA and *mT* produced the highest number of microshoots across all basal salt treatments. Both medium and cytokinin type independently influenced mean leaf number ( $P < 0.01$  and 0.01, respectively) with MS medium and BA generally producing the most leaves per microshoot.

In the present study, MS medium with 2  $\mu\text{M}$  BA produced high quality microshoot growth responses, with high microshoot regeneration and longer microshoots. Similarly, Cheng (5) achieved effective microshoot regeneration with MS medium and 5  $\mu\text{M}$  BA for *A. platanoides* 'Crimson Sentry'. In other studies, low concentrations of TDZ have induced microshoot regeneration of *A. platanoides* wild type and *A. platanoides* 'Crimson King' in combination with WPM and LS medium (19, 23). However, TDZ at 2  $\mu\text{M}$  in the present study was one of the poorest performing cytokinins on *A. platanoides* 'Crimson Sentry' (Table 1). Therefore, different responses of *A. platanoides* to cytokinins could be attributed to genetic variation between cultivars.

Genetic diversity within species often leads to variability of in vitro responses of different cultivars. Studies on a diverse range of species have shown that genotype was one of the most significant factors influencing the development of tissue culture protocols (17, 24, 28, 33). For *Malus Tourn. ex L.*, genotype was a significant factor influencing in vitro cold storage and regeneration (17). Similarly, significant variation regarding microshoot development and subsequent root formation was observed among 32 cultivars of *Vitis vinifera* L. (33).

**Cytokinin concentration.** Regression analysis of microshoot data revealed BA concentration had no significant effect on microshoot number (Fig. 1A). However, microshoot length increased from  $17.40 \pm 0.44$  mm at 0  $\mu\text{M}$  BA to  $27.43 \pm 2.07$  mm at 2  $\mu\text{M}$  BA before declining linearly with



**Table 1.** In vitro growth responses of *Acer platanoides* ‘Crimson Century’ on medium supplemented with 2 µM cytokinin.

Media <sup>a</sup>	Cytokinin	Shoot number <sup>y</sup>	Shoot length (mm) <sup>y</sup>	Leaves <sup>y</sup>	Nodes <sup>y</sup>
MS	BA	3.2 ± 0.3ab <sup>x</sup>	30.6 ± 1.4a	8.1 ± 1.0ab	3.6 ± 0.2a
	TDZ	0.9 ± 0.0c	10.8 ± 0.5g	2.4 ± 0.4g	1.2 ± 0.1h
	2iP	1.9 ± 0.7d	12.0 ± 0.9fg	6.3 ± 1.1cde	2.2 ± 0.2ce
	mT	3.1 ± 0.2ae	21.4 ± 2.4bc	7.5 ± 0.6ac	2.9 ± 0.2b
	Kin	1.8 ± 0.2d	9.9 ± 1.1g	5.5 ± 0.6de	1.9 ± 0.2ef
WPM	BA	3.9 ± 0.5ab	22.1 ± 1.8bc	7.8 ± 0.8ac	2.9 ± 0.2b
	TDZ	0.9 ± 0.0c	12.0 ± 0.9f	2.4 ± 0.4g	1.4 ± 0.1gi
	2iP	2.1 ± 0.3df	15.0 ± 0.8ef	4.9 ± 0.2ef	2.1 ± 0.1de
	mT	2.9 ± 0.4af	19.7 ± 3.1cd	6.8 ± 0.5bcd	2.6 ± 0.2bc
	Kin	2.3 ± 0.1def	10.9 ± 0.5g	4.5 ± 0.2ef	1.8 ± 0.1e
QL	BA	3.5 ± 0.2ab	24.8 ± 3.0b	8.9 ± 0.5a	3.5 ± 0.2a
	TDZ	0.9 ± 0.1c	10.3 ± 1.6g	2.2 ± 0.4g	1.1 ± 0.2i
	2iP	1.7 ± 0.3cd	9.2 ± 0.2g	3.7 ± 0.5fg	1.6 ± 0.1fgh
	mT	2.5 ± 0.3def	16.9 ± 1.5de	5.9 ± 0.6de	2.4 ± 0.2cd
	Kin	2.4 ± 0.2def	10.1 ± 0.4g	3.6 ± 0.3fg	1.8 ± 0.2eg
Analysis of Variance <sup>w</sup>					
Media		NS	*	**	*
Cyto		**	**	**	**
Media × Cyto		NS	**	NS	*

<sup>a</sup>Key to media abbreviations: MS (Murashige and Skoog basal salts and vitamins) WPM (woody plant medium basal salts and vitamins) QL (Quoirin and Lepoivre salts and Gamborg vitamins), BA (6-benzylaminopurine), TDZ (thidiazuron), mT (*meta*-Topolin), 2iP (6-( $\gamma$ , $\gamma$ -dimethylallylamino) purine), Kin (Kinetin).

<sup>y</sup>Values represent means ± SEM. Means followed by different letters within columns are significantly different,  $P < 0.05$ .

<sup>x</sup>Mean separation within columns by Fishers LSD,  $P < 0.05$ ,  $n = 6$

<sup>w</sup>NS, \*, \*\*: Nonsignificant or significant at  $P < 0.05$  or  $< 0.01$ , respectively.

increasing BA concentration ( $P < 0.01$ ) (Fig. 1B). Similarly, multiplication rate (number of 20 mm microcuttings obtained per subsample after 5 weeks) increased from 1.40 at 0 µM BA to 2.83 at 4 µM BA before exhibiting a negative linear response to increasing BA concentrations ( $P < 0.01$ ) (Fig. 1C). Optimum BA concentration occurred in the range of 2 to 4 µM BA (Fig. 1C). In comparison, 1 µM BA promoted microshoot formation in *A. platanoides* ‘Crimson King’ (22), again suggesting variation between cultivars.

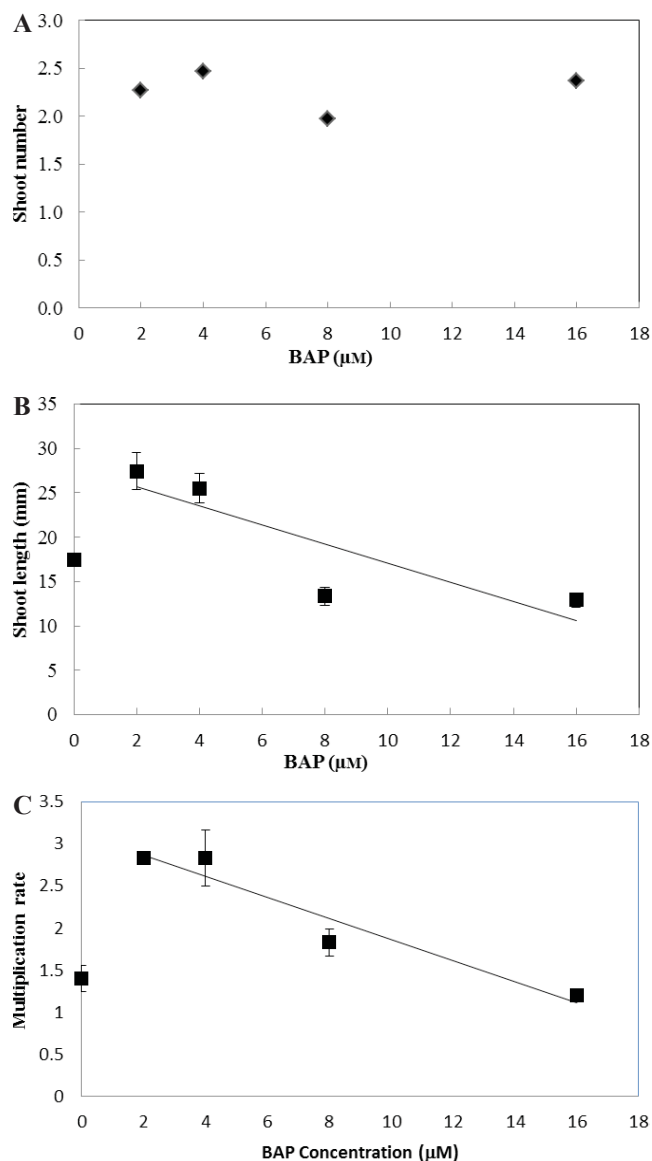
**Rooting.** After 8 weeks, in vitro root formation was observed for all media treatments containing IBA, whereas no root formation was observed in the control (0 µM IBA) treatments. Regression analysis indicated that percent rooting, number of roots per microcutting, and root length exhibited a negative linear response to IBA concentration ( $P < 0.01$ , 0.05, and 0.01, respectively) (Fig. 2A, B and C). While there was a significant negative linear response, the number of roots produced per microcutting was variable with highest root production achieved between 10 and 40 µM IBA ( $3.03 \pm 0.85$  and  $3.37 \pm 1.03$ , respectively). However, the highest rooting percentage ( $77 \pm 13$  and  $70 \pm 12$ , respectively) and longest roots ( $17.13 \pm 3.10$  and  $17.85 \pm 3.47$ , respectively) were achieved at 5 and 10 µM IBA (Fig. 2A and C), suggesting lower IBA concentrations (5 to 10 µM) are optimal for in vitro root production of *A. platanoides* ‘Crimson SENTRY’. This optimal rooting medium contained similar concentrations of IBA compared to previous studies on *A. platanoides* ‘Crimson King’ that utilized 5 µM IBA (23) and 4.9 µM IBA (8).

**Polyploid induction.** Percentage of surviving microcuttings was influenced by BA and oryzalin concentration ( $P <$

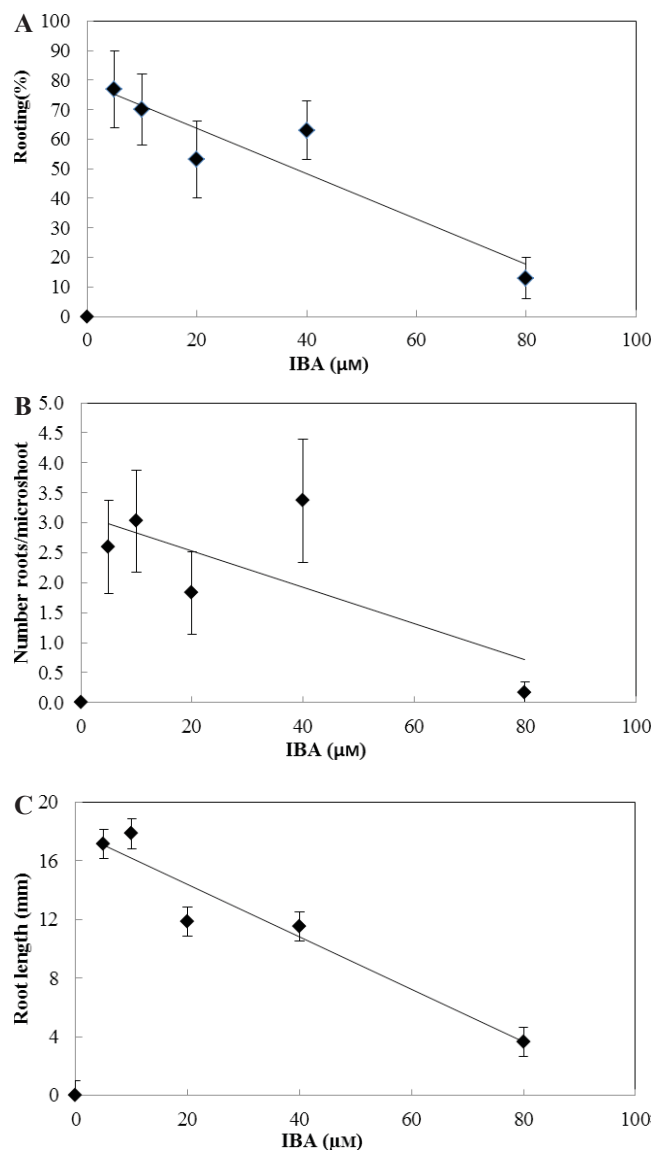
0.01) (Fig. 3), but there were no effects of treatment durations (data not presented). Survival of microcuttings for both 0 and 2 µM BA treatments had a negative quadratic response to increasing oryzalin concentrations ( $P < 0.01$ ) (Fig. 3). Addition of BA to medium increased microcutting fatality at higher oryzalin concentrations, regardless of exposure duration (Fig. 3). No explants survived the treatment combination of 2 µM BA and 45 µM oryzalin. As a cytokinin, BA may act to increase cell cycling, producing cells that more quickly transition from the G2 to M phase (metaphase to anaphase) (39, 41) where they may be susceptible to oryzalin toxicity.

Mixoploids (tissues containing 2× and 4× cells) and what appeared to be homogeneous tetraploids (4×) were induced successfully by oryzalin. However, after 6 months all apparent tetraploids had reverted to diploids. Production of polyploids (% mixoploids and % tetraploids) exhibited a quadratic response to oryzalin concentration [ $y = 1.0 + 2.4x - 0.055x^2$  ( $P < 0.01$ )]. No significant effects were observed from length of exposure, BA concentration, or their interaction (data not shown). The largest percentage (27%) of polyploids was recovered from treatment with 15 µM oryzalin (data not shown). While length of exposure was not significant across all treatments, highest number of polyploids occurred at 3 days (data not shown).

Successful chromosome doubling has been achieved for several woody plant species using similar oryzalin concentrations (7). For example, Stanys et al. (42) achieved successful chromosome doubling for *Chaenomeles japonica* using oryzalin concentrations between 10 and 50 µM. One possible explanation for instability of tetraploids in our study may be a high propensity for diplontic selection within the apical meristem. Every initial cell within all three histogenic lay-



**Fig. 1.** Influence of BA concentration on microshoot development of *Acer platanoides* 'Crimson Sentry'. Symbols represent means,  $n = 7$ ,  $\pm$  SEM. A) Number of microshoots produced per microcutting; B) Length of longest microshoot;  $y = 27.86 - 1.076x$ ,  $r^2 = 0.74$  and C) Multiplication rate defined as the number of 20 mm microcuttings produced per subsample after 5 weeks;  $y = 15.57 - 0.63x$ ,  $r^2 = 0.93$ . Regression equations and lines do not include control treatments.



**Fig. 2.** Influence of IBA concentration on in vitro root development of *Acer platanoides* 'Crimson Sentry'. Values are means,  $n = 6$ ,  $\pm$  SEM. A) Number of microcuttings producing in vitro roots;  $y = 0.788 - 0.0076x$ ,  $r^2 = 0.86$  B) Number of in vitro roots per microcutting;  $y = 3.14 - 0.03x$ ,  $r^2 = 0.53$  and C) Length of the longest root in vitro;  $y = 17.98 - 0.17x$ ,  $r^2 = 0.92$ . Regression equations and lines do not include control treatments.

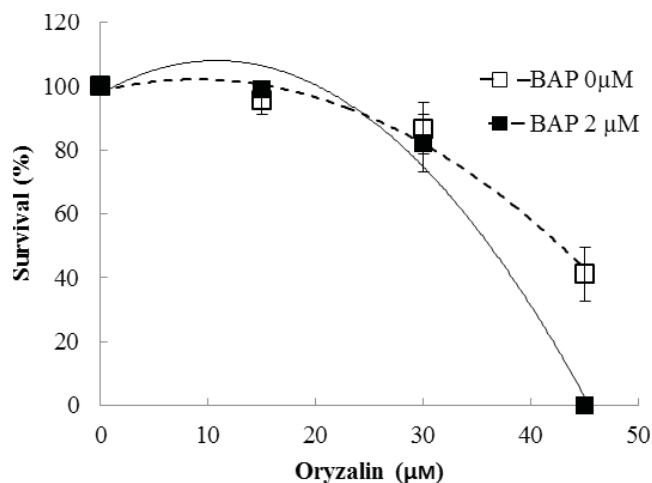
ers of the apical meristem must be affected by oryzalin for successful homogeneous tetraploids to be formed (6, 13). If all initial cells were not affected, a cytochimeric mosaic of ploidy levels (mixoploid) can form. Diplontic selection may result if diploid cells, having less DNA content, take less time to replicate and divide than competing polyploid cells (13, 15). Over time, the proportion of diploid cells to polyploid cells may increase resulting in a loss of converted cells.

In the second trial, addition of auxins to pre-treatment medium significantly influenced production of polyploids ( $P < 0.05$ ) (Table 2). Although mixoploids were induced in all treatments, homogeneous tetraploids only developed when pre-treatment media was supplemented with IAA (Table 2).

In contrast to the first trial, all tetraploids have remained stable for 10 months.

To facilitate production of stable tetraploids, addition of IAA to pretreatment medium may work indirectly to aid oryzalin. During the cell cycle, auxins interact synergistically with cytokinins to regulate expression of cyclin-dependant kinases (CDKs). More specifically, auxins induce CDKs involved in G1 to S and G2 to M transitions (34). Exogenous application of auxins, particularly IAA, may facilitate initiation of the cell cycle in quiescent cells thereby ensuring cells enter M phase where oryzalin is most active.

Auxins, particularly IAA, have also been associated with an increase in the ubiquitous signal molecule nitric oxide (32).



**Fig. 3.** Survival rates of microshoots of *A. platanoides* 'Crimson Sentry' as a factor of oryzalin concentration and treatment with 0 or 2  $\mu\text{M}$  BA. Lines represent trends fitted using quadratic regression analysis. For 0  $\mu\text{M}$  BA,  $y = 99 + 0.70x - 0.04522x^2$ ,  $r^2 = 0.94$ . For 2  $\mu\text{M}$  BA,  $y = 97 + 1.90x - 0.0892x^2$ ,  $r^2 = 0.91$ .

Nitric oxide can react with tyrosine to form nitrotyrosine (30). Jovanovic et al. (13) demonstrated that nitrotyrosine increased sensitivity of *Nicotiana tabacum* L. cell cultures to oryzalin. Therefore, in the present study, addition of IAA to the medium may have indirectly led to an increase in sensitivity to oryzalin.

Results of this research provide effective protocols for micropropagation and polyploid induction of *A. platanoides* 'Crimson Sentry'. Tetraploid plants induced in this study will be used in future plant improvement programs with the objective of developing seedless, triploid *A. platanoides* 'Crimson Sentry'. These methods also provide a platform for future improvement of other cultivars of *A. platanoides*.

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**Table 2.** Influence of growth regulator pretreatment on percent polyploid induction of micropropagated shoots of *Acer platanoides* 'Crimson Sentry' treated with 15  $\mu\text{M}$  oryzalin.

Growth regulators <sup>a</sup>	Diploid (%)	Mixoploid (%)	Tetraploid (%)
4 $\mu\text{M}$ BA	67 $\pm$ 0b <sup>y</sup>	33 $\pm$ 0ab	0 $\pm$ 0b
4 $\mu\text{M}$ BA + 1 $\mu\text{M}$ IBA	78 $\pm$ 11ab	22 $\pm$ 11bc	0 $\pm$ 0b
4 $\mu\text{M}$ BA + 1 $\mu\text{M}$ IAA	33 $\pm$ 12c	50 $\pm$ 7a	17 $\pm$ 5a
4 $\mu\text{M}$ BA + 1 $\mu\text{M}$ NAA	94 $\pm$ 5c	6 $\pm$ 5c	0 $\pm$ 0b

<sup>a</sup>Key to media and plant growth regulators: BA (6-benzylaminopurine), IBA (indole-3-butyric acid), IAA (indole-3-acetic acid), NAA (1-naphthaleneacetic acid).

<sup>y</sup>Values represent means  $\pm$  SEM. Means followed by different letters within columns were significantly different,  $P < 0.05$ .

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