ABSTRACT

JONES, JEFFREY ROBERT. Investigating Prevalence, Induction, and Fertility of Polyploid *Rhododendron* L. and the Development of Protocols for Vegetative Propagation. (Under the direction of Dr. Thomas G. Ranney.)

Studies were conducted to determine the ploidy levels of specific *Rhododendron* taxa, to develop a simple and effective *ex-vitro* method for inducing polyploidy in *Rhododendron* seedlings, and to evaluate the effect of increased ploidy level on pollen fertility. A diverse collection of species, hybrids, and cultivars in the *Hymenanthes* (elepidote rhododendrons), *Rhododendron* (lepidote rhododendrons), *Pentanthera* (deciduous azaleas), and *Tsutsusi* (evergreen azaleas) subgenera were surveyed to determine ploidy level and relative genome size using flow cytometry. In instances where ploidy levels were inconsistent with past literature, chromosome counts were made on young root tips to substantiate findings. Mean 2C holoploid genome sizes varied as a function of subgenus and ploidy level. Relative genome sizes (2C) within ploidy level, for a given subgenus, had a narrow range providing clear distinction among ploidy levels. Mean 1Cx monoploid genome size was conserved across ploidy levels within a subgenus. Polyploidy was found to be common in the genus *Rhododendron* and considerably more prevalent in the subgenus *Pentanthera* than previously known. Particularly noteworthy were the findings that *R. occidentale* includes both diploid and tetraploid individuals and that *R. atlanticum* and *R. austrinum* are predominantly tetraploid species. Induction studies were then completed with the goal of obtaining artificial tetraploids from diploid × diploid hybridizations. The effectiveness of using repeated treatments of an oryzalin suspension in a warm agar solution applied directly to apical shoots

of *Rhododendron* seedlings to induce polyploidy was tested. Apical meristems of hybrid seedlings were subjected to 1, 2, 3, or 4 applications of oryzalin separated by 4 day intervals or left untreated (control). The results of this study demonstrated that the method of applying a suspension of oryzalin in warm, semi-solid agar to the shoots of *Rhododendron* seedlings was an effective method for inducing polyploidy. Although single applications resulted in some polyploid plants, multiple applications increased efficacy for some of the taxa studied. Treatments resulted in a range of ploidy levels, from 2*x* to 8*x*, including mixaploids (cytochimeras). The effect of increased ploidy level on pollen fertility and the occurrence of unreduced gametes in triploid taxa were also studied. Pollen viability was compared between corresponding progenitor and polyploid taxa by staining pollen with 1% acetocarmine (w/v) for 15 minutes. The existence of unreduced gametes in triploid taxa was determined by the presence of dyad and/or monad pollen grains. The results demonstrated that the fertility of polyploid *Rhododendron* can be highly variable and that the induction of polyploidy may either enhance or compromise fertility. Moreover, some triploids produced viable, unreduced pollen (as high as 5%), allowing for the possible utilization of these plants in breeding programs. Documentation of polyploid taxa, improved methods for inducing polyploidy, and information on fertility and reproductive biology of polyploid *Rhododendron* will be valuable to plant breeders. Methods for improving propagation of stem cutting of North American deciduous azaleas were also examined. Influence of dormant hedging, a range of rooting hormone concentrations (0, 2500, 5000, 7500, 10,000 ppm K-IBA), and tissue growth stage on rooting of *Rhododendron austrinum* (Small) Rehder and *R. flammeum* (Michx.) Sarg. were evaluated. Softwood stem cuttings of both species rooted from 70-90%

while semi-hardwood cuttings rooted from 20-70% Cuttings from hedged stock plants rooted at higher percentages and possessed higher root system indexes compared to the unhedged counterparts in *R. flammeum,* but the effect of hedging was less evident in *R. austrinum*. Increasing IBA concentration increased rooting percentage of softwood cuttings of *R. flammeum*, but had no beneficial effect on rooting of semi-hardwood cuttings of *R. flammeum* or on *R. austrinum,* regardless of growth stage.

Investigating Prevalence, Induction, and Fertility of Polyploid *Rhododendron* L. and the Development of Protocols for Vegetative Propagation

by
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A thesis submitted to the Graduate Faculty of
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APPROVED BY:

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Dr. Anthony V. LeBude Dr. Dennis J. Werner

DEDICATION

To my entire family.

It is because of them I am who I am.

My character, values, and passions were shaped by their love and guidance.

For that I am undeniably grateful.

BIOGRAPHY

Jeffrey Robert Jones was born January 26, 1982 in Winston-Salem, North Carolina and raised just outside the metropolis in the quaint village of Tobaccoville. One would expect stories of long, sun burnt days of priming that floral gold but the days of endless tobacco fields ended prior to his youth. Sports, outdoor, and church activities encompassed his days. He spent his younger years exploring the land surrounding his homestead as trouncing through the woods was never discouraged. It was outdoors working in fields and gardens and on tractors and tillers that he gained his appreciation for plant life and the ornamental diversity of landscape plants. Jeff graduated from North Forsyth High School in 2000 and subsequently continued the family legacy by enrolling at the North Carolina State University in August of the same year.

During the first semester of his freshman year, the decision to follow his interests in the plant sciences was affirmed after taking an introductory Horticulture class with Mr. Bryce Lane. Plant identification and propagation classes with Drs. Paul Fantz and Dennis Werner, respectively, along with the invaluable experience of working with Dr. Todd Lasseigne and Jon Roethling at the J.C. Raulston Arboretum allowed Jeff to hone his passion and gain experience working with woody plant materials. Jeff graduated summa cum laude in the Fall of 2004 with a B.S. in Horticultural Science and a B.S. in Botany. Following graduation, Dr. Dennis Werner graciously allowed Jeff to work on the breeding of *Buddleia davidii* hybrids for a year. It was in this year of hands-on breeding, that Jeff gained a new perspective on the science behind research.

In 2006, Jeff entered graduate school at N.C. State to pursue a Master of Science degree in Horticultural Science working with Dr. Thomas G. Ranney. Two summers were spent in the mountains of western North Carolina at the Mountain Horticultural Crops Research Station and Extension Center in Fletcher and classes were begrudgingly taken in Raleigh, NC. Upon completion of his degree, Jeff will look to pursue opportunities in ornamental plant breeding, evaluation, and introduction.

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Beginning the acknowledgements is quite a feat for the gratitude should be spread far and wide. Many tremendous opportunities and experiences have rained down upon me. Being able to engage in the glory and immaculate detail of the natural world has been an indescribable blessing. The foundation of my interest in plants was laid at home but an introductory Horticulture class taught by Mr. Bryce Lane cemented by educational fate. The knowledge and passion combined with an intentional communication style is appreciated. There was a time in the not too distant past in which a conversation was had between a student and his advisor regarding the endless possibilities in the world of ornamental plants. A statement was made regarding the tedious, non-viable option of research. For listening and providing opportunities to see fruitful research, I sincerely thank Dr. Dennis Werner. That would not be the first time I stuck my foot squarely in my mouth.

The decision to pursue a Masters degree did not come without some apprehension. For some reason, Dr. Tom Ranney decided to give me a chance even after I expressed my indifference to a project solely centered around plant breeding and for that I am grateful. You gave me a well-rounded project that opened my eyes to the many applications involved in plant breeding. Through many a conversation, walks around the station, and bike rides through the mountains, Tom taught me the need to expand my thinking in breadth and depth, loosen my biases, and to take a plethora of pictures. He also suggested my third committee member, a fresh, energetic professor in the adjacent office with the quickest wit this side of the French Broad. I know the propagation portion of the project was not always in the

spotlight, but many thanks to Dr. Anthony LeBude for his valuable contributions and thorough questioning throughout.

I could not have asked for a better group of people to work with in the project. To the crew in Fletcher at the Mountain Horticultural Crops Research Station I am indebted. I knew and still only know so little about the ins and outs of research, but you all guided me along the entire way. To Joel Mowrey, who collected pollen, kept track of confusing crosses, and contributed to my personal collection of plants, and the venerable Tom Eaker, who graciously allowed me to get away from research with a backpack sprayer and organized many portions of the project, I thank. Please let me know when the cookout is planned at the grad house. I echo the sentiments given by Dr. Richard Olsen in saying that through the skills learned in the lab and the knowledge he possesses, Nathan Lynch should receive an honorary graduate degree of some kind. I will most certainly never have as many projects or insights into so many realms of conversational topics, and I know I would have been utterly lost in the lab without his expertise. To the entire crew working at the station, I extend a big thank you for the enjoyable and at many times comical working environment, for providing the chance to spike a volleyball, and introducing me to the mountains, trails, and cornucopia of activities that dwell within these places. Perhaps a new mountain bike will one day be in my future, thus removing my excuse for braking down hills.

There are many others in the department whom have had a significant influence on my time here. Working with Dr. Todd Lasseigne at the J.C. Raulston Arboretum was the equivalent of walking around with a plant identification manual. I only hope to know a tenth of the plants he thinks about in a given day. Many have said it before but, Rachel

McLaughlin is truly is a blessing on the first floor of Kilgore Hall. It was indeed refreshing to have someone to chat with about non-horticultural topics like pottery, the glory of Hillsborough St., and family. Providing unlimited, beckoning chocolate never hurt. If there were a recommendation form for a raise, I would fill it out. Past graduate students Richard Olsen and Ryan Contreras showed me the ropes and were always there to explain difficult materials. Brian Krug filled the office with music over my shoulder and lent valuable aid on anything computer related, and Jessica Barb was frequently available for critical chats. For the sarcastic breaks from reality and inspiring me to challenge my conventional thinking, many thanks to Colleen Brannen and for engaging me in debates over the fruits of life in the Great Plains and providing many a laugh I thank her and, the wise-beyond his years, Ryan Pekarek. To my colleague in ornamental breeding, Lis Meyer, I sincerely thank for the numerous humorous, professional, and personal conversations. You will make an excellent teacher.

My time in Raleigh has certainly led to the accumulation of a plethora of magnificent friendships. To all those who questioned exactly what horticulture was, I thank you for keeping me sharp and giving me opportunities to explain my interests. Accountability, encouragement, and solid fellowship were a constant.

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GENERAL INTRODUCTION

The genus *Rhododendron* L., of the Ericaceae, contains tremendous diversity with approximately 900 species in 8 subgenera. Rhododendrons are native throughout the world, but have especially dense distributions in China, Japan, and the eastern United States (Galle, 1987; Leach, 1961). The genus is highly regarded for its rich ornamental diversity. Foliage and flower characteristics can vary in shape, size, texture, color, fragrance, and timing. This diversity, along with a range of hardiness, growth habit, adaptability and broad crossability make the potential exciting for breeding *Rhododendron*.

Most *Rhododendron* are diploid ($2n = 2x = 26$). However, polyploidy occurs naturally in several subgenera of rhododendron with the ploidy level ranging from four to twelve (Ammal, 1950; Ammal et al., 1950; Barlup, 2002). Polyploidy, as defined by Ramsey and Schemske (2002) is "the genome-wide multiplication of chromosome number" or the condition of having more than 2 sets of chromosomes. Polyploidy is generally more common in the lepidote rhododendron species (subgenus *Rhododendron*), e.g., *R. augustinii, R. maddenii, R. triflorum, R. lepidotum, R. glaucum, R. yunnanense,* and *R. lapponicum* and some deciduous azalea species (subgenus *Pentanthera*), e.g., *R. calendulaceum, R. canadense,* and *R. luteum* (Ammal, 1950; Ammal et al., 1950). However, there has been relatively little published work on chromosome numbers of specific cultivars (Barlup, 2002). Recognition of polyploidy is paramount to the breeding efforts in the genus.

Polyploidy is common in plants and can arise through somatic chromosome doubling in the meristem, through fertilization with unreduced gametes, or through artificial chemical induction (Harlen and deWet, 1975; Soltis et al., 2003). Autopolyploids typically arise from

within a single species or from two closely related species. Fertility may be lacking due to unequal segregation and unbalanced pairing associated with polysomic pairing of the homologous chromosomes (Riesberg, 2001). In contrast, allopolyploids arise from wide hybridizations between distinct species where the presence of homeologous chromosomes allows nonrandom, disomic pairing of the sets of homologs. For this reason, allopolyploids are often fertile. Generally, allopolyploids are desirable due to greater heterozygosity; however, preferential pairing of homologous chromosome pairs in allopolyploids can result in fixed heterozygosity and minimal intergenomic exchange in later generations (Ramsey and Schemske, 2002; Ranney, 2006).

Other traits associated with polyploidy include enlarged flowers, thicker leaves and petals, compact growth habit, delayed flowering, longer juvenility periods, extended flower retention, and a wider range of pigmentation when compared to diploid progenitors (Kehr, 1996). Allopolyploids may exhibit a degree of adaptation allowing for environmental stress tolerance and resistance. This flexibility may be attributed to the combination of two distinct genomes. The ability to produce diverse enzymes from both parental species may extend environmental adaptability (Roose and Gottleib, 1976; Soltis and Soltis, 1993). However, studies of cold hardiness of induced tetraploid rhododendron did not find such results (Krebs, 2005; Väinölä and Repo, 1999).

The induction of artificial polyploids has several useful applications including the restoration of fertility in wide hybrids through the development of allopolyploids, the development of sterile triploids, the enhancement of crossability and heterozygosity, the creation of novel gene combinations, and the transfer of genes among and within ploidy

levels (Contreras et al., 2007; Olsen et al., 2006; Ranney, 2006). Chemical polyploid induction has been attempted on many crops, *Rhododendron* included, with varying degrees of success and is accomplished through the use of mitotic inhibitors (Hancock, 1997; Pryor and Frazier, 1968). Inhibiting cell division results in chromosome doubling but lacks the addition of any new genetic material.

Many *Rhododendron* cultivars of ornamental significance are the result of commercial polyploid induction (Jones et al., 2007). Fertility of these taxa depends highly on their polyploid origins. The significance of polyploid induction and fertility is evident in the case of *R.* 'Fragrant Affinity', a sterile, inter-subgeneric hybrid. Through the creation of an allopolyploid from the diploid progenitor cultivar, male and female fertility was restored (Contreras et al., 2007).

Additionally, the incorporation of triploid taxa into breeding programs is possible through the examination of fertility. In many cases, triploids are sterile, having uneven sets of chromosomes that yield aneuploid gametes (Ramsey and Schemske, 1998). Many triploids possess desirable ornamental characteristics in growth, flower, and fruit morphology (Allard, 1960), yet the reproductive biology of triploids in the genus is not well understood (Barlup, 2002). Unreduced gametes may be associated with triploids (Veilleux, 1985) and can be utilized in the recognition of triploid fertility. Fertile triploids can then be utilized as bridges for gene transfer between polyploid levels and in sexual polyploidization (Bretagnolle and Thompson, 1995; Ramsey and Schemske 1998). Unreduced gametes are the products of irregularities in the meiotic process and contain a chromosome complement

equal to (or in rare instances, greater than) that of the sporophytic chromosome number (Veilleux, 1985).

Along with concerns regarding the breeding of polyploid taxa within the genus, challenges to propagation among the deciduous azaleas also exist. The native, North American azaleas (subgenus *Pentanthera*) have tremendous ornamental merit and can serve as outstanding landscape plants. Unfortunately, many of these species can be difficult to propagate from stem cuttings (Galle, 1987). Stock plant manipulation has commonly been utilized in improving the rooting potential of difficult-to-root species. Both pre- and post-removal techniques have proven advantageous (Hartmann et al., 2002). The development of dependable and efficient propagation protocols can greatly impact the commercial potential within this group.

This thesis presents the results of studies surveying the significance of polyploidy within the genus *Rhododendron* through recognition and documentation, chemical induction, and fertility analysis of polyploid taxa. Protocols for effective stem cutting propagation of two North American, native deciduous azaleas are also reported.

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Chapter 1

Ploidy Levels and Relative Genome Sizes of Diverse Species, Hybrids, and Cultivars of *Rhododendron*

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

Ploidy Levels and Relative Genome Sizes of Diverse Species, Hybrids, and Cultivars of Rhododendron

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Introduction

Polyploidy has been an important pathway in the evolution of plants and can contribute to reproductive isolation, increased heterozygosity, novel gene combinations, modified gene expression, enzymatic multiplicity, and ultimately divergence and speciation (Soltis and Soltis, 1993; 2000; Wendel, 2000). The origins, adaptive significance, and genetic implications of polyploidy continue to be an active field of research (Bennett, 2004; Soltis et al., 2003; Chen and Ni, 2006).

For plant breeders, ploidy level is an important consideration because it can influence male and female fertility, cross fertility, plant vigor, and gene expression (Chahal and Gosal,

2002; Contreras et al., 2007; Ranney, 2006; Thomas, 1993). In some cases, polyploid plants, including rhododendrons, can have desirable characteristics including thicker leaves, enhanced vigor, and larger flowers with thicker petals that persist longer (Barlup, 2002; Hosoda et al., 1953; Kehr, 1996a; Leach, 1961). As a result, there continues to be interest in identifying naturally occurring polyploids and inducing (through mitotic doubling agents) artificial polyploids as a component of rhododendron breeding programs (Barlup, 2002; Kehr, 1996b; Paden et al., 1990; Pryor and Frazier, 1970; Leach, 1961).

Most of the more than 800 *Rhododendron* species have been reported to be diploid with $2n = 2x = 26$. However, polyploidy occurs naturally in some rhododendron species, particularly within the *Pentanthera* and *Rhododendron* subgenera, with ploidy levels ranging from three to twelve (Ammal, 1950; Ammal et al., 1950). Sax (1930) completed one of the first surveys of chromosome numbers of rhododendron, including sixteen species, and determined a base chromosome complement of $x = 13$ for the genus and identified both *R. calendulaceum* and *R. canadense* (deciduous azaleas in subgenus *Pentanthera*) as natural tetraploids. Nakamura (1931) surveyed fifteen Japanese species of rhododendron and found them all to be diploid. Ammal et al. (1950) completed an extensive survey of chromosome numbers and ploidy levels in 360 species of rhododendron and found the elepidote rhododendrons (subgenus *Hymenanthes*), evergreen azaleas (subgenus *Tsutsusi*), and the deciduous azaleas (with the exception of the tetraploid *R. calendulaceum* and *R. canadense*) to be predominantly diploid. Ammal et al. (1950) further reported a high frequency of polyploids in the scaly-leaved species of subgenus *Rhododendron*, with taxa ranging from triploids to dodecaploids. In a survey of fifteen deciduous azaleas from Eastern North

America, Li (1957) reported that all of the species were diploid with the exception of the tetraploid *R. calendulaceum.* However, a single triploid *R. atlanticum* was also identified among the otherwise diploid species. Among lepidotes, chromosome counts for 27 species in the tropical subgenus *Rhododendron* section *Vireya* indicated that they were uniformly diploid (Atkinson et al., 2000).

Published information on chromosome counts of specific cultivars or clones of rhododendron is less extensive. Hosada et al. (1953) completed chromosome counts on twelve cultivars of Satsuki azaleas (*R. lateritium*) and identified diploid, triploid ('Bangaku'), and tetraploid ('Banka', 'Taihei', and 'Wakō') plants. Pryor and Frazier (1970) determined that the evergreen azalea hybrids 'Redwing' and 'Ablaze' were triploids and also documented the existence of mixed ploidy cytochimeras resulting from colchicine treatment. Heursel and DeRoo (1981) completed chromosome counts on 47 cultivars of evergreen azaleas and found they were all diploid with the exception of the triploid, 'Euratom'.

The chromosomes in rhododendron are small and can be difficult to view and count (Eiselein, 1994; Tolstead and Glencoe, 1991). Light microscopy is therefore not a practical method for determining ploidy levels of large numbers of individual cultivars and clones. However, flow cytometry can provide a fast and accurate determination of nuclear DNA content (genome size) that is related directly to ploidy level among closely related taxa (de Laat et al., 1987; Doležel, 1991; Doležel et al., 1998; Galbraith et al., 1983). Flow cytometry is also effective for detecting mixaploidy or cytochimeras and individual histogenic layers can be analyzed by sampling appropriate tissue (DeSchepper et al., 2001). Flow cytometry has been used successfully to determine relative genome size and ploidy levels of

Rhododendron spp. (DeSchepper et al., 2001; Eeckhaut et al., 2004; Sakai et al., 2003, 2004a, 2004b, 2006; Ureshino and Miyajima, 1998; Väinölä, 2000). De Schepper et al. (2001), for example, determined the ploidy level for six species and 88 cultivars within the evergreen azalea subgenus *Tsutsusi* using flow cytometry. The vast majority were found to be diploid with the exception of three triploids ('Red Wing', 'Euratom', and 'Euratom Orange') and one mixaploid ('Casablanca Tetra') that was found to be diploid in the LI and LII layers and tetraploid in the LIII. Eeckhaut et al. (2004) studied various Ghent and Rustica deciduous azalea hybrids by using flow cytometry and found them to be either triploid ('Mina Van Houtte', 'Daviesii', 'Quadricolor', 'Gloria Mundi', 'Van Houtte Flore Pleno', 'Norma', and 'Phebe') or tetraploid ('Nancy Waterer', 'Unique', 'Narcissiflorum', 'Jozef Baumann', 'Maja', 'Rosetta', 'Semiramis', 'Souvenir du Press. Carnot', 'Marie Verschaffelt', 'Batholo Lazarri', 'Guelder Rose', 'Coccinea Major', 'Raphaël De Smet', 'General Trauff', 'Graff von Meran', 'Goldlack', 'Fenelon', and 'Racine'). In contrast to the survey by Ammal et al. (1950), Eeckhaut et al. (2004) found three clones of *R. luteum* to be tetraploid, not diploid. Sakai et al. (2006) identified twenty-three diploid, six triploid ('Dalsetsuzan', 'Goka', 'Horiuchikanzaki', 'Isshonoharu', 'Melcho', and 'Yuhime'), nine tetraploid ('Ayaka', 'Eiko', 'Hoshuku', 'Hoshun', 'Sachinoharu', 'Shunka', 'Taihei', 'Taikonotsuki', and *R. kiusianum* × *R. eriocapum* No. 5) and four mixaploid ('Koyo', 'Miharu', 'Shinsen', and 'Sulsen') evergreen azaleas and eight diploid and five tetraploid ('Golden Flare', 'Golden Sunset', 'Klondyke', 'Melford Yellow', and *R. japonicum* f. *flavum* No. 6) deciduous azaleas. Although flow cytometry can be used to directly compare relative genome sizes of tissue from related taxa, inclusion of an internal standard with a known genome size allows the

calculation of the sample genome size (Doležel and Bartoš, 2005), which enables comparisons among studies of more divergent taxa.

The objectives of this project were to determine the ploidy level and relative genome size of a diverse collection of species, hybrids, and cultivars of rhododendron by using a combination of flow cytometry and traditional cytology in order to: 1) determine the ploidy level of suspected, but unconfirmed, polyploid taxa (both naturally occurring and chemically induced), 2) increase sampling among and within species, and 3) develop an extensive database for specific cultivars and clones for use by rhododendron breeders.

Materials and Methods

Flow cytometry. Holoploid, 2C genome sizes (i.e., DNA content of the entire non-replicated, chromosome complement irrespective of ploidy level) were determined via flow cytometry (de Laat et al., 1987; Doležel, 1991; Galbraith et al., 1983; Greilhuber et al., 2005). Diverse species and cultivars were acquired from various sources that included taxa from the *Hymenanthes, Rhododendron, Tsutsusi*, and *Pentanthera* subgenera along with several inter-subgeneric hybrids (Table 1). Approximately 1 cm^2 of newly expanded leaf or petal tissue was finely chopped with a razor blade in a Petri dish with 500 □L of nuclei extraction buffer (CyStain UV Precise P Nuclei Extraction Buffer, Partec, Münster, Germany). The solution was incubated for 1 to 2 min at approximately 24 °C then filtered through Partec CellTrics™ disposable filters with a pore size of 50 µm to remove tissue debris. Nuclei were stained with 1.5 mL 4', 6-Diamidino-2-phenylindole (DAPI) staining buffer (CyStain UV Precise P

Staining Buffer, Partec). Stained nuclei were analyzed with a flow cytometer (Partec PA-I, Partec) to determine relative genome size. Counts exceeded a minimum of 3000 cells per sample. Genome sizes were determined by comparing mean relative fluorescence of each sample with an internal standard, *Pisum sativum* L. 'Ctirad', with a known genome size of 9.09 pg (Bennett and Smith, 1976; Doležel et al., 1998) and calculated as: 2C genome size of sample = 9.09 pg × (mean fluorescence value of sample/ mean fluorescence value of standard). The relationship between ploidy levels and genome sizes was initially determined for plants with documented chromosome numbers including diploid *R.* 'Fragrant Affinity', triploid *R.* 'Redwing' azalea, and the tetraploid Ilam azalea #HA L49-520 (Contreras et al., 2007; De Schepper et al., 2001; Krebs, 1997). Genome sizes were also determined for a range of species where ploidy levels and chromosome counts have been previously reported. Mean 1Cx monoploid genome size (i.e., DNA content of the non-replicated base set of chromosomes with x = 13) was calculated as 2C genome size / ploidy level. Data were subjected to analysis of variance and means separation by using the Waller procedure (PROC GLM; SAS version 8.02, SAS Institute., Cary, N.C.; SAS Institute, 1988).

Chromosome counts. In situations where cytometric results were not consistent with published research, chromosomes were counted by using standard cytological techniques (Contreras et al., 2007). Chromosomes were counted in mitotic cells from young root tips of rhododendron cuttings. Roots were collected before 11 a.m. and root tips were placed in a pre-fixative solution of 2mM 8-hydroxyquinoline for 4 hours at 12 °C in the dark. Root tissue was then fixed in a 1 : 3 solution of propionic acid : 95% ethanol solution for 24 hours at room temperature and then hydrolyzed in 1N HCl for 15 minutes at room temperature and

for 25 minutes at 60 °C, followed by a rinse in distilled water. Root tips were excised and placed on a glass microscope slide with a drop of 1% acetocarmine. Slides with tissue samples were heated to approximately 70°C for 10 to 15 s, squashed with a coverslip, and viewed under a light microscope (Nikon Eclipse 80i, Nikon, Melville, NY) at 1,500× using oil immersion.

Results and Discussion

Flow cytometry was an effective method for determining genome sizes and ploidy levels of rhododendron. Mean 2C holoploid genome sizes varied as a function of subgenus and ploidy level (Tables 1 and 2). Analysis of variance demonstrated significant effects of both subgenus and ploidy level on 2C genome size ($P<0.05$). Genome sizes (2C) within ploidy levels for a given subgenus had a narrow range providing clear distinction among ploidy levels. Mean 1Cx monoploid genome size was conserved across ploidy levels within a subgenus, ranging from 0.72 to 0.75 pg for subgenus *Hymenanthes,* 0.67 to 0.83 pg for subgenus *Rhododendron,* 0.63 to 0.67 pg for subgenus *Tsutsusi,* and 0.80 to 0.83 for subgenus *Pentanthera* (Table 2). There did not appear to be a consistent reduction in base 1Cx genome size with increasing ploidy level (i.e, genome downsizing) in rhododendron as has been commonly found in other genera with polyploid series (Leitch and Bennett, 2004). These results were based on cytometry methods using DAPI staining that provides consistent determination of relative genome size. However, it should be noted that other methods and stains may provide slightly different values and ranges (Doležel and Bartoš, 2005).

Hymenanthes

Genomic sizes (2C) in this subgenus ranged from 1.4 to 1.6 pg for diploids, from 2.1 to 2.2 pg for triploids, and from 2.9 to 3.4 pg for tetraploids (Table 2). As expected from earlier reports (Ammal et al., 1950; Nakamura, 1931), all of the sampled species fell within the diploid group (Table 1). However, some hybrids derived from species within this subgenus exhibited polyploidy. Barlup (2002) speculated on the possible polyploid nature of 'Taurus', ('The Honorable Jean Marie de Montague' × *R. strigillosum*) and we found it to be triploid, which most likely explains its low fertility. 'Hallelujah' ('The Honourable Jean Marie de Montague' × 'Kimberly') and an unnamed hybrid [('Nancy Evans' × ('Whopper' × 'Lem's Cameo')) × 'Point Defiance'] were also found to be triploids. These triploids may have arisen from either interploid crosses (particularly when the tetraploid 'Point Defiance' was a parent) or from an unreduced gamete from a diploid parent. Hybridity has been shown to increase formation of unreduced gametes even when the parental species might not exhibit the same characteristic (Ramsey and Schemske, 1998; Widrlechner et al. 1982). Other tetraploids arising from interspecific hybridization in this subgenus included 'Horizon Monarch' ('Nancy Evans × 'Point Defiance'), 'Lem's Monarch' ('Anna' × 'Marinus Koster'), 'Point Defiance' ('Anna' × 'Marinus Koster'), and 'Gentle Giant' ('Point Defiance' × 'Platinum Pearl'). 'Vulcan' tetraploid arose as somatic mutation (i.e., branch sport) on 'Vulcan' (Harold Greer, Eugene, Ore., per. comm.). Interestingly, we found 'Vulcan' tetraploid to be a 2x + 4x mixaploid that apparently arose from a mitiotic doubling event within a single histogenic layer.

Several chemically-induced tetraploids were also confirmed including 'Everlasting Tetra', 'Super Nova', 'Briggs Red Star', and *R. fortunei* (NCSU 2005-175). 'Everlasting Tetra' was developed from 'Everlasting' ('No Suchianum') (see Grant et al., 2004 for more history on this cultivar) at N.C. State University based on methods described by Contreras et al. (2007). 'Super Nova' resulted from *in-vitro* colchicine treatment of 'Nova Zembla' at Briggs Nursery, Olympia, Wash. (Dan Meier, Olympia Wash., per. comm.). 'Briggs Red Star' was developed similarly at Briggs Nursery, but was found to be a 2x + 4x mixapoloid. *R. fortunei* NCSU 2005-175 was a colchicine treated plant developed by Dr. Max Byrkit, Williamsport, Md. (Kehr, 1996 b).

Rhododendron

Concordant with previous findings, polyploidy was prevalent among species and their hybrid derivatives from subgenus *Rhododendron* (Ammal et al., 1950). Genome sizes (2C) for diploids ranged from 1.3 to 1.9 pg, there was one triploid at 2.0 pg, tetraploids ranged from 2.8 to 3.3 pg, and hexaploids ranged from 4.4 to 4.6 pg (Table 2) The relationship between genome size and ploidy level above the hexaploid level was less clear. Two *R. maddenii* clones had genome sizes ranging from 5.4 to 5.8 pg that are most likely octoploids, but the plant with 5.4 pg could possibly be heptaploid. The only triploid found was 'White Ruffles', a cross made by Dr. August Kehr between the tetraploid *R. carolinianum* 'Epoch' (Kehr, 1996b) and *R. mucronulatum*. *Rhododendron augustinii* was found to be tetraploid as reported previously (Ammal et al., 1950) as were Dr. Kehr's *augustinii* hybrids: 37-1, 37-4, and 37-7 (Dr. Kehr, per. comm.). 'Shorty', a cross between a selfed 'Epoch' and 'High

Tech' (Henry Schannen, Jackson, N.J., per. comm.), was a tetraploid indicating that 'High Tech' is either a tetraploid or produced unreduced pollen. 'Bubblegum' and 'Northern Starburst' were both tetraploids and were developed from *in-vitro* colchicine treatment of 'Weston Aglo' and 'PJM', respectively, at Briggs Nursery (Dan Meier, Olympia Wash., per. comm.).

Pentanthera

Genome sizes for species and hybrids in subgenus *Pentanthera* ranged from 1.5 to 1.7 pg for diploids, 2.3-2.6 for triploids, 3.0-3.9 for tetraploids, and 6.3-6.5 for octoploids. The majority of deciduous azaleas, including *R. arborescens, alabamense, canescens, cumberlandense, japonicum, molle, periclymenoides, prinophyllum, prunifolium, serrulatum, vaseyi,* and *viscosum* were found to be diploids as has been reported previously (Ammal, 1950; Li, 1957; Sax, 1930). The more recently discovered *R. eastmanii* was also found to be a diploid (Kron and Creel, 1999). Also agreeing with past literature (Ammal et al., 1950; Li, 1957; Sax, 1930) was the confirmation of *R. calendulaceum* as a tetraploid, though one triploid *R. calendulaceum,* NCSU 2000-164, was found that most likely resulted from a natural hybrid with a diploid species. Three wild-collected accessions of Gregory Bald Hybrids were found to be diploids, confirming that their parentage does not include the tetraploid *R. calendulaceum.*

Our cytometric evidence suggests that natural polyploidy may be more prevalent among deciduous azalea species than previously thought. The data obtained for two selections of the Pontic azalea, *R. luteum* 'Bumb' and 'Golden Comet' (Table 1) substantiate

a finding by Eeckhaut et al. (2004) that this Central Asian species has tetraploid forms. All of the *R. atlanticum* and *R. austrinum* accessions tested in this study (Table 1) had polyploid genome sizes (mostly tetraploid and a few triploid), as did some of the *R. flammeum* and *R. occidentale* samples. This is notable because in all earlier reports, only one instance of polyploidy (triploid) in these four North American species has been reported (Ammal, 1950; Li, 1957; Sax, 1930). Cytometric results in the present study were confirmed by chromosome counts on somatic cells from fifteen accessions of both *R. atlanticum* and *R. austrinum*, which showed that they were tetraploids, $2n = 4x = 52$ (Figs. 1 and 2). Indirect evidence of tetraploidy in *R. atlanticum* is provided by the observation that *R. atlanticum* H2004-055 and H2004-056 readily hybridize with *R. calendulaceum* and produce fertile hybrids (Dr. Jim Ballington, N.C. State University, Raleigh, N.C., per. comm.). Fertile hybrids have also resulted from crosses between *R. calendulaceum* × *austrinum*, *R. calendulaceum* × *atlanticum*, *R. calendulaceum* × 'Marydel' (Mr. Ray Head, Rutherfordton, N.C., per. comm.).

No diploid *R. austrinum* or *R. atlanticum* was found despite extensive sampling of taxa from diverse sources and geographical origins (26 *R. austrinum* and 30 *R. atlanticum* accessions collected throughout the Southeast). The assessment of these species as diploids in previous studies was based on a much more limited sampling (Ammal, 1950; Li, 1957; Sax, 1930). Therefore it seems unlikely that the lack of diploid forms of *R. atlanticum* and *R. austrinum* in this survey represents a sampling limitation, but rather a predominance of polyploids in these species. This appears to be the case for *R. calendulaceum* as well, where there are no reports (present study included) of diploid populations. The two triploid *R.*

austrinum accessions observed here (Table 1) may have resulted from a natural interploid cross between sympatric diploid and tetraploid populations – if so it would be informative to sample again from the areas where they were collected in order to document the presence of more diploid forms of this species.

The best example of a natural polyploid series in *Rhododendron* species appears to be *R. occidentale*, where both diploid and tetraploid accessions were observed (Table 1). All samples originated from the same stand at the Stage Coach Hill Azalea Reserve, CA (Red Cavender, per. comm..). These data suggest there is a range of ploidy levels found within these species as is naturally found in many other species, e.g., *Galax aphylla* (Nesom, 1983), representing an evolutionary progression (Arnold, 1997; Briggs and Walters, 1997). Multiple ploidy levels were observed for *R. flammeum*, and *an* interspecific triploid hybrid between *R. flammeum* and *R. canescens* (TNCA 1994-2*F) was documented. However, the majority of the samples observed were diploids. Much more extensive geographic sampling of ploidy levels, morphological identication, and molecular characterization of native populations of *R. flammeum* would be desirable to elucidate the presence, prevalence, and evolutionary significance of polyploidy in this species.

Many hybrid cultivars within this subgenus were found to be polyploids; most likely resulting from the hybridization of polyploid parents. Three Exbury azaleas of unknown parentage, 'Gibraltar', 'Gold Dust', and 'Klondyke', were tetraploids as were 'My Mary' ('Nacoochee' × 'Austrinum Gold'), 'Lemon Lights' (Northern Lights Series, unknown parentage), 'Admiral Semmes' (Confederate Series, 'Hotspur Yellow' × *R. austrinum*), 'Marydel'(*R. atlanticum* or possible hybrid with *R. periclymenoides*) and an unnamed Ilam

hybrid (HA L-46-520; unknown parentage) (Dirr, 1998; Galle, 1987). The tetraploid 'Washington State Centennial' is a hybrid of *R. occidentale* and *R. cumberlandense* then pollinated with 'Santiam' (a hybrid between *R. occidentale* and the Knapp Hill azaleas), thus further suggesting polyploidy in *R. occidentale* and the presence of unreduced gametes in the subgenus. 'Snowbird' was determined to be a tetraploid and is believed to be a natural hybrid between *R. atlanticum* and *R. canescens* (Galle, 1987), suggesting an unreduced gamete from the *R. canescens* parent. 'Fragrant Star', developed through *in-vitro* colchicine treatment of 'Snowbird' at Briggs Nursery (Dan Meier, Olympia Wash., per. comm.) was found to be an octoploid as were open pollinated (selfed) seedlings from 'Fragrant Star'.

Tsutsusi

The ranges for 2C genome sizes in subgenus *Tsutsusi* were consistently lower than the other subgenera with the diploids ranging from 1.2 to 1.3 pg, the triploids from 1.9 to 2.0 pg, and the tetraploids from 2.6 to 2.8 pg. We found 'Red Wing' to be a triploid which was consistent with the findings of Pryor and Frazier (1970), but contrary to the findings of Heursel and Roo (1981), who found it to be a diploid, suggesting that multiple clones may exist under the same name. The purple-leaved 'Crimson Majesty', a sport of 'Red Formosa' was also found to be a triploid as was an unnamed hybrid between 'Pink Gloria Tetra' × 314-1 (NCSU 2000-171). The clone 314-1, a colchicine-treated seedling (open-pollinated seedling of 'Perle de Swynaerde' × 'Pryor Dwarf') developed by Dr. August Kehr, was also found to be a tetraploid, as was the unnamed hybrid 'Anytime Tetra' × 314-1 (NCSU 2000-167). We did not have access to 'Pink Gloria Tetra' and could not determine its ploidy.

However, upon further investigation, we found the original 314-1 specimen, provided by Dr. Kehr, to be a mixture of diploid and tetraploid shoots with diploid shoots arising from below the treated crown. If flowers from these diploid shoots were used in breeding with the presumed tetraploid 'Pink Gloria Tetra', a triploid could have resulted.

Inter-subgeneric Hybrids

Several hybrids were examined that were the result of crosses between subgenera. In agreement with Contreras et al. (2007), we confirmed that 'Fragrant Affinity' (*R. viscosum* × *R. ponticum*) was a diploid and its allopolyploid complement, 'Fragrant Affinity Tetra', was a tetraploid. The hybrids *R. calendulaceum* × '314-1' and 'Briggs Red Star' × 'Fragrant Affinity Tetra' were tetraploids as expected given that all parents were also tetraploids.

Conclusion

This study provides extensive information on genome sizes and ploidy levels for a broad range of species, cultivars, and hybrids of rhododendron including naturally occurring and induced polyploids. Flow cytometry was an efficient and effective method for determining genome size of rhododendron. Genome sizes (2C) within ploidy levels for a given subgenus had a narrow range providing clear distinction among ploidy levels. Polyploidy was found to be common in the genus *Rhododendron* and considerably more prevalent in the subgenus *Pentanthera* than previously known. Particularly noteworthy were the findings that *R. occidentale* includes both diploid and tetraploid individuals and that *R. atlanticum* and *R. austrinum* are predominantly tetraploid species. This information provides

further insights into the genetics, evolution, and reproductive biology of rhododendron as well as serving as a valuable database for breeder.

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Table 1. Relative genome size and estimated ploidy level, determined by flow cytometry, for a diverse collection of rhododendron species and cultivars.

Taxa	Source[1]	Relative 2C genome size (pg)[2]	Estimated ploidy (*x*)
Subgenus Hymenanthes			
Species			
catawbiense 'Catalgla'	HA	1.44±0.03	2
fortunei	NCSU 2003-144	1.55±0.02	2
maximum	NCSU 2006-281	1.44±0.01	2
maximum	NCSU 2005-243	1.53±0.12	2
ponticum (variegated)	NCSU 2006-047	1.46±0.01	2
sinogrande	NCSU 2006-038	1.54±0.04	2
Hybrids			
'Cheyenne'	NCSU 2002-086	1.41±0.03	2
'Everlasting'	NCSU 2000-162	1.52±0.03	2
'Fantastica'	NCSU 2004-285	1.45±0.00	2
'Goldflimmer'	JCRA 040681	1.64±0.01	2
'Janet Blair'	NCSU 2004-291	1.44±---	2
'Maxicat'	NCSU 2005-238	1.52±0.00	2
'Nova Zembla'	NCSU 2006-093	1.53±0.01	2
'Polar Bear'	NCSU 2002-089	1.55±0.02	2
'Puget Sound'	NCSU 2005-015	1.47±0.00	2
'Queen Anne' × 'Gold Dust'	NCSU 2000-270	1.43±0.02	2
'Vulcan'	NCSU 2006-095	1.49±0.03	2
'Vulcan's Flame'	NCSU 2004-134	1.55±0.01	2
'Taurus'	NCSU 2006-026	2.06±0.06	3
'Hallelujah'	NCSU 2005-009	2.22±0.05	3
[Nancy Evans × (Whopper × Lem's Cameo)] × Point Defiance	Brockenbrough	2.22±0.06	3
'Gentle Giant'	NCSU 2006-020	3.37±0.11	4
'Grand Slam'	NCSU 2006-021	3.03±0.02	4
'Horizon Monarch'	NCSU 2006-022	2.89±0.07	4
'Horizon Monarch' × 'Point Defiance' (clone R)	Brockenbrough	2.93±0.00	4
'Lem's Monarch'	Brockenbrough	2.90±0.01	4
'Point Defiance'	Brockenbrough	2.93±0.02	4

Table 1 (continued).

'Vulcan Tetraploid'	NCSU 2004-103	1.51±0.02 3.03±0.07	2+4
Induced polyploids			
'Briggs Red Star'	NCSU 2002-260	1.53±0.02 3.04±0.05	2+4
'Everlasting Tetra'	NCSU 2005-149	2.86±0.02	4
'Super Nova'	NCSU 2002-263	2.98±0.04	4
fortunei	NCSU 2005-175	3.14±0.03	4
Subgenus Rhododendron			
Species			
edgeworthii 'Bodnant'	NCSU 2005-361	1.75±0.01	2
edgeworthii 'Ice'	NCSU 2006-053	1.76±0.04	2
augustinii	NCSU 2000-170	3.10±0.01	4
maddenii	NCSU 2006-162	4.41±0.04	6
maddenii	NCSU 2006-160	4.45±0.02	6
maddenii subsp. crassum	NCSU 2006-256	4.39±0.01	6
maddenii subsp. maddenii	NCSU 2006-037	4.47±0.01	6
maddenii	NCSU 2006-161	5.97±0.01	8
maddenii subsp. crassum	NCSU 2006-258	5.42±0.01	8
Hybrids			
'Aglo'	NCSU 2006-045	1.49±0.05	2
'April Rose'	NCSU 2006-018	1.36±0.02	2
'California Gold'	NCSU 2006-259	1.71±0.02	2
'Coastal Spice'	NCSU 2005-355	1.86±0.01	2
'Dora Armateus'	NCSU 2005-222	1.62±0.06	2
'Improved Fragrantissimum'	NCSU 2002-088	1.72±0.05	2
'McNabii'	NCSU 2006-039	1.61±0.02	2
'Mysterious Maddenii'	NCSU 2006-262	1.82±0.00	2
'PJM'	NCSU 2006-012	1.32±---	2
'Reine Long'	NCSU 2006-264	1.76±0.02	2
'Southern Cloud'	NCSU 2006-265	1.65±0.04	2
'White Ruffles'	NCSU 2006-113	2.01±0.03	3
'Blue Target'	NCSU 2000-168	3.10±0.00	4
'Epoch' × *augustinii*	NCSU 2006-044	3.25±0.02	4
'Gletschernacht'	NCSU 2003-143	2.78±0.07	4
37-1	NCSU 2000-267	3.11±0.02	4
37-4	NCSU 2000-169	3.12±0.10	4
37-7	NCSU 2000-269	3.19±0.00	4

Table 1 (continued).

'Shorty'	NCSU 2006-042	3.22±0.04	4
'Bernice'	NCSU 2006-255	4.57±0.01	6
'Pink Trumpets'	NCSU 2006-263	4.61±0.02	6
Induced polyploids			
'Bubblegum'	NCSU 2006-046	2.90±0.07	4
'Northern Starburst'	NCSU 2006-011	2.81±---	4
Subgenus Pentanthera			
Species			
alabamense	2004-114	1.66±0.05	2
arborescens	NCSU 1998-454	1.65±0.05	2
austrinum (OP)	BE	1.59±0.00	2
austrinum (pale yellow)	BE	1.64±0.02	2
canescens 'Crains Creek'	TNCA 1995-26*B	1.72±0.02	2
canescens 'Sp. Found'	TNCA 1989-60*A	1.61±0.01	2
canescens 'White Canescens'	TNCA 1989-59*A	1.65±0.01	2
cumberlandense	TNCA 1994-10*B	1.63±0.02	2
eastmanii (Newberry, SC)	Cantrell	1.58±.04	2
eastmanii (York Co, SC)	Cantrell	1.60±.04	2
flammeum (ES Selection)	TNCA 1994-454*B	1.68±0.01	2
flammeum 'Salmon Form'	TNCA 1995-31*B	1.72±0.02	2
flammeum (Thompson Co, GA)	Towe	1.52±0.01	2
flammeum (Transplant Nursery)	Ballington	1.71±0.01	2
flammeum OP	Ballington	1.70±0.02	2
flammeum B-218	Ballington	1.69±0.01	2
flammeum (Cobb Co, GA)	Head	1.49±0.01	2
flammeum 'Hazel Hamilton'	Head	1.69±0.03	2
japonicum	Cantrell	1.48±0.00	2
molle	Cantrell	1.49±0.01	2
occidentale 'Homboldt Picotee'	Cavender	1.51±.04	2
occidentale 'Tatum's Deep Pink'	Cavender	1.51±.06	2
periclymenoides (*nudiflorum*)	NCSU 2000-419	1.68±0.00	2
prinophyllum	TNCA 1994-20*A	1.64±0.00	2

Table 1 (continued).

prunifolium	TNCA 1994-22*B	1.56±0.01	2
prunifolium	NCSU 1998-455	1.58±0.00	2
serrulatum	TNCA 1989-78*A	1.74±0.03	2
vaseyi	NCSU 1998-447	1.56±0.01	2
viscosum	TNCA 1995-460*A	1.67±0.04	2
austrinum	TNCA 1989-40*A	2.52±0.05	3
austrinum 'Firecracker'	TNCA 1995-451*A	2.48±0.01	3
calendulaceum	NCSU 2000-164	2.30±0.07	3
atlanticum	JCRA 050431	3.01±0.01	4
atlanticum	TNCA 1998-0103a	3.05±---	4
atlanticum	NCBG 1994-0093b	3.10±---	4
atlanticum	NCBG 1986-2041a	3.12±---	4
atlanticum	NCSU H2004-054-002	3.15±0.01	4
atlanticum	TNCA 1994-9*B	3.16±0.00	4
atlanticum	NCSU H2004-056-002	3.20±0.00	4
atlanticum	NCSU H2004-055-003	3.20±0.02	4
atlanticum	NCSU H2004-055-001	3.21±0.01	4
atlanticum	NCSU H2004-056-001	3.24±0.00	4
atlanticum	NCSU H2004-055-002	3.24±0.02	4
atlanticum	NCSU H2004-054-004	3.24±0.07	4
atlanticum	NCSU H2004-054-001	3.26±0.00	4
atlanticum	NCSU H2004-054-003	3.26±0.01	4
atlanticum	TNCA 1989-33*A	3.27±0.00	4
atlanticum #1	HA	3.16±0.02	4
atlanticum #1 (Del Mar Peninsula)	HA	3.13±0.06	4
atlanticum #2	HA	3.10±---	4

Table 1 (continued).

atlanticum #2	HA	3.33±0.02	4
atlanticum #3	HA	3.10±0.01	4
atlanticum #3	HA	3.29±0.01	4
atlanticum #4	HA	3.12±0.03	4
atlanticum #4	HA	3.14±0.01	4
atlanticum #5	HA	3.08±.00	4
atlanticum #6	HA	3.06±---	4
atlanticum #7	HA	3.19±0.05	4
atlanticum #8	HA	3.32±---	4
atlanticum 'Choptank Pink &White'	TNCA 1995-467*B	3.22±0.01	4
atlanticum 'Tetra Form'	TNCA 1998-34*A	3.20±0.02	4
atlanticum 'Winterthur'	JCRA 000609	3.18±0.02	4
austrinum	JCRA 020494	3.11±0.03	4
austrinum	NCBG 1991-0301a	3.12±---	4
austrinum	TNCA 1996-0374a	3.21±---	4
austrinum	JCRA L20	3.21±0.05	4
austrinum	TNCA 1994-339*B	3.24±0.01	4
austrinum	TNCA 1989-221*E	3.43±0.03	4
austrinum	NCBG 1998-0104a	3.47±---	4
austrinum	TNCA 1989-221*A	3.41±0.01	4
austrinum	NCBG 1998-0188a	3.31±---	4
austrinum	NCSU 2005-062	3.33±0.03	4
austrinum	NCSU 2006-223	3.34±0.04	4
austrinum	NCSU 2004-117	3.36±0.01	4
austrinum	NCSU 2005-063	3.37±0.02	4
austrinum #1 (Nat. For. Ala.)	HA	3.33±0.00	4
austrinum #10	HA	3.37±0.09	4
austrinum #12	HA	3.30±0.00	4
austrinum #2	HA	3.23±0.01	4
austrinum #3	HA	3.27±0.05	4
austrinum #4	HA	3.88±0.59	4
austrinum #5	HA	3.32±0.00	4

Table 1 (continued).

austrinum #6	HA	3.29±0.02	4
austrinum 'Austrinum Gold'	TNCA 1989-38*A	3.28±0.01	4
austrinum 'Flame'	TNCA 1990-22*A	3.28±0.01	4
austrinum 'Millie Mac'	TNCA 1993-327*A	3.33±0.07	4
calendualaceum 'Deliverance'	TNCA 1989-55*A	3.28±0.01	4
calendulaceum	NCSU H2000-048	3.14±0.09	4
flammeum	NCSU 2007-001	3.14±0.03	4
flammeum 'Pink Surprise'	TNCA 1994-332*B	3.24±0.03	4
luteum 'Bumb'	NCSU 2005-101	3.00±0.01	4
luteum 'Golden Comet'	NCSU 2006-006	3.00±0.01	4
occidentale 'Double Dig Twelve'	Cavender	2.94±.08	4
Hybrids			
'August Beauty'	NCSU 2006-118	1.58±0.00	2
'Lemon Drop'	NCSU 2006-119	1.51±0.04	2
'Millenium'	NCSU 2005-122	1.61±0.03	2
'Popcorn'	NCSU 2005-123	1.51±0.01	2
'Summer Lyric'	NCSU 1998-453	1.64±0.04	2
'Weston's Parade'	NCSU 2005-121	1.57±0.06	2
Gregory Bald Hybrid	TNCA 1992-515*M	1.62±0.03	2
Gregory Bald Hybrid	TNCA 1992-212*E	1.65±0.01	2
Gregory Bald Hybrid	TNCA 1992-213*B	1.67±0.03	2
flammeum × *canescens*	TNCA 1994-2*F	2.60±0.05	3
'Admiral Semmes'	NCSU 2005-081	3.15±0.04	4
flammeum × *calendualaceum*	TNCA 1996-325*A	3.39±0.01	4
'Gilbralter'	NCSU 2005-356	3.38±0.01	4
'Gold Dust'	NCSU 2005-111	3.27±0.04	4
'Ilam' hybrid	HA L49-520	3.17±0.01	4
'Klondyke'	NCSU 2005-357	3.26±0.16	4
'Lemon Lights'	NCSU 2005-113	3.03±0.00	4
'Marydel'	NCSU 1998-456	3.43±0.03	4
'My Mary'	NCSU 2006-117	3.15±0.08	4
'Snowbird'	NCSU 2006-048	3.24±0.03	4

Table 1 (continued).

'Washington State Centennial'	NCSU 2007-091	3.28±0.03	4
Induced polyploids			
'Fragrant Star'	NCSU 2004-293	6.32±0.03	8
'Fragrant Star' selfed	NCSU H2006-007-003	6.39±0.11	8
'Fragrant Star' selfed	NCSU H2006-007-001	6.41±0.08	8
'Fragrant Star' selfed	NCSU H2006-007-004	6.46±0.03	8
Subgenus Tsutsusi			
Species			
stenopetalum 'Linearifolium'	JCRA 050534	1.27±0.01	2
Hybrids			
'Conles' Autumn Express ™	NCSU 2002-237	1.27±0.02	2
'Glacier'	NCSU 2005-064	1.24±0.03	2
'Hardy Gardenia'	NCSU 2005-023	1.22±0.00	2
'Polar Bear'	NCSU 2005-196	1.26±0.00	2
'Secret Wish'	NCSU 2005-097	1.30±0.01	2
'Crimson Majesty'	NCSU 2004-245	1.94±0.03	3
'Pink Gloria Tetra' × '314-1'	NCSU 2000-171	1.98±0.01	3
'Redwing'	NCSU 2006-094	1.88±0.02	3
'Anytime Tetra' × '314-1'	NCSU 2000-167	2.75±0.07	4
Induced polyploids			
'314-1'	NCSU 2000-165	2.60±0.01	4
Inter-Subgeneric			
Hybrids			
'Fragrant Affinity'	NCSU H2003-003	1.59±0.12	2
'Briggs Red Star' × 'Fragrant Affinity Tetra'	NCSU H2005-085	2.99±0.03	4
calendulaceum × '314-1'	NCSU H2006-008-001	2.81±0.00	4
Induced polyploids			
'Fragrant Affinity Tetra'	NCSU H2003-002	3.11±0.04	4

Table 1 (continued).

[1]BE = Biltmore Estate, Asheville, N.C.
Ballington = Dr. Jim Ballington, North Carolina State University, Raleigh, NC.
Brockenbrough = Mr. Ned Brockenbrough, Hunts Point, Wash.
Cantrell = Mr. Allen Cantrell, Chesnee, SC.
Cavender = Mr. Dick 'Red' Cavender, Sherwood, Oregon.
HA = Holden Arboretum, Kirtland and Madison, Ohio.
Head = Mr. Ray Head, Rutherfordton, NC.
Towe = Mr. Clarence Towe, Walhalla, SC.
TNCA = The North Carolina Arboretum, Asheville, N.C.
NCBG = North Carolina Botanical Garden, Chapel Hill, NC.
NCSU = North Carolina State University, Mountain Horticultural Crops Research and Extension Center, Fletcher, N.C.
[2]Values represent mean 2C holoploid genome size ± SEM for two samples. Values with no SEM indicate only sample was analyzed.

Table 2. Summary of means and ranges for 2C, holoploid genome size (ρg) and 1Cx monoploid genome size (pg) by subgenus and ploidy level.

	Ploidy level				
	Diploid (2x)	Triploid (3x)	Tetraploid (4x)	Hexaploid (6x)	Octoploid (8x)
Hymenanthes	2C = 1.50 ± 0.01 A (1.41-1.64) 1Cx = 0.75 ± 0.01 A (0.71-0.82)	2C = 2.17 ± 0.05 B (2.06-2.22) 1Cx = 0.72 ± 0.02 A (0.69-0.74)	2C = 3.01 ± 0.04 C (2.89-3.37) 1Cx = 0.75 ± 0.01 A (0.72-0.84)	NA	NA
Rhododendron	2C = 1.65 ± 0.05 A (1.32-1.86) 1Cx = 0.83 ± 0.02 A (0.66-0.93)	2C = 2.01 ± -- B (NA) 1Cx = 0.67 ± -- B (NA)	2C = 3.06 ± 0.05 C (2.78-3.25) 1Cx =0.77 ± 0.01 AB (0.70-0.81)	2C = 4.48 ± 0.04 D (4.39-4.61) 1Cx = 0.75 ± 0.01 AB (0.73-0.77)	5.70 ± 0.28 E (5.42-5.97) 1 Cx = 0.72 ± 0.03 AB (0.68-0.75)
Pentanthera	2C = 1.61 ± 0.01 A (1.48-1.74) 1Cx = 0.81 ± 0.01 A (0.74-0.87)	2C = 2.48 ± 0.06 B (2.30-2.60) 1Cx = 0.83 ± 0.02 A (0.77-0.87)	2C = 3.23 ± 0.02 C (3.00-3.88) 1Cx = 0.81 ± 0.00 A (0.75-0.97)	NA	2C = 6.40 ± .03 D (6.32-6.46) 1Cx =0.80 ± 0.00 A (0.79-0.81)
Tsutsusi	2C = 1.26 ± 0.01 A (1.22-1.30) 1Cx = 0.63 ± 0.01 A (0.61-0.65)	2C = 1.93 ± 0.03 B (1.88-1.98) 1Cx = 0.65 ± 0.01 AB (0.63-0.66)	2C = 2.68 ± 0.08 C (2.60-2.75) 1Cx = 0.67 ± 0.02 B (0.65-0.68)	NA	NA

[1]Values represent means ± SEM followed by (ranges) derived from Table 1. Means followed by different letter, within a row, are significantly different, $P<0.05$.

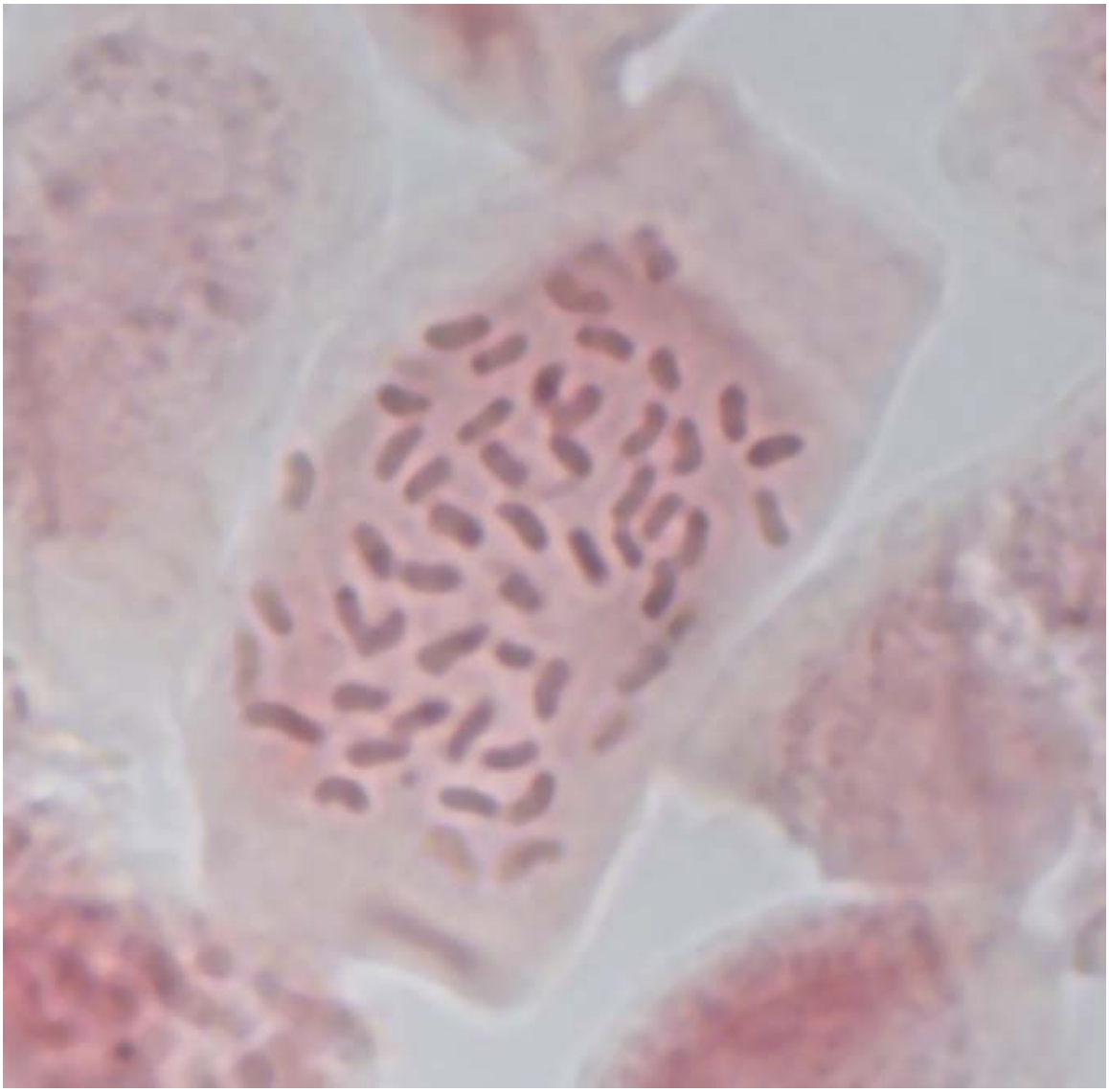

Figure 1. Photomicrograph of root tip cell of *R. austrinum* (2006-223) in prophase with 2*n* = 4x = 52 somatic chromosomes.

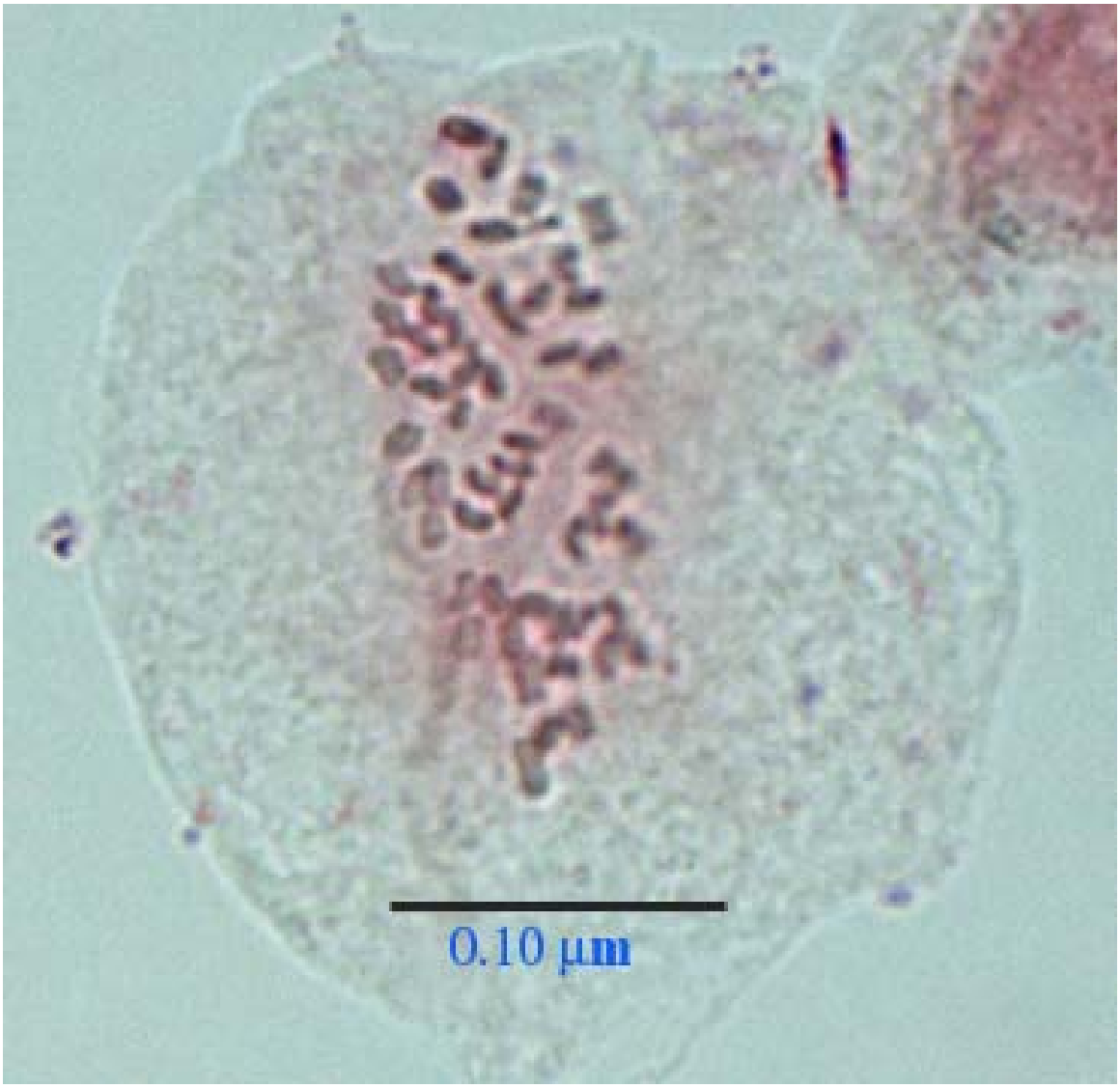

Figure 2. Photomicrograph of root tip cell of *R. atlanticum* (H2004-054-002) in prophase with $2n = 4x = 52$ somatic chromosomes.

Chapter 2

A Novel Method for Inducing Polyploidy in *Rhododendron* Seedlings

(In the format appropriate for submission to the

Journal of the American Rhododendron Society)

A Novel Method for Inducing Polyploidy in *Rhododendron* Seedlings

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Introduction

Polyploidy (having three or more complete sets of chromosomes) is relatively common in plants. By some estimates as many as 70% of all angiosperms are natural polyploids (Masterson, 1994). The importance and prevalence of polyploidy is especially evident in the genus *Rhododendron*. Polyploidy occurs naturally in many species and hybrids of *Rhododendron*, particularly in the *Rhododendron* and *Pentanthera* subgenera (Ammal et al., 1950; Jones et al., 2007).

Polyploidy is considered to be a major pathway for plant evolution and can contribute to reproductive isolation and abrupt speciation (Ramsey and Schemske, 1998; Soltis et al., 2003; Wendel, 2000). The effects of polyploidy on plant traits are also important to horticulturists and plant breeders. Ploidy levels can influence crossability, fertility of hybrids, plant vigor, and gene expression (Ranney, 2006). The induction of artificial polyploids has been utilized in the development of allopolyploids to restore fertility in sterile hybrids, enhance crossability and fertility of progeny, create seedless triploids, produce novel gene combinations, and increase the expression and diversity of secondary metabolites (Chen and

Ni, 2006; Contreras et al., 2007; Olsen et al., 2006; Soltis and Soltis, 1993; Wendel, 2000). In some cases, polyploids may also have additional desirable ornamental characteristics including thicker leaves and larger flowers with thicker petals that persist longer (Barlup, 2002; Hosoda et al., 1953; Kehr, 1996a; Leach, 1961).

Polyploidy can arise naturally through multiple pathways including spontaneous chromosome doubling in somatic meristem cells and sexual fertilization with unreduced gametes (Jones et al., 2007; Ramsey and Schemske, 1998; Widrlechner et al., 1982). Polyploidy can also be induced through the use of various chemical doubling agents (mitotic inhibitors) (Contreras et al., 2007; Sanford, 1983; van Tuyl, 1992). Colchicine (N-(5,6,7,9-tetrahydro-1,2,3,10-tetra-methoxy-9-oxobenzo(a)heptalen-7-yl)acetamide) was first discovered as an effective mitotic inhibitor in 1937 and has been extensively utilized for inducing polyploidy in a wide range of species (Eigsti and Dustin, 1955; Hancock, 1997). The dinitroaniline herbicide oryzalin (3,5-dinitro-N4,N4-dipropylsufanilamide) has also been effectively utilized as a doubling agent and is considerably less toxic than colchicine (van Tuyl, 1992). Both agents have a similar mode of action; inhibiting microtubule polymerization and arresting mitosis at metaphase thus preventing the replicated chromosomes from separating into daughter cells. When mitosis resumes, a lineage of polyploidy cells with double the normal chromosome number can be established. Studies comparing the effectiveness of colchicine versus oryzalin as induction agents have produced mixed results. Oryzalin is more effective at lower dosages than colchicine due to a higher specificity for tubulin binding sites in plant material (Eeckhaut et al., 2001; Eiselein, 1994; Morejohn et al., 1987; van Tuyl, 1992). Undesirable side effects of colchicine, including

sterility, abnormal growth, and deformed tissue can be avoided when using oryzalin (Bouvier, 1994; van Tuyl, 1992). Eeckhaut et al. (2001) found that *in-vitro* treatment of seedlings of rhododendron with 0.05% and 0.25% colchicine had no effect on ploidy while treatment with 0.01% and 0.05% oryzalin yielded some polyploids and numerous cytochimeras. Väinölä (2000) compared the efficacy of colchicine (0.025% or 0.05%) and oryzalin (0.001% or 0.005%) for 24 or 48 hour durations on chromosome doubling in *Rhododendron* seedlings. Plant survival was higher with colchicine, but oryzalin was more efficient in the induction of polyploidy (18% of the surviving plants at 0.005% with the 24 hr exposure). The use of mitotic inhibitors often produces polyploids that are cytochimeras (mixaploids) whereby different cells or histogenic layers vary in ploidy level (Pratt, 1983; Pryor and Frazier, 1968; Väinölä, 2000). The ploidy of the LII histogenic layer is crucial to breeding as this layer gives rise to reproductive tissue (Pratt, 1983; Ranney, 2006).

Polyploidy has been induced in many woody ornamental plant genera such ×*Chitalpa* (Olsen et al., 2006), *Citrus* (Lee, 1988), *Rosa* (Semeniuk and Arisumi, 1968), *Prunus* (Dermen, 1953), and *Pyrus* (Kadota and Niimi, 2002). Attempts to induce polyploidy in rhododendrons, through an assortment of methods both *ex-vitro* and *in-vitro,* have been met with varying degrees of success (Contreras et al., 2007; Eeckhaut et al., 2001; Eiselein, 1994; Kehr, 1996b; Paden, 1990; Pryor and Frazier, 1968; Sakai, et al., 2004; Väinölä, 2000). Mitotic inhibitors only affect actively dividing cells; therefore, prolonged contact with the apical meristem is crucial for inducing polyploidy, yet over-exposure results in death (Kehr, 1996a). Pryor and Frazier (1968) successfully applied colchicine to actively growing shoots to obtain tetraploid azaleas. Kehr (1996b) developed a protocol for misting seedlings with

colchicine after the cotyledons developed but before the first true leaves were evident; however, efficacy of the treatment was never determined. Contreras et al. (2007) developed an allotetraploid form of *Rhododendron* 'Fragrant Affinity' by submerging actively growing shoots tips in 150μM oryzalin for 24 hours. Eiselein (1994) compared single applications of 1% colchicine for 0, 24, 48, 72, and 96 hours with repeated applications of a 24 hour exposure, interrupted by 2-5 day recovery periods, totaling 96 hours of treatment. Percentage of tetraploids (determined on root tips - no data presented on shoots) with the single treatments averaged approximately 20% with no effect of duration, while the repeated applications resulted in a 79% conversion rate. The higher conversion rate for the repeated applications may have resulted from impacting a greater number of cells in metaphase, over time, while allowing for periodic recovery periods (Eiselein, 1994).

Confirmation of ploidy levels in treated seedlings is essential to determine efficacy of these techniques. Although determination of ploidy level in *Rhododendron* by counting chromosomes is possible, the chromosomes of *Rhododendron* are small and particularly difficult to view and discern using standard cytological techniques (Eiselein, 1994; Tolstead and Glencoe, 1991). Flow cytometry provides a fast and efficient method for determining relative genome size and associated ploidy level of *Rhododendron* (De Schepper et al., 2001; Eeckhaut et al., 2004; Jones et al., 2007). An additional advantage of flow cytometry for evaluating efficacy of mitotic inhibitors is the ability to sample thousands of cells and also determine the presence of cytochimeras (De Schepper et al., 2001; Jones et al., 2007).

The objectives of this project were to 1) develop a simple and effective, *ex-vitro* method for inducing polyploidy in *Rhododendron* seedlings, 2) evaluate the effectiveness of using repeated treatments of an oryzalin suspension in a warm agar solution applied directly to apical shoots of *Rhododendron* seedlings to induce polyploidy, and 3) develop a population of new polyploid rhododendrons and azaleas for use in future breeding projects.

Materials and Methods

Controlled pollinations were completed to produce new hybrids with desirable ornamental characteristics for use in this study. All parents were confirmed diploids (Jones et al, 2007). Seeds were obtained from the following crosses:

1) *R.* 'Summer Lyric' (*R. prunifolium* × *R. arborescens*) [pollinated with either *R.* 'Millennium' (*R.* 'Weston's Sparkler' x *R.* 'Weston's Parade') or *R.* 'August Beauty'(*R. prunifolium* × *R. arborescens*)] with the goal of producing tetraploid deciduous azaleas (subgenus *Pentanthera*) with fragrant flowers, a range of flower colors, and late-season flowering.

2) *R.* 'Cheyenne' (*R.* 'Jalisco' × *R.* Loderi Group) × *R.* 'Capistrano' (*R.* 'Hindustan' × {{[*R. catawbiense* × (*R. fortunei* ssp. *discolor* × *R.* Fabia Group)] × (*R.* 'Russell Harmon' × *R.* 'Goldsworth Orange')} × *R.* 'Golden Gala'}) with the goal of producing a tetraploid elepidote rhododendron (subgenus *Hymenanthes*) with fragrant, yellow flowers.

3) *R.* 'Kimberly' (*R. williamsianum* × *R. fortunei* ssp. *fortunei*) × *R.* 'Nestucca' (*R. fortunei* ssp. *fortunei* × *R. degronianum* ssp. *yakushimanum*) with the goal of producing a tetraploid elepidote rhododendron (subgenus *Hymenanthes*) with a compact habit, good cold hardiness, and fragrant flowers.

Seedlings from each cross were germinated in five separate pots with approximately 100 seeds per pot. When seedlings were at the cotyledon stage, all of the plants (subsamples) in an individual pot were either treated with 1, 2, 3, or 4 applications of oryzalin separated by 4 day intervals or left untreated (control). The preemergent herbicide Surflan® A.S. (40.4% oryzalin) was diluted to produce a suspension containing 50μM oryzalin with 5.5g/L agar at 50 °C. Concentrations of oryzalin and agar were based on preliminary studies (data not presented). A single drop (2-4 μL) of warm (~40 °C) oryzalin suspension was then pipetted on top of the cotyledons of each seedling to cover the emerging shoot. Pots were placed in a high humidity (approximately 100% relative humidity) growth chamber at 23ºC under constant light to preserve the integrity of the agar droplet. Subsequent applications were made after the 4 day interval. After treatment, plants were grown under standard greenhouse conditions prior to analysis. The experimental design was completely randomized. Flow cytometry was utilized to determine ploidy levels approximately 3 months after treatment using methods described by Jones et al. (2007). Data on percent death and ploidy level were subjected to regression analysis (PROC REG; SAS version 8.02, SAS Institute., Cary, N.C.; SAS Institute, 1988).

Results and Discussion

The semi-solid agar appeared to be an effective medium for applying oryzalin to the apical shoots of rhododendron seedlings (Figure 1). The agar drop typically rested on the cotyledons and solidified around the elongating shoots, thus allowing for sufficient contact between the oryzalin and the meristem. Drops persisted for 2 to 3 days before deteriorating. There were no visual phytotoxic symptoms over the treatment periods.

Treatment of 'Summer Lyric' seedlings resulted in a broad range of ploidy levels including mixaploids (Table 1). Induction percentages for the different classes of polyploids varied as a function of number of applications. The percentage of homogeneous tetraploids (of primary interest) followed a quadratic trend in response to increasing number of applications with the highest percentage, 41%, resulting after two successive applications of oryzalin. A few higher level polyploids, including octoploids, and mixaploids were also recovered as the number of applications increased. Although a quadratic response was significant for seedling death, the increasing number of applications did not increase death over that of the control. Overall, it appeared that 2 to 3 applications were ideal for inducing tetraploids.

A few polyploids resulted from the oryzalin treated *R.* 'Cheyenne' × 'Capistrano' hybrids (Table 2). Mixaploids increased with number of applications, while there was no significant trend for tetraploids. The percentage of dead plants increased linearly with increasing application number, suggesting a sensitivity to oryzalin. Oryzalin treatments did result in some polyploids, but it remains unclear if there was any benefit from multiple applications to induce tetraploid plants.

Among the *R.* 'Kimberly' × 'Nestucca' seedlings, oryzalin treatment resulted in a range of polyploids including tetraploids, several octoploids, and three classes of mixaploids (Table 3). Diploids decreased linearly with each additional application, and conversely, the percentage of 2x+4x mixaploids and solid tetraploids increased linearly. The octoploid, higher level mixaploids, and death percentages were random in their distribution with no significant trend. For induction of tetraploids, 2-4 applications were optimal.

The shoot apical meristem is comprised of zones. The central zone includes a group of cells at the distal end of the meristem. These cells function as intitial cells that give rise to other cells, other regions of the meristem and ultimately the shoot (Francis, 1997; Kerstetter and Hake, 1997; Tax and Durbank, 2006). Within the central zone lay multiple histogenic layers: L1, L2, and L3, that are distinct and give rise to separate cell lines and tissues (Hudson and Goodrich, 1997). Development of homogeneous polyploids requires the successful doubling of the initial cells, in all histogenic layers, within the central zone of the apical meristem. In constrast, mixaploids appeared to be a conglomeration of cells of varying ploidy levels, among or within the histogenic layers, resulting from incomplete doubling of initial cells within the meristem. As suggested by Eiselein (1994), only a certain percentage of meristematic cells may be affected by any single application of a mitotic inhibitor. Pryor and Frazier (1968) also observed mixaploids following a single application of colchicine on evergreen azaleas. Poor penetration or asynchronous cell cycling within the meristem could result in only partial doubling of the meristem. A gradient of cell size, relative growth rates and cell cycling times can exist within a meristimatic zone (Francis, 1997). Because the cell cycle is not synchronized among all the cells in the meristem,

multiple applications may induce polyploidy in different populations of cells. In some cases, e.g., 'Summer Lyric' and 'Kimberly ×'Nestucca' seedlings, increasing the number of applications (from 2 to 3 or 1 to 2, respectively) increased the number of homogeneous tetraploids. Thus, repeated applications over time most likely allowed for doubling of different initial cells during several asynchronous cell cycles.

Stability of the mixaploids developed here and the specific nature of their chimeral arrangement is uncertain. If all the cells in an individual histogenic layer are uniformly one ploidy level, e.g., a periclinal chimera, the confirmation may be more stable. Limited sampling two months after initial testing revealed that many of the higher level mixaploids eventually reverted to their lower ploidy level (data not presented). Väinölä (2000) reported similar results in which one third of the induced mixaploids shifted to diploidy. If the meristem is composed of a mosaic of cells with different ploidy levels mixed within histogenic layers, some cell types may multiply faster (those cells of lower ploidy) and effectively overrun the other cell type (those of higher ploidy) in a phenomenon known as diplontic selection (Broertjes and Keen, 1980; Pratt, 1983). Cell types of higher DNA content typically take longer to cycle through mitosis (Singh, 1993) and selection will then favor reversion to the faster proliferating, lower ploidy level, cytotype. The higher level polyploids (e.g., octoploids) likely resulted from mitotic inhibition of multiple cell cycles whereby diploid meristematic cells became tetraploid, and those tetraploid cells were doubled again to become octoploid. Such occurrences have been previously noted in polyploid induction of apple (Tilney-Bassett, 1986).

The results of this study demonstrate that the method of applying a suspension of oryzalin in warm, semi-solid agar to the shoots of *Rhododendron* seedlings is an effective method for inducing polyploidy. Although single applications resulted in some polyploid plants, multiple applications increased efficacy for some of the taxa studied. Polyploid plants developed in this study will be further evaluated for desirable traits and incorporated into an ongoing rhododendron breeding program.

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Table 1. Ploidy levels and death of seedlings from *Rhododendron* 'Summer Lyric' following treatment of apical shoots with 0, 1, 2, 3, or 4 applications of 50µM oryzalin in agar separated by 4 day intervals.

	Number of Applications					
Ploidy	**0**	**1**	**2**	**3**	**4**	**Trend**
2x	89[Z]	59	24	31	19	Q[Y]***; R^2=0.95
2x + 4x	0	21	26	26	31	Q**; R^2=0.92
4x	0	12	41	33	24	Q***; R^2=0.86
4x + 8x	0	0	4	0	8	L[X]**; R^2=0.49
8x	0	0	1	2	0	NS[W]
2x + 8x	0	0	1	0	5	L**; R^2=0.53
2x + 4x + 8x	0	0	0	0	3	Q*; R^2=0.85
Dead	11	8	2	9	10	Q*; R^2=0.66

[Z]Data in percent.
[Y]Q=quadratic trend.
[X]L=linear trend.
[W]NS=trend not significant.
*significant, $P \leq 0.10$
**significant, $P \leq 0.05$
***significant, $P \leq 0.01$

Table 2. Ploidy levels and death of *Rhododendron* 'Cheyenne' × *R.* 'Capistrano' seedlings following treatment of apical shoots with 0, 1, 2, 3, or 4 applications of 50µM oryzalin in agar separated by 4 day intervals.

	Number of Applications					
Ploidy	**0**	**1**	**2**	**3**	**4**	**Trend**
2x	69[Z]	59	24	31	19	Q[Y]***; R^2=0.88
2x + 4x	0	8	7	6	2	Q**; R^2=0.88
4x	0	2	7	8	4	NS[W]
Dead	31	68	65	68	80	L[X]***; R^2=0.70

[Z]Data in percent.
[Y]Q=quadratic trend.
[X]L=linear trend.
[W]NS=trend not significant.
*significant, $P \leq 0.10$
**significant, $P \leq 0.05$
***significant, $P \leq 0.01$

Table 3. Ploidy levels and death of *Rhododendron* 'Kimberly' × *R.* 'Nestucca' seedlings following treatment of apical shoots with 0, 1, 2, 3, or 4 applications of 50µM oryzalin in agar separated by 4 day intervals.

	Number of Applications					
Ploidy	**0**	**1**	**2**	**3**	**4**	**Trend**
2x	67[Z]	30	43	33	20	L[X]***; R^2=0.67
2x + 4x	0	6	9	11	11	L**; R^2=0.85
4x	0	4	12	11	12	L***; R^2=0.81
4x + 8x	0	0	0	1	1	NS[W]
8x	0	0	0	4	0	NS
2x + 8x	0	0	0	0	1	NS
Dead	33	60	36	40	55	NS

[Z]Data in percent.
[X]L=linear trend.
[W]NS=trend not significant.
*significant, $P \leq 0.10$
**significant, $P \leq 0.05$
***significant, $P \leq 0.01$

Figure 1. Oryzalin treatment of hybrid *Rhododendron* seedling.

Chapter 3

Fertility of Neopolyploid *Rhododendron* and Occurrence of Unreduced Gametes in Triploid Cultivars

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

Fertility of Neopolyploid *Rhododendron* and Occurrence of Unreduced Gametes in Triploid Cultivars

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Introduction

Polyploidy, defined as an organism with three or more complete sets of chromosomes, is common in plants and widely recognized as an important mechanism of adaptation and speciation (Ramsey and Schemske, 2002; Soltis et al., 2003; Wendel, 2000). The prevalence of polyploidy in the genus *Rhododendron* has previously been documented (Jones et al., 2007). In some cases, polyploid plants, including *Rhododendron*, can have desirable features including enhanced vigor, thicker leaves, and larger flowers with thicker petals that persist longer (Kehr, 1996). Polyploidy can also have a profound influence on reproductive biology, including fertility (Allard, 1960; Ramsey and Schemske, 1998). As such, a greater understanding of reproductive behavior of polyploids is valuable to plant breeders.

The potential for utilizing polyploids in a breeding program is dependent upon fertility of specific taxa. The origin of polyploids can be a major factor in determining fertility. If a polyploid arises from somatic doubling in a meristem (spontaneous endoreduplication or chemical induction) or from the union of unreduced gametes from two

closely related diploid parents, it will have four similar (homologous) versions of each chromosome and is often referred to as an autotetraploid (or polysomic tetraploid) (Ramsey and Schemske, 1998; Sanford, 1983). Autopolyploids may lack fertility due to the presence of multiple homologous chromosomes that can result in multivalent pairing and unequal segregation in meiosis (Ranney, 2006; Riesberg, 2001; Stebbins, 1950). Polyploids that result from somatic doubling in a hybrid or from unreduced gametes from **different species** are referred to as allopolyploids (or sometimes amphidiploids or disomic polyploids). Allopolyploids are often fertile due to nonrandom, disomic pairing between two distinct sets (pairing among chromosomes derived from each parental species, but not between species) of chromosomes during meiosis (Ramsey and Schemske, 2002; Ranney, 2006). In many cases, however, polyploids fall somewhere between an autopolyploid and an allopolyploid; where there is partial chromosome homology resulting in a combination of disomic and polysomic pairing; these polyploids are referred to as segmental allopolyploids. Surveying a broad range of plant species, Ramsey and Schemske (2002) noted that fertility varied considerably within both neoauto- and neoallopolyploids (newly formed polyploids), emphasizing the need to determine fertility on a plant-by-plant basis.

Triploids are often found to be highly infertile, if not sterile (Allard, 1960; Ramsey and Schemske, 1998). Infertility of triploids results from the fact that three sets of chromosomes cannot be divided evenly during meiosis yielding unequal segregation of the chromosomes often resulting in aneuploid gametes or meiotic failure. A number of triploid ($2n = 3x = 39$) *Rhododendron* have been documented. De Schepper et al. (2001) confirmed triploidy in ‘Euratom’, ‘Euratom Orange’, and ‘Red Wing’, and Jones et al. (2007) found

'Hallelujah', 'Taurus', 'White Ruffles', and 'Crimson Majesty' to be triploids. However, little is known about the fertility of triploid *Rhododendron*.

Although triploids are often infertile, meiotic restitution can circumvent this limitation resulting in functional, unreduced gametes (Dweikat and Lyrene, 1988; Ramsey and Schemske, 1998). Triploids that produce unreduced gametes can be utilized as bridges for the development of tetraploids by crossing them back with diploids (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998). Moreover, hexaploids may also be produced by intercrossing among triploids that produce viable unreduced gametes (Ehlenfeldt and Vorsa, 1993). Frequencies of unreduced gamete production are highly variable, may differ considerably among individuals, and vary with environmental factors such as temperature (Bretagnolle and Thompson, 1995; Dweikat and Thompson, 1988; Ramsey and Schemske, 1998). Unreduced pollen has previously been documented within the genus in diploid clones including the elepidote *R.* 'Hexe' (De Schepper et al. 2001), evergreen azaleas (Eeckhaut et al., 2006), and deciduous azaleas with hybrids involving *R. kosterianum* and *R. prinophyllum* displaying a range of unreduced pollen production from 0 – 21% (Widrlechner et al., 1982). Circumstantial evidence also supports the role of unreduced gametes in the formation of various polyploid cultivars (Jones et al., 2007; Li, 1957; Widrlechner et al., 1982; Willingham, 1976). Unreduced gametes may also be more prevalent in triploids than in diploids (Dweikat and Lyrene, 1988; Veilleux, 1985). Examination of the fertility of specific clones is therefore necessary to determine the usefulness of incorporating triploids into a breeding program.

The pollen structure in *Rhododendron* and many *Ericaceous* species is typically a tetrad of grains that are tightly grouped at maturity (Copenhaver, 2005; Widrlechner et al., 1982). However, the pollen of unreduced gamete producers is often a mix of sporads containing tetrads, dyads, and monads (Ortiz et al., 1992). The dyad grain structure is representative of 2*n* (the somatic chromosome complement) pollen that results from irregularities of the first or second meiotic division (Bretagnolle and Thompson, 1995; Widrlechner et al., 1982). Unreduced pollen grains are also typically larger than reduced pollen grains (Bretagnolle and Thompson, 1995; Widrlechner et al., 1982). Pollen staining, using acetocarmine, can be used to assess male fertility and reflects the potential for pollen to germinate and contribute to fertilization (Dafni and Firmage, 2000). Acetocarmine stains nuclei within the cells and has been utilized previously in studies of *Rhododendron* pollen (Contreras et al., 2007; Sakai et al., 2004).

The objectives of this project were to: 1) evaluate the effect of increased ploidy level on pollen fertility of selected *Rhododendron* and 2) evaluate pollen fertility of naturally occurring triploids found in the genus.

Materials and Methods

Comparing fertility between ploidy levels. Newly formed polyploids and their progenitor taxa were chosen to compare the fertilities between ploidy levels within the same genotype. The ploidy levels of all plants were determined by Jones et al. (2007) (Table 1.). The tetraploid *R.* 'Super Nova' and the octoploid *R.* 'Fragrant Star' were chemically induced

at Briggs Nursery, Olympia, Wash. from *R.* 'Nova Zembla' and *R.* 'Snowbird', respectively (Dan Meier, Olympia, Wash., per. comm.). *Rhododendron* 'Vulcan' tetraploid is a 2x + 4x mixaploid (Jones et al., 2007) and the result of natural somatic endoreduplication in a shoot of *R.* 'Vulcan' (Harold Greer, Eugene, Ore., per. comm.). *Rhododendron* 'Briggs Red Star' is also a 2x + 4x mixaploid (Jones et al., 2007) developed at Briggs Nursery through the chemical induction of *R.* 'The Honourable Jean Marie de Montague'. Both mixaploids appear to be tetraploid in the LII histogenic layers since they breed as tetraploids (Dr. Thomas G. Ranney, N.C. State University, NC, per. comm.) and gametogenesis occurs within the LII layer (Pratt, 1983; Ranney, 2006). *Rhododendron* 'Fragrant Affinity Tetra' was chemically induced from *R.* 'Fragrant Affinity' (Contreras et al., 2007). Dr. Max Byrkit chemically induced *R. fortunei* to produce the tetraploid form (Kehr, 1996). *Rhododendron* 'Northern Starburst' and *R.* 'Bubblegum' are both chemically induced tetraploids of *R.* 'PJM' and *R.* 'Weston's Aglo', respectively, developed at Briggs Nursery (Dan Meier, Olympia, Wash., per. comm.).

Pollen viability was determined using staining procedures similar to those described by Contreras et al. (2007) and Olsen et al. (2006). All pollen was collected at anthesis from plants at the Mountain Horticultural Crops Research Station, dried at ~21ºC for 24 hrs., and stored at -25ºC until testing. Pollen was placed on glass microscope slides, and the grains were stained with 1% acetocarmine (w/v) for 15 minutes. Pollen grains that stained a distinct red-pink color were scored as viable (Dafni and Firmage, 2000; Ramsey and Schemske, 2002). The tetrad nature of *Rhododendron* pollen required each individual grain in the tetrad to be analyzed. Each tetrad has the potential to contain four viable grains. The experimental

design was a randomized complete block with ten replicates blocked over time. A minimum of 50 tetrads were randomly selected and analyzed per replicate. Pollen was observed at 300× using a light microscope (Nikon Eclipse 80i, Nikon, Melville, NY). Pollen viability percentages were calculated and the data were subjected to analysis of variance and pairwise means comparisons between ploidy levels for a given genotype (LSMEANS option, PROC GLM; SAS version 8.02, SAS Institute., Cary, N.C.; SAS Institute, 1988).

Triploid fertility. The cultivars *R.* 'Hallelujah', *R.* 'Red Wing', and *R.* 'Taurus' were confirmed as triploids by Jones et al. (2007). Recent analysis also determined that the azaleodendrons (crosses of an unnamed 'Ilam' azalea by the evergreen rhododendron 'Catalga' provided by Dr. Steven Krebs at the Holden Arboretum, Kirtland, Ohio) 94-28/2, 94-28/3, 94-28/7, and 94-28/14 were also triploids (Krebs et al., in preparation). Pollen was collected at anthesis, and the frequency of viable, unreduced gametes in the triploid taxa was determined using pollen staining as described above and observed under a light microscope at 300×. The experimental design was a randomized complete block with 5 replicates blocked over time. At least 50 sporads (tetrads, dyads, or monads) were randomly selected and analyzed per replicate. Pollen was considered viable and unreduced if there was a well-stained monad or dyad and the pollen diameter was visibly larger (>120%) than normal (Shoemaker-Megalos and Ballington, 1988). The frequency of unreduced gametes was determined using the equation from Ortiz et al. (1992):

Unreduced pollen frequency = [(2 × # of dyads) + (# of monads)] / (# of total grains).

Data were then subjected to analysis of variance and means compared using least significant differences (LSD) (PROC GLM; SAS version 8.02, SAS Institute., Cary, N.C.; SAS Institute, 1988).

Results and Discussion

Comparing fertility between ploidy levels. Pollen grains were readily apparent as being stained or unstained (Figure 1.). Pollen viabilities for all taxa ranged from 1.5 – 63.8 % (Table 1). There was a significant effect of ploidy level (P<0.001), genotype (P<0.0001), and a ploidy-genotype interaction (P<0.0001) on fertility. The significant interaction indicated the effect of polyploidy on fertility depended on genotype.

In the genotypes, *R. fortunei*, 'The Honourable Jean Marie de Montague', and 'Snowbird', the increased ploidy level reduced fertility. *Rhododendron fortunei* Tetraploid is an induced autotetraploid and its reduced fertility most likely results from problems associated with polysomic chromosome pairing commonly found in autotetraploids. The diploid *R.* 'The Honourable Jean Marie de Montague' is a hybrid of unknown parentage believed to involve *R. griffitianum*. The reduced fertility of the tetraploid *R.* 'Briggs Red Star' suggests that it functions more like an autotetraploid or segmental allopolyploid and the increased ploidy level compromised gamete formation. *Rhododendron* 'Snowbird' is a naturally occurring tetraploid believed to be a hybrid between *R. atlanticum* and *R. canescens* (Galle, 1987); most likely the result of a normally reduced gamete from *R. atlanticum* combined with an unreduced gamete from *R. canescens.* The induced octoploid *R.* 'Fragrant Star' would thus be considered an autoallopolyploid, that contains four homologous

chromosomes from each species, which may still result in polysomic chromosome pairing leading to reduced fertility.

In the genotypes, *R.* 'PJM', 'Weston's Aglo', and 'Fragrant Affinity', the increased ploidy level significantly increased fertility. Both *R.* 'PJM' and 'Weston's Aglo' are interspecific hybrids between *R. minus* x *R. dauricum*. The induced polyploids of these taxa, *R.* 'Northern Starburst' and 'Bubblegum', represent allopolyploids, and the increased fertility most likely results from restoration of chromosome homology and improved disomic pairing. The increased fertility of *R.* 'Fragrant Affinity Tetra' over 'Fragrant Affinity' represents a similar case as previously described by Contreras et al. (2007).

For the remaining genotypes, *R.* 'Nova Zembla' and *R.* 'Vulcan', increased ploidy level had no apparent effect on fertility. Both of these genotypes are complex hybrids and the induced tetraploids most likely function as segmental allopolyploids with moderate to high fertility, regardless of ploidy level.

These results demonstrate that fertility of polyploid *Rhododendron* can be highly variable and that induced polyploidy may either enhance or compromise fertility. The effect of polyploidy on fertility most likely results from the level of homology among the chromosome sets and subsequent impacts on chromosome pairing during meiosis. Information on the fertility of specific cultivars will assist breeders in utilizing these plants in future breeding programs.

Triploid fertility. Viable dyad and monad grains (Figures 2 and 3) were observed in pollen samples from triploid taxa, ranging from 0.2 to 5.3% (Table 2), indicating the

presence of unreduced pollen. The increased size of unreduced pollen diameter was also clearly evident (Figure 3.). As in other triploid *Ericaeous* taxa (Ehelnfeldt and Vorsa, 1993; Vorsa and Ballington, 1991), the small percentage of viable stained pollen was largely limited to monad or dyad grains. There was a significant effect ($P<0.0001$) of taxa on the percentage of unreduced pollen. *Rhododendron* 'Red Wing' had the highest percentage of unreduced pollen at 5.3%, followed by *R.* 'Hallelujah' at 2.9%, while the remaining taxa were similar with less than 1.1% unreduced gametes. In studies of *Vaccinium* spp., also in the *Ericaceae*, significant differences in frequencies of unreduced pollen among taxa have also been reported (Ortiz et al., 1992; Shoemaker-Megalos and Ballington, 1988).

Dyad pollen grains are typically expected to be 2*n* and can result from a variety of meiotic irregularities including first or second division restitution (Bretagnolle and Thompson, 1995; Islam and Shepherd, 1980; Widrlechner, 1982). Monad pollen grains could result from different pathways including double restitution (4*n*) or the combination of a restitution event and abnormal nuclear fusion of individual grains producing a 2*n* monad (Bretagnolle and Thompson, 1995; Veilleux, 1985).

The production of unreduced pollen by triploid taxa indicates the potential for utilizing certain taxa in breeding programs. The greater frequency of unreduced pollen found in *R.* 'Red Wing' and 'Hallelujah' may allow for successful hybridizations given adequate numbers of pollinations. Additional breeding studies with triploid parents would help elucidate the level of functional unreduced pollen and the presence of unreduced female gametes (Ehlenfeldt and Vorsa, 1993).

Overall, the influence of polyploidy on fertility in *Rhododendron* is highly variable and appears to be influenced by the ploidy level, degree of homology among chromosomes, and in the case of triploids, the frequency of unreduced gamete formation. A greater understanding of fertility mechanisms in polyploid *Rhododendron* and information on fertility of specific clones will better allow breeders to incorporate and utilize polyploids in plant improvement programs.

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Table 1. Pollen viability of polyploid *Rhododendron* and progenitor taxa.

Taxa	Genotype	Ploidy Level	Viability (%)[1]	Contrast[2]
'Nova Zembla'	1	2*x*	33.4 ± 1.8	NS[3]
'Super Nova'	1	4*x*	30.4 ± 2.5	
'Vulcan'	2	2*x*	48.0 ± 1.3	NS
'Vulcan' Tetraploid	2	4*x*	46.5 ± 6.4	
'Snowbird'	3	4*x*	63.8 ± 3.5	*P*<0.0001
'Fragrant Star'	3	8*x*	16.4 ± 1.5	
'Fragrant Affinity'	4	2*x*	1.5 ± 0.2	*P*<0.0001
'Fragrant Affinity Tetra'	4	4*x*	19.9 ± 2.3	
fortunei	5	2*x*	47.4 ± 4.0	*P*<0.0001
fortunei Tetraploid	5	4*x*	7.7 ± 1.8	
'PJM'	6	2*x*	31.6 ± 1.4	*P*<0.0001
'Northern Starburst'	6	4*x*	47.0 ± 1.3	
'The Honourable Jean Marie de Montague'	7	2*x*	28.7 ± 2.8	*P*<0.0001
'Briggs Red Star'	7	4*x*	11.0 ± 1.1	
'Weston's Aglo'	8	2*x*	19.6 ± 0.9	*P*<0.0001
'Bubblegum'	8	4*x*	58.8 ± 3.2	

[1]Values represent means ± SEM for 10 replications.
[2]Contrast represents LSD, $_{0.05}$ mean separations between common (highlighted) genotypes of different ploidy levels.
[3]NS = Not significant.

Table 2. Percent unreduced gametes in selected triploid *Rhododendron* taxa.

Taxa	Viable Unreduced Gametes (%)[1]
'Hallelujah'	2.87 ± 0.55 B
'Red Wing'	5.31 ± 0.81 A
'Taurus'	1.09 ± 0.13 C
'White Ruffles'	0.65 ± 0.19 C
Azaleodendron 94-28/2	0.45 ± 0.19 C
Azaleodendron 94-28/3	0.98 ± 0.27 C
Azaleodendron 94-28/7	0.60 ± 0.23 C
Azaleodendron 94-28/14	0.23 ± 0.01 C

[1]Values represent means ± SEM. Means followed by a different letter are significantly different at $P<0.05$.

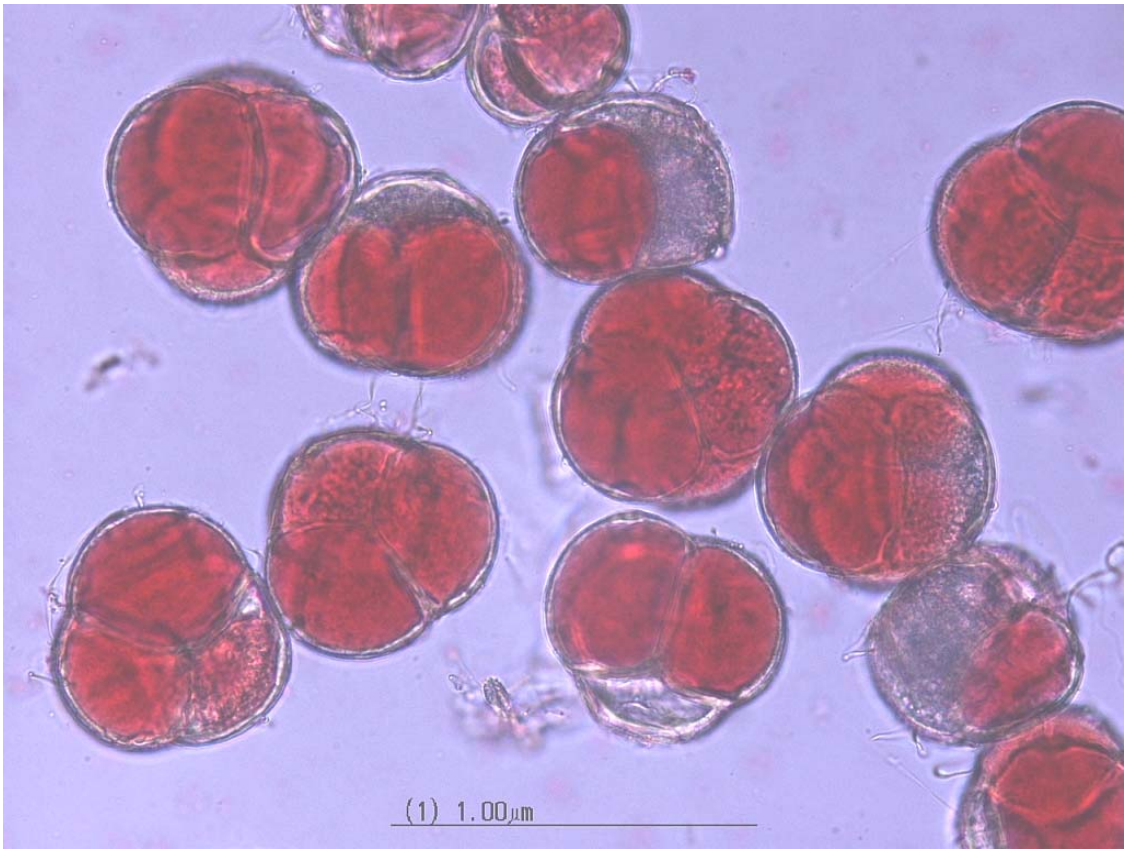

Figure 1. Photomicrograph of tetrad pollen grains of the tetraploid *Rhododendron* 'Northern Starburst'

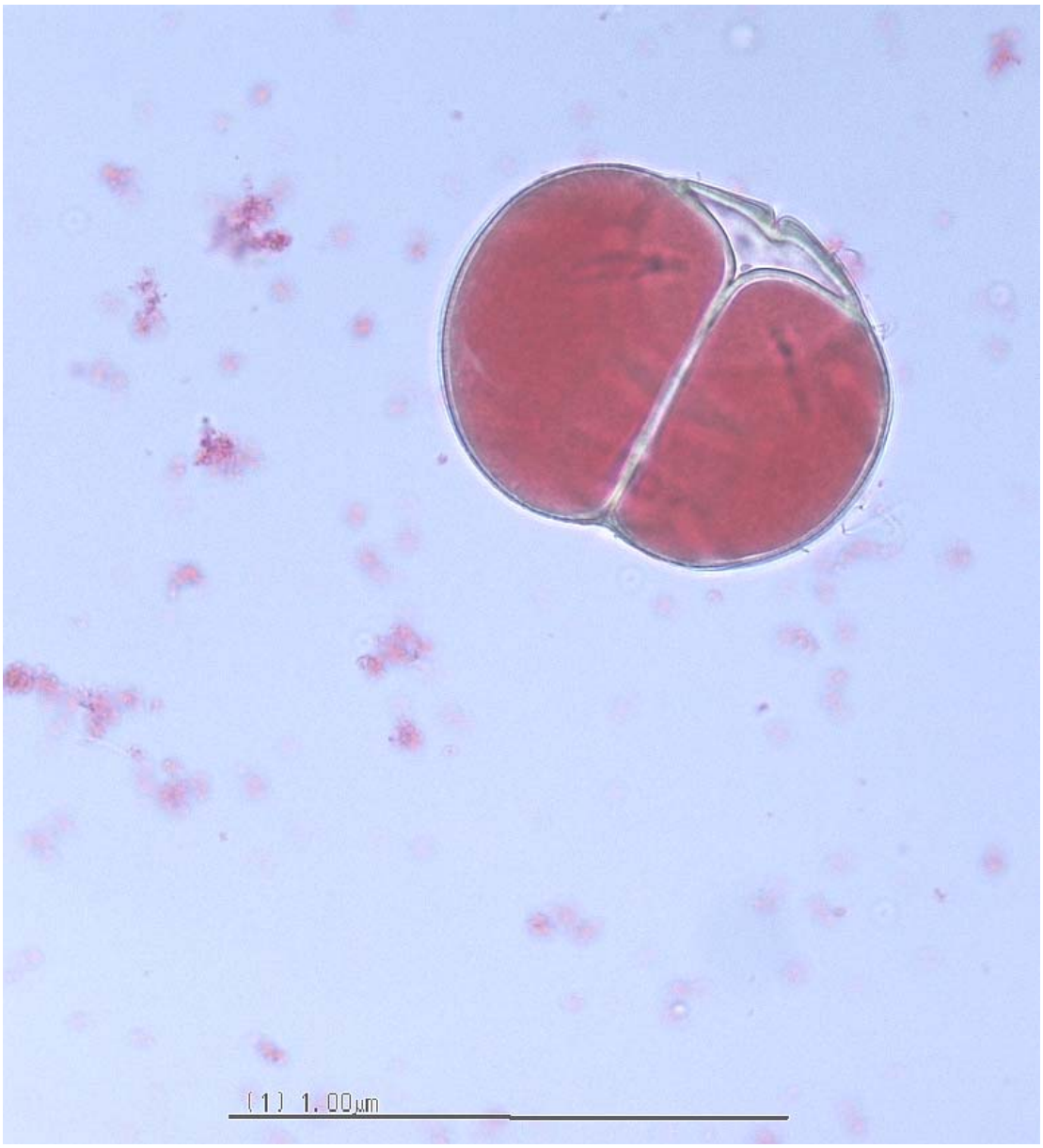

Figure 2. Photomicrograph of dyad pollen grain of the triploid *Rhododendron* 'Red Wing'

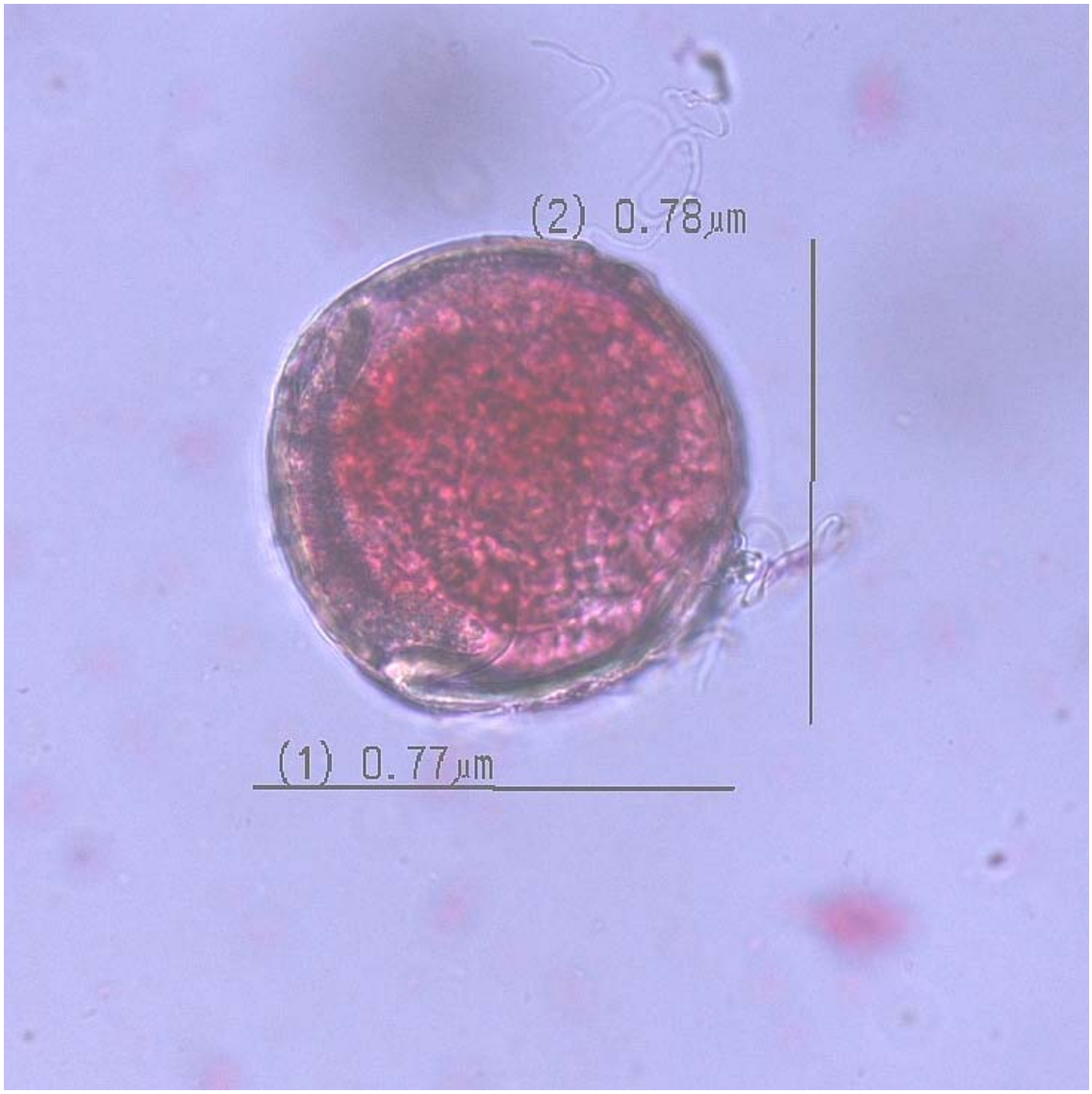

Figure 3. Photomicrograph of monad pollen grain of the triploid *Rhododendron* 'Hallelujah' illustrating size dimensions.

Chapter 4

Vegetative Propagation of *Rhododendron austrinum* (Small) Rehder and *Rhododendron flammeum* (Michx.) Sarg. by Stem Cuttings

(In the format appropriate for submission to the

Journal of Environmental Horticulture)

Vegetative Propagation of *Rhododendron austrinum* (Small) Rehder and *Rhododendron flammeum* (Michx.) Sarg. by Stem Cuttings

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Abstract: Eastern North American deciduous azaleas (*Rhododendron* L.) offer diverse flower colors and bloom times combined with a fine to medium foliage texture, which imparts a subtle, natural beauty to the landscape. The presence of these natives in the nursery trade, however, can be limited by the lack of propagation and production protocols. Therefore, the objective of this study was to use a combination of severe winter pruning (hedging) of stock plants, collection of either softwood or semi-hardwood stem cuttings, and a range of auxin concentrations to improve the rooting percentage and root system size (measured as the length × width of the overall root system) of stem cuttings *of Rhododendron austrinum* (Small) Rehder and *Rhododendron flammeum* (Michx.) Sarg. Stem cuttings were dipped for 3 s in 0, 2500, 5000, 7500, or 10,000 ppm of the potassium salt of Indole-3-butyric acid (K-IBA). In both species, rooting percentages were higher for softwood stem cuttings than for semi-hardwood stem cuttings. Cuttings rooted without the use of rooting hormones, but the utilization of 7,500-10,000 ppm K-IBA significantly increased the root system size of both species.

Cuttings from hedged stock plants rooted at higher percentages and possessed larger root system sizes compared to the unhedged counterparts in *R. flammeum,* but the effect of hedging was less evident in *R. austrinum.*

Index Words: *Rhododendron austrinum, R. flammeum*, deciduous azalea, cutting propagation, hedging, K-IBA, timing

Species Used in Study: *Rhododendron austrinum* and *Rhododendron flammeum*

Chemicals Used in Study: K-IBA (indole-3-butyric acid with potassium salt)

Significance to the Nursery Industry

Stem cutting propagation is the principle method for mass production of clonally derived plant material (Hartmann et al., 2002), however, vegetative propagation of many of the deciduous azaleas native to the Eastern United States has been difficult. The utility of these plants in the landscape on a commercial scale is dependent upon reliable, productive propagation protocols. Among the home gardener and hobby collector, many anecdotal methods for propagation exist (Towe, 2004), but challenges to commercial cutting propagation have forced the production of deciduous azaleas by grafting (in Europe), micropropagation, and seed propagation (Galle, 1987; Skinner, 1954). Both grafting and seed propagation are time consuming, and type variability exists among individuals propagated by seed. Micropropagation can yield rapid results, yet be a costly endeavor. Softwood stem cuttings taken from hedged stock plants and treated with 7,500-10,000 ppm

K-IBA resulted in excellent rooting percentages (up to 85%) and root system size for both species.

Introduction

Native North American deciduous azaleas (*Rhododendron* L. spp.) have tremendous ornamental diversity in habit, flower size and color, fragrance, and bloom time (Galle, 1987). Native species serve as outstanding landscape plants because species flower throughout the spring into late summer. Unfortunately, several of our native azaleas can be difficult to propagate from stem cuttings (Galle, 1987; Nolde and Coartney, 1985; Shelton and Bir, 1980; Skinner, 1961) and tremendous inconsistencies exist both among and within species for the ability to form adventitious roots from stem cuttings.

Rhododendron austrinum (Small) Rehder, Florida flame azalea, is a fragrant, early blooming shrub with orange-golden blossoms. The plant is native to southwest Georgia, northwest Florida, southern Alabama, and southeast Mississippi. *Rhododendron flammeum* (Michx) Sarg., Oconee azalea, is another early flowering, medium sized shrub with yellow-orange to red blossoms. The Oconee azalea is native to parts of northwestern and southern South Carolina and western Georgia. The two species can be distinguished from one another by the presence of fragrance in *R. austrinum*, a floral blotch in *R. flammeum*, and flowering times, as the Oconee azalea blooms after the Florida flame (Galle, 1987). Both species can be moderately difficult to root (Knight et al., 2005; Skinner, 1961). This difficulty in propagation has greatly limited the potential for selecting, breeding, and producing superior

cultivars (e.g. *R. flammeum* 'Hazel Hamilton') (Mr. Ray Head, Rutherfordton, N.C., pers. comm. December 2005) as well as subsequent commercial significance.

Maintaining stock plants for propagation provides ample supplies of cutting material and maximizes rootability while maintaining healthy, uniform, correctly identified blocks (Hartmann et al., 2002; Howard, 1994). A number of stock plant management techniques exist, including severe winter pruning (hedging), that have been identified as increasing rooting potential among difficult-to-root taxa (Cameron et al., 2001; Howard, 1994). Rhododendron stock plants have been manipulated successfully in the past to obtain favorable adventitious root formation on stem cuttings (Apine and Kondratovičs, 2005). Cameron et al. (2001) noted that hedging stock plants of several taxa (*Corylopsis* and *Syringa*) strongly increased rooting performance.

Hedging of young plants or severe serial pruning of older plants can be utilized to reinvigorate and maintain juvenile-type, vegetative characteristics. Cuttings from juvenile plants are typically easier to root from stem cuttings than are cuttings from older, mature plants (Preece, 2003). After hedging, adventitious bud break occurs at the bases of plants, thereby producing an abundance of vigorous growing, non-flowering shoots, mimicking the juvenile growth state (MacDonald, 1986). These shoots tend to grow faster and longer into the growing season than those on non-hedged individuals, while also yielding thinner cuttings with the capacity to root faster (Hartmann et al., 2002). Thus, hedging allows for the preservation of the vegetative growth phase which is correlated to an enhanced effect on rooting (Hartmann et al., 2002; Howard, 1994; MacDonald, 1986).

Along with stock plant management, the use of rooting hormones has the potential to positively impact rooting of deciduous azaleas. Nawrocka-Grześkowiak and Grześkowiak (2003) observed an increase in rooting percentages and root ball size among azaleas with the use of rooting hormones (in the form of indolebutyric acid, IBA), however optimal concentrations were variable. Skinner (1954) reported rooting success among Ghent and Mollis hybrids, respectively, both with and without the use of rooting hormones but noted that rooting percentages were increased up to 100%, root system quality was improved, and time required for rooting was generally shorter with the use of hormones (IBA). For deciduous azaleas, Dirr and Heuser (1987) suggest a hormone rate of 4,000 ppm IBA, while Sommerville (1998) recommended a higher rate of 8,000 ppm IBA. Knight et al. (2005) studied the influence of hormone (K-IBA) concentration on the rooting of *R. austrinum* and *R. canescens* (Michx.) Sweet, mountain azalea, but found no differences in rooting percentage associated with hormone concentrations from 0-10,000 ppm K-IBA. The response to K-IBA was variable for both species and this was attributed to the genetic and physiological differences associated with collecting stem cuttings from wild plant populations.

The timing of the collection of cutting material may also play a critical role in propagation success (Nawrocka-Grześkowiak and Grześkowiak, 2003; Skinner, 1961; Leach 1962). Recommendations vary from April to June as the optimum time for collection of stem cuttings (Dirr and Heuser 1987; Knight et al. 2005; Sommerville 1998; Apine and Kondratovičs 2005; Towe, 2004). Rather than adhere to strict calendar dating, timing may be classified according to the physiological growth stage of the stock plant from softwood to

semi-hardwood to hardwood. Softwood cuttings are taken from the distal end of the current season's growth and are the youngest tissue in terms of development; whereas, hardwood cuttings have ceased growth and contain the oldest, most lignified tissue (Howard, 1994; MacDonald, 1986). The degree of lignification in the secondary xylem is closely associated with the growth state and the date of collection resulting in pronounced effects on the degree of rooting (Nawrocka-Grześkowiak and Grześkowiak, 2003). Burton and Webster (1971) suggested it was best to take cuttings after the completion of growth when the tissue was most lignified.

The objectives of this project were to develop and optimize protocols for cutting propagation of R. *flammeum* and R. *austrinum* by evaluating effects of hedging stock plants and a range of auxin concentraions for both softwood and semi-hardwood stem cuttings.

Materials and Methods

In Fall 2005, 50 plants of each species (East Fork Nursery, Sevierville, Tenn.) were planted on center 1 m × 2.5 m (3 ft × 8 ft) in field beds at the Mountain Horticultural Crops Research and Extension Center. Each species was arranged separately in 5 blocks of 10 plants each. In March 2006 (March 2007 for *R. austrinum*), five plants in each block were randomly chosen to be hedged severely to 15 cm (6 in) above ground level, leaving the remaining 5 plants in each block as unhedged controls. The five plants served as sub-samples of each plot for the hedged or unhedged treatment in each block.

Each species represented a separate experiment with a randomized complete block design. There were two tissue types (softwood and semi-hardwood), 2 hedging treatments, and 5 rates of the potassium salt of indolebutyric acid (K-IBA). Terminal stem cuttings approximately 7.6 cm (3 in) or longer were collected in June (softwood) and September (semi-hardwood) of 2006 (*R. flammeum* only) and 2007 (both species). Thirty stem cuttings were collected from the sub-sample of five plants in each plot. Stem cuttings were recut to 7.6 cm (3 in) and the basal 1 cm dipped for 3 s in either 0, 2500, 5000, 7500, or 10000 ppm K-IBA and immediately inserted into 38 cm × 38 cm × 15 cm (15 in × 15 in × 6 in) trays (Anderson flats, Anderson Die & Manufacturing Co., Portland, Ore.) containing a substrate of 2 peat: 3 perlite (v/v) and placed under intermittent mist in a propagation greenhouse. Intermittent mist was applied using a Superior Controller **(**Superior Controls, Co., Inc. Valencia, Calif.**)**. Between 6:00 a.m. and 10:00 a.m. mist was applied every 20 min for 8 s.; between 10:00 a.m. and 6:00 p.m. mist was applied every 10 min for 8 s; between 6:00 p.m. and 10:00 p.m. mist was applied every 15 min for 8 s and every 240 min for 8 sec between 10:00 p.m. and 6:00 a.m. Cuttings were spaced 4 cm (1.6 in) x 4 cm (1.6 in) in trays and allowed to root for approximately 7 weeks prior to recording data. Data recorded included total number of possible terminal stem cuttings greater than or equal to 7.6 cm (3 in) (recorded on each stock plant prior to removing cuttings), rooting percentage, and root system size (expressed as the length × width of the overall root system measured at the largest points). Root system size was determined to give a relative indication of the root system without destroying the root systems. Plants were immediately potted after rooting for future evaluation.

Data for rooting percentage and root system size were transformed using arcsine square root and the natural logarithm, respectively, when they were not normally distributed. Data for *R. flammeum* were analyzed as a split-split plot design with year as the main plot, growth stage as a sub plot, and IBA and hedging as sub-sub plots. Data for *R. autrinum* was analyzed as a split plot design with growth stage as the main plot and IBA and hedging as sub plots. All data were subjected to analysis of variance (PROC GLM) and regression analysis (PROC REG) (SAS version 8.02, SAS Institute., Cary, N.C.; SAS Institute, 1988).

Results and Discussion

Rooting percentage of stem cuttings of *R. flammeum* was affected by growth stage (tissue type) ($P<0.01$), hedging ($P<0.01$), and K-IBA rate ($P<0.10$). Additionally, the interactions tissue by rate ($P<0.01$) and tissue by hedge ($P<0.01$) affected rooting percentage. Overwhelmingly, selecting stem cuttings in the softwood tissue stage had the greatest effect on improving rooting percentage (Figure 1). For softwood stem cuttings, increased rates of K-IBA increased rooting percentage ($r^2=0.81$, $P<0.01$) in a linear fashion. Rooting percentage of semi-hardwood stem cuttings, however, was not affected by K-IBA rate (Fig 1). Hedging stock plants increased rooting percentage for semi-hardwood stem cuttings, but not for softwood stem cuttings of *R. flammeum*. Softwood stem cuttings had a mean rooting of 85%, regardless of hedging. Semi-hardwood stem cuttings collected from hedged stock plants had a mean rooting of 35%, whereas, those collected from unhedged plants rooted only 0.1%. The addition of K-IBA to either hedged or unhedged semi-hardwood stem cuttings did not improve rooting percentage considerably.

Root system size was affected by tissue (P<0.01), hedging (P<0.01) and K-IBA rate (P<0.01). Softwood stem cuttings had a greater mean root system size at 15.5 ± 1.6 cm2 (2.4 ± 0.25 in2) than semi-hardwood cuttings at 0.95 ± 0.72 cm2 (0.15 ± 0.11 in2). Rooted stem cuttings originating from all hedged stock plants had a mean root system size index of 11.8 ± 1.6 cm2 (1.8 ± 0.25 in2), whereas root system size was 4.6 ± 1.6 cm2 (0.71 ± 0.24 in2) for rooted cuttings originating from unhedged stock plants. Root system size increased linearly with increasing K-IBA rate for both softwood (r^2=0.78, *P*=0.04) and semi-hardwood (r^2=0.91, *P*=0.01) stem cuttings when collected from hedged stock plants (Fig 2).

The improved response of rooting percentage and root system size to increased rates of rooting hormone suggests the use of softwood cuttings with a K-IBA rate of 10,000 ppm for optimal rooting and root growth. Hedging was an effective method of stock plant manipulation on *R. flammeum* in that the quality of the root system was increased in cuttings from hedged plants when compared to those of rooted cuttings from unhedged plants. When rooting semi-hardwood cuttings of the Oconee azalea, the hormone concentration was irrelevant in regards to rooting percentages; however, increasing the rate provided a root ball of larger size.

Rooting percentage for stem cuttings of *R. austrinum* was affected by tissue (*P*<0.01), rate (*P*<0.05) and the interactions of tissue by rate (*P*<0.01), tissue by hedge (*P*<0.01), and tissue by hedge by rate (*P*<0.01). Softwood stem cuttings collected from either hedged or unhedged stock plants rooted at 96% regardless of rooting hormone application. Semi-hardwood stem cuttings collected from hedged stock plants had a mean rooting of 31%. The interaction of tissue with hedge and rate is observed in the rooting percentage of semi-

hardwood stem cuttings collected from unhedged stock plants having a negative linear relationship (r^2=0.74; P<0.10) with K-IBA rate (Fig 3). The highest rooting (73%) occurred at 0 ppm K-IBA (no rooting hormone) applied to stem cuttings.

For rooted cuttings of *R. austrinum*, root ball size index was affected by tissue (P<0.01) and K-IBA rate (P<0.01), but not by the hedging treatment. Root system size for rooted cuttings from softwood tissue was significantly higher than that of semi-hardwood cuttings, 10.2 ± 0.69 cm^2 (1.5 ± 0.11 in^2) and 2.9 ± 0.51 cm^2 (0.50 ± 0.08 in^2), respectively. Root system size of both tissue types combined increased linearly with increasing K-IBA rate (r^2=0.85, P<0.05) (Fig 4.).

The difference in rooting percentage between semi-hardwood stem cuttings collected from hedged (30%) and unhedged stock plants (75%) and not treated with rooting hormone (0 ppm K-IBA) is puzzling (Fig. 3). This suggests that hedging stock plants can decrease rooting percentages in semi-hardwood stem cuttings. Two hypotheses are that hedging decreases endogenous auxin levels or hedging increases the factors associated with inhibiting adventitious root formation in stem cuttings, or, perhaps, hedging contributes to both scenarios. If so, then the effects of hedging might be alleviated by the application of exogenous auxins and a subsequent increase in rooting percentage. There was no relationship, however, between K-IBA application and rooting percentage of semi-hardwood stem cuttings collected from hedged stock plants. Additionally, the rooting percentage of stem cuttings from unhedged stock plants decreased with increased rates of K-IBA. It is possible that semi-hardwood stem cuttings have a sensitivity to high auxin concentrations, particularly K-IBA, and hedging increases this sensitivity. Usually, softwood stem cuttings,

which are less lignified than semi-hardwood stem cuttings, are more sensitive to high rates of auxin. This sensitivity in softwood stem cuttings was not noted in the study herein or in Knight et al. (2005). Moreover, root system size increased with increasing rates of K-IBA and was not affected by the hedging treatments. Because of these conflicting data, the experiment with *R. austrinum* will be conducted again.

Severe winter hedging did affect ($P<0.01$) the total number of cuttings available for collection from both azalea species. *R. flammeum* plants grown under the hedged regime produced 19 ± 2 stem cuttings at either softwood or semi-hardwood collection times, whereas unhedged plants produced 37 ± 7 stem cuttings. In contrast, the hedged treatment in *R. austrinum* produced 21 ± 1 cuttings at either tissue type, while the lack of hedging yielded 11 ± 1 stem cuttings. For commercial propagation purposes, hedging is recommended in association with *R. austrinum* to increase cutting numbers. Although not an effective strategy for producing more stem cuttings in *R. flammeum*, hedging produced more uniform rooted cuttings with larger root systems.

The effect of rooting hormone on percentage and quality of rooting was observed both visually and statistically. Rooting may occur without the use of auxin, but the addition of exogenous auxin resulted in higher quality root systems and more rooted cuttings overall, especially in softwood stem cuttings. Nolde and Coartney (1985) also observed a positive effect of hormone on rooting. Even in plant species with adequate levels of endogenous auxin for regeneration of roots, auxin applications can dramatically increase the root quality (De Klerk et al., 1999). Similarly, the use of higher rates of rooting hormones was quite beneficial in *R. austrinum* and *R. flammeum*. Knight et al. (2005) observed rooting in

softwood stem cuttings of *R. austrinum* at all rates applied, however the highest rooting percentages (100%) were obtained using higher rates of K-IBA (7,500 and 10,000 ppm K-IBA). Additionally, they noted that root number and length of rooted softwood stem cuttings was greatest when using 10,000 ppm K-IBA.

In conclusion, treating softwood stem cuttings of both *R. flammeum* and *R. austrinum* with 10,000 ppm K-IBA produced the highest number of rooted cuttings with the largest root systems. In R. flammeum, hedging improved the rooting percentage of semi-hardwood stem cuttings and also improved the root system size of rooted cuttings of both growth stages. In *R. austrinum*, hedging had less of an effect on rooting percentage and root growth, but did increase the number of stem cuttings produced per plant. Utilizing higher rates of rooting hormone with softwood stem cuttings, and perhaps maintaining hedged stock plants, may improve vegetative propagation and commercial availability of these two species.

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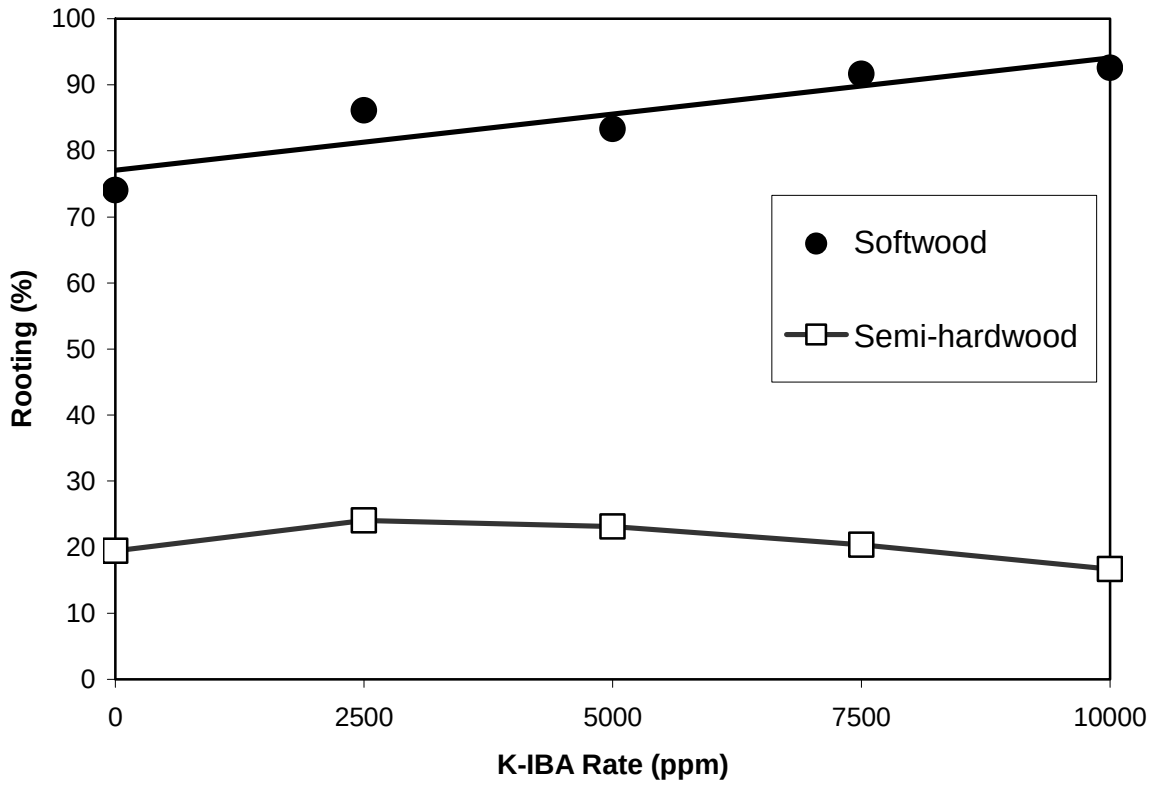

Figure 1. Rooting percentage of stem cuttings of *Rhododendron flammeum* collected in the softwood or semi-hardwood stage and dipped in the potassium salt of indolebutyric acid (K-IBA). Rooting percentage (softwood stem cuttings) = 77.04 + 0.0017(K-IBA); r^2 = 0.81; *P*< 0.01. The relationship between semi-hardwood stem cuttings and K-IBA rate was not significant.

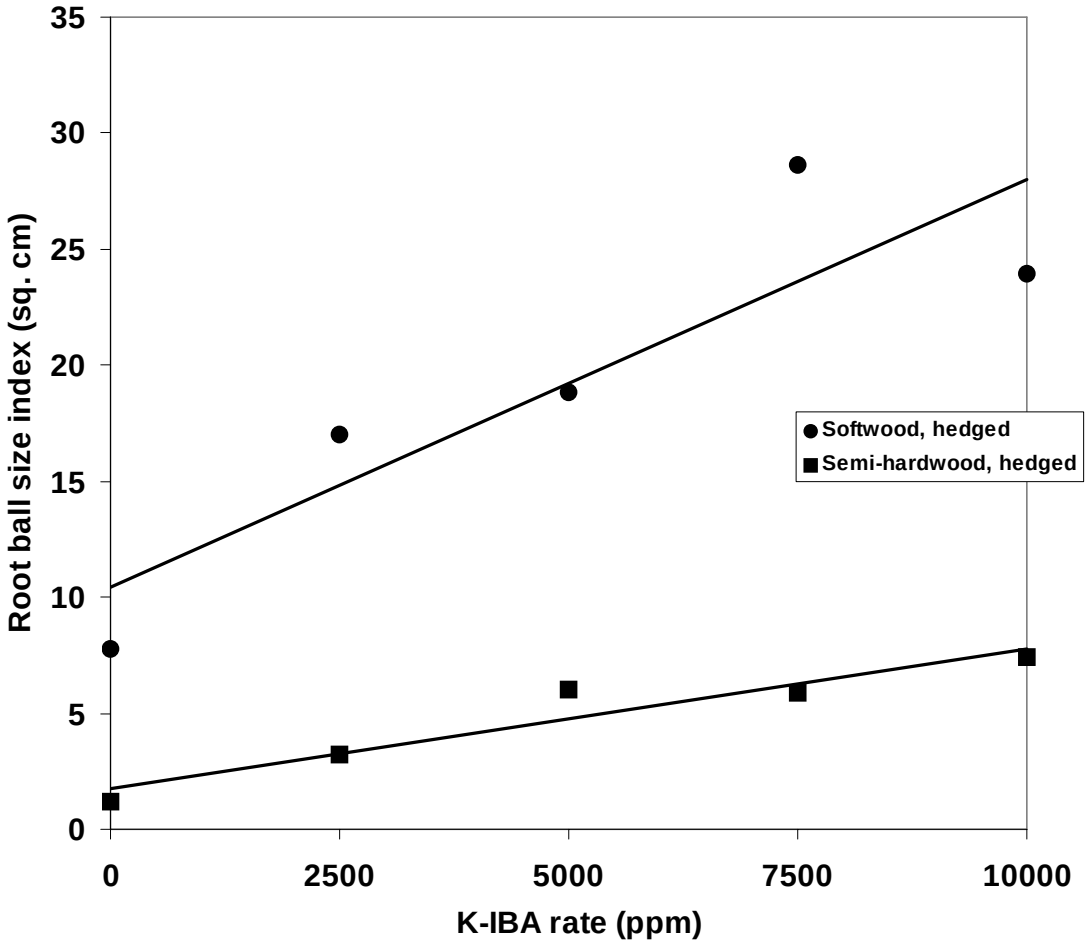

Figure 2. Mean root system size index (length times width of the root) for softwood and semi-hardwood stem cuttings collected from hedged stock plants of *Rhododendron flammeum* and then dipped in the potassium salt of indolebutyric acid. Root system size index of softwood stem cuttings = 10.44 + 0.0018(K-IBA rate), r^2=0.78, *P*=0.04. Root system size of semi-hardwood stem cuttings = 1.72 +0.0006(K-IBA rate); r^2=0.91, *P*=0.01.

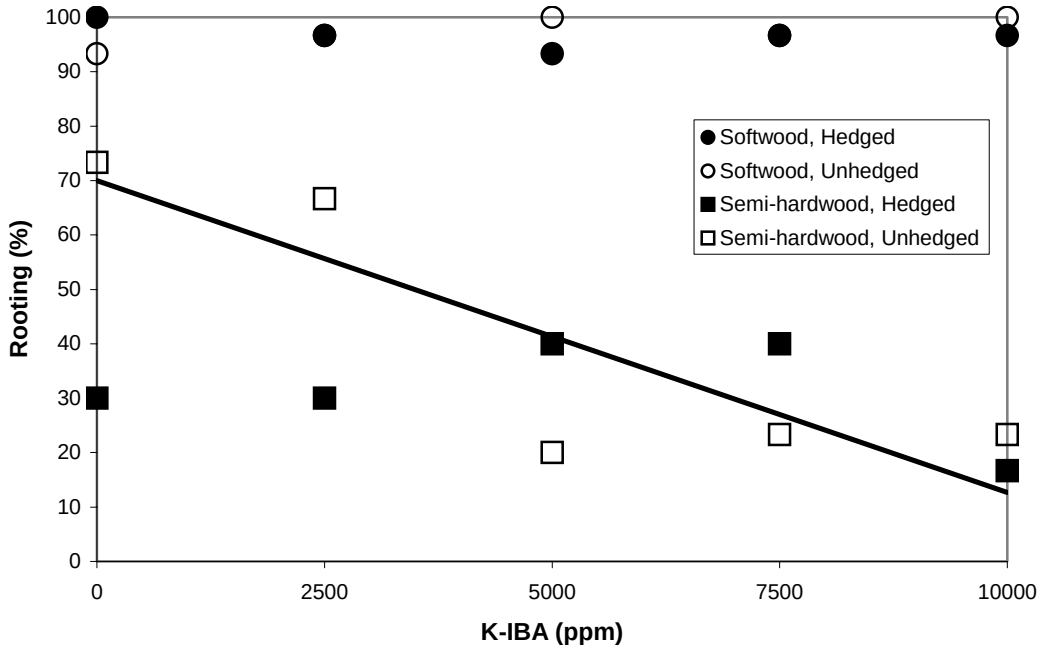

Figure 3. Rooting percentages of stem cuttings collected in the softwood or semi-hardwood growth stage from hedged or unhedged stock plants of *Rhododendron austrinum* and dipped in the potassium salt of indolebutyric acid (K-IBA). Rooting percentage (Semi-hardwood, Unhedged) = -0.006 (K-IBA) + 70; r^2=0.74; *P*<0.05.

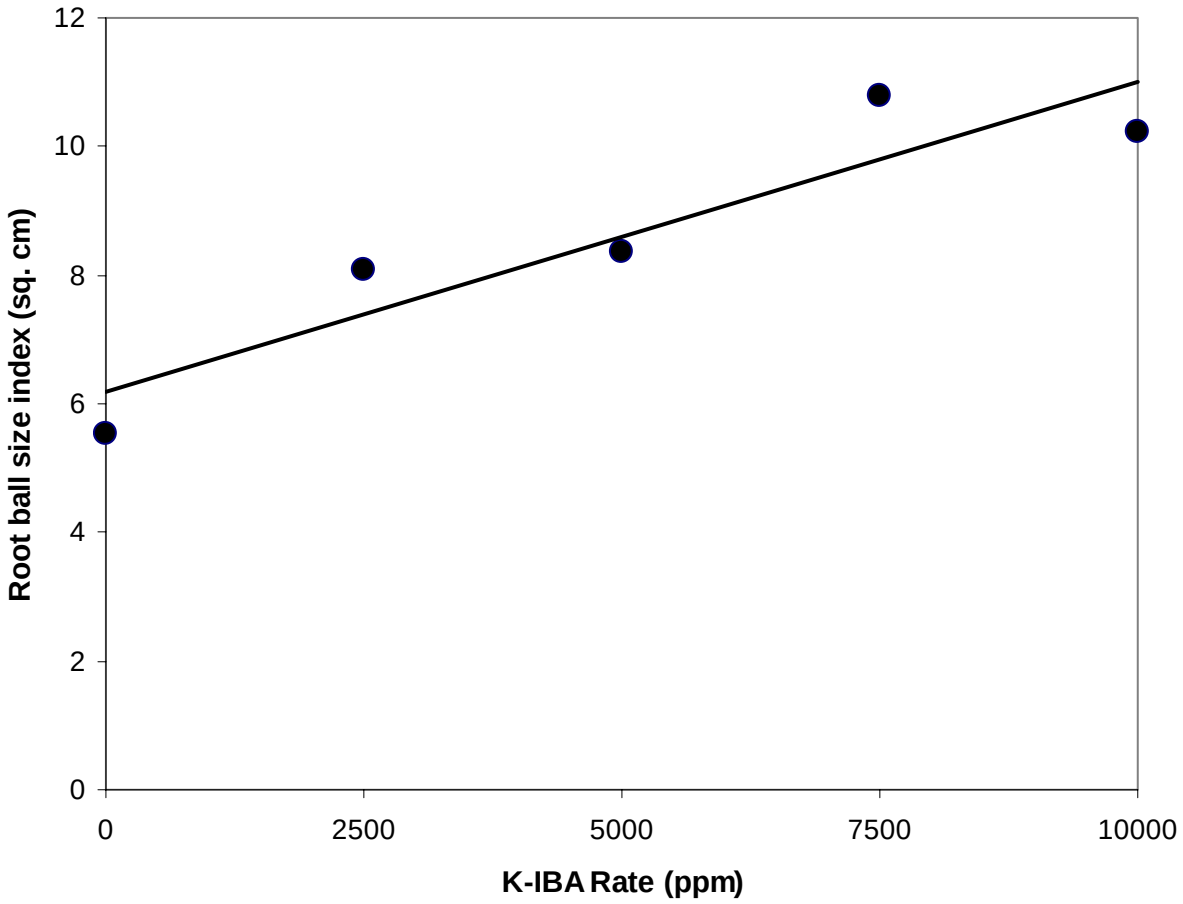

Figure 4. Root system size index (calculated by multiplying the length and width of the excavated root ball) of rooted stem cuttings of *Rhododendron austrinum* treated with the potassium salt of indolebutyric acid (K-IBA). Data points represent the mean of two collection times and two hedging treatments. Root ball size index = 0.0005(K-IBA rate) + 6.18; r^2 = 0.85; *P*<0.05.