ABSTRACT

ROUNSAVILLE, TODD JEFFREY. Cytogenetics, Micropropagation, and Reproductive Biology of *Berberis*, *Mahonia*, and *Miscanthus*. (Under the direction of Thomas G. Ranney).

Research was conducted to determine the genome sizes and ploidy levels for a diverse collection of *Berberis* L. and *Mahonia* Nutt. genotypes, develop a micropropagation protocol for *Mahonia* 'Soft Caress', and examine the fertility and reproductive pathways among clones of diploid and triploid *Miscanthus sinensis* Andersson.

Berberis and *Mahonia* are sister taxa within the Berberidaceae with strong potential for ornamental improvement. Propidium iodide (PI) flow cytometric analysis was conducted to determine genome sizes. Mean $1C_X$ genome size varied between the two *Mahonia* subgenera (*Occidentales* = 1.17 pg, *Orientales* = 1.27 pg), while those of *Berberis* subgenera were similar (*Australes* = 1.45 pg, *Septentrionales* = 1.47 pg), but larger than those of *Mahonia*. Traditional cytology was performed on representative species to calibrate genome sizes with ploidy levels. While the majority of species were determined to be diploid with $2n = 2x = 28$, artificially-induced autopolyploid *Berberis thunbergii* seedlings were confirmed to be tetraploid and an accession of *Mahonia nervosa* was confirmed to be hexaploid. Genome sizes and ploidy levels are presented for the first time for the majority of taxa sampled and will serve as a resource for plant breeders, ecologists, and systematists.

Mahonia 'Soft Caress' is a unique new cultivar exhibiting a compact form and delicate evergreen leaves, though propagation can be a limiting factor for production. Micropropagation protocols for *M.* 'Soft Caress' were developed to expedite multiplication and serve as a foundation for future work with other *Mahonia* taxa. Sucrose at 30 or 45 $g \cdot L^{-1}$ in conjunction with Gamborgs B5 (B5), Quoirin and Lepoivre (QL), and Murashige and

Skoog (MS) basal media, and other growth regulator treatments were evaluated as multiplication media. Rooting of microcuttings was conducted in vitro using indole-3-butyric acid (IBA) at 0, 2, 4, 8, or 16 µM under either light or dark. Gamborgs B5 media supplemented with sucrose at 30 $g \cdot L^{-1}$, 5 µM 6-benzylaminopurine (BAP), 5 µM kinetin (Kin), 0.5 µM indole-3-acetic acid (IAA), and 2.5 µM gibberellic acid (GA_3) yielded 2.8 ± 0.1 shoots with a mean length of 14.8 ± 0.6 mm over a 6-week culture period and was the most effective multiplication media. Light treatment and IBA concentration had a significant effect on rooting percentages and microcuttings treated with 8 µM IBA and maintained in the dark resulted in the greatest rooting (70%) and ex vitro establishment.

Miscanthus sinensis is a popular ornamental grass with over 100 cultivars commercially available. Its propensity to rapidly colonize open and disturbed environments has made it a nuisance and weed in some regions in the United States. Infertile forms of *Miscanthus sinensis* would greatly benefit the nursery and horticulture industries. Triploid plants were evaluated for male (pollen viability) and female (seed set and germination) fertility. Pollen viability staining was reduced in triploid plants (mean 60%) ($P < 0.0001$) compared to diploid controls (mean 74%). Relative female fertility of individual triploids clones {(% seed set × % germination for triploid)/ (% seed set × % germination for diploid control)} ranged from 49% to 0.7% for H2008-091-004. Reproductive pathways of triploid plants were evaluated by determining the genome sizes of open pollinated progeny. The progeny arising from triploids were predominantly aneuploid with genome sizes intermediate between diploid and triploids. There was no evidence of apomixis, selfing, or triploid × triploid fertilization events observed among triploid parents. Formation of unreduced gametes was rare for both ploidy levels (~0.01%). The considerable reduction in female

fertility in some triploid clones combined with the limited production of primarily aneuploid progeny, provides highly infertile alternatives to existing diploid cultivars.

Cytogenetics, Micropropagation, and Reproductive Biology of *Berberis*, *Mahonia*, and *Miscanthus*

by
Todd Jeffrey Rounsaville

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

2011

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Dr. Frank A. Blazich	Dr. Dennis J. Werner

DEDICATION

To my grandmother,

Marguerite Ann Rounsaville.

Your love and support are still with me,

Even if you are not.

BIOGRAPHY

Todd Jeffrey Rounsaville was born to Jeffrey and Leslie Rounsaville and raised in southern New Hampshire. He attended the University of Rhode Island for his undergraduate education, and majored in Turfgrass Management for a year before switching to Environmental Horticulture. Todd spent his summers gaining professional experience through internships, first at the International Club in Bolton, MA, followed by the Arnold Arboretum in Boston, MA, and then the US National Arboretum in Washington, DC. He continued moving southward after graduating and spent 2 years in Philadelphia at the Scott Arboretum and another two at Plant Delights Nursery, Juniper Level, NC. In 2009, Todd relocated to the northeastern United States (Cary, NC) to begin graduate school.

ACKNOWLEDGMENTS

Despite numerous meetings with various program administrators and years of contemplation, I could never convince myself to go *back* to school. Getting to graduate school, and getting *through* graduate school, was not without considerable help from the people listed herein. For giving me the opportunity to work with you, and for offering me three exciting and challenging projects, I sincerely thank Dr. Tom Ranney. Thank you for always making yourself available as an advisor and mentor. Your insightful, often unconventional perspective of seemingly simple things has been one of the most valuable lessons I take from graduate school. I thank you for placing your trust in me, and for all of the time you spent editing, offering advice, and just talking plants. I am confident that returning to school was the right step for me, and I wouldn't have done it anywhere else.

The entire staff of the Mountain Crop Improvement Lab in Mills River, NC, has contributed to my graduate education in equal proportion to the formal classes taken in Raleigh. Without exception you all took the time to put aside your own (numerous) responsibilities to answer my questions and assist with my projects, especially in my absence during the academic year. Tom Eaker, I am grateful to have always found you in good spirits with something to give me a hard time about, and for always pointing me in the right direction when I was searching for plants and materials. Nathan Lynch, I remain in awe of your encyclopedic knowledge of, as far as I can tell, everything. Learning something new and exciting during every conversation with you was a highlight of my time at the Research

Station. Your command of all the laboratory equipment and protocols, and your desire to teach them to one graduate student after another, is very much appreciated. To Joel Mowrey, you are a fantastic plantsman with a phenomenal work ethic. I thank you for busting me out of the lab to get my hands dirty once or twice a week, and for always stopping to show me the cool things you are working on. Jeremy Smith likely spent more time than anyone helping with my projects. Thank you for getting me settled in the TC lab, and your many hours subculturing and seed counting. On the subject of seed counting, I am grateful to Irene Palmer for helping me extract thousands of *Miscanthus* seed under a microscope. I will try not to rub it in that you have many thousands more to count for your own research, and simply wish you the very best as you complete your degree. Dr. Darren Touchell, I sincerely believe that you lost sleep at night contemplating some of our experiments (particularly the troublesome early phases of getting *Mahonia* into tissue culture), for that I am deeply grateful. For being a sounding board for many of my ideas, and letting me experiment with both the good and some of the bad ones, thank you. For *not* answering so many of my questions, but instead pushing me to figure it out myself, thank you.

In Raleigh, I was extremely fortunate to have Drs. Frank Blazich and Dennis Werner serve on my graduate committee. Although away from the physical research, I am indebted to them for helping to shape our experiments, and their time editing manuscripts and this thesis. To Dr. Blazich, thank you for all of your advice, and for co-authoring the tissue culture manuscript. Your comments were meticulous and insightful, and they made for a much stronger paper. To Dr. Werner, even though a number of early project ideas never

came to fruition, I thank you for taking the time to discuss potential experiments, and for showing a genuine interest in me and my research. It was a pleasure to work with you. Also in Raleigh, Rachel McLaughlin does the work of several people to keep all the graduate students on track. I thank you for the many, many times you helped me the past few years. To Dr. Brian Jackson, your energy and attitude are inspiring; I thank you for immediately trusting me as a TA. The most rewarding classes I had at NC State were the ones I taught for you. Fellow graduate students Jared Barnes and Elisabeth Meyer have been invaluable friends. Thank you for navigating me through the graduate swamp.

I thank Karen Stever for her candid correspondence as we independently struggled to get *Mahonia* 'Soft Caress' into tissue culture. Ozzie Johnson was remarkably kind in support of my *Mahonia* related projects as well. Thank you Ozzie, for inviting me into your home and garden, and so generously donating plant material to both my research and personal plant collections.

I owe a debt of gratitude to the people who wrote letters of support for my graduate school application, and whom continue to serve as personal references. Dr. Susan Gordon taught my first plant science class at URI and is one of the most influential professors I have ever had. I thank you tremendously for your lessons inside and outside of the classroom. James Adams, you are one of the most driven, hard working people I have ever known. Your humor and wit is unparalleled, and it is always a pleasure to be in your company. I shudder to think where I would be in life had you not forced me to apply to the Scott Arboretum, and I sincerely thank you for it. Swarthmore was a treasure of more dear friends and wonderful

experiences than I can possibly catalog here; I thank Claire Sawyers for hiring me, being one of those friends, and continuing to go to bat on my behalf. Tony Avent, your enthusiasm for plants is extraordinarily contagious. I thank you for the opportunity to be a part of Plant Delights, and for your contributions of plant material for my research. Andrew Bunting was my boss for several years, and I could not have asked for a better supervisor. You gave me carte blanche on dozens of things that you were accountable for, and I flourished. Your superhuman plantsmanship is exceeded only by your unwavering friendship, and for that I can only hope to someday repay you.

To my wife, Melanie, you have been my daily source of strength and inspiration for as long as I have known you. Going back to school required a dramatic shift in our routine and schedules; it was difficult at times, but you remained positive throughout and helped me do the same. During my routine frustrations and struggles, it was your encouragement and constant championing that consistently pulled me through. I remain in awe of the kindness and patience you display in everything you do, and I'm hopeful it will rub off on me someday. I could not have asked for a better person with which to share all the joys and sorrows of life. Thank you so much, you have all of my love. I thank John and Trudy Blandford; and Nick, Susan, Luke, Maebel, and William Blandford for treating me like family before I ever was, and for helping Melanie and me in so many ways.

My family has put in front of me more opportunities than I could ever ask for, and made me who I am today. With each of my grandparents I have had a unique relationship.

In life and in spirit, you remain in my thoughts as models of compassion and courage; it means the world to me, thank you.

I was never expected to work in my mother's garden, but it was a garden impressive enough that I gravitated to it anyway. Mom, thank you for giving me an appreciation of the natural world, and always finding room for the orphan plants I would drag home. Most of all, thank you for always being there for me. My father has been a life-long role model through his words and his actions. Thank you dad for so many life lessons, and for instilling in me an appreciation of the things in life that are truly important. My sister Jamie is 4 years younger than me. She is also smarter, braver, more mature, and probably a better shot than I. Every time I see you I am more impressed, thanks for being you.

I thank my parents for all of their sacrifices, for their steadfast encouragement, and for always being available and willing to offer advice when I needed it. You have continued to provide support in every conceivable way, even though you don't always know it.

Thank you.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 3. Fertility and Reproductive Pathways in Diploid and Triploid *Miscanthus sinensis*

LIST OF FIGURES

Chapter 2. Micropropagation of *Mahonia* 'Soft Caress'

Chapter 3. Fertility and Reproductive Pathways in Diploid and Triploid *Miscanthus sinensis*

Chapter 1

Ploidy Levels and Genome Sizes of *Berberis* L. and *Mahonia* Nutt. Species, Hybrids, and Cultivars

(In the format appropriate for submission to HortScience)

Ploidy Levels and Genome Sizes of *Berberis* L. and *Mahonia* Nutt. Species, Hybrids, and Cultivars

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Received for publication . Accepted for Publication .

Thanks are expressed to Tom Eaker, Nathan Lynch, Joel Mowrey, and the staff of the Mountain Horticultural Crops Research Station for excellent technical assistance. Additional thanks to Tony Avent, Ozzie Johnson, Todd Wiegardt, and the staff of the JC Raulston Arboretum and Bartlett Arboretum for generous donations of sample material.

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Subject Category: Breeding, Cultivars, Rootstocks, and Germplasm Resources – Ornamentals

Ploidy Levels and Genome Sizes of *Berberis* L. and *Mahonia* Nutt. Species, Hybrids, and Cultivars

Additional index words. chromosome number, cytology, DNA content, flow cytometry, genome size, polyploidy, systematics, taxonomy

Abstract. An extensive survey of genome sizes and ploidy levels was conducted for a diverse collection of *Berberis* and *Mahonia* taxa (Berberidaceae). Propidium iodide (PI) flow cytometric analysis was conducted using *Pisum sativum* L. 'Ctirad' (2C DNA = 8.76 pg) as an internal standard to determine genome sizes. Mean $1C_X$ genome sizes varied between the two *Mahonia* subgenera (*Occidentales* = 1.17 ± 0.02, *Orientales* = 1.27 ± 0.01), while those of *Berberis* subgenera were similar (*Australes* = 1.45 ± 0.03, *Septentrionales* = 1.47 ± 0.02), and each significantly larger than those of *Mahonia*. Traditional cytology was performed on representative species to calibrate genome sizes with ploidy levels. Polyploidy among both wild and cultivated taxa was found to be rare. While the majority of species were determined to be diploid with $2n=2x=28$, artificially-induced autopolyploid *Berberis thunbergii* seedlings were confirmed to be tetraploid and an accession of *Mahonia nervosa* was confirmed to be hexaploid. Genome size and ploidy level reports for the majority of taxa sampled are

presented for the first time and are intended to be of use to plant breeders, ecologists, and systematists.

Introduction

The sister genera *Berberis* L. and *Mahonia* Nutt. represent the two largest groups within the *Berberidaceae*, consisting of ~ 400 and 100 species, respectively (Ahrendt, 1961; Kim et al., 2004). This highly ornamental group of shrubs and small trees are valued for their evergreen or multi-colored leaves, brilliant flowers, and often showy fruit. The two genera have also been recognized for their pharmaceutical and medicinal properties (Alvarez et al., 2009), as well as their use in the printing and dyeing industry (Yan-Jun et al., 2006). Distribution of the two genera is nearly worldwide, with centers of diversity in southern Asia as well as Central and South America, and with minor representation in North America, Europe, Africa, and the Pacific Islands (Ahrendt, 1961).

Taxonomic standing of *Mahonia* and *Berberis* as distinct genera has been the subject of much debate among botanists and horticulturists. Prior to the development of DNA-based phylogenetics, morphological characters such as leaf and stem complexity, inflorescence structure, and floral anatomy had served to distinctly separate *Mahonia* from *Berberis*. However, *Mahonia* section *Horridae* Fedde (~9 species), which includes *M. freemontii* (Torr.) Fedde, *M. haematocarpa* (Wooton) Fedde, *M. nevinii* (A. Gray) Fedde, and *M. trifoliolata* (Moric.) Fedde, exhibits a blend of taxonomic features intermediate between *Mahonia* and *Berberis* (Ahrendt, 1961; Whittemore, 1997). These morphological

inconsistencies have led some (Lafferriere, 1997; Marroquin, 1993; Whittemore, 1997) to adopt a unified treatment of all *Berberis* and *Mahonia* species within *Berberis*. Nevertheless, the obvious difference in physical appearance between the two genera, with compound leaves within *Mahonia* vs. simple leaves within *Berberis*, make a unified circumscription difficult to reconcile, and consequently, the horticultural field generally maintains the two groups as separate genera (Ahrendt, 1961; Dirr, 2009; Hinkley, 2009; Huxley et al., 1992; Yan-Jun et al., 2006).

When the taxonomy is viewed *sensu* Ahrendt, *Berberis* and *Mahonia* are each divided into two subgenera, set forth by Schneider (1905) and Ahrendt (1961). Within *Berberis*, the *Australes* C.K. Schneid. includes all the species from Central and South America; the remaining species are placed in the *Septentrionales* C.K. Schneid., and occur entirely in the northern hemisphere save for two in East Africa and one in Java and Sumatra (Ahrendt, 1961). Conversely, *Mahonia* are grouped longitudinally, with those of the eastern hemisphere in subgenus *Orientales* Ahrendt and all those of the western hemisphere (with the notable exception of *M. nervosa* (Pursh) Nutt.) in subgenus *Occidentales* Ahrendt.

Recent phylogenetic analysis based on internal transcribed spacer (ITS) sequencing (Kim et al., 2004) has yielded further insight into the taxonomic relationships and evolutionary history of *Mahonia* and *Berberis*. For example, the postulation of Ahrendt (1961) of *Mahonia* as the progenitor of *Berberis* was supported. Examining the contemporary dispersal of the two genera from South America northward reveals that while the distinctive compound-leaved *Mahonia* is first encountered in Central America, a number

of *Berberis* characters persist within *Mahonia* much further north. These transitional species, representing the aforementioned *Mahonia* section *Horridae*, showed a closer relationship with *Berberis*, and thus a paraphyletic subgenus *Occidentales* (Kim et al., 2004). While ITS phylogeny supported the subgenera proposed by Schneider (1905) and Ahrendt (1961), groupings below the subgeneric levels were not supported (Kim et al., 2004). Furthermore, *M. nervosa* was retained within the *Orientales*, albeit with weak support.

Along with the monotypic herb *Ranzania japonica* T. Ito, *Berberis* and *Mahonia* form a monophyletic clade within Berberidaceae where base chromosome number is x=7 (Kim and Jansen, 1998). As Dermen (1931) noted in his cytological studies, chromosomes amongst widespread species of both genera are of similar size. Furthermore, artificial intergeneric hybridization events between *Mahonia* and *Berberis* originated in Europe as early as 1854 (Dirr, 2009). Despite a number of successful intergeneric hybrids (×*Mahoberberis* C.K. Schneid.), the resulting progeny have been horticultural curiosities at best, typically regarded as inferior to both parent taxa (Phillips and Barber, 1981). ×*Mahoberberis* tend to exhibit numerous leaf-morphs among single plants, and in general flowering and fruiting of the hybrids is known to be rare or nonexistent (Dirr, 2009; Wyman, 1958). In addition, all ×*Mahoberberis* hybrids have been comprised of only one species of *Mahonia* (*M. aquifolium* (Pursh) Nutt.), and the cross appears uni-directional with *Mahonia* only functioning as the maternal parent (Dirr, 2009; personal observation), further suggesting that the two genera are largely incompatible. Conversely, hybrids among species of *Berberis* and among species of *Mahonia* are commonplace (Huxley et al., 1992; personal observation).

Polyploidization is a significant phenomenon in the plant kingdom that can play a role in rapid genomic rearrangement, development of novel traits and adaptations, reproductive isolation, and can ultimately lead to speciation (Adams and Wendel, 2005; Soltis and Burleigh, 2009). Furthermore, polyploidy is an important consideration in plant breeding as it can influence crossability, morphology, fertility, and gene expression (Chen and Ni, 2006; Soltis et al., 2004). Sampling of ploidy levels has been very limited for *Mahonia* taxa. *Mahonia aquifolium*, *M. napaulensis* DC., *M. repens* (Lindl.) G. Don., and *M. japonica* (Thunb.) DC. have been reported to be diploid with $2n=2x=28$ (Dermen, 1931; Xu et al., 1992). In other cases, *M. aquifolium* and *M. nervosa* were reported to be tetraploid with $2n=4x=56$ (Taylor and Taylor, 1997). Reports on 45 *Berberis* species found diploids, $2n=2x=28$, including *B. koreana* Palib., *B. seiboldii* Miq., *B. thunbergii* DC., *B. vulgaris* L. and *B. yunnanensis* Franch., as well as tetraploids, $2n=4x=56$, including *B. buxifolia* Lam., *B. heterophylla* Juss. ex Poir., and *B. turcomanica* Kar. (Bottini et al., 2000; Dermen, 1931).

Independent of variations in ploidy level, information on base genome size (base DNA content) can be used as an indicator of genome evolution and taxonomic relationships (Greilhuber, 1998; Vinogradov, 1994; Zonneveld and Van Iren, 2001; Zonneveld and Duncan, 2010), lending insight into species evolution and potential breeding applications. As it relates to breeding, disparities in genome sizes can reflect differences in chromosome sizes and arrangement that may influence crossability and fertility of hybrid progeny (Zonneveld, 2009). There are no published reports of *Mahonia* genome size, and those of *Berberis* are extremely limited in both number of taxa and species diversity. Previous reports

of genome size among *Berberis* were determined using Feulgen microspectrophotometry, with diploid species constituting a range of 1.5 pg (picograms) for *B. bidentata* Lechler to 3.6 pg for *B. empetrifolia* Lam. (Bottini et al., 2000). A desirable alternative to microspectrophotometry is flow cytometry, which allows for much greater ease in sample preparation, rapid determination of genome size, and can be accurately performed using a variety of plant tissues (Doležel et al., 1998; Doležel and Bartos, 2005). For closely related taxa, where genome sizes are relatively conserved, flow cytometry can also be used for determination of ploidy level. While a number of different fluorochromes may be used to stain DNA, many including 4', 6-Diamidino -2-phenylindole (DAPI), Hoechst 33258 (HO) and olivomycin (OM) are exclusive to either AT or CG base pairs, whereas propidium iodide (PI) is known to be largely non-specific with only a slight preference towards CG (Doležel et al., 1998; Vinogradov, 1994).

Considering the tremendous diversity and crossability found in *Berberis* and *Mahonia*, the potential for breeding improved hybrids is considerable. However, a greater understanding of genome sizes and ploidy levels within these genera would greatly enhance future breeding efforts. While basic information on chromosome numbers, genome sizes, and ploidy levels have been reported for some *Berberis* and *Mahonia*, sampling has been limited and little is known about ploidy levels of specific clones or cultivars. Therefore, the objectives of this research were to conduct an extensive survey of genome sizes and ploidy levels of species, hybrids, and cultivars of *Berberis* and *Mahonia* using a combination of flow cytometry and traditional cytology. Taxa included for this survey exhibit attributes of

value for the ornamental plant breeder, and are representative of each major phylogenetic clade. Due to the unresolved nature of the generic classification, and for purposes of comparison, we accept the treatment of Leslie W. A. Ahrendt (1961), who conducted the last thorough review of the genera.

Materials and Methods

A diverse collection of *Berberis* and *Mahonia* taxa were obtained from various institutions, gardens, and private collectors. All sampled plants from the Mountain Horticulture Crops Research Station (MHCRS) were container grown, while additional sample material shared with the authors was collected from field grown plants (Table 1). Sampled taxa represented species from each of the four subgenera (*Australes*, *Septentrionales*, *Occidentales*, and *Orientales*), many common cultivars including interspecific hybrids, and a few purported artificially-induced autopolyploids.

Flow cytometry was conducted on tissue (0.5 cm^2) taken from recently expanded leaves using a hole punch. Leaf tissue for each sample, as well as an internal standard (*Pisum sativum* 'Ctirad' 2C DNA = 8.76 pg) was finely diced with a razor blade in a petri dish containing 500 µL of nuclei extraction buffer. Upon being filtered into a small test tube using a 50 µm filter, a solution containing 2 mL staining buffer, 6µL RNase A, and 12 µL propidium iodide (PI) (CyStain PI absolute P, Partec, Münster, Germany) was added, and the samples were moved to a refrigerator at 4° C for 1 h. A flow cytometer (Partec PA-II ,

Partec, Münster, Germany) was used to analyze the stained nuclei, with a minimum of 5,000 counts per sample, and two sub-samples conducted for each taxon. Flow cytometry was conducted during the spring and summer of 2009 when fresh leaf material was available for sample. Holoploid, 2C genome size was calculated as: 2C = genome size of standard × (mean fluorescence value of sample/ mean fluorescence value of standard). Genome values presented within Table 1 represent the mean value of two sub-samples conducted for each taxon. The relationship between ploidy levels and genome sizes was determined for plants with documented chromosome numbers (Bottini et al., 2000; Xu et al., 1992). Mean $1C_X$ monoploid genome size (i.e., DNA content of the non-replicated base set of chromosomes with x = 14) was calculated as (2C genome size / ploidy level) to assess variability in base genome size. Data were subjected to analysis of variance and means separation using the Waller procedure to compare means of genera and subgenera (SAS Institute Inc., Cary, NC).

Traditional cytology was conducted to verify previous work and calibrate genome size with ploidy level. Between 8 and 9 am, actively growing root tips approximately 5mm in length were removed from container-grown plants and placed into small vials of 2 mM 8-hydroxyquinoline. The vials were left in the dark for two hours at room temperature followed by two hours in darkness at 4° C. Roots tips were then thoroughly rinsed in cold distilled water, blotted dry, and transferred to a fixative of (3:1) 95% ethanol : propionic acid, overnight at room temperature. After 16-24 h in fixative the root tissue was rinsed with 70% ethanol and transferred to storage in 70% ethanol at 4° C. When time permitted, root tissue

was removed from cold storage and hydrolyzed in a solution of (3:1) 95% ethanol : 12N HCl for 5-10 s. Root tips were then placed into a drop of modified carbol fuschin stain (Kao, 1975) on a glass microscope slide and gently squashed with a coverslip. Chromosomes were counted using oil immersion at 1,500×.

Results and Discussion

Cytology performed on *Mahonia eurybracteata* Fedde 'Soft Caress' (MHCRS 2008-267) and *B. thunbergii* var. *atropurpurea* 'Concorde' confirmed them to both be diploid, providing an additional confirmation and calibration of ploidy level with genome size. Flow cytometry was subsequently found to be an effective method for determining genome sizes and ploidy levels of *Mahonia* and *Berberis* (Table 1). The base, $1C_X$, genome size for *Mahonia* varied for each subgenus with a mean of 1.17 pg for *Occidentales* and 1.27 pg for *Orientales* (Table 2). There was no difference in $1C_X$ genome size between the two subgenera of *Berberis*, but plants in the genus *Berberis* had a significantly higher mean (1.45 pg) than either subgenus of *Mahonia*. These data indicate that a significantly greater (~18%) expansion in genome size occurred during the evolution of *Berberis*, compared to *Mahonia*. The substantial difference in genome size between *Mahonia* and *Berberis* could compromise fertility of intergeneric hybrids due to chromosomal sterility and may explain why these hybrids are typically sterile. Of additional interest are the genome sizes for species in *Mahonia* section *Horridae* (*Occidentales*). Although ITS phylogeny (Kim et al., 2004)

indicated plants in this section were more closely allied with *Berberis* than to *Mahonia*, the mean $1C_X$ value for *M. haematocarpa*, *M. nevinii*, and *M. trifoliolata* (all in section *Horridae*) was 1.19 pg - significantly lower ($P<0.05$) than either subgenus of *Berberis* or *Mahonia* subgenus *Orientales*, but consistent with *Mahonia* subgenus *Occidentales*.

Within *Berberis* subgenus *Septentrionales*, diploid 2C genome sizes ranged from 2.48 pg for *B. wilsonii* var. *stapfiana* (C.K. Schneid.) C.K. Schneid. (JCRA, accession #E41) to 3.36 pg for *B. sieboldii* (MHCRS 2005-179) with a mean of 2.94 pg for the subgenus. Only four taxa were available for sampling from *Berberis* subgenus *Australes* and ranged in 2C genome size from 2.77 pg for *B.* ×*stenophylla* Hort. (MHCRS 2000-210) to 3.02 pg for *Berberis trigona* Kunze ex Poepp. & Endl. 'Orange King' (MHCRS 2003-053), with a mean of 2.90 pg for the subgenus – well within the range found for *Berberis* subgenus *Septentrionales*. No natural polyploids were identified among the *Berberis* sampled in this study. However, artificially-induced autopolyploid seedlings from both *Berberis thunbergii* var. *atropurpurea* and *Berberis thunbergii* 'Rose Glow' yielded mean 2C genome sizes of 5.93 pg, effectively confirming them as tetraploids. While the previous report on *Berberis* genome sizes by Bottini et al. (2000) was exclusive to the *Australes*, this subgenus was only modestly sampled in our study, and therefore there was very little overlap between the studies. Our 2C genome size of 2.92 pg for *B. darwinii* Hook. was congruent with the range of 2.88 to 3.22 pg reported by Bottini et al. (2000) in wild populations of the species. Additionally, *Berberis trigona* (syn. *Berberis linearifolia* Phil.) cultivar 'Orange King', had a

genome size slightly less (3.02 pg) than the reported range of 3.24 to 3.57 pg (Bottini et al., 2000).

Genome sizes of diploid *Mahonia* subgenus *Occidentales* ranged from 1.71 pg (*M.* sp., Mexican origin; MHCRS 2009-010) to 2.66 pg (*M. pallida* (Benth.) Fedde PDN 40338), with a mean of 2.35 pg. Subgenus *Orientales* had a higher mean of 2.55 pg for diploids, ranging from 2.28 pg (*M.* sp.; OJ 04052) to 2.76 pg (*M.* sp.; PDN CPC 6.5.01.1). We have not found other published reports of 2C genome sizes for *Mahonia*. No tetraploid *Mahonia* were identified among the taxa sampled for this project. However, prior reports (Taylor and Taylor, 1997) have documented tetraploid clones of *M. aquifolium* and *M. nervosa*, indicating these species may include a polyploid series. The only polyploid *Mahonia* found in this survey was *M. nervosa* (MHCRS, 2008-062) which was estimated to be hexaploid ($2n=6x=84$), with a genome size of 7.45 pg. Interestingly, *M. nervosa* is the only new world species placed within subgenus *Orientales*, based on both morphology (Ahrendt, 1961) and DNA phylogeny (Kim et al., 2004).

Overall, results herein demonstrate that PI flow cytometry is an extremely useful tool for studying genome sizes and polyploidy in both *Berberis* and *Mahonia.* Substantial differences in base $1C_X$ genome size between *Berberis* and *Mahonia* demonstrate considerable variation in genome evolution between these groups. However, genome sizes were strongly conserved *within Berberis* and *Mahonia* subgenera which allowed for rapid and consistent calibration with ploidy levels. Although polyploidy appears to be uncommon among species of both *Berberis* and *Mahonia*, one accession of *M. nervosa* was found to be

hexaploid. Furthermore, artificially-induced plants of *B. thunbergii* were confirmed to be tetraploids. Data from this study provides insight into evolutionary history, taxonomic treatment, and information on ploidy levels of specific taxa that will aid in breeding and development of new hybrids and serve as a valuable database for plant breeders, systematists, and evolutionary biologists.

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Table 1. Mean 2C genome sizes and ploidy levels of *Berberis* and *Mahonia* species, hybrids, and cultivars.

Subgenus	Taxa[Z]	2C genome size (pg)[Y]	Ploidy level (*x*)
Australes	*Berberis darwinii* Hook. (MHCRS 2007-018)	2.92±0.01	2
Australes	*Berberis* ×*lologensis* Sandwith 'Apricot Queen' (PDN 31368)	2.87±0.03	2
Australes	*Berberis* ×*stenophylla* Hort. (MHCRS 2000-210)	2.77±0.03	2
Australes	*Berberis trigona* Kunze ex Poepp. & Endl. 'Orange King' (MHCRS 2003-053)	3.02±0.06	2
Septentrionales	*Berberis aggregata* C.K. Schneid. (JCRA 001745)	2.62±0.01	2
Septentrionales	*Berberis aristata* Sims (JCRA 001752)	2.83±0.10	2
Septentrionales	*Berberis calliantha* Mulligan (MHCRS 2003-066)	2.63±0.06	2
Septentrionales	*Berberis calliantha* Mulligan (PDN 21861)	2.85±0.04	2
Septentrionales	*Berberis* ×*carminea* Ahrendt 'Pirate King' (MHCRS 2004-033)	2.62±0.03	2
Septentrionales	*Berberis dasystachya* Maxim. (MHCRS 2004-034)	2.91±0.01	2
Septentrionales	*Berberis fendleri* A. Gray (MHCRS 2006-222)	3.07±0.02	2
Septentrionales	*Berberis francisci-ferdinandii* C.K. Schneid. (PDN 31140)	2.91±0.01	2
Septentrionales	*Berberis* ×*frikartii* C.K.Schneid. ex H.J.van deLaar 'Telstar' (MHCRS 2000-155)	3.01±0.04	2
Septentrionales	*Berberis gilgiana* Fedde (JCRA 001680)	2.74±0.08	2
Septentrionales	*Berberis* ×*gladwynensis* E. Anders. 'William Penn' (MHCRS 2000-178)	3.04±0.01	2
Septentrionales	*Berberis henryana* C.K. Schneid (MHCRS 2005-196)	2.87±0.03	2
Septentrionales	*Berberis* ×*interposita* Ahrendt 'Wallich's Purple' (MHCRS 2000-160)	2.66±0.01	2
Septentrionales	*Berberis jamesiana* Forrest & W.W. Sm. (MHCRS 2005-198)	2.97±0.03	2
Septentrionales	*Berberis jamesiana* Forrest & W.W. Sm. (PDN 26519)	3.00±0.04	2
Septentrionales	*Berberis koreana* Palib. (JCRA 971069)	3.03±0.01	2
Septentrionales	*Berberis koreana* Palib. 'Red Tears' (PDN 14990)	3.02±0.02	2
Septentrionales	*Berberis koreana* Palib. 'Red Tears' (MHCRS 200-133)	2.97±0.00	2
Septentrionales	*Berberis lempergiana* Ahrendt (MHCRS 2005-199)	3.06±0.01	2
Septentrionales	*Berberis lycium* Royle (MHCRS 2005-197)	2.89±0.07	2
Septentrionales	*Berberis* ×*media* Groot. ex Boom 'Red Jewel' (MHCRS 2002-162)	3.01±0.10	2
Septentrionales	*Berberis* ×*ottawensis* C.K. Schneid. 'Superba' (MHCRS 2003-063)	3.01±0.01	2
Septentrionales	*Berberis prattii* C.K. Schneid. (MHCRS 2007-207)	3.06±0.04	2
Septentrionales	*Berberis replicata* W.W. Sm. (MHCRS 2007-204)	3.03±0.08	2
Septentrionales	*Berberis sieboldii* Miq. (MHCRS 2005-179)	3.36±0.02	2
Septentrionales	*Berberis soulieana* C.K. Schneid. 'Claret Cascade' (MHCRS 2005-283)	2.93±0.00	2
Septentrionales	*Berberis* sp. (MHCRS China collection, purple flowers)	2.96±0.05	2

Table 1 (continued).

Septentrionales	*Berberis temolaica* Ahrendt (MHCRS 2007-199)	2.59±0.04	2
Septentrionales	*Berberis thunbergii* DC. (MHCRS h2002-030-008)[W]	5.86±0.04	4
Septentrionales	*Berberis thunbergii* DC. (MHCRS h2002-030-009)[W]	5.82±0.06	4
Septentrionales	*Berberis thunbergii* DC. (MHCRS h2002-030-021)[W]	6.05±0.04	4
Septentrionales	*Berberis thunbergii* DC. (MHCRS h2002-030-024)[W]	5.82±0.02	4
Septentrionales	*Berberis thunbergii* DC. (MHCRS h2002-030-025)[W]	6.03±0.02	4
Septentrionales	*Berberis thunbergii* DC. 'Aurea' (MHCRS 2002-168)	3.04±0.04	2
Septentrionales	*Berberis thunbergii* DC. 'Bogozam' (MHCRS 2006-168)	3.21±0.05	2
Septentrionales	*Berberis thunbergii* DC. 'Rose Glow' (MHCRS 2004-105)	3.02±0.01	2
Septentrionales	*Berberis thunbergii* var. *atropurpurea* Chenault (MHCRS h2002-029-006)[W]	6.15±0.06	4
Septentrionales	*Berberis thunbergii* var. *atropurpurea* Chenault (MHCRS h2002-029-013)[W]	5.79±0.03	4
Septentrionales	*Berberis thunbergii* var. *atropurpurea* Chenault (MHCRS h2002-029-022)[W]	6.00±0.06	4
Septentrionales	*Berberis thunbergii* var. *atropurpurea* Chenault (MHCRS h2002-029-024)[W]	5.96±0.11	4
Septentrionales	*Berberis thunbergii* var. *atropurpurea* Chenault (MHCRS h2002-029-028)[W]	5.80±0.01	4
Septentrionales	*Berberis thunbergii* var. *atropurpurea* Chenault 'Concorde' (MHCRS 2000-138)	2.93±0.08	2*
Septentrionales	*Berberis thunbergii* var. *atropurpurea* Chenault 'Helmond Pillar' (MHCRS 2000-139)	3.09±0.03	2
Septentrionales	*Berberis thunbergii* var. *atropurpurea* Chenault 'Royal Cloak' (PDN 5070)	3.10±0.02	2
Septentrionales	*Berberis thunbergii* var. *atropurpurea* Chenault 'Royal Cloak' (MHCRS 2005-076)	3.10±0.09	2
Septentrionales	*Berberis vernae* C.K. Schneid. (MHCRS 2003-088)	2.91±0.03	2
Septentrionales	*Berberis wilsoniae* Hemsl. (PDN 96CSC134)	2.68±0.07	2
Septentrionales	*Berberis wilsonii* var. *stapfiana* (C.K. Schneid.) C.K. Schneid. (JCRA bed E41)	2.48±0.06	2
Occidentales	*Mahonia aquifolium* (Pursh) Nutt. (PDN 32862 blue leaves)	2.40±0.02	2
Occidentales	*Mahonia aquifolium* (Pursh) Nutt. 'Apollo' (MHCRS 2008-315)	2.28±0.03	2
Occidentales	*Mahonia aquifolium* (Pursh) Nutt. 'Apollo' (MHCRS 2009-006)	2.28±0.02	2
Occidentales	*Mahonia aquifolium* (Pursh) Nutt. 'Compacta' (MHCRS 2007-021)	2.31±0.01	2
Occidentales	*Mahonia aquifolium* (Pursh) Nutt. 'Orange Flame' (MHCRS 2008-163)	2.26±0.02	2
Occidentales	*Mahonia gracilis* Benth. (Fedde) (MHCRS 2005-184)	2.42±0.00	2
Occidentales	*Mahonia gracilis* Benth. (Fedde) (MHCRS 2006-105)	2.36±0.00	2
Occidentales	*Mahonia haematocarpa* (Wooton) Fedde (JCRA 090501)	2.32±0.01	2
Occidentales	*Mahonia hartwegii* (Benth.) Fedde (MHCRS 2005-201)	2.41±0.01	2
Occidentales	*Mahonia ilicina* Schltdl. (MHCRS 2005-195)	2.57±0.03	2
Occidentales	*Mahonia lanceolata* (Benth.) Fedde (MHCRS 2005-207)	2.45±0.06	2
Occidentales	*Mahonia lanceolata* (Benth.) Fedde 'El Cielo' (MHCRS 2005-208)	2.53±0.01	2
Occidentales	*Mahonia nevinii* (A. Gray) Fedde (MHCRS 2006-137)	2.47±0.02	2
Occidentales	*Mahonia pallida* (Benth.) Fedde (MHCRS 2005-252)	1.90±0.04	2
Occidentales	*Mahonia pallida* (Benth.) Fedde (PDN 28953)	2.21±0.10	2

Table 1 (continued).

Occidentales	*Mahonia pallida* (Benth.) Fedde (PDN 40338)	2.66±0.01	2
Occidentales	*Mahonia paniculata* Oerst. (MHCRS 2006-144)	2.60±0.01	2
Occidentales	*Mahonia pumila* (Greene) Fedde (MHCRS 2006-138)	2.32±0.02	2
Occidentales	*Mahonia repens* (Lindl.) G. Don (MHCRS 2008-063)	2.31±0.01	2
Occidentales	*Mahonia repens* (Lindl.) G. Don (MHCRS 2009-007)	2.29±0.02	2
Occidentales	*Mahonia* sp. (MHCRS 2009-010)	1.71±0.01	2
Occidentales	*Mahonia* sp. (MHCRS 2009-011)	2.46±0.05	2
Occidentales	*Mahonia trifoliolata* (Moric.) Fedde (JCRA 970613)	2.33±0.01	2
Occidentales	*Mahonia* ×*wagneri* (Jouin) Rehder 'King's Ransom' (MHCRS 1996-169)	2.31±0.03	2
Orientales	*Mahonia bealei* (Fortune) Carrière (BA 2005-0391a)	2.49±0.03	2
Orientales	*Mahonia bealei* (Fortune) Carrière (JCRA bed E19d)	2.50±0.05	2
Orientales	*Mahonia bodinieri* Gagnep. (OJ Yamaguchi/Ogisu collection)	2.75±0.06	2
Orientales	*Mahonia duclouxiana* Gagnep. (MHCRS 2009-130)	2.52±0.04	2
Orientales	*Mahonia duclouxiana* Gagnep. (PDN 03856)	2.58±0.04	2
Orientales	*Mahonia eurybracteata* Fedde Heronswood form (PDN 31413)	2.49±0.03	2
Orientales	*Mahonia eurybracteata* Fedde (MHCRS 2005-180)	2.43±0.03	2
Orientales	*Mahonia eurybracteata* Fedde (MHCRS 2005-193)	2.46±0.00	2
Orientales	*Mahonia eurybracteata* Fedde 'Nari Hira' (MHCRS 2005-182)	2.54±0.01	2
Orientales	*Mahonia eurybracteata* Fedde 'Soft Caress' (MHCRS 2008-267)	2.43±0.01	2*
Orientales	*Mahonia* cf. *fargesii* (PDN CPC 3.5.01.3A)	2.49±0.04	2
Orientales	*Mahonia* cf. *fargesii* (PDN CPC 5.5.01.1B)	2.53±0.01	2
Orientales	*Mahonia* cf. *fargesii* (OJ 04052)	2.34±0.02	2
Orientales	*Mahonia fortunei* (Lindl.) Fedde (BA 2005-0392a)	2.52±0.01	2
Orientales	*Mahonia fortunei* (Lindl.) Fedde 'Curlyque' (MHCRS 2006-143)	2.57±0.00	2
Orientales	*Mahonia fortunei* (Lindl.) Fedde 'Dan Hinkley' (PDN 39659)	2.62±0.01	2
Orientales	*Mahonia gracilipes* (Oliv.) Fedde (PDN DJHC-755)	2.66±0.01	2
Orientales	*Mahonia gracilipes* (Oliv.) Fedde (MHCRS 2005-181)	2.63±0.00	2
Orientales	*Mahonia gracilipes* (Oliv.) Fedde (MHCRS 2006-142)	2.67±0.05	2
Orientales	*Mahonia gracilipes* (Oliv.) Fedde (MHCRS 2008-005)	2.57±0.04	2
Orientales	*Mahonia gracilipes* (Oliv.) Fedde (OJ 94008)	2.67±0.00	2
Orientales	*Mahonia gracilipes* (Oliv.) Fedde (OJ 94058)	2.59±0.07	2
Orientales	*Mahonia japonica* (Thunb.) DC. (OJ 97001)	2.55±0.03	2
Orientales	*Mahonia leptodonta* Gagnep. (PDN 34396)	2.68±0.02	2
Orientales	*Mahonia leptodonta* Gagnep. (OJ Yamaguchi/Ogisu collection)	2.50±0.01	2
Orientales	*Mahonia* ×*lindsayae* Yeo 'Cantab' (MHCRS 2005-189)	2.54±0.00	2
Orientales	*Mahonia longibracteata* Takeda (PDN 26555)	2.74±0.02	2

Table 1 (continued).

Orientales	*Mahonia longibracteata* Takeda (OJ Yamaguchi/Ogisu collection)	2.38±0.02	2
Orientales	*Mahonia* ×*media* C.D.Brickell 'Lionel Fortescue' (MHCRS 2005-190)	2.52±0.01	2
Orientales	*Mahonia napaulensis* DC. (MHCRS 2006-139)	2.52±0.01	2
Orientales	*Mahonia napaulensis* DC. (MHCRS 2008-300)	2.61±0.02	2
Orientales	*Mahonia napaulensis* DC. Grayswood Hybrid (MHCRS 2005-203)	2.52±0.01	2
Orientales	*Mahonia napaulensis* DC. 'Maharajah' (MHCRS)	2.53±0.02	2
Orientales	*Mahonia nervosa* (Pursh) Nutt. (MHCRS 2008-062)	7.45±0.04	6
Orientales	*Mahonia* cf. *nitens* (MHCRS 2005-205)	2.59±0.01	2
Orientales	*Mahonia nitens* C.K. Schneid. (JCRA 041803)	2.69±0.01	2
Orientales	*Mahonia nitens* C.K. Schneid. (MHCRS 2005-204)	2.59±0.02	2
Orientales	*Mahonia nitens* C.K. Schneid. (OJ 94010)	2.55±0.00	2
Orientales	*Mahonia nitens* C.K. Schneid. (OJ 94044)	2.57±0.06	2
Orientales	*Mahonia nitens* × *eurybracteata* (PDN#12191)	2.57±0.04	2
Orientales	*Mahonia oiwakensis* Hayata (JCRA MWT-112)	2.46±0.04	2
Orientales	*Mahonia oiwakensis* Hayata (PDN 33792)	2.55±0.01	2
Orientales	*Mahonia* cf. *pallida* (MHCRS 2005-191)	2.56±0.02	2
Orientales	*Mahonia polyodonta* Fedde (MHCRS 2005-200)	2.52±0.02	2
Orientales	*Mahonia polyodonta* Fedde (PDN OJ04CH123)	2.53±0.01	2
Orientales	*Mahonia* ×*savilliana* (PDN 29590)	2.51±0.01	2
Orientales	*Mahonia* sp. (PDN CPC 6.5.01.1)	2.76±0.00	2
Orientales	*Mahonia* sp. (OJ 04052)	2.28±0.09	2

[Z]Taxa (source and accession). BA = Bartlett Arboretum, Charlotte, NC; JCRA = JC Raulston Arboretum, Raleigh, NC; MHCRS = Mountain Horticultural Crops Research Station, Mills River, NC; OJ = Mr. Ozzie Johnson, Atlanta, GA; PDN = Plant Delights Nursery, Juniper Level Botanical Garden, Raleigh, NC

[Y]Values are means ± SEM.

[W]Taxa represent open pollinated seedlings of *B. thunbergii*, that were treated with oryzalin to induce polyploidy.

[*]Chromosome number and ploidy level was confirmed via cytology.

Table 2. Mean $1C_X$ genome size among the 4 subgenera of *Berberis* and *Mahonia*

Genus	Subgenus	$1C_X$ genome size (pg)	Taxa sampled (#)
Berberis	*Australes*	1.45 ± 0.03 C[Z]	4
	Septentrionales	1.47 ± 0.02 C	48
Mahonia	*Occidentales*	1.17 ± 0.02 A	24
	Orientales	1.27 ± 0.01 B	48

[Z]Values are means ± SEM. Values followed by different letters, within a column, are significantly different, $P \leq 0.05$.

Chapter 2

Micropropagation of *Mahonia* ‘Soft Caress’

(In the format appropriate for submission to HortScience)

Micropropagation of *Mahonia* 'Soft Caress'

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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 the trade names in this publication does not imply endorsement by the NCARS of products named, not criticism of similar ones not mentioned. Technical assistance of Jeremy Smith is gratefully acknowledged. The research is from a MS thesis by the senior author.

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Subject Category: Propagation and Tissue Culture

Micropropagation of *Mahonia* 'Soft Caress'

Additional index words. *Berberis*, benzylaminopurine, indole-3-butyric acid, kinetin, plant growth regulators, propagation, tissue culture

Abstract. Mahonia 'Soft Caress' is a unique new cultivar exhibiting a compact form and delicate evergreen leaves. Protocols for micropropagation of *M.* 'Soft Caress' were developed to expedite multiplication and serve as a foundation for future work with other taxa of *Mahonia* Nutt. Combinations of sucrose at 30 or 45 $g \cdot L^{-1}$ in conjunction with Gamborg B5 (B5), Quoirin and Lepoivre (QL), and Murashige and Skoog (MS) basal media, as well as other selected growth regulator treatments, were evaluated as multiplication media. Rooting of microcuttings was conducted in vitro using combinations of indole-3-butyric acid (IBA) at 0, 2, 4, 8, or 16 µM under either light or dark. Quick dip treatments with aqueous solutions of the potassium (K) salt (K-salt) of IBA at 0, 5.2, 10.4, 20.7, or 41.4 µM were tested in a second experiment for ex vitro rooting. Media containing B5 basal salts and vitamins supplemented with sucrose at 30 $g \cdot L^{-1}$, 5 µM 6-benzylaminopurine, 5 µM kinetin, 0.5 µM indole-3-acetic acid, and 2.5 µM gibberellic acid yielded 2.80 ± 0.14 microshoots with a mean length of 14.76 ± 0.63 mm over a 6-week culture period and was an optimal multiplication media. Light treatment and IBA concentration had a significant effect on

rooting percentages and microcuttings treated with 8 µM IBA and maintained in the dark resulted in the best rooting (70%) and ex vitro establishment.

Introduction

Many years ago Hibberd (1862) noted "the time is fast coming when gardens of any pretensions to beauty will be judged by their collections of *Berberis*, for there is not any other class of evergreen shrub which affords so many points for interesting observation". Now 150 years later, the genus *Mahonia* Nutt. (syn. *Berberis* L; for review see: Rounsaville and Ranney, 2010) is finally making a transition from the gardens of collectors into mainstream horticulture. *Mahonia* have tremendous ornamental potential, owing to their durability, showy displays of brilliant-colored flowers and evergreen foliage. Nevertheless, few selections of the genus have been introduced commercially. *Mahonia* ×*media* cultivars dominate retail sales, though these selections are less than desirable to many consumers due to their spindly growth and spine-covered leaflets.

Mahonia 'Soft Caress' (PP20183) is a new cultivar which has shown great promise as a landscape plant. This cultivar arose as a seedling from an open pollinated *M. eurybracteata* Fedde (Ozzie Johnson, personal communication). While *M. eurybracteata* is notable for its relative compactness and narrow evergreen foliage, 'Soft Caress' grows considerably denser and was aptly named for its delicate linear leaflets. Hardy to USDA zone 7, 'Soft Caress' is adorned with terminal yellow racemes during autumn and early winter.

While *Mahonia* sp. can be successfully propagated by stem cuttings, plants tend to have few lateral branches and thus a limited number of shoots available for propagation. Development of in vitro propagation methods would provide a desirable option for more rapid multiplication. In addition to propagation, establishing tissue culture protocols provides an ideal platform for manipulating ploidy level, harvesting chemical compounds, and initiating embryogenesis (Alvarez et al., 2009; Herbert et al., 2010; Pierik, 1997).

Studies on micropropagation of ornamental varieties of *Berberis* and *Mahonia* have been limited to *Berberis thunbergii* DC. 'Crimson Pygmy' (Uno and Preece, 1987); *M. aquifolium* 'Apollo' and 'Undulata' and *M.* ×*media* 'Winter Sun' (Daguin et al., 1992b); and *M. trifoliata* (Mackay et al., 1996). These studies have demonstrated that protocols necessary for shoot growth and development can vary considerably among genotypes and different basal salts and plant growth regulator combinations may be necessary. Woody Plant Medium (WPM) (Lloyd and McCown, 1980) and Murashige and Skoog media (MS) (Murashige and Skoog, 1962) were utilized for *Berberis* and *Mahonia* cultures by Uno and Preece (1987) and Daguin et al. (1992b), respectively. Additionally, Mackay et al. (1996) used a combination of WPM salts and MS vitamins for *M. trifoliata*.

In most micropropagation studies, 6-benzylaminopurine (BAP) has been an effective cytokinin for shoot growth for *Mahonia* and *Berberis* species when provided at 5 to 10 µM (Daguin et al., 1992b; Mackay et al., 1996; Uno and Preece 1987). Mackay et al. (1996) found BAP alone was sufficient for shoot proliferation of *M. trifoliata*; however, for several other *Mahonia* sp., low concentrations of kinetin (Kin) and the auxin, indole-3-acetic acid

(IAA), were necessary to stimulate shoot initiation and multiplication (Daguin et al., 1992b). Daguin et al. (1992b) also found it necessary to use higher concentrations of sucrose (45 vs. 30 $g \cdot L^{-1}$), which are sometimes beneficial for younger tissues (Pierik, 1997). Furthermore, Daguin et al. (1992b) incorporated activated charcoal, which is often used to bind organic compounds secreted by plants in vitro, despite the undesirable binding of crucial growth regulators, particularly BAP (Thomas, 2008).

The ability of gibberellic acid (GA_3) to break dormancy, induce bud growth, and promote internodal elongation (Pierik, 1997) make it a valuable compound for in vitro culture of small bud explants. Daguin et al. (1992b) and Uno and Preece (1987) used GA_3 at 0.6 or 10 µM for shoot multiplication of *Mahonia* and *Berberis* cultures, respectively. GA_3 is also known to promote ethylene-induced necrosis and leaf abscission (Morgan, 1976), which can be a particular problem for cultures in sealed vessels. However, use of ethylene inhibitors such as cobalt(II) chloride hexahydrate ($CoCl_2$) or silver nitrate ($AgNO_3$) can be effective for mediating the negative impacts of GA_3 promoted ethylene (Ma et al., 1998; Misra and Chakrabarty, 2009).

Rooting microcuttings of *Berberis* and *Mahonia* has been successfully achieved both in vitro and ex vitro using a variety of auxins. Mackay et al. (1996) achieved nearly 100% rooting of *M. trifoliata* in vitro using 1.0 µM naphthaleneacetic acid (NAA) on cultures younger than 6 months. However, as culture age increased, microshoots required a cytokinin free subculture followed by increased levels of auxin, which ultimately yielded lower (68%) rooting percentages. Conversely, Daguin et al (1992b) transferred microcuttings of *Mahonia*

directly to glasshouse flats following treatment of the microcuttings with a low concentration of indole-3-butyric acid (IBA), and achieved 80% rooting within 6 weeks. Karhu and Hakala (1990) treated microcuttings of *Berberis thunbergii* for 7 days in the dark, using liquid media with low levels (2 to 6 µM) of either IAA or IBA (Karhu and Hakala, 1990). Interestingly, a subsequent transfer of the microcuttings to an auxin-free media gave significantly better rooting (85%) than those transferred to media with low auxin levels (35%) (Karhu and Hakala, 1990).

With development and introduction of new selections of *Mahonia*, it would be advantageous to have micropropagation protocols to facilitate rapid production and commercialization of these plants. Therefore, the objective of this research was to develop a rapid micropropagation protocol for *Mahonia* 'Soft Caress'.

Materials and Methods

Initiation. During late summer, actively elongating shoots, ≈ 9 cm long, were collected at 0900 HR from containerized plants maintained in a glasshouse. Leaves were removed without damaging the petiole base and shoots were rinsed under tap water for 4 h. Shoots were surface sterilized in a 20% (v/v) Ultra Clorox (6.15% NaOCl) solution and two to three drops of Tween 20, periodically agitated for 15 min, and subjected to three rinses in sterile distilled water for 5 min each. Explants consisted of axillary buds excised from the base of leaf petioles.

Excised buds were placed in culture tubes containing 10 mL of initiation media. Based on preliminary trials (data not presented), the initiation media consisted of Gamborg's B5 basal salts and vitamins (Gamborg et al., 1968) supplemented with sucrose at 45 $g \cdot L^{-1}$, 5 µM BAP, 5 µM Kin, 0.5 µM IAA, 2.5 µM GA_3, 25 µM $CoCl_2$, myoinositol at 0.1 $g \cdot L^{-1}$ MES monohydrate at 0.1 $g \cdot L^{-1}$, solidified with agar at 7.5 $g \cdot L^{-1}$, and pH adjusted to 5.75 ± 0.03. Filter sterilized GA_3 was added to cooled autoclaved media. Excised buds were placed on media and incubated in the dark at 23 ± 2 °C for 2 weeks before being placed under standard culture conditions [23 ± 2 °C and a 16 h photoperiod of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (400-700 nm) provided by cool-white fluorescent lamps].

Shoot proliferation. In the first shoot proliferation experiment, the influence of basal media and sucrose concentration on shoot growth were investigated. Treatments included a factorial combination of three media types, B5 salts and vitamins, QL salts (Quoirin and Lepoivre, 1977) with MS vitamins, and MS salts and vitamins, each in combination with sucrose at 30 or 45 $g \cdot L^{-1}$, and the same growth regulators as listed for initiation (Table 1). Media were prepared in 180 mL glass jars. Five shoots, ≈ 10-15 mm long, were placed in each jar and incubated under standard culture conditions. Following 6 weeks of culture, explants were scored for shoot number and shoot length (longest shoot). The experiment was then repeated by subculturing single shoots (10-15 mm long) on fresh identical media to minimize any residual effects from initiation media. Each treatment consisted of six replications (jars) containing five subsamples (explants) each, for a total of 30 explants per treatment. All treatments were arranged in a completely randomized design. Data were

subjected to analysis of variance (ANOVA), and mean separation using Fischer's LSD with SAS Version 9.1 (SAS Inst. Inc., Cary, NC).

To further improve multiplication rates and shoot growth, a second shoot proliferation study was conducted using B5 basal media with sucrose at 30 $g \cdot L^{-1}$ (one of the best media from experiment 1) with selected media components (see Table 2). Media were prepared as described for experiment 1 and shoots were incubated under the standard culture conditions. Data were collected on shoot number and shoot length (longest shoot) following 6 weeks of culture. Each treatment consisted of six replications arranged in a completely randomized design. Each replicate contained five subsamples each, for a total of 30 explants per treatment. Data were subjected to ANOVA, and mean separation using Fischer's LSD with SAS Version 9.1 (SAS Inst. Inc., Cary, NC).

Rooting. Experiments were conducted to study both in vitro and ex vitro rooting of *M.* 'Soft Caress'. Media used for in vitro rooting consisted of B5 salts and vitamins and sucrose at 30 $g \cdot L^{-1}$, supplemented with 0, 2, 4, 8, or 16 µM IBA. Microshoots $\approx$ 10 mm long were subcultured into 180 mL jars, half of which were subjected to a 'dark' treatment achieved by fully enclosing individual jars in aluminum foil. Each of the 10 treatments consisted of six replications with five subsamples each. All jars were arranged in a complete randomized design under standard culture conditions. Foil was removed 4 weeks after the cultures were placed in the dark. Following an additional 2 weeks of culture, microcuttings were scored for rooting percentage and root length (longest root) (Table 3). Microcuttings (rooted and unrooted) were then transferred directly to ex vitro cell-flats containing a

substrate of 1 peat : 1 vermiculite (by vol.) with 1.19 $kg \cdot m^{-3}$ micronutrients (Micromax®, Scotts Company LLC, Marysville, OH). Microcuttings were arranged in a completely randomized design and grown in a glasshouse under intermittent mist for 6 weeks, and scored for rooting percentage, root number, root length, and shoot length. Data were subjected to regression analyses using SAS Version 9.1 (SAS Inst. Inc., Cary, NC).

An additional experiment was designed to study ex vitro rooting of microcuttings. Microshoots growing on multiplication media were trimmed from the bases to ≈ 10 mm in length and the basal portions dipped for 5 to10 s in 0, 5.2, 10.4, 20.7, or 41.4 µM aqueous solutions of K-IBA. Microcuttings were arranged in a completely randomized design with six replications and five subsamples per replication. Cell trays and rooting substrate were identical to the aforementioned experiment. Microcuttings were set under intermittent mist in a glasshouse, and scored for rooting percentage, root number, root length (longest root), and shoot length (longest shoot) after 6 weeks.

Results and Discussion

Shoot proliferation. For both subculture periods during the first shoot proliferation experiment, there were significant interactions between basal media and sucrose that affected the number of shoots (Table 1). During both subcultures, cultures produced a higher number of shoots on B5 and QL media containing sucrose at 30 $g \cdot L^{-1}$, while cultures on MS media tended to produce more shoots with sucrose at 45 $g \cdot L^{-1}$. These interactive effects on shoot number were especially pronounced during the second subculture period, during which

cultures on B5 with sucrose at 30 $g \cdot L^{-1}$ and QL with sucrose at 30 $g \cdot L^{-1}$ produced a greater number of shoots (2.6 ± 0.3 and 2.4 ± 0.4, respectively) than cultures on MS with sucrose at 30 $g \cdot L^{-1}$ and QL with sucrose at 45 $g \cdot L^{-1}$ (1.6 ± 0.1 and 1.5 ± 0.2, respectively).

Media, sucrose, and a media × sucrose interaction all significantly impacted shoot length during the first subculture period ($P \leq 0.01$, 0.05, and 0.01, respectively). Mean shoot lengths recordered during the first subculture again favored B5 with sucrose at 30 $g \cdot L^{-1}$ (17.9 ± 1.3 mm) and QL with sucrose at 30 $g \cdot L^{-1}$ (22.2 ± 2.1 mm) which were significantly greater than MS at either sucrose concentration. Shoot length following the second subculture was not different among treatments.

Overall, microcutting acclimatization following 12 weeks of culture indicated the rates of shoot multiplication among treatments were consistent and significantly different, while shoot lengths had stabilized and were not significantly different. The B5 basal media with sucrose at 30 $g \cdot L^{-1}$ was selected for future experiments because it resulted in the highest shoot multiplication rates during both subcultures, as well as good shoot elongation following the first subculture. Furthermore, the treatment consisting of the B5 media with sucrose at 30 $g \cdot L^{-1}$ was observed to produce higher quality plantlets with light green expanded leaves compared with other treatments which tended to produced yellow shoots and narrow folded leaflets. In previous studies on *Mahonia*, total N and the ratio of NH_4^+/NO_3^- were suggested as factors that influenced in vitro growth (Daguin et al. 1992a). Total N and NH_4^+/NO_3^- ratios in B5, QL, and MS basal salts used in our study were 27.1 mM, 36.2 mM, and 60.3 mM and 8.2%, 16.1%, and 52.3%, respectively. The high total N

and NH_4^+/NO_3^- ratios in MS basal salts may have inhibited growth of microcuttings of *M.* 'Soft Caress'. Similarly, Daguin et al. (1992a) reported uptake of critical NO_3^- ions was limited when the NH_4^+/NO_3^- ratio was > 50% resulting in poor growth of *Mahonia* sp.

In the second shoot proliferation experiment, shoot multiplication remained high for all media with no significant improvement compared to the media selected in experiment 1 (Table 2). Conversely, shoot elongation was significantly influenced by media composition. The treatment containing 5 μM BAP, 5 μM Kin, 0.5 μM IAA, and 2.5 μM GA_3 yielded the greatest mean shoot length at 14.8 ± 0.6 mm. By contrast, addition of the ethylene inhibitor $CoCl_2$ to the aforementioned treatment significantly reduced shoot length (10.8 ± 0.7 mm). In beberine-producing plants, such as *Mahonia* sp., ethylene has been shown to promote berberine production (Kobayashi et al., 1991, Sato et al. 1990). Inhibition of ethylene led to a decrease in endogenous berberine synthesis and an increase in growth in cell cultures of *Thalictrum minus* L. (meadow rue) (Kobayashi et al. 1991). Surprisingly, in the present investigation, use of $CoCl_2$ caused a decrease in shoot length and an increase in chlorosis, suggesting that in *Mahonia*, ethylene may play a role regulating a desirable level of berberine biosynthesis. Alternatively, the concentration of $CoCl_2$ used may have had a phytotoxic effect, as excess cobalt is reported to inhibit photosynthesis (Palit et al., 1994). Results herein also showed a significant decrease in shoot length in those treatments supplemented with thidiazuron (TDZ) (Table 2). TDZ is often an effective cytokinin for in vitro woody plant culture because of its ability to induce adventitious shoots; however, it also tends to reduce apical dominance thereby leading to poor shoot elongation (Murthy et al., 1998).

Rooting of microcuttings. In vitro root initiation was observed within 3 weeks of culture. Regression analysis indicated that microcuttings subjected to dark treatments had a quadratic response to IBA concentration for both rooting percentage and average root length (Table 3). Treatment of microcuttings with 8 μM IBA/dark yielded the highest rooting percentage (37%) and the longest mean root length (36.5 ± 3.5 mm). Nontreated microcuttings (0 μM IBA) in light or dark as well as 2 or 4 μM IBA under light failed to produce any roots.

Microcutting (rooted and unrooted) on in vitro rooting media were transferred ex vitro. After 6 weeks, root formation was observed in every treatment. Analysis of variance indicated light and IBA treatment and their interaction had a significant effect on rooting percentage ($P < 0.05$) (Fig. 1). Regression analysis showed the percentage of microcuttings forming roots from in vitro dark treatments increased linearly with IBA concentration, while those plantlets from in vitro light treatments had a quadratic response with IBA concentration. All plantlets initiated in the dark gave better rooting percentages than those under lights at corresponding IBA concentrations, while the IBA control yielded identical results under both light regimes. Based on regression analysis, the dark treatment at 16 μM gave the highest estimated rooting percentages with 78% of microcuttings forming roots.

The effect of light on root initiation of *M.* 'Soft Caress' is similar to that of *B. thunbergii*, which benefited from an induction period in darkness during rooting (Karhu and Hakala, 1990). Conversely, *M. trifoliolata* was found to root quite readily in vitro with light (Mackay et al., 1996). While it is known that many woody plant species benefit from

darkness during the early stages of in vitro rooting (Rugini et al., 1993), the complexities of the effects are still not fully understood (Nor Aini et al., 2009). Additionally, auxin is only required during the early stages of root growth and development in vitro, and continued exposure to auxin may actually inhibit further growth (De Klerk et al., 1999). While this was not a factor with *M. trifoliolata* for Mackay et al. (1996), Karhu and Hakala (1990) observed rooting percentages increase from 35% to 85% in *B. thunbergii* after microcuttings were transferred to an auxin-free media after 1 week compared to continual exposure on 4 µM IAA.

By comparison, in vitro rooting percentages in the current investigation were similar to those observed in 21 month old cultures of *M. trifoliolata* (Mackay et al. 1996). A cytokinin-free subculture followed by transfer onto media with high concentrations of IBA (4.9 or 12.3 µM) was sufficient to increase rooting percentages from 2% to 79% and 67%, respectively, in *M. trifoliolata* (Mackay et al. 1996). Mackay et al. (1996) interpreted this as residual BAP accumulation in older cultures, as microcuttings < 6 months old displayed 100% rooting. While overall rooting percentages of *M.* 'Soft Caress' were comparable to that of *M. trifoliata* over the same duration, improved rooting may be achieved by a cytokinin free subculture or reduced exposure to plant growth regulators.

IBA had a significant effect on number of roots produced depending on the light treatment ($P < 0.05$) (Fig. 2). For microcuttings with in vitro dark treatments, the number of roots increased linearly with IBA concentration. There was no significant effect of IBA on number of roots for plantlets in the light treatment. Based on regression analysis, dark

treatment with 16 µM IBA gave the estimated maximum of 1.6 roots. IBA concentration and light did not significantly affect root or shoot lengths.

Regression analysis performed on ex vitro rooting data revealed no significant linear or quadratic trends. The highest concentration of K-IBA (41.4 µM) yielded the highest rooting percentage, at 37 ± 9.2 % (data not presented). By comparison, the highest rooting percentage observed during the first 6 weeks of the in vitro study was also 37% (8 µM IBA dark treatment). Interestingly, root morphology appeared to differ after 6 weeks of growth between experiments. Roots formed in vitro under the influence of IBA (and those formed following transfer ex vitro without additional treatment) were considerably thicker, ≈ 1-2 mm in diameter, and in nearly all cases appeared to arise from the basal callus of the microshoots. By comparison, those treated with the K-IBA dip were finer, ≤ 0.5 mm in diameter, and did not appear to initiate from callus. Proliferation of nonorganized callus can be affected by many variables including BAP, type of explant, and basal media (Pierik, 1997; Swamy et al. 2004). While callus formation on microcuttings is known to promote rooting among some species (Mukherjee et al. 2010), it can drastically decrease rooting capacity in others, including *B. thunbergii* (Karhu and Hakala, 1990; Puddephat et al. 1999; Thakur and Kanwar, 2008). Adventitious root initiation on *M.* 'Soft Caress' does not appear to require callus formation, due to lack of callus on ex vitro plantlets forming roots as well as those in vitro at low IBA concentrations.

In conclusion, a successful micropropagation protocol was developed for *M.* 'Soft Caress'. Shoot culture media provided good multiplication rates, and proved effective for

maintaining cultures over extended periods of time. Continued studies will focus on improving rooting response of microcuttings, specifically to decrease overall rooting time. These protocols provide a basis for rapid propagation of *M.* 'Soft Caress' and may provide a foundation for other species and hybrids within the genus.

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Table 1. Effect of nutrient media and sucrose concentration on shoot proliferation of *M.* 'Soft Caress'. All treatments included 5 µM BAP[z], 5 µM Kin, 0.5 µM IAA, 2.5 µM GA_3, and 25 µM $CoCl_2$. Data were recorded after 6 weeks for each subculture.

	Subculture 1		Subculture 2	
Treatment	Number of shoots	Shoot length (mm)	Number of shoots	Shoot length (mm)
B5 + 30 g•L^{-1}	2.83 ± 0.27 a[y]	17.93 ± 1.26 ab	2.57 ± 0.26 a	12.00 ± 1.06 a
B5 + 45 g•L^{-1}	2.13 ± 0.23 ac	16.40 ± 1.95 bd	1.83 ± 0.32 ab	11.03 ± 1.95 a
QL + 30 g•L^{-1}	2.57 ± 0.17 a	22.17 ± 2.13 a	2.40 ± 0.36 a	12.87 ± 2.13 a
QL + 45 g•L^{-1}	2.07 ± 0.44 ac	14.23 ± 1.04 bc	1.47 ± 0.18 bc	11.20 ± 1.05 a
MS + 30 g•L^{-1}	1.50 ± 0.18 bc	10.27 ± 1.52 c	1.55 ± 0.13 bc	10.70 ± 1.52 a
MS + 45 g•L^{-1}	2.20 ± 0.35 a	12.10 ± 2.09 cd	2.03 ± 0.30 ac	12.60 ± 2.09 a
ANOVA[x]				
Media	NS	**	NS	NS
Sucrose	NS	*	NS	NS
Media x sucrose	*	**	*	NS

[z]Key to media and growth regulator abbreviations: BAP (6-benzylaminopurine), Kin (kinetin), IAA (indole-3-acetic acid), GA_3 (gibberellic acid), $CoCl_2$ (cobalt(II) chloride hexahydrate), B5 (Gamborg's B5 basal salts and vitamins), QL (Quoirin and Lepoivre basal salts and Murashige and Skoog vitamins), MS (Murashige and Skoog basal salts and vitamins).

[y]Mean separation within columns by Fischer's LSD at $P < 0.05$. The mean values represent six replications with five subsamples each.

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

Table 2. Effect of selected growth regulator treatments on microshoot proliferation of *M.* 'Soft Caress'. Data were recordered following 6 weeks on treatment media.

Treatment (µM)	Number of shoots	Shoot length (mm)
5 BAP, 5 Kin, 0.5 IAA, 2.5 GA_3, 25 $CoCl_2$	2.92 ± 0.30a[y]	10.80 ± 0.72 bc
5 BAP, 5 Kin, 0.5 IAA, 2.5 GA_3	2.80 ± 0.14 a	14.76 ± 0.63 a
5 TDZ, 0.5 IAA, 2.5 GA_3	2.76 ± 0.26 a	8.60 ± 0.99 c
5 TDZ, 2.5 GA_3	2.88 ± 0.27 a	8.64 ± 1.00 c
10 Kin	2.28 ± 0.29 a	9.40 ± 0.51 c

[z]Key to media and growth regulator abbreviations: BAP (6-benzylaminopurine), Kin (kinetin), IAA (indole-3-acetic acid), GA_3 (gibberellic acid), $CoCl_2$ (cobalt(II) chloride hexahydrate), TDZ (thidiazuron). All growth regulator concentrations are listed in µM.

[y]Mean separation within columns by Fischer's LSD at $P < 0.05$. Mean values represent six replications with five subsamples each.

Table 3. Effect of IBA concentration and light treatment on in vitro rooting percentage and root length of *M.* 'Soft Caress'. Data were recordered following 6 weeks on treatment media.

Light treatement	IBA[z](µM)	Rooting (%)	Mean root length (mm)
Light	0	0 ± 0.0	-[y]
Light	2	0 ± 0.0	-
Light	4	0 ± 0.0	-
Light	8	10 ± 4.47	11.7±4.6
Light	16	3 ± 3.33	11±0
Dark	0	0 ± 0.0	-
Dark	2	13 ± 6.67	19.3±10.8
Dark	4	13 ± 4.22	10.0±5.0
Dark	8	37 ± 12.02	36.5±3.5
Dark	16	27 ± 9.54	20.4±4.2
ANOVA[x]			
IBA - linear		**	**
IBA - quadratic		*	*
Light x IBA - linear		NS	*
Light x IBA - quadratic		*	*

[z]Indolebutyric acid.

[y]Microcuttings failed to produce roots.

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

The mean values represent six replications with five subsamples each ± SEM

- Shoots failed to produce roots

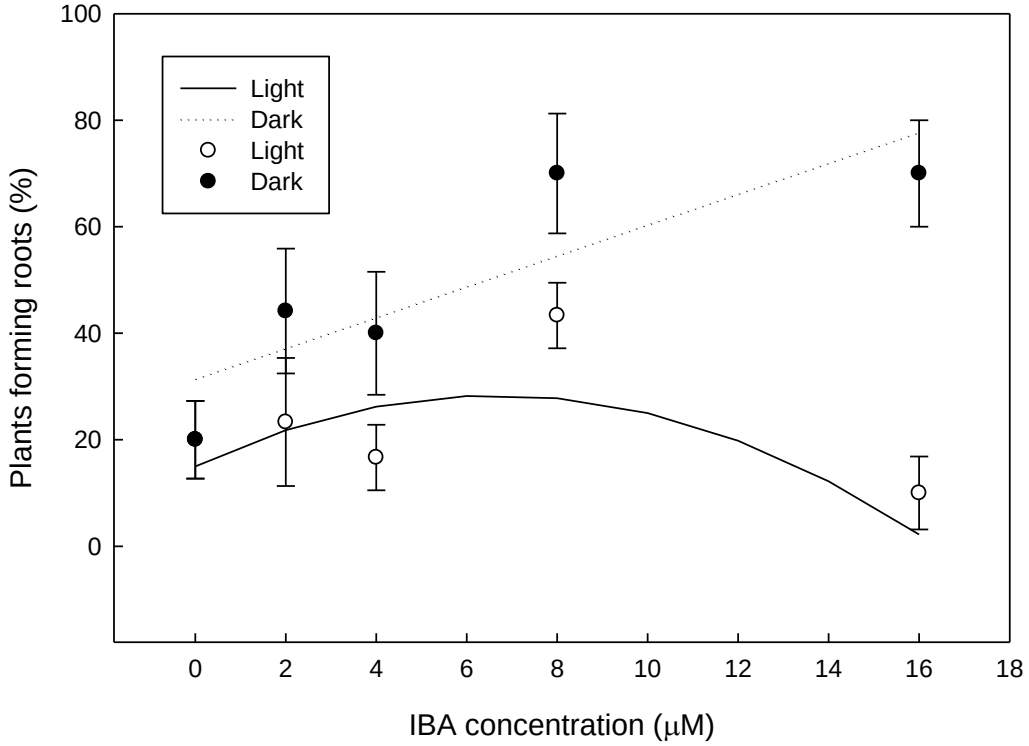

Fig 1. Effect of IBA and light on percentage of microcuttings of *M.* 'Soft Caress' forming roots. Dotted and solid lines represent trends fitted using linear and quadratic regression analyses: Dark (●) = 0.315 + 0.029(IBA), $r^2 = 0.29$, $P < 0.05$; Light (○) = 0.15 + 0.049(IBA) – 0.003(IBA^2), $r^2 = 0.15$, $P < 0.05$. Vertical lines = ± 1 SE.

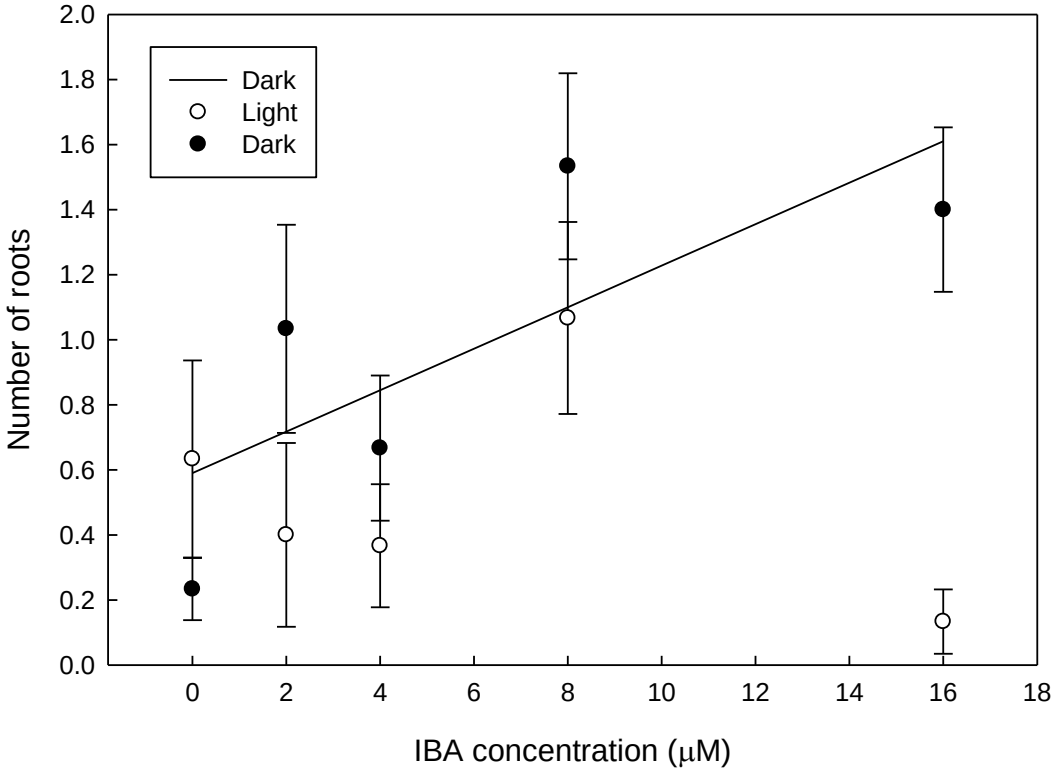

Fig. 2. Effect of IBA and light on number of roots produced on microcuttings of *M.* 'Soft Caress'. Solid line represents a linear trend fitted using linear regression analysis: Dark (●) = 0.59 + 0.064(IBA), $r^2 = 0.25$, $P < 0.05$. Vertical lines = ± 1 SE.

Chapter 3

Fertility and Reproductive Pathways in Diploid and Triploid *Miscanthus sinensis*

(In the format appropriate for submission to HortScience)

Fertility and Reproductive Pathways in Diploid and Triploid *Miscanthus sinensis*

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Received for publication . Accepted for Publication .

This research was funded, in part, by the North Carolina Agricultural Research Service (NCARS), Raleigh, NC, 27695-7643, and the U.S. Department of Agriculture Agricultural Research Service, Floriculture and Nursery Research Initiative, Beltsville, MD. Thanks are expressed to the North Carolina Biotechnology Center, the Biofuels Center of North Carolina, the Kenan Institute, and Hoffman Nursery for their support of this research. Technical assistance of Kelly Gaskill, Irene Palmer, and Jeremy Smith is gratefully acknowledged. This research is from a MS thesis by the senior author.

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Subject Category: Breeding, Cultivars, Rootstocks, and Germplasm Resources

Fertility and Reproductive Pathways in Diploid and Triploid *Miscanthus sinensis*

Additional index words. aneuploidy, invasive, polyploidy, plant breeding, propagule pressure, reproductive biology, seedlessness, exotic plants

*Abstract***.** *Miscanthus sinensis* Andersson is a popular ornamental grass and has additional potential as a bioenergy crop. In some regards, the ability of *M. sinensis* to withstand a broad range of climatic and cultural conditions is desirable, but its propensity to rapidly colonize open and disturbed environments has allowed it to naturalize and become weedy in some regions in the U.S. Considering the value of this crop, the development and documentation of infertile clones would be desirable. Triploid plants were evaluated for male (pollen viability staining) and female (seed set and germination) fertility. Pollen viability staining, seed set, and seed germination from triploid plants was reduced compared to diploids, but varied considerably within each cytotype. Overall, relative female fertility of individual triploids clones {(% seed set × % germination for triploid)/(% seed set × % germination for diploid control)} was reduced substantially and ranged from 49% to 0.7%. Additionally, we examined the reproductive pathways of triploid plants by evaluating the 2C genome sizes of progeny derived from open pollination. The limited progeny arising from open pollinated triploids were predominantly aneuploids with 2C genome sizes intermediate between

diploids and triploids. There was no clear evidence of apomixis, selfing, or triploid × triploid fertilization events observed among triploid parents. Formation of unreduced gametes was rare for both ploidy levels (~1%). The considerable reduction in female fertility in some triploid clones combined with the limited production of primarily aneuploid progeny provides highly infertile alternatives to existing diploid cultivars.

Introduction

The genus *Miscanthus* Andersson contains approximately 20 species of perennial C4 grasses predominately from Asia (Hodkinson et al., 2002; Glowacka and Jezowski, 2009). Genetic diversity is high within Miscanthus and has allowed some species to evolve populations adapted to highly diverse environments (Heaton et al., 2010). Miscanthus have long been recognized for their potential as ornamentals, and M. sinensis (Maiden grass, Silver grass) has been grown in the United States since being introduced in the late nineteenth century (Quinn et al., 2010). *Miscanthus sinenesis* is native to China and Japan, and like many *Miscanthus* is notable for its high water use efficiency and the ability fix nitrogen through a bacterial symbiotic relationship (Eckert et al., 2001; Stewart et al., 2009). Several hundred years of cultivation has given rise to over 100 *M. sinensis* cultivars, featuring a range of sizes, foliage types, and variegations (vertical or horizontal banding) (Darke, 2007). In North America, *M. sinensis* is one of the most planted of all ornamental grasses due to its colorful and dense inflorescences, durability, and a capacity to thrive in USDA hardiness zones 4-9 (Meyer and Tchida, 1999; Wilson and Knox, 2006). The merits

of *M. sinensis* have led to over 50 new selections introduced within the past 25 years (Wilson and Knox, 2006). More recently, *M. sinensis* has shown promise as a potential crop for bioenergy applications, particularly in more northern latitudes were less cold hardy species and hybrids of *Miscanthus* are unreliable (Glowacka and Jezowski, 2009; Heaton et al., 2010).

Miscanthus sinensis is strictly a clump forming grass which spreads naturally over short distances by wind-blown seed (Darke, 2007; Stewart et al., 2009). Naturalization of this species, predominately in open or disturbed environments, can be significant and problematic (Barney and DiTomasa, 2008; Meyer and Tchida, 1999). The first documented naturalization in the United States was reported in Washington DC, New York, and Florida in 1913 (Quinn et al., 2010). Wilson and Knox (2006) noted that *M. sinensis* has since naturalized in at least 25 states, of which many include it on invasive watch lists. Although *M. sinensis* is an early successional, pioneer species that typically colonizes open disturbed sites (Stewart et al., 2009; Tsuyuzaki and Hase, 2005; Ohtsuka et al., 1993), it can be a prolific weed in managed landscapes such as roadsides and pastures (Hirata et al., 2007).

Reproductive biology of *M. sinensis* has been studied in both wild populations and cultivars of diploid plants. Stewart et al. (2009) documented seed set (percentage of flowers setting seed) among wild populations in Japan between 3%-84% depending on plant density as well as genetic and environmental factors. Among cultivated varieties, seed set and germination has been shown to vary as a function of location and genotype (Meyer and Tchida, 1999; Wilson and Knox, 2006). *M. sinensis* cultivars are generally regarded as self-

incompatible, and effective pollination and seed set requires different genotypes in close proximity (< 4 m) with ample pollen load and overlapping bloom times (Chiang et al., 2003; Stewart et al., 2009). *Miscanthus* pollen grains are trinucleate, which is generally indicative of rapid germination on stigmas and short-lived viability (Friedman and Barrett, 2011). There are no published reports on male (pollen) fertility for *Miscanthus* species.

M*iscanthus* have a base chromosome number n = 19 and wild *Miscanthus sinensis* varieties and cultivars have been reported as diploid with $2n = 2x = 38$ (Rayburn et al., 2009). Hybridization between species as well as across ploidy levels has been documented, including *M.* ×*giganteus* ($2n = 3x = 57$), a naturally occurring triploid hybrid between diploid *M. sinensis* and tetraploid *M. sacchariflorus* (Glowacka et al., 2009). Due to the inability of three chromosome sets to divide equally during meiosis, triploid plants often suffer from reduced fertility or complete sterility (Ramsey and Schemske, 1998). For example *M.* ×*giganteus* is widely reported to be completely infertile (Glowacka et al., 2009; Meyer and Tchida, 1999; Rayburn et al., 2009). However, in some cases triploids can have limited fertility resulting from the formation of apomictic embryos, unreduced gametes, and the union of aneuploid gametes (Lim et al., 2003; Lo et al., 2009; Ramsey and Schemske, 1998; Sears, 1953). Determination of 2C genome size of progeny from triploid parents can elucidate the reproductive pathway by which seedlings originated (Doležel and Bartos, 2005).

Development of new triploid *M. sinensis* may produce infertile plants with low reproductive potential. Infertile forms of *M. sinensis* would be valuable alternatives for landscape and bioenergy applications . Researchers at the North Carolina State University, Mountain Crop Improvement Lab™ (MCI Lab), Mills River, NC, previously developed a population of triploid *M. sinensis,* of which, eighteen clones were selected for having desirable ornamental and/or growth characteristics. Unlike the full sterility documented in *M. ×giganteus,* some of these triploid *M. sinensis* were observed to produce both pollen and low numbers of seed. In order to evaluate the reproductive potential of these plants, it is necessary to evaluate and document the fertility among each of the selected clones. The objectives of this study were to evaluate selected triploid clones of *Miscanthus sinensis* for male and female fertility and determine the 2C genome size and reproductive pathways of seedlings derived from these plants.

Materials and Methods

Plant material. The taxa used in this study comprised 5 diploid (2x) and 18 triploid (3x) clones of *Miscanthus sinensis* (Table 1). Diploid plants include 4 commercially available cultivars and one hybrid developed at the MCI Lab. Tetraploid plants were developed by hybridizing *M. sinensis* 'Variegatus' with 'Strictus', followed by in vitro polypoid induction using procedures similar to Herbert et al. (2010). Triploid plants were generated by crossing these autotetraploids with selected diploids, and subsequently

recovered by embryo rescue. Triploid taxa selected for this study were chosen from 700 genotypes following 2 years of field trials.

Experimental design. Divisions of each genotype were field planted in mid-May using a randomized complete block design, consisting of 5 replicated blocks. Each block contained 38 plants, including one of each triploid clone and four (subsamples) of each diploid clone to ensure adequate pollination. Plants were spaced on 46 cm centers in cultivated (mounded) rows with 1.2 m between rows and irrigated as needed throughout the summer using drip irrigation.

Evaluating male fertility. Male fertility was assessed by determining the viability of fresh pollen with acetocarmine staining. Pollen was collected over a 2-week period in late August. Sampling was conducted randomly within blocks, one block at a time. Inflorescences were collected between 0800-1000 HR, placed in a sealed plastic bag, and immediately brought into the lab for assessment. Fresh pollen was dusted onto a microscope slide by gently shaking the inflorescence. Pollen grains were stained by adding one to two drops of 1% acetocarmine to the microscope slide and incubated for 30 min at room temperature (23º C). Pollen grains that were well-stained and well-formed were scored as viable, with ≥ 100 grains scored per replicate. Pollen viability was calculated by number of grains scored as viable / number of grains counted.

Evaluating female fertility. Female fertility was assessed by determining percentage of seed set and percentage of viable seeds based on germination. Whole inflorescences (up to 10 per replicate) were collected in October and stored in large paper bags at room

temperature until dry. For each replicate, spikelets were randomly sampled from different inflorescences. Up to 1,000 individual flowers were examined per replicate (or until 100 seeds were extracted). Seed set was calculated by number of seed counted / number of flowers examined.

Seeds collected during the seed-set experiment were germinated one block at a time. Seeds were de-hulled, placed on blotter paper (SDB3.5, Anchor Paper Co., St. Paul, Minn.), and moistened with distilled H_2O in plastic petri dishes (100 mm × 15 mm). Petri dishes were sealed with parafilm and allowed to germinate at 23 ± 2 °C with a 16 h photoperiod of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (400-700 nm) provided by cool-white fluorescent lamps. Germination was tallied after four weeks using radical emergence as the criterion. Total germination was calculated as seeds germinated / total seeds. Overall female fertility (% viable seed set) was determined by: {[seed set (%) × seed germination (%)] /100}. Additionally, relative female fertility (overall female fertility of each clone in relation to the diploid control with the highest overall female fertility) was determined as: {(% seed set × % germination for given clone) / (% seed set × % germination for diploid control)}.

Determining 2C genome size and ploidy level. Flow cytometric analysis was used to determine the 2C genome size and ploidy level of all parents and offspring. Young leaf tissue (~0.5 cm^2) was finely chopped within 0.4 mL of nuclei extraction buffer along with *Pisum sativum* L. 'Ctirad', an internal standard with a known 2C genome size of 8.75 picograms (pg) (Doležel et al, 1998). Nuclei were filtered using a 50 µm nylon filter and stained using 1.4 mL 4', 6-diamidino-2-phenylindole (DAPI) staining buffer. Genome size

(DNA content of the nonreplicated chromosome complement) was analyzed with a Partec PA-II flow cytometer (Partec, Münster, Germany). Holoploid, 2C genome size was calculated as: 2C = genome size of standard × (mean fluorescence value of sample/ mean fluorescence value of standard).

Evaluating reproductive pathways. From each germination trial for 3 blocks, 6 germinated seedlings were chosen at random and moved to a fresh petri dish. After another 1-2 weeks of growth, 568 seedlings were subject to flow cytometric analysis following the procedures previously described. Data from all studies were subjected to analysis of variance (ANOVA) procedures and mean separated using Fischer's LSD with SAS Version 9.1 (SAS Inst. Inc., Cary, NC).

Results and Discussion

The mean 2C genome size of the 5 diploid genotypes was 4.02 ± 0.03 (SEM) pg, while the mean for the 18 triploid genotypes was 5.75 ± 0.03 (SEM) pg. The genome size of triploids was slightly < 1.5 times that of the diploids, yet intermediate between diploid and tetraploid parents (tetraploids had a mean 2C genome size of 7.52 ± 0.04 (SEM) pg). The decrease in 1Cx genome size (genome size of one complete set of chromosomes) with increasing ploidy level may indicate genomic downsizing, a phenomenon often observed following chromosome doubling (Buggs et al., 2009; Leitch et al., 2008; Soltis et al., 2003).

Male fertility. Pollen staining in triploid plants ranged from 50% to 72% (60% average) compared to 73% to 77% (74% average) for the diploid controls (Table 1).

Although acetocarmine staining is routinely used to estimate pollen viability, this dye indiscriminately stains DNA and cytoplasmic proteins and may stain pollen grains that are not fully functional (MacFarlane Smith et al., 1989; Slomka et al., 2010) and can thereby overestimate pollen viability. Pollen germination is a preferable method for more accurately assessing viability. However, like many grass species, pollen of *Miscanthus* is short lived and efforts to develop reliable germination protocols have been unsuccessful.

Female fertility. Seed set was significantly lower in triploids compared to diploids ($P < 0.0001$). Seed set also differed within diploid cultivars and within triploid cultivars ($P < 0.0001$) (Table 1). Seed set data for diploid plants ranged from 50% for 'Gracillimus' to 84% for 'Variegatus', demonstrating that diploid cultivars can vary substantially in this trait. Among triploid plants seed set varied considerable by clone, ranging from 0.7% for H2008-091-004 to 52% for H2008-061-001. Of the 18 triploids used in this experiment, six had < 10% seed set.

Miscanthus seed began germinating within 3 to 4 days and after 4 weeks no further germination was detected. Similar to seed set, there was a significant overall reduction in germination in triploids compared to diploids ($P < 0.0001$). Further, germination differed among both diploid cultivars and triploid cultivars ($P < 0.0001$) (Table 1). Germination in diploid plants ranged from 45% for 'Gracillimus' to 75% for H2006-026-006. We observed higher germination rates among 'Variegatus' (84%) and 'Zebrinus' (74%) compared with Meyer and Tchida (1999) who reported a maximum of 16% and 60%, respectively.

Germination rates among triploid plants ranged from 16% for H2008-067-021 to 69% for H2008-065-003.

Overall female fertility reflects the percentage of viable seed produced among flowers (Table 1). Overall female fertility ranged from 25% to 55% among diploids, compared to 0.4% to 27% for triploids. The diploid cultivar 'Zebrinus' had the highest overall female fertility, even though it did not have the highest individual seed set or viability. Relative female fertility reflects overall female fertility relative to the most fertile diploid control, 'Zebrinus'. Relative female fertility also showed significant reductions in triploid plants, with eight triploids having < 10% relative fertility and four triploids having < 5% relative fertility. The most significant reduction in fertility was observed with H2008-091-004, which had a relative fertility of 0.7% of the diploid control.

Reproductive pathways. Genome sizes (2C) recorded from 568 progeny of open pollinated diploid and triploid cytotypes provide insight into their reproductive pathways (Fig. 1). The majority (99%) of seedlings derived from open pollinated diploid maternal parents were diploids with a mean 2C genome size of 4.13 ± 0.02 (SEM) pg. This value is slightly higher (~0.11 pg) than the diploid parents measured at different times and under different conditions, and may vary due to subtle variation in tissue chemistry and pigments that can interact with the DAPI fluorochrome (Doležel et al., 1998; Ochatt et al., 2011). Fertilization of diploid maternal parents with reduced pollen from triploids was rare; however, in four instances (1.4%), diploid seedlings displayed 2C genome size between 4.55 - 4.76 pg, suggesting diploid × triploid hybridization. One extreme outlier with 2C genome

size = 6.15 pg (~3x) was observed among the progeny from diploid maternal parents (0.4% of seedlings). This fertilization event was most likely the result of an unreduced (2n) gamete pairing from one diploid parent with a reduced (n) diploid gamete from a second diploid parent.

The majority of progeny from maternal triploids had 2C genome sizes intermediate between triploid and diploid parents with a mean of 4.67 ± 0.01 (SEM) pg (Fig. 1). We found no clear evidence of triploid × triploid hybridization (assuming random chromosome assortment in meiosis) or apomixis among triploids which would result in progeny with 2C genome sizes of approximately 5.8 – 6.3 pg. In some plant species, triploids are able to produce viable gametes that contain an exact haploid (1x) or diploid (2x) chromosome complement (Sears, 1953). If this were occurring we would have expected to observe 3x and 4x (in addition to 2x) progeny which were not evident. Rather, triploid progeny displayed a normal distribution of genome sizes indicative of triploid × diploid hybridization following random chromosome segregation and aneuploid formation (Norrmann and Keeler, 2003; Tel-Zur et al. 2004). In three cases seedlings had 2C genome sizes of 6.39, 6.71, and 7.32 pg; substantially greater than their triploid maternal parents. These three instances (1.1%) most likely represent the formation of unreduced gametes from one of the parents. The frequency of unreduced gametes among all progeny was 1.4%.

Propagule pressure is an appraisal of vegetative and gametic structures differing abilities to advance naturalization of a species (Martínez-Ghersa and Ghersa, 2006). Assessing propagule pressure is an important measure of naturalization potential because

unlike the majority of predictive factors, it has a consistent positive association with the establishment of a self-sustaining population (Colautti et al., 2006). Vegetatively, *Miscanthus sinensis* poses very little risk of spread as a result of its strong clumping habit. Native clumps in Japan had radial expansion rates an average of 2.4 $cm^2 \cdot yr^{-1}$ over a 9-year period, while at the same time shoot density in the center of clumps declined year by year (Stewart et al., 2009). Gametic propagule pressure (seed) poses a far greater risk for naturalization of *Miscanthus* and several factors must be taken into consideration. While our study shows that seed set of diploids can reach 84% with numerous nearby pollen donors, seed production in wild populations has been reported to be < 20% among plants > 4 m apart (Stewart et al., 2009). However, seed set can be prolific, with a mean of 1,051 seeds per plant reported in the wild, and a mean of 657 seeds (per 3 inflorescences) from cultivated plants in the U.S. (Meyer and Tchida, 1999; Stewart et al., 2009).

Decreasing fertility through reductions in pollen viability, seed set, and seed germination, mitigates propagule pressure and the potential for plants to naturalize. In particular, curbing fecundity to a point where more individuals are dying than are being produced will inevitably lead to a decline in population growth (Jaquart and Knight, 2010; Ramula et al., 2008). For example, reducing fecundity and propagule survival by 60% was sufficient to cause population decreases in several invasive annual grasses (Tozer at al., 2008). In a study of scotch broom (*Cytisus scoparius* (L.) Link), Sheppard et al. (2002) estimated that a 62% reduction in seed set was needed to reverse dominance of this species in native grasslands. Similarly, populations of *Carduus nutans* L. were estimated to begin

decreasing in size pending a ~69% decrease in seed production (Shea and Kelly, 1998). Populations of the prolific biennial weed garlic mustard (*Alliaria petiolata* (M. Bieb.) Cavara & Grande) began declining when seed reduction caused by weevils was reduced by 49% (Davis et al., 2006). Overall, triploids examined in this study had between 50%-99% reductions in relative fertility compared to diploid controls. This represents a significant decline in fertility which would reduce the potential for these plants to initially naturalize, particularly for highly infertile clones.

There is legislative precedence for exempting infertile cultivars from sales restriction of weedy species. For example, varieties of *Buddleia davidii* Franch. (Butterfly Bush) documented to produce < 2% viable seed are now considered effectively sterile by the Oregon Department of Agriculture (2011). A stipulation in the aforementioned criteria calls for the presence of abundant pollen donors to ensure legitimate results during testing. Our study included an abundance of diploid pollinators (100 plants) in close proximity to triploids. High levels of seed set among diploids confirmed favorable pollination conditions. In another example based on germination alone, *Rhamnus frangula* L. 'Ron Williams' is now being widely marketed as a non-invasive plant based on a germination rate of 14% that of the invasive parent species, *R. frangula* (Jaquart and Knight, 2010). Favorable pollination and germination conditions in our study provided an effective evaluation of triploid reproductive potential.

In addition to reduced fertility of triploid clones of *M. sinensis*, the limited progeny derived from triploid maternal parents were predominantly aneuploids which can lead to

reduced fitness and fecundity (Ramsey and Schemske, 1998). The production of mostly aneuploidy progeny further supports the use of low fertility triploid *Miscanthus* as landscape plants. Despite triploid pollen being > 50% stainable with acetocarmine, our data suggest that other 2x × 3x and 3x × 3x fertilizations were very rare. Therefore, this study provided optimal conditions for fertilization of triploid by being intermixed with diploid pollinators. Seed production by triploid plants isolated by themselves may be extremely rare. This study concludes that some triploid *Miscanthus* display substantial reductions (> 95%) in fertility compared to diploid controls despite being phenotypically similar. Several of these clones with substantial reductions in fertility may provide desirable, non-invasive substitutes to highly fertile diploids.

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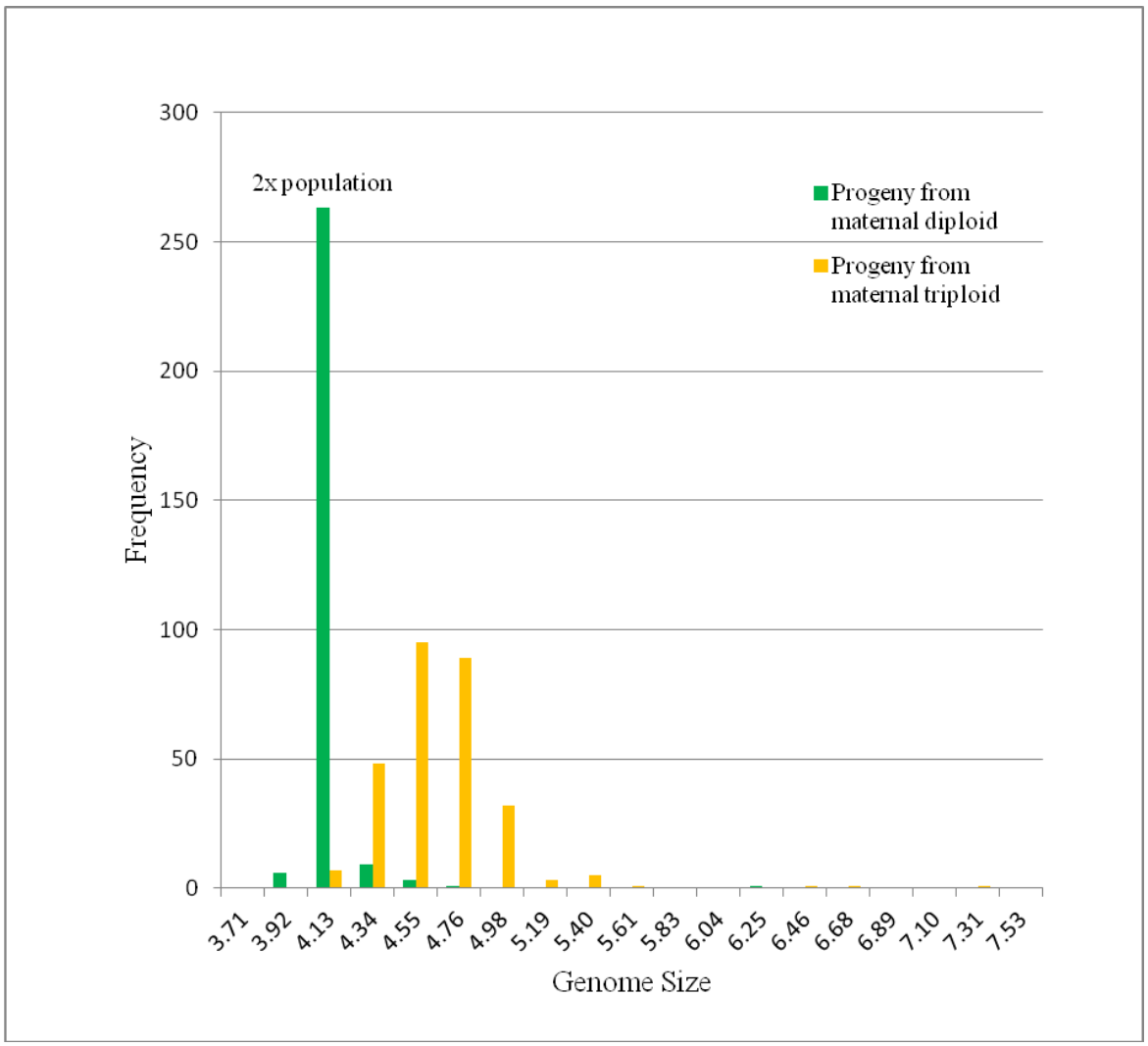

Fig. 1. Frequency distribution of 2C genome sizes of seedlings derived from open pollinated diploid and triploid *Miscanthus sinensis* genotypes. Gate width for columns was based on a 95% prediction interval for diploid plants calculated as the mean ±1.96 SD = 0.21 pg.

Table 1. Ploidy level, 2C genome size, pollen viability, seed set, seed viability, overall fertility, and relative fertility based on diploid control ('Zebrinus') of selected *Miscanthus sinensis* genotypes.

	Ploidy level (x)	2C genome size (pg)[Z]	Pollen viability (%)[Y]	Seed set (%)	Germination (%)	Overall fertility (%)[X]	Relative fertility (% 'Zebrinus')[W]
M. sinensis 'Gracillimus'	2	3.97 ± 0.03[V]	73.4 ± 5.0ad	50.2 ± 6.2c	44.6 ± 4.7d	24.8c	44.8c
M. sinensis'Superstripe' 2006-238	2	4.02 ± 0.01	77.0 ± 25a	69.8 ± 2.5b	71.2 ± 3.4ab	49.7ab	91.0ab
M. sinensis 'Variegatus' 2004-257	2	3.94 ± 0.05	75.0 ± 3.1ac	84.4 ± 2.7a	52.9 ± 3.5d	43.9b	79.4b
M. sinensis 'Zebrinus' 2004-259	2	4.08 ± 0.02	74.0 ± 3.3a	74.4 ± 2.1b	73.4 ± 4.8a	55.3a	100.0a
M. sinensis H2006-026-006	2	4.01 ± 0.00	76.4 ± 3.3ab	66.3 ± 2.9b	75.3 ± 3.3a	49.9ab	91.4ab
M. sinensis H2008-025-002	3	5.68 ± 0.02	72.2 ± 4.9ae	23.0 ± 4.6eh	38.8 ± 5.8d	9.1de	16.5de
M. sinensis H2008-026-010	3	5.82 ± 0.01	60.4 ± 6.4defghi	7.9 ± 1.5hijk	31.7 ± 7.4gj	2.8de	5.1de
M. sinensis H2008-027-003	3	5.91 ± 0.05	60.0 ± 4.2fghi	16.5 ± 1.9e	23.2 ± 1.3ij	3.8de	6.8de
M. sinensis H2008-036-025	3	5.62 ± 0.01	55.6 ± 2.4ghi	7.3 ± 1.2hijk	35.4 ± 4.9egj	2.4de	4.3de
M. sinensis H2008-061-001	3	5.65 ± 0.02	62.0 ± 2.6defhi	52.1 ± 4.6c	50.8 ± 5.9cdg	27.2c	49.2c
M. sinensis H2008-063-011	3	5.47 ± 0.02	53.3 ± 4.5ghi	5.6 ± 3.7hik	31.4 ± 9.9fgj	2.5de	4.4de
M. sinensis H2008-064-009	3	5.73 ± 0.02	55.2 ± 3.8hi	32.6 ± 6.8de	23.0 ± 8.2ij	9.6de	17.3de
M. sinensis H2008-065-003	3	5.81 ± 0.02	63.3 ± 2.8cdefghi	24.9 ± 1.3eg	69.0 ± 8.1ac	17.0cd	30.7cd
M. sinensis H2008-065-006	3	5.80 ± 0.02	59.2 ± 6.7fghi	12.9 ± 1.0fghijk	50.8 ± 5.7cdg	6.5de	11.8de
M. sinensis H2008-067-021	3	5.62 ± 0.03	65.4 ± 3.3ah	27.6 ± 4.5ef	16.0 ± 2.7j	4.3de	7.8de
M. sinensis H2008-071-002	3	5.77 ± 0.01	50.3 ± 6.5i	15.8 ± 3.2fghijk	42.2 ± 3.7di	6.5de	11.7de
M. sinensis H2008-071-009	3	5.65 ± 0.03	54.5 ± 4.3ghi	20.1 ± 3.7ej	48.3 ± 3.1dh	9.9de	18.5de
M. sinensis H2008-072-001	3	5.89 ± 0.04	62.3 ± 0.8cdefghi	12.6 ± 2.8fghijk	52.5 ± 9.0cdef	6.4de	11.6de
M. sinensis H2008-084-002	3	5.80 ± 0.01	70.5 ± 7.0afh	20.9 ± 8.1ei	21.3 ± 7.0ij	6.4de	11.9de
M. sinensis H2008-087-001	3	5.60 ± 0.02	52.0 ± 5.3hi	8.9 ± 1.0ghijk	54.8 ± 8.5cde	5.1de	8.9de

Table 1 (continued).

M. sinensis H2008-091-004	3	5.86 ± 0.02	64.6 ± 5.9cdefgh	0.7 ± 0.1k	56.3 ± 7.1bcd	0.4e	0.7e
M. sinensis H2008-130-001	3	5.87 ± 0.01	58.6 ± 3.0fghi	6.3 ± 1.9ijk	27.5 ± 5.1ij	1.7de	3.1de
M. sinensis H2008-131-001	3	5.91 ± 0.01	67.2 ± 3.3ag	45.9 ± 7.5cd	28.4 ± 5.9hij	12.7ce	23.0ce

[Z]2C DNA values represent the mean value of four sub-samples conducted for each taxon.

[Y]Values followed by different letters, within a column, are significantly different, $P \leq 0.05$.

[X]Calculated as (seed set × seed germination)/100.

[W]Calculated as overall fertility / (0.546); where 54.6 is the percentage fertility of 'Zebrinus', a diploid control.

[V]Values are means ± SEM.