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Optimal concentration of selective agents for inhibiting *in vitro* growth of *Urochloa brizantha* embryogenic calli

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The ability to distinguish transgenic cells of untransformed cell mass is a key step for the production of transgenic plants. Thus, the use of selection marker genes for identification of genetically modified plants is necessary. The aim of this study was to determine the optimal concentration of four selective agents (kanamycin, hygromycin, phosphinothricin and mannose) to inhibit *in vitro* growth of *Urochloa brizantha* cv. Marandu calli. Embryogenic calli were obtained from mature seeds inoculated in MS medium supplemented with 30 g/L sucrose, 3 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 300 mg/L hydrolysate casein and their growth rate was monitored for 74 days by measuring calli fresh weight. It was demonstrated that *U. brizantha* calli are more sensitive to low concentrations of hygromycin than kanamycin (25 and 50 mg/L, respectively). For the herbicide phosphinothricin, 5 mg/L was enough to prevent the calli growth, but allowed escape. Mannose should be used as the only carbon source on the plant tissue culture medium. All selective agents tested here, in the appropriate concentration, could be used in experiments aiming to produce transgenic signal grass. However, mannose selection might reduce environmental concerns about gene flow and development of herbicide resistance in escaped *Urochloa* populations.

Key words: Signal grass, transformation, marker genes, selection.

INTRODUCTION

A number of steps are required for producing transgenic plants, such as the introduction of DNA into cells, identification or selection of cells that have the exogenous DNA integrated into the plant genome and regeneration of the transformed plant cells. Due to the low efficiency of transgene integration, selectable marker genes (SMGs) are routinely used to differentiate transformed cells from a population of untransformed cells, and are typically co-transformed with the gene of interest. Among the commonly used SMGs are those that confer tolerance to antibiotics or herbicides (Ji et al., 2013). Genes providing resistance to these compounds are known as negative selectable markers and have been used to kill or reduce the population of non-transgenic cells (Puchta, 2003).

The SMGs that confer resistance to antibiotics are involved in bacterial detoxification systems and are distinct enough from plant processes, so the interactions

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between the SMGs and the co-processing genes are unlikely (Miki and McHugh, 2004). Aminoglycoside antibiotics include a number of molecules that are very toxic to plant, animal and fungal cells by binding to the ribosomal subunits and inhibiting protein synthesis in eukaryote plastids and mitochondria. The bacterial neomycin phosphotransferase II (NPTII) has been shown to be very effective as a selectable marker in mammalian and yeast cells, and in plants (Miki and McHugh, 2004; Padilha and Burgos, 2010). Currently, 45 events of genetically modified plants have been approved for commercial release containing the *nptII* gene including: oilseed rape, corn, potato, tomato, flax, chicory, papaya, melon, plum, zucchini, sugar beet, rose, tobacco and cotton (CERA, 2015). No risk for humans, animals or on the environment has been related to using NPTII or the *nptII* gene (Fuchs et al., 1993).

Hygromycin is also an antibiotic inhibitor of protein synthesis with a broad spectrum activity against prokaryotes and eukaryotes. The *Escherichia coli* gene aphIV (*hph*, *hpt*), coding for hygromycin B phosphotransferase, confers resistance on bacteria, fungi, animal cells and plant cells by detoxifying hygromycin (Waldron et al., 1985). In plants, this antibiotic is very toxic and has been applied in transformation procedures for various monocot tissues (Sharma et al., 2005).

Amino acid biosynthesis pathways are also a target for selective agents in distinguishing transgenic from non-transgenic events. An example of marker genes used for monocotyledons transformation comprises, respectively, of the *bar* and *pat* genes from *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes*, which confer resistance to the herbicide phosphinothricin (PPT), also known as ammonium glufosinate (Thompson et al., 1987; Strauch et al., 1988). This herbicide inhibits glutamine synthetase, a key enzyme in nitrogen assimilation, causing ammonia accumulation, damage of cell membranes and inhibition of photosynthesis and, eventually, plant death.

Among the alternative methods to produce transgenic plants without the use of antibiotic or herbicide marker genes are the so-called positive selection systems, which are defined as those that allow the growth of transformed tissues (Joersbo et al., 1998; Miki and McHugh, 2004). In this system, substances that are not normally metabolized by plants are used as selective agent– for example, the carbohydrate mannose. The *manA* gene from *E. coli*, which codes the phosphomannose isomerase enzyme (PMI, E.C. 5.3.1.8) converts mannose-6-phosphate into fructose-6-phosphate, so transformed plant cells can assimilate mannose via glycolysis while non-transformed cells cannot metabolize this carbohydrate (Reed et al., 2001). The selective mode of action of this system has been suggested to be mediated by the Pi sequestration by phosphorylating mannose into mannose-P (Brouquisse et al., 2001) and/or by the inability of the plant cell to utilize mannose as a carbon source (Stoykova and Stoeva-Popova, 2011). It has been demonstrated that the use of the *manA* gene enhanced the efficiency of transformation of monocots as compared to traditional selection on herbicide containing medium (Wright et al., 2001). The *manA* gene has successfully been applied as a selectable marker in plant transformation for several dicot and monocot plants including wheat and maize (Wright et al., 2001), sorghum (Gurel et al., 2009), oil palm (Bahariah et al., 2013), sugarcane (Zhang et al., 2014) and rice (Gui et al., 2014), among others.

In addition to the choice of the appropriate SMG, the establishment of its correct concentration on the culture media is a very important step in the transformation process. Low concentrations of SMGs may allow escapes to regenerate, whereas too high concentrations impose a stringent process capable of killing the transformed plants expressing moderate levels of resistance (Ijaz et al., 2012). Therefore, the optimum concentration of selective agents has to be determined a priori by testing a variety of concentrations in the laboratory.

The *Urochloa* genus belongs to the Poaceae family, which also covers important species such as rice, wheat and maize, that together account for about half the world's food production (Bennetzen and Freeling, 1993). Although significant research progress has been made concerning *in vitro* plant regeneration and genetic transformation in grasses (Giri and Praveena, 2015), this has not been the case for *Urochloa* species. Transient expression of glucuronidase gene (*gus*) under several heterologous promoters has been first reported in *U. brizantha* (signal grass), however, no transgenic plant was regenerated (Silveira et al., 2003). The only report that describes the regeneration of *Urochloa* transgenic plants used a genotype of *U. ruziziensis* (congo grass). In that study, a vector containing the *bar* and *gus* genes, the former conferring resistance to phosphinothricin was introduced into embryogenic callus by particle bombardment, but only two transformed plants were regenerated (Ishigaki et al., 2012).

Recently, our group published a paper that described an improvement of the protocol for *in vitro* regeneration of different *Urochloa* species (Takamori et al., 2015). Here, we reported results of the optimal concentrations of four selective agents (kanamycin, hygromycin, phosphinothricin and mannose) to restrict the *in vitro* growth of *Urochloa brizantha* cv. Marandu embryogenic callus as a part of the establishment of a transformation system for a recalcitrant species like signal grass.

MATERIALS AND METHODS

Plant material and callus induction media

Mature seeds of *U. brizantha* cv. Marandu were used as initial explants for callus induction. First, the seeds were scarified by immersion in concentrated sulfuric acid in a glass Becker and mixed with glass rod for 15 min. The seeds were then rinsed in running water to remove the acid and dried at room temperature. The scarified seeds were manually peeled and sterilized by immersion

in 70% ethanol (v/v) for 5 min and in sodium hypochlorite 5% (v/v) containing 3 drops of Tween 80™ per 20 min, followed by 5 rinses in autoclaved double distilled water.

The medium for inducing callus (MIC) was composed of MS salts (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 3 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 300 mg/L hydrolysate casein, and solidified with 8 g/L agar. The media pH was adjusted to 5.8 ± 0.1 and autoclaved for 20 min at 121 ± 1°C. Ten seeds were inoculated per Petri dishes and the plates were kept in the dark at 25 ± 1°C. The calli were subcultured into fresh medium every 14 days and maintained under the same conditions.

Determination of the optimal concentration of selective agents

After 35 days of seed inoculation, the pro-embryogenic calli were transferred to the MIC medium containing different concentration of the selective agents as follows: 0, 25, 50 and 100 mg/L for the aminoglycoside antibiotics kanamycin and hygromycin, and 0, 5, 10, 20 and 40 mg/L for the herbicide phosphinothricin. In the case of mannose as the selective agent, the calli were cultivated in the MIC media containing various concentrations of mannose as the sole carbon source, or in combination with sucrose, in the following mixtures: 0:30; 10: 20; 15:15; 20:10; 30:0 g/L of mannose: sucrose. The Petri dishes were kept in the dark at 25 ±1°C.

After 30 days, the calli were weighed and transferred to MS media without 2,4-D and casein, supplemented with 30 g/L sucrose and the corresponding concentrations of kanamycin, hygromycin, phosphinothricin and Mannose:sucrose. All petri dishes were kept under light (30 µmol/m^2/s^1) with photoperiod 16/8 (light/dark) for 14 days (44 days under selection). After this period, they were weighed again and subcultured into half strength MS salts supplemented with 2 mg/L benzyladenine and kept under the same light conditions for 30 days (74 days under selection), when the last weighing was done on a precision scale. Callus relative growth rate was determined on a fresh weight basis according to the formula: [(initial weight - final weight / initial weight)] (Dennehey et al., 1994).

Statistical analysis

All the experiments were composed of a control (without selective agent) and different concentrations of the selective agents. The treatments were arranged in a completely randomized design with six replicates, each replicate consist of a Petri dish with six calli (150 mg each). The experiments were repeated three times.

Raw data were subjected to analysis of variance (ANOVA) to detect significant differences between means. Mean separation was conducted by Tukey's test ($p < 0.05$) using the statistics software SISVAR Version 5.3 (Ferreira, 2011).

RESULTS AND DISCUSSION

Calli of *U. brizantha* cv. Marandu were induced from scarified mature seeds. After 35 days in induction medium (MIC), the calli were weighed and subcultured onto media containing different concentrations of the selective agents (kanamycin, hygromycin, phosphinothricin or mannose: sucrose combinations).

Mature seeds of *U. brizantha* are known to be good explant source for callus induction. Explants cultured on modified MS medium containing 2,4-D produced embryogenic callus, characterized by whitish globular structures surrounded by friable calli (Takamori et al., 2015). Calli maintained in medium without the addition of the selective agents (control treatment) increased their mean fresh weight as much as 5 times (724 mg fresh weight) over that of the initial value at the time of inoculation in the MIC medium at the end of the experiment.

Antibiotics

In order to determine the optimal inhibitory concentration of the aminoglycoside antibiotics, kanamycin and hygromycin, the growth of *U. brizantha* cv. Marandu calli was assessed at 30, 44 and 74 days after inoculation in the different media (Figure 1A and B).

In medium with kanamycin, there was a progressive restriction in callus growth up to the concentration of 75 mg/L. After 74 days under selective conditions, the growth reduction caused by kanamycin was 37, 57 and 66% relative to the control at the concentrations of 25, 50 and 75 mg/L, respectively (Figure 1A). There were no significant difference in the magnitude of calli growth reduction between the higher concentrations (75 and 100 mg/L) of kanamycin. Treatment of *U. brizantha* callus with increasing concentrations of kanamycin produced a progressive darkening in color from white to pale-yellow. After 44 days under selection at the concentration of 25 mg/L kanamycin, some albino shoots with purple pigmentation on the leaves were visible, yet any of these chlorotic shoots were able to further elongate and regenerate into plants. There was no shoot formation at any other kanamycin concentration tested (Figure 4A).

In contrast, the presence of hygromycin in the medium caused a dramatic reduction on growth of *U. brizantha* calli already at 30 days of selection. Even at the lowest concentration of hygromycin (25 mg/L), a severe callus growth reduction (66.5%) was observed as compared to the control cultures. There was no significant differences in callus growth among all hygromycin treatments (Figure 2B), as no further increase in the concentration elicited any greater response. There was no visual morphology of calli among the hygromycin concentrations (Figure 4).

Antibiotics are extensively used as a selection agent from the beginning of plant transformation. The popularity of these selection systems is reflected on the efficiency, availability and applicability of their use across a wide range of plant species and its regenerative efficacy in plant tissue culture systems (Sundar and Sakthivel, 2008).

The susceptibility to antibiotics varies among species, genotypes and explant source (Padilha and Burgos, 2010). Generally, dicotyledonous plants are most sensitive to kanamycin than monocots. For example, a low concentration of kanamycin (50 mg/L) allowed the regeneration of transgenic adventitious buds from epicotyl sections of the citrus rootstock, Swingle citrumelo

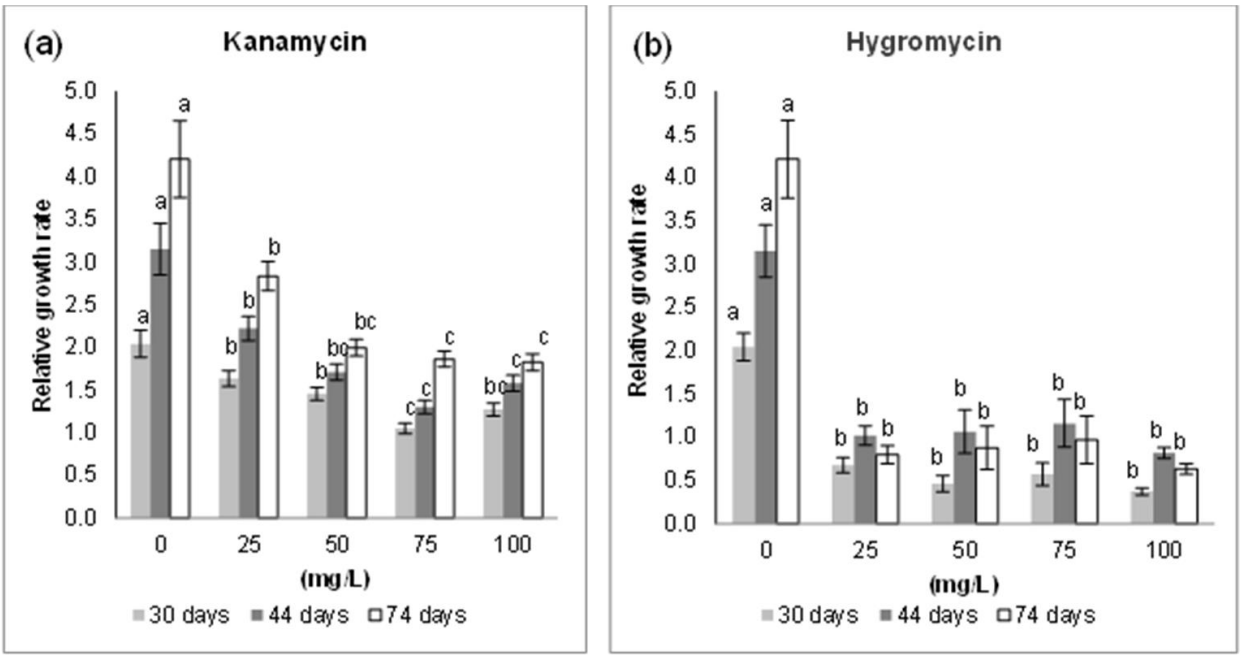

Figure 1. Relative growth rate of *U. brizantha* Marandu calli in medium containing different concentrations of kanamycin (A) and Hygromycin (B) over 74 days. Columns followed by the same letter in each sampling time did not differ significantly by Tukey's test ($P < 0.05$).

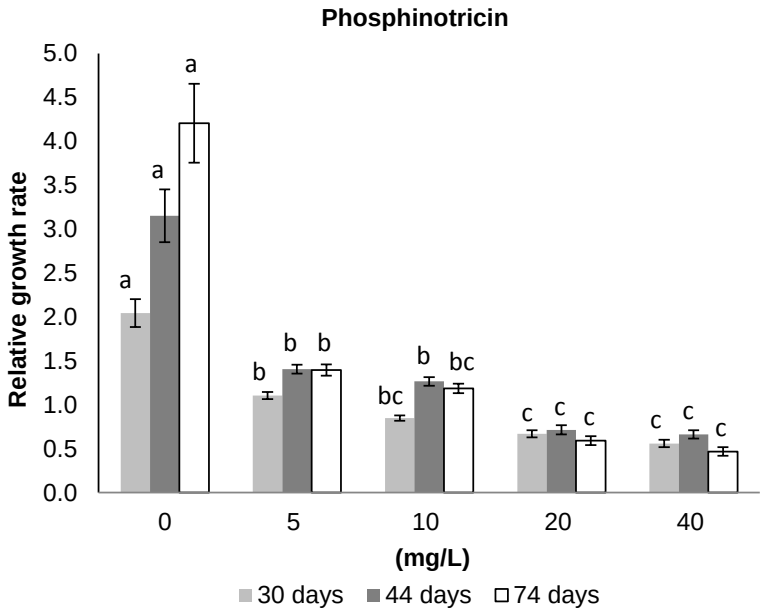

Figure 2. Relative growth rate of *U. brizantha* cv. Marandu calli in medium containing different concentrations of glufosinate ammonium over 74 days. Columns followed by the same letter in each sampling time did not differ significantly by Tukey's test ($P < 0.05$).

transformed via *Agrobacterium* (Molinari et al., 2004). Even lower concentration of kanamycin (20 mg/L) was used to recover transgenic shoots of *Jatropha curcas* (Pan et al., 2010).

In contrast, some monocots such as *Triticum monococcum*, *Panicum maximum*, *Pennisetum americanum* and a hybrid between *Pennisetum americanum*, *Pennisetum purpureum* and *Pennisetum*

squamulatum have been shown to be resistant to kanamycin selection requiring high antibiotic concentrations (800 mg/L) to inhibit 30% of growth as compared to the control (Hauptmann et al., 1988). Despite that, the antibiotic kanamycin, together with the selective marker gene neomycin phosphotransferase (*nptII*), has allowed high frequency recovering of transgenic monocots (Cheng et al., 2003; Liu et al., 2007; Gasparis et al., 2008; Liu and Goodwin, 2012). In this work, kanamycin at 50 mg/L appears to be sufficient to restrict the calli growth in *U. brizantha* cv. Marandu. This result was similar to those obtained for Caucasian bluestem (*Bothriochloa ischaemum*), a warm-season perennial grass in which calli growth was not completely suppressed but considerably reduced at the concentration of 50 mg/L kanamycin (Franklin et al., 1990).

The selectable marker gene hygromycin phosphotransferase (*htp*) is also reported to be suitable for selection of monocot transformants (Hiei and Komari 2008; Ozawa 2009) and are commonly used when *nptII* is ineffective (Miki and McHugh 2004). The growth rates of *U. brizantha* cv. Marandu calli in medium containing different concentrations of hygromycin were dramatically reduced. The concentration of 25 mg/L hygromycin was sufficient to restrict the calli growth. Similar data were reported by Ramamoorthy and Kumar (2012), who demonstrated that low concentrations of hygromycin (25 and 50 mg/L) were sufficient to restrict cell proliferation of calli of *Panicum virgatum* as compared to the control. On the other hand, another report showed that all *P. virgatum* plants regenerated on selective medium containing 25 mg/L of hygromycin escaped. Only a concentration of 75 mg/L was able to select transgenic events with the *hptII* gene (Xi et al., 2009). In maize, hygromycin was shown to be a better selective agent as compared to kanamycin, inhibiting cellular growth and proliferation at 30 mg/L (Ishida et al., 2007). In this study, it should be noted that *U. brizantha* cv. Marandu calli are more sensitive to hygromycin than kanamycin. With this latter antibiotic, some escapes occurred at the concentration of 50 mg/L kanamycin, despite the fact that the small plant shoots became chlorotic and died within few weeks.

Phosphinothricin (PPT)

The herbicide Finale® (Bayer CropScience SG), which contains in its commercial formulation, 20% of the phosphinothricin, was used at 0, 5, 10, 20 and 40 mg/L of the active ingredient in this experiment. Within the first 30 days in the selective medium, there was a cessation of active growth and slight browning of the callus surface in all concentrations tested (Figure 2). The longer the callus remained in medium containing PPT, the darker they became (Figure 5).

At 5 and 10 mg/L of PPT, the callus growth rate decreased by circa 70% as compared to the control after 74 days in the selective medium. However, the lowest concentration of the herbicide allowed the few escapes. With higher concentrations of PPT (20 and 40 mg/L), the growth rate was reduced by a factor of 4 at the end of the experimental period.

In monocots, principally, a herbicide phosphinothricin was used as the selection system to distinguish transgenic from non-transgenic events (Ishida et al., 2007; Molinari et al., 2007; Sandhu and Alpeter, 2008; Han et al., 2009). In *Paspalum notatum*, the concentration of 1.0 mg/L increased the recovery of transgenic plants and minimized the amount of escapes (approximately 10%). This result validated the use of phosphinothricin as a robust and effective selective method to obtain a transformation frequency of 64.2% in this species (Mancini et al., 2014). Three selective agents (phosphinothricin, hygromycin and paromomycin) were tested for transformation of tall fescue (Long et al., 2011). Growth of non-transformed calli was completely inhibited on callus induction medium supplemented with 100 mg/L paromomycin without any signs of newly developing callus structures, while non-transformed calli cultured on 2 mg/L PPT or 100 mg/L hygromycin grew normally for the first 1-2 weeks. After 2 weeks of selection, only transgenic calli continued to grow, while non-transformed calli displayed progressive necrosis. The *bar* gene with PPT was considered the most efficient combination for selecting transformed cells. Similar conclusions have been reported for other monocot transformation protocols (Somleva et al., 2002; Luo et al., 2004; Gondo et al., 2005).

Presently, the only transformed plant in *Urochloa* genus was achieved in *U. ruziziensis* using phosphinothricin as a selective agent. After 8-9 weeks in selective media containing 10 mg/L PPT, most of the calli were killed, and only four resistant calli (1.4% efficiency) showed strong GUS expression and remained highly embryogenic (Ishigaki et al., 2012). In this study, a complete inhibition of regeneration of *U. brizantha* cv. Marandu calli with 10 mg/L of PPT was also observed in the medium. However, as shown in *U ruziziensis,* this concentration of the herbicide can affect the development of the plants, which tend to be sterile (Ishigaki et al., 2012). Thus, it may be preferable to use a concentration of 5 mg/L PPT even if some escapes occur.

Mannose

In all three sampling periods, calli cultivated only in sucrose (30 g/L) or in combinations of mannose: sucrose (10:20, 15:15, 20:10) continued to grow and produced shoot initials. At the end of the experiment (74 days), the highest growth rate was recorded for the combination 10:20 g/L mannose: sucrose with about 3 times the initial weight (Figure 3).

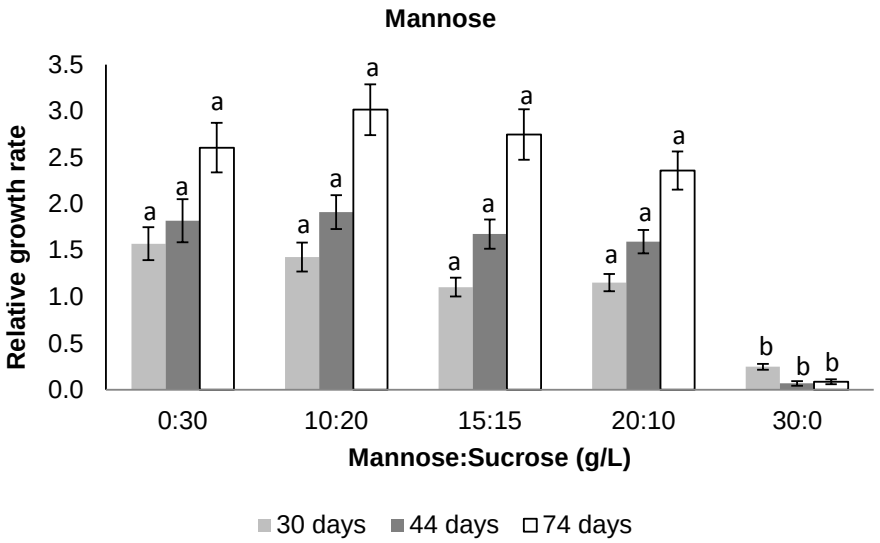

Figure 3. Relative growth rate of *U. brizantha* cv. Marandu calli cultured in medium supplemented with different combinations of sucrose and mannose over 74 days. Columns followed by the same letter in each sampling time did not differ significantly by Tukey's test ($P < 0.05$).

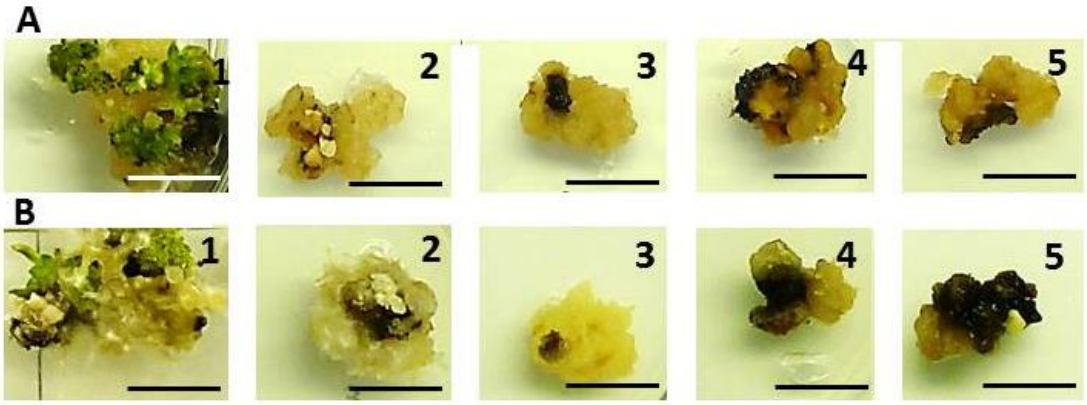

Figure 4. Morphology of *U. brizantha* cv. Marandu calli grown on media containing the antibiotics kanamycin are show in the upper row (A) while calli grown on medium containing hygromycin are shown in lower row (B) after 74 days of cultivation. The calli treated with different concentrations of antibiotics are shown in the photos marked 1 (0 mg/L – control), 2 (25 mg/L), 3 (50 mg/L), 4 (75 mg/L) and 5 (100 mg/L). Bars = 1 cm.

Independently of the sampling period, a significant difference was only detected when the calli were cultivated with mannose as the solely carbon source (Figure 3). The calli cultivated on medium containing only mannose (30 g/L) grew very poorly since the beginning of the selection procedure. A reduction in weight was observed thereafter, probably due to water loss and cells shrinkage. Regarding the morphology of the calli growing only on mannose, there was a change in color from cream to brown and gelatinous consistency with the increasing permanence of the calli in a selective media. Interestingly, root proliferation occurred at combination of 0:30, 20:10, 10:20 and 15:15 g/L mannose: sucrose, but they were not observed on the media containing mannose only (30:0) (Figure 6).

The positive selection system using phosphomannose isomerase gene (*manA*) and its correspondent selectable agent mannose have been widely used for identification and selection of transgenic cells/tissues in several monocot species (Giri and Praveena, 2015). Using mannose as selective agent demands preliminary studies to determine the best concentration of a selective agent and the need for supplemental carbon source. The toxic effect of mannose to plant cells increases with a

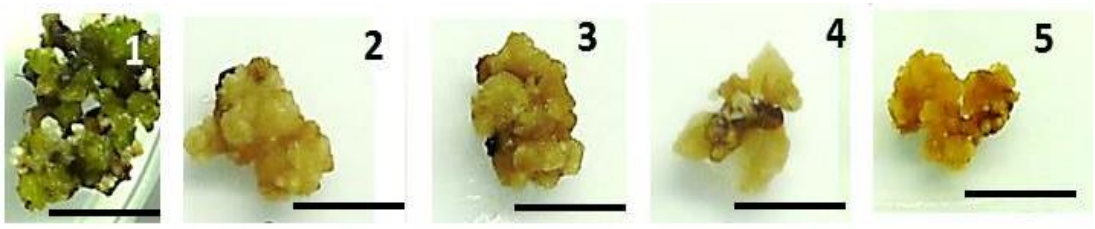

Figure 5. Morphology of *U. brizantha* cv. Maradu calli on medium containing phosphinothricin after 74 days of cultivation. A- without herbicide control, B– 5 mg/L, C– 10 mg/L, D– 20 mg/L and E– 40 mg/L. Bars = 1 cm.

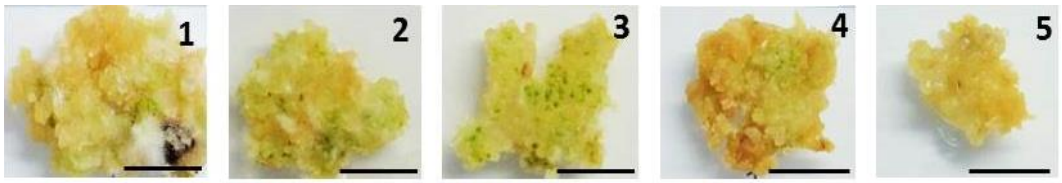

Figure 6. Morphology *U. brizantha* calli on media supplemented with different concentrations (g/L) of the mannose: sucrose A- 0:30, B – 10:20, C – 15:15, D – 20:10, E –30:0. Bars = 1 cm.

decreasing concentration of sucrose in the medium, indicating that there is an interaction between these carbohydrates (Joersbo et al., 1998). Furthermore, it has been reported that high sucrose concentrations have an additive effect in inhibiting the formation of shoots when combined with high levels of mannose (Kim et al., 2002). The addition of sucrose to selective medium containing mannose seems to have a positive effect on the recovery of transgenic corn and wheat and reduced escapes. Transformation frequency was three times higher when sucrose was added to the medium during selection (Reed et al., 2001; Wright et al., 2001).

In the first attempt to prevent *U. brizantha* calli formation using mannose as selective agent, it was demonstrated that 5 g/L mannose greatly inhibited callus formation and development of embryos even when sucrose (15 g/L) was added to the media (Silveira et al., 2003). In this study, we confirm that the use of mannose as the sole source of carbohydrate severely restricted the growth of calli and no shoots were regenerated. The present data suggest that *U. brizantha* cv. Marandu do not have the capability to metabolize mannose, which is different from the findings of Bahariah et al. (2012) who observed that palm cells are partially able to use mannose as a carbon source as indicated by the ability to form shoots. The inhibitory effect of mannose was alleviated by adding sucrose to the medium. In all other combinations of mannose and sucrose, it was observed that, the emergence of shoots, showing that medium containing only mannose should be used when *manA* is chosen as a selective marker gene for *U. brizantha* transformation.

Conclusion

In this study, the authors determined the optimal concentration of four selective agents- kanamycin, hygromycin, glufosinate ammonium and mannose– for inhibiting the *in vitro* growth of *U. brizanha* cv. Marandu embryogenic calli. All selective agents tested here, in the appropriate concentration, could be applied in experiments aiming to produce transgenic signal grass.

Although, the use of antibiotic marker genes have already been proven to be safe and very effective for transgenic plant selection on a variety of species, such SMGs from microbial origin may still cause public concerns (Breyer et al., 2014). In the case of genes conferring resistance to herbicides, as the *pat* and *bar* genes used in combination with phosphinothricin for selecting transformed plants, the main concerns are related to the introgression of the transgene in wild populations. Despite being an apomictic forage grass, the observation that *Urochloa* species exist in nature in the form of agamic complex and are cross-compatible with related species (Renvoize et al., 1996), gene flow can lead to the development of resistant volunteer plants, which may present management challenges for producers in different agricultural systems. In addition, with the transgenic trait for phosphinothricin resistance, management of volunteer signal grass could become costly. In this way, the use of mannose as a selective agent in *Urochloa* seems to be more appropriate for the development of transgenic plants for commercial purposes. The *manA* gene is considered a biosafe selectable marker due to its absence in plant genomes

and because its product, ManA, is not toxic (Stoykova and Stoeva-Popova, 2011).

Finally, this work provides information on the choice of the proper concentrations of selective agents for the establishment of more efficient transformation protocols for *U. brizantha* since no transgenic plant of this species has been regenerated so far.

Conflict of Interests

The authors have not declared any conflict of interests.

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