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ORIGINAL ARTICLE

# Regeneration of fertile, hexaploid, interspecific hybrids of elephantgrass and pearl millet following treatment of embryogenic calli with antimitotic agents

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**Abstract** Elephantgrass (*Pennisetum purpureum,* 2n = 4x = 28) produces large amounts of biomass in tropical and subtropical regions and is considered a prime candidate for lignocellulosic biofuel production. Interspecific hybridization between elephantgrass and pearl millet (*Pennisetum glaucum*, 2n = 2x = 14) may allow improvement of drought tolerance and biomass quality. These interspecific hybrids are male and female sterile due to their triploid genome (2n = 3x = 21). Chromosome doubling of the triploid hybrids may restore fertility, permitting a backcross with the recurrent or other elephantgrass parents to enhance biomass yield and persistence. In this study, chromosome doubling of productive interspecific hybrids was performed in vitro. Immature inflorescence cross-sections of five interspecific hybrids with good agronomic performance were used as explants for establishment of embryogenic tissue cultures. These calli were treated with different antimitotic agents, oryzalin or trifluralin. Chromosome doubling was achieved in all five interspecific hybrids and a total of 74 plants with altered ploidy were confirmed by flow cytometry. Stomatal size determination was a suitable screening tool for identification of hexaploid events. Genotypes MN18 and MN51 had the highest number of plants with altered ploidy of 29 and 27, respectively. Oryzalin at 5 µM was the most effective antimitotic treatment and produced 55 of the 74 plants with altered ploidy. The most vigorous lines were grown to maturity and produced viable pollen.

**Keywords** Elephantgrass · Chromosome doubling · Tissue culture · Oryzalin · Trifluralin · Stomata size · Flow cytometry · Biofuel feedstock · Forage

## Introduction

Elephantgrass (*Pennisetum purpureum* Schum.) is one of the most productive biomass plants in tropical and subtropical climates and it is an important forage and bioenergy crop (Martha et al. 2004; Strezov et al. 2008). Its relative, pearl millet (*Pennisetum glaucum* (L.), grows well on soils with low fertility, is drought tolerant and produces a high biomass quality (Serraj et al. 2005). Sexual compatibility between elephantgrass (2n = 4x = 28) and pearl millet (2n = 2x = 14) (Burton 1944) permits development of interspecific, triploid hybrids and introgression of favorable alleles by traditional plant breeding (Sidu and Gupta 1973; Hanna and Monson 1980; Hanna et al. 1984).

In contrast to elephantgrass, which produces large amounts of wind dispersed seeds, interspecific hybrids between elephantgrass and pearl millet are male and female sterile due to their triploid (2n = 3x = 21) genome causing irregular chromosomal segregation (Techio et al. 2006). Male and female sterility of interspecific hybrids will facilitate their biological containment. However,

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interspecific hybrids cannot be used as parents for hybridization due to sterility. Chromosome doubling of the triploid hybrids can restore fertility (Hanna et al. 1984; Campos et al. 2009) to enable subsequent backcrosses with elephantgrass and may enhance biomass production and persistence in the resulting pentaploid hybrids.

Common antimitotic agents used for chromosome doubling are colchicine, trifluralin and oryzalin (reviewed by Dhooghe et al. 2011). Colchicine has high affinity to animal tubulin dimers, which makes it highly toxic to humans. Plant tubulin binding antimitotic agents such as oryzalin and trifluralin can be employed to reduce mammalian toxicity (Petersen et al. 2003). Antimitotic agents have been used in various plants for doubling the chromosome number of sterile hybrids which restored their fertility (Jan et al. 1988; Lu and Bridgen 1997; Adaniya and Shirai 2001; Kermani et al. 2003) including *Pennisetum* hybrid species (Campos et al. 2009). However, the latter study used in vitro grown seedlings as targets for application of an antimitotic agent resulting in a high frequency of chimerism. This method also excluded an assessment of agronomic performance of individual genotypes prior to antimitotic treatment. Petersen et al. (2003) also observed high frequency of chimerism in *Miscanthus* following the application of antimitotic agents on rooted in vitro seedlings. In contrast regeneration of plants via somatic embryogenesis supports production of non-chimeric plants (Zimmerman 1993) and callus can be induced from selected genotypes after evaluation of their agronomic performance. Somatic embryogenesis has been studied in many species of grasses (Conger et al. 1987; Vasil 1987) including elephantgrass (Haydu and Vasil 1981; Wang and Vasil 1982) and an interspecific hybrid of elephantgrass and pearl millet (Vasil and Vasil 1981). Genotype is one of the key factors affecting tissue culture response in grasses (Vasil 1987).

In this study, we selected seven productive interspecific hybrids of elephantgrass and pearl millet and evaluated them for callus induction, somatic embryogenesis, and plant regeneration responses. Embryogenic cultures were then treated with antimitotic agents and regenerated plants were evaluated for chromosome doubling by stomatal size measurements, flow cytometry and restoration of fertility.

Determination of ploidy level in plants may be performed directly by counting chromosome numbers or by flow cytometry (Doležel and Bartos 2005; Sandhu et al. 2009; Ochatt et al. 2011). Determination of stomata number and size provide a reliable screening tool for identifying altered ploidy. Vandenhout et al. (1995) and Quesenberry et al. (2010) successfully used stomatal density as the indirect measure of altered ploidy in banana and bahiagrass, respectively.

## Materials and methods

### Genotypes

Seven interspecific hybrids (MN12, MN18, MN22, MN51, MN54, MN55 and MN70) were selected on the basis of their superior biomass production and persistence as reported by Kannan et al. (2010). All these genotypes were triploid ($2n = 3x = 21$), male and female sterile interspecific hybrids which were derived from crosses between diploid pearl millet ($2n = 2x = 14$) and tetraploid elephantgrass ($2n = 4x = 28$). Male sterility of pearl millet genotype DMP3A4 facilitated interspecific hybridization with fully fertile elephantgrass genotype 'Merkeron'.

### Tissue culture

Leaf whorls containing immature inflorescences were collected from seven interspecific hybrids at the University of Florida, Plant Science Research and Education Center (UF PSREU), Citra, FL. After surface sterilization of leaf whorls with 70 % ethanol, immature inflorescences of 1–3 cm in length were excised from the leaf whorl and cut into 1–2 mm cross-sections. These cross-sections were placed on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 2 mg $l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.3 % Phytagel (Phytotechnology Laboratories, Shawnee Mission, KS), 3 % sucrose, 7.8 µM CuSO4, pH 5.8, and cultured in the dark at 27 ± 2 °C. Each replication was represented by 18 explants in two petri dishes for a total of 5 replications that were tested in a Randomized Complete Block Design (RCBD). Thirty days after culture initiation, the percentage of explants with callus (CI30), the amount of embryogenic callus (EC30), and the level of necrosis (NI30) were evaluated.

After 30 days, the cultures were either subcultured to the same media or to media supplemented with 4 mg $l^{-1}$ 2,4-D and 0.3 mg $l^{-1}$ 6-Benzylaminopurine (BAP). Sixty days after culture initiation, these cultures were evaluated for the effect of media supplementation with 4 mg $l^{-1}$ 2,4-D and 0.3 mg $l^{-1}$ BAP on the formation of embryogenic callus. After 60 days, a sub-set of embryogenic calli (Fig. 1a) were transferred to regeneration media (MS with 20 g $l^{-1}$ sucrose, 0.1 mg $l^{-1}$ 2,4-D, 0.5 mg $l^{-1}$ BAP, 0.3 % Phytagel, 7.8 µM $CuSO_4$, pH 5.8) and cultured under 100 µmol $m^{-2}$ $s^{-1}$ light intensity with 16 h (light):8 h (dark) photoperiod at 28 ± 2 °C (Fig. 1b, c) for 20 days prior to evaluation of number of shoots regenerated per callus.

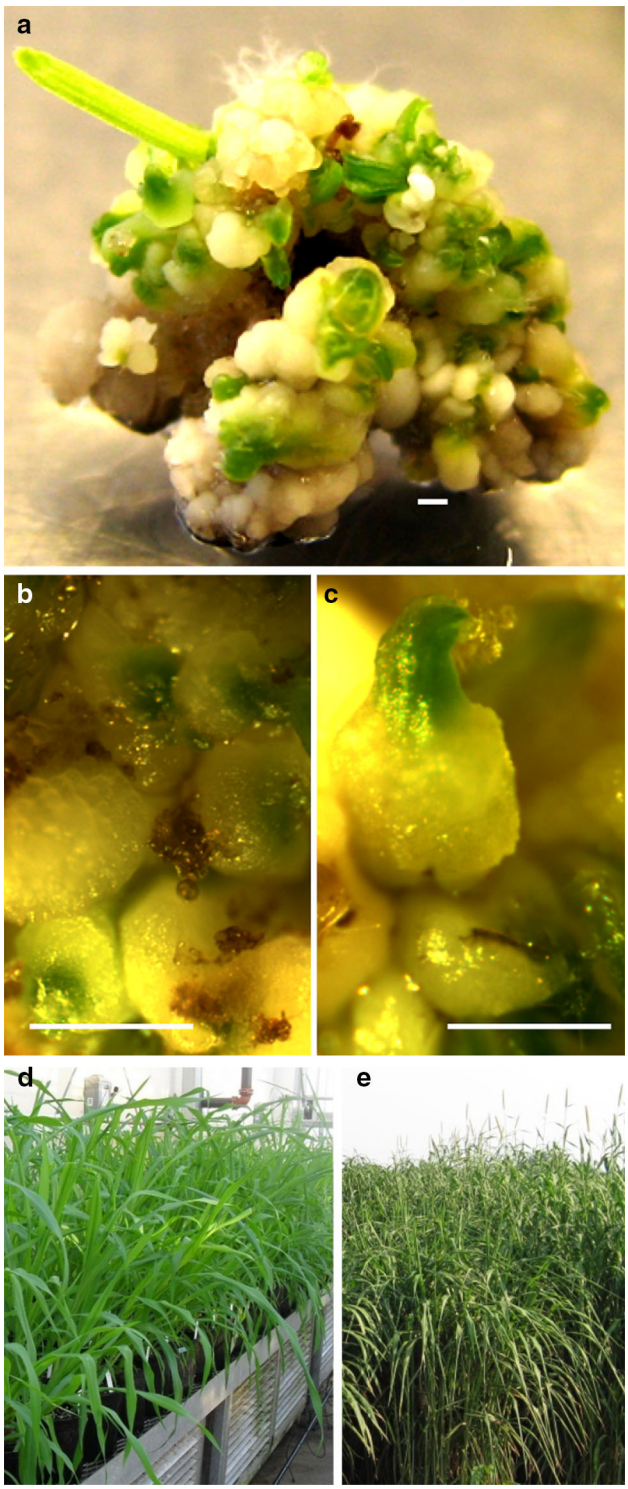


**Fig. 1** Regeneration of interspecific hybrids of elephantgrass and pearl millet through somatic embryogenesis. **a** Embryogenic callus. **b** and **c** Germinating embryos. **d** Regenerated plants after transfer to soil. **e** Flowering hexaploid plants. *Bar* (in all figures) 500 μm

### Treatments with the antimitotic agents oryzalin or trifluralin

Five (MN12, MN18, MN51, MN54 and MN55) of the seven interspecific hybrids were selected for treatment with antimitotic agents based on their superior tissue culture response, including formation of embryogenic callus and low level of necrosis. Embryogenic calli of the five selected hybrids were utilized as explant sources for treatments with antimitotic agents. Antimitotic agents were applied 60 days after culture initiation.

A 0.012 mol $l^{-1}$ stock solutions of oryzalin (3,5-dinitro-*N*4,*N*4-dipropylsulfanilamide) or trifluralin (a,a,a-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) (Fisher Scientific, Atlanta, GA) was prepared by dissolving 0.041 mg of oryzalin or 0.040 mg of trifluralin in 1 ml of dimethyl sulfoxide (DMSO) (Fisher Scientific, Atlanta, GA), filter sterilized and final volume made up to 10 ml using sterile water. 416 or 833 μl of stock solution of antimitotic agent were added to 1 l callus induction medium after autoclaving to obtain the final concentrations of 5 or 10 μM. The concentration range and incubation time for the antimitotic agents were similar to an earlier described protocol (Quesenberry et al. 2010) for bahiagrass.

Calli from the interspecific hybrids were divided into 5 mm pieces, dipped in petri plates with callus induction medium with antimitotic agent or callus induction medium without antimitotic agent. Ten calli distributed in two plates were used for each treatment (5 genotypes × 2 antimitotic agents × 2 concentrations). The calli were treated for 48 h after which calli were rinsed with callus induction medium without antimitotic agent dried on blotting paper and re-plated on callus induction media with Phytagel for 1 week. The calli were then transferred to regeneration media and cultured under 100 μmol $m^{-2}$ $s^{-1}$ light intensity with 16 h (light):8 h (dark) photoperiod at 28 ± 2 °C and subcultured to fresh media every 14 days. Regenerated shoots were transferred to regeneration media without 2,4-D and BAP for root formation. Regeneration date of plants was recorded and grouped into 30 days and earlier, 31–60 days and 61–90 days after treatment with antimitotic agents. Regenerated plants were transferred to soil and covered with a Magenta box for five days in a plant growth room with 16 h photoperiod and 500 μmol $m^{-2}$ $s^{-1}$ light intensity at 28 ± 2 °C day and 25 ± 2 °C night temperature. Subsequently plants were transferred to an air conditioned greenhouse and grown under natural photoperiod and 28 ± 3 °C day and 25 ± 3 °C night temperature (Fig. 1d).

### Measurement of stomata size

Stomatal longitudinal and transverse dimensions and stomatal area measurements were recorded for abaxial and adaxial stomata of all regenerated and acclimated plants 30 days after being transferred to the soil. The stomata size measurements were compared with flow cytometry data to determine whether such measurements can serve as a screening tool. Leaf surfaces were coated with clear nail polish, which was allowed to dry, peeled off, and dry mounted on a microscope slide. Twelve stomata from each leaf were measured using the Infinity Analyze Software® at 100× magnification.

### Ploidy determination via flow cytometry

The ploidy level of acclimated, regenerated plants was confirmed using a Partec PAII Ploidy Analyzer® and Partec CyStain UV Precise P Kit®. The protocol described by Sandhu et al. (2009) was followed for flow cytometry analysis. The top of each plant was cut and the old leaves were removed. The meristematic region located above the upper node was carefully removed and a 1–2 cm piece of the soft meristem was excised and placed in a 55 mm plastic petri dish with 400 µl extraction buffer (Partec CyStain UV Precise P). The tissue was minced for 1 min with a sharp razor blade to release intact nuclei. The solution with intact nuclei was filtered through a Partec 50 µm disposable filter into a test tube. Afterwards, 1.6 ml of the Partec Staining Buffer, which contains DAPI fluorophore, was added into the test tube and the solution was incubated for 3 min prior to analysis in the flow cytometer using the blue fluorescent channel. The gain of the Partec PAII ploidy analyzer was adjusted daily to produce peaks for all samples in the same spectrum. UV light source were used for fluorescence excitation of the samples and blue laser detected the fluorescence emission. The flow cytometry profiles were displayed with log scale to accommodate large ploidy variations. At least 5000 nuclei were analyzed in each sample. The analyzer was arbitrarily calibrated using the known triploid (interspecific hybrid) and tetraploid ('Merkeron') elephantgrass to determine the ploidy level of all test samples.

### Pollen staining

Pollen staining is an indirect measure of pollen viability. The pollen staining procedure described by Sandhu et al. (2009) was followed. Pollen grains were collected from chromosome doubled plants after growing them to maturity (Fig. 1e) and dusted over a clean glass slide. A drop of aceto-carmine was added over the pollen grains and a cover slip was gently placed. The excess stain was removed using blotting paper. The slide was observed under microscope (WILD Haerbrugg, Switzerland) fitted with camera (Infinity *1–3c*) (Lumenera Corp., Ottawa, Canada) with 400× magnification. Pollen grains of triploid (sterile) and tetraploid (fertile) plants were used as a control.

### Variables and statistics

The variables measured were CI30, EC30 and NI30. CI30 were expressed as percentage, whereas EC30 and NI30 were evaluated using a visual rating scale of 1–9. 1 represents non-embryogenic or non-necrotic calli and 9 represents highly embryogenic or completely-necrotic calli for EC30 and NI30, respectively. Analysis of variance (ANOVA) was performed according to the randomization structure (RCBD design), and means were separated by Tukey's test at the 5 or 1 % level of significance. The relationship between tissue culture responses (variables) and explant characteristics were determined by Pearson correlation, using the GENES program (Cruz 2006).

## Results

### Genotype response to tissue culture

Analysis of variance showed significant genotype effects for percentage of explants with callus 30 days after culture initiation (CI30), amount of embryogenic callus 30 days after culture initiation (EC30), and necrosis intensity 30 days after culture initiation (NI30) at $p < 0.05$ (Table 1). The genotypes that induced the most calli, 30 days after culture initiation were MN22, MN55 and MN54 (Table 2). There were no significant differences ($p < 0.01$) among genotypes for CI30. More than 50 % of the explants had induced callus across genotypes. Genotypes with a large amount embryogenic callus 30 days after

**Table 1** Analysis of variance of callus induction, embryogenic callus and necrosis intensity of interspecific hybrids on tissue culture media

| Source of variation | *df* | Mean squares | | |
|---|---|---|---|---|
| | | CI30 (%) | EC30[a] | NI30[a] |
| Genotype | 6 | 1063.3* | 8.23** | 7.98** |
| Error | 28 | 399.1 | 1.44 | 0.65 |

CI30 (percentage of explants with callus 30 days after culture initiation); EC30 (amount of embryogenic callus 30 days after culture initiation); NI30 (necrosis intensity 30 days after culture initiation)

[a] Scored as 1–9, 1 represents non-embryogenic, no necrosis and 9 represents highly embryogenic, completely necrotic for EC30 and NI30, respectively

*, ** Significant *F*- values at 5 and 1 % level of probability, respectively

**Table 2** Effects of genotype on callus induction, amount of embryogenic callus and necrosis intensity 30 days after culture initiation from of immature inflorescence cross-sections

| Genotype | CI30 (%) | EC30[a] | NI30[a] |
|---|---|---|---|
| MN12 | 55.0a | 5.7a | 4.6ab |
| MN18 | 67.0a | 4.0ab | 3.4bc |
| MN22 | 90.0a | 2.4b | 4.2bc |
| MN51 | 81.0a | 4.6ab | 4.8ab |
| MN54 | 82.0a | 2.6b | 2.4c |
| MN55 | 89.0a | 3.3ab | 5.1ab |
| MN70 | 57.0a | 5.3ab | 6.4a |
| Mean | 74.4 | 4.0 | 4.4 |
| SD | 14.7 | 1.3 | 1.3 |

CI30 (percentage of explants with callus 30 days after culture initiation); EC30 (amount of embryogenic callus 30 days after culture initiation); NI30 (necrosis intensity 30 days after culture initiation). Means followed by the same letter in the column are not significantly different according to the Tukey test at the 1 % level of significance; *SD* standard deviation; n = 90

[a] Scored as 1–9, 1 represents non-embryogenic or no necrosis and 9 represents highly embryogenic or completely necrotic for EC30 and NI30, respectively

culture initiation (EC30) were MN12 and MN70 and MN51. The genotype with the highest level of necrosis was MN70, while MN54 had the lowest level of necrosis (Table 2).

Genotypes differed in their response to propagation on media with 2 mg $l^{-1}$ 2,4-D or media with 4 mg $l^{-1}$ 2,4-D and 0.3 mg $l^{-1}$ BAP (Table 3). The embryogenic callus percentage was 100 % for MN12, MN22 and MN51 on media with 2 mg $l^{-1}$ 2,4-D. However, subculture on media with 4 mg $l^{-1}$ 2,4-D and 0.3 mg $l^{-1}$ BAP increased the percentage of embryogenic callus for genotypes MN54 and MN55. A sub-set of calli from all seven interspecific hybrids was evaluated for the regeneration of shoots per callus 20 days after transfer to regeneration media. MN22 and MN51 showed the highest regeneration response with 17 and 15 shoots per callus, respectively after subculture on media with 2 mg $l^{-1}$ 2,4-D. In contrast MN55 did not produce any shoots following callus subculture on media with 2 mg $l^{-1}$ 2,4-D. However, this genotype produced 4 shoots per callus following subculture on media with 4 mg $l^{-1}$ 2,4-D and 0.3 mg $l^{-1}$ BAP. The addition of BAP in the subculture medium decreased the number of shoots per callus for MN18, MN22, MN12 and MN51 (Table 3).

Two genotypes with a low percentage of callus induction (MN12 and MN70) produced a higher amount of embryogenic callus 30 days after culture initiation (EC30). Genotypes MN22, MN55 and MN54 displayed higher callus induction and reduced amount of embryogenic callus 30 days after culture initiation. This observation was confirmed by significantly negative correlation −0.86) between CI30 and EC30 (Table 4). In contrast to these genotypes, MN51 displayed both a high level of callus induction (81.0 %) and a moderately high amount of embryogenic callus (4.6) (Table 2). There was a significantly positive correlation found between percentage of embryogenic callus and the number of shoots following subculture on media with 2,4-D (2 mg $l^{-1}$). However, no significant correlation was found between these two traits on media with 2,4-D (4 mg $l^{-1}$) and BAP (0.3 mg $l^{-1}$) (Table 4).

**Table 3** Percentage of embryogenic calli and average number of shoots per callus from immature inflorescence cross-sections of seven interspecific hybrids

| Genotype | Embryogenic callus (%)[a] | | Number of shoots per callus[b] | |
|---|---|---|---|---|
| | 2,4-D (2 mg $l^{-1}$) | 2,4-D (4 mg $l^{-1}$) + BAP (0.3 mg $l^{-1}$) | 2,4-D (2 mg $l^{-1}$) | 2,4-D (4 mg $l^{-1}$) + BAP (0.3 mg $l^{-1}$) |
| MN12 | 100 | 60 | 6 | 4 |
| MN18 | 60 | 40 | 9 | 3 |
| MN22 | 100 | 60 | 17 | 5 |
| MN51 | 100 | 80 | 15 | 7 |
| MN54 | 20 | 40 | 6 | 7 |
| MN55 | 0 | 60 | 0 | 4 |
| MN70 | 33 | 40 | 3 | 5 |
| Mean | 59.0 | 54.3 | 8.0 | 5.0 |
| SD | 42.3 | 15.1 | 6.2 | 1.5 |

*SD* standard deviation

[a] 60 days after culture initiation; (n = 90)

[b] 20 days of regeneration (100 μmol $m^{-2}$ $s^{-1}$ light intensity with 16 h light photoperiod); (n = 12)

**Table 4** Pearson correlation among variables in tissue culture for seven interspecific hybrids

| Variable | Significant correlations |
|---|---|
| CI30 (%) | −0.86** EC30 |
| EC30[a] | −0.86** CI30 (%) |
| NI30[a] | ns |
| EC2,4-D (%) | 0.80** S/EC2,4-D (%) |
| EC2,4-DBAP (%) | ns |
| S/EC2,4-D | 0.80** EC2,4-D (%) |
| S/EC2,4-DBAP | ns |

CI30 (%) (percentage of explants with callus 30 days after culture initiation); EC30 (amount of embryogenic callus 30 days after culture initiation); NI30 (necrosis intensity 30 days after culture initiation); EC2,4-D (%) [percentage of embryogenic callus after transferred to 2,4-D (2 mg $l^{-1}$)]; EC2,4-DBAP (%) [percentage of embryogenic callus after transferred to 2,4-D (4 mg $l^{-1}$) + BAP (0.3 mg $l^{-1}$)]; S/EC2,4-D [number of shoots regenerated per callus after transferred to 2,4-D (2 mg $l^{-1}$)] and S/EC2,4-DBAP [number of shoots regenerated per callus after transferred to 2,4-D (4 mg $l^{-1}$) + BAP (0.3 mg $l^{-1}$)]

*ns* non-significant

** Significant at 1 % level of probability by 't' test

[a] Scored as 1–9, 1 represents non-embryogenic, no necrosis and 9 represents highly embryogenic, completely necrotic for EC30 and NI30, respectively

## In vitro chromosome doubling

Following treatments of embryogenic callus with oryzalin or trifluralin, a total of 328 regenerated plants were obtained, 74 of which were verified as having altered polidy and normal plant development. The 328 regenerated plants were obtained from three successive rounds of regeneration, 30 days and earlier, 31–60 and 61–90 days after treatment with antimitotic chemical agents. The number of plants regenerated after the first, second and third round were 95, 137 and 96 plants, respectively. None of the tissue culture derived plants that were not treated with antimitotic agents displayed altered ploidy.

There was a wide variation in stomata measurements among the regenerated plants of interspecific hybrids obtained from embryogenic calli treated with antimitotic agents (data not shown). However, the coefficients of variation were low for all of the measurements and large correlation coefficients between the measurements using 12 stomata from each of the 328 regenerated plants (data not shown). Therefore, only the stomatal longitudinal diameter evaluated in abaxial leaf surface was analyzed as a screening approach for altered ploidy.

The mean of stomatal longitudinal diameter of non-treated triploid plants was ca. 100 µm. Plants with stomatal longitudinal diameter up to 109.9 µm did not display altered ploidy (Fig. 2). 30 % of plants with stomata longitudinal diameters 110–119.9 µm displayed altered ploidy. The percentage of plants with altered ploidy was 81 % in the class of stomata longitudinal diameter 120–129.9 µm (Fig. 2). Considering all plants with stomatal longitudinal diameters higher than 120 µm, the percentage of plants with altered ploidy was 70 %.

When we grouped the stomata measurements into plants derived from the 3 cycles of regeneration, the first cycle had only 9 plants (9.5 %) with increased stomatal longitudinal diameters greater than 120 µm, and only 4 (4.2 %) had altered ploidy levels. In the second and third rounds of regeneration, the numbers of plants with altered ploidy were 44 (32.1 %) and 26 (27.1 %), respectively (Fig. 3a).

Regenerated plants with altered ploidy were obtained for all five superior interspecific hybrids (Fig. 3b). Hybrids MN51 and MN18 regenerated 143 and 83 plants, respectively, which were the highest numbers of regenerated plants among all hybrids. These two genotypes also produced the highest numbers of plants with altered ploidy, 29 and 27 plants for MN18 and MN51, respectively.

An analysis of the efficiency of antimitotic treatments demonstrated that among 268 regenerated plants obtained after treatment with 5 µM oryzalin, 85 (31.7 %) had stomatal longitudinal diameters greater than 120 µm, and 55 (20.5 %) displayed altered ploidy (Fig. 3c). After treatment with 10 µM oryzalin, 42 regenerated plants were obtained, 26 (61.9 %) of which had stomatal longitudinal diameters greater than 120 µm, and 13 (30.9 %) of which displayed altered ploidy. After treatment with 5 µM trifluralin 9 regenerated plants were obtained and none of them displayed altered ploidy. While after treatment with 10 µM trifluralin, 9 regenerated plants were obtained, of which 6 displayed altered ploidy (Fig. 3c).

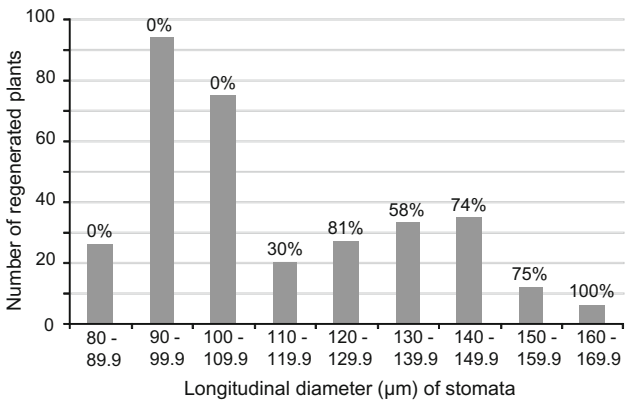


**Fig. 2** Frequency distribution of regenerated interspecific hybrids of elephantgrass × pearl millet obtained from embryogenic calli treated with antimitotic agents grouped into different classes based on stomatal longitudinal diameters. The percentage above the *bars* corresponds to the percentage of regenerated plants of the respective class with altered ploidy

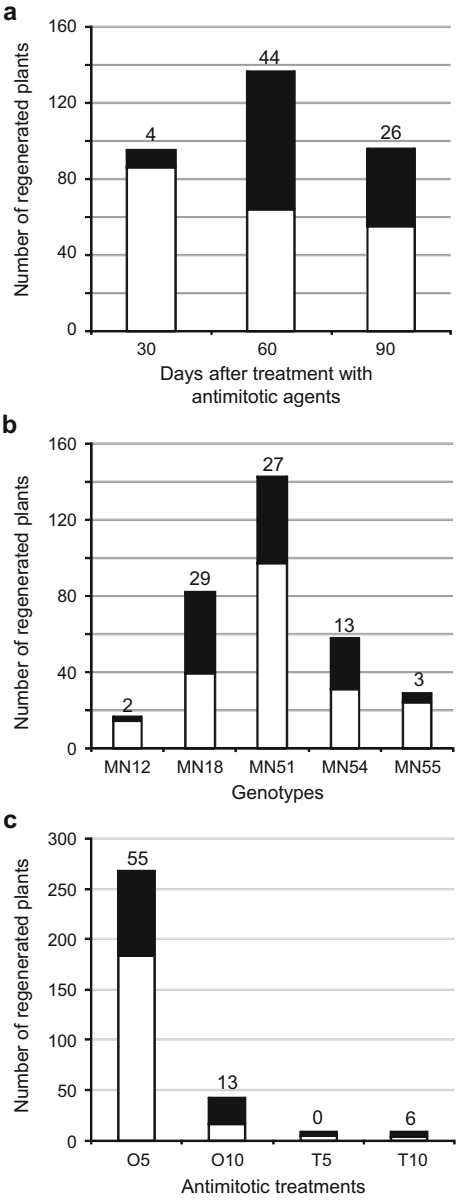


**Fig. 3** Number of regenerated plants with stomatal longitudinal diameter (SLD) higher than 120 µm (*filled square*) and lower than 120 µm (*open square*) obtained after, **a** three cycles of plant regeneration obtained 30, 31–60 and 61–90 days after treatment with antimitotic agents from embryogenic calli of, **b** five interspecific hybrids of elephant grass and pearl millet (MN12, MN18, MN51, MN54 and MN55) treated with, **c** the following antimitotic treatments 5 µM oryzalin (O5), 10 µM oryzalin (O10), 5 µM trifluralin (T5) or 10 µM trifluralin (T10). The number above the bars corresponds to the plants with normal development and altered ploidy

Treatment with 5 µM oryzalin effectively altered ploidy in 4 of the 5 triploid hybrids. However, this treatment was not effective to alter the ploidy of the MN55 hybrid (Fig. 4). This genotype had its ploidy altered with the 10 µM trifluralin treatment. The best antimitotic treatment for hybrid MN54 was 10 µM oryzalin. MN55 and MN12

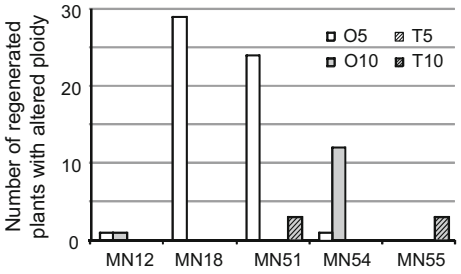


**Fig. 4** Number of regenerated plants with normal development and altered ploidy of five (MN12, MN18, MN51, MN54 and MN55) interspecific hybrids of elephantgrass and pearl millet obtained from embryogenic calli and following treatments with 5 µM Oryzalin (O5), 10 µM Oryzalin (O10), 5 µM Trifluralin (T5) or 10 µM Trifluralin (T10) antimitotic agents

hybrids regenerated only 3 and 2 plants with altered ploidy, respectively.

A total of 152 plants were selected for flow cytometry analysis which include all plants with stomatal longitudinal diameters greater than 120 µm (113 plants) and 39 plants with less than 120 µm. Of the 113 plants, which had stomatal longitudinal diameters greater than 120 µm plants, 14 plants displayed abnormal growth and development. These plants were excluded from the analysis. Figure 5a and b show stomatal impressions of triploid and hexaploid plant with normal development and Fig. 5c shows stomatal impression of plants with abnormal development. Flow cytometry analysis indicated that 74 plants have altered ploidy with normal growth and development. The relative nuclear DNA content of 61 plants was consistent with a hexaploid genome while 13 plants displayed greater nuclear DNA contents. Figure 6 shows typical triploid (3x) and hexaploid (6x) flow cytometry histograms with tetraploid (4x) control. Hexaploid plants were grown to maturity (Fig. 1e). Different tillers of the most vigorous hexaploid plant were also evaluated by flow cytometry and all tillers displayed consistent peak patterns (Fig. S1, Supplementary file 1).

Fertility restoration of chromosome doubled plants was confirmed via pollen staining (Fig. 7). The viable pollen is deeply stained and has a round shape with dark red color as in the case of pollens from a fertile tetraploid plant (Fig. 7c). In contrast, non-viable pollen of a sterile triploid plant remain unstained, shriveled and translucent (Fig. 7a). In this study, most of the pollen grains of chromosome doubled plants were deeply stained and round in shape with dark red color (Fig. 7b). However, very few unstained pollen grains were also observed.

## Discussion

Interspecific hybridization of elephantgrass (*Pennisetum purpureum*) and pearl millet (*Pennisetum glaucum*) allows selection of genotypes which combine high yield and high

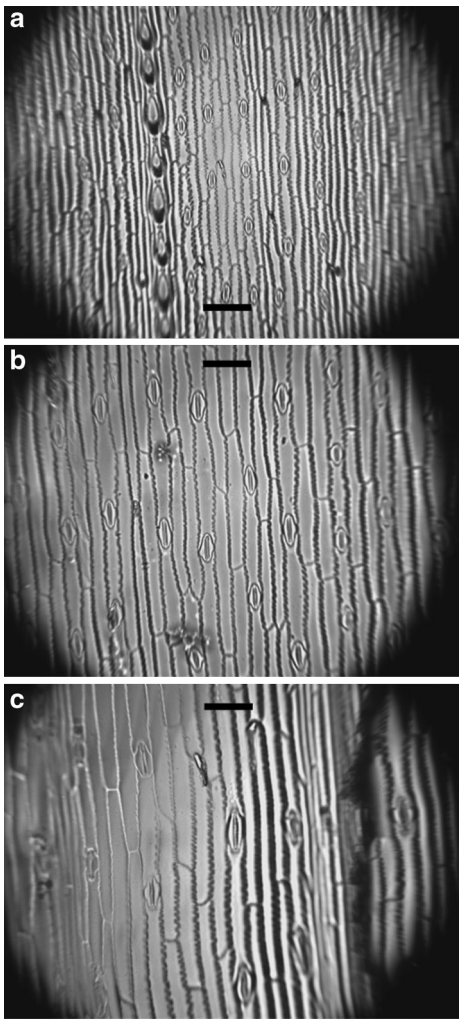


**Fig. 5** Stomatal impressions and flow cytometry histograms. **a** from MN18 plant number 148 (triploid hybrid), **b** from MN18 plant number 36 (hexaploid), **c** from MN18 plant number 36 (chromosome number doubled with abnormal development). *Bar* (in all figures) 200 µm

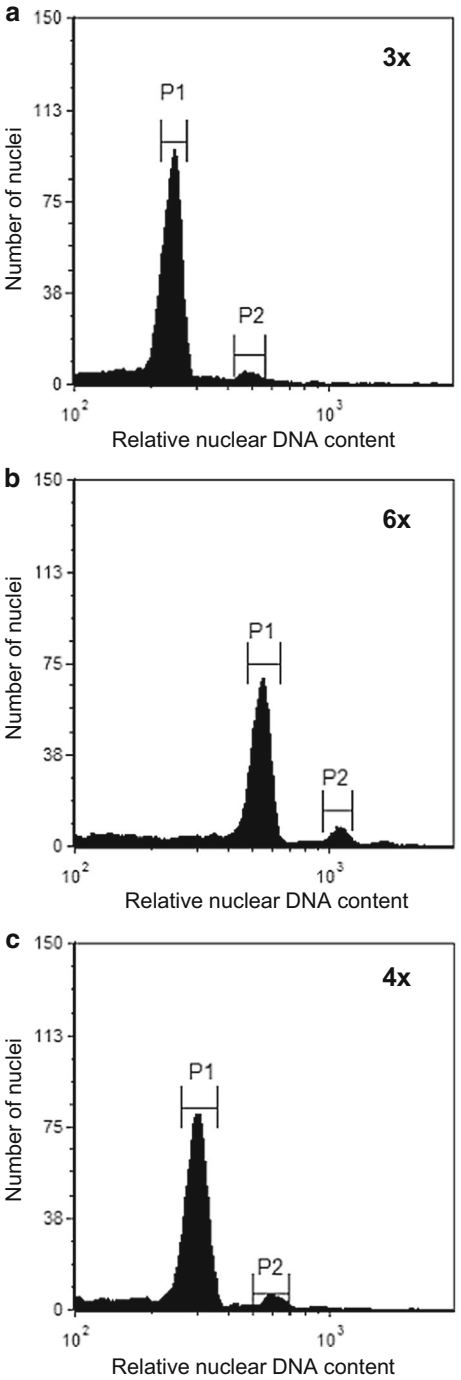


**Fig. 6** Flow cytometry histograms. **a** triploid interspecific hybrid (3x). **b** hexaploid (6x). **c** tetraploid (4x) elephantgrass control. P1 and P2: Peak 1 and Peak 2

biomass quality (Sidu and Gupta 1973; Hanna and Monson 1980; Hanna et al. 1984). However, the interspecific hybrids are triploid (2n = 3x = 21 chromosomes) and the resulting reproductive sterility complicates the use of this germplasm in breeding programs. In vitro chromosome doubling of triploid, interspecific hybrids has the potential to restore fertility and produce non-chimeric hexaploid plants. However, regeneration via somatic embryogenesis is often limited by genotypic differences in the tissue culture response (Vasil 1987; Bhaskaran and Smith 1990; Christou 1993; Lambe et al. 1999) making it difficult to apply such protocols to specific genotypes with good agronomic performance.

We recently identified 7 interspecific hybrids of elephantgrass and pearl millet with good agronomic performance (Kannan et al. 2010). Here we describe genotype specific modifications of the tissue culture protocol and antimitotic treatment supporting chromosome doubling in five of the seven selected genotypes.

In this study a significant effect of the genotype of interspecific hybrids was observed for callus induction, amount of embryogenic callus and necrosis intensity.

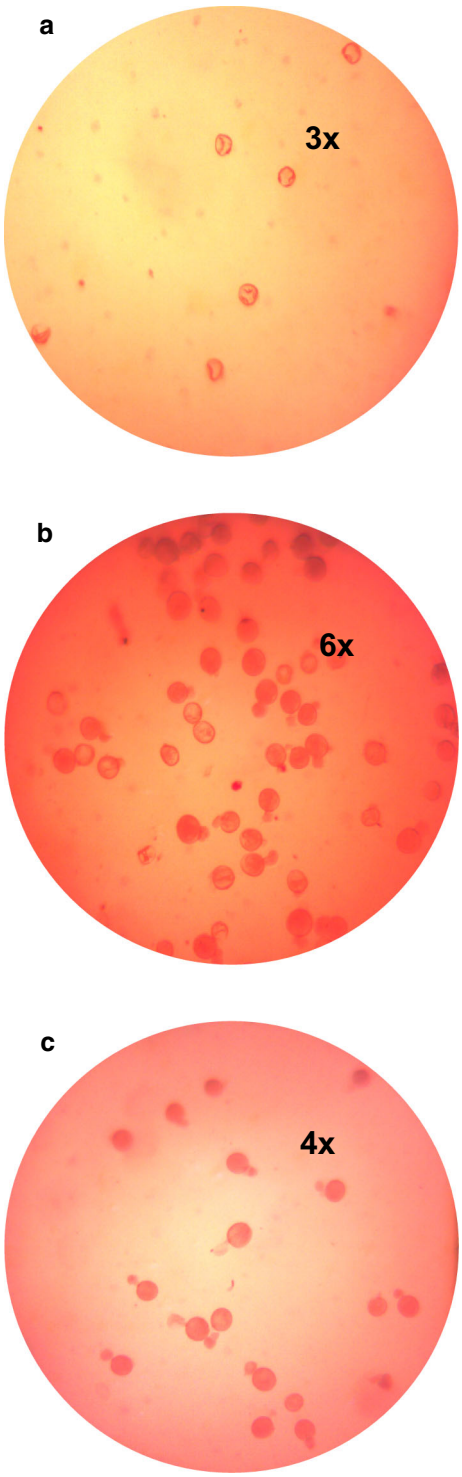


**Fig. 7** Evaluation of pollen viability, **a** before, **b** after chromosome doubling of interspecific hybrids, **c** as compared to tetraploid elephantgrass control. Magnification ×400

Genotype MN51 displayed the highest callus induction frequency and produced the most embryogenic callus of the seven evaluated genotypes. Propagation of calli in media supplemented with both 2,4-D and BAP gave rise to a higher percentage of embryogenic calli and supported plant regeneration from recalcitrant genotypes. This medium modification supported induction of embryogenic callus and plant regeneration from all seven interspecific hybrid genotypes in this study. Vasil and Vasil (1981) reported regeneration of a single interspecific hybrid of pearl millet and elephantgrass via somatic embryogenesis following callus induction on 2,4-D containing culture media lacking BAP. The tissue culture protocol described here provided the basis for development of an in vitro chromosome doubling protocol to produce hexaploid hybrids from a range of triploid genotypes. The vast majority of the plants regenerated via somatic embryogenesis are uniform and genetically stable (Rajasekaran et al. 1986; Shenoy and Vasil 1992; Vasil 1994).

Treatment of embryogenic tissue cultures with different concentrations of oryzalin or trifluralin supported regeneration of 61 independent hexaploid events from five different genotypes. Among these five genotypes, MN18 and MN51 displayed a higher frequency of regeneration and altered ploidy. Campos et al. (2009) reported successful chromosome doubling after exposing shoot and root portions of interspecific hybrid seedlings of *P. purpureum* and *P. glaucum* to colchicine. In contrast to our protocol this method may favor development of chimeric events, since it does not involve plant regeneration via somatic embryogenesis. Also the procedure described by Campos et al. (2009) does not support the chromosome doubling of specific genotypes.

Among the two evaluated antimitotic agents, oryzalin induced significantly more events with altered ploidy than trifluralin. However, treatments with 10 µM trifluralin resulted in the highest percentage of plants (67 %) with ploidy alterations among the regenerated plants (ploidy alteration rate). For chromosome doubling in bahiagrass, trifluralin and colchicine resulted in more events with altered ploidy than oryzalin. However, oryzalin supported the highest ploidy alteration rate, facilitating the identification of events with chromosome doubling (Quesenberry et al. 2010). For wheat, colchicine was more effective in chromosome doubling than oryzalin and trifluralin (Hassawi and Liang 1991). In contrast, oryzalin and trifluralin induced a higher ploidy alteration rate than colchicine in onion (Geoffriau et al. 1997) and in *Brassica napus* (Hansen and Andersen 1996).

In regard to concentration of the antimitotic agent, 5 µM oryzalin generated more events with altered ploidy than 10 µM oryzalin. Similarly, more ploidy altered bahiagrass and rose plants were obtained from treatments with 5 µM oryzalin than using higher concentrations (Kermani et al. 2003). Higher concentrations of oryzalin may cause toxicity as suggested by the lower number of plants regenerated with this treatment.

Assessment of stomatal density was reported as a reliable screening procedure to identify events with doubled chromosomes (Liu et al. 2007; Campos et al. 2009; Quesenberry et al. 2010; Aina et al. 2012). Flow cytometry is used widely for confirmation of altered ploidy (Ochatt et al. 2011). In agreement with these earlier reports, stomata of triploid and hexaploid interspecific hybrids were readily discernible. The vast majority of plants (70 %) displaying stomatal longitudinal diameters 20 % or more (>120 µm) than wildtype were confirmed by flow cytometry as having altered ploidy. Production of viable pollen was confirmed by pollen staining in mature hexaploid plants.

In conclusion, following chromosome doubling and somatic embryogenesis fertile, interspecific hybrids of elephantgrass and pearl millet were regenerated. The superior plants can be used in the elephantgrass breeding program.

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