ABSTRACT

LATTIER, JASON DANIEL. Cytogenetics, Micropropagation, and Polyploid Induction of Selected Nursery Crops. (Under the direction of Dr. Thomas G. Ranney).

Protocols were developed for micopropagation and polyploid induction of Norway Maple (*A. platanoides* 'Crimson Sentry'). Basal salts including Murashige and Skoog Medium (MS), Woody Plant Medium (WPM), and Quoirin and Lepoivre Medium (QL) were supplemented with 2 µM 6-benzylaminopurine (BAP), *meta*-Topolin (*m*T), 6-(γ,γ-dimethylallylamino) purine (2iP), kinetin (Kin), or thidiazuron (TDZ) to evaluate microshoot proliferation. The combination of MS and 2 µM BAP yielded the most microshoots (3.2) and longest microshoots (30.6 mm) per subsample after 5 weeks. The influence of BAP concentration on proliferation was evaluated at 0, 2, 4, 8, or 16 µM. Optimal multiplication was achieved at 2 to 4 µM BAP producing 2.83 microcuttings per subsample after 5 weeks. To induce in vitro rooting, half-strength WPM was supplemented with 0, 5, 10, 20, 40, or 80 µM 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 µM IBA after 8 weeks. To induce polyploidy, microcuttings were pretreated for 7 days on MS with 4 µM BAP alone or combined with 1 µM IBA, indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA) prior to treatment in liquid MS containing 15 µM oryzalin (OZ) for 3 days. Homogenous tetraploids were only obtained from shoots pretreated with IAA.

Rhododendron canadense represents a morphologically unique and taxonomically complicated species of azalea. Previous studies have yielded confusing reports of unusually small genome sizes and both diploid and tetraploid individuals. This study utilized flow cytometry and traditional cytology to provide evidence that *R. canadense* exists primarily as

a diploid species. Determination of relative genome sizes revealed that *R. canadense* has approximately 26% less DNA than most other deciduous azaleas. Also, a further cytological investigation indicated that *R. canadense* may possess a lower base chromosome number with $2n = 2x = 24$ compared to most other diploid *Rhododendron* with $2n = 2x = 26$.

Liriope and *Ophiopogon* (collectively liriopogons) represent important evergreen groundcovers cultivated throughout the world. Breeding efforts are constrained by taxonomic confusion and lack of information on ploidy levels and cytogenetics. This study examined an extensive collection of liriopogons by identification of taxa, cytometric determination of genome sizes, and cytological evaluation of ploidy. *Liriope* taxa examined formed diploid, tetraploid, and hexaploid groups. The diploid group included *L. graminifolia*, *L. longipedicellata*, *L. minor*, and some of the *L. platyphylla*. The tetraploid group included *L. muscari* and the remaining *L. platyphylla*. The hexaploid group included *L. exiliflora*. *Ophiopogon* taxa examined formed two ploidy groups. The diploid group included *O. intermedius*, *O. jaburan*, *O. planiscapus*, and *O umbraticola*. The tetraploid/hypotetraploid group included *O. japonicus*. This research will aid future taxonomic revisions and assist in developing breeding strategies for liriopogons.

Micropropagation protocols were developed for the hybrid dogwood H2007-017-154 (*Cornus kousa* 'Miss Satomi' x *Cornus hongkongensis* 'Summer Passion'). Basal salts including Driver and Kuniyuki Walnut (DKW), Schenk and Hildebrandt (SH), MS, WPM, and QL media were each supplemented with 5 µM BAP to evaluate microshoot proliferation. Cytokinins were evaluated on WPM combined with zeatin (Ztn), BAP, *m*T, or Kin at 0.625, 1.25, 2.5, 5, or 10 µM. Combining WPM with 5 µM BAP produced an optimal multiplication rate of 4.97 microcuttings per subsample after 5 weeks. The addition of 0.5

μM IAA to the multiplication medium produced a 19% increase in shoot length after 5 weeks. In vitro treatments of WPM supplemented with IAA or NAA at 0.625, 1.25, 2.5, 5, or 10 μM were not efficient to induce in vitro rooting.

© Copyright 2012 by Jason Daniel Lattier

All Rights Reserved

Cytogenetics, Micropropagation, and Polyploid Induction of Selected Nursery Crops

by
Jason Daniel Lattier

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Horticultural Science

Raleigh, North Carolina

2012

APPROVED BY:

_____	_____
Dr. Thomas G. Ranney Committee Chair	Dr. Paul R. Fantz
_____	_____
Dr. Dennis J. Werner	Dr. Darren H. Touchell

DEDICATION

For my loving parents, Richard Oscar Lattier & Jan Permenter Lattier.

BIOGRAPHY

Jason Daniel Lattier was born in the loving home of Jan and Richard Lattier and raised in Raleigh, North Carolina. Jason's love of horticulture began at an early age. As a child, he had the good fortune of having his grandparents, Oscar and Louise, living next door. His frequent walks through their gardens began his fascination with the natural world. Many cherished days were spent harvesting corn and snap peas with Oscar or cutting roses and planting bulbs with Louise. However, it was not until late in his undergraduate career at NC State University that Jason rediscovered his love of plants. With the love and support of his parents, Jason graduated with undergraduate degrees in botany and horticultural science. Jason's passion for plants then took him around the world in pursuit of greater learning opportunities.

In the years after graduation, Jason performed several research internships, attended numerous trade shows and lectures, published research papers, and developed an insatiable urge to travel and study horticulture abroad. Jason got his first taste of applied horticultural science in the research greenhouses of Longwood Gardens as a tissue culture and plant breeding intern. His next journey took him to the cloud forests of Costa Rica, where he worked as a visiting scientist at the Nectandra Institute. While there, he conducted ecotours and established a tissue culture conservation program focused on rare and endangered cloud forest plants. Upon returning to the United States, Jason's interest in plant breeding took him back to Pennsylvania to work for Garden Genetics, LLC.

During his internship with Garden Genetics, a life-changing opportunity arose when he was selected for the Martin F. McLaren Scholarship through the Garden Club of America and the Royal Horticulture Society. He was able to embark on an incredible scholarly journey through the varied and timeless gardens of the United Kingdom, including Tresco Abbey Garden in the Isles of Scilly, the Eden Project, RHS Edinburgh, RHS Wisley, the Conservation Biotechnology Unit at RBG Kew, and the U.S. Ambassador's Residence (Winfield House and Garden, London). Living and working in one of the birthplaces of gardening gave Jason a new perspective on the value of ornamental plants. Besides working in laboratories and gardens in the United Kingdom, Jason developed a passion for the great Victorian plant explorers and the history of ornamental plant introduction.

One such great plantsman, E.H. Wilson, once said, "Agriculture has led the march of civilization through the ages. Horticulture is her twin sister whose mission is to gratify man's aesthetic needs when agriculture has satisfied his bodily necessities." Jason returned to the North Carolina and brought with him a wealth of inspiration from his studies and travels. He rededicated himself to horticulture with the belief that plants not only satisfy our aesthetic desires, but can also play a critical role in recapturing generations of people becoming increasingly detached from nature. Jason started longing for a career where he could inspire people to see beauty and value in their own landscapes, hopefully leading to a broader desire to become stewards of the natural world. This desire to share new and exciting plants with the world led Jason back to NC State University to begin a master's degree in plant breeding.

Jason began his master's degree under the direction of Dr. Tom Ranney at the Mountain Crop Improvement Lab and the Mountain Horticultural Crops Research and Extension Center. On the campus of NC State University and at the research station, Jason was exposed to a diverse range of plant breeding programs ranging from food and biofuel crops to ornamental trees and shrubs. Many of these breeding programs utilize techniques that were new and exciting to Jason, such as polyploid induction, RNA interference, irradiation, mutagenesis, wide hybridization, and much more. During his time as a student, Jason also had the opportunity to give lectures at local gardens and arboreta, teach an ornamental plant identification lab, and present research at several research competitions.

Now that Jason is nearing graduation, his next move will be across the country to Corvallis, Oregon where he will begin his PhD in ornamental plant breeding at Oregon State University under the direction of Dr. Ryan Contreras. Jason looks forward to starting this next phase in his life with his fiancé, Kim Shearer, and they both long for a life filled with new plants, new opportunities, and new friends.

ACKNOWLEDGMENTS

Somehow, I have come full circle and found my way back to NC State. After several exhausting, rambling, eye-opening years of horticultural travels around the world, I returned to the place of my birth like the proverbial salmon. Back to the place that spawned my interest in science, nature, plants, food, music, and everything truly good in this world. It was hard to imagine just a few short years ago when Dr. Tom Ranney was touring me through his fields of carefully selected botanical jewels, I would have the absolute privilege of spending the next few years of my life working with him. His intellect, patience, and long-suffering good humor have well-prepared Dr. Ranney for the constant stream of horticultural pilgrims who routinely break the solitude of his mountain retreat in search of plant knowledge. Thank you, Tom, for giving me a home in your research program, for constantly challenging me, and for always encouraging me to meet my potential. I treasure the time spent learning from you, laughing with you, and becoming your friend.

Equally influential on my education at NCSU was the incomparable tissue culturalist and all-around brilliant Aussie, Dr. Darren Touchell. Walking into his tissue culture lab the first time, I caught Darren looking at me much like a bit of rough plant material that needed to be dissected, incubated, and transformed under his care for a few years to produce a new-and-improved version of my former self. No one is better or more qualified at this task than you, Darren, be it plants or graduate students. I have learned volumes working with you, and I could not have asked for a better coauthor, mentor, and friend.

However, my interest in tissue culture was actually spawned nearly a decade ago in the classroom of one of my favorite teachers, Dr. Dennis Werner. In his classrooms and greenhouses was where I first discovered how much joy one can get out of scribbling down data in a lab notebook that is stained with "soilless media" and bits of plant debris. And equally, how much joy can be gained out of regenerating dozens of new orchids and ferns from just a few small seeds, spores, or bits of stem tissue. I am forever grateful for your kindness, and willingness to share plant propagation with me so many years ago. It is an honor to have you on my thesis committee.

Another influential member from my undergraduate days is the tenacious taxonomist, Dr. Paul Fantz—a man who braved the elements, the undergrads, and the occasional attack from a plant (think *Maclura pomifera*) to teach plant identification to an entirely new generation of horticulturalists. Out of the 500 or so plants you taught me over the course of a year, the very first was an *Ophiopogon*. How could I have known, standing there gazing into that perfectly cobalt blue seed, that one day I would be researching the same plant with you for my graduate thesis? I am grateful that you came out of retirement to help me on this project, and grateful that you spurred my interest in the mysterious world of liriopogons.

Now carrying the horticultural baton passed by Paul, Dr. Brian Jackson gave me an opportunity to share my love of plants in the same classroom in which I sat so many years ago. Brian, the year spent teaching the ornamental plant identification lab with you was one of the most rewarding experiences of my life. Thank you for giving me a chance, and allowing me to learn from your prodigious skills of pedagogy. I am now consumed by the

desire to have my own classroom one day, and if I can muster a small percentage of the energy, enthusiasm, and love that you brought to the classroom every day, I know I will be a success. Good luck with your career, thank you for your friendship, and keep changing lives one plant at a time.

Of course my acknowledgements would be incomplete, along with my thesis, without the tireless efforts of the staff at the Mountain Crop Improvement Lab and the Mountain Horticultural Crops Research and Extension Center. To Jeremy Smith, thank you for all the laughs while sitting elbow to elbow in the flow hood. I can't imagine the research station without you. Your easy-going demeanor and many hours of assistance on graduate projects are a gift to every graduate student coming through the program. I know we will be life-long friends. To Nathan Lynch, you are a true Renaissance man. You are as easily at home behind the lens of a microscope or the eyepiece of a telescope as you are in the shade of a spinnaker. You have been instrumental in my success as a graduate student, and I will forever be in awe of your skills as a scientist.

To Joel Mowrey, I would never know where to find anything on the station without your help. You are an inspiring multitasker, horticulturalist, family man, and entrepreneur with a profound ability to juggle all that life throws at you. You are a wealth of information and advice for all graduate students who come through the program, and I hope I never find myself without one of your hot sauces on hand. To Tom Eaker, you are a true patriarch of the research station, father figure to the graduate students, and barbeque master extraordinaire. Thank you for all your help and all the kind words over the years. Please

enjoy every minute of your well-deserved retirement. And to Andy Whipple, Andra Nus, and all of the interns and graduate students, I treasure all the moments of hard work, dirty hands, and hearty laughs during our brief time together in the mountains. I wish you all the best and a lifetime of happiness.

During the course of my research, I also had the pleasure of working some of the areas consummate plantsmen. To Tony Avent and the entire staff at Plant Delights Nursery and Juniper Level Botanic Garden, as well as Mark Weathington and the staff of the J.C. Raulston Arboretum, thank you all for your support of plant material and expertise. Having the support of local gardens, nurseries, and arboreta was invaluable during my time at NC State. I wish you all good plant hunting and good gardening.

Though my colleagues in horticulture have irrevocably shaped my life and the course of my career, without great parents none of my dreams would have ever been realized. To my parents, Jan and Richard Lattier, you have been a continual source of strength and comfort to me, and I always carry you with me wherever I go. Thank you for giving me a loving home for so many years and for always supporting my dreams. And to my brother, Josh, thank you for the decades of light-hearted sibling rivalry, and for being a great friend. I am proud of you, proud of the great business you built with your beautiful wife, and proud to call you my brother.

And finally, there is Kimberly Shearer, what can I say? We met just a year ago, hiked and biked the Appalachians, fell in love, and now we are engaged to be married. The late nights in the lab, working on our projects and talking about the future, I wouldn't trade those

memories for anything in the world. I know one of these days we will travel the world together, one forest, one garden, one plant at a time. Thank you for letting me share the rest of my life with you. I look forward to our new life on the west coast, and will be counting the days until we get to walk hand in hand through the redwood forest together, with Maybelle in tow of course.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1. Micropropagation and Polyploid Induction of *Acer platanoides* 'Crimson Sentry'

CHAPTER 1

Micropropagation and Polyploid Induction of Acer platanoides ‘Crimson Sentry’

(In the format appropriate for submission to HortScience)

Micropropagation and Polyploid Induction of *Acer platanoides* 'Crimson Sentry'

Jason D. Lattier[1], Darren H. Touchell[2], Thomas G. Ranney[3], and Jeremy C. Smith[4]

Mountain Crop Improvement Lab, Department of Horticultural Science, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, 455 Research Drive, Mills River, NC 28759-3423

Received for publication . Accepted for publication .

This research was funded, in part, by the North Carolina Agricultural Research Service (NCARS), the Garden Club of America, the Royal Horticultural Society (UK), the J. Frank Schmidt Family Foundation, and the U.S. Department of Agriculture Agricultural Research Service, Floriculture and Nursery Research Initiative. Use of trade names in this publication does not imply endorsement by the NCARS of products named nor criticism of similar ones not mentioned. Assistance of the staff of the Mountain Horticultural Crops Research and Extension Center, including Nathan Lynch, is gratefully acknowledged. From a thesis submitted by J. D. Lattier in partial fulfillment of the requirements for the MS degree.

[1]Graduate Research Assistant.

[2]Research Associate and corresponding author. E-mail:darren_touchell@ncsu.edu.

[3]Professor.

[4]Research Specialist.

Subject Category: Propagation and Tissue Culture

Micropropagation and Polyploid Induction of *Acer platanoides* 'Crimson Sentry'

Additional index words: 6-benzylaminopurine, auxins, Norway maple, oryzalin, polyploidy

Abstract. Protocols were developed for micropropagation and induction of autopolyploids in a fastigiate cultivar of Norway maple (*A. platanoides* L. 'Crimson Sentry'). Basal salts including Murashige and Skoog Medium (MS), Woody Plant Medium (WPM), and Quoirin and Lepoivre Medium (QL) were supplemented with 2 µM 6-benzylaminopurine (BAP), *meta*-Topolin (*m*T), 6-(γ,γ-dimethylallylamino) purine (2iP), kinetin (Kin), or thidiazuron (TDZ) to evaluate microshoot proliferation. The combination of MS and 2 µM BAP yielded the most microshoots (3.2) and longest microshoots (30.6 mm) per subsample after 5 weeks. The influence of BAP concentration on proliferation was evaluated at 0, 2, 4, 8, or 16 µM. Optimal multiplication rate was achieved at 2 to 4 µM BAP producing 2.83 microcuttings per subsample after 5 weeks. To induce in vitro rooting, half-strength WPM was supplemented with 0, 5, 10, 20, 40, or 80 µM 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 µM IBA after 8 weeks. To induce polyploidy, microcuttings were pretreated for 7 days on MS with 4 µM BAP alone or combined with 1 µM IBA, indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA) prior to treatment in liquid MS containing 15 µM 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.

Introduction

Norway maple (*Acer platanoides* L.) was introduced to North America by Bartram in 1756 (Nowak and Roundtree, 1990) and has served as an important substitute for American elm (*Ulmus americana* L.) following introduction of Dutch elm disease (Wangen and Webster, 2006). Its attractive architecture, pest and disease resistance, and tolerance of poor soils and pollution have made it a successful municipal street tree (Bertin et al., 2005). Following initial success, *A. 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 (Kloeppel and Abrams, 1995; Reinhart et al., 2006a, 2006b; Wangen and Webster, 2006).

Although *A. platanoides* can be propagated by seed, plants tend to display a diverse range of phenotypes. Therefore, elite cultivars with improved ornamental characteristics are typically propagated asexually by budding onto seedling rootstocks (Lindén and Riikonen, 2006). On the other hand, micropropagation can provide an alternative means for rapid propagation of valuable ornamental cultivars, as well as a platform for improving ornamental traits. Studies on micropropagation of *A. platanoides* have focused on wild types (Ďurkovič,

1996; Lindén and Riikonen, 2006) or the popular cultivar, ‘Crimson King’ (Marks and Simpson, 1990, 1994), with a single report on cultivar ‘Crimson Sentry’ (Cheng, 1978). Initial studies on micropropagation of *A. platanoides* ‘Crimson Sentry’ utilized Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) for microshoot regeneration (Cheng, 1978). More recent studies employed Linsmaier and Skoog (LS) medium (Linsmaier and Skoog, 1965) and Woody Plant medium (WPM) (Lloyd and McCown, 1980) for *A. platanoides* ‘Crimson King’ (Marks and Simpson, 1990, 1994) and wild type *A. platanoides* (Ďurkovič, 1996; Lindén and Riikonen, 2006), respectively.

The cytokinin, 6-benzylaminopurine (BAP), used solely or in combination with other plant growth regulators, is the most commonly used cytokinin for in vitro propagation of *A. platanoides*. Marks and Simpson (1990) reported 1 µM BAP alone promoted microshoot formation in *A. platanoides* ‘Crimson King’. Additional studies showed that microshoot proliferation could be increased in *A. platanoides* ‘Crimson King’ when media containing BAP were supplemented with low concentrations of thidiazuron (TDZ); however, significant callus formation was also observed (Marks and Simpson, 1994). Similarly, Lindén and Riikonen (2006) demonstrated significant microshoot proliferation of apical and axillary buds of *A. platanoides* with low concentrations (0.01 to 0.1 µM) of TDZ. Kinetin (Kin) has also proven effective for inducing microshoot regeneration in *A. platanoides* (Ďurkovič, 1996; Lindén and Riikonen, 2006). For *A. platanoides* ‘Crimson Sentry’, in vitro cultures were established using BAP in combination with IBA; however, exact proliferation rates were not provided (Cheng, 1978).

Topolins are a group of natural aromatic cytokinins having a similar structure to BAP yet have not been associated with hyperhydricity, heterogeneity of growth, or inhibition of rooting (Amoo et al., 2011; Bairu et al., 2007). However, effects of *meta*-Topolin (*m*T) on in vitro growth and development of *A. platanoides* have yet to be investigated. Whereas toxicity of BAP is the result of its stable nature within the plant, *m*T and its metabolites are easily degradable, and accumulation of *m*T is prevented by its rapid translocation in plant tissues (Amoo et al., 2011; Bairu et al., 2007). In addition, *m*T's origin in *Populus* may indicate a greater activity in woody plants (Bairu et al., 2007; Tarkowská et al., 2003), 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 media supplemented with IBA (Cheng, 1978; Marks and Simpson, 1994). Marks and Simpson (1994) achieved rooting for cultivar 'Crimson King' when microcuttings were cultured for 7 days on half-strength modified LS medium containing 5 µM IBA, followed by half-strength modified LS media free of hormones. Similarly, Ďurkovič (1996) found microcuttings of *A. platanoides* successfully rooted on half-strength WPM supplemented with 5 µM 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 (Ranney, 2006). Polyploidy is usually achieved by treating plant tissues with a spindle fiber inhibitor such as colchicine, oryzalin, or trifluralin (Ranney, 2006). 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 (Dhooge et al., 2010; Zhang et al., 2007). However, colchicine's affinity for animal microtubules makes it toxic to humans (Dhooge et al., 2010; Morejohn et al., 1984) while its weak affinity for plant tubulins necessitates higher doses to maintain effectiveness in plant tissues (Dhooge et al., 2010). In contrast, oryzalin (3,5-dinitro-N4,N4-dipropylsufanilamide) is a mitosis-inhibiting dinitroaniline herbicide with a high affinity for plant tubulin dimers (Morejohn et al., 1987). This affinity allows for effective use of oryzalin at low concentrations with reduced toxicity and incidences of mutation or abnormal growth (Bajer and Molebajer, 1986; Dhooge et al., 2010; Hugdahl and Morejohn, 1993; Morejohn et al., 1987). Alterntively, oryzalin has been used successfully to create polyploid lines of woody and semi-woody plants including *Rosa* L. (Kermani et al., 2003), *Rhododendron* L. (Väinölä, 2000; Jones et al., 2008; Hebert et al., 2010), *Chaenomeles* Lindl. (Stanys et al., 2006), *Hypericum* L. (Meyer et al., 2009) and *Berberis* L. (Lehrer et al., 2008).

Development of efficient micropropagation protocols 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 to 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 cultures. Actively growing shoots were collected from a containerized plant maintained in a glasshouse and rinsed under tap water for 4 h. Explants were surface-sterilized 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). Explants were agitated periodically for 17 min followed by three 5-min rinses in sterile distilled water. Explants were cultured on regeneration media consisting of MS basal salts and vitamins supplemented with BAP at 2 µM, *myo*-Inositol at 100 $mg \cdot L^{-1}$, 2-(N-Morpholino) ethanesulfonic acid (MES) monohydrate at 100 $mg \cdot L^{-1}$, and sucrose at 30 $g \cdot L^{-1}$. Media were solidified with agar at 7.5 $g \cdot L^{-1}$ and adjusted to a pH of 5.75. Regenerated microshoots were maintained by transfer to fresh regeneration media every 4 to 6 weeks and incubated under standard culture conditions [23 ± 2 °C and a 16 h photoperiod of 75 µmol $m^{-2}s^{-1}$ (400-700 nm) 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 QL salts (Quoirin and Lepoivre, 1977) and Gamborg vitamins (Gamborg et al., 1968) each in combination with BAP, TDZ, 2iP, *m*T, or Kin at 2 µM. All media were supplemented with *myo*-Inositol at 100 $mg \cdot L^{-1}$, MES at 100 $mg \cdot L^{-1}$, and sucrose at 30 $g \cdot L^{-1}$, solidified with agar at 7.5 $g \cdot L^{-1}$ and adjusted to a pH of 5.75. Media (25 mL) was dispensed to 180-mL glass jars. Five microcuttings (10-20 mm long) were place vertically in

each jar. Six replicates of each media composition were incubated under standard culture conditions, as described for microshoot initiation, 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) (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Cytokinin concentration. To further improve microshoot proliferation of *A. platanoides* 'Crimson Sentry', a second study was conducted to optimize cytokinin concentration in the best resulting media from the first experiment. Media consisted of MS salts and vitamins in combination with BAP at 0, 2, 4, 8, or 16 μM. Media were prepared as described for the first experiment. 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 for microshoot initiation). 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. An additional experiment was conducted to examine in vitro rooting of *A. platanoides* 'Crimson Sentry'. Media used for in vitro rooting consisted of half-strength WPM salts and vitamins supplemented with 0, 5, 10, 20, 40, or 80 µM IBA, sucrose at 30 $g \cdot L^{-1}$, MES at 100 $mg \cdot L^{-1}$, and *myo*-Inositol at 100 $mg \cdot L^{-1}$. Media were solidified with agar at 7.5 $g \cdot L^{-1}$ and adjusted to a pH of 5.75. Microcuttings 10 to 20 mm long were subcultured into 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 for microshoot initiation. 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 (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Polyploid induction. A 2×3×4 complete factorial design was used to test effects of BAP, oryzalin concentration, and oryzalin exposure time on polyploid induction of *A. platanoides* 'Crimson Sentry'. Liquid media (adjusted to pH of 5.75) containing MS salts and vitamins, *myo*-Inositol at 100 $mg \cdot L^{-1}$, MES at 100 $mg \cdot L^{-1}$, and sucrose at 30 $g \cdot L^{-1}$ were supplemented with 0 or 2 µM BAP and 0, 15, 30, or 45 µM oryzalin. Microshoots 10-20 mm long were cultured horizontally on regeneration media (as described for microshoot initiation) 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. Samples were then transferred to jars containing liquid oryzalin media and placed on a platform shaker in the dark at 23°C for 3, 5, or 7 days. Microshoots were then washed for 24 h in

liquid regeneration media to remove excess oryzalin and cultured on solidified regeneration media. Each treatment consisted of six replicates with five subsamples 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 media consisting of MS salts and vitamins, *myo*-Inositol at 100 $mg \cdot L^{-1}$, MES at 100 $mg \cdot L^{-1}$, sucrose at 30 $g \cdot L^{-1}$ and pH at 5.75. Pretreatment media was supplemented with either 4 µM BAP, 4 µM BAP + 1 µM IBA, 4 µM BAP + 1 µM indole-3-acetic acid (IAA) or 4 µM BAP + 1 µM 1-naphthaleneacetic acid (NAA) for 7 days. Samples were then transferred to jars containing liquid MS media supplemented with 15 µM oryzalin (as described above) for 3 days. Following treatment, microshoots were washed for 24 h in liquid regeneration media to remove excess oryzalin and then cultured on solidified regeneration media. Each treatment consisted of six replicates with five subsamples each. All jars were completely randomized under standard culture conditions as described for microshoot initiation.

After 8 weeks, data were recorded on percent survival and number of microshoots produced. Ploidy level was determined via flow cytometry. For each sample, several expanded leaves were place in a petri dish containing 400 µL 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 buffer.

The resulting solution was filter sterilized through a Partec CellTrics™ disposable filter with a pore size of 50 µm. Then, 1.2 mL of a nucleotide 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 of basal salts and cytokinins. 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). The combination of MS and 2 µM BAP produced the longest mean microshoot length (30.6 ± 1.4 mm) and there was a general trend of BAP producing longer microshoots among all media types. The most nodes per microshoot were produced with MS and BAP (3.6 ± 0.2), as well as QL and BAP (3.5 ± 0.2). Microshoot number was influenced only by cytokinin ($P < 0.01$). In general, BAP and *m*T produced the highest number of microshoots across all basal salt treatments. Both media and cytokinin type independently influenced mean leaf number ($P < 0.01$ and 0.01, respectively) with MS and BAP generally producing the most leaves per microshoot.

In the present study, MS combined with BAP produced high quality microshoot growth responses, with high microshoot regeneration and longer microshoots. Similarly, Cheng (1978) achieved effective microshoot regeneration with MS media and BAP 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 media (Lindén and Riikonen, 2006; Marks and Simpson, 1994). However, TDZ at 2 µM 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 is one of the most significant factors influencing the development of tissue culture protocols (Kovalchuk et al., 2009; Matt and Jehle, 2005; Mori et al., 2005; Péros et al., 1998). For *Malus* Tourn. ex L., genotype was a significant factor influencing in vitro cold storage and regeneration (Kovalchuk et al., 2009). Similarly, significant variation regarding microshoot development and subsequent root formation was observed among 32 cultivars of *Vitis vinifera* L. (Péros et al., 1998).

Cytokinin concentration. Regression analysis of microshoot data revealed BAP concentration had no significant effect on microshoot number (Fig. 1A). However, microshoot length increased from 17.40 ± 0.44 mm at 0 µM BAP to 27.43 ± 2.07 mm at 2 µM BAP before declining linearly with increasing BAP concentration ($P < 0.01$) (Fig. 1B).

Similarly, multiplication rate (number of 20 mm microcuttings obtained per subsample after 5 weeks) increased from 1.40 ± 0.16 at 0 µM BAP to 2.83 ± 0.33 at 4 µM BAP before exhibiting a negative linear response to increasing BAP concentrations ($P < 0.01$) (Fig. 1C). Optimum BAP concentration occurred in the range of 2 to 4 µM BAP (Fig. 1C).

Rooting. After 8 weeks, in vitro root formation was observed for all media treatments containing IBA, whereas no root formation was observed in control (0 µM IBA) treatments. Regression analysis indicated that percent rooting, number of roots per rooted microcutting, and root length exhibited a negative linear response to IBA concentration ($P < 0.01$, 0.05, and 0.01, respectively) (Fig. 2). 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 ± 0.85 and 3.37 ± 1.03, respectively). However, the highest rooting percentage and longest roots (17.13 ± 3.10 and 17.85 ± 3.47, respectively) were achieved at 5 and 10 µM IBA (Fig. 2), suggesting lower IBA concentrations (5 to 10 µM) are optimal for in vitro root production of *A. platanoides* ‘Crimson Sentry’. This optimal rooting media contained similar concentrations of IBA compared to previous studies on *A. platanoides* ‘Crimson King’ that utilized 5 µM IBA (Marks and Simpson, 1994) and 4.9 µM IBA (Ďurkovič, 1996).

Polyploid induction. Percentage of surviving microcuttings was influenced by BAP 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 BAP treatments had a negative quadratic response to increasing oryzalin concentrations ($P < 0.01$)

(Fig. 3). Addition of BAP to media increased microcutting fatality at higher oryzalin concentrations, regardless of exposure duration (Fig. 3). No explants survived the treatment combination of 2 µM BAP and 45 µM oryzalin. Interestingly, addition of BAP to media reduced microcutting survival at higher oryzalin concentrations. As a cytokinin, BAP may act to increase cell cycling, producing cells that more quickly transition from the G2 to M phase (metaphase/anaphase) (Richard et al., 2002; Stals and Inzé, 2001) where they may be susceptible to oryzalin toxicity.

Mixaploids (tissues containing 2x and 4x cells) and what appeared to be homogeneous tetraploids (4x) were induced successfully by oryzalin. However, after 6 months all apparent tetraploids had reverted to diploids. Production of polyploids (mixaploids + tetraploids) exhibited a quadratic response to oryzalin concentration [polyploidy = 0.01 + 0.024(oryzalin) – 0.00055(oryzalin^2) ($P < 0.01$), (data not presented)]. No significant effects were observed from length of exposure, BAP concentration, or their interaction. 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 presented).

One possible explanation for instability of apparent tetraploids may be a high propensity for diplontic selection within the apical meristem. Every initial cell within all three histogenic layers of the apical meristem must be affected by oryzalin for successful homogeneous tetraploids to be formed (Jones et al., 2008). If all initial cells are not affected, a cytochimeric mosaic of ploidy levels (mixaploid) can form. Diplontic selection may result

if diploid cells, having less DNA content, take less time to replicate and divide than competing polyploid cells (Jones et al., 2008; Klekowski et al., 2003). 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 pretreatment media significantly influenced production of polyploids ($P < 0.05$) (Table 2). Although mixaploids 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 media 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/S and G2/M transitions (Perrot-Rechenmann, 2010). 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 (Pagnussat et al., 2002). Nitric oxide can react with tyrosine to form nitrotyrosine (Neill et al., 2003). Jovanovic et al. (2010) demonstrated that nitrotyrosine increased sensitivity of *Nicotiana tabacum* L. cell cultures to oryzalin.

Therefore, in the present study, addition of IAA to media 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*.

Literature Cited

Amoo, S.O., J.F. Finney, and J. Van Staden. 2011. The role of meta-topolins in alleviating micropropagation problems. Plant Growth Regulat. 63:197-206.

Bairu, M.W., W.A. Stirk, K. Dolezal, and J.V. Staden. 2007. Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: can *meta*-topolin and its derivatives serve as replacement for benzyladenine and zeatin? Plant Cell Tissue Organ Cult. 90:15-23.

Bajer, A.S. and J. Molebajar. 1986. Drugs with colchicine-like effects that specifically disassemble plant but not animal microtubules. Ann. New York Acad. Sci. 466:767-784.

Bertin, R.I., M.E. Manner, B.F. Larrow, T.W. Cantwell, and E.M. Berstene. 2005. Norway maple (*Acer platanoides*) and other non-native trees in urban woodlands of central Massachusetts. J. Torrey Botanical. Soc. 132:225–235.

Cheng, T.-Y. 1978. Clonal propagation of woody plant species through tissue culture techniques. Combined Proc. Intl. Plant Propagators Soc. 28:139-155.

Dhooge, E., K.V. Laere, T. Eeckhaut, L. Leus, and J.V. Huylenbroeck. 2010. Mitotic chromosome doubling of plant tissues in vitro. Plant Cell Tissue Organ Cult. 104:359-373.

Ďurkovič, J. 1996. In vitro regeneration of Norway maple (*Acer platanoides* L.). Biologia Plantarum 38:303-307.

Gamborg, O.L., R.A. Miller, and K. Ojima. 1968. Nutrient Requirements of suspension cultures of soybean root cells. Experimental Cell Res. 50:151-158.

Hebert, C.J., D.H. Touchell, T.G. Ranney, and A.V. LeBude. 2010. In vitro shoot regeneration and polyploidy induction of *Rhododendron* 'Frangrantissimum Improved'. HortScience 45(4):801-804.

Hugdahl, J.D. and L.C. Morejohn. 1993. Rapid and reversible high-affinity binding of the dinitroaniline herbicide oryzalin to tubulin from *Zea mays* L. Plant Physiol. 102:725-740.

Jones, J.R., T.G. Ranney, and T.A. Eaker. 2008. A novel method for inducing polyploidy in *Rhododendron* seedlings. J. Amer. Rhododendron Soc. 62:130-135.

Jovanović, A.M., S. Durst, and P. Nick. 2010. Plant cell division is specifically affected by nitrotyrosine. J. Experimental Bot. 61:901-909.

Kermani, M.J., V. Sarasan, A.V. Roberts, K. Yokoya, J. Wentworth, and V.K. Sieber. 2003. Oryzalin-induced chromosome doubling in *Rosa* and its effect on plant morphology and pollen viability. Theoretical Appl. Genet. 107:1195-1200.

Klekowski, E.J. 2003. Plant clonality, mutation, diplontic selection and mutational meltdown. Biological J. Linnean Soc. 79:61-67.

Kloeppel, B.D. and M.D. Abrams. 1995. Ecophysiological attributes of native *Acer saccharum* and the exotic *Acer platanoides* in urban oak forests in Pennsylvania, USA. Tree Physiol. 15:739-746.

Kovalchuk, I., Y. Lyudvikova, M. Volgina, and B.M. Reed. 2009. Medium, container and genotype all influence in vitro cold storage of apple germplasm. Plant Cell Tissue Organ Cult. 96:127-136.

Lehrer, J.M., M.H. Brand, and J.D. Lubell. 2008. Induction of tetraploidy in meristematically active seeds of Japanese barberry (*Berberis thunbergii* var. *atropurpurea*) through exposure to colchicine and oryzalin. Scientia Hort. 119:67-71.

Lindén, L. and A. Riikonen. 2006. Effects of 6-benzylaminopurine, thidiazuron and type of explant on in vitro shoot development of *Acer platanoides* L. Prop. Ornamental Plants 6:201-204.

Linsmaier, E.M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue culture. Physiol. Plant. 18:100-127.

Lloyd, G. and B.H. McCown. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Combined Proc. Intern. Plant Propagators Soc. 30:421-427.

Marks, T.R. and S.E. Simpson. 1990. Reduced phenolic oxidation at culture initiation in vitro following the exposure of field-grown stockplants to darkness or low levels of irradiance. J. Hort. Sci. 65:103-111.

Marks, T.R. and S.E. Simpson. 1994. Factors affecting shoot development in apically dominant *Acer* cultivars *in vitro*. J. Hort. Sci. 69:543-537.

Matt, A. and J.A. Jehle. 2005. In vitro plant regeneration from leaves and internode sections of sweet cherry cultivars (*Prunus avium* L.). Plant Cell Rpt. 24:468-476.

Meyer, E.M., D.H. Touchell, and T.G. Ranney. 2009. In vitro shoot regeneration and polyploid induction from leaves of *Hypericum* species. HortScience 44:1957-1961.

Morejohn, L.C., T.E. Bureau, J. Molebajer, A.S. Bajer, and D.E. Fosket. 1987. Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. Planta 172:252-264.

Morejohn, L.C., T.E. Bureau, L.P. Tocchi, and D.E. Fosket. 1984. Tubulins from different higher-plant species are immunologically non-identical and bind colchicine differentially. Proc. Natl. Acad. Sci. USA 81:1440-1444.

Mori, S., Y. Adachi, S. Horimoto, S. Suzuki, and M. Nakano. 2005. Callus formation and plant regeneration in various *Lilium* species and cultivars. In Vitro Cellular Developmental Biol. – Plant 41:783-788.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Neill, S.J., R. Desikan, and J.T. Hancock. 2003. Nitric oxide signaling in plants. New Phytol. 159:11-35.

Nowak, D.J. and R.A. Rowntree. 1990. History and range of Norway maple. J. Arboriculture 16(11):291–296.

Pagnussat, G.C., M. Simontacchi, S. Puntarulo, and L. Lamattina. 2002. Nitric oxide is required for root organogenesis. Plant Physiol. 129:954-956.

Péros, J.P., L. Torregrosa, and G. Berger. 1998. Variability among *Vitis vinifera* cultivars in micropropagation, organogenesis and antibiotic sensitivity. J. Experimental Bot. 49:171-179.

Perrot-Rechenmann, C. 2010. Cellular responses to auxin: Division versus expansion. Cold Spring Harbor Perspectives Biol. 2:1-15.

Quoirin, M. and P. Lepoivre. 1977. Improved medium for in vitro culture of *Prunus* sp. Acta. Hort. 78:437-442.

Ranney, TG. 2006. Polyploidy: From evolution to new plant development. Combined Proc. Intl. Plant Propagators Soc. 56:137-142.

Reinhart, K.O., J. Gurnee, R. Tirado, and R.M. Callaway. 2006. Invasion through quantitative effects: intense shade drives native decline and invasive success. Ecol. Applications 16:1821-1831.

Reinhart, K.O., F.T. Maestre, and R.M. Callaway. 2006. Facilitation and inhibition of seedlings of an invasive tree (*Acer platanoides*) by different tree species in a mountain ecosystem. Biol. Invasions 8:231-240.

Richard, C., M. Lescot, D. Inzé, and L. De Veylder. 2002. Effect of auxin, cytokinin, and sucrose on cell cycle gene expression in *Arabidopsis thaliana* cell suspension cultures. Plant Cell Tissue Organ Cult. 69:167-176.

Stals, H. and D. Inzé. 2001. When plant cells decide to divide. Trends Plant Sci. 6:359–364.

Stanys, V., A. Weckman, G. Staniene, and P. Duchovskis. 2006. In vitro induction of polyploidy in Japanese quince (*Chaenomeles japonica*). Plant Cell Tissue Organ Cult. 84:263-268.

Tarkowská, D., K. Dolezal, P. Tarkowski, C. Astot, J. Holub, K. Fuksová, T. Schmülling, G. Sandberg, and M. Strnad. 2003. Identification of new aromatic cytokinins in *Arabidopsis thaliana* and *Populus* ×*canadensis* leaves by LC-(+)ESI-MS and capillary liquid chromatography frit-fast atom bombardment mass spectrometry. Physiol. Plant. 117:579-590.

Väinölä, A. 2000. Polyploidization and early screening of *Rhododendron* hybrids. Euphytica 112:239-244.

Wangen, S.R. and C.R. Webster. 2006. Potential for multiple lag phases during biotic invasions: reconstructing an invasion of the exotic tree *Acer platanoides*. J. Appl. Ecol. 43:258-268.

Zhang J., M. Zhang and X. Deng. 2007. Obtaining autotetraploids in vitro at high frequency in *Citrus sinensis*. Plant Cell Tissue Organ Cult. 89:211-216.

Table 1. In vitro growth responses of *A. platanoides* 'Crimson Sentry' to three in vitro culture media and five cytokinins.

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

[z] Values represent means ± SEM. Means followed by different letters within columns are significantly different, $P < 0.05$.

[y] Mean separation within columns by Fishers LSD, $P < 0.05$, n = 6

[x] NS, *, **: Nonsignificant or significant at $P < 0.05$ or <0.01, respectively.

Table 2. Influence of growth regulator pretreatments on percent polyploid induction of micropropagated shoots of *A. platanoides* ‘Crimson Sentry’.

Growth regulators	Diploid (%)	Mixaploid (%)	Tetraploid (%)
4 µM BAP	67 ± 00 b[z]	33 ± 00 ab	0 ± 00 b
4 µM BAP + 1 µM IBA	78 ± 11 ab	22 ± 11 bc	0 ± 00 b
4 µM BAP + 1 µM IAA	33 ± 12 c	50 ± 07 a	17 ± 05 a
4 µM BAP + 1 µM NAA	94 ± 05 c	6 ± 05 c	0 ± 00 b

[z] Values represent means ± SEM. Means followed by different letters within columns are significantly different, $P < 0.05$.

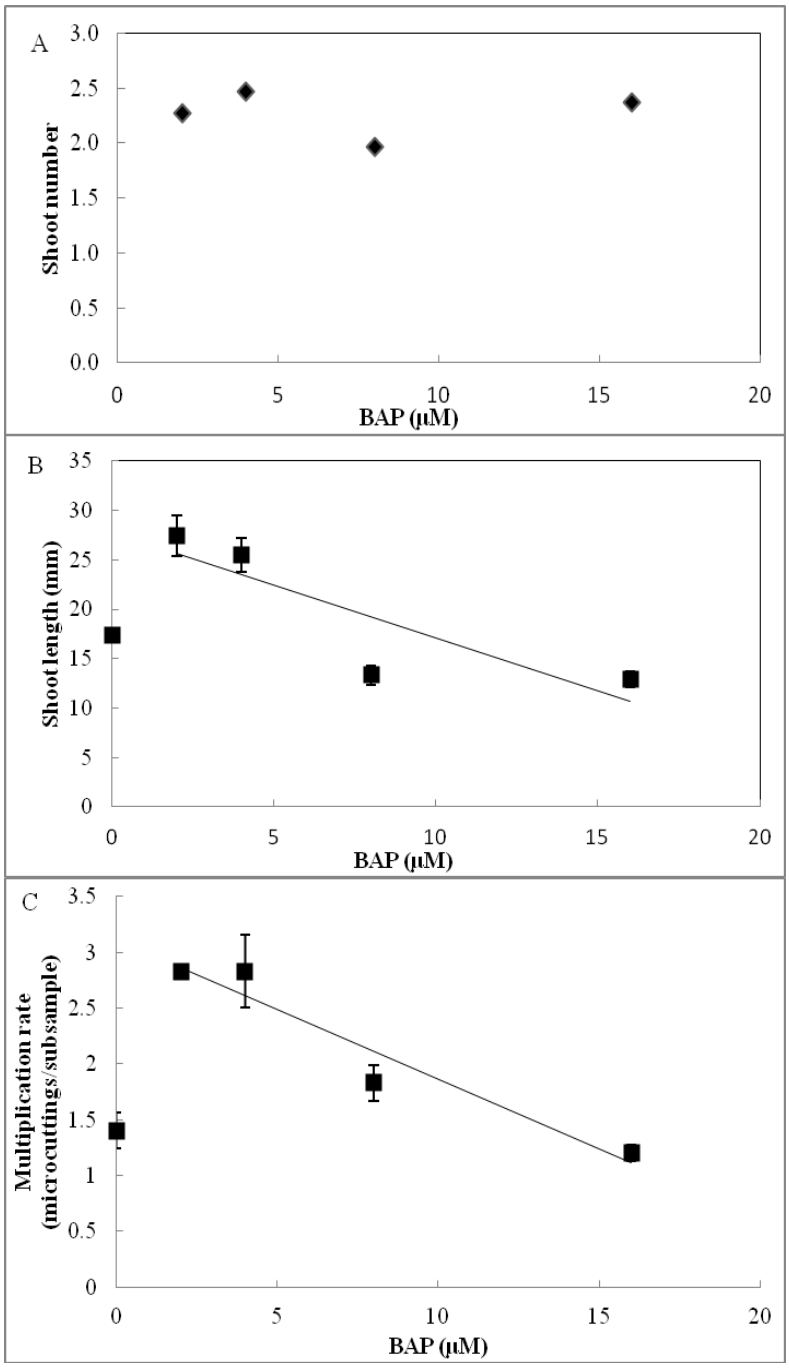

Figure 1. Influence of BAP concentration on microshoot development of *Acer platanoides* 'Crimson Sentry'. Symbols represent means, n=6, ± SEM. A) Number of microshoots produced per microcutting; B) Length of longest microshoot; y = 27.86 – 1.076x, r^2 = 0.74 and C) Multiplication rate defined as the number of 20 mm microcuttings produced per subsample after five weeks; y = 3.1 – 0.12x, r^2 = 0.93. Regression equations and lines do not include control treatments.

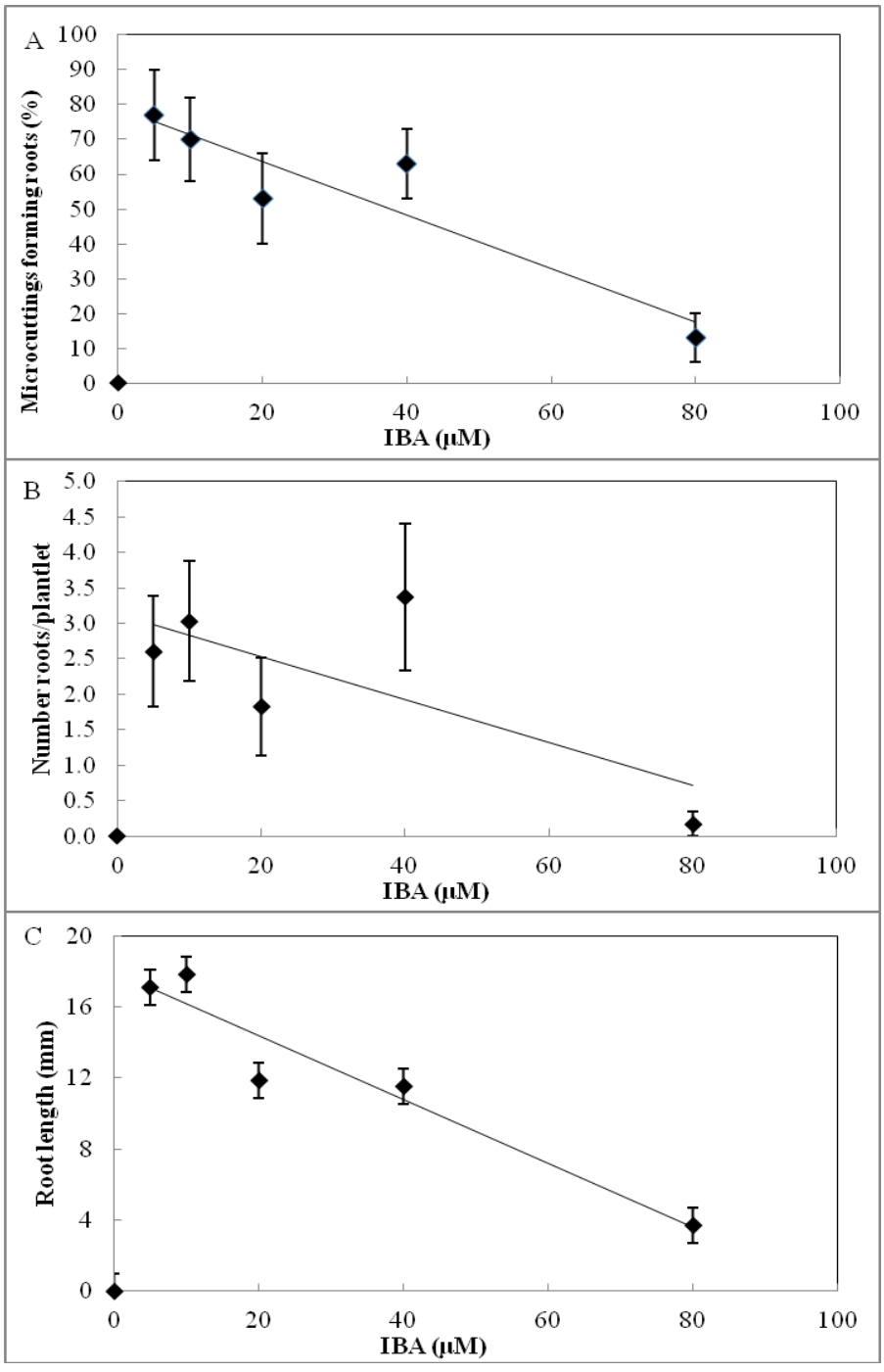

Figure 2. Influence of IBA concentration on in vitro root development of *Acer platanoides* 'Crimson Sentry'. Values are means, n=6, ± SEM. A) Number of microcuttings producing in vitro roots; y = 0.788 – 0.0076x, r^2 = 0.86 B) Number of in vitro roots per microcutting; y = 3.14 – 0.03x, r^2 = 0.53 and 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.

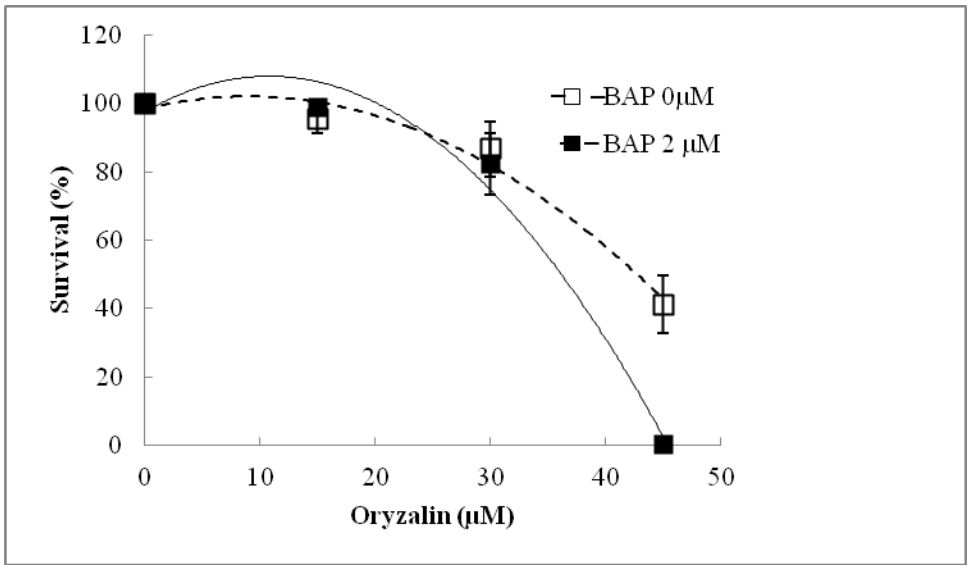

Figure 3. Survival rates of microshoots of *A. platanoides* 'Crimson Sentry' as a factor of oryzalin concentration and treatment with 0 or 2 µM BAP. Lines represent trends fitted using quadratic regression analysis. For 0 µM BAP, Survival = 99 + 0.70 (oryzalin) - 0.04522 (oryzalin^2), r^2 = 0.94. For 2 µM BAP, Survival = 97 + 1.90 (oryzalin) – 0.0892 (oryzalin^2), r^2=0.91.

CHAPTER 2

History and Cytological Reassessment of *Rhododendron canadense*

(In the format appropriate for submission to the Journal of the American Rhododendron Society)

History and Cytological Reassessment of *Rhododendron canadense*

Jason D. Lattier[1], Thomas G. Ranney[2], and Nathan P. Lynch[3]

Mountain Crop Improvement Lab, Department of Horticultural Science, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, 455 Research Drive, Mills River, NC 28759-3423

Received for publication . Accepted for publication .

This research was funded, in part, by the North Carolina Agricultural Research Service (NCARS), the Garden Club of America, and the Royal Horticultural Society (UK). Use of trade names in this publication does not imply endorsement by the NCARS of products named nor criticism of similar ones not mentioned. From a thesis submitted by J. D. Lattier in partial fulfillment of the requirements for the MS degree.

[1]Graduate Research Assistant and Corresponding Author. Email: jdlattier@gmail.com

[2]Professor.

[3]Research Specialist

Summary

Rhododendron canadense represents a morphologically unique and taxonomically complicated species of azalea. Early attempts at classification commonly placed *R. canadense* and *R. vaseyi* together in clades based upon odd features such as extra stamens and tubeless flowers. However, more recent molecular studies suggest *R. canadense* shares a closer relationship with other deciduous azaleas including *R. luteum, molle,* and *calendulaceum*. Cytological and cytometric studies have yielded equally confusing results by reporting inordinately small genome sizes (DNA content) and both diploid and tetraploid individuals of *R. canadense*. This study utilized flow cytometry and traditional cytology to provided conclusive evidence that *R. canadense* exists primarily as a diploid species. A survey of relative genome size of *R. canadense* also revealed that this species has approximately 26% less DNA than most other deciduous azaleas. Further cytological investigation indicated that *R. canadense* may also possesses a lower base chromosome number with $2n = 2x = 24$ compared to most other *Rhododendron* with $2n = 2x = 26$. Additional investigation into *R. canadense* must be undertaken to reveal the full complexities of its cytogenetics, evolutionary history, and relationships within *Rhododendron*.

Introduction

Rhododendron canadense (L.) Torr., commonly known as rhodora, is an unusual, primitive azalea that has been plucked from the rising grassy tussocks of sodden bogs for use in ornamental landscapes. Suited to the cold, wet conditions of northeast North America, *R.*

canadense has charmed countless gardens with its bare, low-growing stems topped with trusses of rose-pink to purple flowers in the spring (Judd and Kron, 1995). In addition to providing a distinctive feature in the garden, *R. canadense* has also played an important cultural role in its native New England. After spending years exploring the natural landscape of Massachusetts, the renowned poet Ralph Waldo Emerson was so moved by this "rival of the rose" that he penned one of his most famous poems, "The Rhodora", in its honor (Wilson and Rehder, 1921). The rhodora was again honored in Massachusetts by becoming the namesake of the journal of the New England Botanical Club, a prestigious Harvard-affiliated scientific journal.

Rhododendron canadense has several morphological features that make it conspicuous among the deciduous azaleas. In April to early July, flowers occur before (or occasionally with) the leaves, and are borne on terminal, umbellate racemes of 3-9 rose-purple to pink (rarely white) flowers (Judd and Kron, 1995). The distinct feature of the *R. canadense* flower is the complete lack of a floral tube due to lack of fusion in its corolla. Flowers are two-lipped, with the fusion of the upper three corolla lobes contrasted by the flaring two lower lobes (Judd and Kron, 1995). Not only does *R. canadense* have the only tubeless flower of the deciduous azaleas, it also typically has ten or seven stamens instead of the usual five (though *R. vaseyi* Gray and *R. schlippenbachii* Maxium are nearly tubeless with more than five stamens) (Galle, 1987; Judd and Kron, 1995; Towe, 2004).

Rhododendron canadense typically grows as a rhizomatous shrub reaching a height of less than 1m. This unique species is the most northern of all the eastern North America

azaleas, and can be found in glaciated areas along river banks, in moist woods, and in swamps from sea level to 1900 m. The geographic distribution of *R. canadense* stretches from Labrador and Newfoundland to southwestern Quebec, continuing down through New England into central New York, northern New Jersey, and northeastern Pennsylvania (Galle 1982; Judd and Kron, 1995; Towe, 2004).

Before its classification as *R. canadense* (Torrey, 1839), rhodora underwent several classifications including *Rhodora canadensis* (Linnaeus, 1762), *Hochenwartia canadensis* (Von Crantz, 1766), *Rhododendron rhodora* (Gmelin, 1791), and *Azalea canadensis* (Kuntze, 1891). The construction of phylogenies for *Rhododendron* and the particular placement of *R. canadense* have historically proven difficult. The first major classification of *Rhododendron* performed by George Don (1834) divided the genus into sections, with *R. canadense* belonging to section *Pentanthera* G. Don. Wilson and Rehder's monograph (1921) recognized the rank of subgenera, with subgenus *Anthodendron* (Endl.) Rehder section *Rhodora* (L.) G. Don containing *R. vaseyi*, *R. canadense*, *R. nipponicum, R. pentaphyllum* Maxim, and *R. albrechtii* Maxim. Philipson W. (1980), Philipson M. (1980), and Judd and Kron (1995) placed *R. canadense* in subgenus *Pentanthera* (G. Don) Poyarkova and further reduced section *Rhodora* to only *R. vaseyi* and *R. canadense*, taxa exhibiting a two-lipped corolla with a glabrous inner surface.

In addition to morphological studies of *Rhododendron*, studies using macromolecular data have helped determine relationships within the genus (Gao et al., 2002; Goetsch et al., 2005; Kurashige et al., 1998, 2001). Kurashige et al. (2001) and Gao et al. (2002)

constructed phylogenies based on chloroplast matK and trnK intron sequences and nuclear ITS sequences, respectively; both placed *R. canadense* in subgenus *Pentanthera* section *Rhodora*, sister to section *Pentanthera*. More recently, Goetsch et al. (2005) evaluated 87 species based on analysis of the RPB2-I gene and found *Rhododendron canadense* was nested within section *Pentanthera* and more closely allied with *R. luteum* (L.) Sweet, *molle* (Bl.) G. Don, and *calendulaceum* (Michx.) Torr.

Rhododendron canadense does not hybridize readily with other species (Galle, 1987). However, limited hybrids have been reported including *R.* ×*seymourii* Herbert ex Lindley (*R. canadense* × *luteum*) and *R.* ×*fraseri* W. Watson (*R. canadense* × *japonicum* (A. Gray) Sur.) (Wilson and Rehder, 1921).

Studies on genome sizes and ploidy levels provide insights into the cytogentics of *Rhododendron*. Early efforts to verify ploidy level relied on counting chromosomes, though cytological studies of *Rhododendron* are notoriously difficult. The counting of *Rhododendron* chromosomes has historically proven a laborious, time consuming, difficult task because of the friability and small size of roots, the inordinately small chromosomes, interference of tannin granules, and the difficulty of obtaining meiotic pollen mother cells in the proper stage. (Galle 1970, McAllister, 1993, Li 1957). For these reasons, relatively few studies have been conducted on cytogenetics and chromosome numbers of *Rhododendron*.

The earliest cytological investigation into *Rhododendron* was performed by Moringa et al. (1929) on *R. quinquefolium* var. *speciosum* N. Yonez., finding the base chromosome compliment of *Rhododendron* to be $x = 13$. Sax (1930) confirmed this finding in a study that

tested pollen mother cells of 16 species. Sax (1930) found all samples tested to be diploid ($2n = 2x = 26$) with the exception of 2 tetraploids ($2n = 4x = 52$), *R. calendulaceum* and *R. canadense*. In a large-scale study in 1950, Ammal et al. investigated 360 species of *Rhododendron* completing over 550 counts. They confirmed Sax's earlier findings of tetraploidy in *R. calendulaceum* and *R. canadense*, finding them to be the only polyploid *Rhododendron* in North America. However, for such a monumental study, no methods or materials were published, and subsequent studies have reached contradicting conclusions for certain species (Eeckhaut, 2004; Jones et al., 2007; Li, 1957; Zhou et al., 2008).

Numerous studies have utilized flow cytometry to quantify relative genome size and ploidy level in *Rhododendron* (De Schepper et al., 2001; Jones et al., 2007; Väinölä, 2000; Zhou et al., 2008). Flow cytometry measures fluorescence of individual nuclei, stained with a fluorescent dye, while suspended in a precise stream of fluid as a means to determine genome size (DNA content) relative to an internal standard (Doležel and Bartoš, 2005). Zhou et al. (2008) utilized flow cytometry to confirm tetraploidy in *R. luteum*, *R. atlanticum* Rehder, *R. calendulaceum, R. colemanii* R. Miller, and *R. austrinum* (Small) Rehder, but found *R. canadense* to be a diploid. However, only a single clone of *R. canadense* was tested, and the authors proposed further investigation into the ploidy of *R. canadense* to confirm their findings (Zhou et al., 2008).

Due to the contradictions in the literature regarding the phylogeny and ploidy level assessment of *R. canadense*, the objective of this study was to perform a survey of genome size and ploidy level of *R. canadense* samples from throughout its natural range.

Methods and Materials

Flow Cytometry. To determine relative genome size of *R. canadense*, a survey composed of 11 *R. canadense* samples from across its natural range (Table 1) was conducted using flow cytometry. For each sample, approximately 1 cm^2 of newly expanded leaf tissue was placed in a petri dish containing 500 µL of nuclei extraction buffer (CyStain ultraviolet Precise P Nuclei Extratcion Buffer®; Partec, Münster, Germany) and chopped finely with a razor blade until sample was completely incorporated into the buffer. After 1-2 minute incubation at room temperature (22^oC), the resulting solution was filtered through a Partec CellTrics^{TM} disposable filter with a pore size of 50 µm. Then, 1.2 mL of a nucleotide staining buffer (4', 6-diamidino-2-phenylindole; CyStain ultraviolet Precise P Staining Buffer; Partec) (DAPI) was added to the filtered solution. The resulting stained nuclei were analyzed with a flow cytometer (Partec PA II; Partec) with counts exceeding a minimum of 3,000 cells per analysis. The mean fluorescence for each sample was compared with an internal standard of known genome size (*Pisum sativum* 'Ctirad' 2C genome size = 8.75 pg) and holoploid, 2C genome size (i.e., DNA content of entire non-replicated chromosome compliment irrespective of ploidy) was calculated as 2C = DNA content of standard × (mean fluorescence of sample / mean fluorescence of standard).

Cytology. Over the course of a year, actively growing root tips were collected following a sunny day from rooted stem cuttings and mature plants of both *R. canadense* (NCSU 2009-164, Long Pond, PA) and *R. canadense* (NCSU 2009-173, Jaffrey, NH). Root tips containing mitotic cells were collected before 10AM and placed in freshly made vials of

pre-fixative solution (2 mM 8-hydroxyquinoline + 70 mg L^{-1} cycloheximide). The compound 8-hydroxyquinoline has proven an effective method to condense chromosomes and destroy the mitotic spindle in order to arrest cells at metaphase in somatic plant cells (Grant and Owens, 1998; Grant and Owens, 2001; Watanabe and Orrillo 1993). Baszczynski et al. (1980) demonstrated that cycloheximide acts in the G2 phase and late metaphase, altering the movement of cells through the nuclear cycle as well as causing an increase in the mitotic index, an increase in metaphase frequency, a decrease in anaphase frequency, and an inhibition of the spindle fiber apparatus assemblage. After remaining in the dark for 3 hours, all vials were moved into a dark refrigerator at approximately 4^{o}C for 3 hours yielding a total pre-fixative treatment of 6 hours. All root tips were rinsed with distilled water and transferred to a freshly made 1:3 fixative solution of propionic acid : 95% ethanol, and left overnight at room temperature (22^{o}C). The next day, a 1:3 hydrolysis solution of 12M HCl : 95% ethanol was made for the root squash procedure.

For each root squash, a fresh root was removed from the fixative and hydrolyzed for approximately 30 seconds before being moved to a clean slide. The root tip was excised under a dissecting microscope and placed on a separate, clean slide with a drop of modified carbol-fuchsin stain. The stain used was a modification of the Carr and Walker (1961) carbol-fuchsin stain for human chromosomes (Kao, 1975). The slide was tilted until the stain completely encompassed the excised root tip, and left for several minutes. Then, a clean cover slip was placed over the droplet of stain containing the excised root tip. A clean sheet of bibulous paper was placed over the cover slip while gently applying pressure with a pencil

eraser (sometimes thumb) to squash the cells. The slide was then observed and photographed using a light microscope (Nikon Eclipse 80i, Nikon, Melville, NY). Layered images containing multiple depths of field were composed using Photoshop CS4 (Adobe Systems, San Jose, CA).

Results and Discussion

Flow Cytometry. All 11 samples of *R. canadense* were found to be diploid (Table 1), in contrast to previous reports of tetraploid individuals (Ammal et al., 1950; Sax, 1930). Relative genome sizes (2C) in our study ranged between 1.07 pg and 1.23 pg (mean 1.15 pg). The fluorescent stain DAPI used in this study binds to adenine-thymine rich regions of DNA, though other stains are available that may provide slightly different estimates of absolute genome size (Doležel and Bartoš, 2005).

Though the sampling distribution covered multiple locations across nearly the entire geographic distribution of *R. canadense*, no tetraploids were found. These results augment the findings of Zhou et al. (2008) and further demonstrate that *R. canadense* is primarily a diploid species. In addition, relative genome sizes (2C) of all *R. canadense* tested proved unusually small (1.07 to 1.23 pg) when contrasted with diploid genome sizes of other deciduous azaleas in previous studies (Jones et al., 2007; Zhou et al. 2008). In the study by Zhou et al. (2008), diploid azalea genome sizes (not including *R. canadense*) ranged between 1.44 pg and 1.57 pg, and in Jones et al. (2007) the diploid azaleas ranged between 1.51 pg and 1.74 pg.

After the flow cytometry results revealed significant discrepancies in relative genome size (2C) between *R. canadense* and other diploid azaleas from previous studies, a separate cytometric analysis was performed by combining leaf tissue from both *R. canadense* (NCSU 2009-164, Long Pond, PA) and *R. periclymenoides* (NCSU 2011-027), as well as *Pisum sativum* 'Ctirad' (internal standard) into a single sample. The resulting histograms showed there to be a significant difference in relative genome size between *R. canadense* and *R. periclymenoides* (Fig. 2), with *R. canadense* having approximately 26% less total DNA than *R. periclymenoides*.

Previous studies indicate that evolution of base genome size (i.e., DNA content of one complete set of chromosomes: 1Cx value) in angiosperms is a dynamic process of increases and decreases, with the general trend of small genome size representing the ancestral state and larger genome size representing derived state (Leitch et al., 1998, 2005; Soltis et al., 2003). Surprisingly, however, 1Cx genome size in *Rhododendron* (not including *R. canadense*) is highly conserved with 1Cx values between 0.61- 0.97 pg for the entire genus and between 0.72 – 0.97 pg for subgenus *Pentanthera* (Jones et al., 2007). We found *R. canadense* to have a particularly low relative (1Cx) genome size of 0.54-0.62 pg, considerably below the typical range for subgenus *Pentanthera*. Interestingly, Zhou et al. (2008) also found *R. luteum* to have a somewhat low 1Cx genome size of 0.65 pg, a species reported to be fairly closely allied to *R. canadense* in phylogenies based on macromolecular data (Gao et al., 2002; Goetsch, 2005; Kurashige et al., 2001). These results suggested further investigation into base chromosome number was warranted. Therefore, two samples

of *R. canadense* (NCSU 2009-164, Long Pond, PA and NCSU 2009-173, Jaffrey, NH) were selected for further study using traditional cytological techniques to determine the true diploid chromosome number of *R. canadense*.

Cytology. Despite extensive cytological examination performed over the course of a year, including observation of >20 cells with well-resolved chromosomes, no diploid cells were found where more than 24 chromosomes could be discerned (Fig. 3 and 4). These results confirm *R. canadense* to be a diploid and further suggest a base chromosome number of $x = 12$.

The plants in the genus *Rhododendron* have been thought to have a uniform base chromosome number of $x = 13$, with the exception of plants in the basal subgenus *Therorhodion* (Maxim.) Small with $x = 12$ (Kron and Judd, 1990; Kurashige et al., 2001; Gao et al., 2002, Stevens, 1971). Curiously, *R. canadense* (subgenus *Pentanthera*) and *R. camtschaticum* Pall. (subgenus *Therorhodion*) share certain traits, such as rhizomatous growth, July blooms of speckled pink/purple flowers, connate petals with a tubeless corolla, and 10 stamens (Hutchinson, 1921). Perhaps *R. canadense* retained the ancestral chromosome number of $2n = 2x = 24$, similar to plants in subgenus *Therorhodion*, but that is unlikely, based on recent phylogenetic analyses (Kurashige et al., 2001; Gao et al., 2002; Goetsch et al., 2005). Alternatively, various processes such as translocations, inversions, insertions or deletions can lead to chromosome fusion (Da Silva, 2005; Luo et al., 2009; Lysak et al., 2006; Schubert, 2007) resulting in reduced chromosome numbers. This may also be the case in *R. canadense*.

Acknowledgements

We gratefully acknowledge John and Sally Perkins, Salem, NH; Jim and Bethany Plyler, Natural Landscapes Nursery, West Grove, PA; Stephen Krebs, Holden Arboretum, Kirtland, OH; Bruce Clyburn, Cape Brenton, NS; and George Newman, Bedford, NH for their cooperation and contributions of plant material for this study. Assistance of the staff of the Mountain Horticultural Crops Research Station is gratefully acknowledged. Thanks also to L. Clarence Towe, Walhalla, SC, Andrew Whipple, Upland, IN, and Ron Miller, Pensacola, FL, for reviewing this manuscript.

Literature Cited

Ammal, E.K.J, I.C. Enoch and M. Bridgwater. 1950. Chromosome numbers in species of rhododendron. Rhododendron Yrbk. 5: 78-91.

Baszczynski, C.L., D.B. Walden and B.G. Atkinson. 1980. Cycloheximide-induced nuclear alteration in maize root tips. Can. J. Genet. and Cytol. 22 (3): 319-331.

Carr, D.H. and J.E. Walker. 1961. Carbol fuchsin as a stain for human chromosomes. Stain Technology 36: 233-236.

De Schepper, S., L. Leus, M. Mertens, E. Van Bockstaele and M. De Loose. 2001. Flow cytometric analysis of ploidy in Rhododendron (subgenus Tsutsusi). HortScience 36 (1): 125-127.

Doležel, J. and J. Bartoš. 2005. Plant DNA flow cytometry and estimation of nuclear genome size. Ann. Bot. 95: 99-110.

Don, G. 1834. A general history of Dichlamydeous plants. Vol. 3, Calyciflorae. J.F. and F. Rivington, London.

Eeckhaut, T.G.R., L.W.H. Leus, A.C. De Raedt and E.J. Van Bockstaele. 2004. Occurrence of polyploidy in *Rhododendron luteum* Sweet, Hardy Ghent, and Rustica hybrids. The Azalean 26: 32-37.

Galle, F.C. 1970. 'Bear Facts' on Gregory Bald. J. Amer. Rhododendron Soc. 24 (4): 199-200.

Galle, F.C. 1987 (Reprinted 1991, 1995). Azaleas: Revised and Enlarged Edition. Timber Press; Portland, Oregon.

Gao , L.M., D.Z. Li, C.Q. Zhang and J.B. Yang. 2002. Infra-generic and sectional relationships in the genus Rhododendron (Ericaceae) inferred from ITS sequence data. Acta Botanica Sinica 44: 1351-1356.

Grant, W. F. and E. T. Owens. 1998. Chromosome aberration assays in Crepsis for the study of environmental mutagens. Mutation Res. 410: 291-307.

Grant, W. F. and E. T. Owens. 2001. Chromosome aberration assays in Pisum for the study of environmental mutagens. Mutation Res. 488: 93-118.

Gmelin, J.F. 1791. Systema Naturae. ed. 1791 (2)1: 694.

Goetsch, L.A., A.J. Eckert and B.D. Hall. 2005. The molecular systematic of Rhododendron (Ericaceae): a phylogeny based upon RPB2 gene sequences. Sys. Bot. 30: 616-626.

Hutchinson, J. 1921. The genus Therorhodion. Bulletin of Miscellaneous Information (Royal Gardens, Kew) 5: 201-205.

Jones, J.R., T.G. Ranney, N.P. Lynch and S.L. Krebs. 2007. Ploidy levels and relative genome sizes of diverse species, hybrids and cultivars of Rhododendron. J. Amer. Rododendron Soc. 61(4): 220-227.

Judd, W.S. and K.A. Kron. 1995. A revision of Rhododendron VI. Subgenus Pentanthera (Sections Sciadorhodion, Rhodora and Viscidula). Edinburgh J. Bot. 52: 1-54.

Kao, K.N. 1975. A nuclear staining method for protoplasts. In: O.L.Gamborg and L.Z. Wetter (ed.) Plant Tissue Culture Methods. L.R. National Research Council of Canada. Praire Regional Labratory. Saskatoon, Saskatchewan, Canada 1975, p. 60-64.

Kron, K. A. and W.S. Judd. 1990. Phylogenetic Relationships within the Rhodoreae (Ericaceae) with Specific Comments on the Placement of Ledum. Systematic Bot. 15(1): 57-68.

Kuntze, O. 1891. Revisio Generum Plantarum. Vol. 2. Leipzig. Pg. 386.

Kurashige, Y., M. Mine, N. Kobayashi, T. Handa, K. Takayangi and T. Yukawa. 1998. Investigation of sectional relationships in the genus Rhododendron (Ericaceae) based on matK sequences. J. Jpn. Bot. 73: 143-154.

Kurashige, Y., J.I. Etoh, T. Handa, K. Takayanagi and T. Yukawa. 2001. Sectional relationships in the genus Rhododendron (Ericaceae): evidence from matK and trnK intron sequences. Plant Systematics and Evolution 228: 1-14.

Li, H. 1957. Chromosome studies in the azaleas of eastern North America. Am. J. Bot. 44(1): 8-14.

Linnaeus, C. 1762. Species plantarum, ed. 2. Stockholm. Pg 561.

McAllister, H.A. 1993. Chromosome numbers in rhododendrons. In: Rhododendrons with camellias and magnolias. Royal Hort. Soc. London, 24-30.

Moringa, T., E. Fukushima, T. Kanui and Y. Tamasaki. 1929. Chromosome numbers of cultivated plants. Botanical Mag. (Tokyo) 43: 589-594.

Philipson, M.N. 1980. Cotyledons and Rhododendron classification. In: Luteyn, J. and M. O'Brien (eds) Contributions toward a classification of Rhododendron. Proc. Intl. Rhododendron Conf., pp. 75-88. New York Botanical Garden, Bronx, NY.

Philipson. W.R. 1980. Problems in classification of the azalea complex. In: Luteyn, J. and M. O'Brien (eds) Contributions toward a classification of Rhododendron. Proc. Intl. Rhododendron Conf., pp. 53-62. New York Botanical Garden, Bronx, NY.

Sax, K. 1930. Chromosome stability in the Genus Rhododendron. Amer. J. Bot. 17(4): 247-251.

Stevens, P.F. 1971. A classification of the Ericaceae: subfamilies and tribes. Bot. J. Linnean Soc. 64: 1-53.

Torrey, J. 1839. Catalogue of Plants. In: Geological Survey, New York Assembly 50: 151.

Towe, L.C. 2004. American Azaleas. Timber Press, Portland and Cambridge, 146pp.

Väinölä, A. 2000. Polyploidization and early screening of *Rhododendron* hybrids. Euphytica 112: 239-244.

Von Crantz, H.J.N. 1766. Institutiones rei herbariae: juxta nutum natur naturae digestae ex habitu. Vol. II. Vienna. Pg 469.

Wilson, E.H. and A. Rehder. 1921. A monograph of the azaleas. Publ. of the Arnold Arboretum 9: 125-206.

Watanabe, K. N. and M. Orrillo. 1993. An alternative pretreatment method for mitotic chromosome observation in potatoes. Amer. J. Potato Res. 70 (7): 543-548.

Zhou, W., T. Gibbons, L. Goetsch, B. Hall, T. Ranney, R. Miller. 2008. Rhododenron colemanii: A new species of deciduous azalea (Rhododendron section Pentanthera; Ericaceae) from the coastal plain of Alabama and Georgia. J. Amer. Rhododendron Soc. 62(4): 72-78.

Table 1. Genome sizes and ploidy levels of *Rhododendron canadense* samples from throughout its natural range.

Location/Source	2C Genome size (pg)[Z]	Ploidy Level (*x*)
Lincoln Bog, Ashburnham, MA, USA. Perkins[K].	1.13 ± 0.02	2*x*
Tobyhanna State Park. Tobyhanna, PA. Perkins[K].	1.07 ± 0.03	2*x*
Exit 1, Interstate 93. Salem, NH, USA. Perkins[K].	1.16 ± 0.02	2*x*
Exit 1, Interstate 93. Salem, NH, USA. Perkins[K].	1.14 ± 0.02	2*x*
Ponemah Bog. Amherst, NH, USA. Perkins[K].	1.17 ± 0.02	2*x*
Bradford Bog. Bradford, NH, USA. Perkins[K].	1.15 ± 0.04	2*x*
Mt. Kearsarge Bog. Warner, NH, USA. Perkins[K].	1.11 ± 0.03	2*x*
Saco Heath Bog. Saco, Maine, USA. Perkins[K].	1.13 ± 0.05	2*x*
Cape Brenton, Nova Scotia, Canada. Clyburn[W].	1.17 ± 0.03	2*x*
Mt. Monadnock, Jaffrey, NH. Newman[X] via Perkins[K]. NCSU 2009-164	1.23 ± 0.02	2*x**
Long Pond, Poconos Mts., PA. Plyler[Q] via Krebs[V]. NCSU 2009-173	1.22 ± 0.01	2*x**

[K]Perkins – Sally and John Perkins, Salem, NH.
[Q]Plyler – Jim and Bethany Plyler, Natural Landscapes Nursery, West Grove, PA.
[V]Krebs – Stephen Krebs, Holden Arboretum, Kirtland, OH.
[W]Clyburn – Bruce Clyburn, Cape Brenton, NS.
[X]Newman – George Newman, Bedford, NH.
[Z]8.75 picograms was use as the genome size for the internal standard, *Pisum sativum* 'Ctirad'.
*Ploidy confirmed with cytology.

Figure 1. Locations of *R. canadense* sampling sites. Map compiled using Google Earth (Google Inc., Mountain View, CA)

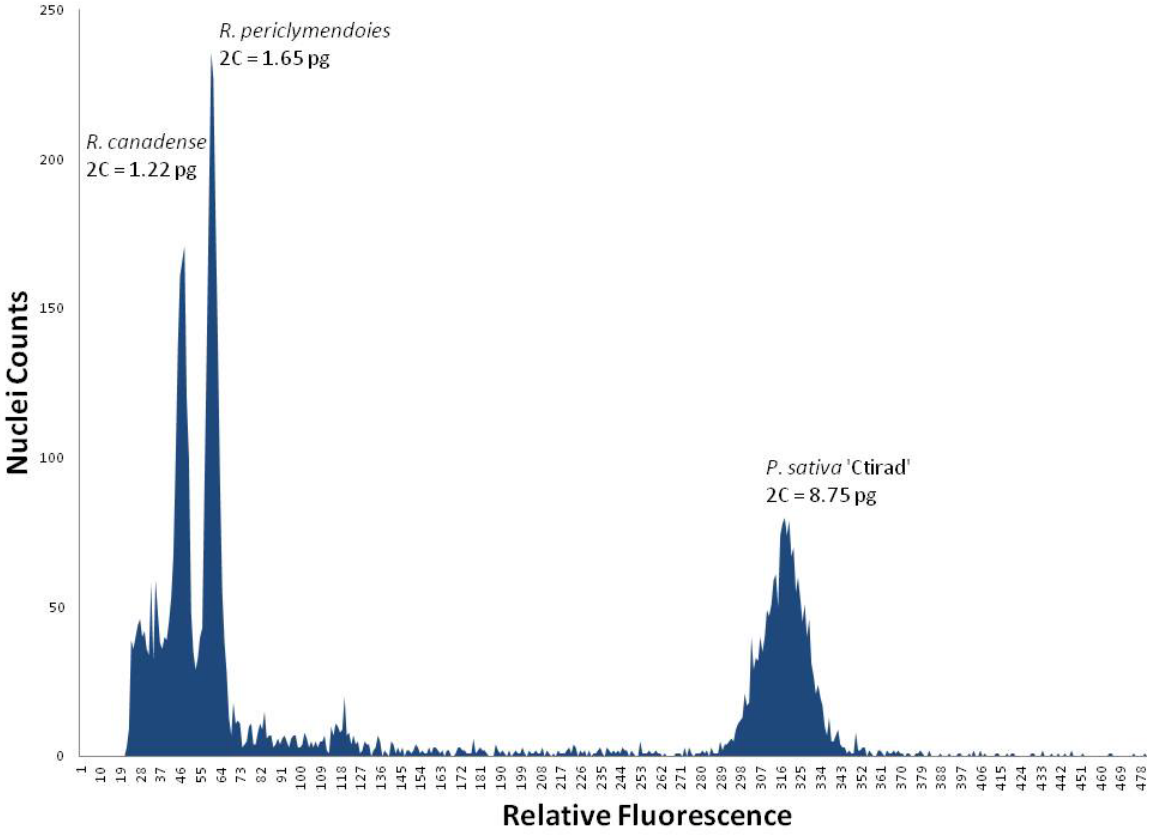

Figure 2. Comparison of genome size of *R. canadense* L. (NCSU 2009-164) and *R. periclymenoides* Michx. (NCSU 2011-027)

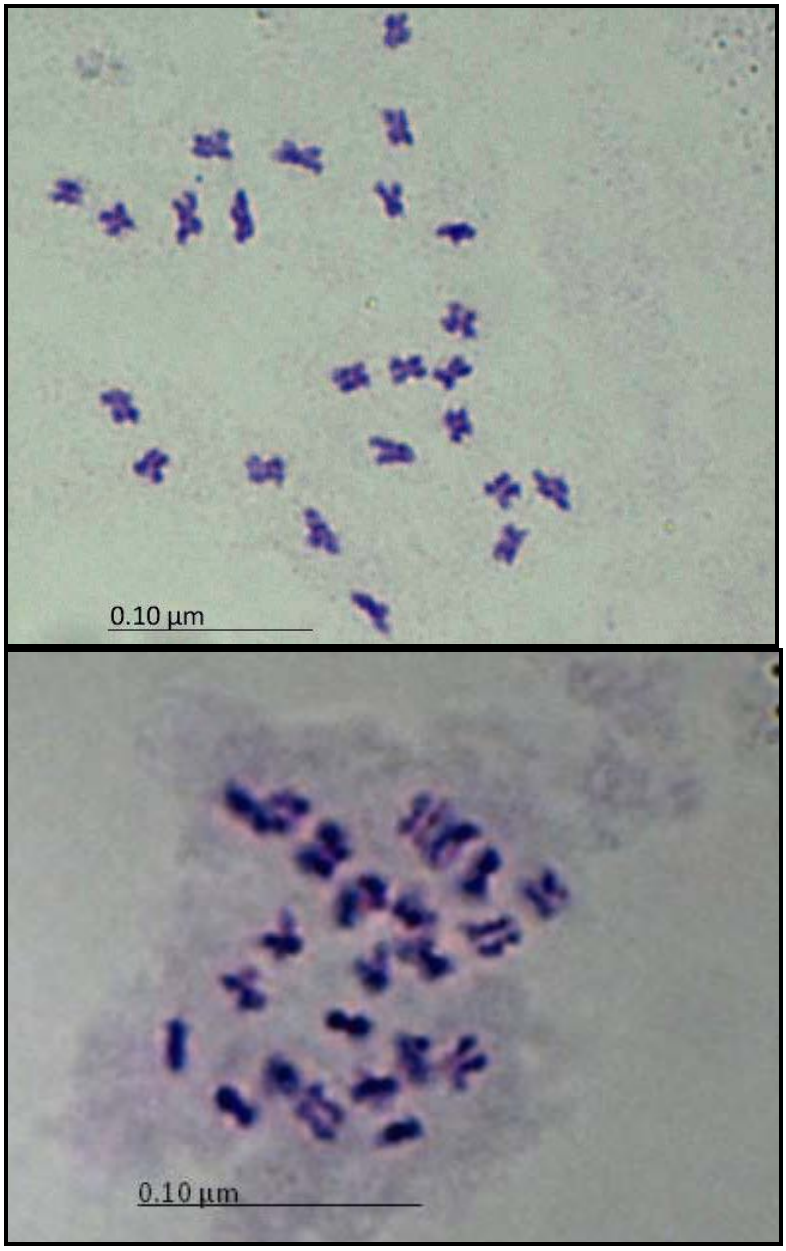

Figure 3. Photomicrographs of metaphase chromosomes from root tip cell of *R. canadense* L. (NCSU 2009-164) collected on Mt. Monadnock, Jaffrey, NH. Viewed at 1000x.

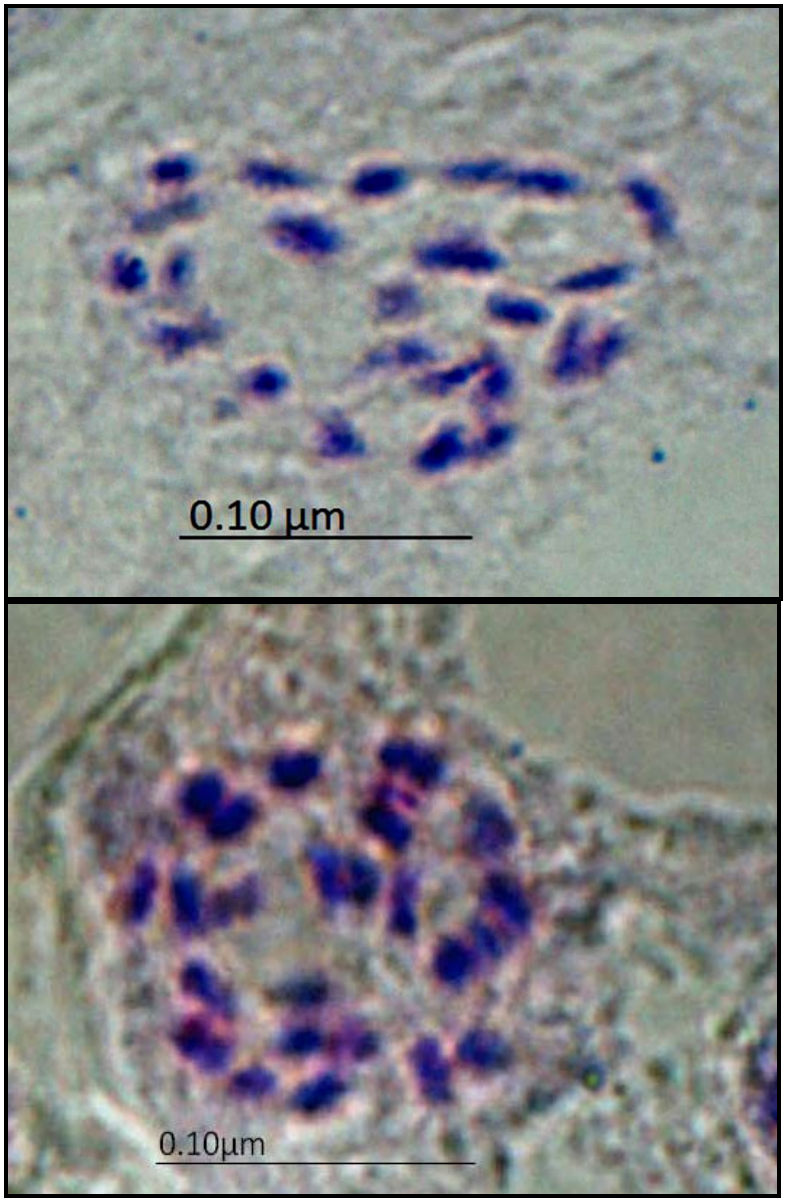

Figure 4. Photomicrographs of metaphase chromosomes from root tip cell of *R. canadense* L. (NCSU 2009-173) collected in the Pocono Mountains, Long Pond, PA. Viewed at 1000x.

CHAPTER 3

Survey of Genome Sizes and Ploidy Levels of *Liriope* and *Ophiopogon* Taxa

(In the format appropriate for submission to HortScience)

Survey of Genome Sizes and Ploidy Levels of *Liriope* and *Ophiopogon* Taxa

Jason D. Lattier[1], Thomas G. Ranney[2], Paul R. Fantz[3], and Tony Avent[4]

Mountain Crop Improvement Lab, Department of Horticultural Science, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, 455 Research Drive, Mills River, NC 28759-3423

Received for publication . Accepted for publication .

This research was funded, in part, by the North Carolina Agricultural Research Service (NCARS), Raleigh, NC, 27695-7643. Use of trade names in this publication does not imply endorsement by the NCARS of products named nor criticism of similar ones not mentioned. We gratefully acknowledge Mark Weathington, J.C. Raulston University, Raleigh, NC; Alexander Krings, NC State University Herbarium, Raleigh, NC; Tony Avent, Dennis Carey, and Jeremy Schmidt, Plant Delights Nursery, Raleigh, NC; Darrell Probst, Garden Vision Epimediums, Hubbardston, MA; Ron Rabideau, RareFind Nursery, Jackson, NJ; Paul Jones, Sarah P. Duke Gardens, Durham, NC; Nathan Lynch and Kim Shearer, Mountain Crop Improvement Lab, Mills River, NC for their cooperation, technical assistance, and contributions of plant material for this study. From a thesis submitted by J.D.L. in partial fulfillment of the requirements for the MS degree.

[1]Graduate Research Assistant and Corresponding Author. Email: jdlattier@gmail.com

[2]Professor

[3]Professor Emeritus, Department of Horticultural Science and Research Taxonomist, North Carolina State University Herbarium (NCSC), Department of Plant Biology, North Carolina State University, Raleigh 27518

[4]Owner, Plant Delights Nursery; Founder, Juniper Level Botanic Garden; Raleigh, NC 27603

Abstract

Liriope Lour. and *Ophiopogon* Ker Gawl., collectively known as liriopogons, represent important evergreen groundcovers grown throughout the world for their variety of ornamental features and medicinal qualities. Nursery practices including sexual propagation of cultivars, plant substitution, mislabeling of cultivars, and seedling invasion of stock plants have resulted in cultivar degradation within the nursery industry. Due to the wide range of ornamental traits found in liriopogons and evidence of interspecific and intergeneric hybridization, there is considerable potential for breeding and improvement of liriopogons. However, breeding efforts are currently constrained by confusion over proper identification and taxonomy, and lack of information on ploidy levels and cytogenetics of individual clones and cultivars. The objectives of this study were to validate the identification and nomenclature, as well as determine genome sizes and ploidy levels for an extensive reference collection of species and cultivars of liriopogons. Identification was accomplished using existing keys. Calculation of genome size was accomplished by extracting nuclei, staining them with propidium idodide, and analyzing using a flow cytometer. Ploidy levels for each

species were confirmed by traditional cytology. Based on our results, *Liriope* examined fit into three ploidy groups, with the exception of a ploidy series of *L. platyphylla*. The diploid group consisted of *L. graminifolia*, *L. longipedicellata*, *L. minor*, and half of the *L. platyphylla*. The tetraploid group consisted of *L. muscari* and half of the *L. platyphylla*. The hexaploid group consisted of *L. exiliflora*. *Ophiopogon* included in this study formed two ploidy groups. The diploid group contained *O. intermedius, O. jaburan*, *O. planiscapus*, and *O umbraticola*. The tetraploid/hypotetraploid group contained *O. japonicus*. There was a general trend across all liriopogons of increased vigor and fertility at higher genome sizes and ploidy levels. Improving breeding strategies for liriopogons requires a resource of plants with similar genome sizes and ploidy levels to enable selections for controlled crosses. This reference collection will aid future taxonomic revisions, as well as assist in efforts to develop new breeding strategies for liriopogons.

Introduction

Liriope Lour. and *Ophiopogon* Ker Gawl. (*Ruscaceae* Hutch, formerly assigned to *Convallariaceae* Horan., *Asparagaceae* Juss., Haemodoraceae Arnot, *Ophiopogonaceae* Kunth, and *Liliaceae* Juss.) are collectively known as liriopogons (Skinner, 1971) and comprise a class of important evergreen groundcovers grown throughout the world. Liriopogons are native to China, India, Japan, Korea, Philippines and Vietnam with *Liriope* consisting of approximately 8 species and *Ophiopogon* consisting of approximately 54 species (Li et al., 2011; Xia, 2012). The popularity of liriopogons is due in part to their

tolerance of pollution (Li et al., 2011) and versatility in the landscape, easily filling the roles of groundcovers, foundation plants, edging and massing plants, and understory plants (Fantz, 1993).

The complex taxonomy of liriopogons has been evolving since the initial designation of *Convallaria japonica* by Thunberg (1780). Centuries following resulted in many genera designations (*Anemarrhena* Bunge, *Chloopsis* Blume, *Convallaria* L., *Flueggea* Rich., *Liriope*, *Mondo* Adans., *Ophiopogon*, *Polygonastrum* Moench, and *Slateria* Desv.) and trade names (Aztec grass, bordergrass, lilyturf, liriope, mondo grass, monkeygrass, and snakesbeard) (Fantz, 1993; Nesom 2010). Nevertheless, liriopogons' attractiveness, resistance to pest and diseases, hardiness, and utility in the landscape made them a valuable nursery crop. Wholesale values of liriopogons in North Carolina are estimated to be over $41 million for 2009 (Trueblood, 2009).

Much of the confusion surrounding liriopogons lies in morphological similarities between the two genera. Both *Liriope* and *Ophipogon* are acaulescent, evergreen herbs that set summer/fall racemes of small pink to purple or white flowers. Floral whorls are found in multiples of three (dichasia to compound dichasia to small cymes) (Fantz, 2008a). The perianth has 6 indistinguishable sepals and petals, and 6 stamens. Seeds of liriopogons are blue/black and berry-like or a three celled capsule (Fantz, 2008a).

Anatomical studies by Cutler (1992) and Rudall (2000) as well as a molecular marker investigation by Mcharo et al. (2003) concluded that similarities between *Liriope* and *Ophiopogon* were too great, and separation into two genera was unwarranted. However,

morphological studies by Bailey (1929), Hume (1961), Skinner (1971), and Conran and Tamuri (1998), molecular phylogenetic studies by Kim et al. (2010), and a molecular marker study by Li et al. (2011) provided evidence supporting separation of *Liriope* and *Ophiopogon*.

A recent overview of *Liriope* and *Ophiopogon* cultivated in the United States reported by Nesom (2010) found floral characteristics the best method of distinguishing between *Liriope* and *Ophiopogon*, supporting Fantz (2008a). Flowers belonging to *Liriope* are erect with corollas cupulate to rotate, superior ovaries, and free anthers with apical poricidal openings and long filaments. In contrast, flowers of *Ophiopogon* are nodding with corollas campanulate, inferior to semi-inferior ovaries, and connate anthers in a column which narrow apically, dehisce longitudinally, and have short filaments (Nesom, 2010).

In addition to the historically complex taxonomy of liriopogons, nursery practices including sexual propagation of cultivars, plant substitution, mislabeling of cultivars, and seedling invasion of stock plants have resulted in cultivar degradation within the nursery industry (Fantz, 1994). Fantz (1994) investigated 22 named species and 88 labeled cultivars of *Liriope* and *Ophiopogon* collected from nurseries that resulted in 17% of germplasm misidentified to genus and 36% misidentified to species.

A variety of ornamental features, such as flower color, inflorescence height, branched and fasciated inflorescences, seed color, and foliar variegation, and valuable medicinal qualities such as steroidal glycosides in tubers (Cheng, et al., 2006; Wang et al., 2012; Yu et al., 1996) indicate a high potential for breeding and improvement of liriopogons. However,

breeding systems and cytogenetics of liriopogons are complex. Previous karyological studies have demonstrated the basic chromosome number for liriopogons to be $x = 18$ with high levels of polyploidy in many species (Table 1). Also, Fukai et al. (2008) successfully investigated ploidy level and relative genome size via flow cytometry of six species (plus cultivars) of liriopogons (Table 1). Oinuma (1946) reported polyploid forms of liriopogons exhibit increased vigor and grow over a wider geographic distribution than diploid forms. In addition to various ploidy levels, many studies have shown liriopogons to be uniquely tolerant of high amounts of aneuploidy (abnormal number of chromosomes) and cytochimerism (different chromosome numbers in tissues of the same plant) (Table 1). Therefore, evaluating the cytogenetics of individual clones and cultivars is critical to developing a breeding strategy for liriopogons.

Little evidence exists indicating that intergeneric crosses would be possible in a breeding program for liriopogons. However, in a recent study by Zhou et al. (2009), a cytological investigation of *L. spicata* var. *prolifera* Y. T. Ma and its proto-variety *L. spicata* (Thunb.) Lour. demonstrated that hybridization between *Liriope* and *Ophiopogon* may be occurring naturally in the wild. They confirmed these two taxa to be separate species with *L. spicata* being a tetraploid ($2n = 4x = 72$) and *L. spicata* var. *prolifera* being a triploid ($2n = 3x = 54$). In Hubei, China, *L. spicata* and *Ophiopogon* occur together in the wild with synchronized anthesis. Their karyotypes are similar, both having the same base chromosome belonging to the symmetrical 2B type (Darlington and Wylie, 1955; Tamura, 1995). Also, both exhibited mostly metacentric chromosomes with 24 additional submetacentric

chromosomes (Zhou et al., 2009). Their study concluded that hybridization between tetraploid *L. spicata* and diploid *Ophiopogon* is likely occurring. This conclusion may reveal new possibilities for breeding beyond the genus barrier in liriopogons.

Due to the wide range of ornamental traits found in liriopogons and evidence of interspecific and intergeneric hybridization, there is considerable potential for breeding and improvement of liriopogons. However, these efforts are constrained by confusion over proper taxonomy, lack of information on ploidy levels, and lack of information on cytogenetics of individual clones and cultivars. The objectives of this study were to 1) validate the identification and nomenclature and 2) determine genome sizes and ploidy levels for an extensive reference collection of liriopogons.

Methods and Materials

Plant Material. Accessions of diverse species and cultivars of liriopogons were collected from nurseries, arboreta, and various individuals (Table 2). Containerized and field specimens of liriopogons were examined in this study including *L. exiliflora* (L.H. Bailey) H. H. Hume, *L. gigantea* H. H. Hume, *L. graminifolia* (L.) Baker, *L. longipedicellata* F.T. Wang and T. Tang, *L. minor* (Maxim.) Makino, *L. muscari* (Decne.) L. H. Bailey, *L. platyphylla* F. T. Wang and T. Tang, *O. intermedius* D. Don, *O. jaburan* (Siebold) Lodd., *O. japonicus* (L. f.) Ker Gawl., and *O. umbraticola* Hance. Multiple herbarium vouchers were collected for nearly all taxa and identified based on previous descriptions and available keys for liriopogons (Broussard, 2007; Cutler, 1992; Chen and Tamura, 2000a, 200b; Fantz, 2008a,

2008b, 2009; Hasegawa, 1968; Liu et al. 2007; Nesom, 2010; Tamura, 1990; Tanaka, 2000, 2001a, 2001b, 2001c; Zhang, 1998). The primary collection will be deposited at the North Carolina State University Herbarium (NCSC), Department of Plant Biology, Raleigh and the Herbarium of the U.S. National Arboretum (NA). Images of the collected taxa as well as chromosome micrographs are archived at: http://www.ces.ncsu.edu/fletcher/mcilab/index.html.

Survey of genome size and ploidy level. Genome sizes and ploidy levels were determined by traditional cytology in combination with flow cytometry. To prepare samples for flow cytometry, leaf tips from expanded leaves (approximately 1 cm^2) from each cultivar were placed in a petri dish containing 500 µL of nuclei extraction buffer (CyStain ultraviolet Precise P Nuclei Extraction Buffer®; Partec, Münster, Germany) and chopped finely with a razor blade until completely incorporated into buffer. The resulting solution was pipette through a Partec CellTrics™ disposable filter with a pore size of 50 µm. Then, 2 mL of a nucleotide staining buffer solution combined with 6 µL RNase A and 12 µL propidium iodide (CyStain PI absolute P; Partec, Münster, Germany) was added to the filtered solution. Samples were refrigerated (4°C) and incubated for over 30 min, and the resulting stained nuclei were analyzed with a flow cytometer (Partec PA II; Partec) with counts exceeding a minimum of 3,000 cells per analysis.

The mean fluorescence for each sample was compared with an internal standard of known genome size (*Pisum sativum* L. 'Ctirad' 2C DNA = 8.76 pg), and holoploid, 2C genome size (i.e., DNA content of entire non-replicated chromosome compliment

irrespective of ploidy) was calculated as 2C = DNA content of standard × (mean fluorescence of sample / mean fluorescence of standard). Mean 1Cx monoploid genome sizes (i.e., DNA content of the non-replicated base set of chromosomes with $2n = 2x = 18$) were calculated for each sample as (2C genome size / ploidy level).

Chromosome counts were performed on selected species of liriopogons to confirm ploidy levels and to allow for further calibration of ploidy level with genome size (Table 2, Fig. 1). A root squash technique was employed that allowed for direct counting of chromosomes. Actively growing root tips were collected from liriopogons before 10:00 AM following a sunny day, and placed in freshly made vials of pre-fixative solution (2 mM 8-hydroxyquinoline + 70 $mg \cdot L^{-1}$ cyclohexamide) at room temperature ($22^{o}C$). After remaining in the dark for 3 hours, all vials were moved into a dark refrigerator at approximately $4^{o}C$ for 3 hours yielding a total pre-fixative treatment of 6 hours. Then, all root tips were rinsed with distilled water and transferred to a freshly made fixative of 1 : 3 propionic acid : 95% ethanol, and left at room temperature overnight. The following day, a 1:3 hydrolysis solution of 12M HCl : 95% ethanol was made for the root squash procedure.

For each root squash, a fresh root was removed from the fixative and hydrolyzed for 12-20 seconds before being moved to a clean slide. Then, the root tip was excised using a dissecting microscope (StereoZoom 6 Photo, Leica Microsystems GmbH, Wetzlar, Germany) and placed on a separate, clean slide with a drop of modified carbol-fuchsion stain. The stain used was a modification of the Carr and Walker (1961) carbol fuchsin stain for human chromosomes (Kao, 1975). Then, the slide was tilted until stain completely encompassed the

excised root tip, and allowed to rest for several minutes before attempting the squash. To perform the squash, a cover slip was placed over the droplet of stain containing the excised root tip, and a clean sheet of bibulous paper was placed over the slide while gently applying pressure with a pencil eraser. An average of 10 highly resolved cells per specimen were used to visualize the total number of chromosomes using a light microscope (Eclipse 80i, Nikon, Melville, NY). Extended depth of field was achieved by layered images containing multiple focal points using Photoshop CS4 (Adobe Systems, San Jose, CA).

Results and Discussion

Liriope exiliflora. Two cultivars ('Silver Dragon' and 'Quail Garden') acquired from nurseries and a single specimen of *L. exiliflora* (MCI 2011-100) collected from the campus of NC State University were included in the present study. The relatively large genome size of *L. exiliflora* ranged from 24.89 to 26.18 pg. This range of genome size falls between that of *L. gigantea* and *L. muscari*. Cytology determined *L. exiliflora* (MCI 2011-100) to be a hexaploid at $2n = 6x = 108$ (Fig. 1). Possibly, former cytological studies included *L. exiliflora* under a different synonym, though no former studies were found to compare with our findings. Our results lend evidence for the treatment of *L. exiliflora* as a separate species in agreement with Fantz (2008b), and in contrast to claims of synonymy with *L. muscari* by Nesom (2010). Cultivars 'Silver Dragon' and 'Quail Garden' were originally acquired as *L. spicata*, and were reassigned according to (Fantz, 2008b) and based on similarity of genome size and ploidy level with known *L. exiliflora*.

Liriope gigantea. Often confused with *L. muscari*, four specimen of *L. gigantea* including four cultivars (‘Green Giant’, ‘Evergreen Giant’, ‘Lynn Lowrey’, and ‘Merton Jacobs’) were tested and found to have significantly larger genome sizes (26.23 to 28.86 pg) than any *L. muscari* (17.64 to 21.53 pg) included in this study. Cytology determined *L. gigantea* MCI 2011-099 to be a tetraploid at $2n = 4x = 72$. Possibly, former cytological studies included *L. gigantea* under a different synonym, though no former studies were found to compare with our findings. The cultivars ‘Green Giant’, ‘Evergreen Giant’ and ‘Lynn Lowrey’ were originally acquired as *L. muscari*. However, these cultivars were reassigned according to Fantz (2008b) and Nesom (2010), and based on similarity of genome size and ploidy level with known *L. gigantea*.

Liriope graminifolia. This grass-like species of *Liriope* is characterized by thin, soft leaves with heavy flowering inflorescences hidden amongst or just topping the leaves (Chen and Tamura, 2000a). *Liriope* matching this description in the present study included one cultivar (‘Porcupine’), an early-blooming cultivar (MCI 2010-063) blooming in April in NC, and a wild-collected clone (MCI 2012-098) from Sichuan, China (D. Probst, Personal Communication). Though the leaf form was similar for all specimens in this group, inflorescence length varied. Some specimens exhibited flowers blooming well above the foliage (initially thought to be a narrow leaf form of *L. platyphylla*) and some specimens exhibited flowers blooming among the foliage. The genome size for *L. graminifolia* ranged from 10.44 to 11.08 and cytology determined *L. graminifolia* Clone B (MCI 2012-098) and *L. graminifolia* ‘Porcupine’ (MCI 2010-056) to be a diploids with $2n = 2x = 36$. In addition

to diploids, previous research has reported *L. graminifolia* to be tetraploid (2*n* = 72) and hexaploid (2*n* = 108) (Table 1). *Liriope graminifolia* Clone B was originally acquired as *O. intermedius* and reassigned according to Nesom (2010) and Chen and Tamura (2000a).

Liriope longipedicellata. As the name suggests, the most identifiable feature of *L. longipedicellata* is its extended pedicels (Chen and Tamura, 2000a) giving the inflorescence a bottle-brush appearance. Otherwise, the species resembles the narrow-leafed *L. graminifolia*, and similar genome sizes and ploidy level further suggest a close relationship. The cultivar 'Grape Fizz' was found to have a genome size of 11.10 pg and the wild-collected *L. longipedicellata* MCI 2012-092 was found to have a genome size 12.31 pg, just outside the observed range for *L. graminifolia*. Cytology demonstrated *L. longipedicellata* MCI 2012-092 to be a diploid with 2*n* = 2*x* = 36. Although it is possible that former cytological studies included *L. longipedicellata* under a different synonym, no former studies were found to compare with our findings. The cultivar 'Grape Fizz' was originally acquired as *O. intermedius* 'Grape Fizz', and was reassigned according to Chen and Tamura (2000a).

Liriope minor. This species represents a spreading, dwarf *Liriope* with narrow leaves occasionally blotched yellow with leaf and inflorescence length less than 20 cm (Chen and Tamura, 2008a; Fantz, 2008b). Two *Liriope* matching this description were investigated in the present study including one cultivar ('Torafu'). Both specimens in this study exhibited yellow blotched variegation. Cytometry revealed genome sizes from 11.08 to 11.31 pg, placing it in a similar range as *L. graminifolia*. Further cytological examination of both specimens revealed *L. minor* to be a diploid 2*n* = 2*x* = 36 (Fig. 1A), concurring with previous

studies also reporting *L. minor* to be a diploid (Table 1). The similar appearance, genome size, and ploidy of *L. minor* revealed in this study suggests a close relationship between *L. graminifolia* and *L. minor*, with *L. minor* having a smaller stature. The cultivar 'Torafu' was originally acquired as *L. muscari* 'Torafu' and reassigned according to Chen and Tamura (2000a) and Fantz (2008b).

Liriope muscari. Seventeen cultivars ranged in genome size from 17.64 to 21.53 pg (Table 2). A general trend was observed that cultivars such as 'Superba' (21.53 pg) and 'Big Blue' (21.40 pg), representing the larger genome sizes, had the most vigor and fertility of all *L. muscari* tested (J. Lattier, Personal Observation). A possible explanation for this is suggested in an earlier study by Westfall (1950) where many *L. muscari* studied were found to be sterile or only partially fertile due to high levels of aneuploiy. In addition, hypotetraploid lines were found to have high levels of variation in inflorescence morophology, leaf width, vigor, and fertility (Westfall, 1950). Our results indicate a similar trend with all the green leaf forms (except for the white-flowering 'Monroe White') having a genome size greater than 20 pg, and all variegated forms or forms with abnormal inflorescences having a genome size less than 20 pg. Another possible explanation this trend is that the fertile *L. muscari* may be hybridizing with *Liriope* of larger genome sizes, such as *L. gigantea*. Cytology of 'Silvery Sunproof' confirmed it to be a tetraploid with $2n = 4x = 72$, contrasting one report of a diploid at $2n = 36$ and one report of a rare specimen at $2n = 112$ for *L. muscari* (Table 1).

Liriope platyphylla. Recent taxonomic studies including Nesom (2010) have treated the broad-leaved *L. platyphylla* to be synonymous with *L. muscari*, or a variety of *L. muscari*, based on the merger of these two species by Hsu and Li (1981) and Hara (1984). Molecular studies have also indicated a close relationship between *L. muscari* and *L. platyphylla* (Wu, 1998). However, the combination of the two species has led to much confusion in interpretation of former literature and confusion on identification of this distinctive *Liriope* (Fantz, 2008b). In the present study, *Liriope platyphylla* was treated as a separate species in contrast to the most recent report by Nesom (2010). Samples in the present study were easily distinguishable from *L. muscari* in agreement with Fantz (2008b) based on their wide, leathery leaves and elongated inflorescence extending well above the foliage. Eight specimens were tested including one with a branching rachis (MCI 2012-095), one particularly large form from the Atlanta Botanical Garden with a nearly 4' tall inflorescence (MCI 2012-125), and one cultivar ('Korean Giant'). Flow cytometry revealed a ploidy series including four specimens with genome sizes from 10.02 to 10.71 pg and four specimens with genome sizes from 19.04 to 19.95 pg. All *L. platyphylla* proved highly fertile, and there was a general trend of the tetraploid specimens exhibiting more vigorous growth and larger overall sizes. A further cytological study was conducted finding MCI 2010-019 to be a diploid with $2n = 2x = 36$ while MCI 2010-051 was found to be a tetraploid with $2n = 4x = 72$. The only other reported ploidy level for *L. platyphylla* was found to be hexaploid with $2n = 108$, though it is likely that cytological studies have been conducted under different synonyms (Table 1).

Ophiopogon intermedius. Cytometric analysis of ‘Aztec’, ‘Twisted Variegated’, and 2 samples of ‘Variegatus’ showed a range of genome sizes from 11.10 to 11.21 pg. Cytology determined ‘Aztec’ to be a diploid with $2n = 2x = 36$. However, previous studies have reported wide ranges of ploidy levels for *O. intermedius* at $2n = 36$, 68, 72, 108, and 112 (Table 1). The cultivar ‘Aztec’ was originally acquired as *L. spicata* ‘Aztec’ and was reassigned in agreement with Fantz (2009) and Nesom (2010). The cultivar ‘Aztec’ is commonly misidentified in the trade as *L. muscari* or *O. jaburan* (Fantz, 2009).

Ophiopogon jaburan. Cytometry of *O. jaburan* ‘HOCF’, ‘Crystal Fan’, ‘Vittatus’, ‘Ursala’s Blue Fruit’, and ‘Wuhan Variegated’ found a range of genome sizes from 16.08 to 16.49 pg. Cytology of ‘Crystal Fan’ documented it to be a diploid with $2n = 2x = 36$. Interestingly, *O. jaburan* was found to have the largest average 1Cx value at 8.15 pg. Compared to other liriopogons in this study, *O. jaburan* had a surprisingly consistent cytological record of existing primarily as a diploid at $2n = 36$ (Table 1) in agreement with our findings.

Ophiopogon japonicus. Cytometry of eleven taxa of *O. japonicus* showed two distinct ranges of genome sizes. The majority of *O. japonicus* samples ranged from 20.39 to 22.04 pg while ‘Tears of Gold’ and ‘Seulitary Man’ ranged from 25.24 to 25.44 pg. Originally thought to be a hyperploid specimen, cytology confirmed ‘Seoulitary Man’ to be a tetraploid with $2n = 4x = 72$ (Fig. 1B), indicating possible misidentification in the trade. Ploidy analysis remains inconclusive for other *O. japonicus*, likely due to high levels of hypotetraploidy as reported in previous studies (Table 1). A wide range of ploidy levels have

been previously reported for *O. japonicus* including 2*n* = 36, 67, 68, 70, 72 (Table 1). In the present study, four samples examined using flow cytometry (‘Fiuri Gyoku Ryu’, ‘Shiroshima Ryu’ ‘Tama Ryu Nishiki’, and‘Variegatus’) yielded multiple fluorescent peaks, with hypotetraploid peaks being associated with the variegated tissue. This phenomenon was only observed in variegated cultivars of *O. japonicus*, and confirmed earlier findings of Wang and Xu (1990) which found some specimen of *O. japonicus* to be cytochimeras with diploid, triploid, and tetraploid cells existing in the same plant.

Ophiopogon planiscapus. Genome sizes for all eight taxa of *O. planiscapus* tested ranged from 12.15 to 12.66 pg. Cytology of the cultivar ‘Nigrescens’ showed it to be 2*n* = 2*x* = 36. The majority of former cytological studies of *O. planiscapus* agree with the present study, however tetraploid forms (2*n* = 72) have been reported (Table 1).

Ophiopogon umbraticola. Often mislabeled *O. chingii* in the nursery industry (P. Fantz, Personal Communication), two samples of *O. umbraticola* (MCI 2010-059, JCRA 990336) had genome sizes ranging from 14.41 to 14.45 pg, which represents a unique range of genome size compared to all liriopogons tested in the present study. Further cytological study of *O. umbraticola* (MCI 2010-059) revealed it to be a diploid at 2*x* = 2*x* = 36, in contrast to one previous report of hypotetraploidy, 2*n* = 68 (Table 1). *O. umbraticola* MCI 2010-059 was originally acquired as *O. chingii* and reassigned according to Chen and Tamura (2000b) and Tanaka (2001a).

Conclusion

This study demonstrates the development of an extensive collection of both living specimens and herbarium vouchers for liriopogons. Several source names were misidentified to genus, and many were misidentified to species as in a previous report by Fantz (1994). Genome size and ploidy level were determined for all taxa in this study. Results confirm the basic chromosome number of x = 18 for liriopogons. Based on our results, *Liriope* examined fit into three ploidy groups, with one exception of a ploidy series of *L. platyphylla*. The diploid group consists of *L. graminifolia, L. longipedicellata, L. minor*, and half of *L. platyphylla*. The tetraploid group consists of *L. muscari* and half of *L. platyphylla*. The hexaploid group consists of *L. exiliflora*. Though controversy surrounds the maintenance of *L. gigantea*, *L. graminifolia*, and *L. exiliflora* as separate species, our relative genome size data of *Liriope* lends evidence for maintaining *L. gigantea* as a distinct species as suggested by Fantz (2008b) and Nesom (2010), *L. exiliflora* as suggested by Fantz (2008b), and *L. graminifolia* as suggested by Nesom (2010) and Chen and Tamura (2000).

Ophiopogon included in this study formed two ploidy groups. The diploid group includes *O. intermedius*, *O. jaburan*, *O. planiscapus*, and *O umbraticola*. The tetraploid/hypotetraploid group includes *O. japonicus*. Base 1Cx genome size varied significantly among ploidy groups indicating genome size divergence of liriopogons; however, within ploidy groups, a low variation in 1Cx values was observed for all liriopogons with the exception of the diploid *Ophiopogon*.

Improving breeding strategies for Liriopogon requires a resource of plants with similar genome sizes and ploidy levels to enable selections for controlled crosses. Compatibility of the genomes/chromosomes is necessary for meiosis to function properly when developing progeny from controlled crosses (Parris et al., 2010). Therefore, a smaller difference in genome size may indicate greater compatibility between two potential liriopogons. In addition, since liriopogons in the present study exhibited increased vigor and fertility at higher genome sizes and higher ploidy level, and tolerance of aneuploidy and cytochimeras (Nagamatsu and Noda, 1971; Westfall, 1950), inducing polyploidy may also be a means of creating new breeding opportunities. Previous studies have shown induced polyploids have proven effective 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 (Ranney, 2006).

Although breeding efforts in the past have been limited by confusion over proper identification of germplasm and lack of information on ploidy levels and cytogenetics of available clones and cultivars, the reference collection established in this study will aid future revisions, as well as assist in the development of breeding strategies for liriopogons.

Literature Cited

Bailey, L.H. 1929. The case of *Ophiopogon* and *Liriope*. Gentes Herb. 2:1-37.

Broussard, M.C. 2007. A horticultural study of *Liriope* and *Ophiopogon*: Nomenclature, morphology, and culture. Ph.D. dissertation, Louisiana State University, Baton Rouge.

Cao, Y., D.-r. Qiao, Y.-x. Li, H.-x. Xing and Y. Jiang. 2002. Karyotypical Analysis of Erective and Creepy *Ophiopogon japonicus* in Mianyang of Sichuan. Journal of Sichuan University (Natural Science Edition) 39 (2): 345-348.

Carr, D.H. and J.E. Walker. 1961. Carbol fuchsin as a stain for human chromosomes. Stain Technology 36: 233-236.

Chen, S.C. and M.N. Tamura. 2000a. *Liriope*. In: Z. Y. Wu and P. H. Raven (eds.). Flora of China 24: 250-251. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

Chen, S.C. and M.N. Tamura. 2000b. *Ophiopogon*. In: Z. Y. Wu and P. H. Raven (eds.). Flora of China 24: 250-251. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.

Cheng, Z.H., T. Wu, Y.L. Guo, B.Y. Yu, and L.S. Xu. 2006. Two new steroidal glycosides from *Liriope muscari*. Chinese Chemical Letters Vol. 17 (1): 31-34.

Conran, J.G. and M.N. Tamura. 1998. Convallariaceae. *In* K. Kubitzki (ed.). The Families and Genera of Vascular Plants 3: 186-198. Springer, Berlin.

Cutler, D. F. 1992. Vegetative anatomy of *Ophiopogoneae* (*Convallariaceae*). Bot. J. Linnean Soc. 110: 385-419.

Darlington, C.D. and A.P. Wylie. 1955. Chromsome Atlas of Flowering Plants. George Allen & Unwin, Ltd., London, 13-14 pp.

Denda, T., K. Nakamura and M. Yokota. 2006. Karyotype of *Ophiopogon reversus* (*Convallariaceae*) from Taiwan and the Southern Ryukyus. Taiwania 51 (2): 117-122.

Dudgeon, W. 1923. Section of Botany, Proceedings of the 9th Indian Science Congress. Proc. Asiat. Soc. Bengal 18: 95-124.

Fantz, P.R. 1993. Taxonomic problems in cultivated Liriopogons. HortTechnology 3: 146-149.

Fantz, P.R. 1994. Taxonomic Problems in cultivated Liriopogons. HortTechnology 3: 146-150.

Fantz, P.R. 2008a. Macrophytogeography of cultivated Liriopogons and genera delineation. HortTechnology 18: 334-342.

Fantz, P.R. 2008b. Species of *Liriope* cultivated in the southeastern United States. HortTechnology 18: 33-348.

Fantz, P.R. 2009. Names and species of *Ophiopogon* cultivated in the Southeastern United States. HortTechnology 19(2): 385-394.

Fu, C.X. and D.Y. Hong. 1989. Cytotaxonomical studies on *Liliaceae* (S.1.); (2) Report on chromosome numbers and karyotypes of 8 species of 8 genera from Zhejiang, China. Acta Phytotaxon. Sin. 27(6): 439-450.

Fukai, S., T. Shimomura, and T. Kondo. 2008. Convallariaceae ground cover plants native to Asia. ISHS Acta Horticulturae 769: XXVII International Horticultural Congress – – IHC2006: International Symposium on Asian plants with unique horticultural potential.

Ge, C., Y. Li and Y. Zhou. 1987. Observations on the chromosome numbers of medicinal plants plants of Shandong province. Acta Botanica Yunnanica 9 (3): 333-338.

Hara, H. 1984. Comments on the East Asiatic plants (13). J. Jap. Bot. 59(2): 33-41.

Hasegawa, K.M. 1968. Cytotaxonomic studies on the genera *Liriope* and *Ophiopogon* in Japan. J. Japanese Bot. 43: 141-155.

Hsu, C.C. 1971. Preliminary chromosome studies on the vascular plants of Taiwan (IV). Counts and some systematic notes on some monocotyledons. Taiwania 16: 123-136.

Hsu, P.S. and L.C. Li. 1981. Critical notes on the classification of the *Liriope muscari* complex. Acta Phytotax. Sini. 19: 456-461.

Hume, H.H. 1961. The *Ophiopogon-Liriope* Complex. Baileya 9: 135-158.

Kao, K.N. 1975. A nuclear staining method for protoplasts. In: O.L.Gamborg and L.Z. Wetter (ed.) Plant Tissue Culture Methods. L.R. National Research Council of Canada. Praire Regional Labratory. Saskatoon, Saskatchewan, Canada 1975, p. 60-64.

Kim, J.-H., D.-K. Kim, F. Forest, M.F. Fay and M.W. Chase. 2010. Molecular phylogenetics of Ruscaceae *sensu lato* and related families (Asparagales) based on plastid and nuclear DNA sequences. Annals of Botany 106: 775-790.

Ko, S.C., Y.O. Kim and Y.S. Kim. 1985. A cytotaxonomical study on the tribe *Ophiopogoneae* in Korea. Kor. J. Plant Tax. 15: 111-125.

Kondo, K., K. Taniguchi, R. Tanaka and Z. Gu. 1992. Karyomorphological studies in Chinese plant-species involving the Japanese floristic elements, I. American Camellia Yearbook: 131-156.

Larsen, K. 1963. Sudies in the flora of Thailand (IV). Counts and some systematic notes on some monocotyledons. Taiwania 16: 123-136.

Li, G., W.-H. Ra, J.-W. Park, S.-W. Kwon, J.-H. Lee, C.-B. Park and Y.-J. Park. 2011. Developing EST-SSR markers to study molecular diversity in *Liriope* and *Ophiopogon*. Biochemical Systematics and Ecology 39: 241-252.

Liang, G., M. Yang and Y. Yan. 1998. Karyotypical analysis of *Ophiopogon japonicus* in Sichuan. Journal of Southwest Agricultural University 20 (1): 307-310.

Liu, W.-Q., J.-H. Jin, and W.-B. Liao. 2007. *Ophiopogon acerobracteatus* (Convallariaceae), a new species from southern China. Ann. Bot. Fennici 44: 492-494.

Liu, Y., S.A. Zhang, P.S. Hsu and L.C. Li. 1985. Chromsome numbers of several cultivated plants from Shanghai. J. Wuhan Bot. Res. (Wuhan Zhiwuxue Yanjou) 3(3): 225-228.

Malik, C.P. 1961. Chromosome number in some Indian Angiosperms: Monocotyledons. Sci. and Cult. 27(4): 197-198.

Matsuura, H. and T. Suto. 1935. Contributions to the idiogram study in phanerogamous plants. I. J. Fac. Sci. Hokkaido Imp. Univ., Ser. 5, Bot. 5.5: 33-75.

Mcharo, M., E. Bush, D. LaBonte, C. Broussard, and L. Urbatsch. 2003. Molecular and morphological investigation of ornamental Liriopogons. J. Amer. Soc. Hort. Sci. 128: 575-577.

Nagamatsu, T. and S. Noda. 1964. Chromsome constitution of *Ophiopogon japonicus*. Bull. Osaka Gakuin Univ. 3: 185-190. (in Japanese with English Summary)

Nagamatsu, T. and S. Noda. 1971. Balanced hypotetraploids in *Ophiopogon japonicus* and *O. ohwii*. Cytologia 36: 332-340.

Nesom, G.L. 2010. Overview of *Liriope* and *Ophiopogon* (Ruscaceae) naturalized and commonly cultivated in the USA. Phytoneuron 56: 1-31.

Oinuma, T. 1944. Chromosome number of *Ophiopogon planiscapus* Nakai. Jap. J. Genet. 20: 130-131. (in Japanese).

Oinuma, T. 1946. Karyotype analysis of *Liriope* and *Ophiopogon*. La Kromosomo 2: 71-75. (in Japanese with English summary).

Oinuma, T. 1949. Further studies on chromosomes of *Ophiopogonaceae*. Jap. J. Genet. Suppl. 2: 29-34. (in Japanese with English résumé).

Parris, J.K., T.G. Ranney, H.T. Knap, and W.V. Baird. 2010. Ploidy levels, relative genome sizes, and base pair composition in magnolia. J. Amer. Soc. Hort. Sci. 135(6): 533-547.

Roy, S.C., S. Ghosh and A. Chatterjee. 1988. A cytological survey of eastern Himalayan plants. II. Cell Chromosome Res. 11: 93-97.

Rudall, P. 2000. Systematics of Ruscaceae/Convallariaceae: a combined morphological and molecular investigation. Botanical Journal of the Linnean Society, 134: 73-92.

Sarkar, A.K., R. Datta and M. Raychowdhury. 1974. In IOPB chromosome number reports XLVI. Taxon 23:801-812.

Sato, D. 1942. Karyotype alteration and phylogeny in *Liliaceae* and allied families. J. Jpn. Bot. 12 (1-2): 57-161.

Sen, S. 1973. Structural hybridity intra- and interspecific levels in *Lilliales*. Folia Biol. (Cracow) 21: 183-197.

Sharma, A.K. and M. Chaudhuri. 1964. Cytological studies as an aid in assessing the status of *Sanseviera*, *Ophiopogon*, and *Curculigo*. Nucleus 7(1): 43-58.

Sheriff, A. and B.K.S. Singh. 1975. Hexaploid wild populations of *Ophiopogon intermedium* from Shiradi Ghats, Karnataka State. Proc. Indian Sci. Congr. Assoc. 62: 117.

Skinner, H.T. 1971. Some liriopogon comments. J. Royal Hort. Soc. 96(8):345-350.

Tamura, M.N. 1990. Studies on the genus *Ophiopogon* (Liliaceae) of Phu Kradung in Thailand. Acta Phytotax. Geobot. 41: 1-6.

Tamura, M.N. 1995. A karyological review of the orders *Asparagales* and *Liliales* (*Monocotyledonae*). Feddes Repert. 106: 83-111.

Tanaka, N. 2000. Taxonomic notes on *Ophiopogon* of South Asia VII. J. Jpn. Bot. 75: 191-212.

Tanaka, N. 2001a. Taxonomic notes on *Ophiopogon* (Convallariaceae) of East Asia (I). J. Jpn. Bot. 76: 59-76.

Tanaka, N. 2001b. Taxonomic notes on *Ophiopogon* (Convallariaceae) of East Asia (II). J. Jpn. Bot. 76: 151-165.

Tanaka, N. 2001c. Taxonomic notes on *Ophiopogon* (Convallariaceae) of East Asia (III). J. Jpn. Bot. 76: 205-218.

Terasaka, O. and R. Tanaka. 1974. Cytological studies on the nuclear differentiation in microspore division of some angiosperms. Bot. Mag. Tokyo 87: 209-217.

Thunberg, C.P. 1780. *Convallaria japonica*. Nov. Act. Reg. Soc. Upsala 3:208.

Trueblood, C.E. 2009. An estimate of the commercial value of potentially invasive nursery crops grown in North Carolina. In: An Invasive Species Assessment System for the North Carolina Horticultural Industry. MS Thesis. North Carolina State University, Raleigh, NC, pp. 63-75.

Wang, K.W., X.Y. Ju, L. Zhang, W. Wang, and L.Q. Shen. 2012. A novel C27-steroidal glycoside sulfate from *Liriope graminifolia*. Yao Xue Xue Bao 47(5): 619-623.

Wang, S.-F. and J.-M. Xu. 1990. Report on Karyotypes of *Smilacina tatsienensis* and *Ophiopogon japonicus*. Acta Phytotaxonomica Sinica 28(3): 207-210.

Westfall, J.J. 1950. Aneuploidy in *Liriope muscari* Bailey. Amer. J. Bot. 37: 667.

Xia, G.-H., S.-H. Jin, D.-D. Ma and G.-Y. Li. 2012. *Liriope zhejiangensis* (*Asparagaceae*), a new species from eastern China. Ann. Bot. Fennici 49: 64-66.

Yamashita, J. and M. N. Tamura. 2001. Karyotype analysis of six species of the genus *Ophiopogon* (*Convallariaceae-Ophiopogoneae*). J. Jpn. Bot. 76: 100-119.

Yang, Y.-P., H. Li., X.-Z. Liu and K. Kondo. 1990. Karyotype study on the genus *Ophiopogon* in Yunnan. Acta Bot. Yunnan., Suppl. 3: 94-102. (in Chinese with English abstract).

Yu, B.-Y., S.-X. Qiu, K. Zaw, G.-J. Xu, Y. Hirai, J. Shoji, H.S. Fong. and A. Douglas. 1996. Steroidal glycosides from the subterranean parts of *Liriope spicata* var. *prolifera*. Phytochem. 43: 201–206. Zhang, D.M. 1998. Systematics of the tribe Ophiopogoneae (Liliaceae s. l.) with special reference to karyotypes and chromosomal evolution. Cathaya 10: 1–154.

Zhang, D.M. 1998. Systematics of tribe Ophiopogoneae (Liliaceae s.l.) with special reference to karyotypes and chromosomal evolution. Cathaya 10: 1-154.

Zhou, Q., J. Zhou, J. Chen and X. Wang. 2009. Karyotype analysis of medicinal plant *Liriope spicata* var. *prolific*. Biologia 64(4): 680-683.

Table 1. Previous cytological and cytometric analyses of liriopogons.

Taxa	Synonyms[z]	Previous findings	References
L. graminifolia	*L. angutissima*, *L. crassiuscula*, *L. spicata*	2*n* = 36	Zhang, 1998
		2*n* = 72	Zhang, 1998
		2*n* = 108	Zhang, 1998
L. minor	*L. cernua*, *L. graminifolia* var. *minor*, *L. spicata* var. *minor*	2*n* = 36	Fukai et al., 2008; Ge et al., 1987; Hasegawa, 1968; Matsuura and Suto, 1935; Oinuma 1946, 1949; Sato 1942
L.muscari	*L. graminifolia* var. *densiflora*	2*n* = 36	Fu and Hong, 1989; Ge et al., 1987; Zhang, 1998
		2*n* = 72	Oinuma, 1946; Westfall, 1950; Zhang, 1998
		2*n* = 112*	Zhang, 1998
L. platyphylla	*L. muscari*, *L. muscari* var. *communis*, *L. graminifolia* var. *communis*	2*n* = 72	Fukai et al., 2008; Hasegawa, 1968; Oinuma 1946, 1949
		2*n* = 108	Oinuma, 1946, 1949
L. spicata	*L. koreana*	2*n* = 36	Hasegawa, 1968; Zhang, 1998
		2*n* = 45*	Kondo et al. 1992
		2*n* = 72	Kondo et al., 1992;Yang, 1990; Zhang, 1998, Zhou et al., 2009
		2*n* = 88*	Zhang (1998)
		2*n* = 90	Zhang (1998)
		2*n* = 108	Fukai et al., 2008; Hasegawa, 1968; Liu et al., 1985; Terasaka and Tanaka, 1974; Zhang, 1998
O. intermedius	*O. aciformis*, *O. wallichianus*	2*n* = 36	Larsen, 1963; Malik, 1961; Roy et al., 1988, Sarkar et al., 1974; Sharma and Chaudhuri, 1964; Yang et al., 1990; Zhang, 1998
		2*n* = 68*	Sen, 1973
		2*n* = 72	Zhang, 1998
		2*n* = 108	Malik, 1961; Sheriff and Singh, 1975; Zhang, 1998
		2*n* = 112*	Dudgeon, 1923
O. jaburan		2*n* = 36	Denda et al., 2006; Hasegawa, 1968; Ko et al., 1985; Matsuura and Suto, 1935; Sato, 1942; Yamashita and Tamura, 2001
O. japonicus	*O. stolonifer*, *O. argyi*, *O. checkiangensis*	2*n* = 34*	Zhang, 1998
		2*n* = 36	Sato, 1942; Wang and Xu; 1990; Zhang, 1998
		2*n* = 67*	Nagamatsu and Noda, 1964, 1971
		2*n* = 68*	Cao et al., 2002; Ge et al., 1988, Liang et al., 1998; Liu et al., 1985; Nagamatsu and Noda, 1964, 1971; Zhang, 1998
		2*n* = 70*	Yamashita and Tamura, 2001
		2*n* = 72	Chaudhuri, 1964; Fukai et al., 2008; Hsu, 1971; Ko et al., 1985; Liu et al., 1985; Oinuma, 1944, 1946, 1949; Sato, 1942; Sharma and Hasegawa, 1968; Yang et al., 1990; Zhang, 1998
		2*n* = 108	Zhang, 1998
O. planiscapus		2*n* = 36	Fukai et al., 2008; Hasegawa, 1968; Hsu, 1971; Oinuma, 1946, 1949; Yamashita and Tamura, 2001
		2*n* = 72	Sato (1942), Oinuma (1944, 1946, 1949)
O. umbraticola	*O. japonicus* var. *umbraticola*, *O. chingii*	2*n* = 68*	Zhang, 1998

[z] Synonyms for taxa obtained from cited studies, from Tropicos®, Missouri Botanic Garden, or from common misidentifications in the US nursery industry.

*Possible hypoploid or hyperploid specimen based on basic chromosome number of *x* = 18.

Table 2. Genome size and estimated ploidy level of cultivated *Liriope* and *Ophiopogon*.

Taxa	Accession[Z]	2C Genome Size[Y]	Ploidy[X]	1Cx Genome Size (pg)[W]
L. exiliflora	MCI 2011-100	24.89 ± 0.87	6x*	4.15
L. exiliflora 'Silver Dragon'	MCI 2010-039	25.81 ± 0.38	6x	4.30
L. exiliflora 'Quail Garden'	MCI 2010-043	26.18 ± 0.48	6x	4.36
L. gigantea 'Green Giant'	MCI 2011-099	27.90 ± 0.20	4x*	6.89
L. gigantea 'Evergreen Giant'	MCI 2010-041	28.86 ± 0.40	4x	7.22
L. gigantea 'Lynn Lowrey'	JCRA 042084	28.05 ± 0.64	4x	7.01
L. gigantea 'Merton Jacobs'	PDN JL12001	26.23 ± 0.04	4x	6.56
L. graminifolia (Early Blooming)	MCI 2010-063	11.08 ± 0.19	2x*	5.54
L. graminifolia 'Porcupine'	MCI 2010-056	10.73 ± 0.13	2x*	5.37
L. graminifolia 'Porcupine'	PDN JL12002	11.04 ± 0.27	2x	5.52
L. graminifolia (Clone B)	MCI 2012-098	10.44 ± 0.14	2x*	5.22
L. longipedicellata	MCI 2012-092	12.31 ± 0.10	2x*	6.16
L. longipedicellata 'Grape Fizz'	MCI 2012-100	11.10 ± 0.19	2x	5.55
L. minor	MCI 2011-101	11.08 ± 0.05	2x*	5.54
L. minor 'Torafu'	MCI 2010-023	11.31 ± 0.14	2x*	5.65
L. muscari 'Big Blue'	MCI 2010-033	21.40 ± 0.10	4x	5.35
L. muscari 'Bigun' Cleopatra™	MCI 2007-183	20.38 ± 0.09	4x	5.09
L. muscari 'Blue Giant'	JCRA 020548	20.66 ± 0.02	4x	5.17
L. muscari 'Hawk's Feather'	JCRA xx0048	18.27 ± 0.17	4x	4.57
L. muscari 'John Burch'	PDN JL12003	17.64 ± 0.10	4x	4.41
L. muscari 'Marant' Marc Anthony®	MCI 2007-182	18.45 ± 0.28	4x	4.61
L. muscari 'Monroe's White'	MCI 2010-040	18.16 ± 0.11	4x	4.54
L. muscari 'Okina'	JCRA 950596	17.94 ± 0.44	4x	4.49
L. muscari 'Pee Dee Ingot'	MCI 2010-042	19.98 ± 0.16	4x	5.00
L. muscari 'Samona'	MCI 2011-143	18.17 ± 0.29	4x	4.54
L. muscari 'Sideswiped'	MCI 2012-097	18.36 ± 0.18	4x	4.59
L. muscari 'Silvery Sunproof'	MCI 2010-046	18.56 ± 0.35	4x*	4.64
L. muscari 'Sno Cone'	JCRA 080869	18.49 ± 0.07	4x	4.62
L. muscari 'Snow Dragon'	PDN JL12004	18.16 ± 0.09	4x	4.54
L. muscari 'Superba'	JCRA 020549	21.53 ± 0.62	4x	5.38
L. muscari 'Tokai Waname'	PDN JL12005	18.50 ± 0.11	4x	4.63
L. muscari (Yellow Splash)	JCRA 031562	19.01 ± 0.42	4x	4.75
L. platyphylla	MCI 2012-124	10.02 ± 0.12	2x	5.01
L. platyphylla	MCI 2010-019	10.23 ± 0.14	2x*	5.11
L. platyphylla	MCI 2010-048	10.36 ± 0.01	2x	5.18
L. platyphylla (branched infl.)	MCI 2012-095	10.71 ± 0.21	2x	5.35

Table 2 Continued.

L. platyphylla	MCI 2010-153	19.55 ± 0.25	4x	4.89
L. platyphylla	MCI 2010-051	19.90 ± 0.27	4x*	4.97
L. platyphylla (ABG Form)	MCI 2012-125	19.04 ± 0.24	4x	4.76
L. platyphylla 'Korean Giant'	MCI 2011-127	19.95 ± 0.26	4x	4.99
O. intermedius 'Aztec'	MCI 2011-098	11.10 ± 0.06	2x*	5.55
O. intermedius 'Twisted Variegated'	MCI 2010-236	11.21 ± 0.17	2x	5.60
O. intermedius 'Variegata'	PDN JL12006	11.16 ± 0.01	2x	5.58
O. jaburan 'HOCF' Crystal Falls	MCI 2007-146	16.08 ± 0.06	2x	8.04
O. jaburan 'Crystal Fan'	MCI 2010-047	16.30 ± 0.18	2x*	8.15
O. jaburan 'Vittatus'	MCI 2010-045	16.45 ± 0.45	2x	8.22
O. jaburan 'Ursala's Blue Fruit'	PDN JL12007	16.21 ± 0.12	2x	8.11
O. jaburan 'Wuhan Variegated'	MCI 2010-045	16.49 ± 0.42	2x	8.25
O. japonicus	MCI 2010-054	20.64 ± 0.50	~4x	5.16
O. japonicus 'Aritake'	PDN JL12008	20.39 ± 0.22	~4x	5.10
O. japonicus 'Bluebird'	MCI 2010-055	20.95 ± 0.25	~4x	5.24
O. japonicus 'Comet'	MCI 2010-061	21.21 ± 0.01	~4x	5.30
O. japonicus 'Fiuri Gyoku Ryu'	MCI 2010-058	NA	Mix[V]	NA
O. japonicus 'Gyoku Ryu'	JCRA 981407	22.04 ± 0.36	~4x	5.51
O. japonicus 'Shiroshima Ryu'	MCI 2012-099	20.05 ± 0.56	Mix[V]	5.01
O. japonicus 'Tama Ryu Nishiki'	MCI 2010-111	NA	Mix[V]	NA
O. japonicus 'Vareigatus'	MCI 2010-053	21.22 ± 0.49	Mix[V]	5.30
O. japonicus var. *caespitosa* 'Seoulitary Man'	MCI 2010-057	25.44 ± 0.13	4x*	6.36
O. japonicus var. *gracilis* 'Tears of Gold'	MCI 2010-064	25.24 ± 0.09	~4x	6.31
O. planiscapus 'Arabicus'	MCI 2010-024	12.66 ± 0.15	2x	6.33
O. planiscapus 'Black Dragon'	MCI 2010-037	12.63 ± 0.18	2x	6.31
O. planiscapus 'Ebknizam'	MCI 2010-038	12.46 ± 0.06	2x	6.23
O. planiscapus 'Edge of Night'	PDN JL12009	12.15 ± 0.32	2x	6.07
O. planiscapus 'Haku Ryo Ko'	MCI 2010-110	12.54 ± 0.07	2x	6.27
O. planiscapus 'Little Tabby'	MCI 2010-062	12.36 ± 0.02	2x*	6.18
O. planiscapus 'Nigrescens'	MCI 2010-043	12.45 ± 0.06	2x*	6.23
O. planiscapus var. *leucanthus*	PDN JL12010	12.33 ± 0.00	2x	6.16
O. umbraticola	MCI 2010-059	14.41 ± 0.19	2x*	7.20
O. umbraticola	JCRA 990336	14.45 ± 0.09	2x	7.23

[Z] JCRA = J.C. Raulston Arboretum, Raleigh, NC; PDN = Plant Delights Nursery, Raleigh, NC; MCI = Mountain Crop Improvement Lab, North Carolina State University, Mills River, NC.
[Y] Holoploid genome sizes were determined using propidium iodide as the flourochrome stain. Values are $\bar{x}$ ± SEM, n=2-3.
[X] Ploidy levels, 2x = diploid, 4x = tetraploid, 5x = pentaploid, 6x = hexaploids, Mix = mixaploid
[W] 1Cx values were calculated as 2C value/ploidy level.
[V] Cytochimera of multiple ploidy levels (mixaploid) confirmed via flow cytometry, 1Cx values calculated using largest 2C value.
[~] Possible hypoploid specimens based high variability in attempted chromosome counts
*Ploidy levels confirmed via cytology.

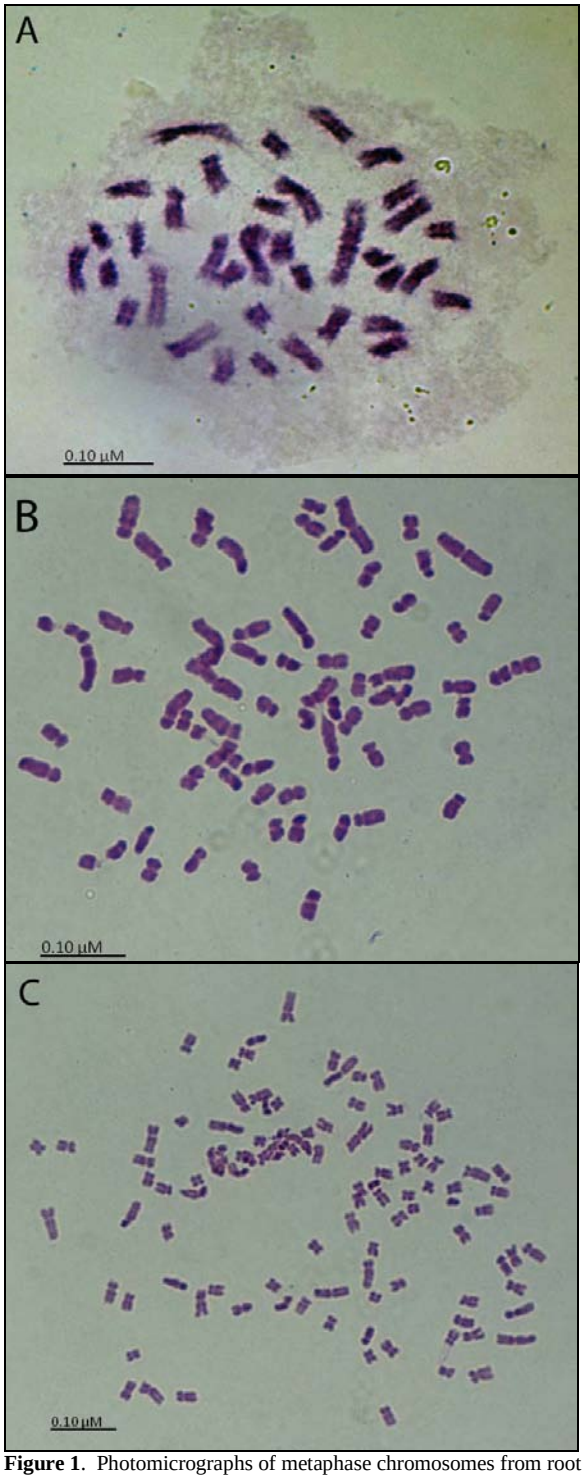

Figure 1. Photomicrographs of metaphase chromosomes from root tip cell of liriopogons viewed at 1000x: A, *L. minor* (2*n* = 2*x* = 36); B, *Ophiopogon japonicus* var. *caespitosa* 'Seoulitary Man' (2*n* = 4*x* = 72); C, *Liriope exiliflora* (2*n* = 6*x* = 108).

CHAPTER 4

Micropropagation of the Hybrid Asian Dogwood (*Cornus kousa* ‘Miss Satomi’ × *Cornus hongkongensis* ‘Summer Passion’)

(In the format appropriate for submission to HortScience)

Micropropagation of the Hybrid Asian Dogwood (*Cornus kousa* 'Miss Satomi' × *Cornus hongkongensis* 'Summer Passion')

Jason D. Lattier[1], Darren H. Touchell[2], and Thomas G. Ranney[3]

Mountain Crop Improvement Lab, Department of Horticultural Science, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, 455 Research Drive, Mills River, NC 28759-3423

Received for publication . Accepted for publication .

This research was funded, in part, by the North Carolina Agricultural Research Service (NCARS), Raleigh, NC, 27695-7643, North Carolina Nursery and Landscape Association, North Carolina Biotechnology Center and the Kenan Insitute. Use of trade names in this publication does not imply endorsement by the NCARS of products named nor criticism of similar ones not mentioned. Assistance of the staff of the Mountain Horticultural Crops Research and Extension Center, particularly, Jeremy Smith is gratefully acknowledged. From a thesis submitted by J. D. L. in partial fulfillment of the requirements for the MS degree.

[1]Graduate Research Assistant.

[2]Research Associate and corresponding author. E-mail:darren_touchell@ncsu.edu.

[3]Professor.

Subject Category: Propagation and Tissue Culture

Micropropagation of the Hybrid Asian Dogwood (*Cornus kousa* 'Miss Satomi' × *Cornus hongkongensis* 'Summer Passion')

Additional index words: auxins, cytokinins, elongation, microshoots, multiplication, phenolics

Abstract. Protocols for micropropagation were developed for the production of a novel hybrid dogwood H2007-017-154 (*Cornus kousa* Buerger ex Miq. 'Miss Satomi' x *Cornus hongkongensis* Hemsl. 'Summer Passion'). Murashige and Skoog Medium (MS), Lloyd and McCown Woody Plant Medium (WPM), Driver and Kuniyuki Walnut medium, Quoirin and Lepoivre Medium (QL), and Schenk and Hildebrandt (SH) basal salts 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 were investigated to optimize cytokinin type and concentration. Woody Plant Media supplemented with 5 µM BAP produced 4.27 microshoots with a mean length of 8.58 mm and a multiplication rate of 4.97 microcuttings per subsample after 5 weeks. The effect of supplementing multiplication media with IAA at 0, 0.1, 0.5, or 1.25 µM on microshoot elongation was evaluated. The addition of IAA at 0.5 µM produced a 19% increase in microshoot length per subsample over 5 weeks. In vitro rooting was evaluated

with WPM supplemented with IAA or NAA at 0.625, 1.25, 2.5, 5, or 10 μM. However, in vitro rooting was not efficiently achieved in microshoots of H2007-017-154.

Introduction

Dogwoods are valuable nursery and landscape crops that are grown throughout the world (Cappiello, 2006). Unfortunately, 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 the elite F_2 hybrid H2007-017-154 of *C. kousa* 'Miss Satomi' x *C. hongkongensis* 'Summer Passion'. This hybrid dogwood is a shrub form, semi-evergreen (down to -15^{o}C) with dense branching, red fall foliage, and excellent resistance to both dogwood anthracnose and powdery mildew.

Asexual propagation of dogwood cultivars can be achieved from budding, greenwood stem cuttings, or from micropropagation (Hadziabdic, 2005). However, cultivars regenerated from seed may exhibit a wide range of phenotypes and disease resistance (Fordham, 1990), and cultivars regenerated from cuttings frequently lack vigor (Hartmann et al., 2002). Micropropagation may prove useful for maintaining vigorous juvenile tissue and increasing

the multiplication rate of new elite cultivars. In addition, micropropagation provides a platform for further cultivar improvements through ploidy manipulation, mutation treatments, and transgenic applications (Touchell et al., 2008).

Micropropagation 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* (Declerck and Korban, 1994; Kaveriappa et al., 1997; Sharma et al., 2005; Trigiano et al., 1989; Wedge and Tainter, 1997), *C. mas* L. (Ďurkovič, 2008), *C. officinalis* Torr. ex Dur. (Lu, 1984; Lu, 1985; Xue et al., 2003), *C. capitata* Wall. (Ishimaru, 1998), and *C. kousa* (Hadziabdic, 2005; Ishimaru et al., 1993; Ishimaru et al., 1998). Basal salt 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 salt compositions have been used for *C. kousa*. Ishimaru et al. (1993) successfully established callus cultures using Murashige and Skoog Medium (MS) (Murashige and Skoog, 1964). 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 Medium (BW) (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 salts and vitamins.

In most micropropagation studies on dogwoods 6-benzylaminopurine (BAP) at 2 to 8 µM has been used as an effective cytokinin for microshoot proliferation. While BAP alone

was sufficient for microshoot proliferation of *C. nuttallii* (Edson, et al., 1994), *C. florida* (Declerck 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 NAA for *C. mas* 'Macrocarpa'. Xue et al. (2003) reported proliferation of *C. officinalis* microshoots on media containing BAP at 0.5 μM, zeatin (Ztn) at 0.5 μM, and NAA at 0.5 μM, and elongation on media containing BAP at 4.4 μM, Ztn at 0.5 μM, NAA at 0.5 μM, and gibberellic acid (GA) at 2.3 μM.

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. For *C. nuttallii,* 62% of microcuttings produced roots ex vitro with a 4.5% IBA talc dip, and 70% of rooted plants survived after 1 year (Edson et al., 1994). Similarly, for *C. canadense*, 50% of microcuttings formed roots in vitro using 0.5 μM IBA (Feng et al., 2009). For *C. florida,* in vitro rooting was optimized using 4.9 μM IBA over 4 weeks (Kaveriappa et al., 1997; Sharma et al 2005). In contrast, NAA and IAA have been more effective in ind\ucing in vitro rooting in Asian dogwoods. Ďurkovič (2008) reported an optimized in vitro rooting medium of using 2.7 μM NAA for *C. mas* 'Macrocarpa'. For *C.kousa,* NAA and indole-3-acetic acid (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 micropropagation of *C. kousa* and its cultivars, few studies have been conducted on *C. kousa* hybrids with evergreen dogwoods.

Currently, no reports on the micropropagation of the evergreen *C. hongkongensis* 'Summer Passion' exist. Therefore the objectives of this study were to evaluate a range of basal media compositions, cytokinins and auxin to optimize micropropagation protocols for a new elite interspecific hybrid H2007-017-154.

Methods and Materials

Plant Material. In vitro cultures of H2007-017-154 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-sterilized 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). Explants were agitated periodically for 17 min followed by three 5-min rinses in sterile distilled water. Explants were cultured on regeneration media consisting of WPM basal salts and vitamins supplemented with BAP at 5 µM, *myo*-Inositol at 100 $mg \cdot L^{-1}$, 2-(N-Morpholino) ethanesulfonic acid (MES) monohydrate at 100 $mg \cdot L^{-1}$, and sucrose at 30 $g \cdot L^{-1}$. Media were solidified adjusted to a pH of 5.75 and solidified with agar at 7.5 $g \cdot L^{-1}$. Regenerated microshoots were used as stock cultures for all experiments (unless stated otherwise) and maintained by transferring to fresh regeneration media (25 mL in 180-mL glass jars) every 4 to 6 weeks 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].

Basal Salts. The effects of MS salts and vitamins, WPM salts and vitamins, Driver and Kuniyuki salts (DKW) and vitamins (Driver and Kuniyuki, 1984), Quoirin and Lepoivre salts (QL) (Quoirin and Lepoivre, 1977) and Gamborg B5 vitamins (Gamborg et al., 1968), and Schenk and Hildebrandt salts (SH) and vitamins (Schenk and Hildebrandt, 1972) on microshoot proliferation of H2007-017-154 were examined. All media treatments were supplemented with BAP at 5 μM, *myo*-Inositol at 100 $mg \cdot L^{-1}$, MES at 100 $mg \cdot L^{-1}$, and sucrose at 30 $g \cdot L^{-1}$. Media were adjusted to a pH of 5.75, solidified with agar at 7.5 $g \cdot L^{-1}$, and dispensed at 25 mL into 180-mL glass jars. Five microshoots (5-10 mm long) were place vertically in each jar. Ten replicates of each media composition were incubated under standard culture conditions in a completely randomized design. Two subcultures were performed at 5 week intervals over a total treatment duration of 10 weeks. Data were collected on number of microshoots, microshoot length (of the longest shoot), and multiplication (number of 5-10 mm microcuttings obtained per subsample per 5 weeks). Total 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 $\geq$ 4 mm. Data sets were subjected to analysis of variance (ANOVA) and means were separated using Fisher's least significant difference (LSD) (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Cytokinins. Effects of BAP, Ztn, *m*T, and Kin at 0.625, 1.25, 2.5, 5, or 10 μM on microshoot proliferation were examined. Woody plant medium was used for all treatments, and media were prepared as described for the first experiment. Zeatin was added to cooled

autoclave media prior to dispensing. The experiment consisted of 8 replicates with five subsamples (5-10 mm microshoots) per treatment. Jars were arranged in a completely randomized design under standard culture conditions (as described for microshoot initiation). To reduce phenolics, two subcultures were performed at 5 week intervals for a total treatment duration of 10 weeks. Data were collected on number of microshoots, microshoot length (of the longest shoot), and multiplication (number of 5-10 mm microcuttings obtained per subsample per 5 weeks). Total phenolics were scored per replicate as described for basal salts. Data were subjected to regression analysis (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Elongation. Microshoot elongation was examined by combining IAA at 0, 0.1, 0.5, or 1.25 µM with WPM and 2 µM BAP. Media were prepared as described for the first experiment. The experiment consisted of 10 replicates with five subsamples (5-10 mm microshoots) per treatment. Jars were arranged in a completely randomized design under standard culture conditions (as described for microshoot initiation). After 5 weeks, data were collected on number of microshoots, microshoot length (of the longest shoot), multiplication (number of 5-10 mm microcuttings obtained per subsample per 5 weeks), and total phenolics (as described for basal salts). Data were subjected to regression analysis (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Rooting. The effects of auxins on the in vitro rooting of H2007-017-154 were evaluated. Media treatments included WPM salts and vitamins combined with IAA or NAA at 0.625, 1.25, 2.5, 5, or 10 µM. Unrooted microshoots mainatianed on WPM supplemented

with BAP at 5μM or Ztn at 5 μM were used to examine possible lag effects of cytokinins. Media were prepared as described for the first experiment. The experiment consisted of 10 replicates with five subsamples (5-10 mm microshoots) per treatment. Jars were arranged in a completely randomized design under standard culture conditions (as described for microshoot initiation). After 8 weeks, data were collected on survival, number of roots, and root length (of the longest root). Data sets were subjected to analysis of variance (ANOVA) and means were separated using Fisher's least significant difference (LSD) (Proc GLM, SAS Version 9.1; SAS Inst., Cary, NC).

Results and Discussion

Basal Salts. Microshoot regeneration was achieved for all basal salt treatments. For both subculture periods, basal salts had a significant effect on microshoot number ($P < 0.01$), microshoot length ($P < 0.05$), multiplication ($P < 0.01$), and phenolics ($P < 0.01$). During both subcultures, WPM supplemented with BAP produced the most number of microshoots, the longest microshoots, and the highest multiplication after 5 weeks (Table 1). The low salt media, SH, produced the smallest amount of phenolics (7.50 ± 0.93) after 5 weeks (Table 1).

Microshoot number, length, and multiplication rate for most basal salt treatments remained stable over the two subcultures (Table 1). However, phenolics and oxidative browning was substantially reduced for all treatments in the second subculture. When phenolic compounds are oxidized, toxic quinones are produced that lead to destructive oxidative browning of 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 (Baaziz et al., 1994; El Hadrami, 1995).

In the present study, WPM basal medium produced high quality microshoot regeneration and growth. The production of microshoots was heavily influenced by nitrogen levels in the basal media. Woody plant media containing lowest nitrogen (12.5 mM) produced the most microshoots (4.27 ± 0.34) after the second subculture (Fig. 1). Also, WPM had the least amount of variability in number of microshoots, microshoot length, and multiplication rate between the two subcultures (Table 1). Several former studies yielded similar results using WPM. Declerck 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 regenerated on combinations of WPM and BAP.

Cytokinins. Data is presented for second subculture only. Cytokinin concentration and the interaction between cytokinin type and concentration both significantly affected number of microshoots ($P < 0.01$ and $P < 0.01$, respectively) and multiplication rate ($P < 0.01$ and $P < 0.01$, respectively). Cytokinin type, cytokinin concentration, and their interaction significantly affected microshoot length ($P < 0.01$, $P < 0.01$, and $P < 0.01$,

respectively.) Cytokinin type and the interaction between cytokinin type and concentration both significantly affected the production of phenolics ($P < 0.01$ and $P < 0.01$, respectively).

After the second subculture, both number of microshoots and multiplication exhibited a quadratic response to BAP. Regression analysis predicted 9 μM BAP to be the optimal BAP concentration with an estimated 4.28 microshoots produced at a multiplication rate of 4.35 microcuttings per subsample 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 microshoots (data not shown). Also, BAP at 1.25 μM produced the longest microshoots (8.98 mm ± 0.87) after 5 weeks. Similar responses were observed in Ztn, with number of microshoots and multiplication exhibiting quadratic responses to Ztn. Regression analysis predicted 8 μM Ztn to be the optimal the optimal Ztn concentration with the 3.19 microshoots produced at a multiplication rate of 3.45 microcuttings per subsample over 5 weeks (Figs. 2A and 2C).

Because of hyperhydration at higher concentrations, 5 μM BAP was considered the optimized 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 (Bairu et al., 2007;

Leshem and Sachs, 1985; Leshem et al. 1988; Teramoto et al. 1993). Therefore, Ztn was included in the rooting experiment to compare possible cytokinin lag effects with BAP.

Elongation. Microshoot regeneration was achieved for all elongation treatments. Microshoot length exhibited a quadratic response to IAA concentration ($P < 0.05$). A 19% increase in microshoot 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 microshoot number. In a previous study, IAA was also successfully utilized in microshoot 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 induce 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 microshoot elongation was obtained without a reduction in multiplication rate.

Rooting. In vitro rooting was achieved, but remained low. In both NAA and IAA treatments, survival decreased with increasing auxin concentration ($P < 0.05$) (Table 2). Highest in vitro rooting was achieved with 10 μM IAA (Table 2). Almost no rooting was

achieved in plantlets obtained from media containing Ztn, indicating increased lag effects associated with Ztn. These results may indicate higher auxin concentrations, or other treatments such as half strength salts or reduced sucrose, may be necessary to achieve efficient rooting.

Low production of in vitro rooting in the present study was surprising. Former studies on Asian dogwood by Ďurkovič (2008) and Hadzibdic (2005) produced efficient rooting with NAA and IAA, in contrast to the present study. However, studies on a diverse range of species have shown that genotype is one of the most significant factors influencing the development of micropropagation protocols (Kovalchuk et al., 2009; Matt and Jehle, 2005; Mori et al., 2005; Péros et al., 1998). For instance, significant variation regarding microshoot development and subsequent root formation was observed among 32 cultivars of *Vitis vinifera* L. by Péros et al. (1998). Therefore, further study must be undertaken including different treatments such as multiple auxin types, higher auxin concentrations, half-strength basal salts, and reduced sucrose.

This research provides effective protocols for in vitro multiplication and elongation of hybrid Asian dogwood H2007-017-154 (*C. kousa* ‘Miss Satomi’ × *C. hongkongensis* ‘Summer Passion’), yet development of efficient in vitro rooting will require further study. These protocols will also provide a platform for further cultivar improvements through ploidy manipulation, mutation treatments, and transgenic applications.

Literature Cited

Bairu, M.W., W.A. Stirk, K. Dolezal, and J. Van Staden. 2007. Optimizing the micropropagation protocol for the endangered *Aloe polyphylla*: can *meta*-topolin and its derivatives serve as a replacement for benzyladenine and zeatin? Plant Cell Tiss Organ Cult 90: 15-23.

Baziz, M., F. Aissam, Z. Brakez, K. Bendiab, I. El Hadrami, and R. Cheikh. 1994. Electrophoretic patterns of acid soluble proteins and active isoforms of peroxidase and polyphenoloxidase typifying calli and somatic embryos of two reputed date palm cultivars in Moroco. Euphytica 76: 159-168.

Branca, C., G. Bucci, P. Domiano, A. Ricci, A. Torelli, and M. Bassi. 1991. Auxin structure and activity on tomato morphogenesis in vitro and pea stem elongation. Plant Cell Tiss and Organ Culture 24: 105-114.

Cappiello, P. 2006. Return of the Dogwoods. Horticulture 103(3):58-63.

Chalupa, V. 1984. *In vitro* propagation of Oak (*Quercus robur* L.) and Linden (*Tilia cordata* Mill.). Biol. Plant. 26: 374-377.

Declerck, V. and S.S. Korban. 1994. Effects of source of macronutrients and plant growth regulator concentrations on proliferation of Cornus florida. Plant Cell, Tissue and Organ Culture 38: 57-60.

Dirr, M., A. 1998. Manual of woody landscape plants: Their identification, ornamental characteristics, culture, propagation and uses. Stipes Publishing L.L.C, Champaign, IL.

Dobranszki, J., and J. Teixeira da Silva. 2010. Micropropagation of apple – a review. Biotechnol. Adv. 28: 462-488.

Driver, J.A. and A.H. Kuniyuki. 1984. In vitro propagation of Paradox walnut rootstock. HortScience 19: 507-509.

Ďurkovič, J. 2008. Micropropagation of mature *Cornus mas* 'Macrocarpa'. Trees 22: 597-602.

Edson, J.L., D.L. Wenny and A. Leege-Brusven. 1994. Micropropagation of Pacific Dogwood. Hortscience 29 (11): 1355-1356.

El Hadrami, I. 1995. L'embryogeneèse somatique chez *Phoenix dactylifera* L.: quelques facteurs limitants et marqueurs biochimiques. Thèse de Doctorat d'Etat. Université Cadi Ayyad, Faculté des Sciences-Semlalia, Marrakech.

Feng, C.-M., R. Qu, L.-L. Zhou, D.-Y. Xie and Q.-Y. Xiang. 2009. Shoot Regeneration of dwarf dogwood (*Cornus canadensis* L.) and morphological characterization of regenerated plants. Plant Cell Tiss Organ Cult 97:27- 37.

Fordham, A.J. 1990. Propagation techniques of Cornus kousa and Hamamelis taxa - 1940s vs. 1980s. Comb. Proc. Intl. Plant Prop. Soc. 40: 524-527.

Gamborg, O.L., R.A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

Hartmann, H.T., D.E. Kester, F.T. Davies, and R.T. Geneve, 2002. Plant Propagation: Principles and Practices, Pearson Education, Inc., Upper Saddle River, New Jersey.

Hadzibdic, D. 2005. *In Vitro* Regeneration of *Cladrastis kentukea* (American yellowwood) and *Cornus kousa* (kousa dogwood). Univ. of Tenn., Knoxville, MS Diss. 46-100.

Ishimaru, K., H. Arakawa and S. Neera. 1993. Polyphenol production in cell cultures of *Cornus kousa*. Phytochemistry 32(5): 1193-1197.

Ishimaru, K., N. Tanaka, T. Kamiya, T. Sato, and K. Shimomura. 1998. Cornus kousa (Dogwood): In Vitro Culture, and the Production of Tannins and Other Phenolic Compounds. In Bajaj, Y.P.S. (Ed.). Biotechnology in Agriculture and Forestry 41. Medicinal and Aromatic Plants X. Springer-Verlag. New York. P. 113-131.

Kaveriappa, K.M., L.M. Phillips and R.N. Trigiano. 1997. Micropropagation of flowering dogwood (*Cornus florida*) from seedlings. Plant Cell Reports 16: 485-489.

Leshem, B. and T. Sachs. 1985. 'Vitrified' Dianthus- teratomata in vitro due to growth factor imbalance. Ann Bot 56: 613-617.

Lesham, B. D.P. Shaley, and S. Izhar. 1988. Cytokinin as an inducer of hyperhydricity in melon. Ann Bot 61: 255–260.

Lloyd, G. and B.H. McCown. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Combined Proc. Intern. Plant Propagators Soc. 30:421-427.

Lu, W.X. 1984. Tissue culture of immature embryos of Cornus officinalis. Zhong Yao Tong Bao 9(1):7-8. (Article in Chinese)

Lu, W.X. 1985. Rapid propagation of plants from the immature embryos of Cornus officinalis. Zhong Yao Tong Bao 10(10): 9-10. (Article in Chinese)

Madhulatha, P., M. Anbalagan, S. Jayachandran, and N. Sakthivel. 2004. Influence of liquid pulse treatment with growth regulators on in vitro propagation of banana (Musa spp. AAA). Plant Cell Tissue Organ Cult. 76:189-192.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Quoirin, M. and P. Lepoivre. 1977. Improved medium for in vitro culture of *Prunus* sp. Acta. Hort. 78:437-442.

Ranney, T.G., L.F. Grand, and J.L. Knighten. 1995. Susceptibility of cultivars and hybrids of kousa dogwood to dogwood anthracnose and powdery mildew. J. Arboric. 21:11-16.

Sharma, A.R., R.N. Trigiano, W.T. Whitte and O.J. Schwarz. 2005. Scientia Horticulturae 103: 381-385.

Schenk, R.U. and A.C. Hildebrandt. 1972. Medium and Techniques for Induction and Growth of Monocotyledonous and Dicotyledonous Plant Cell Cultures. Can. J. Bot. 50: 199-204.

Teramoto H, E. Momotani , and H. Tsuji. 1993. Benzyladenine-induced changes in the translatable mRNA population in excised cucumber cotyledons. Physiol Plant 87:584–591

Trigiano, R.N., M.H. Ament, M.T. Windham, and J.K. Moulton. 2004. Genetic profiling of red-bracted Cornus kousa cultivars indicates significant cultivar synonomy. HortScience. 39: 489-492.

Trigiano, R.N., R.M. Beaty and J.T. Dietrich. 1989. Somatic embryogenesis and plantlet regeneration in Cornus florida. Plant Cell Reports 8: 270-273.

Touchell, D., J. Smith, and T.G. Ranney. 2008. Novel applications of plant tissue culture. Proc. Combined Inter. Plant Propagators' Soc. 58:196-199.

Vernoux, T. F. Besnard, and J. Traas. 2010. Auxin at the Shoot Apical Meristem. Cold Spring Harbor Perspectives in Biology Epub: a001487.

Wedge, D.E. and F.H. Tainter. 1997. In vitro detection of *Cornus florida* callus insensitive to toxic metabolites of *Discula destructiva*. In Vitro Cell. Dev. Biol. – Plant 33: 142-146.

Xue, J.P., A.M. Zhang, Y.H. Wang, and W. Sheng. 2003. Study on plant tissue culture of Cornus officinalis. Zhongguo Zhong Yao Za Zhi 28(2): 118-121.

Zhao Y., S.K. Christensen, C. Fankhauser, J.R. Cashman, J.D. Cohen, D. Weigel, and J. Chory. 2001. A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science 291: 306–309.

Table 1. Effect of basal salts and vitamins on in vitro shoot proliferation and phenolic production of H2007-017-154 (*C. kousa* 'Miss Satomi' x *C. hongkongensis* 'Summer Passion').

	Subculture 1			
Treatment[z]	**Number of shoots**	**Shoot length (mm)**	**Multiplication rate[x]**	**Phenolics[w]**
MS	1.54 ± 0.40 b[y]	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
	Subculture 2			
Treatment[z]	**Number of shoots**	**Shoot length (mm)**	**Multiplication rate[x]**	**Phenolics[w]**
MS	1.83 ± 0.36 b[y]	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

[z] Key to media and hormone abbreviations: MS (Murashige and Skoog basal salts and vitamins); WPM (Lloyd and McCown woody plant medium and vitamins), DKW (Driver and Kuniyuki walnut medium and vitamins), QL (Quoirin and Lepoivre basal salts and Gamborg B5 vitamins), and SH (Schenk and Hildebrandt basal salts and vitamins), and BAP (6-benzylaminopurine). All media treatments supplemented with BAP at 5 μM.

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

[x] Multiplication rate defined as the number of 20 mm microcuttings produced per subsample after five weeks

[w] Total 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.

Table 2. Effects of cytokinin pretreatment and auxins on in vitro rooting of H2007-017-154 (*C. kousa* 'Miss Satomi' x *C. hongkongensis* 'Summer Passion').

Cytokinin Prereatment[z]	Auxin	Auxin concentration (µM)	Percent Survival	Root number	Root Length (mm)
Ztn	IAA	0.625	0.83 ± 0.05 ab[z]	0.09 ± 0.06 b	0.97 ± 0.72 b
Ztn	IAA	1.25	0.78 ± 0.08 ab	0.00 ± 0.00 c	0.00 ± 0.00 c
Ztn	IAA	2.5	0.70 ± 0.10 abc	0.00 ± 0.00 c	0.00 ± 0.00 c
Ztn	IAA	5	0.70 ± 0.11 abc	0.00 ± 0.00 c	0.00 ± 0.00 c
Ztn	IAA	10	0.76 ± 0.08 ab	0.02 ± 0.02 bc	0.42 ± 0.42 b
Ztn	NAA	0.625	0.71 ± 0.06 abc	0.00 ± 0.00 c	0.00 ± 0.00 c
Ztn	NAA	1.25	0.83 ± 0.06 ab	0.00 ± 0.00 c	0.00 ± 0.00 c
Ztn	NAA	2.5	0.80 ± 0.07 ab	0.00 ± 0.00 c	0.00 ± 0.00 c
Ztn	NAA	5	0.48 ± 0.07 cd	0.00 ± 0.00 c	0.00 ± 0.00 c
Ztn	NAA	10	0.43 ± 0.08 cd	0.00 ± 0.00 c	0.00 ± 0.00 c
BAP	IAA	0.625	0.80 ± 0.07 ab	0.08 ± 0.05 bc	0.55 ± 0.38 bc
BAP	IAA	1.25	0.73 ± 0.06 abc	0.00 ± 0.00 c	0.00 ± 0.00 c
BAP	IAA	2.5	0.65 ± 0.06 bc	0.03 ± 0.03 bc	0.13 ± 0.13 bc
BAP	IAA	5	0.43 ± 0.12 cd	0.14 ± 0.08 ab	0.69 ± 0.36 bc
BAP	IAA	10	0.56 ± 0.09 cd	0.24 ± 0.09 a	1.93 ± 0.71 a
BAP	NAA	0.625	0.60 ± 0.11bcd	0.10 ± 0.05 bc	0.63 ± 0.33 bc
BAP	NAA	1.25	0.60 ± 0.08 bcd	0.00 ± 0.00 c	0.00 ± 0.00 c
BAP	NAA	2.5	0.89 ± 0.04 a	0.14 ± 0.11 ab	0.71 ± 0.54 bc
BAP	NAA	5	0.73 ± 0.07 abc	0.00 ± 0.00 c	0.00 ± 0.00 c
BAP	NAA	10	0.40 ± 0.06 d	0.06 ± 0.04 bc	0.49 ± 0.37 bc

[z] Mean separation within columns by Fischer's LSD test at $P < 0.05$.

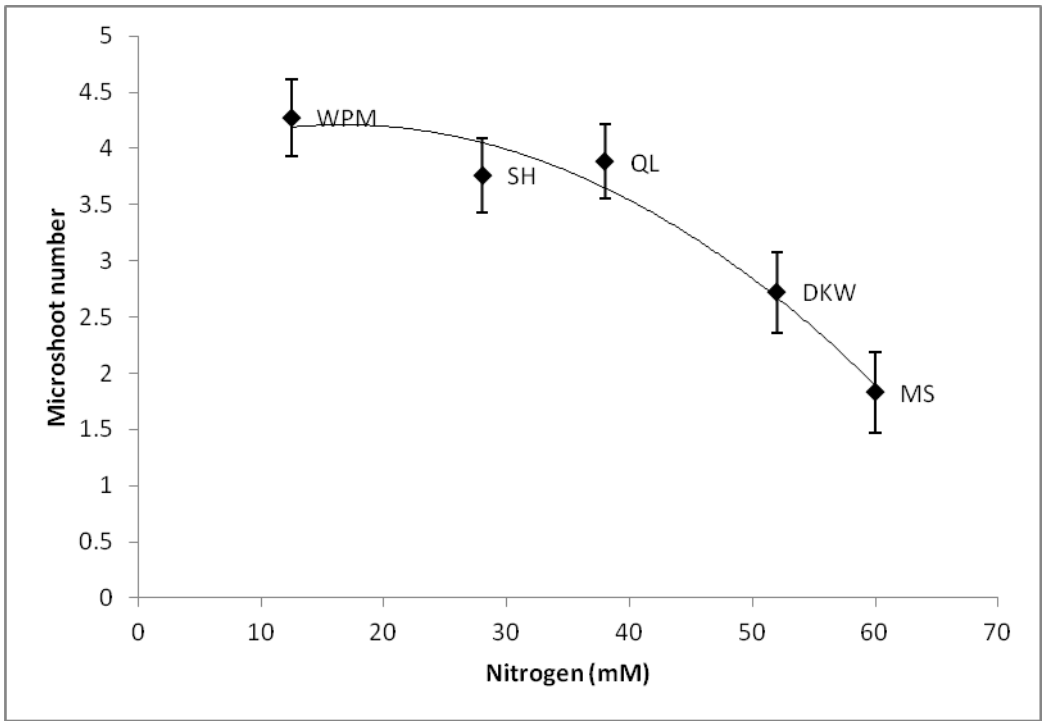

Figure 1. Influence of basal media nitrogen levels on microshoot production of H2011-017-154 (*C. kousa* 'Miss Satomi' × *C. hongkongensis* 'Summer Passion'). Symbols represent means, n=6, ± SEM. Microshoot number is measured as length of the longest microshoot: $y = -0.0012x^2 + 0.041x + 3.8689$, $r^2 = 0.96$. Key to media 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). A) Number of microshoots produced per microcutting; B) Length of longest microshoot; and C) Multiplication rate defined as the number of 20 mm microcuttings produced per subsample after five weeks.

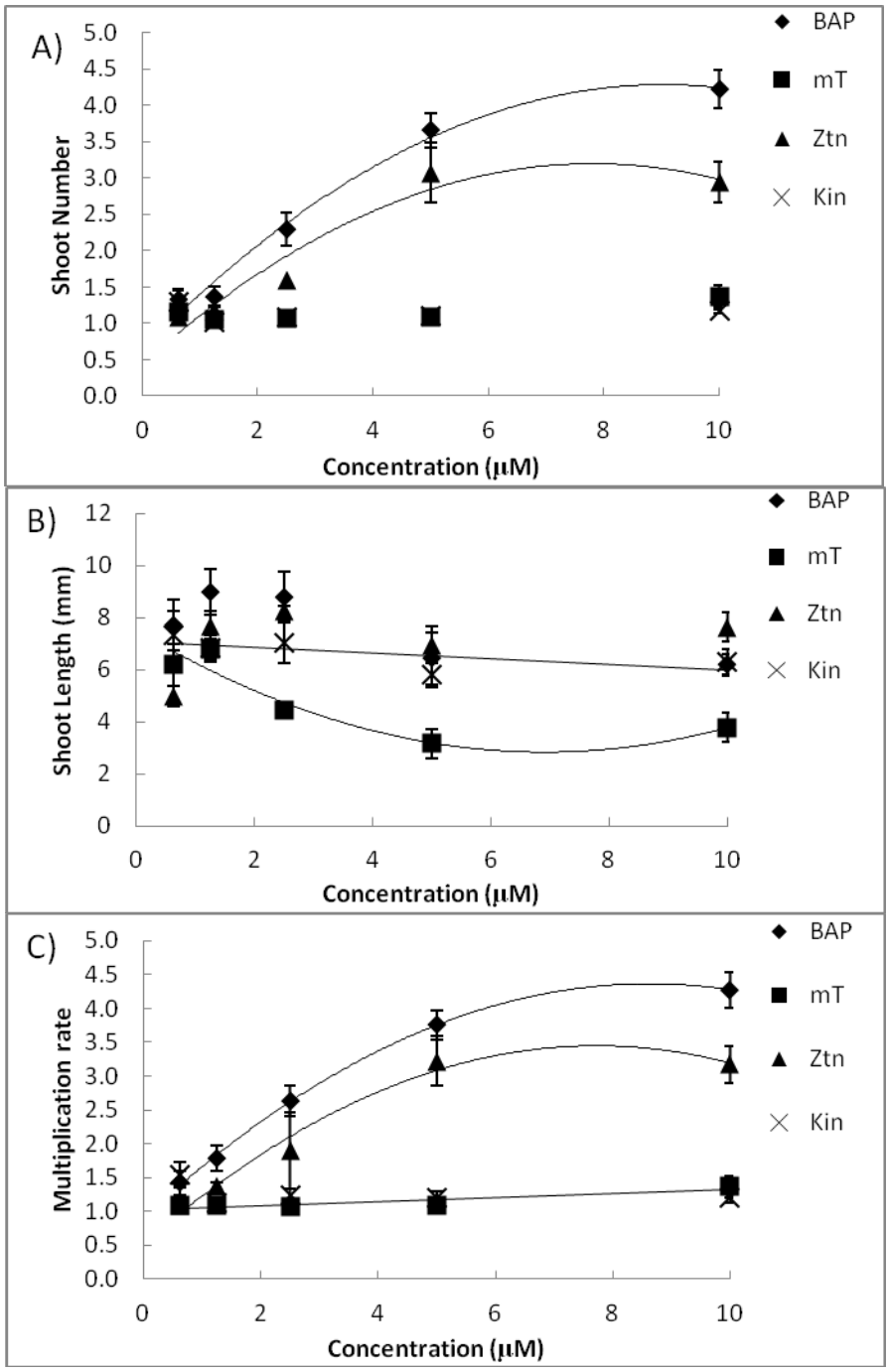

Figure 2. Influence of cytokinin type and concentration on microshoot development of H2011-017-154 (*C. kousa* 'Miss Satomi' × *C. hongkongensis* 'Summer Passion'). Symbols represent means, n=6, ± SEM. A) Number of microshoots produced per microcutting; B) Length of longest microshoot; and C) Multiplication rate defined as the number of 20 mm microcuttings produced per subsample after five weeks.