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RESEARCH REPORT

Plant regeneration from embryogenic callus and cell suspensions of *Brachiaria brizantha*

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Introduction

In monocots, *in vitro* plant regeneration can be obtained through somatic embryogenesis (Vasil and Vasil 1980, 1982; Vasil 2005). Evidence of more than one morphogenic response in the same explant, under the control of different auxin/cytokinin ratios and concentrations, was observed in species of the Poaceae family such as sorghum (*Sorghum bicolor*, (L.) Moench.), minor millet (*Paspalum scrobiculatum* L.), sugar cane (*Saccharum officinarum* L.), and baby bamboo (*Pogonatherum paniceum* Lam. Hack.) (reviewed by Wang *et al.* 2008).

Brachiaria callus has been induced using seeds as explants from *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf, *Brachiaria decumbens* Stapf, *Brachiaria ruziziensis* Germain et Evrard and *Brachiaria dictyoneura* [*Brachiaria humidicola* (Rendle) Schweick vr. Lanero, ex *B. dictyoneura*] (Tohme *et al.* 1996). Subsequently, using the same methodology, calli were induced from 76% of isolated embryos from seeds of *B. brizantha* (Lenis-Manzano 1998). The formation of somatic embryos and multiple shoots from seedling apical meristems was demonstrated for *B. ruziziensis* (Ishigaki *et al.* 2009). In this system, mature seeds were used as source of explants and cultured on a medium containing 4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Ishigaki *et al.* (2012) reported that embryogenic calli were more suitable than shoot apex explants for genetic transformation of ruzigrass.

B. brizantha cv. Marandu is the most important and cultivated forage grass in Brazil. It is tolerant to spittlebugs (Homoptera: Cercopidae complex, mainly genera *Deois* and *Zulia*), an economically important pest for *Brachiaria* (Felismino *et al.* 2012). This cultivar is considered as the key forage in beef cattle production in Brazil. Breeding is hindered by its apomictic reproductive mode (the production of clones of the mother plant), which reduces the possibility of being used for hybridizations (Valle and Savidan 1996). Alternatives to conventional breeding of *B. brizantha* would include genetic modification *via* transformation. However, methods of transformation and tissue culture of these plants are not yet established.

The development of embryogenic calli has been reported from 73% of the *B. brizantha* cv. Marandu isolated seed embryos in culture and 67% of calli regenerated plants (Silveira *et al.* 2003). More recently, the histology of embryos isolated from the *in vitro* cultivated seed was shown (Lenis-Manzano *et al.* 2010). The formation of multiple shoots was reported in cultured basal segments from micropropagated plantlets of this cultivar (Pinheiro *et al.* 2000), a system that was efficient for in vitro chromosome duplication in *Brachiaria*. Somatic embryogenesis and organogenesis in *B. brizantha* is influenced by several factors such as genotype, explant type, and culture conditions (Cabral *et al.* 2011).

A high rate of cell division in explants used for genetic transformation of monocots is a prerequisite for the integration of exogenous DNA (Hiei *et al.* 1997; Vasil 2005). Rapid cell proliferation is a characteristic of embryogenic tissue. Cell suspension cultures (CS) are well known for their cell division capacity and the possibility of developmental-stage

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synchronization. This study describes the development and characterization of *B. brizantha* somatic embryogenesis from embryogenic callus and cell suspension cultures.

Material and Methods

Plant material. Seeds of the apomictic genotype *B. brizantha* (Syn. *Urochloa brizantha*) cv. Marandu (Embrapa's collection number BRA 000591) were kindly provided by Embrapa Beef Cattle, MS, Brazil.

Callus induction. Mature seeds were peeled and disinfested in a 70% ethanol solution for 5 min, followed by 30 min in a 2.5% sodium hypochlorite solution with 0.1% Tween 20, followed by five rinses in sterile distilled water. Three to 400 seeds were inoculated in Petri dishes (12 seeds per plate) containing one of the following induction media: M1.3 pH 4, NB pH 5.8, or NBBAP either at pH 4 or pH 5.8 (Table 1). The cultures were kept in the dark at 25±2 °C for 4 wk. After this period, embryogenic calli were identified and isolated, with the aid of a stereomicroscope, and transferred to fresh medium with the same composition, 30 to 50 pieces of embryogenic tissue per plate. The remaining calli were transferred to regeneration medium, half onto MS3 pH 4 and the other half onto NBreg pH 5.8 (Table 1). After 10 d, half of the calli were transferred to pre-regeneration medium (PRM pH 5.8, Table 1) for 8 to 10 d, and then transferred to regeneration medium, either MS3 or NBreg, while the other half was transferred directly to regeneration medium (MS3 or NBreg) (Table 1).

Callus induction for obtaining embryogenic cell suspension. - Calli obtained on each solid induction medium, M1.3 pH 4 or NBBAP at pH 4 or pH 5.8, were subcultured to fresh medium, and after 15 d were transferred to liquid medium with the same composition. In each 250-mL Erlenmeyer flask, containing 30 mL of medium, 10 to 15 calli were inoculated and maintained under agitation on an orbital shaker at 100 rpm in the dark. Calli were cultured for 3 to 4 wk for cell suspension (CS) proliferation, with weekly renewal of the medium accomplished by allowing cells to settle for a few minutes before pipetting the old medium out and adding new medium. After this period, the calli and differentiated embryos were removed by sedimentation. In a second sedimentation step, the settled cell volume (SCV) was measured using a conic centrifuge tube (Falcon 50 mL).

To evaluate multiplication and regeneration, the CS was then divided into three parts. One part was maintained under agitation, for additional cell suspension proliferation and confirmation of embryogenic capacity. A second part was transferred to pre-regeneration medium and subsequently to regeneration medium, 200 to 300 mg or 1 mL SCV, to confirm the ability of the cell aggregates to differentiate into embryos and/or shoots. Pre-regeneration was carried out either in DD1 or PRM (Table 1) liquid medium at pH 5.8, under agitation, for 1 wk in the dark, followed by subculture to regeneration medium, either NBreg pH 5.8 or MS3 pH 4, solidified with Phytagel™ and agar, respectively. The cell aggregates were cultivated for 3 d under moderate light (around 30 μmol/m^2/s), and thereafter transferred to higher light conditions (75 μmol/m^2/s), both at a 16-h photoperiod. The third part of the CS was cultured directly onto solidified regeneration medium, for 3 to 4 wk, with liquid regeneration medium (MS3) being added after 2 wk to renew the medium components without subculture, to avoid contamination. All cultures were maintained at 25±2 °C. Regenerated shoots were transferred to test tubes for elongation and rooting in MMP medium (Table 1). After 1 mo, the plantlets were acclimatized in the greenhouse, initially in vermiculite, and subsequently transferred to soil.

Statistical analyses. The free access R statistical program (R Core Team 2013) was used for all analyses, using an error rate of $p<0.05$. The effect of different culture media on morphogenesis was compared using the binomial model (ratio) or Poisson model (count or score). To correct the high variability (overdispersion) present in the data, the Quasi-likelihood method was used for estimating the heterogeneity factor.

The effect of pH in the somatic embryogenesis induction medium and the effect of two induction media in the observation times were compared by two-way ANOVA. The post hoc Tukey HSD multiple comparison test was conducted to access differences in settled cell volume (SCV) and observation times. The quality of the adopted model was measured by graphical analysis of the residues, as well as through tests of normality (Shapiro-Wilk test) and homoscedasticity of variances (Bartlett test).

The growth of *B. brizantha* cells in suspension culture was measured by the increase of SCV and was analyzed by the logistic curve. The logistic curve describes the changes in the volume increase (Y) with respect to the culture time (t), represented by the equation $Y_t = A(1 + be^{-kt})^{-1}$. The parameters of this function are as follows: A represents the stationary phase, b is the scale parameter (constant), and k represents the growth rate. The inflection point (change curve) occurs in (b/k, A/2). The fitness of the model was measured by the R^2 statistics. The plateau point was estimated using the segmented regression model.

Light and scanning electron microscopy. Embryogenic calli were collected at different developmental stages and processed for light microscopy (LM) and scanning electron microscopy (SEM). For LM, samples were fixed in paraformaldehyde (4% *v/v*), dehydrated in an ethanol series (30 to 100%) at 30-min intervals, followed by propanol (100%) for 8 h, and then butanol (100 %) overnight. Infiltration was done at 4 °C

Table 1 Media used for in vitro culture of *Brachiaria brizantha* in the present work

Embryogenesis step	Medium and pH	Culture medium composition	References
Induction	M1.3 pH 4.0	MS basal medium, 300 mg/L casein hydrolysate, 3 mg/L 2,4-D, 0.2 mg/L BAP	Cabral *et al.* (2011)
	NB pH 5.8	N6 major salts, B5 minor salts and vitamins, 100 mg/L Inositol, 500 mg/L proline, 500 mg/L glutamine, 300 mg/L casein hydrolysate, 2.5 mg/L 2,4-D, 3% sucrose, 0.3% Phytagel	Sallaud *et al.* (2003)
	NBBAP pH 4.0 or 5.8	NB medium supplemented with 0.2 mg/L BAP	–
Pre-regeneration	PRM pH 5.8	NB medium supplemented with 5 mg/L BAP, 1 mg/L NAA, 2 mg/L ABA, 0.7% agarose type I	Sallaud *et al.* (2003)
	DD1 pH 5.8	MS basal medium, 10 mg/L ascorbic acid, 1.1 mg/L 2,4-D, 0.2 mg/L zeatin, 3% sucrose	Matsumoto (2006)
Regeneration	MS3 pH 4.0	MS basal medium, 300 mg/L casein hydrolysate, 0.5 mg/L NAA, 1 mg/L BAP, 2.5 mg/L kinetin	Cabral *et al.* (2011)
	NBreg pH 5.8	NB medium supplemented with 3 mg/L BAP, 0.5 mg/L NAA, 0.6% Phytagel	Sallaud *et al.* (2003)
Elongation and rooting	MMP pH 5.8	MS basal medium, ½ strength major salts, 100 mg/L casein hydrolysate, 0.5 mg/L kinetin, 0.2 mg/L NAA, 0.5 mg/L GA_3, 2% sucrose, 0.7% agar	Cabral *et al.* (2011)
All media	pH was adjusted to 5.8 with 1 N KOH or to 4.0 with 1 N HCl prior to autoclaving. Agar concentration was 7 g/L, except for pH 4, which was 14 g/L. Medium was autoclaved at 121 °C for 20 min. When ABA and GA_3 were supplemented media were filter sterilized		

ABA abscisic acid; *BAP* 6-benzylaminopurine; *GA3* gibberellic acid; *NAA* naphthaleneacetic acid; *2,4-D* 2,4-dichlorophenoxiacetic acid; *MS* Murashige and Skoog (1962)

using butanol/infiltration medium (glycol methacrylate, Historesin kit (Leica, Heidelberg, Germany) (2:1, 1:1, 1:2, minimum of 2 h each step), followed by infiltration in 100% infiltration medium for 48 h. Polymerization was done at room temperature for 24 to 48 h. Histological serial sections (5-μm thick) were obtained in a rotary microtome (Leica RM 2155, Nussloch, Germany), stained with 0.05% (*w/v*) toluidine blue in water, mounted in Entellan® synthetic resin (Merck, Darmstadt, Germany), covered with coverslips, and observed under an optical microscope Zeiss Axioskop 40 HBO 50 A/C (Carl Zeiss, Jena, Germany). For SEM, embryogenic units were fixed in paraformaldehyde (4% *v/v*), dehydrated to 100% ethanol, critical point dried using liquid CO_2, mounted on metal stubs, sputter coated with gold, and observed under a LEO 435 VP (Carl Zeiss, Jena, Germany) at 20 keV.

Results and Discussion

Embryogenic callus. Embryogenic calli were obtained after 3 to 4 wk of culture in M1.3 or NBBAP induction media. Seeds cultured in M1.3, with slightly higher 2,4-D concentration, showed significantly higher callus induction than those in NBBAP medium (Table 2, $p=0.0002$). In M1.3 medium, hyperhydric or watery non-embryogenic callus was not observed, contrasting with a frequency of 19% of this type of callus in NBBAP medium. Moreover, larger calli—with embryogenic callus, roots, and differentiated somatic embryos (DSE)—were formed in M1.3 medium at a frequency of 57%, compared to 15% in NBBAP (Table 2, $p<2.2e-16$).

After removal of visible embryogenic callus from 2,4-D/BAP-containing induction media (M1.3 or NBBAP), the remaining calli were plated onto regeneration medium (MS3 or NBReg), with cytokinin(s) only. Regardless of the induction medium, MS3 regeneration medium, produced a higher number of regenerants (Fig. 1). The combination of M1.3/MS3 media was the most efficient for shoot regeneration (100%; Fig. 1*A*). These results are in accordance with previous observations showing high efficiency of the M1.3/MS3 media combination for inducing and regenerating plants *via* somatic embryogenesis in *B. brizantha* (Cabral *et al.* 2011). The profuse development of roots, regardless of the induction medium, was more frequent in NBreg than in MS3 medium (Fig. 1*B*).

Table 2 Morphogenesis of *Brachiaria brizantha* cv. Marandu mature seeds cultivated on somatic embryogenesis induction media at pH 4

Culture medium	No. of seeds		No. of calli		
	Total	Without callus±SD	Ø ≤5 mm, with roots and DSE	Ø >5 mm, with roots and DSE	Hyperhydric
M1.3	400	38±5.6 b	81±18.4 b	227±20.8 a	0
NBBAP	352	51±4.2 a	165±39.6 a	53±11 b	68±14
LR Chisq	–	13.38	15.739	133.44	
p value	–	0.0002	7.27e-05	<2.2e-16	

Data represent means±SD (n=3); Values followed by different *letters* in the same *column* are significantly different (p<0.05)

LR likelihood ratio, *DSE* differentiated somatic embryos (with visible scutellum), *SD* standard deviation, *Ø* diameter

The high frequency of shoot development on MS3 medium could have been favored by a synergistic effect of the combination of the cytokinins kinetin (2.5 mg/L) and 6-benzylaminopurine (BAP; 1 mg/L). Similar behavior was observed in bud formation in *Brachiaria* spp. on micropropagation medium (Cabral *et al.* 2011). The induction/regeneration media combination NB/NBreg was originally used for rice somatic embryogenesis (Sallaud *et al.* 2003), and even though these media are richer in free amino acids than M1.3/MS3, the best response for *B. brizantha* was obtained with the M1.3/MS3 combination. In the composition of NBreg medium, only BAP is present (3 mg/L). Likewise, in *Solanum surattense*, the combination of cytokinins was more efficient for regeneration of buds and shoots than separately (Yadav *et al.* 2010). The development of shoots and somatic embryos from calli after removing visible embryogenic tissue indicates the unsynchronized induction in the same callus, including embryogenic structures that were not perceptible.

In *B. brizantha*, three different phases were characterized during embryogenic callus formation (Fig. 2). In the first phase, the tissue was compact, hard and round, 1 to 2 mm in

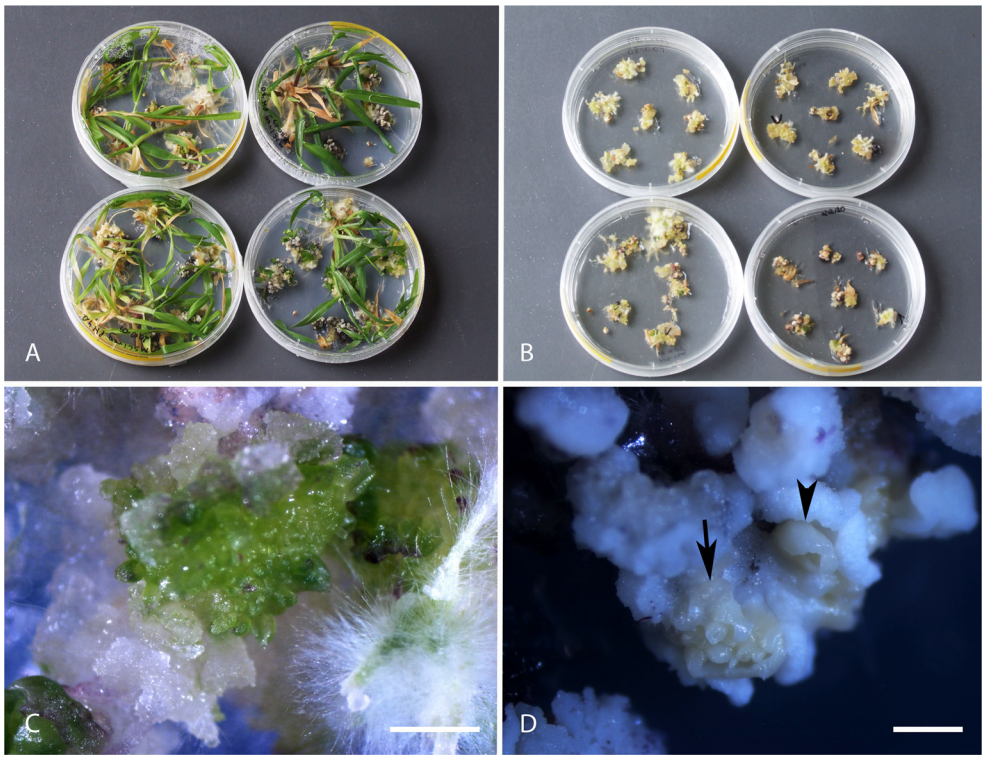

Figure 1. Morphogenetic response of *Brachiaria brizantha* callus derived from mature seeds, after removal of visible embryogenic callus, cultured in different regeneration media. *A* Shoot development in MS3 medium. *B* Root proliferation in NBreg medium. *C–D* Calli in MS3 regeneration medium showing areas with shoot bud formation (*C*) and areas with somatic embryos in different patterns, one scutellum surrounding several embryo axes (*arrow*) and a well-differentiated somatic embryo with one embryo axis and its scutellum (*arrowhead*), showing white scutellum and cream-colored embryo axis (*D*). *Bars C* 2 mm, *D* 1 mm.

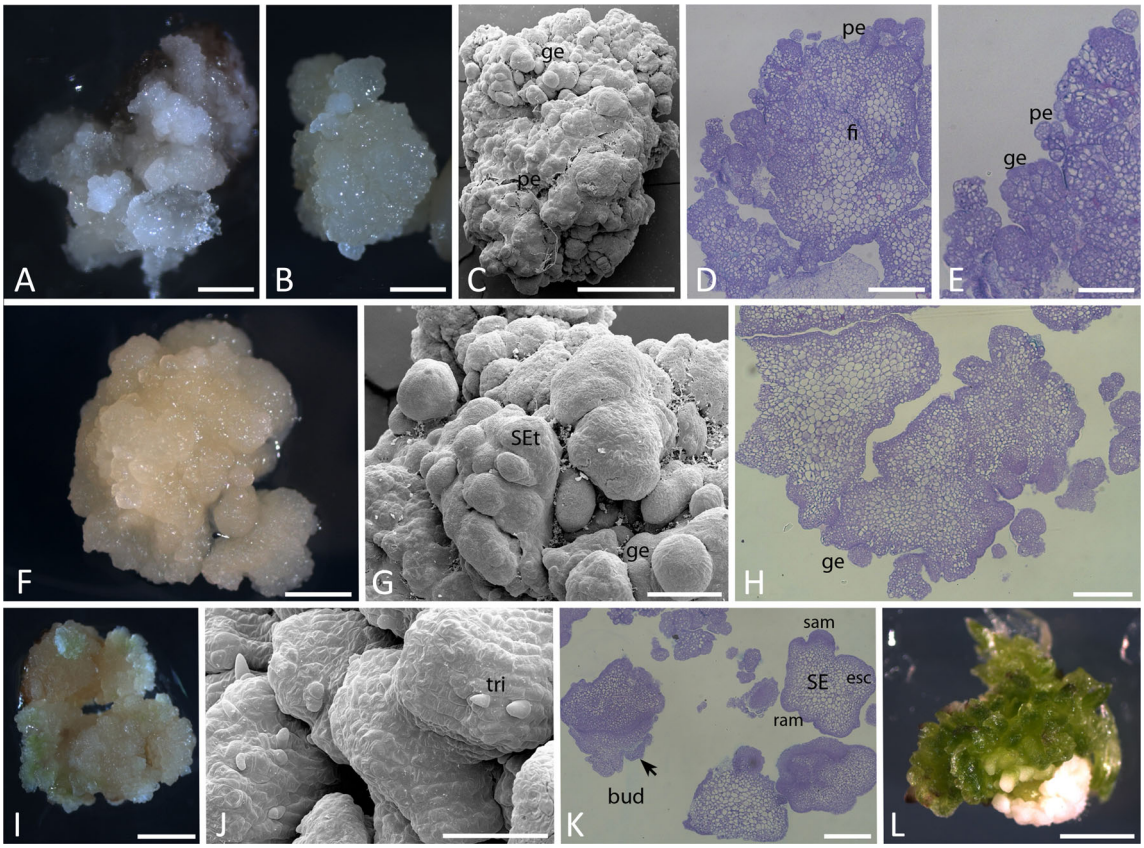

Figure 2. Development of *Brachiaria brizantha* embryogenic callus in M1.3/NBBAP/NBreg sequence of culture media. Micrographs obtained under the stereomicroscope (*A*, *B*, *F*, *I*, *L*); histological sections (*D*, *E*, *H*, *K*); and scanning electron micrographs (*C*, *G*, *J*). *A–E* Phase I showing clumps of embryogenic and non-embryogenic callus (*A*); isolated embryogenic callus (*B*); embryogenic callus with proembryos and globular embryos (*C*); histological section of embryogenic callus showing proembryos and fissures frequently observed in embryogenic callus (*D*), detail of proembryos and globular embryos from *D* (*E*). *F–H* In phase II, embryogenic callus with differentiating embryo axes and mature somatic embryos. *I–K* Embryogenic callus in phase III, showing differentiated structures (*I*), embryogenic callus with leafy structures and scutellum-leafy trichomes (*J*), and somatic embryo with well-developed scutellum, with sam and ram, and shoot bud proliferation (*arrow*) (*K*). *L* Embryogenic tissue with green shoot buds and shoots with a whitish well-differentiated scutellum. *Bars A, B, C, F* 1 mm; *D*, *J* 100 μm; *E* 50 μm; *G, H, I, K, L* 200 μm; *bud* adventitious bud, *sam* shoot apical meristem, *ram* root apical meristem, *esc* scutellum, *fi* fissure, *ge* globular embryo, *pe* proembryo, *SEt* somatic embryo in torpedo stage, *tri* trichome.

diameter, and had a smooth surface (Fig. 2*A, B*). Intensely proliferating meristematic regions were observed, as well as proembryos and somatic embryos at the globular stage (Fig. 2*C–E*). In detail, it was possible to observe that the embryogenic tissue surface presented small cell clumps or small cells with dense cytoplasm, indicating the high metabolic activity and high division cell rate (Fig. 2*D, E*) that characterize somatic embryogenesis-competent cells. Another characteristic observed on the surface of embryogenic tissue was the presence of fissures, indicating regions of embryo detachment,

Table 3 Effects of different induction media on the multiplication of *Brachiaria brizantha* embryogenic callus after 10 d of culture in fresh induction medium

Culture medium	Total	Embryogenic callus number with (%)			
		Browning callus	Friable callus	Friable callus + EC + DSE	Friable callus + DSE
M1.3	152	14 (9) b	30 (20) b	18 (12) c	90 (59) a
NB	268	45 (17) a	104 (39) a	77 (29) b	42 (15) b
NBBAP	306	21 (7) b	52 (17) b	196 (64) a	37 (12) b

Each treatment was carried out with three independent replications, in which at least 50 explants were considered as one replicate. Means in a *column* sharing the same *letter* are not significantly different ($p<0.05$)

% percentage ratio, *DSE* differentiated somatic embryos (with visible scutellum), *EC* embryogenic callus

Table 4 Summary of the best conditions to obtain embryogenic callus followed by plant regeneration from *Brachiaria brizantha*

In vitro step	Best culture medium	Culture period	Culture conditions
Induction	M1.3	30 d	dark
Multiplication	NBBAP	10 d	dark
Regeneration	NBreg	30 d	light

M1.3 and NBBAP induction medium, *NBreg* regeneration medium

Table 5 Effect of the induction medium (M1.3 or NBBAP) in *Brachiaria brizantha* cell suspension proliferation measured by the settled cell volume (SCV) during 10 wk of culture at pH 4

Week in culture	Induction medium	
	M1.3	NBBAP
4	4.80±1.01 aA	4.70±0.48 aA
6	15.43±4.25 aB	6.40±2.22 bB
8	29.40±5.44 aC	15.95±3.48 bC
10	42.87±5.76 aD	31.50±4.96 bD

Data represent means±SD (n=5). Different *lowercase letters* indicate that induction medium differ significantly, while different *uppercase letters* indicate that time of culture differ significantly (p<0.05) based on Tukey's test

giving rise to other embryos. In phase II, the embryogenic tissues were less compact and more differentiated (Fig. 2*F*), exhibited a more rough surface, measured 3 to 4 mm in diameter, and contained globular and cotiledonary somatic embryos (Fig. 2*G, H*). In phase III, the embryos became green and possessed scutellum-like structures with trichomes (Fig. 2*J*). At this stage, it was common to observe different stages of embryo development and buds, a characteristic of repetitive systems (Fig. 2*K, L*).

The embryogenic tissue showed distinct morphogenic responses during multiplication in the different induction media (Table 3). In NBBAP medium, 64 % of embryogenic tissue proliferated friable calli, plus differentiated somatic embryos (DSE), compared to only 12% on M1.3 medium. Moreover, in NBBAP medium, 12% of embryogenic tissue proliferated friable callus plus differentiated somatic embryos (DSE), compared to 59% in M1.3 medium. This indicates that, despite being a very effective medium for somatic embryogenesis induction in the mature seed scutellum of *B. brizantha*, NBBAP is not suitable for maintaining the meristematic state and potentially embryogenic cultures. In this medium, differentiation of somatic embryos occurs rapidly from embryogenic callus, with embryos showing scutellum differentiation (Fig. 2). In NB medium, the embryogenic tissue showed higher rates of oxidative browning (17%) than in the other two media, M1.3 and NBBAP, with approximately 30% of calli showing potential for repetitive cultures. Our results indicate that NBBAP medium was the most appropriate for maintenance of the embryogenic/meristematic potential of the embryogenic tissue.

Independently of the induction medium, the regeneration medium NBreg favored shoot development (Fig. 2*L*, Supplemental Table 1). Likewise, the embryogenic tissues obtained

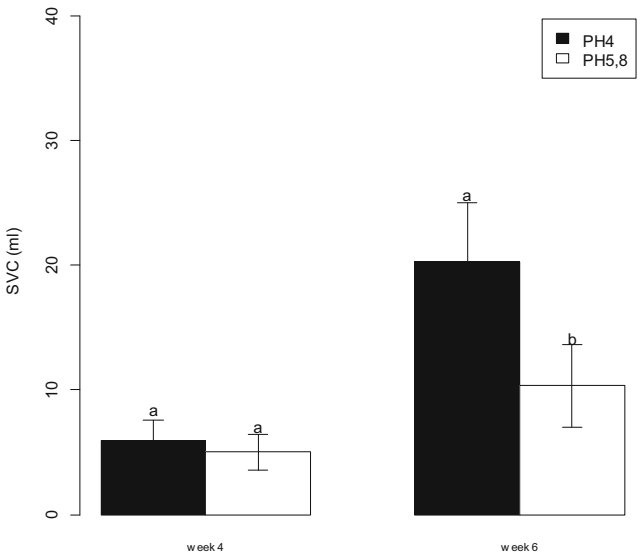

Figure 3. Effect of the pH of NBBAP induction medium on cell suspension proliferation, measured by the settled cell volume (*SCV*) after 4 and 6 wk of culture. *Vertical bars* represent the standard error of the mean (n=6 flasks per treatment). Different *letters* indicate significant differences at 5% level of probability according to Tukey's test.

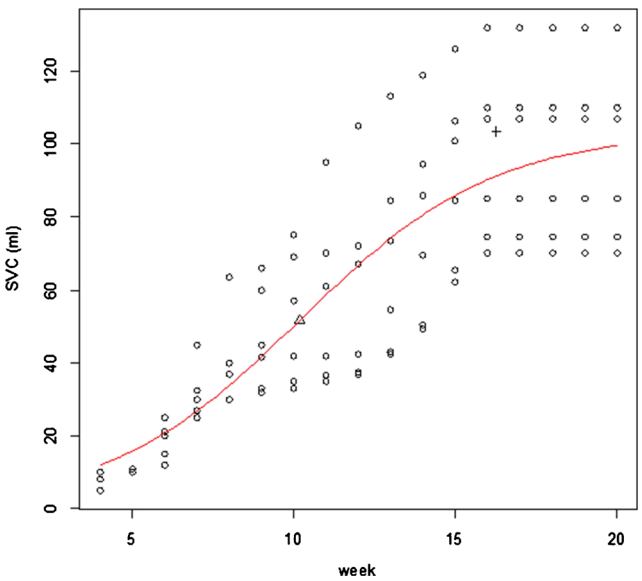

Figure 4. A cell suspension growth curve obtained by weekly subcultures in M1.3 medium in the course of 20 wk measured by the increase of SCV (settled cell volume) and analyzed by the logistic curve. The explanatory power of the logistic curve was 72.37% (R^2). Δ inflection point; + plateau point.

in NB medium generally showed high rates of root formation, either in NBreg (36%), or in the pre-regeneration medium before subculture to the regeneration medium NBreg (44%) (Supplemental Table 1). In summary, the NB induction medium did not favor embryos or shoot formation but led to root proliferation. NBreg medium favored shoot development from callus, regardless of the induction medium used, and was more effective when used directly, without a pre-regeneration medium (Supplemental Table 1).

Higher efficiency regeneration systems take less time (Table 4), possibly resulting in lower costs. The reduction of one culture step (pre-regeneration) can be advantageous, because of the higher chance of the occurrence of somaclonal variation with extended time in culture (Taparia *et al.* 2012) and also with exposure to growth regulators (Cassells and Curry 2001). Recently, Ishigaki *et al.* (2014) showed that 4-mo-old embryogenic callus generated polyploids, while all regenerants derived from 2-mo-old embryogenic calli were diploid, suggesting that extended times in culture can lead to duplication of chromosomes in rapidly proliferating embryogenic callus of ruzigrass (*B. ruziziensis*). Cabral *et al.* (2011) showed in *B. brizantha* that 4-mo-old embryogenic callus from mature seeds regenerated only albino plants. These data indicate that 2-mo-old or younger embryogenic calli are best suited for *Brachiaria* spp. In general, it is not difficult to induce callus from grasses (Wang *et al.* 2001). It is often the multiplication/maintenance of embryogenic state with further regeneration of green plants that is the challenge (Wu *et al.* 2014). In this study, high frequencies of embryogenic tissue induction in M1.3 medium (70%),

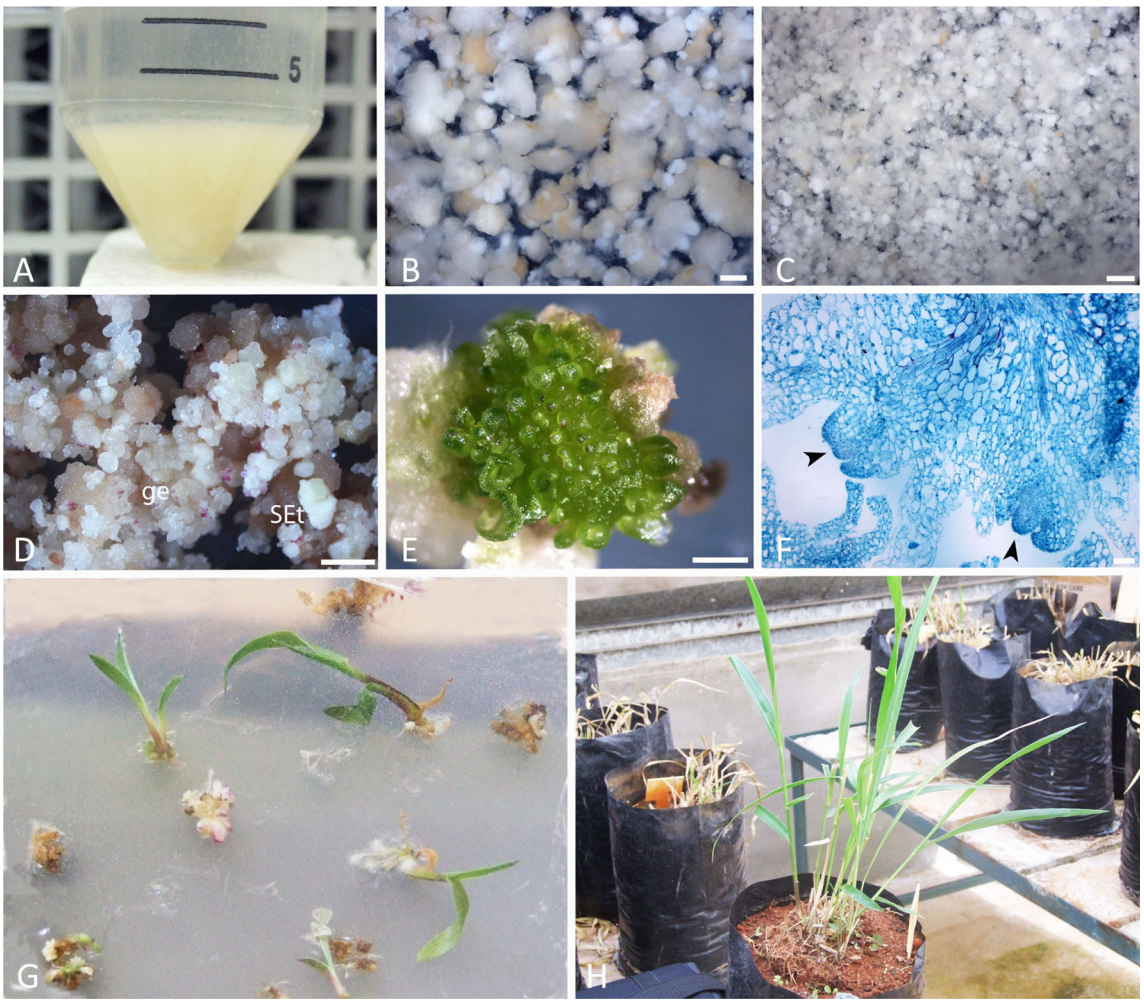

Figure 5. Induction in M1.3 or NBBAP medium and regeneration of *Brachiaria brizantha* embryogenic cell suspensions (*CS*) obtained in DD1 medium followed by NBreg solid medium covered with MS3liq to complement medium components. *A* Cell suspension obtained after a 4-wk culture in M1.3 medium, pH 4. *B–C* CS after 8 wk in liquid medium M1.3, pH 4 (*B*) and NBBAP, pH 4 (*C*). *D–E* CS in NBreg solid medium presenting SE with or without differentiated scutellum (*D*) and with bud proliferation (*E*). *F* Histological section of a cell aggregate presenting multiple buds showing the shoot apical meristem (*arrowheads*) and vascular tissue attached to the original callus. *G* Isolated shoots from CS. *H* plants obtained from CS in the greenhouse. *ge* globular embryo, *SE* somatic embryo, *M1.3 and NBBAP* induction media, *NBreg* regeneration medium. *Bars B, C, E* 1 mm, *D* 2 mm, *F* 100 μm, *G* 5 mm.

multiplication in NBBAP medium (64%), and plant regeneration in NBreg (up to 84%) were achieved from 2-mo-old cultures.

Induction and regeneration of cell suspension. Comparisons of pH in the NBBAP induction medium for cell suspension cultures showed that use of media at pH 4 resulted in a fourfold increase in settled cell volume (SCV) at 6 wk (p=1.14e-14) as compared to pH 5.8 (Fig. 3), which may be a result of a higher cell multiplication rate. This result, in addition to the response observed in callus (Cabral *et al.* 2011), indicates a positive effect of an acidic pH in cell proliferation in *B. brizantha*.

After 6 wk in culture, the SCV was significantly higher in M1.3 than in NBBAP (p=2e-16; Table 5). Cell suspensions obtained in different culture media (Table 5; Figs. 3, 4, and 5*A*) showed a similar lag phase of 4 wk before SCV began to increase (Fig. 5*A*). The lag phase was the time from callus inoculation into liquid medium to the stage when a rapid increase in cell volume was observed. A CS growth curve was obtained by weekly subcultures in M1.3 medium in the course of 20 wk, and we observed that SCV increased slowly until 10 wk (51.74 mL of SCV), followed by a faster increase in SCV and a stabilization around 16 wk at 103.48 mL of SCV Fig. 4. The curve indicated an inflection point at 10 wk and a plateau point at 16 wk that delimited the exponential or log phase as a period of high cell division rate. The increase in SCV may also reflect the elevated cell proliferation due to the larger cell clumps observed in M1.3 medium (Fig. 5*B*), compared to NBBAP medium (Fig. 5*C*). The suspension cell clusters obtained and cultivated in M1.3 were very similar to the embryogenic tissue at phase I described earlier (Fig. 2*B–E*), having a high proliferative capacity with a high proportion of small, dense, yellowish, isodiametric cells, as also observed in *Colocasia esculenta* CS (Deo *et al.* 2010). Moreover, the plateau point indicated the beginning of a stationary phase, the arrest in cell multiplication that can be an effect of endogenous factors causing cell division inhibition, or cell death.

When M1.3 was used for induction, the number of shoots obtained was almost fourfold (328) higher than for NBBAP (83). Furthermore, the percentage of albino shoots was lower in M1.3 (34%), than in NBBAP medium (69%). After transferring CS to pre-regeneration or regeneration media, regeneration of somatic embryos, multiple buds, shoots, and roots was evident (Fig. 5*D–G*). In some treatments, a high percentage of albino shoots was observed. The largest number of green shoots, as well as the highest conversion of cell aggregates into somatic embryos, were obtained in CS cultured in DD1 pre-regeneration medium followed by NBreg medium (Supplemental Table 1). The efficiency of DD1 as a pre-regeneration medium may be due to its lower 2,4-D concentration, the use of zeatin, and consequently, a lower auxin/cytokinin ratio, as compared to the induction media, which favors somatic embryo differentiation. Somatic embryo germination may have been enhanced in the presence of cytokinins in the regeneration medium, or culture conditions may have stimulated the bud differentiation and proliferation (Fig. 5*E, F*). The NB medium was initially described for rice and tested in this work for *B. brizantha* because of its content of amino acids, such as proline and glutamine. However, the original medium without BAP failed to induce regeneration in *Brachiaria*. NB medium supplemented with 0.2 mg/L BAP for induction resulted in regeneration of green shoots, regardless of the pre-regeneration and regeneration medium used. The proliferation of undifferentiated cells in different media combinations was higher in CS cultured in NBBAP than in M1.3 medium.

The media combination M1.3/DD1/NBreg/MS3liq was the most efficient for achieving multiplication and regeneration from CS (Fig. 5*A–G*). Isolated and multiple shoots, as well as embryogenic calli, were obtained from cell suspensions. Regenerated plantlets, when acclimatized in the greenhouse (Fig. 5*H*), showed morphological patterns of growth, flowering, and seed production that were, similar to tiller-propagated plants (data not shown).

In this study, a somatic embryogenesis system was established from embryogenic callus of *B. brizantha* cv. Marandu, using M1.3 medium for induction, NBBAP medium for embryo multiplication, and NBreg for plant regeneration. This system was efficient for plant regeneration, and embryogenic callus in phase I showed a high cell division rate. Additionally, a methodology for induction, maintenance, and regeneration of *B. brizantha* by cell suspensions was established, using a sequence of steps in M1.3, DD1, NBreg, and MS3 media.

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