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Doubling the chromosome number of bahiagrass via tissue culture

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Abstract Crop improvement in bahiagrass (*Paspalum notatum* Flüggé) is limited by apomixis in most natural tetraploids, however, diploid sexual types occur. Production of sexual tetraploids by chromosome doubling will allow hybridization with apomictic tetraploids. Diploid bahiagrass (*Paspalum notatum* Flüggé) embryogenic callus tissue was exposed to three concentrations of three antimitotic chemical agents, colchicine, oryzalin and trifluralin. Callus was generated to plants and ploidy was evaluated by stomata size, mitotic chromosome counts, and flow cytometry. A total of 310 plants were verified as tetraploid of 1,432 plants that reached transplanting size. All treatments yielded 4*x* plants. The mean percentage success over all treatments was 22%, with means of 31% for oryzalin, 24% for colchicine and 16% for trifluralin. The high rates of success indicate that all agents can be successfully used to double chromosome numbers in bahiagrass. The percentage of 4*x* plants ranged from 9% (20 μM trifluralin) to 43% (20 μM oryzalin). Several treatments adversely affected regeneration. Mitotic chromosome counts are difficult and labor intensive in bahiagrass. Therefore, leaf stomata measurements were used as a preliminary screen. Data gave a bimodal distribution with overlapping tails and based on chromosome counts would have given an error rate of 12%. Flow cytometry analysis of regenerated plants resulted in mean nucleus fluorescence distributions consistent with control diploid or tetraploid values. These values agreed with chromosome counts, and this method is recommended for determining bahiagrass ploidy level. Research goals and available resources should be taken into consideration when selecting a treatment for chromosome doubling in bahiagrass.

Keywords Polyploidy · Chromosome doubling · *Paspalum* · Tissue culture

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Introduction

Most bahiagrass (*Paspalum notatum* Flüggé) germplasm collected worldwide is tetraploid and apomictic, however the type introduced into the southeastern USA and naturalized as 'Pensacola' is diploid and sexual (Burton 1948, 1955). The development of sexual tetraploid lines would facilitate crosses to more effectually utilize variability in the world germplasm collections. A limited number of sexual tetraploids have been generated by treating both diploid seed and tissue cultured calli with colchicine (Forbes and Burton 1961; Quarin et al. 2001). Research with other apomictic tetraploid grasses has shown that when cross-compatible sexual germplasm

can been identified for use as females, significant variability is released in the F_1 when apomictic lines are used as males (Miles and do Valle 1996).

Although methods of chromosome doubling using colchicine have been known for over 70 years (Blakeslee 1939; Bradley and Goodspeed 1943; Brewbaker 1952), only in the past two decades has the use of other mitotic spindle poison agents, such as oryzalin and trifluralin, been investigated (Morejohn et al. 1987; Hugdahl and Morejohn 1993; Hansen and Andersen 1996). Some reports have used in vitro tissue culture regeneration protocols for chromosome doubling (Kermani et al. 2003). These procedures have been especially effective when applied to haploid tissues such as microspores (Hansen and Andersen 1996), ovules (Hansen et al. 1998) or plantlets (Hassawi and Liang 1991). When using alternative methods and antimitotic agents, it is important to identify concentrations that will effectively double chromosomes without interfering with the culture and regeneration of plants.

Another successful method of chromosome doubling is treatment of just fertilized zygotes with nitrous oxide under pressure. An advantage of this method compared to colchicine treatment of vegetative tissues is a much higher success rate (ca. 70% with N_2O vs. <10% with colchicine in red clover) and absence of sectoral chimeras since the N_2O treatment targets the first mitotic division of the zygote (Taylor et al. 1976). A disadvantage of this method with cross-pollinated species is that the exact genotype of an individual cannot be duplicated at the 4*x* level since hybrid zygotes are the target tissue. In vitro treatments of early developmental stage somatic embryos would allow chromosome doubling of an exact genotype and might also reduce or eliminate sectoral chimeras. Methods for in vitro somatic embryogenesis in bahiagrass have been reported (Smith et al. 2002).

The objectives of this research were to double the chromosome number of diploid bahiagrass using in vitro embryogenic cultures and selected antimicrotubular chemicals, and to investigate concentrations of the antimicrotubular chemicals that would effectively double bahiagrass chromosomes without interfering with tissue culture regeneration of plants. The three antimicrotubular chemicals were selected based on reports of their successful use in doubling the chromosomes of haploid cultures.

Materials and methods

Tissue culture and antimitotic treatments

Seed of the improved diploid sexual bahiagrass cultivar 'Tifton 9' (Burton 1989) ($2n = 2x = 20$) was treated with concentrated H_2SO_4 for 15 min to break seed dormancy, rinsed several times with sterile water, air dried for 1 h, and then disinfected in a closed desiccator with vapors from 20 ml Clorox mixed with 10 ml glacial acetic acid for 60 min. Callus was initiated by germinating the disinfected seeds at 28°C, in the dark on plates of initiation/maintenance (IM) medium containing 4.3 g l^{-1} MS salts (Murshige and Skoog 1962), 1 ml l^{-1} Gamborg's B5 vitamins (Gamborg et al. 1968), 1.13 mg l^{-1} BAP (6-benzylaminopurine), 6.6 mg l^{-1} dicamba (3,6-dichloro-*o*-anisic acid), 30 g l^{-1} sucrose and 2 g l^{-1} Phytagel (Sigma, St. Louis, Mo.), pH 5.7 (Smith et al. 2002). Seed germination occurred in about 5 days and calli appeared at the shoot meristem of most seedlings in 4–5 weeks. The calli were transferred every 14 days to fresh medium and with each transfer embryogenic callus was selected.

Three antimitotic agents, colchicine, {(S)-*N*-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo[*a*]heptalen-7-yl) acetamide}, oryzalin (3,5-dinitro-*N*4,*N*4-dipropylsulfanilamide), and trifluralin (a,a,a-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) were used to treat the calli. Data reported by Hassawi and Liang (1991), Geoffriau et al. (1997), Wan et al. (1991), Hansen and Andersen (1996), and Hansen et al. (1998) were used to select a range of treatment concentrations and exposure time of the chemicals. Our objective was to determine concentrations that would induce chromosome doubling without inhibiting plant regeneration. Colchicine was used at 313, 626 and 900 μM, which was prepared by adding a 0.0125 mol l^{-1} stock solution (in water) to IM medium without gelling agent. Oryzalin and trifluralin were used at 5, 10 and 20 μM concentrations which were prepared by adding the proper amounts of a 0.012 mol l^{-1} stock solutions of those chemicals (in DMSO or Dimethyl sulfoxide) to IM medium without gelling agent.

Replicates of each antimitotic chemical treatment/concentration were made by adding a layer of sterile gel blot paper (Scheiche and Schell, Keene, NH) and 12 ml of the proper treatment solution to each petri dish (100 mm dia). This amount of treatment solution

provided some free solution on top of the blotter paper. Calli were broken into 2 mm pieces and distributed at random to the plates. All treatments lasted 48 h after which the treatment solution was rinsed from the callus pieces by dipping them in IM solution without an antimitotic chemical. Calli were replated in clusters of four on IM medium (with gelling agent) for 5–7 days. The calli were then transferred to a regeneration medium (RM) which was similar to IM except dicamba was removed and 1 μM GA_3 (Gibberellic acid) was added. Culture was under white light (16 h day^{-1}) at 26°C and calli were transferred to new media every 14 days. After shoots were formed, 0.5 mg l^{-1} 1-naphthaleneacetic acid (NAA) was added to RM for 7–14 days to initiate rooting, then shoots were transferred back to RM without NAA. After a sufficient number of roots had formed, medium was washed off the roots and individual plants were transferred to Fafard #3B potting mix in 7.5 cm pots, conditioned in high humidity for several days and transferred to the greenhouse. All regenerated plants (ca. 1440) were transplanted to a field nursery in summer 2002 and allowed to grow for the entire season. Data on percentage of regenerated plants from each treatment were log transformed prior to regression analysis of rates of antimitotic agents.

Ploidy evaluation methods

Measurement of stomata

Stomate measurements were made first on a group of 160 plants selected as suspected tetraploids in the greenhouse based on phenotypic traits (broader leaves and larger plant size) to evaluate whether stomate size was a useful predictor of ploidy level. Size of plant stomates in both the greenhouse and field were measured. Leaf surfaces were coated with clear nail polish, which was allowed to dry, peeled off, and dry mounted on clean microscope slides. Ten stomates from each of two leaves were measured using an ocular micrometer at 200× magnification. The sum of the 20 measurements was used to predict ploidy level.

Mitotic chromosome determinations

Determination of mitotic chromosome numbers in many C_4 tropical grasses including bahiagrass is difficult due to small chromosome size and often low mitotic index, thus chromosome numbers were determined on a subset of suspected 4*x* plants with known 2*x* individuals used as laboratory controls. The suspected ploidy level was not disclosed to laboratory personnel involved in chromosome number determinations. Actively growing root tips were pretreated for 4–5 h in saturated aqueous 8-hydroxyquinoline at 4°C, and fixed in 3:1, v:v, 95% ethanol:glacial acetic acid for 24 h. After fixation, tissues were transferred to 70% ethanol. Root tips were then excised, macerated on a clean slide in a drop of 40% acetic acid, and stained for 4–24 h in acetorcein under a coverslip. After staining, preparations were squashed further, blotted of excess stain and observed under 400× or 1000× magnification to determine chromosome numbers.

Ploidy determination via flow cytometry

During the period of this research a Partec PAII Ploidy Analyzer® was acquired and subsequently used in evaluation of the ploidy level of over 450 individual plants. Actively growing shoot meristems were excised in the field with an attached piece of stolon, tagged, and placed into a container of water for transport to the lab. Developed leaves were removed and a small piece (ca. 0.5–1 cm) of the soft meristem just above the upper node was excised. This tissue was chopped with a single edged razor blade in a plastic petri dish over ice in 500 μl of extraction buffer (Partec CyStain UV Precise P) to release intact nuclei. Released nuclei were washed into the tilted side of the dish, siphoned up with a micro-pipet and filtered through a 50 μm filter into an analysis vial. Then 1500 μl of staining buffer (Partec DAPI: 4′,6-diamidino-2-phenylindole) was added to the vial and samples were allowed to stain for 3 min prior to analysis. Known 2*x* and 4*x* clones were sampled as reference standards each day a group of plants were processed for ploidy determination. The gain of the ploidy analyzer was adjusted daily to produce peaks at near 100 and 200 for the diploid and tetraploid standards, respectively.

Results and discussion

A total of 310 plants were ultimately verified as tetraploid among a total of 1,432 plants that reached transplanting size under controlled greenhouse conditions

(21.7%). Tetraploid plants were verified from all nine antimitotic chemical-concentration treatment combinations with a range of doubled plants from 9% (20 μM trifluralin) to 43% (20 μM oryzalin) (Table 1). The mean percentage success in chromosome doubling over all treatments of 22% is high when compared with the 1–10% success rate reported using somatic tissue chromosome doubling methods (Bradley and Goodspeed 1943; Brewbaker 1952). When averaged over rates of each antimitotic agent there were differences ($P < 0.05$) in percentage tetraploids produced, with mean values for oryzalin (31%), greater than trifluralin (16%), but not different from colchicine (24%) (Table 1). Colchicine, the antimitotic agent most commonly used for ploidy manipulation research with intact meristems in plants, gave percentage successful chromosome doubling intermediate between oryzalin and trifluralin when used for treatment of somatic embryos.

Although oryzalin yielded the highest mean percentage of 4*x* plants, it apparently had a detrimental effect ($P = 0.05$) on plant regeneration efficiency using this in vitro system for bahiagrass. Only 96 plants were recovered from the three oryzalin treatments compared to 561 and 775 from the trifluralin and colchicine treatments, respectively. Time and labor required to confirm the identity of 4*x* individuals from a mixed population of 2*x* and 4*x* plants is a consideration in any ploidy manipulation research. Choosing an agent such as oryzalin, that reduces the total number of plants to be screened while yielding a high frequency of successfully doubled chromosome number plants, may be beneficial if only lower numbers of 4*x* plants are the desired outcome.

The colchicine treatments yielded the highest number of plants (775) and the highest number of verified 4*x* plants (189) but with an intermediate success rate of 24% (Table 1). When averaged over rates, the mean number of regenerated plants was not different ($P > 0.05$) between colchicine and trifluralin. Nevertheless, the reduced number of verified 4*x* plants from the trifluralin treatments, indicates that, although successful, this antimitotic agent would be the less desirable choice for use with bahiagrass in vitro chromosome doubling research.

Regression analysis of percentage plants doubled data showed an increasing linear effect of concentration among the three colchicine treatments ($P = 0.04$, $r^2 = 0.92$). Due to the fact that more total plants (395) were successfully recovered from the 626 μM colchicine treatment and that this treatment had a high percentage tetraploids (26%), the largest number of 4*x* plants (102) were obtained from this treatment. There was a trend for the 626 and 900 μM colchicine treatments to produce a higher percentage of tetraploids compared to the 313 μM level (Table 1).

Increasing concentrations of oryzalin from 5 to 20 μM also resulted in a significant linear increase ($P = 0.05$, $r^2 = 0.90$) in percentage chromosome doubled plants recovered. The 20 μM oryzalin treatment resulted in 43% 4*x* plants among the 14 plants recovered from this treatment (Table 1). Additional

Table 1 Effects of types and concentrations of antimitotic agents on in vitro total plant production and 4*x* plant production in bahiagrass

Antimitotic agent	Concentration (μM)	No. of plants produced	No. of verified 4*x* plants	Percentage 4*x* plants (%)
Colchicine	313	157	31	20
Colchicine	626	395	102	26
Colchicine	900	223	56	25
Colchicine summary		775	189	24
Trifluralin	5	184	44	24
Trifluralin	10	114	24	21
Trifluralin	20	263	23	9
Trifluralin summary		561	91	16
Oryzalin	5	64	19	30
Oryzalin	10	18	5	28
Oryzalin	20	14	6	43
Oryzalin summary		96	30	31
Overall summary		1432	310	21.7

research may also be justified to define the optimum concentrations of this antimitotic agent for bahiagrass in vitro chromosome doubling research. Our data suggests that rates of oryzalin higher than 20 μM are likely to be phytotoxic to in vitro plant regeneration, but slightly higher rates could possibly result in even higher frequency of chromosome doubling among any regenerated plants. If large numbers of 4*x* plants from variable genotypes are the desired outcome, then colchicine at the 626 μM rate would seem to be a more desirable choice.

Increasing concentrations of trifluralin from 5 to 20 μM resulted in a significant quadratic decrease ($P = 0.01$, $r^2 = 0.99$) in percentage 4*x* plants recovered. The 5 μM trifluralin treatment resulted in 24% tetraploids among the 184 plants recovered from this treatment. Although the two higher concentrations of trifluralin yielded lower percentages of 4*x* plants, these concentrations did not appear to have any consistent phytotoxic effect on overall in vitro bahiagrass plant regeneration. The 20 μM concentration yielded the second highest total number of regenerated plants. Additional research should be conducted to define the optimum lower limit of concentrations of this antimitotic agent for bahiagrass tissue culture treatment.

Measurements of leaf stomata can be used as a preliminary screen when large numbers of plants need to be evaluated for ploidy level. However, our results (data not shown) indicated that misidentifications (based on microscopic chromosome counts) were a concern. Our results on almost 500 plants showed a distinct bimodal distribution (representing 2*x* and 4*x* populations) of stomata length frequency distribution, but with overlapping tails of the 2*x* and 4*x* stomata length distributions. Based on microscopic chromosome counts or flow cytometry verification of ploidy level, this would have resulted in an error rate of about 12%. The majority of the misclassified plants had stomata measurements would have indicated as 2*x*, but that were later determined to be 4*x* by chromosome counts or flow cytometry. Figure 1 shows typical 2*x* and 4*x* leaf stomata.

Chromosome numbers from bahiagrass root tips verified the numbers of $2n = 2x = 20$ for untreated

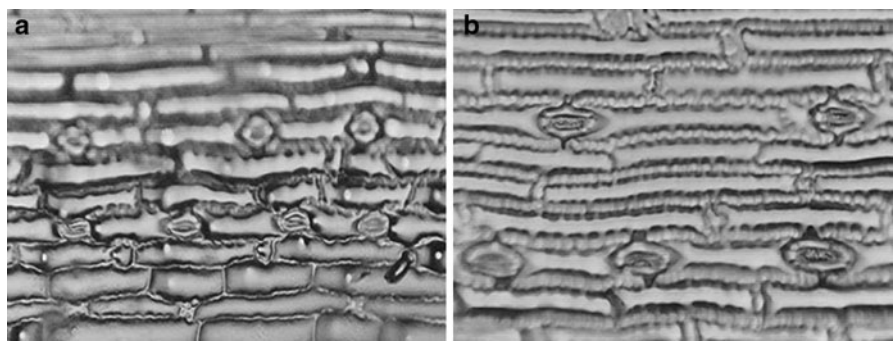

Fig. 1 Stomata from $2n = 2x = 20$ (**a**) and from $2n = 4x = 40$ (**b**) bahiagrass

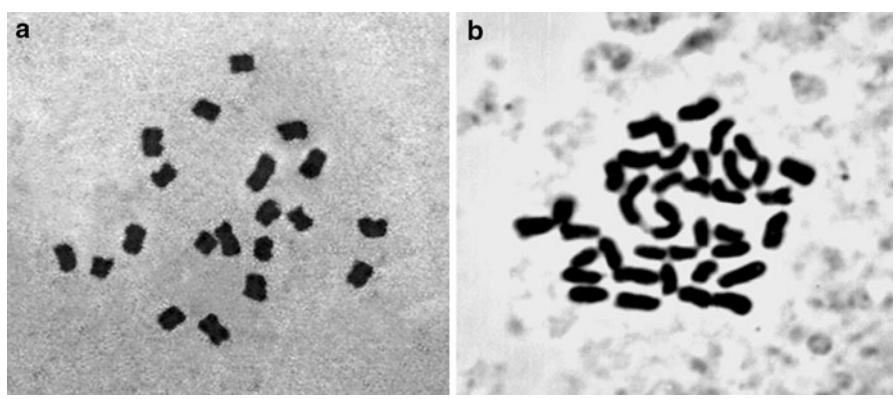

Fig. 2 Chromosomes from $2n = 2x = 20$ (**a**) and from $2n = 4x = 40$ (**b**) bahiagrass. Magnification ×1000

plants or from unsuccessful treatments. Successfully doubled chromosome numbers were $2n = 4x = 40$ (Fig. 2). It was difficult to separate and count the tetraploid chromosome number, but no consistent counts of aneuploid numbers were observed. Mitotic index (frequency of countable metaphase cells in a root tip squash) was consistently low requiring a large time commitment for microscopic chromosome number verification. Chromosome numbers were determined on over 120 $4x$ plants and 80 $2x$ plants.

After a season of growth in the field, suspected $4x$ plants usually showed morphological traits that

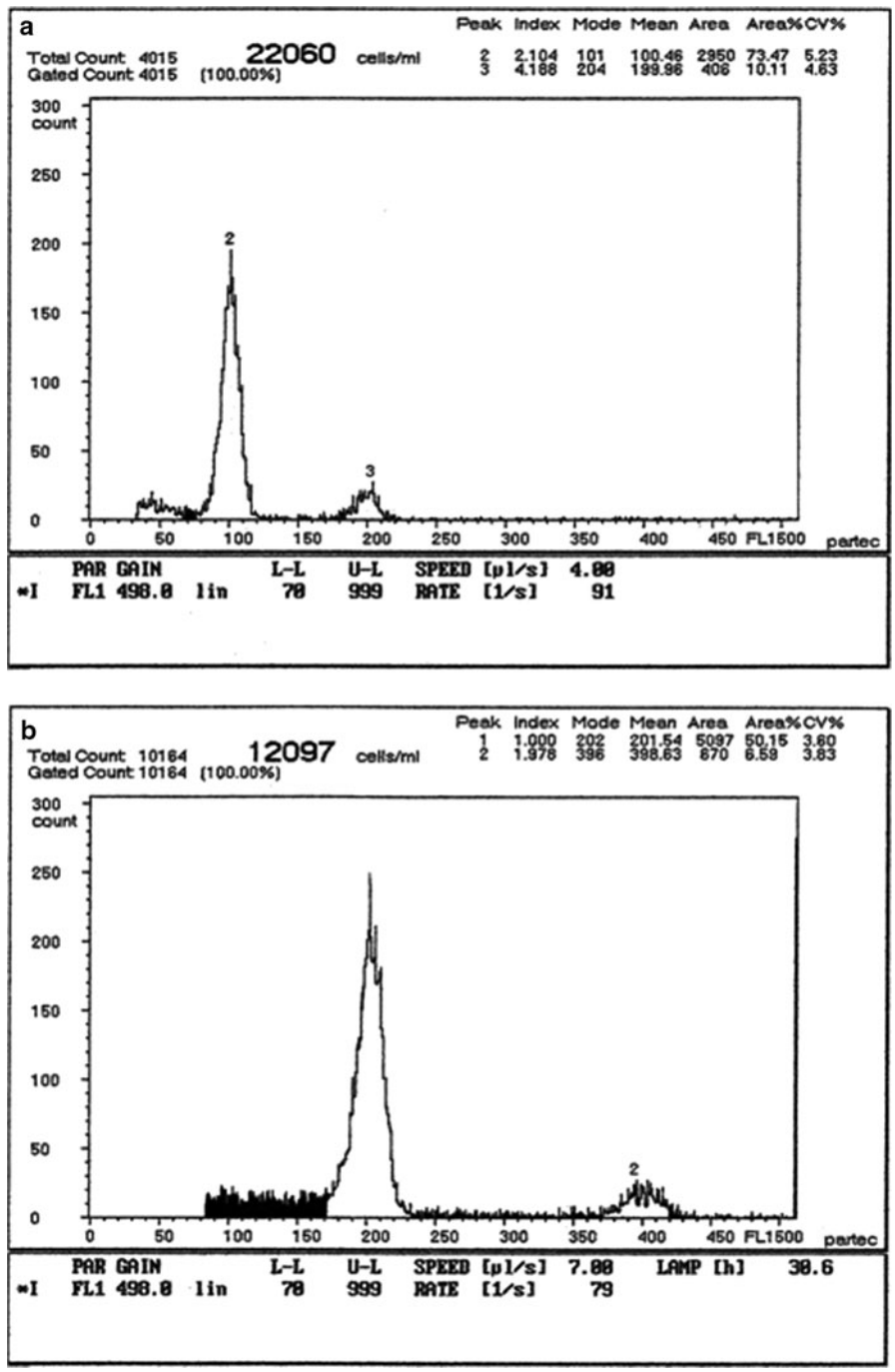

Fig. 3 Flow cytometry histograms from $2n = 2x = 20$ (**a**) and from $2n = 4x = 40$ (**b**) bahiagrass

suggested increased ploidy (more compact upright growth, reduced flower head number, and slight differences in leaf color). Flow cytometry was used to evaluate ploidy level of all plants suspected as tetraploid based on field morphology and a sample of other assumed diploid plants. Mean nucleus fluorescence distributions varied around the control diploid and tetraploid values of 100 and 200, but there were no samples that appeared to be intermediate (triploid). The analysis of ploidy level using the flow cytometer was consistently in agreement with ploidy determined by chromosome counts (50 plants) except for two cases where subsequent chromosome counts validated the flow cytometry results. Therefore, flow cytometry can be confidently used to determine the ploidy level of bahiagrass clones. The use of flow cytometry allows for a cost effective method to determine the ploidy level of multiple shoots of an individual in order to rule out the existence of sectoral chimeras. Figure 3 shows a printout of a 2*x* and 4*x* individual bahiagrass plant.

Finally, the group of 310 4*x* plants represents a key to release the genetic variability contained in the tetraploid bahiagrass germplasm for breeding purposes. In fact, a group of 20 of the most vigorous clones had already been evaluated for mode of reproduction and they were classified as mainly sexual (Acuña et al. 2007). In addition, eleven of these sexual clones had been also used in crosses as female parents with different apomictic 4*x* clones as male parents, and a high genetic diversity was released for traits of agronomic importance, such as mode of reproduction, seasonal growth and growth habit (Acuña et al. 2009). This additional information demonstrates that these 310 clones can be used in a breeding program with the objective of fixing superior apomictic 4*x* hybrids.

Summary and conclusions

In vitro chromosome doubling using embryogenic callus of bahiagrass is an efficient method that resulted in higher percentages of 4*x* plants among recovered plants than previously reported whole plant meristem treatment methods in other species. The overall percentage of 4*x* plants recovered was 22% with the best treatment yielding 43% 4*x* plants. More than 300 4*x* plants were recovered for field evaluation. Additionally, no evidence of ploidy level chimeras within plants was detected, an additional benefit for in vitro methods compared to whole plant methods.

Addition of colchicine, oryzalin, and trifluralin as antimitotic agents to diploid bahiagrass in vitro plant regeneration media resulted in successful recovery of 4*x* plants. Treatments yielding the top percentages of 4*x* plants were: 20 μM oryzalin (43%), 626 μM colchicine (26%), and 5 μM trifluralin (24%).

Research goals and available resources should be taken into consideration when selecting chromosome doubling and ploidy evaluation methods in bahiagrass. If large numbers of 4*x* plants from variable genotypes are the desired outcome, then colchicine at the 626 μM concentration would seem to be a more desirable choice. Trifluralin at 5 μM resulted in a similar percentage of 4*x* plants, however in vitro plant regeneration was only half that of the colchicine 626 μM concentration. Thus, trifluralin could be an appropriate choice if resources are limited. Although stomatal measurements may provide a preliminary screen, we recommend flow cytometry as the most efficient and reliable method of ploidy analysis in bahiagrass.

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