Creation of Hexaploid and Octaploid Zoysiagrass Using Colchicine and Breeding

Brian M. Schwartz,★ Karen R. Harris-Shultz, Ryan N. Contreras,
Christian S. Hans, and Scott A. Jackson

ABSTRACT

Zoysiagrasses (*Zoysia* Willd.) are a slow-growing, tetraploid ($2n = 4x = 40$) turfgrass that can be successfully managed with less input than many other warm-season grasses. Despite extensive genetic and morphological variation, genotypes with the ability to recuperate quickly from damage are rare. Therefore, a long-term effort to increase vegetative growth rates was initiated during 2009 by first studying the effectiveness of six colchicine seed treatments and breeding for manipulating the ploidy level of 'Zenith' zoysiagrass. Colchicine-treated seedlings were screened using flow cytometry for genome size changes. Four putative octaploids and one cytochimera were identified. Average stomata length of the four colchicine-induced putative octaploids were 28% larger than that of Zenith, but the cytochimera's stomata length was not altered. Pollen diameter of the four putative octaploids was larger than that of Zenith and the cytochimera. Pollen stainability was relatively unchanged by the colchicine treatments. Further self- and cross-pollination of 09-TZ-103 (putative M_0 octaploid) led to the development and verification of M_1 octaploid and M_1 hexaploid genotypes. These results support that DNA content of the L-I (epidermis), L-II (germ line), and L-III (adventitious roots) histogenic layers of *Zoysia* can be manipulated with colchicine and breeding. Future evaluation of the turfgrass performance of these polyploids is the next step in determining the value of this breeding procedure for improvement of zoysiagrass.

B.M. Schwartz, Dep. of Crop and Soil Sciences, Univ. of Georgia, 115 Coastal Way, Tifton, GA 31793; K.R. Harris-Shultz, Crop Genetics and Breeding Res. Unit, USDA-ARS, 115 Coastal Way, Tifton, GA 31793; R.N. Contreras, Dep. of Horticulture, Oregon State Univ., 4017 Agricultural and Life Sciences Building, Corvallis, OR 97331; C.S. Hans and S.A. Jackson, Dep. of Crop and Soil Sciences, Univ. of Georgia, 111 Riverbend Road, Athens, GA 30602. Received 26 Feb. 2013 ★Corresponding author (tifturf@uga.edu).

Zoysiagrasses (*Zoysia* Willd.) have the potential to be used in environments where light (Morton et al., 1991; Wherley et al., 2011), fertilizer (Engelke et al., 1992), and moderate drought (Marcum et al., 1995; White et al., 2001) can limit the performance of other turfgrasses. Newer *Zoysia* cultivars are gaining popularity, albeit rather gradually because of their slow establishment (Okeyo et al., 2011; Patton et al., 2007) and susceptibility to large patch caused by *Rhizoctonia solani* Kühn (AG) 2-2 LP (Green et al., 1994; Obasa et al., 2012). Low mowing frequency requirements (Christians and Engelke, 1994), the same characteristic that benefits home owners and professional turfgrass managers when zoysiagrasses are healthy, can severely restrict the recovery potential of cultivars that have been injured by disease or traffic (Trappe et al., 2011). Several private and public institutions in the United States have had sustained, or initiated new, efforts to address these issues.

The morphological differences, speciation, and instances of reproductive incompatibility observed in the genus *Zoysia* do not appear to be the result of ploidy level shifts but more likely genetic drift driven by geographic isolation over time (Weng et al., 2007). Although a diploid ($2n = 2x = 20$) *Z. matrella* (L.) Merr. accession has been reported (Gould and Soderstrom, 1974), all

other cytological research of *Zoysia* germplasm has characterized these turfgrasses with a chromosome number of $2n = 4x = 40$ (Arumuganathan et al., 1999; Chen and Hsu, 1962; Christopher and Abraham, 1974; Forbes, 1952; Murray et al., 2005; Schwartz et al., 2010; Tateoka, 1955). Meiotic chromosomal associations (Forbes, 1952) and linkage mapping analysis (Yaneshita et al., 1999) support an allotetraploid classification of zoysiagrass.

Poehlman and Sleper (1995) postulated that increased ploidy levels after chromosome doubling can result in plants with increased growth rates and larger vegetative structures. In both turf and forage grasses, there are examples of this not only between species in the same genus but also among varieties within the same species with varying chromosome numbers. The range of morphological characteristics observed in different bermudagrasses [*Cynodon* (L.) Rich)] with different ploidy levels have allowed for their broad adaptability and use (Harlan and de Wet, 1969; Harlan et al., 1970, Wu et al., 2006). The polyploid St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] cultivar Floratam (Horn et al., 1973) has a more aggressive growth habit and larger leaf size than the diploid cultivar Seville (Riordan et al., 1980). Diversity in plant, leaf, and inflorescence structure among diploid individuals within seashore paspalum (*Paspalum vaginatum* Swartz), and between other diploid *Paspalum* species, has been observed (Burson et al., 1973; Evers and Burson, 2004). 'Argentine' is a tetraploid bahiagrass (*Paspalum notatum* Flügge) that has wider leaves and faster regrowth after defoliation when compared with the finer and longer leafed diploid cultivar Pensacola, giving it the potential to out-yield Pensacola and produce higher average daily livestock gains in some environments (Gates et al., 2004). Breeding and selection after doubling the chromosome number of the diploid annual ryegrass (*Lolium multiflorum* Lam.) cultivar Surrey (Prine, 1996) led to the development of 'Jumbo' (Prine et al., 2002), a tetraploid annual ryegrass with larger stems, leaves, seed heads, and seed than Surrey.

In contrast to the accounts above, there are reports of poor correlation between chromosome number and morphology in many grass species or high levels of morphological variability between genotypes of the same species and ploidy. An association between 2C nuclear DNA content, chromosome number, and plant morphology has not been evident in buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.] (Johnson et al., 1998), switchgrass (*Panicum virgatum* L.) (Hultquist et al., 1997), or napiergrass (*Pennisetum purpureum* Schumach.) (Taylor and Vasil, 1987). Discerning the ploidy level of perennial ryegrass (*Lolium perenne* L.) (Wang et al., 2009), fine fescue (*Festuca* spp.) (Huff and Palazzo, 1998), bentgrass (*Agrostis* spp.) (Bonos et al., 2002), and Kentucky bluegrass (*Poa pratensis* L.) (Eaton et al., 2004) has been facilitated through screening germplasm with flow cytometry rather than using leaf, stem,

and inflorescence measurements. On the opposite end of this spectrum, a large degree of variability for plant, root, and inflorescence morphology has been documented for members of the same species in bermudagrass (Harlan and de Wet, 1969) and zoysiagrass (Schwartz et al., 2010) with the same ploidy level.

The University of Georgia's zoysiagrass germplasm collection in Tifton only contains tetraploid genotypes, very few of which have growth and recovery rates faster than those of cultivars that are currently being produced. Therefore, the objective of this research was to induce higher polyploidy through colchicine treatment followed by breeding to derive hexaploid and octaploid zoysiagrass from seed in an effort to increase genetic variation.

MATERIALS AND METHODS
Colchicine Treatment and Seed Germination

'Zenith' zoysiagrass seeds were exposed to six colchicine seed treatments in the laboratory to induce higher polyploidy during February of 2009. These treatments included soaking 25 seeds in 5.0 mL of 0.025%, 0.05%, and 0.1% colchicine solutions in 9-cm glass petri dishes for 1 wk with or without a 1-wk pretreatment soak in 5.0 mL of water before adding colchicine. Seeds in the nontreated control were soaked in 5.0 mL of water for 1 wk to determine the effects of colchicine treatment on germination. Each treatment was replicated three times, and petri dishes were organized in a completely randomized design on a laboratory bench at 21°C. On 4–5 March 2009 seeds were individually transplanted from petri dishes using forceps into steam-sterilized Tifton sandy loam (fine-loamy, kaolinitic, thermic Plinthic Kandiudults) soil in 6.4-cm diameter clay pots in a glasshouse on the University of Georgia Tifton Campus. Each seed was pressed approximately 0.3 cm into the soil and watered as needed to maintain adequate soil moisture. Final germination counts were taken on 21 May 2009. Seeds transplanted from a single petri dish were grouped together to expedite germination counts, but treatments and replications were arranged in a completely randomized design on a greenhouse bench.

An analysis of variance was performed to determine whether germination percentages varied between colchicine seed treatments and the untreated control. A Waller–Duncan K ratio LSD was used to separate mean germination percentages among treatments.

Flow Cytometry

Flow cytometry analysis was used to evaluate all zoysiagrass seedlings for changes in 2C nuclear DNA content as compared with the DNA content of Zenith, the tetraploid ($2n = 4x = 40$) control. To expedite the screening process, bulk samples of up to five seedlings from the same colchicine treatment and Zenith were analyzed at one time. In bulked samples where a single Gap1 (G_1) peak was observed, all plants were characterized as tetraploids. When multiple peaks were observed in a bulked analysis, plants were reanalyzed individually with and without tissue of Zenith to determine their relative DNA content and to infer ploidy level.

Approximately 1 cm² of fresh tissue from a mature leaf from each genotype was evaluated using flow cytometry. Leaf tissue was chopped with a double-edged razor blade in a petri dish with 500 µL of nuclei extraction buffer (CyStain UV Precise P, Partec GmbH) for approximately 60 s. The resulting solution, containing isolated nuclei, was pipetted into a 5-mL test tube through a 50-µm nylon mesh filter cap. Nuclei were stained with 1.6 mL of 4′, 6-Diamidino-2-phenylindole (DAPI) buffer (CyStain UV Precise P, Partec GmbH). Samples were incubated at 21°C for a minimum of 10 min. Flow cytometry analysis was conducted at the University of Georgia Tifton Campus on a PASIII cytometer (Partec). For screening purposes, the DNA peak data were generally based on the fluorescence of between 2000 and 5000 scanned particles.

Stomata and Pollen Grain Measurements

Stomatal lengths of five colchicine-induced (M_0) putative octaploids (09-TZ-101, 09-TZ-102, 09-TZ-103, 09-TZ-104, and 09-TZ-105) and Zenith were measured from plants grown in the greenhouse on 31 Mar. 2010. Clear fingernail polish was applied to the abaxial side of four leaves (replications) from each genotype, pealed from the leaf after 1 min, and secured under a slip cover on a microscope slide. Fifty stomata from each leaf impression were measured under a light microscope (Wild M20, Wild Heerbrugg) with an ocular micrometer at 400X magnification to determine the average stomata length of each genotype.

The starch content of pollen grains from four seedheads of 09-TZ-101, 09-TZ-102, 09-TZ-103, 09-TZ-104, 09-TZ-105, and Zenith grown in the greenhouse was estimated by staining pollen deposited onto microscope slides with 2% iodine–potassium iodide for 10 min before observation on 8–9 Feb. 2010. Percentage of pollen stainability was estimated by classifying 100 pollen grains within the field of view at 100X magnification as good (stained and regularly shaped) or bad (not stained or irregularly shaped). Pollen diameters were measured on the same slides that were prepared to estimate pollen stainability. Twenty-five subsamples (pollen grains) were measured at 400X magnification to determine the average pollen diameter of 09-TZ-101, 09-TZ-102, 09-TZ-103, 09-TZ-104, 09-TZ-105, and Zenith.

The distribution of data from leaf stomata length, pollen stainability, and pollen diameter was assessed with a histogram and normal probability plot for normality. An analysis of variance was used to determine whether genotypes varied for each of these traits. Where the source of variation "genotype" was found to be significant ($P \leq 0.05$), a Waller–Duncan K ratio LSD was used to separate means.

Cross- and Self-Pollinations

Efforts to self– and cross-pollinate the putative octaploid genotype 09-TZ-103 began during February 2010 in the greenhouse. To make these crosses, individual spikes were covered with inverted 2-mL microcentrifuge tubes for pollen isolation before stigma emergence. These inflorescences were hand-pollinated with either 09-TZ-103 or Meyer zoysiagrass when all stigmas had emerged but before anther extrusion. The protogynous flowering habit of these grasses was used to ensure the desired cross without emasculation (Engelke and Anderson, 2003).

Beginning on 5 Apr. 2010, seedheads that had been hand-pollinated were harvested, bulked as either 09-TZ-103 self-pollinations (⊗) or 09-TZ-103 (♀) × Meyer (♂) crosses, and dried at 35°C for 3 d. The bulked seedheads from each group were separately hand-threshed. Each of the two seed lots was then pretreated in a 5% bleach solution for 30 min and planted into steam-sterilized Tifton sandy loam soil in a 30.5-cm pot. Individual seedlings were transplanted into 6.4-cm diameter clay pots as they germinated.

Cytology

The apical meristem from the distal ~2 cm of actively growing roots was excised from plants growing in potting mix. The roots were rinsed in cold water before being treated with nitrous oxide for 75 to 90 min (Kato, 1999). Root tips were immediately fixed in 3:1 ethanol:acetic acid and left at room temperature for up to 1 mo before generating chromosome spreads (Kato et al., 2004; Gill et al., 2009; Findley et al., 2010). Chromosomes were stained and mounted with Vectashield with DAPI (H-1200, Vector Labs) and viewed using a Zeiss AxioImager M2 epifluorescence microscope (Carl Zeiss Microscopy GmbH). Images were captured and analyzed using the attached Zeiss AxioCam MRc camera and analyzed with Zeiss Axiovision Release 4.8 software. Chromosome spreads of at least 15 cells for each genotype were counted to determine final chromosome number.

RESULTS
2009

Differences ($P \leq 0.05$) in germination were observed among colchicine seed treatments, but all were significantly less than observed in the control, which averaged 73% (Table 1). Seed germination in the water–0.1% colchicine seed treatment (24%) was the lowest. The remaining five colchicine seed treatments resulted in statistically equal germination rates from 48 to 52%.

The 55 seedlings that germinated in the control treatment all had 2C nuclear DNA content levels that corresponded with tetraploid ($2n = 4x = 40$) Zenith zoysiagrass (Table 1). The additional 204 zoysiagrass seedlings that germinated after treatment with different concentrations of colchicine, with or without presoak in water, were also evaluated by flow cytometry. Three putative colchicine-induced octaploids (M_0 09-TZ-102, 09-TZ-103, and 09-TZ-104) resulted from the water–0.025% colchicine treatment, and one additional putative octaploid (M_0 09-TZ-105) was found after being treated with water–0.05% colchicine. These four genotypes had 2C nuclear DNA contents twice that of Zenith. A putative cytochimera (M_0 09-TZ-101) was identified from the 0.1% colchicine seed treatment and was characterized as having DNA content that registered on both the tetraploid and octaploid histogram channels.

2010

Stomata lengths of six *Zoysia* genotypes were found to be significantly different ($P \leq 0.0001$) according to an analysis of variance (Table 2). Zenith (Fig. 1A) and 09-TZ-101 had stomata lengths of 26.0 and 25.2 µm, respectively, which

Table 1. Summary of zoysiagrass (*Zoysia japonica*) germplasm developed through colchicine seed treatment in the turfgrass breeding program at the University of Georgia Tifton campus during 2009.

♀ Parent	Method	Number germinated[†]	Number successful[‡]	Identity	Ploidy[§]
Zenith	Water	55	0	–	–
Zenith	0.025% colchicine	36	0	–	–
Zenith	0.05% colchicine	37	0	–	–
Zenith	0.1% colchicine	39	1	09-TZ-101	$4x + 8x$
Zenith	Water then 0.025% colchicine	36	3	09-TZ-102 09-TZ-103 09-TZ-104	$8x$ $8x$ $8x$
Zenith	Water then 0.05% colchicine	38	1	09-TZ-105	$8x$
Zenith	Water then 0.1% colchicine	18	0	–	–

[†]Number of plants that germinated out of a possible 75 treated seeds.

[‡]Number of plants identified with DNA content or ploidy level other than that of tetraploid ($2n = 4x = 40$) zoysiagrass.

[§]Actual chromosome numbers have not been counted. The ploidy level of these genotypes was inferred from results of flow cytometry analysis.

Table 2. Mean stomata length, pollen stainability, and pollen diameter of tetraploid ($2n = 4x = 40$) Zenith zoysiagrass (*Zoysia japonica*) and five colchicine-induced M_0 genotypes.

Genotype	Ploidy level	Stomata length	Pollen stainability	Pollen diameter
		μm	%	μm
Zenith	$4x$	26.0 b[†]	99 a	27.3 e
09-TZ-101	$4x + 8x$	25.2 b	97 b	29.2 d
09-TZ-102	$8x$	31.8 a	92 c	40.0 a
09-TZ-103	$8x$	33.4 a	99 a	38.0 b
09-TZ-104	$8x$	34.7 a	98 ab	38.0 b
09-TZ-105	$8x$	32.9 a	99 a	36.3 c
% CV		6.9	1	2.9

[†]Means within a column followed by the same letter are not different at $K = 100$ (approximates $P = 0.05$) according to Waller–Duncan LSD.
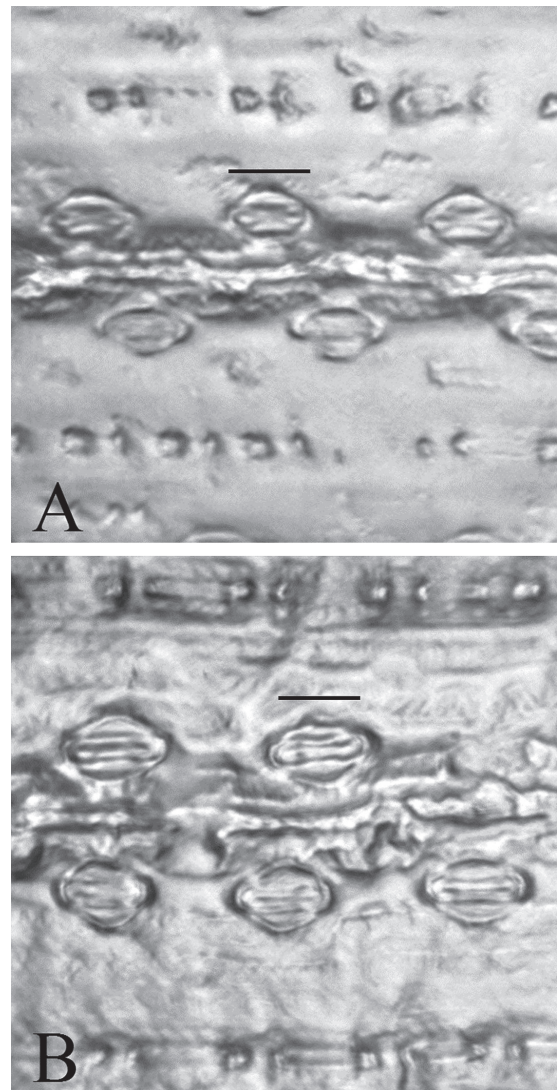

Figure 1. Photomicrographs of leaf stomata at 400X magnification. Scale bars represent 30 μm. (A) Tetraploid ($2n = 4x = 40$) Zenith zoysiagrass (*Zoysia japonica*). (B) Colchicine-induced octaploid ($2n = 8x = 80$) experimental genotype (M_0 09-TZ-103) derived from Zenith zoysiagrass seed.

were smaller than observed from leaves of 09-TZ–102, 09-TZ–103 (Fig. 1B), 09-TZ–104, and 09-TZ–105, which ranged from 31.8 to 34.7 μm. An analysis of variance of pollen starch stainability determined that there were significant differences ($P ≤ 0.0001$) in percentages of good pollen observed on 8–9 Feb. 2010 for these zoysiagrasses (Table 2). The putative octaploid 09-TZ–102 had the lowest number of stained pollen grains (92%), where pollen from the remaining five genotypes stained at between 97 and 99%. Variability ($P ≤ 0.0001$) in pollen diameter was also observed on 8–9 Feb. 2010 (Table 2). There were significant differences among the four putative octaploid genotypes that had pollen grain diameters that ranged from 36.3 to 40.0 μm, but all had larger pollen than Zenith (27.3 μm) and the cytochimera 09-TZ–101 (29.2 μm).

2011

Flow cytometry was used to screen the zoysiagrass plants that germinated from self– and cross-pollination of 09-TZ–103 in 2010 for changes in relative 2C nuclear DNA content. After analysis of the M_1 seedlings that were derived from 09-TZ–103, putative octaploid ($2n = 8x = 80$) and hexaploid ($2n = 6x = 60$) genotypes were identified. Chromosome numbers were determined with root tip spreads from one genotype representing each of these ploidy levels (Fig. 2). 10-TZ–3192, a seedling from self-pollinating 09-TZ–103, was characterized as an octaploid on the basis of chromosome counts (Fig. 2A). Chromosome counts confirmed that 10-TZ–3073, a seedling derived from 09-TZ–103 (♀) × Meyer (♂) crosses, was hexaploid as initially classified by flow cytometry (Fig. 2B).
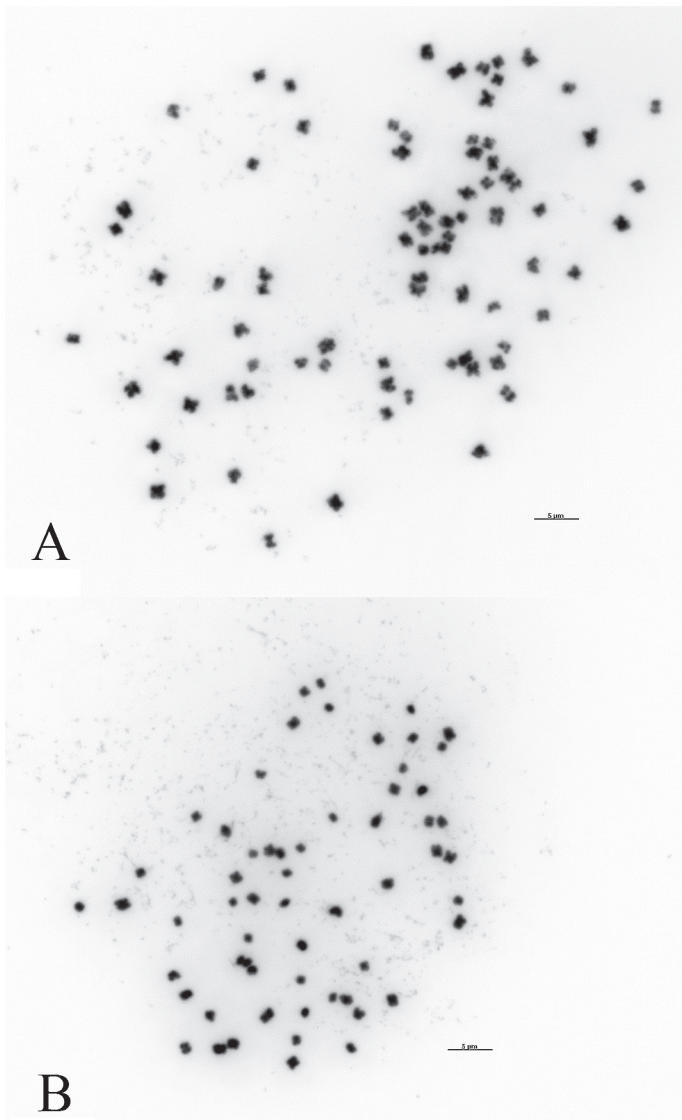
Figure 2. Photomicrographs of root tip spreads taken at 1000X magnification. Scale bars represent 5 µm. (A) Octaploid ($2n = 8x = 80$) seedling (M_1 10-TZ-3192) from self-pollination of M_0 09-TZ-103. (B) Hexaploid ($2n = 6x = 60$) seedling (M_1 10-TZ-3073) derived from cross-pollination of M_0 09-TZ-103 with tetraploid Meyer zoysiagrass.

DISCUSSION

Only five putative octaploid zoysiagrass plants were initially discovered as a result of the colchicine seed treatments, three of these from the water–0.025% solution. Similar success in doubling the chromosome number of soybean [*Glycine max* (L.) Merr.], wheat (*Triticum aestivum* L.), and rice (*Oryza sativa* L.) was achieved with 0.05 to 0.1% solutions of colchicine in one of the first reports of using these methods (Tang and Loo, 1940). We observed a 29 to 67% reduction in germination as compared with the untreated controls (Table 1). Beachell and Jones (1945) also described reduced germination and low incidence of polyploid identification in rice during their research of temperature and 0.0125 to 0.05% colchicine treatments. In a study of the effects of different concentrations of colchicine

on spindle formation during mitosis, Gaulden and Carlson (1946) offered an explanation for the low frequency of chromosome doubling with colchicine among plants that live after treatment in addition to a description of why many seeds never grow after germination. They surmised that only cells that are affected by colchicine treatments at late metaphase or early anaphase will lead to formation of polyploid cells. Our identification of a putative chimera (09-TZ-101) provides further evidence that colchicine treatment may not work if mitosis is not arrested in cells very early in the differentiation process, at exactly the right mitotic stage. Experience and history would suggest that additional zoysiagrass octaploids could be induced through a variety of methods as long as meristematic tissue of a plant in the proper stage of development is exposed to adequate colchicine concentrations long enough to disrupt spindle fibers (Bell, 1950). With additional studies, our understanding of ploidy manipulation, fertility, and inheritance in this species may be expanded.

Stomata lengths and pollen sizes from 09-TZ-101, 09-TZ-102, 09-TZ-103, 09-TZ-104, and 09-TZ-105 were compared with those of tetraploid Zenith to determine which histogenic layers may have been affected by the colchicine treatment, in addition to researching whether or not these two morphological measurements could be used to screen for ploidy changes without the need for flow cytometry analysis or actual chromosome counts (Table 2). Prior research has determined that the L-I histogenic layer gives rise to the epidermis, that the germ line is derived from the L-II, and that adventitious roots originate from the L-III (Dermen, 1960). Results from the measurements and crossing efforts (data not shown) in 2010 do not support the designation of 09-TZ-101 as an octaploid or chimera because epidermal stomata cells and pollen, of the L-I and L-II histogenic layers, respectively, were not larger (Table 2). Furthermore, all progeny from the self- and cross-pollination of 09-TZ-101 were tetraploid (data not shown). Conversely, flow cytometry analysis, stomata lengths, and pollen diameters characterized 09-TZ-102, 09-TZ-103, 09-TZ-104, and 09-TZ-105 as octaploids. Leaves of these octaploids were uncharacteristically thick and "rough" (Fig. 1) as also observed in some of the first colchicine-induced polyploids (Blakeslee and Avery, 1937). Interestingly, seedling production using these four genotypes as the female parent to date has not been difficult and has resulted in plants with 2C nuclear DNA contents that differ from known tetraploid standards (Fig. 2). Additionally, the pollen stainability of all genotypes was greater than 90% (Table 2). Fehr (1987) generalized that autopolyploids should exhibit a reduced frequency of viable pollen and egg cells owing to irregularity of chromosome pairing. Possibly, the iodine–potassium iodide stained pollen grains were only a good measure of starch content and not a true estimate of pollen viability.

Or perhaps there is a genetic mechanism controlling chromosome pairing during meiosis as found in hexaploid wheat (Riley and Chapman, 1958) and tall fescue (*Festuca arundinacea* Schreb.) (Jauhar, 1975). Although stomata lengths and pollen size were indicative of ploidy shifts in the respective histogenic layers for zoysiagrass, these measurements are more time consuming than flow cytometry analysis. Our breeding program has shifted emphasis to screening germplasm with only flow cytometry analysis and root-tip chromosome counts because verification of polyploidy must still be completed, even when stomata lengths or pollen size is known. Morphological measurements could still be used in breeding programs where such laboratory analyses are not feasible.

Our goal in this research was to produce seed-derived M_1 hexaploid and M_1 octaploid plants in an effort to stabilize the ploidy shifts that were induced with colchicine with the desire to increase the genetic variability available for improvement of zoysiagrass. There has been no indication of reversion to the tetraploid chromosome number in M_1 plants that inherited higher ploidy levels through the sexual process. The absence of discussion on ploidy level instability in generations following colchicine treatment in the literature indicates that these changes may be lasting. It is our hope that future crossing and recombination above the tetraploid level will result in heterosis stemming from the accumulation of favorable dominant alleles in repulsion phase that mask recessive phenotypes. In the search for more vigorous zoysiagrasses, it would not be prudent to select new cultivars without the characteristics that currently make *Zoysia* desirable, for example, lower mowing frequency and fertility requirements. Further breeding and evaluation will be necessary to determine the feasibility of increasing growth rates while preserving attributes of lower maintenance. More work should be done to create additional higher ploidy zoysiagrass plants from several populations with different genetic backgrounds than the cultivar Zenith. Additionally, tetraploid × hexaploid and hexaploid × octaploid crosses should be made to create pentaploid ($2n = 5x = 50$) and septaploid ($2n = 7x = 70$) zoysiagrasses, respectively. If vigorous and sterile, these artificially created polyploids could have less potential of becoming contaminated with off-types from self-pollination, a situation that can jeopardize the stability of many currently grown zoysiagrasses, thereby raising the bar for uniformity and performance in these species.

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