Maltose in culture media improves the *in vitro* regeneration of *Urochloa brizantha* cv. 'Marandu' plants

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ABSTRACT

The improvement of the *in vitro* regeneration of *Urochloa brizantha* is an important step to toward the development of transgenic cultivars. The objective of this work was to test the effect of the amino acid proline and the replacement of sucrose by maltose as carbon source in culture media to optimize the somatic embryogenesis. Mature seeds of *U. brizantha* cv. Marandu were used as initial explant. The seeds were scarified in sulfuric acid, manually peeled disinfested and washed. The callus induction media were composed basically by Murashige and Skoog salts supplemented with 30 g l^{-1} sucrose, 3 mg l^{-1} 2,4-dichlorophenoxy acetic acid, 300 mg l^{-1} hydrolyzed casein and 8 g l^{-1} agar. Carbon sources maltose or sucrose at 30, 40 and 50 g l^{-1} was added in the callus induction medium. In another experiment the L-proline was added to the media at 100, 200 and 400 mg l^{-1}. It was evaluated the number of seeds that produced callus, calluses growth after 14 weeks (mg) and number of regenerated plants. The results showed maltose was more efficient than sucrose for regenerate the plants. Nevertheless, the addition of proline to the media not improved it. Therefore, substitution of sucrose by maltose to the *in vitro* culture media of *U. brizantha* increase the plant regeneration.

Keywords: brachiaria, carbon source, somatic embryogenesis

La maltosa en los medios de cultivo mejora la regeneración *in vitro* de plantas de *Urochloa brizantha* cv. 'Marandu'

RESUMEN

La mejora de la regeneración *in vitro* de *Urochloa brizantha* es un paso importante hacia el desarrollo de cultivares transgénicos. El objetivo de este trabajo fue evaluar el efecto del aminoácido prolina y el reemplazo de sacarosa por maltosa como fuente de carbono en medios de cultivo para optimizar la embriogénesis somática. Semillas maduras de *U. brizantha* cv. Marandu fueron utilizados como explante inicial. Las semillas fueron escarificadas en ácido sulfúrico, peladas manualmente, desinfectadas y lavadas. Los medios de cultivo para la formación de callos estuvieron compuestos básicamente por sales de Murashige y Skoog con 30 g l^{-1} de sacarosa, 3 mg l^{-1} de ácido 2,4-diclorofenoxi acético, 300 mg l^{-1} de caseína hidrolizada y 8 g l^{-1} de agar. Se añadieron fuentes de carbono maltosa o sacarosa a 30, 40 y 50 g l^{-1} en el medio de cultivo de formación de callos. En otro experimento, se añadió L-prolina a los medios de cultivo a 100, 200 y 400 mg l^{-1}. Se evaluó el número de semillas que produjeron callos, el crecimiento de callos después de 14 semanas (mg) y el número de plantas regeneradas. Los resultados mostraron que la maltosa era más eficiente que la sacarosa para regenerar las plantas. La adición de prolina a los medios de cultivo no lo mejoró. Por lo tanto, la sustitución de sacarosa por maltosa en los medios de cultivo *in vitro* de *U. brizantha* aumenta la regeneración de las plantas.

Palabras clave: braquiaria, fuente de carbono, embriogénesis somática

INTRODUCTION

Native from Africa, the *Urochloa* (Beauv.) genus belongs to *Poaceae* family and has approximately 100 species (Soreng *et al.*, 2017) that are among the most important for the world livestock. *U. brizantha* popularly known as brachiaria grass is one of the main forage grass to feed cattle in Brazil and feed a herd of 218.23 million head of cattle (IBGE, 2016).

The improvement of *in vitro* regeneration system in this grass is an important biotechnological approach aiming to produce transgenic varieties. Until now, no commercial transgenic plant of *Urochloa brizantha* was produced. In this way, an efficient regeneration system is the first requirement to obtaining such plants. Some modifications on the basic culture media has shown to increase the induction and regeneration efficiency of *in vitro* plants. For example, the addition of the amino acid L-proline and the replacement of sucrose by maltose as carbon sources have improve cereal regeneration and transformation (Hiei *et al.*, 2014). Although sucrose is the most widely used carbon source in tissue culture, the replacement by maltose showed better results in somatic embryogenesis and plant regeneration for a number of species including: indica rice (*Oryza sativa* L.) (Joyia and Khan, 2013), *Pinus* (Pullman and Bucalo, 2014), *Gossypium* spp. (Juturu *et al.*, 2015; Kumar *et al.*, 2015), and sugarcane (*Saccharum officinarum* L.) (Kaur and Kapoor, 2016), in different cultivars of *Triticum aestivum* L. (Ren *et al.*, 2010; Malik *et al.*, 2017) and lemongrass (*Cymbopogon schoenanthus* L.), a medicinal plant (Abdelsalam *et al.*, 2018).

The addition of L-proline in the media for *in vitro* plants regeneration has also demonstrated to improve somatic embryogenesis in different plants such as triticale a hybrid of wheat (*Triticum*), rye (*Secale*) and wheat (*Triticum aestivum* L.) (Asif *et al.*, 2013), *Persea americana* Mill. (Encina *et al.*, 2014), big bluestem (*Andropogon gerardi* Vitman.) (Pantha *et al.*, 2016), sugarcane (Kaur and Kapoor, 2016).

The protocols published for *in vitro* regeneration of Brachiaria grass so far only used sucrose as carbon source (Takamori *et al.*, 2015; Cabral *et al.*, 2018; Yaguinuma *et al.*, 2018). However, the replacement of sucrose by maltose and the addition of different concentrations of L-proline to the culture media was never be assayed for brachiaria *in vitro* plants regeneration. According to that, the aim of this paper was to test the effect of the amino acid proline and the replacement of sucrose by maltose as carbon source in culture media to optimize the somatic embryogenesis of *U. brizantha* cv. 'Marandu'.

MATERIAL AND METHODS

Plant material and callus induction medium

Mature seeds of *U. brizantha* cv. 'Marandu' were used as initial explants for calluses induction as described in Takamori *et al.* (2015). The seeds were scarified by immersion in concentrated sulfuric acid (98%) in a beaker and mixed with a glass stick for 15 minutes. The seeds were washed in tap water to remove the acid and it were dried at room temperature. Scarified seeds were manually peeled and disinfested by immersion in 70% (v / v) ethanol for 5 minutes and in 5% (v / v) sodium hypochlorite containing three drops of Tween 80® for 20 minutes followed by five washes in sterile bidistilled water.

The calluses induction medium (CIM) was composed by MS salts (Murashige and Skoog, 1962) supplemented with 3 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 300 mg l^{-1} casein hydrolyzed, and solidified with 8 g l^{-1} agar. The pH of the medium was adjusted to 5.8 ± 0.1 and it was sterilized by autoclave for 20 minutes at 121 ± 1 °C. As carbon source maltose or sucrose at 30, 40 and 50 g l^{-1} were added to the CIM medium. The calluses were sub-cultured every 14 days under the same conditions and kept in the dark (26 ± 2 °C). In the last sub-culture it remained for 30 days under these conditions. Thereafter, the calluses with embryogenics structures were subcultured to regeneration medium with half-strength MS (MS/2), 2 mg l^{-1} 6-benzylaminopurine (BAP) and the same concentration of each carbon source tested.

In another experiment, the amino acid L-proline was added to the CIM medium at 100, 200 and 400 mg l^{-1}, as described above, and containing 30 g l^{-1} sucrose.

For both, (carbon sources and proline), ten seeds were initially placed in each Petri dish containing CIM medium and the respective concentration to be tested (proline or carbon sources). After 28 days, the number of seeds that produced primary calluses were recorded and the percentage was calculated.

Six pro-embryogenic calluses per treatment were used to evaluate their growth as fresh mass (mg) at 14 weeks after inoculation. For regeneration the *in vitro* plantlets, the callus were transferred to light (30 µmol m^{-2} s^{-1}) at 26 ± 2 °C. The number of regenerated plants were recorded when plants had at least 5 cm in high and had a well-developed roots.

Double staining with acetocarmine and Evans blue was used to distinguish embryogenic from non-embryogenic callus as described in Takamori *et al.* (2015).

Statistical analysis

The experimental design was completely randomized with ten replicates, each repetition consisting of one Petri dish with six calluses each. The data were normalized and subjected to analysis of variance (ANOVA) to detect the significant differences. The mean separation was conducted by Fisher's Least Significance Difference (LSD) (P<0.05) using SISVAR Software Version 5.3 (Ferreira, 2011).

RESULTS AND DISCUSSION

Highly efficient *in vitro* regenerations protocols for *Urochloa brizantha* is a fundamental step to develop transgenic plants. Calluses were induced from mature seeds of *U. brizantha* (Figure 1 A). Double staining showed embryogenic (colored in red) from non-embryogenic callus (colored in blue) (Figure 1 B). This staining have been successfully used in different species to indicate embryogenic cells (Ahn *et al.*, 2017; Varis *et al.*, 2018). *U. brizantha* calluses friable with cream color were highly embryogenic and regenerated morphogenetic normal plants.

In this work, calluses of *U. brizantha* in media supplemented with sucrose showed no difference in primary induction calluses for any concentration (Table 1). The callus growth was damage after 14 weeks in the culture media with sucrose at 50 g l^{-1} (Table 1). Plant regeneration was higher with 30 g l^{-1} sucrose (Figure 2 A, Table 1) while at 40 and 50 g l^{-1} the number of plantlets was progressively reduced (Figure 2 BC, Table 1). At these concentrations there was almost no seedlings (Figure 3 A, Table 1). Due to the heterotrophic condition of the *in vitro* plant culture, the addition of a carbon source is essential in the medium to develop embryogenic calluses. Different kinds and concentrations of sugars in the culture media can change the osmotic pressure and modify the morphological response of the *in vitro* plant tissues (Joyia and Khan, 2013). This occurs probably due to their differential role in vascular differentiation, metabolism, endogenous content of sugars in cultured tissues, and differential sensitivity (Yaseen *et al.*, 2103).
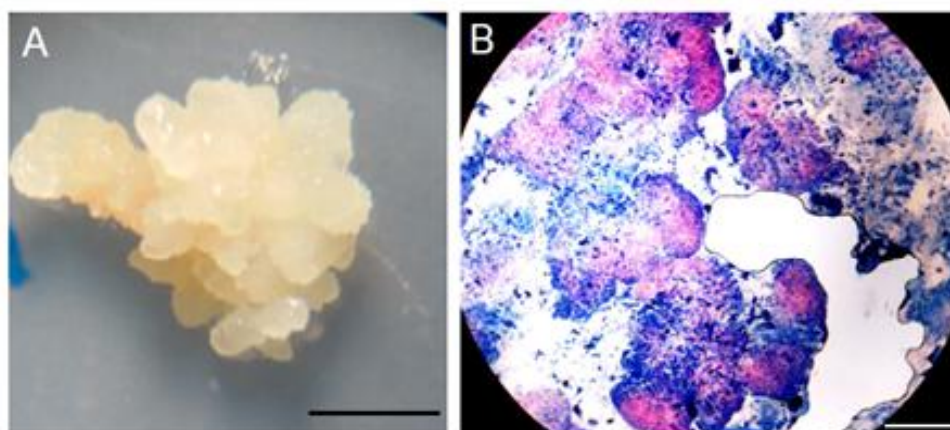

Figure 1. *Urochloa brizantha* cv. 'Marandu' callus (A). Double stained callus with evans blue and Acetocarmine dyes, 40x magnification. Bars 1 cm (A) and 500 µm (B).

Table 1. Calluses of *Urochloa brizantha* cv. 'Marandu' grown in media with maltose or sucrose as carbon sources.

Carbon sources	Concentration (g l⁻¹)	Primary callus induction (%)	Calluses growth after 14 weeks (mg)	Average of regenerated plants
Maltose	30	72.5 ± 8.5 ab	418.08 ± 53.42 a	50 b
	40	60.0 ± 10.8 b	367.27 ±104.78 ab	105 a
	50	62.5 ± 4.78 b	278.67 ± 73.33 ab	119 a
Sucrose	30	80.0± 8.16 ab	347.71 ± 68.02 ab	41 b
	40	90.0± 5.77 a	376.77 ±75.35 ab	2 c
	50	75.0 ± 6.45 ab	185.50 ± 41.77 b	0 c

Means followed by the same letter in each column did not differ significantly by the LSD test ($p<0.05$). Means ± SD
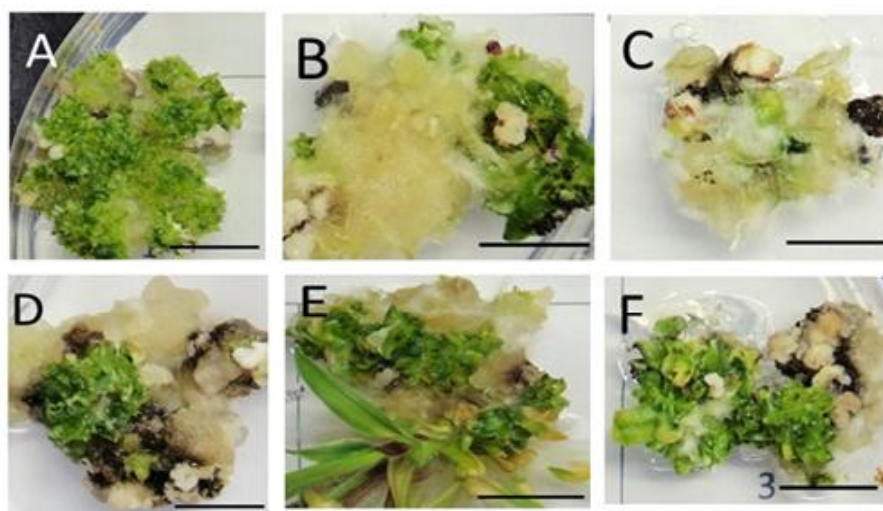

Figure 2. *Urochloa brizantha* cv. 'Marandu' callus morphology cultivated in media supplemented with different concentrations of carbon sources. ABC sucrose and DEF maltose. AD 30 g l⁻¹, BE 40 g l⁻¹ and EF 50 g l⁻¹, respectively. Bars 1 cm.

In media supplemented with maltose there were no differences in induction and growth of calluses in media with different concentrations (Table 1). However, maltose at 40 and 50 g l⁻¹ produced a higher number of regenerated plants (Table 1, Figure 1) increasing progressively according to the maltose concentration (Figure 2 DEF) (Table 1, Figure 3 B). The average number of regenerated plants at 50 g l⁻¹, was almost three times greater than the number of plants regenerated with 30 g l⁻¹ sucrose, usually used in the MS medium (Table 1). These data indicate that concentrations of 40 and 50 g l⁻¹ maltose are beneficial to the regeneration of plants in *U. brizantha* cv. 'Marandu'.

The results demonstrated that the replacement of the sucrose by maltose in culture media improved the number of regenerated plants. In other species, like conifers, it was suggested that during the course of somatic embryogenesis process, specific media composition can be necessary for each phase because the alteration on water potential in the media affect the embryo development (Shimomae *et al.*, 2013; Pullman and Bucalo, 2014).

Brachiaria was able to form callus in both maltose and sucrose although plant regeneration was greatly affected by the source of carbon used. Whereas maltose at 40 and 50 g l⁻¹ regenerated the greater number of plant the same concentration of sucrose inhibited plant regeneration. Sucrose hydrolysis more rapidly leading cells

to storage compounds and fast proliferation whereas maltose has slow hydrolysis and this could be a biochemical signal to induce somatic embryos development (Yaseen *et al.*, 2103). In kodo millet (*Paspalum scorbiculatum* Linn.) plants, maltose compared with others carbon source (glucose, fructose and sucrose) yield both great number of somatic embryos and rapid elongation of shoots for plantlet conversion (Ceasar and Ignacimuthu, 2010). The rapid stimuli of cell differentiation and metabolism provided by maltose generating readily available energy for a longer period as compared to other carbon sources (Kaur and Kapoor, 2016), it could be the reason for this result.

Besides that, maltose in the culture media avoid browning of cells during embryogenesis leading to an improvement of embryogenic calluses formation, somatic embryo germination and shoot length for cotton (*Gossypium* spp.) (Juturu *et al.*, 2015). The slower rate of extracellular hydrolysis of maltose compared to sucrose result in less phenolic secretion into culture medium and this thought to be a reason for the best performance of maltose face to sucrose (Kumar *et al.*, 2015). Although phenolic segregation was not observed in *U. brizantha* for both carbon sources, maltose in culture media improved the plant regeneration compared to sucrose.

The addition of different concentrations of amino acid L-proline in the CIM medium for inducing brachiaria plants regeneration showed less favorable results. Initially, the addition of L-proline to the media do not favored the percentage of seed that produced primary calluses (Table 2). L-proline at 100 mg l^{-1} in the media promoted calluses growth after 14 weeks cultivation and did not differed from other concentrations tested. Although there was increase in calluses weight it not reflected in the average number of regenerated plants (Table 2, Figure 4).

The amino acid L-proline when added to the culture media function as an elicitor and stimulate the pentose phosphate pathway that directs toward the shikimate and phenylpropanoid pathway, leading to the alteration on secondary metabolism (Dias *et al.*, 2016). It takes part in proteins synthesis and in the regulation of very important functions like osmotic adjustment and protection of proteins during stress conditions. It is believed that it participate in proteins structure because deficiency in proline

Figure 3. Regenerated plants of *Urochloa brizantha* cv. 'Marandu' grown in media supplemented with different concentration of sucrose or maltose as carbon sources. A: sucrose, B: maltose, 1, 2 and 3 = 30, 40 and 50 g l^{-1}, respectively.

Table 2. *Urochloa brizantha* cv. 'Marandu' calluses grown and plant regeneration on media with L-proline concentrations

Proline (mg l⁻¹)	Seeds with primary calluses (%)	14 weeks after calluses induction (mg)	Average of regenerated plants
0	82.5 ± 2.5 a	362.90 ± 59.52 b	16 a
100	70.0 ± 5.77 a	574.78 ± 91.20 a	21 a
200	75.0 ± 5.0 a	468.10 ± 55.70 ab	22 a
400	82.5 ± 4.78 a	536.03 ± 55.77 ab	18 a

Means followed by the same letter in each column did not differ significantly by the LSD test (P <0.05). Means±SD
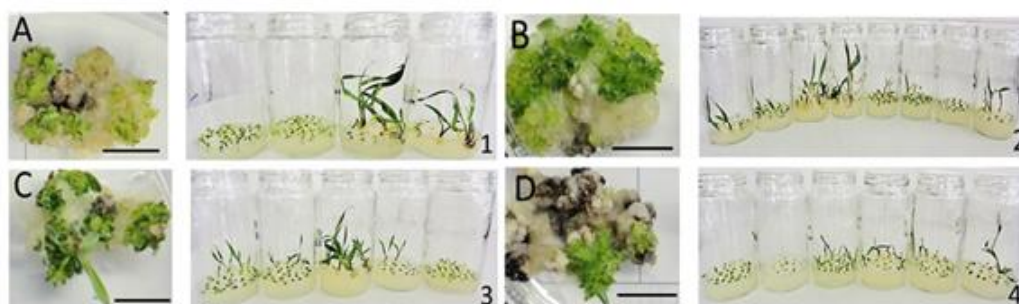

Figure 4. *Urochloa brizantha* cv. 'Marandu' callus morphology cultivated in media supplemented with different concentrations of L-proline. (A) without proline addition, (B) 100 mg l⁻¹, (C) 200 mg l⁻¹, (D) 400 mg l⁻¹ L-proline and regeneration of plants at the eighteenth week of culture at the same concentrations, respectively. (1) Without proline addition, (2) 100 mg l⁻¹, (3) 200 mg l⁻¹, (4) 400 mg l⁻¹.

biosynthesis leads to abnormal plants and cell wall defects (Kishor *et al.*, 2015). In addition to *in vitro* osmoprotection, proline has multiple functions such as protein stabilization, inhibition of protein aggregation and reduction the ROS levels under oxidative stress conditions (Bach and Hiroshi, 2013).

In this work, the regeneration of brachiaria was morphological normal even in the absence of proline in culture media. Average number of regenerated plants was not significant when compared to the ones regenerated in media without proline. This fact could be due to the concentration used. The highest concentration tested was 400 mg l⁻¹ while for regeneration of the grass *Andropogon gerardii,* Pantha *et al.* (2016) added 2 g l⁻¹ of proline. On the other hand, for sweet sorghum (*Sorghum bicolor* L.) the addition of proline (range from 0 – 2.4 g l⁻¹) was not necessary for the calluses induction (Zhao *et al.*, 2010). Like sorghum, the *in vitro* regeneration of brachiaria grass was not improved by the addition of low proline concentration (up to

400 mg l⁻¹). Higher concentrations of proline may be will test in the regeneration media.

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CONCLUSIONS

Maltose replacing sucrose in culture media for somatic embryogenesis of brachiaria improves the number of *in vitro* regenerated plants while the addition of L-proline up to 400 mg l^{-1} did not.

Conflicts of interest
The authors declare no conflicts of interest.

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The authors declare that they have no conflict of interest.

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