ABSTRACT

PALMER, IRENE ELIZABETH. Breeding and Evaluation of Perennial Grasses for Landscape and Bioenergy Applications. (Under the direction of Dr. Thomas G. Ranney.)

In the landscape, perennial grasses (PGs) are highly desired for their year-round interest, range of colors and textures, low resource requirements, and excellent pest resistance. Many PGs also have potential as bioenergy crops. Evaluation of a variety of PGs in different locations is necessary if these crops are to be incorporated into energy production systems. Moreover, study of the reproductive pathways and mechanisms of PGs will aid in continued improvement and biological understanding of these plants.

Biomass yield, nutrient removal, and nitrogen (N) response of seven PGs were evaluated in two North Carolina locations (representing Mountain and Coastal Plain regions) over four years to assess the biomass production, nutrient optimization and regional adaptability of these taxa. Giant miscanthus (Miscanthus × giganteus), M. sinensis ‘Gracillimus’ and MH2006, plume grass (Saccharum arundinaceum), ravenna grass (S. ravennae), switchgrass (Panicum virgatum ‘Alamo’), and giant reed (Arundo donax) field plots were established in 2008, treated with four N fertilizer rates (0, 34, 67, 134 kg N ha\(^{-1}\) yr\(^{-1}\)), and harvested annually in winter from 2008-2011. Giant reed, ‘Gracillimus’, switchgrass, MH2006, giant miscanthus and ravenna grass at the Mountain site produced average yields at maturity (mean for the final two harvests) of 22.8, 21.3, 20.9, 19.3, 18.4, and 10.0 Mg ha\(^{-1}\) year\(^{-1}\) respectively. Average yields at maturity for the Coastal Plain site were 27.4, 20.8, 20.1, 14.3, and 9.4 Mg ha\(^{-1}\) for giant reed, giant miscanthus, switchgrass, ravenna grass, and ‘Gracillimus’ respectively. Increasing N rates did not have a consistent significant effect on biomass production. High yields and high mortality for plume grass across sites indicates its potential as a bioenergy crop and need for continued improvement. Overall, the PGs in this study had low nutrient removal,
although giant reed and plume grass often removed significantly more N, P, K and S compared with *Miscanthus* spp. and switchgrass. Our results indicate that giant reed, giant miscanthus, and switchgrass are well-suited and productive potential bioenergy crops across North Carolina.

Flow cytometry was used to elucidate reproductive pathways and cytotype distributions of successive generations *M. sinensis* originating from a complete factorial of 2x (mean 2C genome size=3.97 pg), 3x (2C=5.67 pg) and 4x (2C=7.52 pg) crosses. Intercrossed triploids obtained from 4x × 2x crosses produced predominantly aneuploid offspring (2C=4.66 pg). Although fertility of these aneuploids was low, the subsequent generation derived from these plants exhibited a smaller mean genome size (2C=4.24 pg) and included aneuploids, some apparent diploids, and one individual with a genome size of 6.99 resulting from an unreduced gamete. The 2x × 3x (G1) (2C=4.13 pg) and 3x × 2x (G1) (2C=4.56 pg) populations produced mostly aneuploids and some apparent diploids. The 2x × 3x (G2) (2C=4.37) and 3x × 2x (G2) (2C=4.29) populations included mostly aneuploids. The 3x × 4x (G1) and 4x × 3x (G1) populations exhibited a wide range of aneuploid offspring with genome sizes ranging from 4.25-7.52 pg (2C=5.84 and 5.67 pg, respectively) and included apparent triploids and tetraploids. In the subsequent generation, the genome size of the 3x × 4x (G2) population shifted upward substantially (2C=6.62 pg) while the genome size of the 4x × 3x G2 population shifted downward (2C=4.24 pg). Although 4x × 4x crosses were performed multiple times over multiple years, no seed was ever recovered. Flow cytometric seed screening of seeds from self-pollinated diploid plants revealed limited self-fertilization, axpomixis, and fertilization from unreduced male gametes. These results demonstrate that *Miscanthus sinensis* has access to diverse reproductive pathways, is able to produce triploids and aneuploids that retain limited fertility in some instances, and indicates that successive generations of triploids and aneuploids drift towards the diploid cytotype.
Irene Elizabeth Palmer was born in the early morning on July 30, 1987 in Asheville, North Carolina; her arrival was six and a half weeks early. Her parents would later say “she couldn’t wait to get here.” Irene was raised in Western North Carolina where she attended Hendersonville Elementary, Middle, and High schools. Irene grew up under the careful watch of her wonderful parents, who tolerated her mischievous and sometimes tumultuous childhood and teenage years with grace and support. They instilled in her a love for the arts and the natural world, and most importantly, encouraged her natural inclination for learning about anything that could find its way into her heart. Her parents (and often her sisters) patiently attended many music recitals, theatre performances, creative problem-solving competitions and mock-trial events; they put up art pieces, sent her to writing, photography and marine-biology camps, and watched as she grew to love so much about this world. All of this culminated in a rather complicated self-designed undergraduate degree in Environmental Studies, with minors in history and international relations, a semester abroad in Mexico, and participation on and off the stage in numerous college plays during her time at Centre College.

The same year that Irene began her undergraduate education, she also started working summers at the Mountain Crop Improvement Lab, located at the Mountain Horticultural Crops Research and Extension Center in Fletcher, North Carolina. Despite having a dismal understanding of plants and being a twiggy teenager that could barely move two one-gallon containers at a time, she was hired back year after year, even after she accidentally unleashed catalpa caterpillars all over the facility. Over the summers, she was eventually given the opportunity to conduct her own research and present her findings at student research competitions; this ultimately culminated in publication.
While at the Mountain Crop Improvement Lab, Irene gained experience in traditional breeding techniques, in-vitro polyploid induction, somatic embryogenesis, mutagenesis, cytology, and propagation of a wide range of plants, while helping to maintain a variety of nursery crops in a greenhouse setting. She considered her summer work to be a second education, and she learned by doing. When she graduated, it was clear that, unlike so many of her fleeting fascinations and interests, plant science and breeding was lasting. When people ask Irene “why plants?” she struggles to communicate how their physiology, genetics, and behavior helped to clarify her vision of the living world. The more she learns about their amazing abilities, the closer she feels to everything.

After graduating and working for one more year at the Mountain Crop Improvement Lab, Irene moved to Swarthmore, Pennsylvania, to serve as a summer intern at the Scott Arboretum. There she spent the weeks working with volunteers and staff to beautify and promote the gardens and collections. On the weekends she visited numerous, breathtaking gardens in the area. While working and visiting gardens she saw first-hand how ornamental plants improve the lives of the public. She saw, in turn, how the love for beauty and communal space connects people, and provides them with a sense of community and peace. Making the beauty of the world more accessible to people, while continuing to explore the genetics, physiology, and sheer diversity of plant systems sounded like a great career to Irene, so she applied to graduate school at North Carolina State University with the goal of obtaining a Master’s Degree in Horticultural Science.

As a graduate student, Irene was given the opportunity to work with perennial grasses, which were very different from the woody shrubs and herbaceous perennials she had become familiar with over her years as a research assistant. However, she quickly became quite the grass enthusiast and enjoyed her work with them greatly. Beyond her graduate research, she was also an
active member of the horticultural honors fraternity Pi Alpha Xi, and served as their Vice President of Woodies in 2011-2012. Irene was also given the opportunity to serve as a teaching assistant for the ornamental plant identification course, which was a challenging, but extremely rewarding, experience. While in graduate school, she was fortunate enough to study in Eastern China for a month, where she hiked mountain parks and reserves, collected and identified plants in the field, and explored large cities and small towns. Irene also gave talks at research conferences and to the public concerning her research focused on bioenergy grasses and was fortunate to win many competitive awards and scholarships for her research. As Irene nears graduation, she looks forward to a life filled with love and learning, and is excited to see where she will find herself next.
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If it takes a village to raise a child, then it takes the population of a small city to raise a graduate student. Without the kindness of the numerous teachers, friends, and colleagues, I certainly would not be where I am today. I am forever indebted to those people who have been willing and committed to helping this student find her way in this world.

I must begin with Dr. Tom Ranney. He is a born educator, a formidable scientist and an all-around brilliant mentor. There is really no way to communicate the impact that Tom has had on my life and education, but maybe designating Tom as a life-changing real-life superhero gets close. When Tom hired me as summer help in 2005, I knew almost nothing about plants, and even less about ornamental breeding. Despite the comedy of errors that was my first few summers, and probably against his better judgment, Tom kept hiring me back. He was the one who suggested that I attempt my own research and was there to navigate the subsequent millions of questions and weeks-worth of editing that followed. He tolerated my easily distractible, often unintelligible, attempts at making sense of what I was doing, and spent countless hours patiently pulling a reluctant scientist out of a liberal arts student. I can say with complete certainty that without his guidance, support, and patience, I would never have discovered how truly amazing plants are or how rewarding it is to work with them. I am very grateful that Tom was willing to put up with me for so many years (and so are my parents). Without Tom, I would still be chasing fleeting, insubstantial, interests and struggling to find an academic path forward, so thank you Tom for all you have done for me and all you continue to do for your students.

Dr. Darren Touchell is another great refiner of budding scientists at the Mountain Crop Improvement Lab, who probably has to deal with more graduate student mistakes and emotional
meltdowns than anyone else in the program. However, he handles his responsibility as main graduate-student wrangler with kindness and humor, and has been an absolutely essential part of my education. He has been especially influential in developing my tissue culture skills and scientific vision. His passion for his work helps fuel our passion for our own, and beyond a strong researcher and adept scientist, he provides wise counsel on life in general. I have learned a great deal from him. He oversaw the bulk of my cytotype project, and put in many hours of editing, performed crosses, and helped me work through puzzles involving statistics, experimental design and flow cytometric seed screening for which I am very thankful.

My two remaining committee members, Dr. Ron Gehl and Dr. Dennis Werner were both a pleasure to work with. Ron’s intuitive, no-nonsense guidance concerning the bioenergy grasses was invaluable, and his humor, drive, and the hours put into editing drafts and harvesting grasses were greatly appreciated. Dr. Werner was not only an inspiring professor, but always a delight to engage in conversation, and I am very glad he was able to serve on my committee. To the Raleigh faculty: your classes were challenging and informative and your dedication to education profound. Dr. Wehner, Dr. Yencho, Dr. Dole, Dr. Perera, Dr. Xiang, Dr. Swallow, Dr. Jordan, Dr. Krings, Dr. Dewey, and Dr. Clouse: your classes in particular were terrific. Dr. Jenny Xiang: thank you for taking such wonderful care of the study abroad group in China. You helped to provide an absolutely life-changing opportunity to students, and studying in China has been one of the most powerful events in my life. Dr. Brian Jackson: thank you for giving me the opportunity to serve as your teaching assistant, you are a brilliant teacher. To my fellow graduate students, past and present: your friendship and support was integral to my success, and deeply appreciated. Jason Lattier, Kimberly Shearer, Kelly Oates, Jared Barnes, Lis Meyer, Mary Archer, Rebecca Pledger and Stephanie Haines, this is particularly true for you. To Heather Bader: you were a wonderful housemate and great
friend. To Ryan Davenport: your support during difficult times will not be forgotten. To all the people who helped, listened and lent support during my graduate career: thank you. It meant more than you know.

However, there was a long period of time before I was a graduate student, back when I was a summer research assistant/intern and confused college student. When I began working for the Mountain Crop Improvement Lab the summer after my senior year of high school, Tom was rather insulated from my mistakes. It was his research specialists Joel Mowery and Nathan Lynch that had to deal with me day-to-day, and bore the brunt of my learning curve. I have learned as much from these two men as from many of my professors, and if it were not for their kindness and patience I would have buckled under the weight of my own incompetence in that first summer. Without them, I probably would not have realized that plants and research were the answer to the great question “what am I supposed to do with my life?” Much of the foundational knowledge I now possess about propagation, the work of breeding, flow cytometry, cytology, plant materials, green house management, reggae, and hot sauce I learned directly from watching and talking with them. As a graduate student, they were directly responsible for the survival of hundreds of plants I used in my research, and their help and advice was critical for the success of my projects. To Nathan and Joel: I really enjoyed working with both of you, and I look back on my summers at the Mountain Crop Improvement Lab fondly.

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CHAPTER 1

Biomass Yield, Nitrogen Response, and Nutrient Uptake of Perennial Bioenergy Grasses in North Carolina

(In the format appropriate for submission to Biomass and Bioenergy)
Biomass Yield, Nitrogen Response, and Nutrient Uptake of Perennial Bioenergy Grasses in North Carolina

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Abstract: Although perennial grasses show considerable potential as candidates for lignocellulosic bioenergy production, these crops can exhibit considerable variation in regional adaptability and yield. An assessment of a variety perennial grasses in different regions for biomass yield, nutrient removal, and nitrogen response is an important first step in the development of the bioenergy sector in the Southeastern US. Giant miscanthus (Miscanthus × giganteus Greef & Deuter), M. sinensis Anderss. ‘Gracillimus’ and MH2006, plume grass (Saccharum arundinaceum Retz.), raven grass (S. ravennae (L.) L.), switchgrass (Panicum virgatum L. ‘Alamo’), and giant reed (Arundo donax L.) field plots were established in 2008, treated with four N fertilizer rates (0, 34, 67, 134 kg N ha⁻¹ yr⁻¹), and harvested annually in winter from 2008-2011. Giant reed, ‘Gracillimus’, switchgrass, MH2006, giant miscanthus and raven grass at the Mountain site produced mean yields of 22.8, 21.3, 20.9, 19.3, 18.4, and 10.0 Mg dry matter (DM) ha⁻¹ yr⁻¹.
respectively (averaged over the last two years). Yields at the Coastal site for giant reed, giant miscanthus, switchgrass, ravenna grass, and ‘Gracillimus’ were 27.4, 20.8, 20.1, 14.3, and 9.4 Mg DM ha\(^{-1}\) yr\(^{-1}\) respectively (averaged over the last two years). Increasing N rates did not have a consistent significant effect on biomass production. High yields coupled with high mortality for plume grass at both sites indicates its potential as a bioenergy crop and need for continued improvement. Overall, the perennial grasses in this study had low nutrient removal, although giant reed and plume grass often removed significantly more N, P, K and S compared with *Miscanthus* spp. and switchgrass. Our results indicate that giant reed, giant miscanthus, and switchgrass are productive bioenergy crops across geographic regions of North Carolina.

**Keywords:** Bioenergy, Giant reed (*Arundo donax* L.), Miscanthus (*Miscanthus* spp. Anderss.), Sugarcane (*Erianthus* spp. (L.) P. Beauv.; *Saccharum* spp. L.), Switchgrass (*Panicum virgatum* L.), nutrient removal.

**Highlights**
- Yield, nutrient removal, and N response reported for bioenergy grasses in NC
- Mean yields (final 2 harvests) span 9-27 Mg ha\(^{-1}\) yr\(^{-1}\) (site/taxa dependent)
- Taxa had low nutrient removal; some removed significantly more than others
- N rate did not consistently significantly impact biomass yield in any taxa
- *Arundo*, giant miscanthus, and switchgrass seem suitable for production in NC
1. **Introduction**

There are many traditional and emerging technologies being explored to support the global transition towards more sustainable, secure, and environmentally sensitive energy systems. Bioenergy, renewable energy derived from biological products, is positioned to be one of the more competitive energy paradigms for two major reasons: investment costs are often lower than other available technologies and there exists a wide range of potential raw materials and transformation processes that can be adapted to a variety of geographical locations, transformation capabilities, and energy needs [1]. One facet of the emerging bioenergy sector in the United States is the biofuels industry, which provided Americans with over a fifth of their renewable energy in 2011, representing a 168% increase in consumption since 2001[2]. In an effort to increase the availability and production of biofuels in the US, the 2007 *Energy Independence and Security Act* (EISA) calls for the production of 136 billion liters per year (BLY) of biofuels by 2022, 60 BLY of which must be derived from cellulosic non-food crop feedstock sources [3]. Biofuels derived from the conversion of non-food crop biomass are of particular interest as new studies question the social and environmental impact of a system that confounds energy and food production [4-8].

Many woody and herbaceous feedstocks exist for potential development and optimization within the emerging bioenergy sector. However, international interest in production systems utilizing perennial grasses has positioned a suite of perennial grasses as strong candidates for dedicated lignocellulosic bioenergy crops. These grasses have high biomass yields, low input requirements [9-11], are productive on marginal land, and provide ecological benefits including soil improvement [12], increased carbon sequestration [13], and wildlife habitat [14,15]. In some cases, perennial grasses have been projected to produce more than twice the amount of ethanol per
hectare than maize grain [16]. While many perennial grasses are being explored for use as bioenergy crops, certain taxa in Miscanthus, Saccharum (Erianthus), Panicum, and Arundo have garnered particular attention as high producing, low-input energy crops, with great potential for improvement and optimization.

Miscanthus contains approximately 14-23 species of tall, warm season C₄ grasses native to East and South East Asia [17, 18]. Miscanthus sinensis Anderss., M. sacchariflorus (Maxim.) Franch., and M. ×giganteus Greef & Deuter, are of significant interest as bioenergy crops for a variety of reasons including high biomass yield and high nitrogen (N) and water use efficiencies [18-20]. Generally, N fertilization does not appear to increase biomass production in Miscanthus [19]. When N fertilizer treatments are found to influence biomass production, they generally have a limited positive effect which is usually apparent only after the first two years of growth [21]. The primary limiting factor for increasing biomass production in Miscanthus is often soil water availability [18,20, 22, 23].

Miscanthus is reported to form symbiotic associations with N₂-fixing bacteria, which may account for the limited N fertilizer responses [24-26].

Giant miscanthus, Miscanthus ×giganteus, (2n = 3x = 57) is a naturally occurring, highly infertile, triploid hybrid derived from diploid Miscanthus sinensis (2n = 2x = 38) and tetraploid Miscanthus sacchariflorus (2n = 4x = 76) [27]. Giant miscanthus produces dry matter (DM) biomass yields ranging from 5-38 Mg DM ha⁻¹ yr⁻¹ in the United States [22, 28-30] and 10-25 Mg DM ha⁻¹ yr⁻¹ in non-irrigated European plots, although yields in excess of 30 Mg DM ha⁻¹ yr⁻¹ have been reported for irrigated stands in southern Europe [31]. Giant miscanthus stands are usually derived from rhizome cuttings, which increases costs associated with propagation and field establishment compared with seed-established crops [19]. However, once established, stands can maintain productivity for at least 14 years [32]. While giant miscanthus has received significant attention in Europe and the US,
costs associated with vegetative propagation and sensitivity to cold temperatures during establishment in some locations [31] limits its ubiquitous use as a bioenergy crop. Chinese silver grass, *Miscanthus sinensis*, has also shown promise, both as a source of genetic material for improvement and as a competitive bioenergy crop [17, 23, 33-35]. Cultivated Chinese silver grass has been reported to reach heights of 1-2 m and produce between 10-40 Mg DM ha$^{-1}$ yr$^{-1}$ in Europe [34]. *Miscanthus sinensis* ‘Gracillimus’ is a popular dense, thin-leaved ornamental that reaches approximately 2m in height and has shown high survival in cooler environments and biomass yields of 2-17 Mg DM ha$^{-1}$ yr$^{-1}$ in Germany and 26.5 Mg DM ha$^{-1}$ yr$^{-1}$ in Italy [35].

A close relative to *Miscanthus*, *Saccharum* contains approximately 6 species, and is dominated by robust rhizomatous C$_4$ cane grasses found mostly along riversides and in valleys or on open hillsides [36, 37]. Certain members of this genus are already successful bioenergy crops, including conventional sugarcane (*Saccharum officinarum* L.). More recently, high fiber energycanes (mostly *Saccharum spontaneum* L. hybrids) [18] have demonstrated yields of from 15.9-32.4 Mg DM ha$^{-1}$ yr$^{-1}$ in Alabama [38], 2-15 Mg DM ha$^{-1}$ yr$^{-1}$ in Arkansas [39], and 18.7 Mg DM ha$^{-1}$ yr$^{-1}$ in Georgia [40]. While sensitivity to winter temperatures limits the use of many *Saccharum* representatives to tropical and sub-tropical regions [38], more cold-hardy members of *Saccharum* (sometimes classified as *Erianthus spp.*) may be useful in temperate regions, including *Saccharum arundinaceum* Retz., plume grass, and *S. ravennae* (L.) L., ravenna grass. These stout, reedy, cold-hardy tufted canes are native to temperate and tropical Asia, India and China and produce large quantities of above ground biomass. However, documentation concerning yield potential and cold hardiness of plume grass and ravenna grass is limited.

*Panicum virgatum* L., switchgrass, is a vigorous, spreading, sod-forming C$_4$ grass, native to North America where it is a common member of the tall grass prairies of the Central Plains. Switchgrass
exhibits considerable genetic diversity [41-43] and includes two distinct ecotypes: the lowland tetraploid (2n=4x=36) ecotype and the upland octaploid (2n=8x=72) ecotype [44]. Improvement and selection of cultivars for conservation and forage purposes in the US began in the 1930s, and in the 1990’s the US Department of Energy began investigating switchgrass as a potential bioenergy crop. Switchgrass was originally selected for conservation purposes and bioenergy production because it is native, perennial, adaptable, palatable to livestock, has documented and well-understood establishment and management practices, and is a crop with which many farmers have some familiarity [45]. Production trials led to the determination of the best commercial varieties, of which ‘Alamo,’ a lowland variety, was selected for its ability to thrive in the Deep South [45]. Switchgrass yields reportedly span 1-40 Mg DM ha\(^{-1}\) yr\(^{-1}\) with the majority of reported yields ranging from 10-14 Mg DM ha\(^{-1}\) yr\(^{-1}\) [46]. Switchgrass has been shown to respond to N fertilizer applications, however annual N requirements are relatively low with optimum yields reported from 40 to 224 kg N ha\(^{-1}\) depending on the site, harvest regime, cultivar, and stand age [45, 47-48, 49]. Switchgrass can be established from seed and stands usually require 3 years to reach productive maturity [44]. As with Miscanthus, N\(_2\) fixing, bacterial endophytes have been implicated in the high N use efficiency of switchgrass [44, 50]. Response to phosphorus (P) is variable, although some reports indicate that switchgrass will respond to P additions in conditions where soil-available P is low [49].

Giant reed, *Arundo donax* L., is a C\(_3\), densely culmed, emergent riparian cane grass that forms clonal stands with an extensive and vigorous root system. Originally native to Asia, giant reed is now naturalized throughout Southern Europe, North Africa, and the Middle East [51]. Giant reed is one of the largest, fastest growing herbaceous grasses on earth [52]. It is of monophyletic origin [51], and has been cultivated for canes, fiber, construction, and musical reeds for thousands of years [52]. Although giant reed rarely produces viable seed and is effectively sterile [53] it is not
difficult to propagate, with stem and rhizome fragments easily producing shoots and roots at nodes [54]. Giant reed tolerates drought and a range of soil types and qualities [55] and its tolerance of soils containing heavy metals and high levels of salinity has encouraged its exploration as a crop for phytoremediation [56]. Culms are thick and persistent with reported heights of 2 to 9 meters [38, 51] and yields ranging from 5-33 Mg DM ha\(^{-1}\) yr\(^{-1}\) have been reported [28, 40, 57]. Reported response of giant reed to N additions varies, but in some cases yield increases in response to N application has been reported [28, 57]. Compared with some other perennial grasses, giant reed exhibits greater nutrient removal [28, 40].

Although these perennial grasses are strong potential candidates for lignocellulosic bioenergy production, they can exhibit substantial differences in adaptability and yield, which can vary considerably by genotype, location, and fertilizer regime [21, 22, 31, 37, 46]. Additionally, information on regional yields and adaptability, N response, and nutrient uptake are often lacking. An assessment of a variety of crops in different geographic regions and soils as well as optimization of nutrient management is an integral component of the transition towards large scale bioenergy production. Thus, the objectives of this study were to evaluate biomass yield, N response, nutrient removal (N, P, K, Ca, Mg, and S) and regional adaptability of selected perennial grasses over four years at two locations in North Carolina as part of a larger, ongoing effort to select and breed improved bioenergy cultivars.

2. Materials and Methods

2.1 Site information

Research was conducted at two sites in North Carolina: The Mountain Horticultural Crops Research Station in Mills River (Mountain site) and the Williamsdale Biofuels Field Lab located near
Wallace (Coastal Plain site). Yearly temperature and precipitation data for both were obtained from weather stations located at each site and compiled by the North Carolina Climate Retrieval and Observations Network of the Southeast (CRONOS) database (Table 1). Data for the 30-year precipitation and temperatures were obtained from the National Oceanic and Atmospheric Administration (NOAA). Soil samples were taken prior to planting and at emergence at both sites annually. Each plot was sampled to a depth of 20 cm using a 1.7-cm ID probe, and 8-10 cores were collected per plot and mixed to form a representative composite sample. Composite samples were sent to the NC Department of Agriculture and Consumer Services (NCDA&CS) Agronomic Division soil testing lab for analysis including: humic matter, soil pH, cation exchange capacity (CEC), and nutrient analysis (Table 2) [58-60].

2.2 Trial establishment

The seven taxa selected for this study were: giant miscanthus, *M. sinensis* H2006-006-001 (MH2006) and cv. ‘Gracillimus’, ravenna grass, plume grass, switchgrass cv. ‘Alamo’, and giant reed. The population of giant reed used in the field was obtained from the Biomass Investment Group (Asheville, NC) as bare-root single-shoot rhizomes with shoots approximately 30cm in height and were planted as such. MH2006 was micropropagated in tissue culture and planted as plugs from 8.25 cm wide x 8.90 cm deep 18 cell trays. Planting stock of ‘Gracilimus’, giant miscanthus, ravenna grass, and plume grass were obtained from Hoffman’s Nursery Inc. (Rougemont, NC) also grown in 18 cell trays to a height of 7cm. Both sites were planted in the spring of 2008.

Site preparation at each location included spring plowing to a depth of 30 cm and 2-3 passes with a rotovator to approximately 20 cm. Prior to the final rotovator pass at the Mountain site, 3.4 Mg ha⁻¹ of agricultural lime was broadcast applied based on soil sample results and per the recommendation from the NCDA&CS. The treatment design was a complete factorial with seven
taxa and four N rates (0, 34, 67, 134 kg N ha\(^{-1}\) yr\(^{-1}\)) arranged in a completely randomized design at each of the two locations. Each plot was 5 x 5m, and contained 25 plants in five rows, with plants spaced 1m apart. An exception to this plant spacing was ‘Alamo’, which was seeded by hand at 4 kg seed ha\(^{-1}\) in 13 rows per plot, with 2.5cm deep furrows spaced 30cm apart. Plots were separated by 2m wide alleys. Both sites received overhead irrigation, at approximately 2.5cm wk\(^{-1}\), for the first 3 months after planting. Nitrogen treatments were surface-broadcast as ammonium nitrate (33.5% N) annually in the spring prior to emergence. Pre-emergence herbicide (S-metolachlor plus atrazine) was applied each year at both sites prior to emergence, and paraquat post-emergent herbicide was spot-sprayed as necessary to minimize weed competition. No significant pests or disease were reported at either site throughout the duration of this study. No plots were replanted after the initial establishment in the spring of 2008.

2.3 Survival, biomass, and nutrient removal

Plant survival and biomass yield were determined annually from harvests in late-December and early-January after stands had undergone a killing frost and subsequent dry-down in the field. Nine plants, selected from the interior 3 x 3m area of each plot, were cut approximately 10 cm above ground and weighed fresh. For ‘Alamo’ the entire interior 3m x 3m square was cut and weighed. Beginning in 2010, as the plants of the rhizomatous species (giant reed and giant miscanthus) had grown together, the entire 3m x 3m interior of each plot was harvested. In instances where record plants from within the 3 x 3m record area recolonized area vacated by a dead plant, survival was readjusted to account for the filling-in of record areas. After sampling the record area of each plot, all remaining, standing biomass was removed from each plot.

Within each record area of each plot, a ~1kg subsample was selected at random, ground, and weighed fresh to determine wet weight. These subsamples were then oven dried at 80°C for 72
hours and reweighed to determine dry weight and to calculate moisture content at harvest. Each subsample was then milled to pass a 2mm sieve and sent to the NCDA&CS plant testing lab to determine nutrient concentrations [61]. Data were analyzed using a generalized linear mixed model (SAS PROC MIXED) with N rate and year treated as continuous factors [62]. In cases where N rate had a significant effect, additional polynomial regression (SAS PROC REG) analyses were completed. Plots that exhibited less than 75% survival were excluded from data analysis (this includes plume grass in 2010 and 2011 at the Mountain site and MH2006 from 2009-2011 and plume grass in 2011 at the Coastal Plain Site).

3. Results and Discussion

Interactions among year, location, and taxa were significant across all variables (Table 3). In some instances, N rate had significant effects on yield, survival and nutrient uptake; however, these effects were generally small, and overshadowed by the main effects associated with year, taxa, and location. Based on these results, simple effects for year × location × taxa are presented in Tables 4 and 5. Regression analysis is presented when there were significant effects for N.

3.1 Survival

At the Mountain site, giant miscanthus, ‘Gracillimus,’ MH2006 and giant reed had greater than 90% survival from time of establishment through the final harvest in late December 2011. Ravenna grass and plume grass had lower total survivability of 69 and 28%, respectively (Table 4). Switchgrass produced dense, even stands at this site, although lodging was observed in some years.

At the Coastal Plain site, giant miscanthus and giant reed had 100% survival at the fourth harvest, while ‘Gracillimus’, MH2006, plume grass and ravenna grass exhibited 81, 22, 33 and 72% survivability, respectively (Table 5). Switchgrass also produced dense, even stands at this site.
In general, giant miscanthus, giant reed and switchgrass produced uniform stands at both locations. For the two rhizomatous species, giant miscanthus and giant reed, limited initial mortality was generally overcome as plants produced rhizome shoots. Although giant miscanthus had 100% final survival at both sites, survival of the two *M. sinensis* representatives, ‘Gracillimus’ and MH2006, was 16% and 70% higher at the Mountain site than the Coastal Plain site, respectively. The Coastal Plain site did not receive substantially less precipitation than the Mountain site (Table 1), and other factors such as high summer temperatures and the sandy soil (Table 2) at the Coastal Plain site may have reduced overall survival of the *M. sinensis* accessions there, indicating that *M. sinensis* may not be as well suited for the Coastal Plain environment compared with other perennial grass options.

Plume grass survival decreased from 97 to 28% at the Mountain site and from 92 to 33% at the Coastal Plain site from 2008-2011. Ravenna grass survival decreased from 83 to 69% at the Mountain site and from 94 to 72% at the Coastal Plain site from 2008-2011. The cause of mortality for these two clumping species is unclear, but stress from harvesting may have been a factor as plants were easily uprooted and dislodged when cut. In addition to stress associated with harvest, high mortality for plume grass at the Mountain site may also be attributable to marginal hardiness to winter temperatures there, as plume grass is generally considered to be cold hardy to USDA zone 7 [36] while the Mountain site is located in USDA 6B/7A. Unlike plume grass, the reduced survival of ravenna grass was probably not the result of cold sensitivity as it is typically considered to be hardy to USDA zone 6 [36] and is commonly found growing as a landscape plant throughout North Carolina.

3.2 Biomass yield

Mean biomass yield for surviving taxa at each site will be presented for the final two harvests, as by the third season many of these grasses were approaching maturity. Excluding the first two
establishment years, giant reed, ‘Gracillimus’, switchgrass, MH2006, and giant miscanthus had similar yields of 22.8, 21.3, 20.9, 19.3, and 18.4 Mg DM ha\(^{-1}\) yr\(^{-1}\) respectively at the Mountain site (Table 4). Ravenna grass yielded significantly less than the others at 10.0 Mg DM ha\(^{-1}\) yr\(^{-1}\). The similar yields for many of these taxa may indicate a common factor, most likely water, was limiting maximum growth at this upland site. Increases in yield occurred for all taxa each year from 2008 through 2010 at the Coastal Plain site, with the exception of MH2006 which exhibited high mortality. However, between the 2010 and 2011 harvests at the Coastal Plain site, yield decreased for all taxa with the exception of switchgrass and giant miscanthus (Table 5). While giant miscanthus yield increased 5% (20.3 to 21.5 Mg DM ha\(^{-1}\) yr\(^{-1}\)) between the 2010 and the 2011 harvests, switchgrass yields increased by 52% (15.9 to 24.2 Mg DM ha\(^{-1}\) yr\(^{-1}\)) indicating that stand maturity may be reached later in switchgrass than giant miscanthus in some environments. Excluding the first two establishment years at the Coastal Plain site, giant reed had the highest mean yield (averaged over the last two harvests) at 27.4 Mg DM ha\(^{-1}\) yr\(^{-1}\). Giant miscanthus and switchgrass produced intermediate yields of 20.8 and 20.1 Mg DM ha\(^{-1}\) yr\(^{-1}\), respectively, and ravenna grass and ‘Gracillimus’ had the lowest yields (14.3 and 9.4 Mg DM ha\(^{-1}\) yr\(^{-1}\), respectively) (Table 5). Although there was considerable mortality over time and plume grass was not included in the 2011 dataset, one of the highest yields in the study was recorded for plume grass at the Coastal Plain site in 2010 (31.7 Mg DM ha\(^{-1}\) yr\(^{-1}\)). In one instance, a single surviving plume grass plant located at the Coastal Plain site in 2011 produced more biomass (53.51 kg fresh weight) than the entire harvested plot of any other taxa at either site in any year. While reduced competition for water and increased access to sunlight may have influenced the growth of the large surviving plume grass accessions at both sites, there exists considerable potential for continued improvement of plume grass as a bioenergy crop if issues of mortality can be addressed.
Average yields for giant miscanthus, excluding the first two seasons, at both sites ranged from 18.4 to 20.8 Mg DM ha$^{-1}$ yr$^{-1}$. While these yields are less than those reported for unfertilized plots in Illinois (24 to 38 Mg DM ha$^{-1}$ yr$^{-1}$) [29], they are greater than those reported from the southern U.S. which range from 4.5 Mg DM ha$^{-1}$ yr$^{-1}$ in unfertilized Texas plots[30] to 3-6 Mg DM ha$^{-1}$ yr$^{-1}$ in fertilized stands in Oklahoma [28]. Our yields are most comparable to those reported from fertilized stands in Kentucky, Nebraska and New Jersey which yielded 16.5-19.5, 15.2-28.0 and 9.3-17.9 Mg DM ha$^{-1}$ yr$^{-1}$, respectively, in their third year [22]. High survivability and high comparative yields indicate that giant miscanthus could serve as a competitive bioenergy crop at the two different sites in North Carolina, while M. sinensis ‘Gracillimus’ and MH2006 appear better suited for the cooler Mountain site environment. Given the yields associated with ‘Gracillimus’, future work exploring the productive capabilities of a wide array of M. sinensis ecotypes and cultivars from their diverse natural range [63] could prove useful in the improvement of bioenergy Miscanthus.

In North Carolina, ‘Alamo’ switchgrass has been found to produce mean yields of 13.4 to 21.6 Mg DM ha$^{-1}$ yr$^{-1}$ [45], which are consistent with our results ranging from 20.1 to 20.9 Mg DM ha$^{-1}$ yr$^{-1}$ when averaged across the final two harvests. Our results are also consistent with switchgrass average yields of 12 to 19 Mg DM ha$^{-1}$ yr$^{-1}$ produced across the south and middle latitudes in the U.S. [45]. A study by Kiniry et al. (2012) determined that some switchgrass cultivars, including ‘Alamo’, can exhibit higher radiation use efficiencies than Miscanthus, and may produce higher biomass yields when located in water-limiting environments [30]. However, our average yields, when excluding the first two years, were not significantly different for giant miscanthus and switchgrass at either site. Overall, switchgrass performed well at both sites, and would make a strong bioenergy candidate in either environment, especially considering reduced costs associated with seed establishment.
Giant reed also produced well at both sites, with yields averaged across the final two harvests of 22.8 to 27.4 Mg DM ha\(^{-1}\) yr\(^{-1}\). High survival coupled with consistently high yields indicates its suitability as a bioenergy crop in North Carolina and other similar environments. While our yields are less than those reported in Oklahoma of 33.2 Mg DM ha\(^{-1}\) yr\(^{-1}\) [28], they are greater than yields reported from non-fertilized stands on marginal land in Georgia which produced 4.8-7.3 Mg DM ha\(^{-1}\) yr\(^{-1}\) [40]. Despite our high yields, nutrient removal for N, P, K, and S were consistently greater for giant reed when compared to other perennial grasses in this study, and the reduced yields reported from unfertilized stands in Georgia indicate that productive stands of giant reed may require more nutrient supplementation than other perennial grasses.

The Billion Ton Study (2005) estimated that 16-24 million hectares of pasture and farmland could be replaced to produce 136-345 million dry tons of biomass assuming annual yields of 11-17 Mg DM ha\(^{-1}\) yr\(^{-1}\) [3]. All taxa, with the exception of the *Saccharum* representatives, were capable of meeting or exceeding this minimum requirement at the Mountain site, and at the Coastal Plain site, giant reed, giant miscanthus, switchgrass, and ravenna grass all met and surpassed this minimum requirement.

### 3.3 Yield response to nitrogen

For a given combination of taxa, location, and year, N rate generally did not have a significant effect on the amount of biomass produced. The Coastal Plain site produced one exception: giant reed during the 2010 growing season exhibited a negative linear response to N rates \((P=0.015)\). As N rate increased, giant reed yields decreased from 31.2 Mg DM ha\(^{-1}\) yr\(^{-1}\) produced by stands treated with 0 kg ha\(^{-1}\) N to 27.9 Mg DM ha\(^{-1}\) yr\(^{-1}\) produced by stands receiving 134 kg N ha\(^{-1}\). This is the first report of a negative association between N application and biomass yield for giant reed, and is not consistent with the literature where a positive response, or the lack of a positive response, is most
often reported [57, 28]. Similarly, both a lack of response and a positive response to N applications are reported for Miscanthus [21, 22, 64]. The general consensus remains that the N requirement for Miscanthus spp. is low due to the life-history characteristics associated with Miscanthus including use of the N-efficient C₄ photosynthetic pathway, characteristic nutrient cycling associated with the rhizome structure of these species [65], and potential associations with N-fixing bacteria [24-26]. Switchgrass is found to exhibit yield increases in response to N application in some instances, though considerations as to the productivity of a specific cultivar, management and harvest regimes and site-specific requirements should be taken into account [66]. In Texas, the optimum N application rate was determined to be 168 kg N ha⁻¹ yr⁻¹ although the achieved yields reported were lower than yields reported here [49], while optimal yield was obtained in Alabama with applications of 224 N kg ha⁻¹ yr⁻¹ [67]. Lower rates of N applications have also been suggested ranging from 56 to 67 kg N ha⁻¹ yr⁻¹ [68, 69]. Continued productivity of these perennial grasses in subsequent years should be assessed in North Carolina to determine if and when N applications will be useful in maintaining high yields.

3.4 Nutrient removal

3.4.1 Nitrogen

Nitrogen removal at the Mountain site increased substantially between the establishment years of 2008 and 2009 for all taxa (Table 4): giant miscanthus removed 27 kg N ha⁻¹ in 2008 and 130 kg N ha⁻¹ in 2009 (381% increase); ‘Gracillimus’ removed 9 kg N ha⁻¹ in 2008 and 172 kg N ha⁻¹ in 2009 (1,811% increase); MH2006 removed 5 kg N ha⁻¹ in 2008 and 137 kg N ha⁻¹ in 2009 (2,640% increase); plume grass removed 60 kg N ha⁻¹ in 2008 and 238 kg N ha⁻¹ in 2009 (297% increase); ravenna grass removed 27 kg N ha⁻¹ in 2008 and 185 kg N ha⁻¹ in 2009 (585% increase); switchgrass removed 20 kg N ha⁻¹ in 2008 and 150 kg N ha⁻¹ in 2009 (650% increase); and giant reed removed 33
kg N ha\(^{-1}\) in 2008 and 252 kg N ha\(^{-1}\) in 2009 (664% increase). After the initial increase in N removal from 2008 to 2009, N removal for all taxa was greatly reduced in 2010 and 2011. Over the course of the 4 year study at the Mountain site, giant miscanthus, ‘Gracillimus’, MH2006, plume grass, ravenna grass, switchgrass and giant reed removed an average of 59, 86, 70, 149 (first two years only), 74, 86 and 112 kg N ha\(^{-1}\) yr\(^{-1}\) respectively.

Taxa at the Coastal Plain site also exhibited an increase in N removal in 2009, followed by a reduction in 2010 and 2011 (Table 5). Giant miscanthus removed 11 kg N ha\(^{-1}\) in 2008 and 102 kg N ha\(^{-1}\) in 2009 (827% increase); ‘Gracillimus’ removed 2 kg N ha\(^{-1}\) in 2008 and 56 kg N ha\(^{-1}\) in 2009 (2700% increase); plume grass removed 51 kg N ha\(^{-1}\) in 2008 and 278 kg N ha\(^{-1}\) in 2009 (445% increase); ravenna grass removed 52 kg N ha\(^{-1}\) in 2008 and 178 kg N ha\(^{-1}\) in 2009 (increase of 242%); switchgrass removed 45 kg N ha\(^{-1}\) in 2008 and 107 kg N ha\(^{-1}\) in 2009 (138% increase); and giant reed removed 9 kg N ha\(^{-1}\) in 2008 and 237 kg N ha\(^{-1}\) in 2009 (2533% increase). The average annual N removal for taxa at the Coastal Plain Site for giant miscanthus, ‘Gracillimus,’ plume grass (first three growing seasons), ravenna grass, switchgrass and giant reed were 47, 45, 161, 97, 77, and 132 kg N ha\(^{-1}\) yr\(^{-1}\), respectively. Kering et al. (2012) also reported significant reductions in N removal between the second and third year of development for giant reed and switchgrass stands located Oklahoma. Many of these crops utilize nutrient cycling systems, whereby nutrients from living above-ground tissues are translocated and stored in underground storage organs for the winter and remobilized in the spring. Whether the reduction between the second and third year N removal indicates maturation of the below-ground nutrient cycling systems in these crops or is the result of site specific phenomena remains unclear.
3.4.2  *Phosphorus, Potassium, Calcium, Magnesium, Sulfur*

Mountain Site: The average annual P removal for giant miscanthus, ‘Gracillimus’, MH2006, plume grass for 2008 and 2009 harvest only), ravenna grass, switchgrass, and giant reed was 3, 5, 5, 13, 7, 6, and 11 kg P ha\(^{-1}\) yr\(^{-1}\), respectively (Table 4). Potassium removal was not significantly different among taxa when averaged across four years at the Mountain Site, with the exception of giant reed and plume grass which removed significantly more K than the other taxa at 166 and 135 kg K ha\(^{-1}\) yr\(^{-1}\), respectively; the remaining taxa removed an average of 58-79 kg K ha\(^{-1}\) annually. Giant reed and the *Saccharum* representatives consistently removed more P and K when compared to the *Miscanthus* taxa.

Giant miscanthus, ‘Gracillimus’, MH2006, plume grass, ravenna grass, switchgrass and giant reed removed an average of 27, 43, 40, 30, 23, 38, and 24 kg Ca ha\(^{-1}\) yr\(^{-1}\) and 8, 14, 11, 12, 8, 18, and 9 kg Mg ha\(^{-1}\) yr\(^{-1}\), respectively. Switchgrass removed significantly more Mg than other taxa for the final two harvests, although plume grass removed the most Mg per year for the two years it was included in analyses. Giant miscanthus, ‘Gracillimus’, MH2006, plume grass, ravenna grass, switchgrass and giant reed removed an average of 6, 8, 6, 14, 11, 8 and 20 kg S ha\(^{-1}\) yr\(^{-1}\). Giant reed removed significantly more S on average annually.

Coastal Plain site: Giant miscanthus, ‘Gracillimus’, plume grass, ravenna grass, switchgrass and giant reed removed an average of 5, 5, 15, 8, 8, and 13 kg P ha\(^{-1}\) yr\(^{-1}\), as well as 47, 36, 206, 104, 70 and 176 kg K ha\(^{-1}\) yr\(^{-1}\), respectively (Table 5). As with the Mountain site, giant reed and plume grass consistently removed more P and K than the other taxa. Giant miscanthus, ‘Gracillimus’, plume grass, ravenna grass, switchgrass and giant reed also removed an average of 29, 23, 55, 34, 30, and 39 kg Ca ha\(^{-1}\) yr\(^{-1}\) and 12, 7, 21, 11, 16 and 17 kg Mg ha\(^{-1}\) yr\(^{-1}\) and 5, 4, 25, 16, 9, and 29 kg S ha\(^{-1}\) yr\(^{-1}\), respectively. Plume grass removed significantly more average Mg per year than the *Miscanthus*
representatives, and both plume grass and giant reed consistently removed more S than *Miscanthus* representatives.

While our results were consistent with removal rates reported for giant reed, giant miscanthus, and switchgrass, stands of giant reed in Oklahoma removed substantially more Mg than giant reed at either of our North Carolina sites [28]. Although Propheter and Staggenborg (2010) did not report a substantial increase in N removal from the first to the second year of growth, they found that total N, P, and K removal for rotated corn was 150-366, 840-1250, and 112-304% greater respectively than giant miscanthus and 219-454, 422-800, and 160% greater respectively than switchgrass [10], indicating that many of these crops remove substantially less nutrients at harvest than maize. However, while these crops may remove relatively less nutrients than high-input annual crops, our data indicates that, over time, biomass harvesting will remove important macronutrients from the soil, and minimum replacement fertilization regimes should be considered when growing perennial grasses for bioenergy use to maintain productive yield. It is worth noting that giant miscanthus stands in Illinois receiving no N fertilization for 5-7 years have been estimated to hold 600 kg N ha\(^{-1}\) after 7 years of growth within the actively growing stands. Almost 200 kg N ha\(^{-1}\) of this in plant N reserve is lost during harvest and leaf drop [70], indicating the possibility of N-fixing associations accounting for the high levels of N accumulation, the presence of which has been confirmed on site [26]. Further research, especially exploring the activity of beneficial bacterial and fungal associations, will be a critical part of enhancing the low-nutrient requirements of these perennial grasses.

4. **Conclusions**

The results of our study indicate that regional adaptability, yield, and nutrient removal can vary significantly among different perennial grasses, environments, and from year to year, and that
optimizing the abilities and requirements of bioenergy systems through taxa selection and nutrient optimization will be an important component in the creation of efficient perennial grass bioenergy infrastructure. More specifically, we determined that giant miscanthus, M. sinensis ‘Gracillimus’ and MH2006, giant reed, and switchgrass ‘Alamo’, can be productive biomass sources in environments similar to those at our Mountain site, while giant miscanthus, switchgrass cv. ‘Alamo’ and giant reed may perform well in locations that are similar to our Coastal Plain site. Not all taxa were equally productive at each site, and Miscanthus sinensis taxa seem better adapted to the environment at the Mountain site than the Coastal Plain site, where they exhibited greater mortality rates and less biomass yield. While plume grass had poor survival in our systems, its substantial biomass producing potential was documented.

Overall, the perennial grasses analyzed in this study were found to have low annual nutrient removal, although giant reed and plume grass were found to have significantly higher removal rates of many nutrients when compared to Miscanthus and switchgrass. While low nutrient removal (minimal inputs) is often desirable, in some instances, such as phytoremediation, high levels of nutrient removal may be desired. Our results indicate a wide range of nutrient removal abilities among evaluated taxa, which will aid in future development of these taxa as bioenergy feedstocks and potentially provide growers with removal options based on specific needs. Future research on longevity of stands, continued analysis of new bioenergy perennial grasses in the region, documentation of the activity and effects of beneficial associations with soilborne bacteria and fungi, continued documentation of nutrient removal overtime by these grasses, as well as multi-environment trials for promising bioenergy candidates will be an important part of optimizing a perennial grass bioenergy system to the specific needs and environmental conditions throughout the Southeastern United States.
Acknowledgements

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Table 1: Monthly precipitation total and mean temperature for 4 years at both sites.

<table>
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<th>Precipitation (mm)</th>
<th>Temperature (°C)</th>
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<td>2009</td>
</tr>
<tr>
<td>January</td>
<td>59</td>
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<table>
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<th>Temperature (°C)</th>
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<tr>
<td>December</td>
<td>55</td>
<td>70</td>
</tr>
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| Total (mm) or Average (°C) | 1091 | 834 | 1324 | 1012 | 1287 | 12.5 | 12.4 | 12.3 | 12.1 | 12.7 |

\(^a\)30 year means derived from quasinormals, source: National Oceanic and Atmospheric Administration (NOAA)
Table 2: Soil characterization and prior use information for the Mountain and Coastal Plain sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>Soil Description</th>
<th>Prior Use</th>
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<th>Harvest dates</th>
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<th>pH</th>
<th>CEC</th>
<th>Ca</th>
<th>Mg</th>
<th>K</th>
<th>P</th>
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<td>Mountain</td>
<td>Bradson gravelly loam</td>
<td>Fescue hay, unfertilized, for &gt;3 years prior to experiment</td>
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<td>02-06-2009</td>
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<td>Fine-loamy, siliceous, subactive, thermic Aquic Paleudults</td>
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Table 3: Analysis of variance for yield, survival, and nutrient removal as a function of taxa, location, nitrogen rate, and year.

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*a* designates significance at p≤0.05  
**b** designates significance at p≤0.01
Table 4. Biomass yield, survival, and nutrient removal for selected taxa over four years at the Mountain site.

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<th>Year</th>
<th>Taxa</th>
<th>Yield&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Survival&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N</th>
<th>P</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
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<td>Mg DM ha&lt;sup&gt;-1&lt;/sup&gt; yr&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>%</td>
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<sup>a</sup>Means within years and in the same column with different letters are significantly different at p<0.05 (Waller-Duncan K-ratio t-test)

<sup>b</sup>Means for yield are derived from the final two harvests to exclude establishment years; means for nutrient removal reflects data from all four years
Table 5. Biomass yield, survival, and nutrient removal for selected taxa over four years at the Coastal Plain site.

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<th>Nutrient removalb kg ha⁻¹</th>
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<th>K</th>
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*a*Means within years and in the same column with different letters are significantly different at p<0.05 (Waller-Duncan K-ratio t-test)

*b*Means for yield are derived from the final two harvests to exclude establishment years; means for nutrient removal reflects data from all four years.

26
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CHAPTER 2

Fertility, reproductive pathways, and cytotype distribution of successive generations of interploid

*Miscanthus sinensis* hybrids

(In the format appropriate for submission to Plant Reproduction)
Fertility, reproductive pathways, and cytotype distribution of successive generations of interploid

*Miscanthus sinensis* hybrids

Irene E. Palmer³*, Darren H. Touchell¹, Thomas G. Ranney³, Todd J. Rounsaville²

¹North Carolina State University, Mountain Crop Improvement Lab, 455 Research Drive, Mills River, NC 28759

*Corresponding author. E-mail: irene.e.palmer@gmail.com

Abstract: Flow cytometry was used to elucidate reproductive pathways and cytotype distributions of successive generations (G1 and G2) of *M. sinensis* originating from a complete factorial of 2x (mean 2C genome size=3.97 pg), 3x (2C=5.67 pg), and 4x (2C=7.52 pg) crosses. Intercrossed triploids obtained from 4x × 2x crosses produced predominantly aneuploid offspring (2C=4.66 pg). The subsequent generation derived from these aneuploids exhibited a smaller mean genome size (2C=4.24 pg) and included aneuploids, apparent diploids, and one individual (6.99 pg) resulting from an unreduced gamete. The 2x × 3x (G1) (2C=4.13 pg) and 3x × 2x (G1) (2C=4.56 pg) populations produced mostly aneuploids and apparent diploids. The 2x × 3x (G2) (2C=4.37) and 3x × 2x (G2) (2C=4.29) populations included mostly aneuploids and one individual with a genome size of 6.63 pg. The 3x × 4x (G1) and 4x × 3x (G1) populations exhibited a wide range of aneuploid offspring with genome sizes ranging 4.25-7.52 pg (2C=5.84 and 5.67 pg, respectively) including apparent triploids and tetraploids. The genome size of the 3x × 4x (G2) population shifted upward (2C=6.62 pg) while the genome size of the 4x × 3x (G2) population shifted downward (2C=4.24 pg). Although 4x × 4x crosses were performed multiple times over multiple years, no seed was recovered. Flow cytometric seed screening of progeny derived from self-pollinated diploid plants revealed potential
limited self-fertility, apomixis, and fertilization from unreduced male gametes. These results
demonstrate diversity in *Miscanthus sinensis* reproductive pathways, production of triploids and
aneuploids that retain limited fertility, and drift of successive generations of triploids and aneuploids
towards the diploid cytotype.

Keywords: *Miscanthus sinensis* · autopolyploidy · aneuploidy · interploid hybridization · unreduced
gametes · flow cytometry
1. Introduction

Polyploidy, the addition and accumulation of complete (euploidy) or incomplete (aneuploidy) sets of chromosomes to a genome, has acted as a driving force in the evolution of higher plants. It is now widely accepted that most, if not all, extant angiosperm families experienced ancient whole genome duplications (Bower et al 2003; Soltis et al 2009; Jiao et al 2011). Whole genome duplications provide genetic preconditions that can lead to modified gene function and subsequent neofunctionalization of duplicate genes, subfunctionalization of duplicate gene activities, selection for modifications of gene product dosage, and masking or removal of nonfunctional or nonbeneficial duplications (McGrath and Lynch 2012). The initial development of a polyploid individual can result in immediate reproductive isolation, and once polyploid lines are established, silencing and modification of copies of ancestral genes can further isolate polyploid populations and subgroups within polyploidy lines, potentially resulting in speciation (Lynch and Conery 2000).

Classical polyploids, as opposed to paleopolyploids, are classified by the homology or homeology of their genomes, though a wide spectrum concerning the homology and homeology of chromosome sets may exist in a single species (Tate et al 2005). Autopolyploids contain multiple sets of homologous chromosomes derived from within a single species via unreduced gametes or mitotic irregularities. Allopolyploids contain two or more functionally different genomes (and thus homeologous chromosomes) and are most often the result of interspecific or intergeneric hybridization in conjunction with WGD. Classical polyploids are found to exist in natural populations (Baack and Stanton 2005; Kao and Parker 2010; Collins et al 2011; Travnicek et al 2011; Husband and Schemske 1998) as well as important crop species including wheat, canola, peanut,
and tobacco (allopolyploids) and alfalfa, potato, watermelon, and strawberries (autopolyploids) (Udall and Wendel 2006). Recent meta-analyses indicate that apomixis, the asexual production of clonal seed as a result of changes in the double fertilization pathway, correlates with polyploidy in angiosperms (Whitton et al. 2008; Carman 1997). It is hypothesized that polyploidy may allow for the stabilization and perpetuation of apomixis by masking deleterious mutations (Comai 2005) and compensating for mutation accumulation that is the result of decreased or absent sexual recombination (Ozias-Akins and van Dijk 2007).

While autopolyploids are often similar in appearance to their diploid predecessors (Soltis et al 2007), they may exhibit morphological changes such as reduced fertility, delayed developmental processes (Yao et al 2011; Deng et al 2012), larger organs and cells (Stupar et al 2007; Annsour et al 2009; Yu et al 2009; Yao et al 2011; Li et al 2012), and increased tolerance to environmental stress (Zhang et al 2010; Deng et al 2012). Changes in gene expression between autopolyploids and their diploid predecessors are often subtle and seem to affect relatively few genes and gene families (Comai 2005; Wang et al 2006; Stupar et al 2007; Yu et al 2010; Li et al 2012). Ploidy manipulation and the creation of synthetic autopolyploids is useful in plant breeding, where they can utilized to facilitate hybridization by removing ploidy-associated reproductive barriers, overcome one-locus gametophytic systems of self-incompatibility (Mabel 2004), enhance ornamental characteristics (Paden et al 1990; Yamaguchi 1989; Takamura and Miyajima 1996), and reduce fertility in potentially invasive or weedy species (Rounsaville et al 2011). Synthetic autopolyploids are also useful in elucidating mechanisms of dosage-dependent gene expression (Wang et al 2006).

Poaceae provides a variety of model species for exploring ancient paleopolyploidy and the reproductive and genetic behaviors of classical polyploids and apomictic species in angiosperms.
Poaceae exhibits all major polyploidy classifications (Levy and Feldman 2002), and the presence of large-scale duplications in grass genomes, which are indicative of a series of ancient grass-specific polyploidy events, have been reported (Levy and Feldman 2002; Wang et al 2005; Jiao et al 2011; Yao et al 2011; Schnable et al 2012; Swamanithan et al 2012). Members of Poaceae have also been found to produce unreduced gametes, a major vehicle for autopolyploid formation, at higher rates than many families (Ramsey and Schemske 1998, supplemental data), and efficient methods of vegetative production in some species may allow for perpetuation of individuals exhibiting reduced fertility, as is the case with the sterile polyploid *Arundo donax* (Balogh et al 2012).

Poaceae also contains many polyploid facultative apomictic model species including members of *Bracharia, Panicum*, and *Paspalum* (Ozias-Akins and van Dijk 2007). Gametophytic apomixis, whereby megagametophytes produce an unfertilized embryo, has been reported to occur in 126 genera, although approximately 75 percent of these genera are located within Poaceae, Rosaceae, and Asteraceae (Carman 1997). In Poaceae, a common pathway for gametophytic apomixis is apospory, whereby somatic nucellar tissue is converted into an unreduced megagametophyte following the degeneration of the megaspore mother cell. This system is often combined with pseudogamy, a reproductive process in which a single sperm nuclei fuses with the polar nuclei to form the endosperm, but no genetic contribution from the male parent occurs (Whitton et al 2008, Ozias-Akins and van Dijk 2007). Diplospory, where the megagametophyte is derived from the megaspore mother cell, and parthenogenesis, where the embryo forms directly from an unfertilized egg cell, has also been reported in grasses, where it couples with pseudogamy (Ozias-Akins and van Dijk 2007 and citations within). Sexual and apomitic
systems of reproduction (as well as different modes of apomixis) are not mutually exclusive, and may occur in a single population (Matzk et al 2005, Ebina et al 2005).

Flow cytometric seed screens (FCSSs) (Matzk et al 2000), anatomical organ clearing, cytological analysis, and progeny screening can be used to elucidate reproductive pathways in many angiosperms (Ozias-Akins, 2006). Flow cytometric seed screens analyze nuclei from a single seed or a seed bulk and allow for rapid ploidy and genome size determination of the embryo and endosperm. The associated ploidy levels can be used to draw conclusions concerning the reproductive pathway of the derived the seeds. For example, in many instances, a 2C embryo and 3C endosperm peak indicate sexual recombination whereby the embryo is the result of the fusion of the egg (n) and a single sperm (n) and the endosperm (assuming double fertilization) is the result of 2n (maternal parent polar nuclei) + n (single sperm) for a 3C peak. The presence of a 2C embryo peak and a 4C endosperm peak, however, often indicates an apomictic event (such as diplospory or apospory). Flow cytometric seed screens can also be used to detect the presence and parental origin of unreduced gametes. For example, in many cases a 3C embryo peak obtained from a seed derived from diploid parents indicates the action of an unreduced gamete; the associated endosperm would be 5C (4n + n) if the maternal parent provided the unreduced gamete, or 4C (2n + 2n) if provided by the male parent. However, not all angiosperms follow this model due to differences in embryo sac structure (Carman 1997), and species-specific considerations must be made to efficiently use FCSSs (Matzk et al 2000).

Miscanthus, classified in the subtribe Saccharinae, has been found to contain a diverse gene pool (Sacks et al 2013), and is able to form intra (Rounsaville et al 2011) and interspecific interploid hybrids (Hodkinson et al 2002b; Nishiwaki et al 2011) as well as hybridize with closely related...
genera, such as *Saccharum* (Hodkinson et al 2002a). *Miscanthus* have a base chromosome number of n=19 and range in ploidy levels from diploid to hexaploid; however, diploid and tetraploid populations appear to be the most prevalent (Clifton-Brown et al 2008 and citations within).

*Miscanthus sinensis* Anderss. is a tall, C₄, warm-season perennial grass native to Japan, the Korean Peninsula, eastern China, and parts of Russia (Stewart et al 2009; Darke 2007) where it serves as a robust, successful pioneer species capable of adapting to variable environmental conditions including poor soils and marginal conditions (Lewandowski et al 2000), and readily colonizes disturbed sites (Kaufman and Kaufman, 2007; Quinn et al 2010). Members of *Miscanthus*, including *M. sinensis*, have long been recognized as important forage, fiber, and ornamental crops (Darke, 2007; Stewart et al 2009; El Bassam 2010) and in recent decades have garnered much attention as potential bioenergy crops (Heaton et al 2008; US Dept. Energy, 2011; Gauder et al 2012). Ploidy levels vary within *M. sinensis*, with reports of diploid, triploid and tetraploid cytotypes (Meyer and Tchida 1999; Clifton-Brown et al 2008). Cultivars of *M. sinensis* appear to be dominated by diploid (2n = 2x = 38) accessions (Clifton-Brown et al 2008; Rayburn et al 2009). In most instances, triploid *M. sinensis* exhibit highly reduced fertility or male sterility (Meyer and Tchida 1999; Rousaville et al 2011), but may occasionally produce fertile aneuploid offspring (Rounsaville et al 2011). Aneuploids in big bluestem (*Andropogon geradii* Vitman) have been reported to exhibit unequal chromosomal segregation as a result of multivalent and univalent formation, as well as lagging chromosomes which results in chromosome loss (Norrmann and Keeler 2003).

While much research has been conducted concerning the reproductive behavior (Sall et al 2005; Liu and Wendel 2002) and response of allopolyploids to genetic shock (Ha et al 2009, Buggs et al 2011, Parisod et al 2009) less is documented regarding autopolyploids (Parisod et al 2010).
Further, autopolyploid cytotype studies often focus on issues of geographic distribution of autopolyploids rather than reproductive behavior and genome size distribution of autopolyploid cytotypes and their progeny (Dijk and Bakx-Schotman 2003; Husband and Schemske 1998). The reproductive potential and cytotype outcomes of offspring derived from triploid and aneuploid parents is also often lacking in the literature, as the reproductive behavior of these cytotypes is often considered to result in genetic dead ends. Thus, the objectives of our study were to analyze the cytotype distributions of successive generations of progeny originating from a complete factorial of 2x, 3x and 4x parents and to use FCSS to explore the reproductive pathways available to diploid *M. sinensis* populations.

2. **Materials and Methods**

2.1 **Plant material**

The taxa used as initial parents in this study included 18 diploid, 15 triploid, and 18 tetraploid accessions of *Miscanthus sinensis* Andersson. The triploid and tetraploid accessions were developed on site at the Mountain Crop Improvement Lab™. Autotetraploids were derived via oryzalin-mediated, in-vitro chromosome doubling using methods similar to Herbert et al (2010). Triploids were obtained by crossing the derived autotetraploids with diploid accessions and were recovered using embryo rescue (Rounsaville et al, 2011). Flow cytometry was used to confirm the ploidy level of each parent based on DNA content following Rounsaville et al. (2011).

2.2 **Diploid crosses and self-pollination**

To assess the frequency of self-pollination or apomixis, individual flower spikes were randomly selected from eight diploid plants and self- and cross-pollinated with other randomly
selected diploid plants. Plants were completely randomized in a greenhouse environment with each plant treated as a block with 2 flower spikes (subsamples) receiving either self or cross pollinations. Flowers of cross-pollinated plants were not emasculated. To facilitate pollinations, flower spikes were placed in paper bags and agitated daily while flowers were receptive. Once mature, a maximum of 100 seed or seed per 1000 parental florets were removed from spikelets. Ten seed from each seed population were randomly selected and analyzed using flow cytometry to determine the genome size of the embryo and endosperm to elucidate the reproductive pathways following Matzk et al. (2000).

2.3 Interploid hybridization experimental design

The initial diploid (2x) by tetraploid (4x) crosses were performed in the summer of 2007 to generate triploids (3x). In 2011 2x, 3x, and 4x cytotypes were intercrossed in a factorial combination in a completely randomized design with n=6 (Fig. 1). For each pairing, an inflorescence of each parent was enclosed in a dry paper bag prior to pollen dehiscence and shaken daily for several days. Due to low fertility, two or more inflorescences were placed in each bag for triploid parents. Following anthesis, inflorescences from each parent were harvested and the seeds collected. First generation (G1) progeny, n=7-14, from each reciprocal cytotype cross were intercrossed the next year in plastic-covered isolation chambers to produce second generation (G2) populations. In instances where 2x parents were used, individual G1 plants that had genome sizes that fell within a 95% confidence interval for diploid 2C genome size were excluded to remove the influence of potential selfs or apomicts. Inflorescences in each chamber were brushed daily during anthesis to better disburse pollen. After 8 weeks, seeds were collected and sown. At least three panicles were collected from each individual plant. A minimum of 10 and a maximum of 24 seedlings recovered
from each plant were analyzed for genome size using flow cytometry, with the exception of some plants in the 3x × 4x and 4x × 3x populations which exhibited low fertility and seed set.

2.4 Seed set and overall fertility

The number of seed per 1000 florets was determined and a minimum of 100 seeds were collected from each replicate cross off each parent for each generation to determine seed set (total seeds per replicate/total number of parental florets counted), germination (number of germinates/total number of seeds), and seedlings per 1000 florets (seed set × germination × 1000). Prior to counting, individual racemes from each panicle associated with a specific individual were removed and randomly selected to create a composite raceme sample. Seeds were removed from the spikelets, de-husked, and placed on moist blotter paper (SDB3.5; Anchor Paper Co., St. Paul, MN) in plastic petri dishes sealed by parafilm. Seed were germinated under a 16-h photoperiod of 30 μmol m⁻² s⁻¹ (400-700nm) provided by cool-white fluorescent lamps at 23 °C. Germination was defined as the emergence of the plumule and radicle and was recorded after 4 weeks. Seedlings were placed in 72 cell packs and grown on a standard seedling mix (1 peat moss : 1 vermiculite (v:v) mix supplemented with 7.2 kg lime, 1 kg Micromax, 1 kg 0-17-0, 0.6 kg KNO₃, 0.2 kg MgSO₄, 30 g B, and 45 g Fe per cubic meter) to an approximate size of 5cm at which point tissue was collected from each seedling and genome size determined via flow cytometry. After non-destructive flow cytometric analysis, seedlings were grown out in green house conditions until they reached approximately 50cm in height. Plants were then transplanted into 5.7L containers containing a standard nursery mix (pine bark supplemented with 1.8 kg lime and 1.0 kg Micromax per cubic meter), fertilized (30g per cubic yard of Osmocote slow release fertilizer), and placed outside on a gravel container pad. These G1 plants received drip irrigation from May through August, at which
point individuals from each cytotype population were randomly selected to serve as parents for the subsequent generation (G2) and moved into isolation chambers.

2.5 Flow cytometry

Holoploid, 2C DNA content for all parental taxa and selected individuals from subsequent generations was determined via flow cytometry (Doležel et al 1998). Approximately 0.5 cm$^2$ of young Miscanthus leaf tissue was chopped using a razor blade in a small petri dish containing 400 µL of DNA extraction buffer (CyStain ultraviolet Precise P; Partec, Münster, Germany). *Pisum sativum* L. ‘Ctirad’ was used as an internal standard with a known 2C genomic size of 8.75 pg (Greilhuber et al 2007). Because *P. sativum* ‘Ctirad’ has a similar genome size to tetraploid Miscanthus, diploid *M. sinensis* ‘Zebrinus’, with a genome size of 4.08 pg, was used as a secondary standard to verify tetraploids. Suspensions were filtered through 50 µm mesh and kept on ice until the addition of the staining buffer containing 4’, 6-diamidino-2-phenylindole (DAPI) (CyStain ultraviolet Precise P; Partec). Nuclei were then analyzed using a Partec PA-II flow cytometer (Partec, Münster, Germany). Holoploid 2C DNA content of Miscanthus samples was calculated as genome size of the standard × (mean fluorescence value of the sample/mean fluorescence value of the standard *P. sativum* ‘Ctirad’). Seed was collected from the diploid self-pollinated and cross-pollinated plants to complete FCSS analysis following Matzk et al. (2000). A single seed was chopped, treated with the extraction buffer and stain and analyzed to determine the relative genome size of the embryo and endosperm. *Pisum sativum* L. ‘Ctirad’ was used as an external standard for these analyses.
3. Results and Discussion

3.1 Diploid crosses and self-pollination

Mean seed set was 19% in cross-pollinated plants and 94% of those seeds exhibited a diploid embryo and a triploid endosperm (Table 1). The remaining 6% of the seeds had triploid embryos and tetraploid endosperm, indicative of a normal reduced embryo sac with fertilization by unreduced 2n male gametophytes. While events of self-pollination or apomixis were limited and occurred in less than 1% of the florets of self-pollinated plants, 100 seed were collected for cytometric analysis. Of those seeds, 94% had a diploid embryo and a triploid endosperm. Two percent of seeds exhibited a diploid embryo and a tetraploid endosperm, indicative of apomixis. The remaining 4% of seeds had a triploid embryo and tetraploid endosperm indicating a reduced embryo sac with fertilization by unreduced 2n male gametophytes.

3.2 2C Genome size of parental euploids and subsequent offspring

The mean (± SEM) 2C genome size of the 2x, 3x and 4x parental taxa were 3.97 ± 0.06, 5.67 ± 0.20, and 7.52 ± 0.20 pg, respectively (n = 12-18). Diploid × diploid G1s exhibited a consistent genome size of 3.97 ± 0.01 pg. The 2x × 2x G1s produced an average of 481 seeds per 1000 parental florets. The 2x × 2x G2s had a consistent genome size of 4.00 ± 0.01 pg and produced an average of 542 seeds per 1000 florets.

The 2x × 4x (G0) population had a unimodal distribution around a mean of 4.02 ± 0.01 pg, which falls within the 99% prediction interval for diploids (3.97 ± 0.07) (Fig. 2). These plants were assumed to be predominantly self-pollinated or apomictic. However, 41% of recovered seedlings fell outside the 99% prediction interval for diploids; these recovered seedlings fell predominantly above
the range for diploids (43 seedlings). Seven seedlings were recovered that had genome sizes smaller
than diploids with genome sizes of 3.33, 3.80, 3.82, 3.86 pg, as well as 3 individuals with a genome
size of 3.89 pg. No triploids were recovered from female diploid parents. The 4x × 2x (G0)
population however, exhibited a unimodal distribution around 5.68 pg, consistent with triploids.
When these 4x × 2x (G0) triploid plants were intercrossed, they produced predominantly aneuploid
offspring. The 3x × 3x (G1) population showed a mean genome size of 4.65 ± 0.03 pg, intermediate
between diploid and triploid levels, although 9 putative triploid seedlings (6%) and a single putative
diploid (0.7%) were recovered, as well as one individual having a genome size of 3.77 pg. The 3x × 3x
(G2) population had an even lower mean genome size of 4.24 ± 0.03 pg, and included 18 individuals
(13%) with genome sizes consistent with diploids, 2 individuals (1%) at triploid levels, and one
individual with a genome size of 6.99 pg that most likely resulted from an unreduced gamete.

The 2x × 3x (G1) produced 52 (42%) seedlings consistent with diploids (most likely self-
pollinated or apomictic), 69 (56%) aneuploid seedlings with intermediate genome sizes between
diploids and triploids, one plant near a triploid level (6.00 pg), and a single haploid (2.00 pg) (Figure
3). This population produced a unimodal distribution around a mean of 4.13 ± 0.02 pg. The 3x × 2x
(G1) also exhibited a unimodal distribution around a mean of 4.56 ± 0.02 pg and included exclusively
aneuploids with one individual with a genome size of 6.63 pg most likely the result of a reduced
triploid gamete fusing with an unreduced diploid gamete. The 2x × 3x (G2) population, which was
derived from only 2x × 3x G1s that fell outside the 95% confidence interval for diploids, had a mean
of 4.36 ± 0.03 pg. This was not significantly different than the mean genome size of 4.29 ± 0.02 for
the 3x × 2x (G2) population. Seven percent of these combined populations exhibited genome sizes
that fell within the 99% prediction interval for diploids; these populations were predominantly aneuploids.

The 3x × 4x (G1) and 4x × 3x (G1) populations exhibited a wide range of mostly aneuploid offspring with genome sizes ranging from 4.25 - 7.52 pg with mean genome sizes of 5.83 ± 0.10 and 5.63 ± 0.11 pg, respectively (Fig. 4). Fertility associated with the parents (G0 euploid crosses) of these populations was low, producing 55 or less seedlings per 1000 parental florets. In the subsequent generation, the genome sizes of the 3x × 4x (G2) population shifted upward with a mean of 6.62 ± 0.06 pg while the genome size of the 4x × 3x G2 population shifted downward with a mean of 4.24 ± 0.12 pg. The fertility of 4x × 3x (G1) was very low: crosses only produced a mean of 4 seedlings per 1000 florets. Three individuals that fell within the 99% prediction interval for tetraploids (7.52 ± 0.24) were recovered in the 4x × 3x (G1) population, and near tetraploids were found in low numbers (1-3 seedlings in each population) in the 3x × 4x (G1 and G2) and 4x × 3x (G1). The 4x × 3x G1 population exhibited abnormally formed inflorescences with reduced spikelet production. Although 4x × 4x crosses were performed multiple times over multiple years, no seed was ever recovered.

3.3 Euploid genome size

The mean of the 2C genome size associated with our triploid and tetraploid accessions were slightly less than 1.5 and 2 times, respectively, greater than the the 2C value of the diploids; both the triploids and the tetraploids were found to be 5-6% smaller than expected. This is consistent with previous research, which reports decreases in 1C genome size in many genera following ploidy increase as a result of genome downsizing (Ozkan et al 2003; Soltis et al 2003; Leitch and Bennett 2004; Buggs et al 2009). Genome sizes larger or smaller than the 99% prediction interval for diploids
(derived from the parental diploid taxa) were recovered in the 2x × 2x G1 and G2 at a greater rate than 1%. The cause of this variation remains unknown. The presence of supernumerary chromosomes, which have been reported in Miscanthus (Weng 1962), should be explored.

3.4 Apomixis and self-compatibility in Miscanthus sinensis

The production of seeds with 2C embryos and 3C endosperm following self-pollination may indicate low self-fertility as has been reported found by Deuter (2000), where putative self-fertility in M. sinensis was documented to range from 1-37%. Fertility under outcrossing conditions was also explored in that study, ranging from 9-80%. Although FCSS can be a useful tool in illuminating reproductive phenomena, FCSS may be unable to distinguish between sexually and apomitically derived individuals if a 2C embryo and 3C endosperm are created regardless of the reproductive pathway used to derive the seed. For example, in the aposporous pseudogamic pathway associated with the Panicum-type embryo sac, the embryo sac is produced with four mitotically-derived, unreduced nuclei including one 2n polar nucleus (Matzk et al 2000). If the polar nucleus undergoes single fertilization (pesudogamy), the seed would have a 2C embryo and a 3C endosperm peak, making it indistinguishable from sexually derived seeds with a reduced embryo sac and double fertilization by reduced male gametophytes (Matzk et al 2000). However, while FCSSs would be unable to detect differences between sexually derived and aposporous pseudogamous reproduction, the recovery of a seed with a 2C embryo and 4C endosperm peak indicates apomixis, with the 4C endosperm produced autonomously or as the result of fusion with an unreduced 2n male gamete. Organ clearing of developing M. sinensis spikelets will be key in determining the nature of the apomictic pathway in Miscanthus. The higher levels of apparent self- fertilization in diploids following cross-pollination with plants of higher ploidy levels (e.g., Fig. 3) may result from
mentor effects where the mixture of self- and cross-pollination leads to a reduction in self-incompatibility systems (Hörandl 2010).

3.5 Unreduced gametes

The recovery of autotriploids from diploid crosses (Table 1) and unusually large genome sizes in other populations (e.g., 6.99 pg in 3x × 3x (G2) – Fig. 2, 6.63 pg in 3x × 2x (G1) – Fig. 3) indicates the occasional presence of unreduced gametes. The recovery of seed containing 3C embryos and 4C endosperm (Table 1) indicates that unreduced gametes were derived from the male parent, while no evidence was found suggesting unreduced gametes from the female parent (i.e., seeds with 3C embryos and 5C endosperm). Rounsaville et al (2011) also found limited progeny (0.4 – 1.1%) derived from unreduced gametes from open pollinated diploid and triploid maternal M. sinensis. Unreduced gametes can result from meiotic irregularities including elimination of the first or second meiotic division, abnormal spindle formation during the second division, or unsuccessful cytokinesis, and has been reported in many plants (Ramana and Jacobsen 2003). Interestingly, no tetraploid offspring were observed in 3x × 2x (G1) or 2x × 3x (G1) populations, in what would be considered to be a triploid bridge route to tetraploid formation.

3.6 Formation and fertility of triploids

The production of autotriploids in M. sinensis seems to be primarily unidirectional with the vast majority of recovered triploids produced by female autotetraploids crossed with diploids (Fig. 2). Reciprocal 2x × 4x crosses resulted in predominantly diploid progeny although a few hypo- and hyperploids were also produced.
Female fertility of *M. sinensis* autotriploids has been previously reported by Rounsaville et al (2011) and appears to be genotype specific; male fertility was also addressed in that study and found that pollen stainability for autotriploids ranged between 52-70%, indicating some male fertility. The initial 2x × 2x cross in our study yielded 481 seedlings per 1000 parental florets, compared with 143, 48, 389 seedlings per 1000 florets recovered in the 4x × 2x (G0), the 3x × 3x (G1), and 3x × 3x (G2), respectively. This indicates that while the fertility of triploids is initially low (~10% of seedling yield associated with diploid crosses), the aneuploid offspring recovered from 3x × 3x crosses may have increased fertility with successive generations as populations migrate closer to the diploid genome size. In *Miscanthus*, synthetic autotetraploids seem to have reduced overall fertility, although pollen staining of autotetraploid pollen revealed a majority of apparently functional grains (data not shown) and these plants were found to set seed, albeit at low levels. Whether this reduction in fertility is the result of polyploidy per se is unclear.

3.7 Production, fertility, and genome size of aneuploids

*Miscanthus* seems to readily produce fertile aneuploids with a wide range of genomic sizes, even in euploid hybridization events. This contrasts results of a similar study done by Peckert and Jun (2006) in *Hieracium* (Asteraceae) where no aneuploids were produced following interploid hybridizations of 2x, 3x and 4x euploids. The genus *Andropogon*, also in the Panicoideae, contains species that are able to maintain odd ploidy as triploids and produce functional euploid gametes of 1x or 2x (Norrmann and Quarin 1987). However triploids in other members of *Andropogon* have been found to produce a normal distribution of aneuploids (Norrmann and Keeler 2003) as is the case in this study. In *Miscanthus*, gametic selection appears to favor aneuploids that exhibit genomic sizes that are more similar to diploids over time, especially in instances where one of the
parents was more closely allied to the diploid genome size. This is consistent with the review by Ramsey and Schemske (2002), where they found that aneuploid-polyploids, surveyed from 26 cytotypes associated with 7 species, tend to produce offspring with cytotypes that are more analogous with euploid chromosome number than those of their aneuploid parents, although large numbers of aneuploids derived from aneuploids are also reported.

Potential diploid convergence in M. sinensis may be the result of univalent exclusion as a result of univalent lag (Ramsey and Schemske 2002), as occurs in the microspores of the permanent odd-ploidy members of dogrose (Lim et al 2005). Diploid convergence of haploid and autotetraploid lines and selection against changes from the historical diploid genome size has been reported in yeast (Gerstein et al., 2006). Selection towards balanced euploid genomes may also be the result of the mal-effects of unbalanced gene dosage. The gene balance hypothesis speculates that dosage imbalances of genes and gene families associated with regulator processes destabilize stoichiometric relationships in protein-complexes thereby disturbing the cellular actions. Aneuploids seem to be more sensitive to these effects than polyploids (Huettel et al 2008). In our study, with the exception of the 1x individual produced from the 2x × 3x cross, and a limited number of individuals produced by the 2x × 3x and 2x × 4x crosses, and the 4x × 2x G1 offspring, the overwhelming majority of aneuploids produced were hyperploids, aneuploids containing additional chromosomes when compared to the diploid euploids. This is consistent with the hypothesis that having less than a complete set of chromosomes is more detrimental to biochemical stoichiometry than having extra chromosomes. The majority of the aneuploids used in our study were phenotypically indistinguishable from their euploid counterparts. However, in some instances, aneuploidy can have a significant impact on phenotype which often greater than the effect of
polyploidy (Birchler et al 2007). In our study, few phenotypic differences among populations were observed, and some of the most vigorous plants in the study were found in the 3x × 3x (G2) population. However, populations derived from triploid-tetraploid crosses were noticeably smaller and exhibited clear floral abnormalities. The vigorous aneuploids derived from the 3x × 3x crosses is consistent with some reports that aneuploids produced in diploid populations generally exhibit sterility and dysfunctional phenotypes, while aneuploids produced from polyploids tend to be more vigorous and are in some instances indistinguishable from their euploid counterparts (Ramsey and Schemske 2002; Arundhati et al 1982).

Putative diploids were recovered in many of the progeny populations including the 2x × 3x (G1), 3x × 2x (G1), 3x × 2x (G2), 3x × 3x (G1), 4x × 2x (G2), and 4x × 3x (G2) populations. The predominance of diploids (82%) in the 2x × 3x (G1) is higher than percentages of diploids retrieved in foxtail millet interploid crosses, where only 20% of 2x × 3x offspring were diploid (Wang et al 1999). It is unknown whether putative diploids obtained in these populations represent true, euploid diploids or aneuploids with a genome size similar to diploids. Revertant diploids derived from autotetraploid pearl millet (Pennisetum glaucum) have been reported (Arundhati et al 1982), and in some instances were more vigorous growers than their associated source diploids. In sugar beet (Beta vulgaris), cytological studies revealed that putative revertant diploids recovered from aneuploids, although containing the correct chromosome number (2n=18), often exhibited abnormal meiotic behavior including univalent formation and lagging chromosomes. Despite these abnormalities, these revertant diploids regularly produced gametes with the correct haploid chromosome number (n=9), as well as an increased number of small or defective pollen grains. While many of these revertant diploids were inferior to source diploids, some accessions were
strong candidates for continued breeding work (Zaykovskaya et al 1980). In pearl millet, gradual “diploidization” of autotetraploid offspring over multiple generations has been reported, and as meiotic behavior shifts from multivalent to bivalent pairing resulting in fertility increases (Gill et al 1969, Jauhar 1981).

The revertant diploids produced in the 4x × 3x G2s are most likely the product of the embryogenesis of a reduced gamete, as is the 1x individual obtained in the 2x × 3x (G1) population. Self-fertile haploids obtained from polyploids were reported in Dichanthium (de Wet and Harlan 1970), Bromus and Parthenium (Kimber and Riley 1963) and Sorghum (de Wet 1971). The production of a haploid individual in the 2x × 3x cross indicates that interploid crosses in Miscanthus may serve as an alternative approach to anther culture or isolated microspore culture to generate haploids.

4. Conclusions:

We have shown that Miscanthus sinensis can produce male unreduced gametes, haploid progeny, fertile autopolyploids, fertile triploids, fertile aneuploids from interploid crosses, and progeny under selfing conditions. We also show a trend toward diploidization in interploid crosses between diploids and triploids, and in crosses between triploids over time, indicating that aneuploid populations tend to drift back towards diploid levels over successive generations, though in limited numbers. Crosses among autotetraploids failed and new tetraploids were not formed in any diploid-triploid cross combinations even though unreduced gametes were observed in diploid parents.

Although apomixis was documented in Miscanthus sinensis, future work including organ clearing is required to determine the particular mechanism that results in apomict formation, as well as determining the frequency with which this phenomena occurs.
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Table 1: Seed set and reproductive pathways of self and cross pollinated diploid *Miscanthus sinensis*.

<table>
<thead>
<tr>
<th>Pollen source</th>
<th>Seed Set (%)</th>
<th>2x embryo 4x endosperm* (%)</th>
<th>2x embryo 4x endosperm** (%)</th>
<th>3x embryo 4x endosperm*** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-pollinated</td>
<td>19 ± 19.8****</td>
<td>94</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Self-pollinated</td>
<td>1 ± 1.5</td>
<td>94</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

*Indicates reduced embryo sac and standard double fertilization by reduced, 1x, male gametophytes.

**Unreduced embryo sac with no fertilization, apomixis.

***Reduced embryo sac and fertilization by unreduced, 2x, male gametophytes.

****Values are means ± SEM.
Figure 1. Development of cytotype populations over successive generations. Populations within circles for generation 1 (G1) were intercrossed to develop populations in generation 2 (G2).
Figure 2: Frequency distributions of 2C genome sizes for 2x × 3x and 3x × 2x G1 and G2 populations. Gate width for columns was based on a 95% prediction interval for tetraploid plants.
Figure 3: Frequency distributions of 2C genome sizes for 2x × 4x, 3x × 3x G1 and G2, and 4x × 2x populations. Gate width for columns was based on a 95% prediction interval for tetraploid plants.
Figure 4: Frequency distributions of 2C genome sizes for 3x × 4x and 4x × 3x G1 and G2 populations. Gate width for columns was based on a 95% prediction interval for tetraploid plants.
REFERENCES


