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ORIGINAL PAPER

Somatic embryogenesis and organogenesis in apomictic and sexual *Brachiaria brizantha*

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Abstract *Brachiaria brizantha* (syn. *Urochloa brizantha*) is an important tropical forage grass widely cultivated in Brazil. In order to optimize tissue culture conditions for *B. brizantha*, in vitro culture of mature seeds, basal segments and leaf segments from in vitro plants of an apomictic and a sexual genotype of *B. brizantha* was performed. When cultured on different media, leaf segments yielded non-embryogenic calluses which formed several roots. Friable calluses from mature seeds and basal segments explants incubated on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid and 6-benzyladenine yielded 80% compact and nodular embryogenic structures. Calluses with such compact embryogenic structures were highly regenerable upon transfer to medium supplemented with kinetin and naphthalene acetic acid. They produced isolated somatic embryos, multiple fused scutelli or isolated scutellum with polyembryos that germinated into isolated or multiple shoots. Green and morphologically normal plants were obtained for the two genotypes. Changing the media from pH 5.8 to pH 4.0 increased the number of explants that formed calluses as well as the number of shoots per explant. When embryogenic calluses from mature seeds were successively sub-cultured for 4 months, aiming at repetitive somatic embryogenesis, all the regenerated plants were albinos. The embryogenic nature of the compact structure was confirmed by scanning electron microscopy.

Keywords Albino plants · Apomixis · Organogenesis · Repetitive somatic embryogenesis · Signalgrass

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Introduction

Brachiaria brizantha (syn. *Urochloa brizantha*; Shirasuna 2010) is a highly productive forage grass that adapts well to different environmental conditions and soils and is widely cultivated in Brazil and other tropical countries. Reproductive characteristics in this genus, however, can impair breeding programs. Apomictic *B. brizantha* cv. Marandu is a fertile tetraploid ($2n = 4x = 36$) (Valle and Savidan 1996; Araujo et al. 2000), pseudogamic plant (Ngendahayo 1988; Alves et al. 2001). Among *B. brizantha* genotypes, a sexual diploid ($2n = 2x = 18$) was found to produce very few viable seeds (Araujo et al. 2007). Due to the general asexual mode of reproduction, *B. brizantha* genetic improvement is restricted to selection of superior cultivars from natural variability (Euclides et al. 2008) or use of the apomictic plant as pollen donor in interspecific hybridization (Valle et al. 2009). The potential use of genetic transformation as a tool for introducing desirable traits in this culture requires an efficient genetic transformation protocol, which in turn relies on a tissue culture protocol, not yet fully investigated.

Callus induction and plant regeneration were first reported by Tohme et al. (1996) using as initial explant seeds of *B. brizantha*, *B. decumbens*, *B. ruziziensis* and

B. dictyoneura. Later, using the same methodology but in isolated embryos from seeds, Lenis-Manzano (1998) reported that 76% of the embryos of *B. brizantha* formed embryogenic callus. It was pointed out that, in some cases, more than one plant could be obtained from one callus. Recently, multiple shoot formation and somatic embryogenesis from seed-derived shoot apical meristem was demonstrated for ruzigrass (*B. ruziziensis*) (Ishigaki et al. 2009). The developed system is based on mature seeds and presented low shoot and embryo efficiency, using 4 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), with the highest frequencies of multiple shoot and somatic embryogenesis being 21 and 17%, respectively.

In *B. brizantha* cv. Marandu, Silveira et al. (2003) reported the response of the isolated seed embryo in tissue culture, comparing the medium combination used by Lenis-Manzano (1998) with other media compositions. Embryogenic calluses were obtained in 73% of the explants and plant regeneration in 67% of the initial explants cultured. Although the frequency was high, the rapid differentiation of friable callus into embryogenic callus was accompanied by the oxidation of the scutelli, which resulted in arrested development (data not published). More recently, the histology of isolated seed embryo cultured using the system previously described by Tohme et al. (1996) was shown (Lenis-Manzano et al. 2010). Multiple shoot formation was reported in cultures of basal segments, which were harvested from a meristematic region just above the roots, of in vitro *B. brizantha* genotypes cultivated in LS medium (Linsmaier and Skoog 1965) supplemented with growth regulators (Pinheiro et al. 2000). This system was efficient for *Brachiaria* spp. in vitro chromosome duplication. However, *B. brizantha* is still considered recalcitrant to in vitro culture and efficient tissue culture methods associated with genetic transformation systems are still lacking in the current literature. The establishment of in vitro tissue culture methods and the understanding of the morphogenetic characteristics of the developing explants will allow the selection of the best pathway of regeneration to be used for *Brachiaria* genetic transformation.

Here we report the potential of using mature seeds for organogenesis as well as for embryogenesis and a regeneration method of the apomictic *B. brizantha*. Somatic embryogenesis is also demonstrated from basal segments of apomictic and sexual plants. The effects of different media and acidic pH on somatic embryogenesis and plant regeneration are discussed. Moreover, for the first time, a detailed morpho-anatomical characterization of the somatic embryogenesis process using both light and scanning electron microscopy is presented for *B. brizantha*. These systems can be amenable to genetic transformation of *B. brizantha*.

Materials and methods

Plant material

Plants of a natural tetraploid apomictic genotype of *Brachiaria brizantha* cv. Marandu (Embrapa's collection number BRA 000591) and a diploid sexual *B. brizantha* (BRA002747) were cultivated in the field of Embrapa Genetic Resources and Biotechnology Centre. Seeds of cv. Marandu were obtained from Embrapa Beef Cattle, MS, Brazil.

Micropropagation

Stems from field-grown plants of both cultivars were collected and leaf blades and sheaths removed. The explants consisted of the node, longitudinally sectioned, with the auxiliary bud and most vascular tissue eliminated without detaching the bud from the stem node. The explants were rinsed in 70% ethanol, surface sterilized in a 5% sodium hypochlorite solution and 0.1% tween 20 in water, for 20 min, followed by three rinses in sterile distilled water. Excess water on thirty explants from each genotype was removed by placing on sterile filter paper before inoculating onto LS medium (Linsmaier and Skoog 1965) supplemented with 1 mg l^{-1} naphthalene acetic acid (NAA), 3 mg l^{-1} kinetin (KIN) (Pinheiro et al. 2000) and 0 or 3 mg l^{-1} 6-benzyladenine (BA), and culturing at 25 ± 1°C under a 14 h photoperiod. The number of explants that sprouted and the mean number of buds obtained per explant was recorded from 30 observations for each treatment in two independent experiments and the Tukey's test at $P > 0.05$ applied. Shoots produced were isolated and transferred to a plant maintenance medium, PMM (Table 1), where roots developed. In vitro plants were subcultured at 6-week intervals and maintained as source of explants for: basal segments, which consist of 0.5 cm segments just above the roots of in vitro plantlets (Pinheiro et al. 2000), and—young leaves, tested for somatic embryogenesis induction.

Organogenesis from mature seeds

Mature seeds of the apomictic genotype were peeled and disinfested in 70% ethanol for 5 min, followed by 40 min in a 5% sodium hypochlorite solution with 0.1% tween 20 and washing five times in sterile distilled water. They were placed in Petri dishes containing direct organogenesis induction medium, OIM (Table 1), supplemented with 3 mg l^{-1} KIN and 1 mg l^{-1} NAA or 3 mg l^{-1} BA and 0.3 mg l^{-1} NAA. Cultures were maintained in a growth chamber at 25 ± 1°C, in the dark, for 38 days.

Table 1 Culture media used for *B. brizantha* micropropagation, organogenesis, somatic embryogenesis induction and plant regeneration

Medium	Composition
LS	MS salts (Murashige and Skoog 1962), 100 mg l^{-1} thiamine, 100 mg l^{-1} myo-inositol, 100 mg l^{-1} casein hydrolysate, 30 g/l sucrose (Linsmaier and Skoog 1965)
PMM	MS salts (major salts ½ strength), MS vitamins, casein hydrolysate 100 mg l^{-1}, sucrose 20 g/l, NAA 0.2 mg l^{-1}, 0.5 mg l^{-1} kinetin, 0.2 mg l^{-1} GA_3
OIM	MS basal medium (Murashige and Skoog 1962), 100 mg l^{-1} casein hydrolysated, 100 mM glutamine
M1	MS basal medium (Murashige and Skoog 1962), casein hydrolysate 100 mg l^{-1}, 2 mg l^{-1} 2,4-D, 0.2 mg l^{-1} BA (Lenis-Manzano 1998)
M1.2	M1 without BA added with a final concentration of 4 mg l^{-1} 2,4-D (Silveira et al. 2003)
M1.3	M1 with a final concentration of 300 mg l^{-1} casein hydrolysate, 3 mg l^{-1} 2,4-D, 0.2 mg l^{-1} BA
MS1	MS basal medium (Murashige and Skoog 1962), 100 mg l^{-1} myo-inositol, 0.1 mg l^{-1} NAA, 0.4 mg l^{-1} kinetin (Lenis-Manzano 1998)
MS2	MS1 without NAA with a final concentration of 4 mg l^{-1} kinetin (Silveira et al. 2003)
MS3	MS basal medium (Murashige and Skoog 1962), 300 mg l^{-1} casein hydrolysate, 0.5 mg l^{-1} NAA, 1 mg l^{-1} BA, 2.5 mg l^{-1} kinetin
All media	pH was adjusted to 5.8 with 1N KOH prior to autoclaving. Agar was 7 g l^{-1}, except for pH 4, which was 14 g l^{-1}. Medium was autoclaved at 121°C, for 20 min, except for GA_3 that was filter sterilized

Somatic embryogenesis

Three types of explants were used to induce somatic embryogenesis as described below.

Mature seeds from apomictic plants: Three hundred mature seeds were peeled and disinfested as described above. The explants were kept in the dark at 25 ± 1°C in Petri dishes containing callus induction medium M1, M1.2 or M1.3 for 4 weeks, and transferred to regeneration medium [Murashige and Skoog (MS)] MS1, MS2 or MS3, respectively (Table 1). They were grown under a 14 h photoperiod at 25 ± 1°C for 4 weeks. The effect of an acidic pH was analyzed evaluating somatic embryogenesis response in M1.3/MS3 medium at pH 5.8 or pH 4.0.

Basal segments from apomictic and sexual plants: Fifty basal segments were obtained from in vitro cultivated plants and induced for somatic embryogenesis as described above for mature seeds. Results were observed after 60 days in culture.

Young leaves from apomictic and sexual plants: Young unexpanded leaves from in vitro sexual and apomictic plants were cultured using a modified method based on Ahmadabadi et al. (2007). Briefly, sections of the basal region of young leaves were placed in callus induction medium for 15, 20 or 30 days, and then transferred to embryogenesis induction medium. Sixty explants were used for each treatment and two independent experiments were carried out.

Repetitive somatic embryogenesis

Calluses obtained from mature seeds in M1.3 medium at pH 5.8 were subcultured for 5 months, at monthly intervals and maintained in the dark at 25 ± 1°C. Callus regeneration ability was verified after 2 and 4 months through transfer of aliquots to MS3 medium.

Plant rooting

For all the experiments, regenerated plantlets were transferred to rooting medium (Medium B; Bourgin et al. 1979) and cultured in a growth chamber under a 14 h photoperiod and light intensity of 35 μmol m^{-2} s^{-1}. Rooted plants were transplanted to plastic pots with vermiculite and kept in the greenhouse for acclimatization.

Light and scanning electron microscopy

Basal segments from in vitro plants and embryogenic calluses obtained from mature seeds were processed for Light Microscopy (LM) or Scanning Electron Microscopy (SEM). Samples were fixed in 4% paraformaldehyde under refrigeration and dehydrated in a graded ethanol series from 30 to 70% ethanol at 30 min intervals and maintained at 4°C until needed. For LM, basal segments were dehydrated in a crescent series of ethanol up to 100% ethanol, transferred to propanol for 8 h and to butanol overnight. Infiltration was done in Historesin (Leica, Heidelberg, Germany), at 4°C, using butanol:infiltration medium (2:1, 1:1, 1:2). Infiltration was completed in 100% infiltration medium for a total of 48 h. Polymerization was done at room temperature for 24–48 h. Serial sections (5 μm thick) were obtained in a rotary microtome, placed onto slides with a drop of water, and dried and stained with 0.05% (w/v) toluidine blue in water. Stained sections were prepared with Entellan (Merck, Darmstadt, Germany) and covered with coverslips. Samples were analyzed and images

obtained under a Zeiss Axioscop 40 HBO 50 A/C (Carl Zeiss, Jena, Germany). For SEM, basal segments and embryogenic calluses from mature seeds were dehydrated in an ethanol series, critical point dried through CO_2, sputter coated with gold and observed under a LEO 435 VP (Carl Zeiss, Jena, Germany), operating at 20 keV.

Results and discussion

In vitro *Brachiaria* plants provide a reliable source of basal segments for micropropagation of *Brachiaria*, as alternative explants to seeds (Pinheiro et al. 2000). Previous assays with detachment of buds from the nodes for in vitro introduction were not efficient, with lack of further development (data not shown). Cutting the nodes in half longitudinally prior to disinfestation exposed the vascular tissue overcoming the endogenous contamination observed when intact nodes from field plants were used (Fig. 1a) and allowing plants to be successfully introduced in vitro. In the induction medium, with or without BA, while some explants did not present new buds or shoots, others had a bunch-type multiple bud proliferation (Fig. 1b) with tillers originating from the crown area that grew upward from the

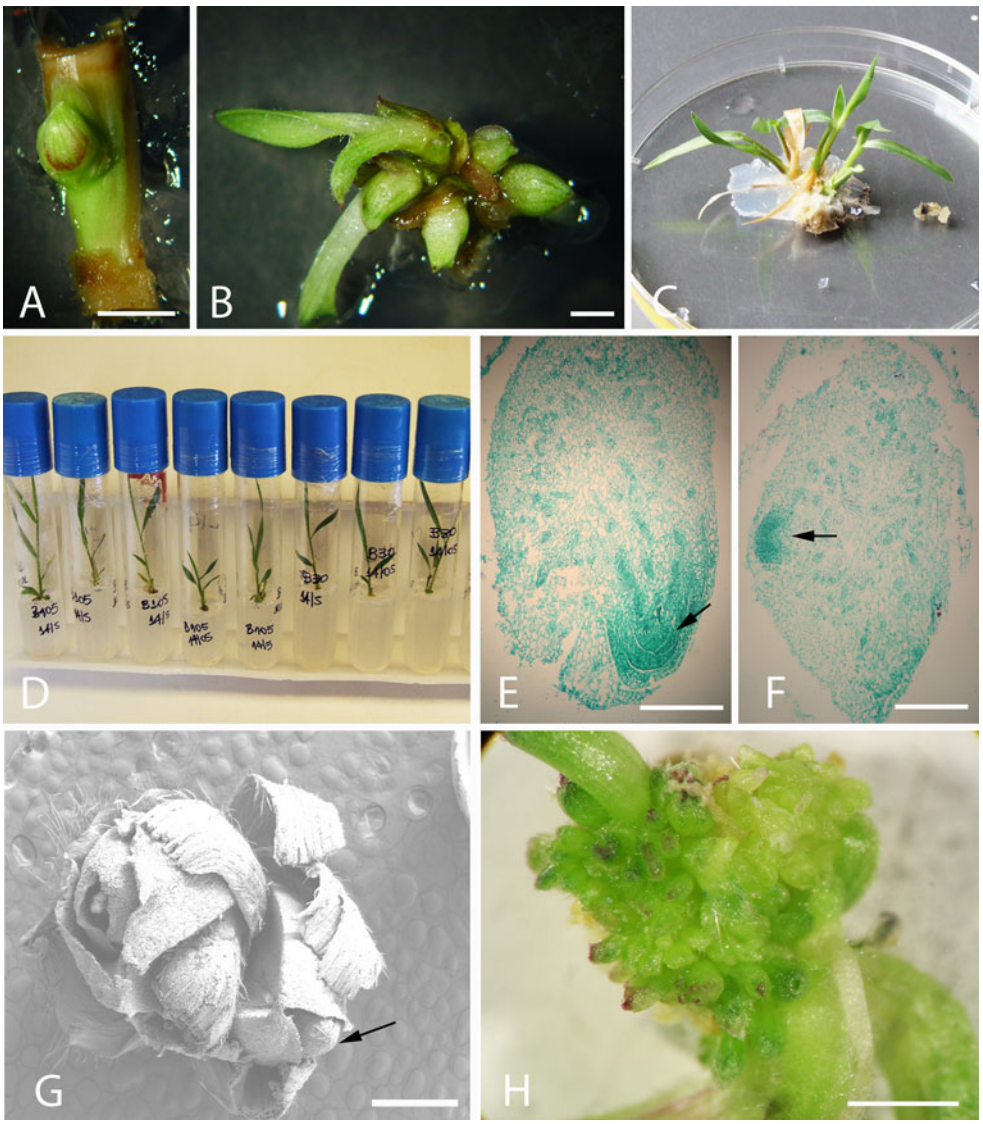

Fig. 1 *Brachiaria brizantha* cv. Marandu micropropagation and organogenesis: **a** in vitro introduced explant consisting of a longitudinally sectioned field grown plant node (half node), with lateral bud; **b**, **c** multiple buds developed from one explant, forming a bunch-type sprouting; **d** a established in vitro plant collection used as source of explants; **e**, **f** histological sections of a basal segment of in vitro grown plant with a shoot bud (**e**, *arrow*) and a shoot bud primordium (**f**, *arrow*) with meristematic cells showing densely stained cytoplasm; **g** scanning electron micrograph of a 10 days in vitro cultivated basal segment, showing the leaf sheath-like structure protecting the shoot meristem (*arrow*); **h** multiple buds that differentiated from a mature seed cultivated in medium containing BA and NAA. *Bars* **a**, **b**, **g**, **h** = 1 mm; **e**, **f** = 200 μm

base of the plant (Fig. 1c). Induced shoots were isolated and developed into plantlets in PMM medium (Fig. 1d). When using basal segments of in vitro cultivated plants, the addition of 3 mg l^{-1} BA to the shoot induction medium favored bud sprouting in the sexual as well as apomictic genotypes. Percent of explants responsive to induction with and without BA was, respectively, 34 and 29% for the apomictic and 27 and 32% for the sexual. In the sexual genotype an average of 6.3 buds per responsive explant was obtained in medium with BA, a number more than four times higher than the 1.5 observed in medium without BA. In the apomictic genotype, an average of 5.8 buds per responsive explant was observed in medium with BA, more than three times higher than the 1.7 found in medium without BA. Histological transverse sections of basal segments of in vitro grown plants in absence of hormones, showed the presence of multiple buds (Fig. 1e, f). In basal segments cultivated in vitro for 10 days in the presence of cytokinins, SEM analysis showed the leaf sheath-like structure protecting the shoot meristem (Fig. 1g), resembling the coleoptile of monocot seeds. The results indicate that multiple buds occur from preexistent bud primordia sprouting, rather than adventitious bud proliferation. Therefore, basal segments, although good micropropagation explants especially for low seed-producing plants, such as the sexual genotype of *B. brizantha,* and suited for chromosome duplication by colchicine (Pinheiro et al. 2000), are not convenient for genetic transformation experiments. The strategy used allowed the introduction, characterization and long term maintenance of *B. brizantha* plants in vitro.

Organogenesis induction from mature seeds

Two combinations of plant growth regulators, KIN/NAA (3:1) and BA/NAA (3:0.3), were tested in an attempt to induce organogenesis from mature seeds of apomictic *B. brizantha*. Fifty-seven percent of the explants in KIN/NAA combination presented one seedling, with absence of coleorrhiza development, while in 28% of the seeds two seedlings developed (Table 2). In *B. brizantha* cv. Marandu 8% of field seeds had two embryos (Alves et al. 2001). The combination of KIN/NAA favored the appearance of two shoots in 28% of the seeds, a percentage that is higher than the 8% observed in nature, indicating that kinetin could have stimulated shoot formation. Friable calluses were observed in 15% of the explants, in the seed embryo mesocotyl region. In the combination of BA/NAA, 76% of the seeds produced only one seedling with coleorrhiza growth; the presence of two seedlings occurred in 6% of the seeds; 9% formed small friable calluses, while 8% formed multiple buds in the mesocotyl region, i.e., the insertion point among the scutellum, the coleoptile and the coleorrhiza in a grass seed embryo (Raven et al. 2007). The multiple buds sprouted into multiple shoots (Fig. 1h) that were separated and rooted. The mesocotyl region in *Brachiaria* seems to be capable of forming multiple buds. Likewise, in wheat in the region around the zygotic embryo proper, a ring of cells is capable of giving rise to shoot meristems (Fischer et al. 1997). This region seems to be responsive to cytokinins in other plants such as *Lotus corniculatus* (Nikolić et al. 2006), *Pogonatherum paniceum* (Wang et al. 2008), *Quercus robur* (Martínez et al. 2008) *Sorghum* and *Trypsacum* (Sairam et al. 2002 and 2005) for which multiple shoots were also obtained. Our results revealed a morphogenic potential for organogenesis in *B. brizantha* mature seeds.

Table 2 *Brachiaria brizantha* cv. Marandu mature seed morphogenetic responses to different hormone combinations

Morphogenetic response	Media hormone composition	
	KIN3:NAA1	BAP3:NAA0.3
Seed with 1 shoot	54 ± 4.24	73.5 ± 2.12
Seed with 2 shoots	24.5 ± 0.07	7.5 ± 0.70
Seed with callus	11 ± 4.24	8 ± 1.41
Seed with buds	0	9 ± 1.41

One hundred seeds were used per treatment and two independent experiments were carried out

Somatic embryogenesis induction from mature seeds

Culturing mature seeds for 5 days in callus induction medium generated a swelling of the scutellum and embryo proper defining the embryo axis (Fig. 2a). The swelling was followed by a proliferation of friable callus on the upper surface of the scutellum, and an opaque white structure was observed (Fig. 2b). This structure is a differentiated scutellum of the somatic embryo (Fig. 2c) and produces new scutelli, repetitively. A more detailed view of the surface of the calluses showed coleoptiles surrounding the shoot apical meristem of the somatic embryos; each coleoptile contains one shoot meristem (Fig. 2d). Several stages of somatic embryo differentiation were shown by the presence of proembryos, typically characterized by a globular structure showing small meristematic cells, and well developed somatic embryos with vascular strands, showing the polarity of the embryo axis (Fig. 2e). Four-week-old explants had a cream-colored embryo proper enveloped with an opaque white-colored, isolated, well differentiated scutellum, and in some cases showed fused scutelli (Fig. 2f, g). Another identified feature is fused embryos proper in one scutellum (Fig. 2h). Two patterns of regeneration were observed after transferring the explants to regeneration medium—multiple shoots produced from multiple fused embryos proper

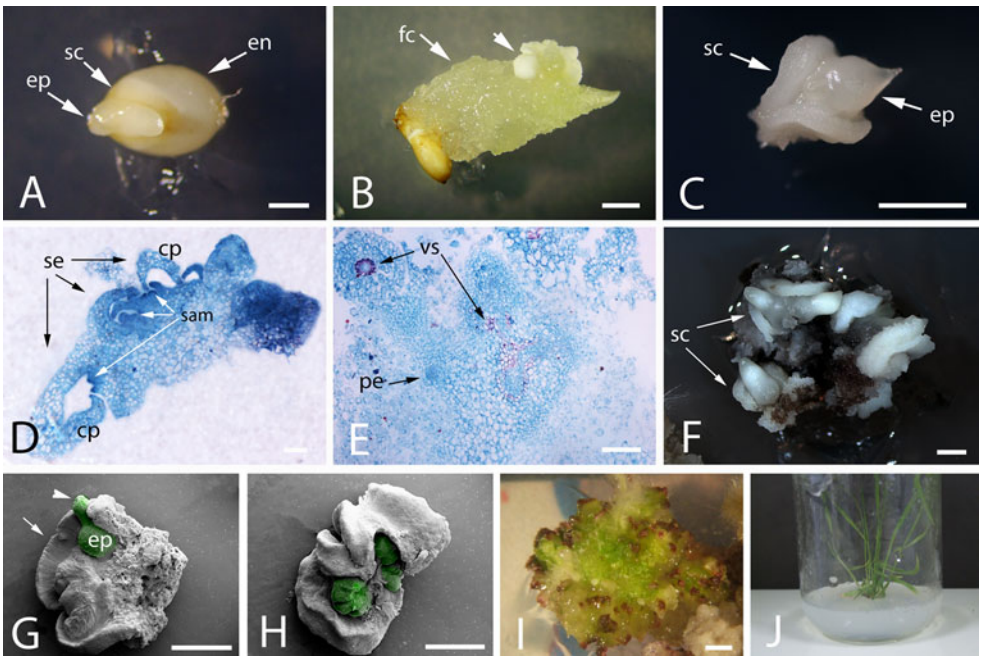

Fig. 2 *Brachiaria brizantha* somatic embryogenesis from mature seeds: **a** 5-day-old explants cultivated in callus induction medium showing swelling of the embryo proper of the apomictic seed embryo; **b** 10-day-old explants cultivated in callus induction medium showing friable callus proliferating from the scutellum of apomictic embryo with white opaque structures (*arrowhead*); **c** detail of an isolated somatic embryo and its well differentiated scutellum from **b**; longitudinal (**d**), and transversal (**e**), histological sections from embryogenic calluses showing in **d** several somatic embryos with the shoot apical meristems enveloped by the coleoptile and in **e** vascular strands and a proembryo; **f** torpedo somatic embryos after 30 days in induction medium; **g**, **h** scanning electron micrographs of somatic embryos showing in **g** fused scutelli (*arrow*), embryo proper colored in *green*, and the coleoptile pore (*arrowhead*) and in H a well developed scutelli with several embryo proper forming polyembryos, colored in *green*; **i** shoot primordia developed from somatic embryos after transferring to regeneration medium; **j** plant obtained from somatic embryogenesis. *ep* embryo proper, *sc* scutellum, *en* endosperm, *fc* friable callus, *se* somatic embryo, *cp* coleoptile, *vs* vascular strand, *sam* shoot apical meristem, *pe* proembryo. *Bars* **a**–**c**, **f**–**h** = 1 mm; **d**, **e** = 200 μm

(Fig. 2i) and a complete plantlet originating from isolated somatic embryos. Under light conditions, a purple pigmentation was observed in the leaf tip of buds and shoots.

The development of somatic embryos has been demonstrated for many monocots like *Pennisetum glaucum* (Vasil and Vasil 1980), *Zea mays* (Lu et al. 1983), *Paspalum scrobiculatum* (Vikrant and Rashid 2003), *Paspalum simplex* (Molinari et al. 2003), *Hordeum vulgare* (Li et al. 2009) and *Cenchrus ciliaris* (Yadav et al. 2009), and mostly primary embryos were formed indirectly via calluses. In *B. brizantha*, somatic embryo development was similar to the well differentiated apomictic seed embryo as observed by Lenis-Manzano et al. (2010). In this work, besides the common characteristics of monocot seed embryos, a variety of other features found during somatic embryo development is shown. The somatic embryos have a distinct scutellum (cotyledon) enveloping the embryo axis where the coleoptile is a typically tubular sheath around the shoot meristem with a terminal pore. The shoot meristem is located at the open end of the scutellum and the root meristem at the closed end forming the embryo proper. The pattern of fused scutelli and polyembryos described here as a result of somatic embryogenesis induction is similar to that reported for wheat zygotic embryo, as a result of a 2,4-D cell accumulation effect (Fischer et al. 1997). Snow and Snow (1937) observed that division of embryonic parts may be due to local accumulation of auxin. When the supply of auxin decreases there is a rapid differentiation into embryos and different patterns of distribution in the same explant may occur due to local accumulations of auxin. Auxin transport and accumulation may also have an influence in somatic embryo differentiation in *Brachiaria*.

Ahmed et al. (2009) showed anthocyanin accumulation in epidermal cells of wheat coleoptiles germinated under white light, suggesting that light is one of the most important environmental stimuli regulating anthocyanin accumulation. Moreover, they demonstrated that anthocyanin biosynthesis is tissue specific and highly conserved among plant species. The purple pigmentation observed in buds and shoots cultivated under light conditions, indicates a stress-induced anthocyanin production in leaf tips of *B. brizantha* cultivated in vitro under light condition.

Table 3 Effect of media combination on somatic embryogenesis of *B. brizantha* mature seeds cultivated for 2 months

Media combination	Total no. seeds	% Seeds with embryogenic callus ± SD	% Seeds that formed shoots + SD
M1/MS1	320	56.25 ± 16.56	22 ± 9.27[a]
M1.2/MS2	470	64.2 ± 23.32	38 ± 22.50[b]
M1.3/MS3	365	77.25 ± 9.74	54.25 ± 19.75[b]

The mean values were obtained from four independent experiments

[a] Presence of 1 or 2 shoots/explant

[b] Presence of shoot bundles/explant

Table 4 Effect of pH on bud regeneration in embryogenic callus obtained from mature seeds in the M1.3/MS3 media combination

Treatment	Bundle type	Mean + SD	Total # explants
pH 4.0	1–5	151 ± 19	195
	6–10	94 ± 31	
	>10	95 ± 31	
pH 5.8	1–5	72 ± 17	189
	6–10	28 ± 3	
	>10	25 ± 16	

Mean value is the mean number of occurrences of each bud clump type (frequency) in three different experiments in regeneration medium. Bundle type refers to the number of buds or shoots found in one clump. They were grouped in three types of bundles: one to five, six to ten and more than ten buds or shoots

The morphogenic response of mature seeds in culture using three combinations of media for callus induction and regeneration of shoots is shown in Table 3. The two media combinations M1/MS1 and M1.2/MS2 showed a lower percentage of seeds with embryogenic callus, as well as a lower percentage of seeds that formed shoots when compared to the M1.3/MS3 media combination. Most of the calluses in M1/MS1 produced one or two shoots per explant, while in the other media combinations multiple shoots were obtained per explant. The M1.3 medium produced the highest percentage of embryogenic calluses (77%) in an intermediate 2,4-D concentration. A M1.3/MS3 media combination produced plant regeneration in up to 54% of the seeds.

In a previous report, 67% of excised embryos from *Brachiaria* mature seeds resulted in embryogenic calluses when cultivated in the media combination M1.2/MS2 (Silveira et al. 2003). In ginseng, excision of mature zygotic embryo is necessary to induce somatic embryogenesis (Choi and Soh 1996). In this study, using the same media combination M1.2/MS2 and intact mature seeds, a similar percentage (64%) of seeds with embryogenic callus was obtained. This result indicates that in *B. brizantha* the excision of the seed embryo is not required for somatic embryo induction, making it a less laborious and time consuming method. In plant tissue culture it is recommended to work with low hormone levels for reducing induced somaclonal variation. M1.3 has an intermediate 2,4 D concentration and MS3 a lower cytokinin concentration compared to the other media tested, and was the best combination to induce embryogenic calluses and recover shoots from cultured seeds.

M1.3/MS3 media combination was used to test the effect of acidic pH. Table 4 presents the effect of pH on plant regeneration in embryogenic calluses. Three types of bundles were observed according to the number of buds or shoots presented in one clump: bundles with one to five, with six to ten and with more than ten buds or shoots. Different bundle types or more than one of the same bundle type could be present in an embryogenic callus. For all three categories of bundle type, at pH 4.0 the frequency of each bundle type increased when compared to pH 5.8. In addition, contamination by endogenous bacteria seemed to be reduced at pH 4.0. The regenerated plantlets at both pH values were all green and morphologically normal (Fig. 2j). Intracellular pH is important for the activity of enzymes at physiological pH that is within 7.4–7.5 in plant cells (Gout et al. 2001). Some studies have demonstrated powerful pH regulatory mechanisms in plant cells. Guern et al. (1986) showed, in *Acer pseudoplatanus* cells cultivated in liquid medium, that acid-loading induces a strong initial acidification of the cytoplasm after which the pH increases. Oscillations in pH seem to be important in controlling the cell cycle and the proliferative capacity of cells (reviewed by Madshus 1988). In *Brachiaria*, reducing the pH of the M1.3/MS3 media combination from pH 5.8 to pH 4.0 increased the number of embryogenic calluses that formed plantlets in the regeneration medium as well as the number of buds in each explant. In addition, the acidic pH was shown to increase shoot recovery from embryogenic callus. Altogether, the embryogenic response of *B. brizantha* mature seeds to culture was very fast and unsynchronized, with 10-day-old explants containing differentiated scutelli.

Somatic embryogenesis induction from basal segments

Morphogenic responses from basal segments from in vitro grown sexual and apomictic plants cultured in three media combination are shown in Table 5. Overall, less than 5% of the explants oxidized and did not form calluses. The exception was the sexual genotype in combination media M1.2/MS2 which showed more than 20% oxidized explants and 10% of friable calluses for the three media combinations tested. Friable calluses producing roots account for the majority of the morphogenetic responses in a percentage varying up to 58% in the M1.2/MS2 media

Table 5 Morphogenetic response of basal segments from in vitro sexual and apomictic genotypes to different media combinations given in mean percentage of explants that showed one of the responses: friable callus, friable callus with roots, friable callus with embryogenic callus or embryogenic callus with shoots

Morphogenetic response	Sexual genotype			Apomictic genotype		
	M1/MS1	M1.2/MS2	M1.3/MS3	M1/MS1	M1.2/MS2	M1.3/MS3
Friable callus	12 ± 1.67	12.5 ± 0.96	14.56 ± 1.53	8.12 ± 4.82	4.14 ± 1.94	16.83 ± 3.72
Friable callus/roots	56 ± 6.83	57.86 ± 8.58	47.41 ± 4.92	67.76 ± 6.75	42.36 ± 7.63	1.98 ± 0.58
Friable callus/embryogenic callus	27 ± 3.15	3.41 ± 1.57	13.28 ± 2.36	2.34 ± 1.01	15.27 ± 4.26	13.41 ± 1.59
Embryogenic callus/shoots	3 ± 1.32	4.29 ± 1.11	21.53 ± 2.74	21.87 ± 7.92	35.31 ± 3.80	64.37 ± 12.11

Average from 50 explants per treatment in two experiments

combination. A higher percentage of basal segments with embryogenic calluses was observed in the M1/MS1 media combination (27%) but less than 5% produced shoots. Less than 10% of the explants produced embryogenic calluses with or without shoots in M1.2/MS2. Approximately 12 and 20% of the explants had embryogenic response in the M1.3/MS3 combination. Explants from the apomictic genotype had a decreasing percentage of friable calluses with roots across the three media combinations. In contrast, an increasing percentage of embryogenic calluses with shoots, 20% in M1/MS1 media combination, 35% in M1.2/MS2 and up to 65% in M1.3/MS3 were observed. For the apomictic genotype, embryogenic calluses associated with friable calluses were observed in 15 and 13% of the explants in M1.2/MS2 and M1.3/MS3, respectively. Similar results were observed for the sexual genotype in M1.3/MS3 combination, that was therefore the best combination for shoot formation. The plantlets recovered were rooted and presented normal appearance and development.

The media combination M1.3/MS3 gave the best result for shoot formation in both the sexual and apomictic genotypes, although the apomictic had a greater percentage. The differences between the apomictic and the sexual genotypes demonstrate a genotype-dependent response in *B. brizantha*. In another grass, *Cenchrus ciliaris*, a genotype-dependent morphogenetic response was also found between apomictic and sexual genotypes (Yadav et al. 2009). Moreover, cultivars differ not only in their embryogenic response to different culture media but also in their interactions with the media during growth (Khanna and Raina 1998; Dai et al. 2011). In *Brachiaria*, similarly to what was observed in Zoysiagrass (Li et al. 2010), the meristematic regions present in the basal segment were possibly responsible for the callus proliferation under the effects of 2,4 D and BA that eventually turned into embryogenic callus and shoots. A somatic embryogenesis system based on the use of basal segments as explants was developed for both the sexual and the apomictic genotypes. Specifically for the sexual genotype, this system constitutes an alternative to seed somatic embryogenesis, which is very important since seeds are not produced on a regular basis.

Somatic embryogenesis induction from young leaves

Table 6 summarizes the morphogenetic responses observed from culture of leaf segments obtained from the basal region of young unexpanded leaves. The results were highly variable and no tendency of responses was observed between the sexual and apomictic genotypes, either as a result of increasing the induction period or altering the pH. Explants in callus induction medium showed a high oxidation rate, and a low number of calluses with nodular compact structure resembling embryogenic callus was obtained (Fig. 3a). When these calluses were transferred to regeneration medium, they gave rise to roots only (Fig. 3b), without further embryo development in all treatments.

In several monocot species, the regeneration of calluses derived from green vegetative tissue seemed to be extremely recalcitrant, and few plantlets were obtained (Chaudhury and Qu 2000). However, *Echinochloa colona* (Samantaray et al. 1997), maize (Ahmadabadi et al. 2007), and barley (Li et al. 2009) have been efficiently regenerated from young leaf bases. Moreover, two genotypes of *Viburnum dentatum*, a popular shrub for landscape use, regenerated shoots when leaf tissues were cultured on woody plant medium (WPM) supplemented with BA or thidiazuron (Dai et al. 2011). Using in *B. brizantha* the same system used for maize, although calluses with embryogenic characteristics were observed, it was not possible to regenerate any plantlet in the tested conditions and only roots profusely proliferated in the callus. It has been reported that meristematic cells of cereals and grasses differentiate rapidly, thus losing their dividing capacity if maintained in the culture medium without the presence of auxin in an adequate concentration. It is possible that the level of auxin in the medium used was not adequate to promote somatic embryogenesis and resulted in induction of root proliferation. On the other hand, changes in the

Table 6 Morphogenic responses of leaf segments from in vitro plants under different induction times, genotypes and pH

Treatments		Induced callus on induction medium % Mean ± SD		Total # of explants in regeneration medium		Explants with callus or roots[a] % Mean ± SD		Calluses with shoots
Days of induction	Genotypes	pH						
		4.0	5.8	4.0	5.8	4	5.8	4 or 5.8
15 days	Apomictic	11.3 ± 0.5	12.5 ± 0.8	48	104	17 ± 4.5	22 ± 8	0
	Sexual	17.5 ± 5.9	16.7 ± 3.4	66	71	30 ± 3	14 ± 1	0
20 days	Apomictic	10 ± 5	5.3 ± 1.72	88	68	23 ± 8.5	15 ± 5	0
	Sexual	0.2 ± 0.1	23.8 ± 1.3	88	90	03 ± 2	21 ± 3	0
30 days	Apomictic	12.5 ± 1.25	8.8 ± 0	50	69	10 ± 3	13 ± 4.5	0
	Sexual	24.9 ± 24.9	10.7 ± 0.7	58	125	19 ± 2	12 ± 3.5	0

Mean of two different experiments

[a] After transferring to regeneration medium

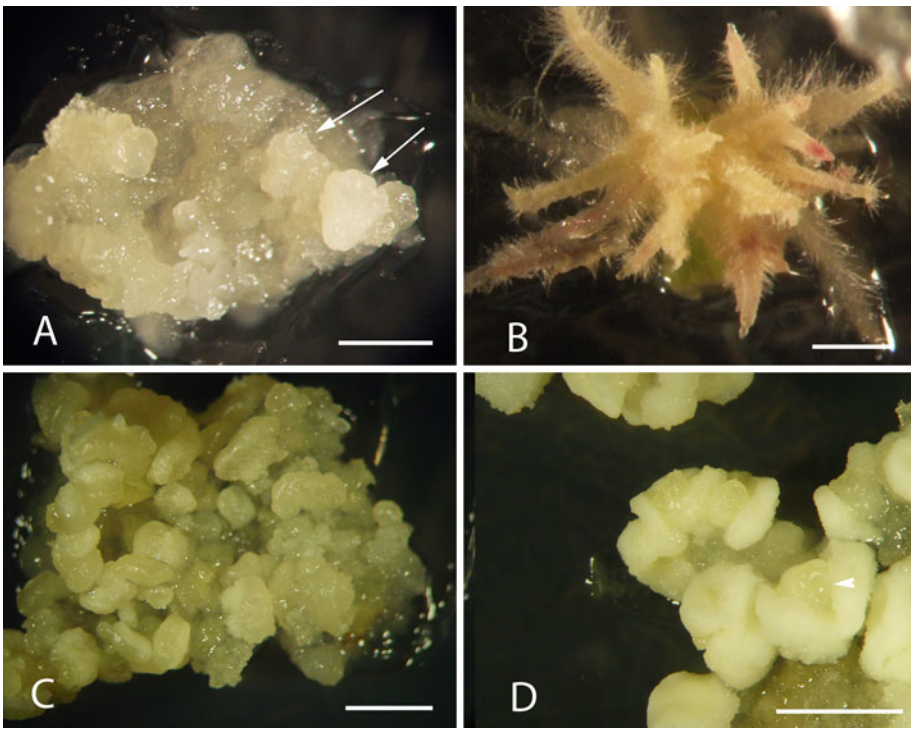

Fig. 3 Morphogenetic response of young leaf segments from in vitro plants, **a**, **b**, and repetitive somatic embryogenesis, **c**, **d**. **a** Callus obtained from apomictic genotype after 15 days on induction and 30 days on regeneration medium, pH 5.8 with globular structures, resembling embryogenic calluses, on callus surface (*arrows*); **b** explant from sexual genotype after 15 days on induction and 30 days on regeneration medium, pH 5.8 with root proliferation. **c** Friable callus proliferating nodular compact structures. **d** Synchronized differentiation of somatic embryos showing the coleoptile pore (*arrowhead*). *Bars* 1 mm

level of endogenous hormones may be involved in differentiation and embryogenic competence (reviewed by Vasil 2005; Sairam et al. 2005). More studies would be necessary to define optimal conditions for embryogenic responses from leaf basal segments.

Repetitive somatic embryogenesis

Embryogenic calluses subcultured at monthly intervals, for 5 months, were composed of friable calluses that originated proliferating compact, nodular structures on the callus surface (Fig. 3c). This nodular structure developed into scutellum-like structures (Fig. 3d) similar to those reported in cereals and other grasses (Ozias-Akins and Vasil 1982, 1983; Vasil and Vasil 1982). These structures generated a well defined scutellum with one embryo proper, in a very homogeneous and synchronized way. Subculturing calluses in regeneration medium for 2 and 4 months resulted in both normal and abnormal development, with embryos germinating in approximately 80 days. From the 4-month-old cultures all the recovered plantlets were albino. Maintaining monocot embryogenic cells at

high levels of 2,4-D by regular subculture to fresh medium kept them in a perpetually meristematic (embryogenic) state (Vasil 2005). Although it became evident that repetitive embryogenic cultures can be obtained in this way for *B brizantha*, the tendency toward albino plant regeneration restricts the possibility of a long-term embryogenic culture for this species. Barley, another monocot, is highly predisposed to somaclonal variation caused by in vitro culture (Cho et al. 1998; Choi et al. 2000). Transgenic and non-transgenic barley cells of most genotypes frequently did not differentiate into plants or only albino plants could be recovered (for review, see Lemaux et al. 1999).

Several factors are known to affect chromosomal stability and induce ectopic changes in plant tissues during in vitro growth, such as the species, genotype, initial ploidy level, explant source, medium composition, growth regulators used, and time in culture (Constantin 1981; Karp 1991; Bregitzer and Campbell 2001). Moreover, prolonged tissue culture, especially in the case of somatic embryogenesis, has been shown to mobilize transposable elements. In this connection, the activation of transposable elements has long been postulated to be a major source of somaclonal variation (Hirochika et al. 1996; Sairam et al. 2002). Kowata et al. (1995) demonstrated that rice plantlets recovered from 1-month-old induced calluses were green while those obtained from 11-month-old calluses were 100% albino. Moreover, Artunduaga et al. (1988) tested three common bermudagrass varieties and obtained 90% albino plants, while the characterization of two micropropagation-derived albino *Bambusa edulis* mutants demonstrated aberrations in their chloroplast genomes, causing reduction or absence of thylakoids, accumulating the lipids in plastoglobules (Liu et al. 2007). *B brizantha* seems to be sensitive to long exposure to 2,4 D in culture, possibly responsible for the high frequency of albinism observed in this grass species in 4-month-old embryogenic callus culture. The use of polyamines to produce green plants has been suggested by several authors, even for monocots (Chiancone et al. 2006; Redha and Suleman 2010; Germanà 2011), and is being tested for *Brachiaria brizantha* (data not shown).

The present work shows for the first time an organogenic potential of *B. brizantha* cultures from mature seeds in addition to the embryogenic potential. Efficient introduction, characterization and long term maintenance of in vitro *Brachiaria* plants was demonstrated. Also, for the first time, a more detailed morphological characterization of the diversity of the somatic embryogenesis responses from friable calluses to plant regeneration in *B. brizantha* was presented. Moreover it was shown that *B brizantha* seems to be sensitive to long-term somatic embryogenesis induction. An alternative system to seed somatic embryogenesis was obtained using basal segments from in vitro plants as explants. The improvement of somatic embryogenesis will reflect directly in the genetic transformation efficiency.

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