Received: 5 December 2022 | Accepted: 20 April 2023

DOI: 10.1002/csc2.21011

Crop Science

ORIGINAL ARTICLE

Turfgrass Science

Creation and characteristics of tetraploid and mixoploid centipedegrass

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Assigned to Associate Editor Bradley Bushman.

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Abstract

Ploidy manipulation has been used in many crop improvement programs to develop plant species with wider adaptability and desirable traits. The objectives of this study were to create stable tetraploid centipedegrass (*Eremochloa ophiuroides* [Munro] Hack.) lines and evaluate them for beneficial traits. To generate polyploid lines, "TifBlair" ($2n = 2x = 18$) seeds were exposed to gamma radiation and callus was generated using tissue culture, exposed to glyphosate and regenerated into plantlets. After 5 years of continuous propagation, two lines, Hongliang Wang (HW)16 and HW123, were found to be tetraploid, and one line, HW61, was found to be a mixoploid using flow cytometry and chromosome counts. Nuclear DNA contents for diploid and tetraploid lines ranged from 1.97 to 2.10 and 4.14 to 4.30 pg $2C^{-1}$, respectively. Ten centipedegrass lines were genotyped using seven simple sequence repeat markers and clustered by origin (derived from TifBlair or the University of Georgia breeding program). Six lines were evaluated for morphological and physiological traits under greenhouse conditions. Trait evaluation showed that tetraploid lines had larger stomata and leaf width and reduced stomatal density. For all other traits evaluated under greenhouse conditions, although significant differences were observed between lines, no consistent differences separated diploid and tetraploid lines. Between the tetraploid lines, HW16 had higher evapotranspiration, percentage green cover, and relative water content than HW123, whereas HW123 had a higher average clipping yield and root width than HW16. In conclusion, irradiation and tissue culture are valuable techniques for the generation of stable polyploid lines, and an increased ploidy level led to larger vegetative structures in centipedegrass.

1 | INTRODUCTION

Turfgrasses, which are composed of a diverse set of plant species in the *Poaceae* family, are an important component of urban and rural lawns and landscapes. Turfgrasses are broadly classified into two major categories: warm- and cool-season grasses. Warm-season grasses utilize the four-carbon enzyme phosphoenolpyruvate carboxylase in photosynthesis and grow best at temperatures between 27 and 35°C, whereas cool-season grasses utilize the three-carbon enzyme Rubisco in photosynthesis and grow best at 15–24°C (Harris-Shultz & Jespersen, 2018). Turfgrasses are primarily used for the development of low-cost surfaces for outdoor sports fields, golf

Abbreviations: HW, Hongliang Wang; MS, Murashige and Skoog; PPM, plant preservative mixture; SSR, simple sequence repeat.

courses, and lawns; soil erosion control; dust stabilization; improving the quality of groundwater; soil improvement; carbon sequestration; and to enhance the beauty of surrounding areas (Beard & Green, 1994).

Centipedegrass ($2n = 2x = 18$; *Eremochloa ophiuroides* [Munro] Hack.) is a perennial warm-season turfgrass native to Southeast Asia and is now widely distributed in different parts of the world (Li et al., 2020; Schwartz, Harris-Shultz, et al., 2013). It was first introduced into the United States from China in 1916 and was referred to as "China grass" (Hanna & Burton, 1978; Islam & Hirata, 2005). Currently, centipedegrass is widely grown in the United States, primarily on the southern Coastal Plain from Texas to South Carolina and north up to Arkansas (Hook et al., 1992; Islam & Hirata, 2005). Centipedegrass is often referred to as "lazy man's grass" or "poor man's grass" mainly because it requires less management and fertilization as compared to other warm-season turfgrasses (Hanna, 1995). In the United States, centipedegrass is commonly used for home lawns, but it is also used in parks, adjacent to greens and fairways, along highways, and in other areas where less care and low maintenance are desired (Haygood & Martin, 1990). Genetic diversity is key for the improvement of any plant species; however, centipedegrass germplasm in the United States is not highly diverse (genetically or morphologically) because most of the centipedegrass germplasm in the United States originated from a single collection (Hanna, 1995; Li et al., 2020).

Ploidy manipulation has been successfully used in many crop improvement programs, mainly to overcome species hybridization barriers, create sterile lines, facilitate interspecific gene transfer, and develop plant species with wider adaptability and desirable traits (Sattler et al., 2016; Singh et al., 1990; Tonosaki et al., 2018). Colchicine has been widely used to manipulate the ploidy of several plant species (Carbajal et al., 2019); however, herbicides trifluralin and oryzalin have been recently used for doubling chromosome numbers due to their lower toxicity, higher affinity to plant tubulins, and efficacy at lower concentrations (Touchell et al., 2020).

It has been reported that increases in ploidy can result in the development of plants with increased growth rates and larger vegetative structures. However, not all plants have improved characteristics after increasing the number of chromosomes. For example, maize (*Zea mays* L.) and banana (*Musa* spp. L.) have maximum vigor at the diploid and triploid levels, respectively, and changes in ploidy for these crops decrease vigor (Poehlman & Sleper, 1995). Compared to diploids, induced polyploids usually show increased cell size, larger organs (leaves, roots, and seeds), larger pollen size, a greater number of chloroplasts per guard cell, larger guard cells and stomates, and less stomatal density (Marinho et al., 2014; Ranney, 2006). Polyploids may also have thicker leaves and larger flowers, and flowering may be later or over a longer period of time as compared to related diploids (Hosoda et al., 1953; Schwartz, Harris-Shultz, Contreras, et al., 2013). When utilizing polyploids in a breeding program, it is important to note that induced polyploids can sometimes be cytochimeras or mixoploids, a condition where ploidy varies for different histogenic layers (Ranney, 2006). Meristems are divided into three histogenic layers: L1 (outer apical layer), L2 (second apical layer), and L3 (third apical layer), where L1 gives rise to the epidermis, L2 gives rise to reproductive tissue, and L3 gives rise to the adventitious roots (Dermen, 1960; Ranney, 2006; Schwartz, Harris-Shultz, et al., 2013).

Ploidy manipulation has been used in many turfgrass species to create sterile triploid lines and induce chromosome doubling. For bermudagrass (*Cynodon* spp. Rich), sterile hybrid triploid cultivars have been developed by crossing tetraploid (*Cynodon dactylon* [L.] Pers.) and diploid (*Cynodon transvaalensis* Burtt-Davy) lines, and these cultivars are widely used because of their superior quality and performance (Mutlu et al., 2020; Reasor et al., 2016). To develop a sterile cultivar that does not produce segregating seedlings in seashore paspalum (*Paspalum vaginatum* Swartz), Schwartz, Contreras et al. (2013) created a stable triploid seashore paspalum line from a diploid cultivar "Sea Spray" using colchicine (although its origin may be from a union of an unreduced and reduced gamete rather than the colchicine treatment). For zoysiagrass (*Zoysia* spp. Willd.), four octoploid zoysiagrasses were generated using colchicine from the tetraploid cultivar Zenith (Schwartz, Harris-Shultz, Contreras, et al., 2013). These octoploid lines had uncharacteristically thick and rough leaves, and the average stomata length and pollen diameter were larger than Zenith. Seven mixoploid (4× and 8×) zoysiagrasses were also identified from an "Empire" field, and the authors conjectured it was likely due to the repeated use of dinitroaniline herbicides (Quesenberry et al., 2021). A year later, the mixoploids

Core Ideas

- Two tetraploid and one mixoploid centipedegrass lines were created using irradiation and tissue culture.
- Nuclear DNA content for diploid and tetraploid lines ranged from 1.97 to 2.10 and 4.14 to 4.30 pg $2C^{-1}$, respectively.
- Tetraploid lines showed increased stomatal size and leaf width but less stomatal density than diploid lines.
- The tetraploid lines differed between each other for multiple morphological and physiological traits.

showed ploidy instability, with two being 4×, two remaining 4×/8×, and three being 8×.

Ploidy manipulation in centipedegrass was previously performed by Schwartz, Harris-Shultz et al. (2013) using colchicine. They created one tetraploid and one cytochimera centipedegrass line from diploid TifBlair seed and studied the variation observed in morphological traits. After 2 years, the tetraploid line and the seed harvested from it had a high rate of reversion back to the diploid state. The authors suggested that, if centipedegrass were to be created with higher levels of ploidy, alternative methods other than using colchicine alone should be explored to ensure ploidy stability. Therefore, the objectives of this study were to create stable tetraploid centipedegrass lines and assess these lines for beneficial morphological and physiological changes as compared to diploid lines.

2 | MATERIALS AND METHODS

2.1 | Generation of polyploid lines

TifBlair centipedegrass seeds (Hanna et al., 1997) were irradiated with 120 Gy using a Gammacell 220 in 2017 (performed by Dr. Matthew Chappell, University of Georgia). Centipedegrass seeds (5 g) were surface sterilized with 5% NaOH for 15 min and washed with running water. Seeds were then treated with 70% (v/v) ethanol for 3 min, followed by a 5 min treatment with 50% (v/v) fresh bleach supplemented with 0.1% Tween-20. Afterward, the seeds were washed in sterile water five times. To further remove and eliminate microbes present in the seed coat, seeds were immersed in half-strength Murashige and Skoog (MS) medium supplemented with 2% plant preservative mixture (PPM, Plant Cell Technology). The pH of the media was adjusted to 6.5 with KOH. The seeds were then put in centrifuge tubes and rotated overnight on a Lab-Line MAXI 4631 (Thermo Fisher Scientific) rotator. Seeds were washed in sterile water five times the next day and immersed in fresh half-strength MS medium supplemented with 2% PPM. Afterward, seeds in centrifuge tubes were rotated overnight again prior to plating. Surface-sterilized seeds were placed onto half-strength MS agar media plates containing 1% sucrose, 0.2% PPM, and 0.8% agar for germination.

After 9–12 days, seedlings were collected and washed with sterile water, followed by overnight treatment with 5% PPM. MS media was made by adding 4.44 g L^{-1} of 1× MS (PlantMedia.com), 30 g L^{-1} mannitol, 3% sucrose, and distilled water. The pH of the media was adjusted to 6.5 with KOH. Note that 1 L media was also supplemented with 1 mL PPM and 7.5 g of agar prior to sterilization by autoclaving. To induce and maintain callus, 2–3 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2, 4-D; filter sterilized) was added to the media after autoclaving. Sterile seedlings were placed onto the MS agar media for callus induction. Callus was induced over multiple days in July of 2017. To select callus with resistance or tolerance to glyphosate, induced callus was transplanted onto the same MS agar media supplemented with 1 mM glyphosate. Glyphosate stock was prepared by diluting Roundup Power Max II (Bayer Crop Science) to 1 M. The diluted Roundup Power Max II was filter sterilized and added to the autoclaved MS agar media containing 2 mg L^{-1} 2,4-D and 1 mL of 1 M glyphosate per liter. Plated seeds and callus were placed in an incubator at 25°C with a 12-h photoperiod. The calluses that survived the selection of glyphosate treatment were continuously propagated on the same media. To regenerate plantlets from callus selected on glyphosate, 2,4-D was excluded from media to induce root formation and shoot growth. After about 4–6 weeks of tissue culture, regenerated plantlets were transferred onto half-strength MS agar media containing 10 g L^{-1} sucrose and 8 g L^{-1} agar. After 2–3 weeks, 500 regenerated plantlets were transplanted into individual pots filled with Pro-mix Bx Biofungicide + Mycorrhizae potting mix (Premier Tech Horticulture) in a greenhouse on June 18, 2018. To maintain high humidity at the beginning, the pots were placed in 2.5-gallon plastic resealable bags that were not sealed for about 2 weeks. Well-established plants were removed from the bags and subjected to regular growth conditions in a greenhouse. The obtained plants were screened using flow cytometry to determine which lines were polyploids. All lines were clonally propagated in a greenhouse.

2.2 | Flow cytometry

Fresh leaf tissue, approximately 3 cm^2, was excised from each centipedegrass line, placed in a 5-cm plastic Petri dish (Thermo Fisher Scientific), and chopped using a double-edge razor blade in 1 mL of Tris $MgCl_2$ nuclei extraction buffer (Pfosser et al., 1995) until well macerated. For 2018–2019 ploidy determination, TifBlair ($2n = 2x = 18$) was used as a standard to represent the diploid level; however, for the 2023 ploidy and 2C nuclear DNA content calculations, each sample was co-chopped with "B73" maize as an internal standard. The B73 was obtained from USDA-ARS Germplasm Resources Information Network (PI 692136). The resulting slurry was then pipetted into a 50-µm CellTrics disposable filter (Partec) sitting on top of a 12 × 75 mm plastic culture tube. The filter was removed, and 250 µL of PI/RNase staining buffer (BD Biosciences) was added to each filtered sample. Samples were then placed on ice for at least 15 min prior to analysis using an Attune NxT flow cytometer (Thermo Fisher Scientific). The flow cytometer was equipped with a 488 nm blue laser to detect propidium iodide fluorescence area signals (FL2A). For each sample, the DNA peak data were obtained

by using a 125 µL acquisition volume at 12.5 µL min^{-1}. The 2C nuclear DNA content, measured in picograms, was calculated for each sample by dividing the mean position of the sample G_1 by the mean position of the internal standard (B73) and then multiplying by the 2C nuclear DNA content of the internal standard, that is, 5.64 pg $2C^{-1}$ (Díez et al., 2013; Quesenberry et al., 2021). G_2 peaks correspond to cells that are actively preparing for mitosis, and these cells contain twice the amount of DNA.

2.3 | Chromosome counting

Mitotic chromosomes were prepared as described by Koo et al. (2018), with minor modifications. Mitotic metaphase spreads were prepared from root tips, which were pretreated with nitrous oxide for 2 h, followed by fixation in 3:1 ethanol:glacial acetic acid overnight. The fixed samples were squashed with a drop of 45% acetic acid. Chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield antifade solution (Vector Laboratories). The images were captured with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy LLC) using a cooled charge-coupled device camera, CoolSNAP HQ2 (Photometrics), and AxioVision 4.8 software. The final contrast of the images was processed using Adobe Photoshop CS5 software.

2.4 | DNA extraction and simple sequence repeat genotyping

Ten centipedegrass lines consisting of six regenerated Hongliang Wang (HW) lines, HW16, HW61, HW107, HW121, HW123, and HW143, and four lines that were selected by Dr. Brian Schwartz of the University of Georgia turfgrass breeding program based on high seed yield, 15-TC-837, 16-TC-2521, 16-TC-1439, and 16-TC-2389, were used for genotyping. Freshly collected leaf tissue from 10 centipedegrass lines was ground to a fine powder by placing microcentrifuge tubes that contained plant tissue and four sterilized metal beads into liquid N_2. Samples were repeatedly ground using a vortex mixer, and samples were not allowed to thaw by repeatedly placing the samples back in liquid N_2. Genomic DNA was extracted using a GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific) following the manufacturer's recommendations. The quantity and quality of the purified DNA were assessed by using a NanoDrop 2000c (Thermo Fisher Scientific) and separation on a 1% agarose gel, respectively. A set of 36 simple sequence repeat (SSR) markers was selected for measuring genetic diversity in centipedegrass lines (Table S1). Of these, 12 markers with the longest repeats were selected from Li et al. (2018), and 24 markers were selected from Harris-Shultz et al. (2012). The polymerase chain reaction reaction for each SSR marker was performed as described previously by Harris-Shultz et al. (2018). Briefly, a 10 µL reaction volume that contained 1 µL of DNA (2.5 ng $µL^{-1}$), 2 µL of 5× clear GoTaq reaction buffer (Promega), 1 µL $MgCl_2$ (25 mM), 0.8 µL deoxynucleotide mix (2.5 mM), 0.5 µL M13-tagged forward primer (1 µM), 2.0 µL of reverse primer (1 µM), 1.8 µL (1 µM) M13 primer (M13-TGTAAAACGACGGCCAGT) fluorescently labeled with the 5′ 6-6-carboxyfluorescein fluorophore (Integrated DNA Technologies), 0.04 µL of GoTaq DNA polymerase (Promega), and 0.86 µL of sterile water. The thermocycler conditions used were as follows: 94°C for 3 min, 39 cycles of 94°C for 30 s, 52.6–65°C (Table 2) for 1 min, 72°C for 1 min and 10 s, and a final elongation step at 72°C for 40 min. Fragment analysis was performed on a SeqStudio Genetic Analyzer (Thermo Fisher Scientific) using a GeneScan 500 carboxy-X-rhodamine size standard (Applied Biosystems). Results were processed and analyzed using GeneMapper v.6.0 software (Applied Biosystems). SSR data were coded as binary with no missing data. The Microsoft Office Excel add-in, GenAlEx v 6.5 (Peakall & Smouse, 2012), was used to generate a principal coordinate analysis (PCoA) based on genetic distance.

TABLE 1 Ploidy determination and 2C nuclear DNA content calculation of the centipedegrass lines.

Genotype	Ploidy	2C nuclear DNA (mean)[a]	SD
HW61(4×)	2×, 4×	4.30A	0.08
HW16	4×	4.22A	0.20
HW123	4×	4.14A	0.07
HW61(2×)	2×, 4×	2.10B	0.13
HW121	2×	2.00B	0.03
HW143	2×	2.00B	0.11
HW107	2×	1.97B	0.08

Note: Within a column, values sharing a common letter are not significantly different among genotypes at $p < 0.01$ according to Tukey's test.
Abbreviation: SD, standard deviation.
[a]The mean 2C nuclear DNA content (pg $2C^{-1}$) value was calculated using four biological replications except for HW121 where only two replicates were available.

2.5 | Morphological and physiological characterization

2.5.1 | Experimental design

Evaluation of physiological and morphological traits was conducted at the Turfgrass Research and Education Center greenhouses located on the University of Georgia Griffin campus, Griffin, GA. The experiment was conducted in the spring of 2021 and repeated in the spring of 2022. The six centipedegrass lines used in this study were HW16, HW123,

TABLE 2 Information on the seven polymorphic simple sequence repeat (SSR) markers used for characterizing centipedegrass lines and allele sizes for each locus.

Locus no.	Primer name	Repeat motifs	Primer sequences (5′–3′)	T_a (°C)	N_a	Size (bp)	Reference
1	TR168608_c0_g4	(AAG)17	F: TGGCAGGCCTCTTTGTTAGT R: ACGCTAGTGCGTGCATGTAA	63.4	4	372, 380, 381, 387	Li et al. (2018)
2	TR99174_c0_g6	(AGA)22	F: GAGGGCTTGTTTAATCCCCTA R: GCTGGACCTGTCTCTCAAGC	63.4	3	175, 207, 216	Li et al. (2018)
3	S98	(TTG)4	F: ACGTATCTGCCATGTCGTTG R: CAGGGACTGGTTCTTTGCTC	63.4	2	126, 165	Harris-Shultz et al. (2012)
4	S69-2	(TG)6	F: ACAGGTCGTCCATGTCGAA R: GCTGATGGTGATGTTGATGTG	63.4	1	176	Harris-Shultz et al. (2012)
5	S101	(CA)4, (GA)6	F: CTGCTGAGACGGACCTCACT R: TCCCCTCCCCTAGTACACCT	65.0	4	159, 161, 163, 167	Harris-Shultz et al. (2012)
6	S4-2	(AC)4	F: TTTCTATGGGAATGCAATGG R: CCTAGCAGAATAGGCCCTGA	52.6	2	306, 307	Harris-Shultz et al. (2012)
7	S38-2	None	F: GGCTTCTCCTGGACCACATA R: CAACCCAACGCTAACTCACA	65.0	2	215, 216	Harris-Shultz et al. (2012)

Note: Repeat motifs: stretch of DNA with dinucleotide or trinucleotide units repeated in a sequence; T_a: annealing temperature; N_a: number of alleles.

HW61, HW107, HW121, and HW143. Centipedegrass plugs were trimmed and washed of soil, then transplanted into 10 cm wide × 40 cm long polyvinyl chloride tubes. A 1:1 sand and calcinated clay mixture was used for the potting media. Plants were allowed to establish in greenhouse conditions for a period of 90 days. During the establishment and experimental periods, 1000-W high-pressure sodium lamps were set to provide supplemental light when natural lightning decreased to less than 400 μmol m^{-2} s^{-2} within the 12-h photoperiod inside the greenhouse. Plants were trimmed every week to 5 cm, fertilized with Miracle Gro soluble fertilizer (N-P_2O_5-K_2O 24-8-16) (Scotts Miracle-Gro) every other week, and watered twice per week. The experiment was divided into two phases: a control phase during which plants were maintained in well-watered conditions for 2 weeks to assess normal growth and water use patterns, followed by a drought phase for 2 weeks when water was withheld completely.

2.5.2 | Stomata morphology

Stomatal analysis was performed to analyze differences in leaf stomatal size and stomatal density among lines based on the methods of Hilu and Randall (1984). Four fully expanded, healthy leaves were taken per pot for both adaxial and abaxial measurements on well-watered plants. Leaf impressions were measured on a compound light microscope (BX51; Olympus). Images were analyzed to determine stomatal area in μm^2 and density using ImageJ software (version 2.3.0).

2.5.3 | Evapotranspiration

The amount of evaporative water loss from the pots was measured gravimetrically by taking pot weights every morning for 5 days a week after bringing the pots to field capacity. Before the initiation of the drought phase, pots were again watered to field capacity, after which pot weights were taken every other day without any irrigation until the end of the study. These data were used to determine differences in average daily evapotranspiration rates of different genotypes under well-watered and drought-stress conditions.

2.5.4 | Percentage green cover

The green tissue coverage of each pot was measured at 5, 10, and 15 days of drought based on the methods of Karcher and Richardson (2013). A digital camera (Canon G9X; Canon) was used to take canopy images in a light box to ensure uniform lighting via light-emitting diodes. Images were processed using ImageJ software (version 2.3.0) to determine the percentage green cover.

2.5.5 | Relative water content

Leaf hydration levels were determined by taking relative water content values at 5, 10, and 15 days of drought using the methods described by Barrs and Weatherley (1962). Fresh leaf

tissue weighing approximately 0.25 g was taken from each pot and immediately measured to determine the fresh weight. The sample was then rehydrated in distilled water overnight at 4°C and weighed to determine the turgid weight. The tissue was subsequently dried in an oven at 72°C for 72 h and weighed again to determine the dry weight. Relative water content was calculated using the formula: relative water content = ([fresh weight − dry weight]/[turgid weight − dry weight]) × 100.

2.5.6 | Tissue harvest

Clippings were collected weekly on well-watered plants by trimming canopies to a height of 5 cm. Leaf tissues were placed between two sheets of polycarbonate plastic and scanned using a flatbed scanner (V550, Epson). The resulting images were used to calculate leaf areas using ImageJ (version 2.3.0). Tissues were oven-dried to determine clipping yields, and clipping leaf area divided by clipping weight was used to calculate specific leaf area. Leaf widths on mature fully expanded leaves and the internode length at the third internode on a stolon were measured with digital calipers under well-watered conditions; at least five measurements per pot were taken.

At the end of the experiment, plants were destructively harvested. Above-ground biomass was separated for each pot and oven-dried at 72°C for 72 h to determine shoot dry weights. Root zones were removed from pots and washed free of soil. Root samples were then separated and scanned for further root analysis using a flatbed scanner (V550, Epson). Root images were analyzed via the software GiA Roots (Galkovskyi et al., 2012) to estimate root parameters such as average root width and total root length. Roots were then dried in an oven as previously described and measured for dry weights. Specific root length was calculated by dividing root length by root dry weight, and root length density was measured by dividing total root length by the volume of soil within a pot.

2.5.7 | Statistical analysis

The experiment was designed as a randomized complete block design with four replicates of each of the six centipedegrass lines. The entire experiment was repeated the next year. Data were analyzed in JMP Pro v16.0 (SAS Institute) using a mixed model, where genotype (and date, where appropriate) were fixed effects and trial year was a random effect. Means were separated by Fisher's protected least significant differences at $\alpha = 0.05$ when significant.

3 | RESULTS

3.1 | Ploidy, 2C nuclear DNA content, and chromosome counting

The use of flow cytometry in 2018 identified four lines of the 500 regenerated plants that were tetraploids (HW16, HW39, HW61, and HW123). In 2019, these lines were examined again for ploidy, and all lines were tetraploids except for HW39, which reverted to a diploid. The material was vegetatively propagated in a greenhouse. In 2023, the three tetraploid lines from 2018 to 2019 (HW16, HW61, and HW123) and three diploid lines (HW107, HW121, and HW143) that were regenerated from tissue culture were tested for ploidy determination and nuclear DNA content by co-chopping each sample with maize line B73. The diploid tissue culture lines were diploids with a single centipedegrass G_1 peak (Figure 1, HW121), two lines were tetraploids (HW16 and HW123) with G_1 peaks double the value of the diploid G_1 peak, and one line was a mixoploid (HW61) with 2× and 4× centipedegrass G_1 peaks (Figure 1). Nuclear DNA contents for diploid and tetraploid lines ranged from 1.97 to 2.10 and 4.14 to 4.30 pg $2C^{-1}$, respectively (Table 1).

The flow cytometry results were confirmed by chromosome counting using root tip cells (Figure 2). HW143 was confirmed as a diploid ($2n = 2x = 18$) (Figure 2a), HW16 (Figure 2b) and HW123 were confirmed tetraploids ($2n = 4x = 36$) (Figures 2C), and HW61 was confirmed as a mixture of diploid and tetraploid cells as some root tip cells were $2n = 2x = 18$ and some were $2n = 4x = 36$ (Figure 2d1 and d2).

3.2 | Genetic relationships among centipedegrass lines

The diploid and tetraploid centipedegrass lines used (HW) were compared to each other using SSR markers to determine if the tetraploids were unique and to determine how this material compared to high seed yielding lines (15 or 16-TC lines) (Figure 3). Of the 36 markers used, only seven were polymorphic (Table 2). In total, 18 alleles were detected across these seven polymorphic loci with an average of 2.57 alleles per locus. The PCoA showed that the lines formed three groups. The first group contained only HW lines, including diploid and tetraploid lines. Tetraploid HW16 shared identical alleles with diploid HW143 for the seven SSR markers used. The second group contained only high-seed-yielding lines, and the third group contained both an HW and a high-seed-yielding line. The two tetraploid lines, HW16 and HW123, were not identical.

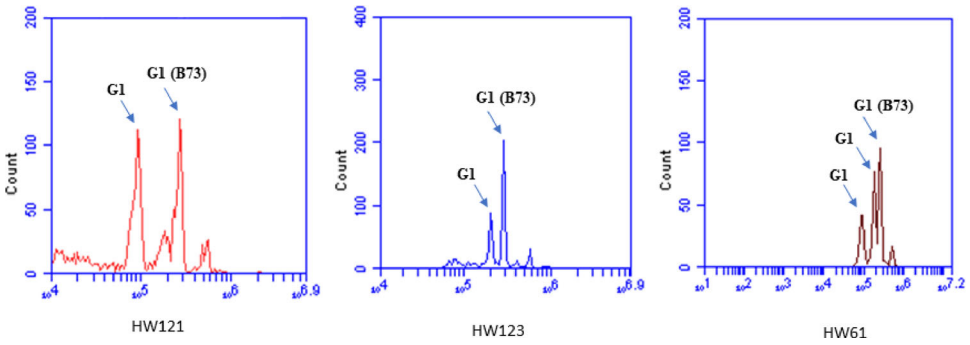

FIGURE 1 Flow cytometry histogram showing the G_1 and G_2 peaks of a diploid HW121, tetraploid HW123, and mixoploid HW61 centipedegrass co-chopped with the "B73" maize standard. HW121 and HW123 have two G_1 peaks (from left to right, the first G_1 peak is the centipedegrass sample and the second is maize) and the smaller peaks not labeled are the G_2 peaks. However, HW61 has three G_1 peaks (from left to right, the first peak is the 2× centipedegrass G_1 peak, the second peak is the 4× centipedegrass G_1 peak, and the third G_1 peak is maize).

TABLE 3 Stomatal traits among centipedegrass lines.

Line[a]	Ploidy[b]	Stomatal size[c] abaxial (μm²)	Stomatal size adaxial (μm²)	Stomatal density abaxial (no./mm²)	Stomatal density adaxial (no./mm²)
HW123	4×	1194.5 (±80.0) A	1118.3 (±70.0) A	59.1 (±1.7) C	65.9 (±5.1) B
HW16	4×	1117.9 (±38.5) A	1168.0 (±67.0) A	60.8 (±2.7) C	65.9 (±7.5) B
HW121	2×	718.7 (±30.5) B	633.7 (±37.6) B	89.6 (±5.1) A	92.9 (±5.8) A
HW143	2×	702.8 (±38.0) B	641.6 (±47.6) B	82.8 (±5.8) AB	91.2 (±1.9) A
HW61	2×, 4×	684.2 (±50.7) BC	635.7 (±37.1) B	86.2 (±5.1) AB	89.6 (±3.2) A
HW107	2×	599.6 (±41.2) C	631.6 (±44.7) B	71.0 (±11.2) BC	81.1 (±7.3) AB
Sig.		****	****	**	**
LSD		91.5	101.7	18.0	16.4

Note: Within a column, values sharing a common letter are not significantly different among genotypes according to Fisher's Least Significant Differences at $p = 0.05$. Values are presented as averages ± standard error; LSD represents Fisher's least significant differences at $p = 0.05$.

[a]Centipedegrass genotype.

[b]Ploidy as determined by flow cytometry and chromosome counts.

[c]Stomatal traits based on four fully expanded leaves per pot.

Sig.: ANOVA significance at $^{**}p = 0.01$, $^{****}p = 0.0001$.

3.3 | Morphological and physiological traits

3.3.1 | Stomatal traits

The tetraploid lines HW16 and HW123 had larger stomatal sizes on both the abaxial and adaxial sides of leaves (Table 3). Tetraploid stomata were larger by more than approximately 50% and 70% than the other diploid lines on the abaxial and adaxial sides of the leaf, respectively. The mixoploid HW61 was similar in stomatal size to the other diploid lines, and few differences were observed among the diploid lines. Stomatal densities had the opposite trend, where the tetraploid lines HW16 and HW123 had lower stomatal densities on both sides of the leaf compared to diploid or mixoploid lines (with the exception of HW107, which had intermediate stomatal densities, not different from most diploid lines or the tetraploid lines).

3.3.2 | Morphological and biomass traits

Differences in leaf traits were noted among centipedegrass lines (Table 4). The lines HW61, HW143, and HW123 had greater clipping yields under well-watered, nonstress conditions compared to the other three lines. The tetraploid lines HW123 and HW16 had greater leaf widths (4.8 and 4.7 mm, respectively) compared to the other lines, which were all 4.0 mm or less. For the internode length of the third node on stolons, HW107 was the only different line and was significantly longer than all other lines. No significant differences

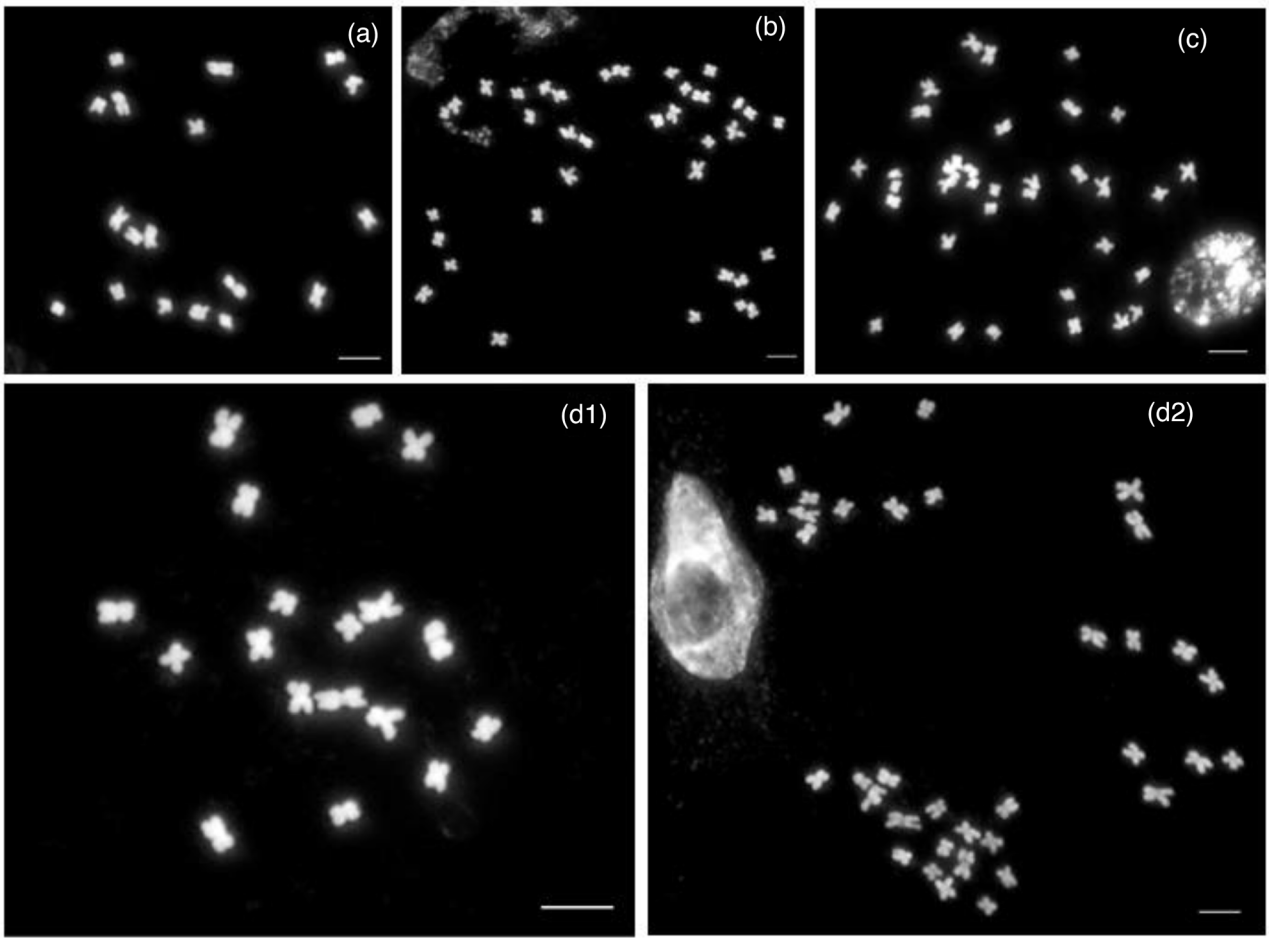

FIGURE 2 4′,6-Diamidino-2-phenylindole (DAPI)-stained mitotic metaphase spreads of HW143 (a), HW16 (b), HW123 (c), and HW61 (d1, d2). Chromosome counting was done using multiple cells derived from the same plant. Bars, 5 μm.

TABLE 4 Leaf traits among centipedegrass lines.

Line[a]	Ploidy[b]	Average clipping yield [c] (mg)	Leaf width[d] (mm)	Internode length (mm)	Specific leaf area (m^2/kg)
HW61	2×, 4×	493.5 (±84.3) A	3.9 (±0.1) B	5.9 (±0.9) B	29.1 (±3.4)
HW143	2×	473.6 (±60.6) A	4.0 (±0.3) B	6.3 (±0.8) B	24.6 (±8.9)
HW107	2×	240.5 (±68.0) B	4.0 (±0.1) B	10.9 (±1.0) A	30.6 (±1.6)
HW16	4×	251.0 (±46.6) B	4.7 (±0.2) A	7.8 (±0.7) B	29.0 (±0.5)
HW121	2×	234.6 (±48.8) B	3.9 (±0.4) B	5.9 (±0.8) B	29.7 (±2.4)
HW123	4×	391.8 (±53.8) A	4.8 (±0.4) A	6.8 (±1.1) B	29.8 (±1.8)
Sig.		****	****	**	n.s.
LSD		124.6	0.4	2.5	–

Note: Within a column, values sharing a common letter are not significantly different among genotypes according to Fisher's Least Significant Differences at $p = 0.05$. Values are presented as averages ± standard error; LSD represents Fisher's least significant differences at $p = 0.05$.

[a]Centipedegrass genotype.

[b]Ploidy as determined by flow cytometry and chromosome counts.

[c]Dry weights of clippings from 1 week of growth.

[d]Other leaf traits based on at least five measurements per pot.

Sig.: ANOVA significance at $^{**}p = 0.01$, $^{****}p = 0.0001$, n.s. = not significant.

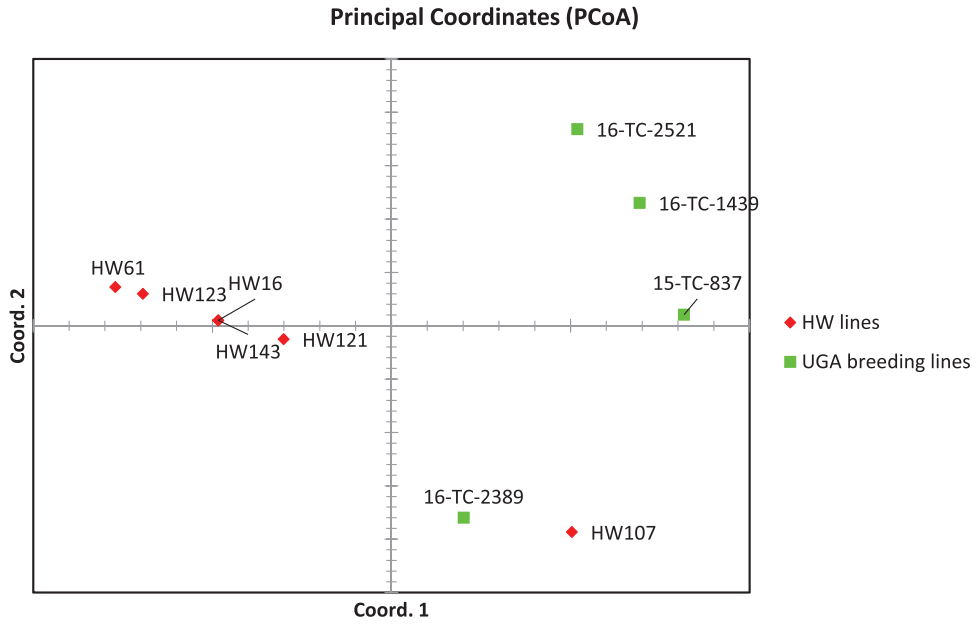

FIGURE 3 Genetic relationships among diploid and tetraploid centipedegrass lines. The first population includes the lines that were regenerated using tissue culture (HW lines), and the second population includes the lines from the University of Georgia (UGA) breeding program with high seed yield.

were observed among lines for specific leaf areas. Differences were identified among lines for biomass and rooting characteristics, but these differences did not necessarily correspond with ploidy levels (Table S2). In general, HW61 had greater biomass, while HW123 had the lowest biomass.

3.3.3 | Plant water relations

Under well-watered conditions, there were limited differences in average daily evapotranspiration rates. Across weeks, HW123 had a significantly lower evapotranspiration rate, with an average evapotranspiration of 6.9 mm day^{-1}, which was significantly lower than HW107, HW61, and HW16 (8.1, 8.1, and 7.8 mm day^{-1}, respectively) (Figure 4). The lines HW143 and HW121 were not significantly different than any other line under well-watered conditions, having average evapotranspiration rates of 7.6 and 7.5 mm day^{-1}. At the end of 15 days of drought, a range in evapotranspiration rates from pots was observed. Average daily evapotranspiration rates were greatest in HW107, HW16, and HW61, which were 7.1, 6.9, and 6.8 mm day^{-1}, respectively. The lowest average evapotranspiration rate was in HW123 (6.3 mm day^{-1}), which was not significantly different from HW143 (6.6) or HW121 (6.4). Under nonstress conditions, all lines maintained greater than 90% relative water content. Under drought, there were no significant differences among centipedegrass lines in terms of relative water content, except at the early stages of stress (Figure S1).

3.3.4 | Percentage green cover

Prior to drought stress, there existed some differences in percentage green cover, with HW123 and HW143 having reduced canopy coverage (likely due to plant density and growth habits) compared to other lines (Figure 5). As the drought progressed, all lines experienced a decline in percentage green cover. By 15 days of drought stress, all lines had declined to 15% green cover or less, with no significant differences among lines. At both 5 and 10 days of drought stress, lines HW16, HW61, and HW107 maintained higher percentages of green cover than HW121, HW123, and HW143.

4 | DISCUSSION

Gamma irradiation (Moutschen-Dahmen & Moutschen-Dahmen, 1970) and tissue culture (Murashige & Nakano, 1966; Ogura, 1990) can generate polyploids in plant species. Tissue culture is a powerful tool for plant breeding and genetic improvement programs. It has been used for diverse purposes, ranging from the investigation of plant developmental processes to the generation of transgenic plants with desirable traits (Loyola-Vargas & Ochoa-Alejo, 2018). In this study, two genetically different, stable tetraploids were generated using gamma irradiation and tissue culture, and a mixoploid centipedegrass line was identified. Mixoploids tend to be unstable and revert back to their lower ploidy cytotypes (Jones et al.,

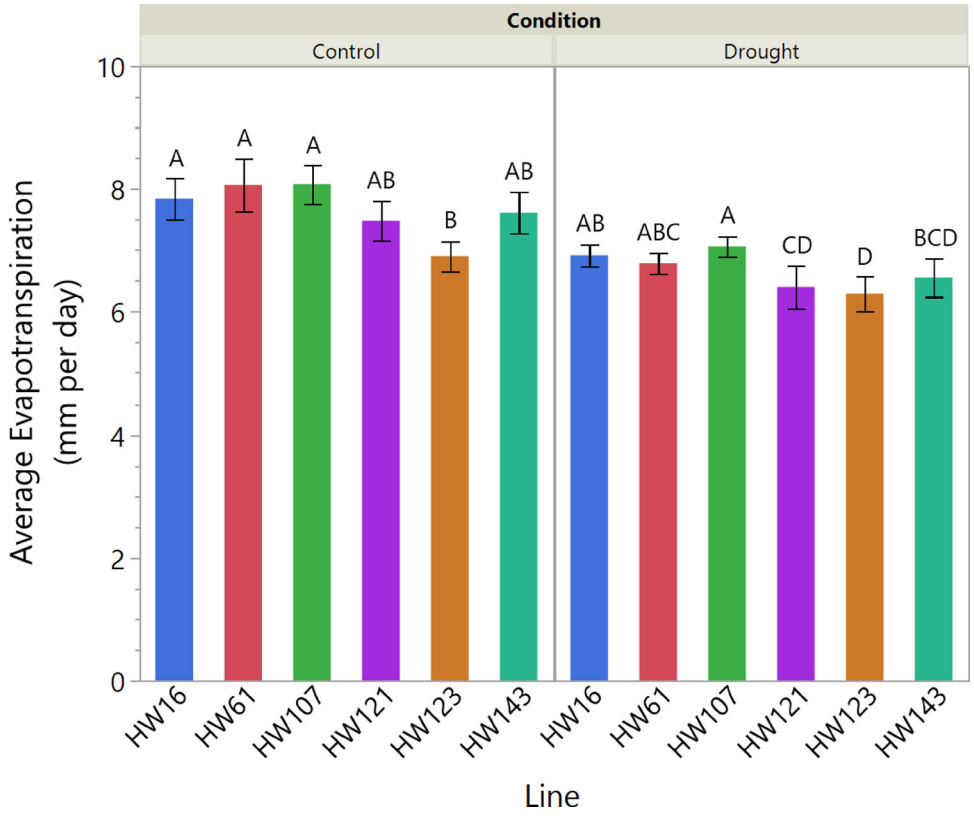

FIGURE 4 Average daily evapotranspiration rates (mm day^{-1}) of centipedegrass lines after 5 days in well-watered control conditions or 15 days of drought. Bars represent standard errors, letters are the least significant difference groupings at $p = 0.05$, lines within a given treatment sharing a common letter are not statistically different. HW16 and HW123 were 4×; HW61 was 2× and 4×; and HW107, HW121, and HW143 were 2×.

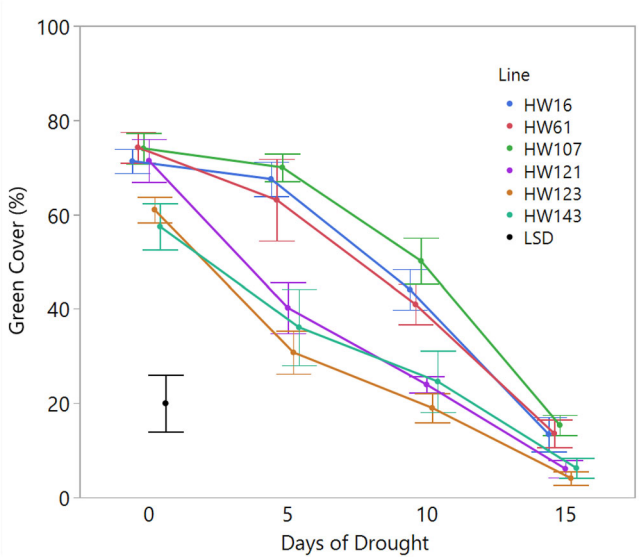

FIGURE 5 Changes in percentage green cover based on digital image analysis over 15 days of drought stress. Colored bars represent standard errors, and the black bar represents the least significant difference interval at $p = 0.05$ for the significant line by day of drought interaction.

2008; Quesenberry et al., 2021). The reason for this reversion is that in a mixoploid, where the histogenic layers are of different ploidy levels, cells of lower ploidy multiply faster and overrun the cells of higher ploidy (diplontic selection) (Broertjes & Keen, 1980; Jones et al., 2008). Cells of lower ploidy multiply faster than cells of higher ploidy because cell types with higher DNA content typically take longer to cycle through mitosis (Van't Hof & Sparrow, 1963), and selection favors the faster proliferating ploidy cytotype (Jones et al., 2008).

Knowledge of the ploidy and 2C nuclear DNA content of turfgrass species facilitates molecular studies, and polyploids may display phenotypic changes as compared to diploids. In this study, using B73 maize as an internal standard, we calculated the 2C nuclear DNA content of the centipedegrass lines, and the values ranged from 1.97 to 2.10 and 4.14 to 4.30 pg 2C^{-1} for diploid and tetraploid lines, respectively (Table 1). As expected, the 2C nuclear content value of 4× lines was twice that of the 2× lines. This result agrees with a previously published report where a 4× centipedegrass line had a 2C nuclear DNA content twice that of the 2× line TifBlair (Schwartz, Harris-Shultz, et al., 2013). In contrast, the nuclear

DNA content of the 2× lines in our study is slightly higher than previously reported results, where the nuclear DNA content of a 2× centipedegrass line was reported to be 1.66 ± 0.02 (Arumuganathan et al., 1999).

Polyploidization, which can occur either naturally or artificially, has been utilized for crop improvement ever since crop plants were first domesticated. Polyploidy provides genome buffering, increases allelic diversity and heterozygosity, and generates novel genotypic and phenotypic variation, all of which are important for crop improvement (Udall & Wendel, 2006). Studies have reported the development of novel phenotypic variation with polyploidization. In an artificially induced polyploid of *Brassica napus*, for example, variation was observed for flowering time and the difference in flowering time was heritable (Schranz & Osborn, 2000). Polyploidization has had an impact on turfgrass breeding for over 50 years and it has been used to generate variation in different turf types (Schwartz, Harris-Shultz, Contreras, et al., 2013).

A total of 36 SSR markers were used to assess genetic diversity among the 10 centipedegrass lines, and only seven markers were polymorphic and produced 18 alleles in total, suggesting low genetic diversity. The two tetraploid lines, HW16 and HW123, and a mixoploid line, HW61, were not genetically identical, but they were genetically similar (Figure 3). In the United States, the genetic base of centipedegrass is narrow because most of the centipedegrass grown originates from a single accession introduced as seed from China collected by Frank Meyer in 1916 (Hanna, 1995). TifBlair, from which the HW lines were derived, was released in 1997 and was selected from irradiated seeds of common centipedegrass that were planted in Blairsville, GA (Hanna et al., 1997), while the TC lines were derived from germplasm obtained during a collection trip to central and southern China in 1999 (Liu et al., 2003). Although SSR markers are a valuable tool for understanding genetic diversity and are commonly used in turfgrasses, marker technology such as single nucleotide polymorphisms that cover the whole genome will give a better measurement of genetic diversity for turfgrass species.

In this study, morphological and physiological traits among diploid, tetraploid, and mixoploid centipedegrass lines were evaluated, and differences among lines were identified. The tetraploid lines HW123 and HW16 had larger stomata, reduced stomatal density, and greater average leaf widths. Increased organ size has been associated with genome size and is a commonly reported effect of polyploidization (Knight & Beaulieu, 2008; Marinho et al., 2014). Schwartz, Harris-Shultz et al. (2013) previously reported that colchicine-induced polypoid centipedegrass had increased stomatal size, and a similar effect was observed in St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) (Carbajal et al., 2019). The tetraploid line HW123 and the mixoploid line HW61 also had greater clipping yield than the other lines (with the exception of HW143), potentially indicating increased growth or leaf elongation rates for these lines.

Despite differences in leaf traits for the polypoid lines, the differences in total accumulated biomass, above or below ground, did not clearly separate based on ploidy levels. Increased vigor and growth have been documented in polyploids, but the phenomenon is not consistent (Innes et al., 2021; Kaensaksiri et al., 2011; Sattler et al., 2016). In some species, induced polyploids have been linked to improved root traits, but observed variations in specific root length and root length density did not directly correspond to ploidy in our study (Kim et al., 2004; Kulkarni & Borse, 2010). The process of inducing callus and maintaining it in tissue culture is commonly used to induce mutations and create novel traits for plant breeding pipelines (Philips et al., 1994). The increased root-to-shoot ratio of HW143 or the increased root length density of HW107 may be desirable traits for future cultivars.

In response to drought conditions, there were relatively few differences among lines. Differences in percentage green cover and evapotranspiration rates were likely due to inherent differences in growth and density among lines and not necessarily due to differences in drought performance. Growth rates and rooting characteristics are important traits for determining water use and drought performance in turfgrasses (Carrow, 1996; Colmer & Barton, 2017; Jespersen & Schwartz, 2018). However, it is worth noting that drought responses may be different under field conditions where rooting is not limited by pot volumes and native soils may have different water holding capacities than the current study.

In conclusion, two stable tetraploid lines were generated in this study. These lines have larger stomata and leaf widths and decreased stomatal density. In addition to the differences observed in polyploid lines, variation among other centipedegrass lines was also observed. Greater quantification of traits, particularly under more realistic field conditions, will be essential to better understanding of the differences in growth and performance among the generated lines. Irradiation and tissue culture appear to be valuable techniques for the generation of more stable polyploids and lines with novel traits in centipedegrass.

AUTHOR CONTRIBUTIONS

Suraj Sapkota: Formal analysis; investigation; methodology; writing–original draft; writing–review and editing. **Ravneet Kaur, Hongliang Wang, and Pheonah Nabukalu**: Investigation; methodology; writing–review and editing. **Karen Harris-Shultz**: Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; visualization; writing–original draft; writing–review and editing. **Dal-Hoe Koo**: Formal analysis; investigation; methodology; writing–review and editing. **David Jespersen**: Conceptualization; formal

analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; writing–original draft; writing–review and editing.

ACKNOWLEDGMENTS

The authors would like to thank Michael Purvis and Mikayla Barela for their assistance in conducting the experiments. The authors are grateful to Dr. Joann Conner for her help with flow cytometry and Dr. Brian Schwartz for providing the TC centipedegrass lines. The U.S. Department of Agriculture (USDA) prohibits discrimination in all its programs and activities on the basis of race, color, national origin, age, disability, and where applicable, sex, marital status, familial status, parental status, religion, sexual orientation, genetic information, political beliefs, reprisal, or because all or part of an individual's income is derived from any public assistance program. (Not all prohibited bases apply to all programs.) Persons with disabilities who require alternative means for communication of program information (Braille, large print, audiotape, etc.) should contact USDA's TARGET Center at (202) 720–2600 (voice and TDD). To file a complaint of discrimination, write to USDA, Director, Office of Civil Rights, 1400 Independence Avenue, S.W., Washington, DC 20250–9410, or call (800) 795–3272 (voice) or (202) 720–6382 (TDD). USDA is an equal opportunity provider and employer.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Sapkota, S., Kaur, R., Harris-Shultz, K., Wang, H., Koo, D.-H., Nabukalu, P., & Jespersen, D. (2023). Creation and characteristics of tetraploid and mixoploid centipedegrass. *Crop Science*, 1–14. https://doi.org/10.1002/csc2.21011