

ABSTRACT

GILLOOLY, DOMINIC ALEXANDER. Genome Sizes and Ploidy Levels in the Genus *Kalmia* and In Vitro Polyploid Induction of *Ophiopogon planiscapus*. (Under the direction of Dr. Thomas G. Ranney).

Kalmia is a highly ornamental genus of shrubs native to North America and Cuba and grown as a valuable nursery crop throughout much of the temperate world. The objective of this study was to determine the relative genome sizes and ploidy levels of species, hybrids, and cultivars of *Kalmia*. Flow cytometry was used to determine the relative genome sizes of 64 accessions representing species, interspecific hybrids, cultivars, and chemically induced polyploids. Traditional cytology was used to calibrate genome sizes with ploidy levels. Results showed that relative genome sizes were conserved with 1Cx values ranging from 0.57 pg to 0.70 pg. Most species were diploid with the exception of *K. angustifolia*, which was primarily tetraploid. An unusual triploid of *K. angustifolia* f. *candida* was also documented. *K. polifolia* included both tetraploid and potentially pentaploid individuals, indicating a ploidy series within this species. *K. latifolia* cultivars also included one triploid, two cytochimeras, and two chemically induced tetraploids. Overall, polyploidy was more prevalent in *Kalmia* than previously reported and varied both within and among species. This broader survey of relative genome sizes and ploidy levels in *Kalmia* provides valuable information for plant breeders and new insights into the systematics and cytogenetics of the genus.

The black-leaved forms of *Ophiopogon planiscapus* are valuable nursery and landscape plants whose slow growth limits production potential by the nursery industry. The creation of autopolyploid black-leaved germplasm has the potential to give rise to more vigorous cultivars. The dinitroaniline herbicide oryzalin is often used as a mitotic inhibitor in

the production of polyploids, but success is often sporadic. More consistent and effective treatments would be desirable. The addition of nitrotyrosine has the potential to give rise to increased numbers of stable polyploids when used in conjunction with oryzalin due to its multiplicative effect on the depolymerization of microtubules during mitosis. The objectives of these studies were to optimize in vitro polyploid induction of *O. planiscapus* and specifically evaluate 1) the combined effects of dose and treatment duration of oryzalin and 2) the combined effects of nitrotyrosine and oryzalin dosages on survival and polyploid induction in vitro. When treated with oryzalin alone, callus survival decreased and the number of mixoploids increased with increasing oryzalin concentration. Some mixoploid shoots kept in tissue culture stabilized as tetraploids at a later date. A number of homogeneous induced tetraploids were successfully recovered evidencing that oryzalin is an effective agent for polyploid induction in *Ophiopogon planiscapus*. Increasing concentrations of oryzalin and nitrotyrosine in combination revealed an interactive effect resulting in decreasing callus survival. Although increasing oryzalin concentrations increased the number of mixoploids, there was no significant effect of nitrotyrosine or an interaction with oryzalin concentration on polyploid induction. Recovery of black-leaved, tetraploid *Ophiopogon planiscapus* will provide new opportunities for breeding improved *Ophiopogon* hybrids.

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Genome Sizes and Ploidy Levels in the Genus *Kalmia* and In Vitro Polyploid Induction of
Ophiopogon planiscapus.

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

Horticultural Science

Raleigh, North Carolina

2016

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BIOGRAPHY

Dominic Alexander Gillooly was born in Fort Myers, Florida to Michael and Cynthia Gillooly and spent most of his first year under the counter of his mother's florist shop, 'Weeds and Things'. He was raised in Asheville, NC where his horticultural future was born, growing up in a home surrounded by a well-tended perennial garden and a greenhouse full of orchids. After graduation from high school he attended the University of North Carolina at Asheville where he earned an undergraduate degree in business management and minored in economics. His last semester as an undergraduate in Asheville he interned at Salomon Smith-Barney where he had a wonderful experience but discovered that a life in financial management would suit neither his temperament nor his passions.

After graduating from UNC Asheville he worked as a cook, waiter, bar tender, call center representative and carpenter. During those years Dominic developed a deeper love of horticulture while pursuing it as hobby, studying bonsai, vegetable and perennial gardening, greenhouses, and orchid culture as an amateur enthusiast.

During the first years of the Great Recession he had to opportunity to move to Boston, Massachusetts to assist in the renovation of a historic home near Harvard University. While in Boston, Dominic made the decision to take a chance and go back to school in pursuit of a career in horticulture as a plant breeder. After much research, he found the best place for him to study was back home in North Carolina at NC State University.

Dominic began an undergraduate program in Horticultural Science at NC State in 2010 and had the privilege to learn from and interact with some of the best professors in horticulture in the nation. During those years, he worked as an assistant to Dr. James

Ballington, as a student worker at the Micropropagation and Repository Unit, and as a research assistant to Dr. Jared Barnes during his PhD research. While studying, he also had the opportunity to meet his wife Dana at the annual Pi Alpha Xi plant sale, for which he is eternally grateful.

During his senior undergraduate year, Dominic was accepted for an internship at the Mountain Horticultural Crops Research and Extension Center with Dr. Tom Ranney. This internship solidified his desire to pursue a career as a plant breeder and his determination to continue his education.

After finishing his internship, Dominic began a master's degree under the direction of Dr. Ranney. His experiences at the research station and on campus at NC State exposed him to many techniques in plant breeding and science, gave him the opportunity to teach students, and the privilege to present research at national research conferences.

Now that Dominic is nearing graduation, he plans to pursue his dream of being a plant breeder and bring amazing new plants into the world. Dominic looks forward to his future with his wife Dana and all that lies before them.

ACKNOWLEDGMENTS

There are many people I owe a debt of gratitude to for their help, support and encouragement over the years; I hope I don't forget anyone. First and foremost I'd like to thank my parents who raised me to be inquisitive, to love nature and all its wonders, and who encouraged me in everything I did. I couldn't be here today without you.

At NC State I've had the opportunity to interact with and be taught by many amazing faculty members who were always patient communicators of their knowledge and good mentors. I'd like to thank, in no particular order, Mr. Bryce Lane, Dr. Dennis Werner, Dr. John Dole, Dr. Julia Kornegay, Dr. Bill Fonteno, Dr. Brian Jackson, Dr. John Williamson, Dr. Todd Wehner, Dr. Craig Yenko, Dr. Gina Fernandez, Dr. Chris Gunter, Dr. Barb Fair, Mr. Will Hooker, Dr. Chad Jordan, and Dr. Alexander Krings. Thank you all for teaching wonderful classes that challenged and excited me.

Also I'd like to acknowledge Ms. Rachel McLaughlin and Ms. Michelle Healey for all their help with administrative matters. Thank you ladies for all your hard work, and for answering the questions of a panicked graduate student.

My undergraduate advisors Mr. Bryce Lane and Dr. Dennis Werner deserve special thanks for helping steer me in the right directions, especially during those early times when I was just finding my feet at the university. Thank you for your time, attention, and kindness.

Dr. James Ballington, thank you for taking me on as a summer employee in your breeding program, I learned an incalculable amount just from being around you and it was a privilege to work with such an accomplished plant breeder. I especially enjoyed watching you ID a rare plant out the window of a moving van at 65 miles an hour - amazing.

Dr. Zvezdana Pesic-VanEsbroeck and Ms. Rose Caldwell, thank you for the opportunity to work at the Micropropagation Unit, the skills I learned there have been invaluable to my work as a graduate student.

Of course many thanks go to Dr. Tom Ranney who gave me the opportunity to intern in his program and then also to work as a graduate student. Your patience and goodwill is legendary Tom, I can never thank you enough, so I'll simply say thank you a thousand times for all the opportunities you've given me. In particular I'd like to thank you for sharing so much knowledge, for exposing all of your students to so many wonderful experiences and for introducing me to so many interesting people. Also, the field trips were always a lot of fun.

Dr. Darren Touchell also deserves many thanks for all his help with my projects. I couldn't have done it without your help, especially all those trips back and forth between labs carrying samples for testing. Thank you for not letting me get discouraged when things didn't work out as planned and helping me persevere to the end.

Thanks also go to Dr. Anthony LeBude for his help getting papers and presentations just right for delivery at the Southern Nursery Association Research Conference and the Southern Region ASHS Research Conference. Your help makes the difference between an okay presentation and a much better, prize-winning one. Not just for me but for many students over the years. Thanks for all your input and advice.

Dr. Dennis Werner, thank you for not only serving as my undergraduate advisor and on my graduate committee, but for your amazing teaching. I learned more in your classes than in any other and I enjoyed every second of it. Thank you also for letting me sit-in during your plant propagation lab for a second time as an undergraduate 'TA' - it was just as much

fun the second time around. Your dedication to science and teaching is exemplary, and it meant a lot to me.

I couldn't have completed my research at the Mountain Horticultural Crops Research and Extension Center without the help of Dr. Ranney's amazing staff. Jeremy Smith, thanks for always making me laugh in the tissue culture lab. Andra Nus, thanks for all your help with my projects and around the lab - you made it a better place to work. Tom Eaker, thanks for all the help in and around the station and for making it fun to be in the field pulling weeds. Joel Mowery, thanks for being such an efficient taskmaster and knowing when to give us a break. Nathan Lynch, thanks for all your help in the lab teaching me flow cytometry and cytology techniques, you're a great teacher and excellent scientist.

I'd also like to thank all my fellow graduate students for their help and support along the way. You rock.

Last, but not least, I'd like to thank my wife Dana for her support during my time in graduate school. I wouldn't have made it without you.

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CHAPTER 1

Genome Sizes and Ploidy Levels in the Genus *Kalmia*

(In the format appropriate for submission to HortScience)

Genome Sizes and Ploidy Levels in the Genus *Kalmia*

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Received for publication _____. Accepted for publication _____.

This research was funded, in part, by the North Carolina Agricultural Research Service, Raleigh, NC and the North Carolina Biotechnology Center, Research Triangle Park, NC.

Technical assistance of Nathan Lynch, Joel Mowrey, Tom Eaker, and Jeremy Smith is gratefully acknowledged. We also thank Kelly Brooks, Southern Highlands Reserve, Lake Toxaway, NC; Geordie Elkins, Highstead, Redding, CT; Eric La Fountaine, University of British Columbia Botanical Garden and Center for Plant Research, Vancouver, BC; Dr. Richard Jaynes, Broken Arrow Nursery, Hamden, CT; Bambi Jones, Hidden Valley Nature Center, Jefferson, ME; Dr. Ron Miller, Pensacola, FL; Cliff Ruth, North Carolina Cooperative Extension Service, Asheville, NC; and Dr. Elizabeth Thompson, University of Vermont, Burlington, VT for their help in locating and procuring the samples used in this study.

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Subject Category: Breeding, Cultivars, Rootstocks, and Germplasm Resources
Genome Sizes and Ploidy Levels in the Genus *Kalmia*

Additional index words. Cytology, DNA content, flow cytometry, *Kalmia angustifolia*, *K. buxifolia*, *K. carolina*, *K. cuneata*, *K. hirsuta*, *K. latifolia*, *K. microphylla*, *K. polifolia*, polyploidy, plant breeding

Abstract. *Kalmia* is a highly ornamental genus of shrubs native to North America and Cuba and grown as a valuable nursery crop throughout much of the temperate world. Although most species of *Kalmia* have previously been found to be diploid with $2n = 2x = 24$, one species, *K. polifolia*, has been found to be tetraploid. However, sampling within the genus has been limited, and information on the ploidy levels of specific cultivars is lacking. The objective of this study was to determine the relative genome sizes and ploidy levels of species, hybrids, and cultivars of *Kalmia*. Flow cytometry was used to determine the relative genome sizes of 64 accessions representing species, interspecific hybrids, cultivars, and chemically induced polyploids. Traditional cytology was used to calibrate genome sizes with ploidy levels. Results showed that relative genome sizes were conserved with $1Cx$ values ranging from 0.57 pg for *K. carolina* to 0.70 pg for *K. latifolia*. Most species of *Kalmia* were diploid including *K. buxifolia* (*Leiophyllum buxifolium*), *K. carolina*, *K. cuneata*, *K. hirsuta*, *K. latifolia*, and *K. microphylla*. Although plants of *K. carolina* (*K. angustifolia* var. *carolina*) were uniformly diploid, the closely related, but more northerly distributed, *K. angustifolia* was primarily tetraploid, providing additional justification for treating these as separate species. An unusual triploid of *K. angustifolia* f. *candida* was also documented. *Kalmia polifolia* included both tetraploid and potentially pentaploid individuals, indicating a

ploidy series within this species. *Kalmia latifolia* cultivars also included one triploid, two cytochimeras, and two chemically induced tetraploids. Overall, polyploidy was more prevalent in *Kalmia* than previously reported and varied both within and among species. This broader survey of relative genome sizes and ploidy levels in *Kalmia* provides valuable information for plant breeders and new insights into the systematics and cytogenetics of the genus.

Introduction

Kalmia L. is a small but diverse genus containing nine to eleven species (Ebinger, 1974; Southhall, 1973; Weakley, 2012) native to North America and Cuba (Jaynes, 1997). Deciduous or evergreen woody shrubs, *Kalmia* spp. have a varied morphology and are each distinct. Some of their diverse characteristics include plants that are “...erect, or ascending, branching shrubs or rarely trees. The leaves are simple, alternate, opposite or whorled, coriaceous, dark green above, light green beneath, entire and often revolute (Southhall, 1973).” *Kalmia latifolia*, and to a lesser degree, *Kalmia angustifolia* L. and *Kalmia polifolia* Wengen., are valuable nursery crops and have been cultivated in North America and Europe since the early 1700’s (Jaynes, 1997).

Cytology was performed on *Kalmia* species by Dr. Richard Jaynes of the Connecticut Agricultural Experiment Station in the late 1960’s (Jaynes, 1969). His examinations found most species of *Kalmia* to be diploid ($2n = 2x = 24$), with the exception of *K. polifolia* that was tetraploid ($2n = 4x = 48$). However, that study only examined material from one or two plants per species to determine ploidy, and in the case of *K. angustifolia*, only samples from

North Carolina were obtained as representatives of that species (Jaynes, 1969). *Kalmia angustifolia* and *K. carolina* Small (*Kalmia angustifolia* var. *carolina* (Small) Fernald) are closely related with North Carolina provenances now generally classified as *Kalmia carolina* (Gillespie and Kron, 2010; Southall and Hardin, 1974; Weakley, 2012). Cytology on Ericaceous plants, including *Kalmia*, is notoriously difficult, due in part to the small chromosomes (Jaynes, 1997). Flow cytometry provides a more efficient approach to broaden sampling within and among species and can provide accurate estimates of ploidy, particularly for Ericaceous plants (Jones et al., 2007).

The objective of this study was to survey genome sizes and ploidy levels of a broad range of species, hybrids, and cultivars of *Kalmia* from throughout North America.

Material and Methods

A diverse collection of *Kalmia* taxa was obtained from cooperators in the United States and Canada (Table 1). The Cuban species *Kalmia ericoides* C. Wright ex Griseb. was not available for analysis. Relative 2C genome sizes were determined using flow cytometry on recently expanded leaves (Greilhuber et al., 2007). Sample tissue was combined with an internal standard (*Pisum sativum* L. 'Ctirad', 2C DNA content = 8.76 pg, Greilhuber et al., 2007) and diced with a razor blade in a petri dish containing 400 μ L of extraction buffer (CyStain Precise P, Partec, Münster, Germany). The nuclei suspension was poured through a 50- μ m filter and stained with 1600 μ L of a nucleotide staining buffer solution (CyStain UV Precise P Staining Buffer, Partec) containing 4', 6-diamidino-2-phenylindole (DAPI). Stained nuclei were analyzed using the PA-II flow cytometer (PA-II; Partec). A minimum of 3,000

nuclei counts were analyzed for each sample with three samples for each taxa . Holoploid, 2C genome sizes for each sample were calculated as: $2C = \text{DNA content of standard} \times (\text{mean fluorescence value of sample} \div \text{mean fluorescence value of the standard})$. The relationship between genome sizes and ploidy levels was based on samples with confirmed chromosome numbers. Mean 1Cx monoploid genome size (i.e., genome size of the non-replicated base set of chromosomes) was calculated as $2C \text{ genome size} \div \text{ploidy}$. Taxa were sampled in a completely randomized fashion. Data for 1Cx values were subjected to analysis of variance and means. Different taxa were separated using Tukey's HSD, $P \leq 0.05$ (SAS version 8.02, SAS Institute, Cary, NC).

Cytology. To confirm ploidy and calibrate with genome size the cultivar *Kalmia latifolia* 'Elf' was selected and traditional cytology was conducted using a root squash technique following procedures outlined in Lattier et al. (2014). Root tips were collected from potted plants and placed in vials of pre-fixative solution (2 mM 8-hydroxyquinoline + 70 mg L⁻¹ cycloheximide). Vials were stored in the dark for two hours at ambient room temperature after which they were placed in a dark refrigerator at approximately 4°C for an additional 2 hours. Following the pre-fixative period, roots were removed and rinsed thoroughly in deionized water. The rinsed root tips were then placed in a 3:1 solution of ethanol and propionic acid and left overnight at ambient room temperature to complete the fixation process. The following day the roots were rinsed with 70% ethanol three times and stored in 70% ethanol at approximately 4°C until root squashes could be performed. Root tips were removed from the ethanol storage solution and placed in a hydrolyzing solution made up of a 3:1 solution of 95% ethanol and hydrochloric acid (12.3 M) for ten minutes.

After hydrolyzing, the root tips were transferred into a modified carbol-fuchsin staining solution for ten minutes. Once stained the terminal end of the root tip was excised under a dissecting stereo microscope and placed on a clean microscope slide in a drop of staining solution. A cover slip was applied, and the root tip tissue was squashed. Slides were observed using a light microscope (Nikon Eclipse 80i, Nikon, Melville, NY). Photographs, taken at multiple focal points, were layered (Photoshop CS6, Adobe, Mountain View, CA) to create an enhanced depth of field.

Results and Discussion

Cytology confirmed that *K. latifolia* ‘Elf’ is a diploid with $2n = 2x = 24$ (Fig. 1) as has been reported previously for that species (Jaynes, 1969). Flow cytometry was an efficient and consistent method for determining relative genome size and ploidy of *Kalmia* (Table 1). Values for multiple subsamples of each replicate were consistent, with the SEM for 2C values ranging from less than 0.01 to 0.1 pg, demonstrating a high level of precision and repeatability and clearly distinguishing between ploidy levels. Results from flow cytometry revealed that genome sizes were relatively conserved with 1Cx values ranging from 0.57 pg for *K. carolina* to 0.70 pg for *K. latifolia*. These values are similar to other Ericaceous plants, including *Rhododendron* that had 1Cx values ranging from 0.63 – 0.83 pg (Jones et al., 2007). Cytometry results also showed there to be considerable variation in ploidy both between and within species (Table 1). Most species of *Kalmia* were predominantly diploid including *K. buxifolia*, *K. carolina*, *K. cuneata*, *K. hirsuta*, *K. latifolia*, and *K. microphylla*. In the case of *K. latifolia*, there were some exceptions. The cultivar *K.*

latifolia ‘Big Boy’ was a triploid, most likely the result of an unreduced gamete from one parent. There were also two mixoploid/cytochimeras found: *K. latifolia* ‘Showtime’ had both diploid and tetraploid tissues, while ‘Silver Dollar’ had a mixture of triploid and hexaploid tissues. These mixoploids most likely arose from mitotic irregularities (endoreduplication) in at least one of the histogenic layers resulting in stable cytochimeras (Joubès and Chevalier, 2000). Naturally occurring mixoploids have been documented in other Ericaceous plants including *Rhododendron* (De Schepper et al., 2001; Jones et al., 2007; Sakai et al., 2006). In the case of ‘Silver Dollar’, this apparently happened in a triploid plant that most likely resulted from the union of an unreduced gamete from one of the parents. Two additional plants of *K. latifolia*, H2014-222-002 and H2014-222-004, were confirmed to be homogeneous tetraploids that resulted from treatment of seedlings with the mitotic inhibitor oryzalin at NC State University.

As previously reported by Jaynes (1969), under the epithet *K. angustifolia* var. *carolina*, the southern sheep laurel, *K. carolina* was confirmed to be diploid. However, samples of *Kalmia angustifolia*, the northern sheep laurel, were all tetraploid, including ‘Hammonasett’, ‘Poke Logan’, ‘Royal Dwarf’, and ‘Wintergreen’. The cultivars ‘Hammonasett’ and ‘Poke Logan’ are known to be wild collected selections from Connecticut and Maine, respectively (Jaynes, 1997). Additionally, a sample of *K. angustifolia* f. *candida*, a white-flowered form, was triploid. Most likely this particular plant is an oddity and not necessarily representative of the white-flowered form in general.

Kalmia polifolia also varied in ploidy. A sample of *K. polifolia* obtained from Hidden Valley Nature Center in Jefferson, Maine was tetraploid. However, wild collected samples

from Alburg, VT had genome sizes consistent with pentaploids, though this could not be confirmed with cytology. The existence of putative pentaploid *K. polifolia* in the wild implies a natural occurring ploidy series within the species, potentially including tetraploid, pentaploid and hexaploid individuals or populations. Additional sampling in this location would be warranted. Kron and King (1996) postulated that *K. polifolia* may be a hybrid between two lineages of *Kalmia* ancestors which are no longer extant. It has often been found that interspecific hybrids have a greater propensity to form unreduced gametes leading to the formation of allopolyploids (Arnold, 2006; Ramsey and Schemske, 1998) and this may be the case for *K. polifolia*. The sample of the hybrid (*K. polifolia* × *K. microphylla* var. *microphylla*) ‘Rocky Top’ was a tetraploid ($2C= 2.37$ pg), suggesting that it formed from the union of an unreduced gamete from the *K. microphylla* parent.

This survey provides new information on the genome sizes and ploidy levels for species, cultivars and hybrids of *Kalmia*. The discovery and confirmation of new natural and induced polyploids within the genus provides basic information for how to best utilize these plants in breeding programs. Although development of interspecific hybrids in *Kalmia* has been challenging (Jaynes, 1997), attempting these crosses at the tetraploid level may result in allopolyploids (amphidiploids) with greater fertility due to disomic chromosome pairing in meiosis (Ranney, 2006), allowing for advanced generations. Alternatively, the development of new triploid hybrids of *K. angustifolia* and *K. carolina* has the potential to help reduce fertility and invasiveness where they have become problematic in European forests (Inderjit and Mallik, 1996).

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Table 1. Relative genome sizes and estimated ploidy levels for 64 accessions of *Kalmia* species, cultivars, and hybrids.

Taxa/cultivar	Source/accession no.	Relative 2C genome size (pg)	Est. ploidy level (x)	Mean relative 1C genome size (pg) by taxa
<i>Kalmia angustifolia</i> 'Royal Dwarf'	NCSU 2003-131 ^Z	2.36 ± 0.02 ^Y	4	0.58 ± 0.00 DE ^X
<i>Kalmia angustifolia</i> 'Hammonasett'	Highstead	2.31 ± 0.01	4	
<i>Kalmia angustifolia</i> 'Royal Dwarf'	Highstead	2.35 ± 0.00	4	
<i>Kalmia angustifolia</i> 'Poke Logan'	Highstead	2.32 ± 0.01	4	
<i>Kalmia angustifolia</i> 'Wintergreen'	Highstead	2.38 ± 0.02	4	
<i>Kalmia angustifolia</i> f. <i>candida</i>	Highstead	1.74 ± 0.00	3	
<i>Kalmia buxifolia</i>	Southern Highlands 1	1.21 ± 0.02	2	0.60 ± 0.00 DE
<i>Kalmia buxifolia</i>	Southern Highlands 2	1.20 ± 0.04	2	
<i>Kalmia buxifolia</i>	Southern Highlands 3	1.21 ± 0.01	2	
<i>Kalmia carolina</i>	NCSU 2003-110	1.17 ± 0.06	2	0.57 ± 0.01 E
<i>Kalmia carolina</i>	NCSU 2012-126	1.15 ± 0.02	2	
<i>Kalmia carolina</i>	BRCC 1	1.12 ± 0.00	2	
<i>Kalmia carolina</i>	BRCC 2	1.10 ± 0.00	2	
<i>Kalmia carolina</i>	BRCC 3	1.12 ± 0.00	2	
<i>Kalmia hirsuta</i>	Miller/NCSU 2013-068	1.35 ± 0.00	2	0.67 ± 0.01 ABC
<i>Kalmia hirsuta</i>	Miller/NCSU 2013-069	1.32 ± 0.01	2	
<i>Kalmia hirsuta</i>	Miller/NCSU 2013-070	1.33 ± 0.02	2	
<i>Kalmia latifolia</i> 'Richard Jaynes'	NCSU 2004-074	1.34 ± 0.02	2	0.70 ± 0.00 A
<i>Kalmia latifolia</i> 'Hearts Desire'	NCSU 2011-011	1.39 ± 0.01	2	
<i>Kalmia latifolia</i> 'Kaleidoscope'	NCSU 2011-153	1.38 ± 0.02	2	
<i>Kalmia latifolia</i> 'Elf'	NCSU 2011-154	1.35 ± 0.10	2	
<i>Kalmia latifolia</i> 'Olympic Fire'	NCSU 2011-156	1.38 ± 0.02	2	
<i>Kalmia latifolia</i> 'Tinkerbelle'	NCSU 2011-157	1.37 ± 0.05	2	
<i>Kalmia latifolia</i> 'Minuet'	NCSU 2011-158	1.39 ± 0.01	2	
<i>Kalmia latifolia</i> 'Sarah'	NCSU 2011-159	1.36 ± 0.04	2	
<i>Kalmia latifolia</i> 'Fire Cracker'	NCSU 2011-160	1.41 ± 0.03	2	
<i>Kalmia latifolia</i> 'Little Linda'	NCSU 2011-161	1.42 ± 0.01	2	
<i>Kalmia latifolia</i> 'Tiddlywinks'	NCSU 2011-162	1.38 ± 0.00	2	
<i>Kalmia latifolia</i> 'Tidewater'	NCSU 2013-053	1.37 ± 0.01	2	
<i>Kalmia latifolia</i>	NCSU H2012-231-008	1.39 ± 0.03	2	
<i>Kalmia latifolia</i>	NCSU H2012-231-009	1.37 ± 0.02	2	
<i>Kalmia latifolia</i> 'Waxy Max'	Jaynes	1.39 ± 0.00	2	
<i>Kalmia latifolia</i>	Jaynes pk-74	1.37 ± 0.01	2	

Table 1. Continued

<i>Kalmia latifolia</i> 'Big Red'	Jaynes	1.42 ± 0.00	2	
<i>Kalmia latifolia</i> 'Emerald Sheen'	Jaynes	1.40 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-1	1.40 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-2	1.45 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-3	1.42 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-4	1.42 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-5	1.43 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-6	1.49 ± 0.03	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-7	1.48 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-8	1.45 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-9	1.44 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-10	1.41 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-11	1.46 ± 0.01	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-12	1.47 ± 0.02	2	
<i>Kalmia latifolia</i>	Jaynes 2-06-13	1.48 ± 0.04	2	
<i>Kalmia latifolia</i> 'Big Boy'	Woodlanders	2.10 ± 0.00	3	
<i>Kalmia latifolia</i> 'Showtime' ^W	Jaynes	1.40 ± 0.00	2	
		2.75 ± 0.00	4	
<i>Kalmia latifolia</i> 'Silver Dollar' ^W	Jaynes	2.09 ± 0.00	3	
		4.21 ± 0.03	6	
<i>Kalmia latifolia</i>	NCSU H2014-222-002	2.77 ± 0.00	4	
<i>Kalmia latifolia</i>	NCSU H2014-222-004	2.74 ± 0.00	4	
<i>(K. latifolia</i> × <i>K. hirsuta</i>) × <i>K. latifolia</i>	Jaynes 1	1.40 ± 0.01	2	0.70 ± 0.01 AB
<i>(K. latifolia</i> × <i>K. hirsuta</i>) × <i>K. latifolia</i>	Jaynes 2	1.41 ± 0.00	2	
<i>(K. latifolia</i> × <i>K. hirsuta</i>) × <i>K. latifolia</i>	Jaynes 3	1.38 ± 0.01	2	
<i>Kalmia microphylla</i> var. <i>microphylla</i>	UBC 1	1.37 ± 0.03	2	0.69 ± 0.01 AB
<i>Kalmia microphylla</i> var. <i>microphylla</i>	UBC 2	1.36 ± 0.03	2	
<i>Kalmia microphylla</i> var. <i>microphylla</i>	UBC DM/2012-008PT 41559	1.41 ± 0.02	2	
<i>Kalmia polifolia</i>	HVNC	2.59 ± 0.01	4	0.65 ± 0.01 BC
<i>Kalmia polifolia</i>	Thompson 1	3.32 ± 0.03	~5	
<i>Kalmia polifolia</i>	Thompson 2	3.23 ± 0.02	~5	
<i>Kalmia polifolia</i>	Thompson 3	3.34 ± 0.00	~5	
<i>Kalmia polifolia</i> × <i>microphylla</i> var. <i>microphylla</i> 'Rocky Top'	Highstead	2.37 ± 0.00	4	0.59 ± 0.00 DE

^YBRCC = Blue Ridge Community College, Flat Rock, NC; Highstead = Highstead Arboretum, Redding CT; HVNC = Hidden Valley Nature Center, Jefferson, ME; Jaynes = Richard Jaynes, Broken Arrow Nursery, Hamden, CT; Miller = Ron Miller, Pensacola, FL; NCSU = North Carolina State University, Mountain Crop Improvement Lab, Mills River, NC; Southern Highlands = Southern Highlands Reserve, Lake Toxaway, NC; Thompson = Elizabeth Thompson, University of Vermont, Burlington, VT; UBC = University of British Columbia, Vancouver, BC, Canada.; Woodlanders = Woodlanders Nursery, Aiken, SC

^ZValues are means (n = 2) ± SEM.

^XMeans followed by a common letter are not significantly different, Tukey's HSD, P ≤ 0.05.

^WMixoploid/cytochimera

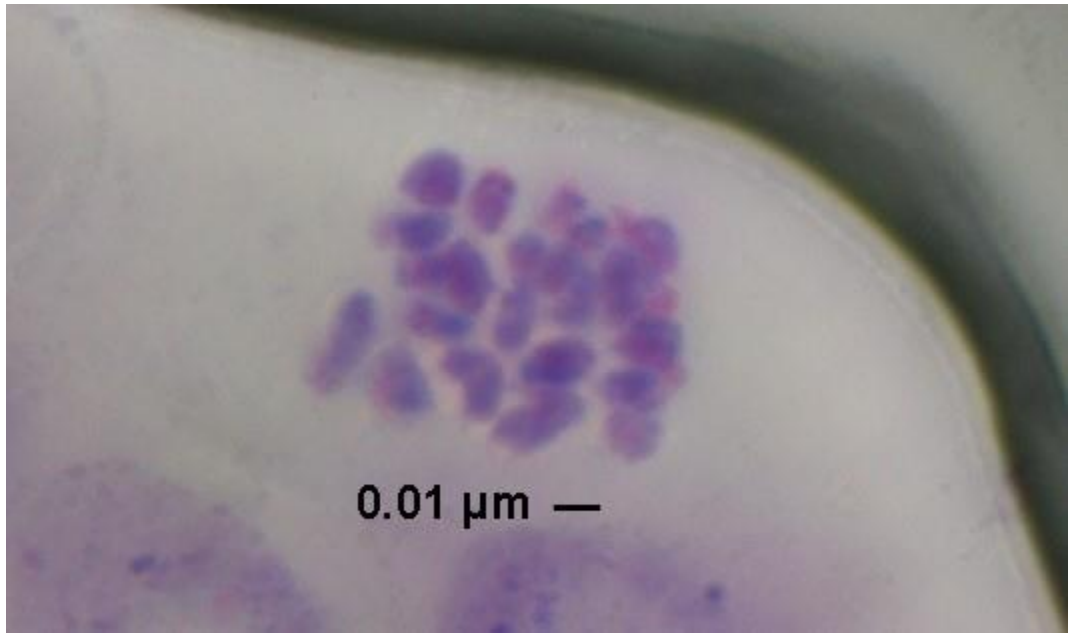


Fig. 1. Condensed chromosomes of *Kalmia latifolia* 'Elf' viewed at 1000× using oil immersion.

CHAPTER 2

In Vitro Polyploid Induction of *Ophiopogon planiscapus* and the Effects of Nitrotyrosine and
Oryzalin

(In the format appropriate for submission to HortScience)

In Vitro Polyploid Induction of *Ophiopogon planiscapus* and the Effects of Nitrotyrosine and Oryzalin

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Received for publication _____. Accepted for publication _____. This research was funded, in part, by the North Carolina Agricultural Research Service, Raleigh, NC, the North Carolina Biotechnology Center, Research Triangle Park, NC, and the Kenan Institute, Raleigh, NC.

Technical assistance of Tom Eaker, Nathan Lynch, Joel Mowrey, Andra Nus, Jeremy Smith and the staff at the Mountain Horticultural Crops Research and Extension Center is gratefully acknowledged. From a thesis submitted by D. A. Gillooly in partial fulfillment of the requirements for the MS degree.

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Subject Category: Propagation and Tissue Culture

In Vitro Polyploid Induction of *Ophiopogon planiscapus* and the Effect of Nitrotyrosine

Additional index words: polyploidy, plant breeding

Abstract. The black-leaved forms of *Ophiopogon planiscapus* are valuable nursery and landscape plants whose slow growth limits production potential by the nursery industry. The creation of autopolyploid black-leaved germplasm has the potential to give rise to more vigorous cultivars. The dinitroaniline herbicide oryzalin is often used as a mitotic inhibitor in the production of polyploids, but success is often sporadic. More consistent and effective treatments would be desirable. The addition of nitrotyrosine has the potential to give rise to increased numbers of stable polyploids when used in conjunction with oryzalin due to its multiplicative effect on the depolymerization of microtubules during mitosis. The objectives of these studies were to optimize in vitro polyploid induction of *O. planiscapus* and specifically evaluate 1) combined effects of dose and treatment duration of oryzalin and 2) combined effects of nitrotyrosine and oryzalin dosages on survival and polyploid induction in vitro. When treated with oryzalin alone, duration had no effect, but callus survival decreased linearly with increasing oryzalin concentration. The mean number of diploid shoots recovered after treatment followed a quadratic model where the percentage of diploid shoots decreased and then began to rise again as oryzalin concentration increased. This trend was seen in reverse in the mean number of mixoploid shoots recovered. As the concentration of oryzalin increased the mean number of mixoploids increased until it began to fall at higher concentrations. No significant effect of concentration on the number of tetraploid shoots

recovered was observed, however tetraploids were successfully recovered from treated callus evidencing that oryzalin is an effective agent for polyploid induction in *Ophiopogon planiscapus*. Mixoploid shoots kept in tissue culture stabilized as tetraploids at a later date. Treatment with oryzalin and nitrotyrosine in combination showed a decrease in callus survival. The number of shoots recovered and ploidy was influenced by oryzalin main effects similarly to the first study with the number of diploids decreasing as oryzalin concentration increased while the number of mixoploids increased. No significant effects of nitrotyrosine alone or interactions with oryzalin were observed. Nitrotyrosine may have a multiplicative effect on cell division in concert with oryzalin but it did not improve conversion rates when used at the concentrations utilized in this study. However, recovery of black-leaved tetraploid *Ophiopogon planiscapus* will provide new opportunities for breeding improved *Ophiopogon* hybrids.

Introduction

Mondo grasses (*Ophiopogon* spp.) are versatile and valuable landscape plants frequently used as groundcovers, foundation plantings, understory plantings, and as massing or edging plants (Fantz, 1993). The cultivar *Ophiopogon planiscapus* 'Nigrescens', commonly known as black mondo grass, is an herbaceous perennial characterized by dark, almost black, grass-like foliage and flowers that are generally more prominent than other *Ophiopogon* (Fantz, 2009). The dark coloration of the foliage is attributable to the high concentrations of anthocyanins and chlorophylls, with the black leaved variety containing ~80% more chlorophyll a and ~30% more chlorophyll b on a fresh weight basis than the green leaved *O. planiscapus* (Hatier, 2007). Anthocyanins present in the black leaved variety were

not detected in significant amounts in the green leaved varieties. Although ‘Nigrescens’ is highly valued for its foliage color, the propagation and production of this cultivar is typically hindered by its slow growth. Artificial doubling of its genome to produce an autotetraploid breeding line may provide opportunities for plant breeders to improve this popular cultivar.

Plant phenotype can be affected by ploidy level in a number of ways. An increase in size and growth rate, larger flowers and longer periods of bloom are often associated with polyploidy. Increased production of secondary metabolites has also been reported (Levin, 2002). Alternatively, the production of artificial autotetraploids may also yield plants which are slower growing than the diploid cytotypes (Levin, 2002). However, induced tetraploids of *O. planiscapus* may provide new opportunities for interspecific hybridization with existing natural tetraploids of *Ophiopogon japonicus* and may prove useful in intergeneric crosses with related tetraploid *Liriope* species *L. gigantea*, *L. muscari*, and *L. platyphylla*. The resultant allotetraploids would have more heterozygosity and potentially exhibit increased vigor (Ranney, 2006).

In vitro techniques provide an ideal platform for the production of polyploids. In vitro polyploidization protocols utilizing tissue culture and treatment with antimetabolic agents was first reported in 1966 and has been an established method since the 1990s (Dhooge et al., 2010). Metaphase inhibitors depolymerize the microtubules, disrupting cytokinesis and leaving cells with a doubled number of chromosomes (Dhooge et al., 2010). Shoots formed from these cells in vitro can be recovered to generate polyploid plants.

Colchicine and dinitroaniline herbicides are commonly used metaphase inhibitors (Dhooge et al., 2010; Ranney, 2006). Colchicine had historically been the preferred agent

due to its ability to retain its effectiveness after autoclaving (Dhooge et al., 2010; Zhang et al., 2007). However there may be side effects including sterility, growth abnormalities, and gene alteration and mutation when colchicine is used. The chemical affinity of colchicine for animal microtubules also makes it a highly toxic substance to humans (Morejohn et al., 1984). Dinitroaniline herbicides have a much higher affinity to plant microtubules and may be used in lower concentrations than colchicine. This reduced affinity to bind to animal microtubules makes them much less toxic to humans, and they are a common substitute for colchicine (Dhooge et al., 2010). Oryzalin is a commonly used dinitroaniline for in vitro polyploid induction and has been used successfully for many species. However, effective concentration and exposure durations can differ greater between taxa. For example, in *Lilium* L., exposure to 150 μ M oryzalin was necessary to induce polyploids (van Tuyl et al., 1992), whereas for *Miscanthus* only 5 or 10 μ M was required (Peterson et al., 2003).

Another chemical which interferes with metaphase is nitrous oxide (Dhooge et al., 2010; Ranney 2006). Although its mode of action is not fully understood it is believed to depolymerize microtubules. In a study which utilized oryzalin as the mitotic inhibitor (Lattier et al., 2013) the addition of the auxin IAA (2-(1H-indol-3-yl)acetic acid) resulted in the production of homogenous stable tetraploids whereas treatments lacking IAA resulted in mixoploid plants only. IAA has been associated with an increase in nitric oxide (Pagnussat et al., 2002). This nitric oxide can react with tyrosine in proteins to form nitrotyrosine (3-nitro-L-tyrosine, NO₂Tyr) (Neill et al., 2003), the addition of which has been shown to act multiplicatively with oryzalin to inhibit cell division, increasing the sensitivity of cells to lower concentrations of oryzalin (Javanovic et al., 2010).

The development of an autopolyploid *O. planiscapus* 'Nigrescens' could enhance ornamental traits and provide nascent lines for future breeding. Therefore two studies were designed, the objectives of which were 1) to develop tetraploid lines of *O. planiscapus* for future breeding and development, and 2) to examine the effects of nitrotyrosine, in combination with oryzalin, on polyploid induction.

Materials and Methods

Embryogenic callus was induced from mature embryos excised from seed collected from *Ophiopogon planiscapus* 'Nigrescens' in September 2012. Embryogenic callus induction and maintenance media consisted of Murashige and Skoog's (MS) basal salts and vitamins (Murashige and Skoog, 1962) supplemented with myo-Inositol at $100 \text{ mg}\cdot\text{L}^{-1}$, 2-(N-Morpholino) ethanesulfonic acid (MES) monohydrate at $100 \text{ mg}\cdot\text{L}^{-1}$, 6-aminopurine sulfate (adenine hemisulfate) at $80 \text{ mg}\cdot\text{L}^{-1}$, sucrose at $20 \text{ g}\cdot\text{L}^{-1}$, $5 \mu\text{M}$ 6-benzylaminopurine (BAP), and $5 \mu\text{M}$ 1-naphthaleneacetic acid (NAA). Media was adjusted to a pH of 5.75 ± 0.03 , solidified with agar at $6.5 \text{ g}\cdot\text{L}^{-1}$ and 25 ml dispensed into 90 mm diameter petri dishes. Mature embryos were placed on the media and incubated in the dark at $23 \text{ }^\circ\text{C}$ to allow embryogenic callus proliferation. These calli were subcultured onto fresh media every 6 to 8 weeks.

Effect of oryzalin concentration and duration on polyploid induction. A liquid media consisting of MS basal salts and vitamins, myo-inositol at $100 \text{ mg}\cdot\text{L}^{-1}$, MES at $100 \text{ mg}\cdot\text{L}^{-1}$ and sucrose at $20 \text{ g}\cdot\text{L}^{-1}$ was prepared. Media was adjusted to a pH of 5.75 ± 0.03 . A 3 mM stock solution of oryzalin (4-(Dipropylamino)-3, 5-dinitrobenzenesulfonamide) was prepared in

95% ethanol. This stock solution was added to cooled autoclaved media. The control solution (0 μM oryzalin) received the addition of 5 ml of 95% ethanol into the 500 ml batch of media. Twenty five milliliters of media was dispensed into 100 ml jars. The experimental design consisted of a completely randomized 3 (duration) \times 4 (concentration) factorial. Calli were submerged in oryzalin concentrations of 0, 7.5, 15 or 30 μM and incubated in the dark on an orbital shaker for 3, 6 or 9 days. Each treatment consisted of five replications with each replication containing five callus pieces (subsamples). After treatment calli were transferred to a liquid MS media (MS basal salts and vitamins, *myo*-Inositol at 100 mgL^{-1} , MES at 100 mgL^{-1} , and sucrose at 20 gL^{-1}). Media was adjusted to a pH of 5.75 ± 0.03 and replaced on the orbital shaker for 24 hours to remove residual oryzalin. Callus pieces were then transferred to an embryogenic maintenance MS media and recovered in the dark. After 28 days, callus survival and shoot formation was scored and resulting shoots analyzed using flow cytometry.

Effects of nitrotyrosine and oryzalin on polyploid induction. In a second study, the effects of nitrotyrosine, in combination with oryzalin, on polyploid induction were investigated. The experimental design was completely randomized with three oryzalin concentrations (0, 7.5 and 15 μM) combined with four separate concentrations of nitrotyrosine (0.0 μM , 0.05 μM , 0.1 μM , or 0.2 μM) in a factorial combination. The exposure duration for all treatments was 6 days. Each treatment consisted of five replications with each replication containing five callus pieces (subsamples).

A liquid MS media consisting of MS basal salts and vitamins, *myo*-Inositol at 100 $\text{mg}\cdot\text{L}^{-1}$, 2-(N-Morpholino) ethanesulfonic acid (MES) monohydrate at 100 $\text{mg}\cdot\text{L}^{-1}$, and sucrose at 20 $\text{g}\cdot\text{L}^{-1}$ was prepared. Media was adjusted to a pH of 5.75 ± 0.03 . Oryzalin and

nitrotyrosine was added to cooled autoclaved media and 25 ml of each solution was dispensed into 100 ml jars. Five callus pieces were placed in each jar, and placed on a rotary shaker in the dark. All jars of each treatment were removed from the shaker after 6 days. Callus were removed from their jars and placed in a liquid MS media (MS basal salts and vitamins, myo-Inositol at 100 mgL⁻¹, MES monohydrate at 100 mgL⁻¹, and sucrose at 20 gL⁻¹ Media adjusted to a pH of 5.75 ± 0.03.) Jars were replaced on the rotary shaker for 24 hours to remove any residual oryzalin or nitrotyrosine. Callus pieces were removed from liquid MS media and re-cultured on the original solidified MS embryogenic maintenance media formulation. After 28 days survival and shoot formation data was collected and resulting shoots analyzed using flow cytometry.

Data Analysis. Data from the all experiments was subjected to ANOVA and multiple regression analysis (Proc GLM, SAS version 9.4; SAS Institute, Cary, NC).

Ploidy analysis. Samples were prepared by placing the *Ophiopogon* tissue in a petri dish with 400 µL of nuclei extraction buffer and chopped finely using a razor blade. The resulting solution was poured through a filter with a pore size of 50 µm. After filtration, 1600 µL of a nucleotide staining buffer solution (CyStain UV Precise P Staining Buffer, Partec, Munster, Germany) containing 4', 6-diamidino-2-phenylindole (DAPI) was added to the solution. Stained nuclei were analyzed using a flow cytometer (Partec, PA-II).

The mean relative fluorescence for each sample was compared with the peak of a confirmed diploid *Ophiogogon* (Lattier et al., 2014) to determine if ploidy levels had been affected by treatment. All polyploid and any strongly mixoploid (>50% tetraploid nuclei) shoots were subcultured into 180 ml jars containing 25 ml of a shoot maintenance media

consisting of MS basal salts and vitamins, myo-inositol at 100 mgL^{-1} , MES at 100 mgL^{-1} , and sucrose at 20 gL^{-1} supplemented with $10 \text{ }\mu\text{M}$ BAP, adjusted to a pH of 5.75 ± 0.03 . Cultures were placed under cool white fluorescent lights ($60 \text{ }\mu\text{mol m}^{-2}\text{s}^{-1}$) with a 16 h photoperiod at $23 \text{ }^\circ\text{C}$. After a period of regrowth these shoots were retested to confirm their ploidy. Diploid and mixoploid shoots were discarded and any tetraploid shoots were subcultured. A population of diploid *O. planiscapus* was also maintained in culture on a solidified MS media containing $10 \text{ }\mu\text{M}$ BAP for use in future morphological studies.

Clones of all tetraploid and selected diploids are currently being multiplied and will subsequently be rooted on a half-strength MS media consisting of Murashige and Skoog's (MS) basal salts and vitamins supplemented with myo-Inositol at $100 \text{ mg}\cdot\text{L}^{-1}$, MES at $100 \text{ mg}\cdot\text{L}^{-1}$, sucrose at $20 \text{ g}\cdot\text{L}^{-1}$, and supplemented with $5 \text{ }\mu\text{M}$ 1-naphthaleneacetic acid (NAA). Media adjusted to a pH of 5.75 ± 0.03 and solidified with agar at $6.5 \text{ g}\cdot\text{L}^{-1}$. Clones will be removed as they root and placed in a greenhouse under intermittent mist to acclimate for use in future breeding work.

Results and Discussion

Effect of oryzalin concentration and duration on polyploid induction. Oryzalin concentration affected callus survival and ploidy level, but there were no effects of duration, nor any interaction between the duration of exposure and the concentration of oryzalin. Callus survival decreased with increasing oryzalin concentration in a linear fashion ($p = 0.0002$) (Fig. 1). Oryzalin concentration also influenced the number of diploid and mixoploid shoots recovered (Fig. 2). The mean number of diploid shoots recovered after

treatment significantly declined with exposure to oryzalin, but remained consistent across all oryzalin concentrations ($p < 0.0001$) (Fig. 2). This trend was seen in reverse in the mean number of mixoploid shoots recovered ($p < 0.0001$). There was an increase in the mean number of mixoploids with exposure to oryzalin, but this remained consistent between 7.5 to 30 μM oryzalin concentrations (Fig. 2). Although there was no significant effect of oryzalin concentration on the number of tetraploid shoots recovered, a limited number of tetraploids were successfully recovered from treated callus (7.5 and 15 μM) evidencing that oryzalin is an effective agent for polyploid induction in *Ophiopogon planiscapus* (Fig. 2). A number of mixoploid shoots that were maintained in tissue culture after their initial testing eventually stabilized as tetraploids after a period of regrowth and acclimation. Seven tetraploids were recovered from shoots initially testing as diploid-tetraploid cytochimeras and five tetraploids were recovered from shoots presenting as tetraploid-octoploid cytochimeras. Due to variations in the rate of cell division and cell fitness, lineages of different cell cytotypes may eventually outcompete one another through this type of endocytotypic selection. However the most common outcome is for these mixoploids to stabilize at the lower ploidy level, as did the tetraploid-octoploid mixes, and it is unusual for stabilization to occur at the higher ploidy as was observed in the diploid-tetraploid mixtures.

Effect of nitrotyrosine on polyploidy induction. Oryzalin and the interaction of oryzalin and nitrotyrosine affected callus survival ($p < 0.05$) whereas number of shoots and ploidy level was affected by oryzalin main effects ($p < 0.05$) and not by nitrotyrosine or interactions. In general, callus treated with oryzalin and 0.2 μM nitrotyrosine had reduced survival (Table 1). In contrast to the first study the effect of oryzalin on the number of diploid

and mixoploid shoots recovered is explained by a linear model ($p < 0.05$). In general, the number of diploids decreased with increasing oryzalin concentration while the number of mixoploids increased.

This interaction of nitrotyrosine and oryzalin on survival may suggest that these chemicals were acting synergistically. However no significant effect could be observed on the number of diploid, mixoploid or tetraploid shoots. In contrast, Jovanovic et al. (2011) used similar concentrations to the present study and found nitrotyrosine increased the sensitivity of cell cultures of *Nicotiana tabacum* to oryzalin. The lack of effect observed in the present study may be explained by the lack of sensitivity of *Ophiopogon planiscaups* to the concentrations of nitrotyrosine administered. At higher concentrations across a range of plant materials a more pronounced effect may have been observed. Further, black-leaved *Ophiopogon planiscapus* retain their slow growing nature in vitro and microtubular dynamics may be similarly slow as well. Because microtubules are eliminated dependent on their innate rates of turnover, lower rates of cell cycling may give rise to microtubules that are not affected by tyrosine and therefore are not eliminated (Jovanovic et al 2010).

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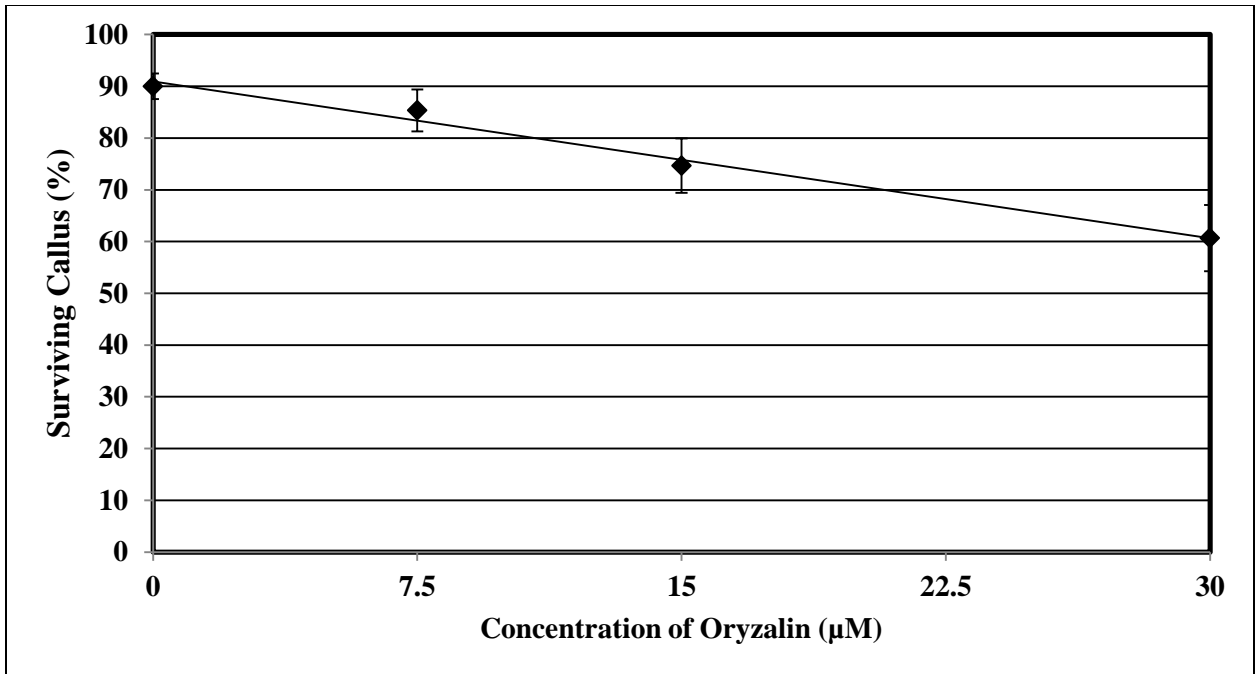


Figure 1: Percentage of callus surviving as a function of oryzalin concentration.

($y = -1.0108x + 90.933$, $R^2 = 0.9882$, $p < 0.0002$).

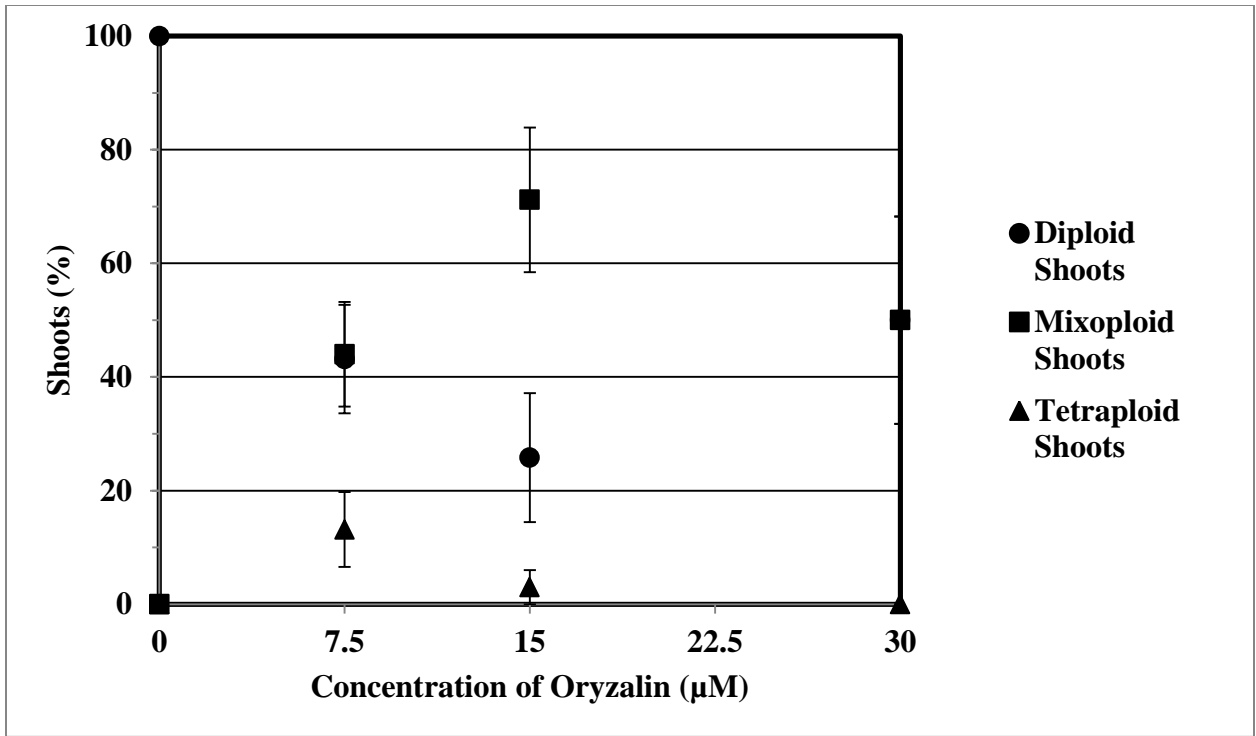


Figure 2: Percentage of diploid, mixoploid and tetraploid shoots recovered as a function of oryzalin concentration.

Table 1: Surviving callus, shoots per replicate and ploidy of shoot in response to a factorial combination of nitrotyrosine and oryzalin.

Conc. Nitrotyrosine (μM)	Conc. Oryzalin (μM)	Surviving Callus (%)	Shoots per Replicate	Ploidy		
				2x (%)	4x (%)	Mixoploid (%)
0	0	64 \pm 7.48 ^Z	4.2 \pm 0.4	100 \pm 0.3	0 \pm 0.0	0 \pm 0.0
0.05	0	52 \pm 20.00	3.0 \pm 0.0	100 \pm 0.4	0 \pm 0.0	0 \pm 0.0
0.1	0	88 \pm 4.90	2.8 \pm 0.6	100 \pm 0.3	0 \pm 0.0	0 \pm 0.0
0.2	0	36 \pm 10.20	3.6 \pm 0.7	100 \pm 0.9	0 \pm 0.0	0 \pm 0.0
0	7.5	16 \pm 10.20	1.5 \pm 0.5	25 \pm 0.0	0 \pm 0.0	75 \pm 0.0
0.05	7.5	52 \pm 5.00	3.3 \pm 0.9	60 \pm 0.4	0 \pm 0.0	40 \pm 0.0
0.1	7.5	64 \pm 4.90	2.8 \pm 0.5	45 \pm 0.5	10 \pm 0.0	45 \pm 0.2
0.2	7.5	52 \pm 4.00	1.5 \pm 0.3	50 \pm 0.0	0 \pm 0.0	50 \pm 0.4
0	15	68 \pm 10.20	2.2 \pm 0.8	43 \pm 0.0	0 \pm 0.0	57 \pm 0.7
0.05	15	68 \pm 11.55	4.0 \pm 3.0	75 \pm 0.0	0 \pm 0.0	25 \pm 0.0
0.1	15	68 \pm 6.67	1.0 \pm 0.0	33 \pm 0.0	33 \pm 0.0	33 \pm 0.0
0.2	15	28 \pm 17.64	2.7 \pm 1.7	0 \pm 0.0	0 \pm 0.0	100 \pm 0.0

Regression Analysis ^Y					
Oryzalin	**	*	*	NS	*
Nitrotyrosine	NS	NS	NS	NS	NS
Oryzalin ² \times Nitrotyrosine ²	**	NS	NS	NS	NS

^ZValues are means (n=25), \pm SEM.

^YSymbols indicate significant linear trend (*), quadratic trend (**), or not significant (NS) at

$\alpha = 0.05$