

Full Length Research Paper

Micropropagation of *Guadua angustifolia* Kunth (Poaceae) using a temporary immersion system RITA[®]

Luís Gonzaga Gutiérrez^{1*}, Rodolfo López-Franco² and Tito Morales-Pinzón³

¹Grupo de Investigación en Biodiversidad y Biotecnología, Facultad de Ciencias Ambientales, Universidad Tecnológica de Pereira, Carrera 27 #10-02 Barrio Alamos, Pereira, Colombia.

²Programa de Biología, Laboratorio de Biotecnología, Centro de Estudios e Investigaciones en Biodiversidad y Biotecnología (CIBUQ), Universidad de Quindío, Carrera 15 Calle 12 Norte, Armenia, Colombia.

³Grupo de Investigación en Gestión Ambiental Territorial, Facultad de Ciencias Ambientales, Universidad Tecnológica de Pereira, Carrera 27 #10-02 Barrio Alamos, Pereira, Colombia.

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The micropropagation of *Guadua angustifolia* Kunth, commonly known as giant bamboo, using semi-solid culture medium, is reported to have low multiplication rates. This study evaluated the multiplication index of *G. angustifolia* in a temporary immersion system (RITA[®]), comparing results with those obtained using a semi-solid culture medium. The treatments consisted of either three or four 2-min immersions per day and use of semi-solid culture medium, which consisted of MS supplemented with 3.0 mg L⁻¹ of the cytokinin benzylaminopurine (BAP). Equipment consisted of 20 vessels for automated RITA[®], each containing 200 ml of culture medium. Immersions were performed for 2 min at two different frequency intervals (6 and 8 h). Large clumps of *G. angustifolia* with 1, 2 or 3 stems were inoculated depending on the treatment. Best results were obtained with four immersion cycles per day (every 6 h), with a multiplication index of 2.7 shoots per original explant (axillary buds) and greater rhizome growth. Overall, the temporary immersion system performed better than the semi-solid medium in terms of shoot multiplication rates and rhizome growth. Further studies should be conducted to develop an application for RITA[®] for use in the commercial production of *G. angustifolia*.

Key words: Giant bamboo, temporary immersion system RITA, rhizome.

INTRODUCTION

Guadua angustifolia, also known as giant bamboo, belongs to the Poaceae family, one of the four largest families of the plant kingdom, harboring from 600 to 700 genera and nearly 10,000 species (Soderstrom et al., 1988). In Colombia, most populations of *G. angustifolia*

grow between 0 and 1800 m altitude, occupying diverse habitats of tropical moist forests (Bh-T) and premontane wet forests (Bmh-P), where they form small patches along rivers or streams (Londoño, 1990).

Because of its widespread distribution, versatility and

*Corresponding author. E-mail: luisgon@utp.edu.co

physical and mechanical characteristics, *G. angustifolia* has played an important environmental, cultural and economic role in Colombia. However, for these same reasons, it has also suffered indiscriminate exploitation. Enormous pressure has been exerted on natural populations of this species, reducing the areas planted and endangering not only the species but also other associated organisms (Cruz, 2009). Furthermore, *G. angustifolia* produces seeds with low germination capacity, which makes sexual reproduction difficult. The deterioration of stands of *G. angustifolia* during vegetative multiplication is further justification for exploring micropropagation approaches to develop efficient mass multiplication methods for this important species using explants (Cruz, 2009).

Propagation studies are appropriate in the case of *G. angustifolia* because *in vitro* multiplication techniques are an alternative for species conservation, providing abundant high-quality planting material. Manzur (1988) reported that *G. angustifolia* plants perform similarly in *in vitro* and *in situ* conditions. As a result, when micro-cuttings from lateral branches are cultured in enriched culture medium, their axillary buds activate the development of intercalary meristems with roots emerging from their basal nodes. The rhizome is immediately formed, which then becomes the fundamental structure in the micropropagation of *G. angustifolia*.

Only a few studies have been reported on the micropropagation of *G. angustifolia* (Manzur, 1988; Marulanda et al., 2005; Jiménez et al., 2006; Daquinta et al., 2007). Most studies conducted on micropropagation of bamboo have focused on Asian species (Muñoz et al., 1989; Saxena, 1990; Gielis and Oprins, 2000; Sood et al., 2002; García et al., 2007; Lin et al., 2007; Ogita et al., 2008; Venkatachalam et al., 2015; Gantait et al., 2016).

In Colombia, several studies have been conducted on the micropropagation of *G. angustifolia*, achieving partial success at different stages of solid culture medium. The first reports by Manzur (1988) showed low multiplication rates (less than 2 shoots/explant); however, Marulanda et al. (2005) subsequently reported a survival rate of 30% during the establishment phase and Acosta and Guzmán (1993) reported a better multiplication rate when 5.0 mg L⁻¹ 6-benzylaminopurine (BAP) was added to the culture medium, supplemented with kinetin 1.0 mg L⁻¹ and indoleacetic acid (IAA) 1.0 mg L⁻¹.

Subsequently, Marulanda et al. (2005) and Jiménez et al. (2006) used BAP (2.5 to 5.0 mg L⁻¹) as growth regulator, reporting low multiplication rates. Large losses have also been recorded during the establishment phase of *G. angustifolia* because of bacteria-induced contamination (Cruz et al., 2007; Ramírez et al., 2009).

Depending on the consistency of the culture medium (liquid, semi-solid and solid) used in plant micropropagation, several systems have been developed to increase the multiplication index and reduce costs. Taking into account the liquid condition of the medium, a system

was initially designed that consisted of a large, elevated culture chamber that could be drained and then refilled with fresh medium (Tisserat and Vandercook, 1985). Subsequently a semi-automated system was developed in which plants were cultured in a large container on a medium that contained no gelling agent, with automatic addition and removal of liquid medium at fixed intervals so the plant material entered into contact with the medium several times throughout the day (Aitken-Christie and Davies, 1988).

Simonton et al. (1991) developed a programmable apparatus, which intermittently applied culture medium to the plants according to a pre-defined schedule, serving as basis for the temporary immersion system (TIS) for plant propagation (Alvard et al., 1993). This system, whose commercial name is RITA for recipient for automated TIS, has been successfully used with many plant species, achieving significant increases in multiplication rates and allowing the semi-automation of processes (Lorenzo et al., 1998). Furthermore, the automation of one or more phases of micropropagation can help reduce not only handling costs but also costs related to laboratory space, while increasing production volume (Castro and González, 2000; Capote et al., 2009).

The frequency and time conditions required to achieve efficiency in TIS processes are determinants for system optimization (Etienne and Berthouly, 2002). Albarrán et al. (2002) confirmed that the massive regeneration of somatic embryos of coffee (*Coffea arabica*) improved when exposure in terms of frequency and duration was optimized. Increases in daily frequency (1×1', 2×1', 6×1') stimulated embryo production without affecting embryo quality.

The TIS (Figure 1A and 1B) offers multiple advantages as compared with propagation using semi-solid culture media (Figure 1C) in terms of shoot multiplication in different plant species (González et al., 2005; Roels et al., 2006; Barberini et al., 2011). In the case of banana, increases have been achieved in high average multiplication rates, with improved plant quality (Albany et al., 2005; Berthouly and Etienne, 2005; Watt, 2012; Pérez et al., 2013). Only a few bamboo species such as *Dendrocalamus latiflorus* (Mongkolsook et al., 2005 cited by García-Ramírez et al., 2014), *Bambusa ventricosa* and *Dracaena deremensis* (Chaille, 2011) and *Bambusa vulgaris* (García-Ramírez et al., 2014) have been propagated by TIS. This study aimed to optimize shoot production for micropropagation of *G. angustifolia* for commercial purposes.

MATERIALS AND METHODS

Plant material

The study was carried out at the Plant Biotechnology Laboratory of the Universidad Tecnológica de Pereira (UTP), where test plants

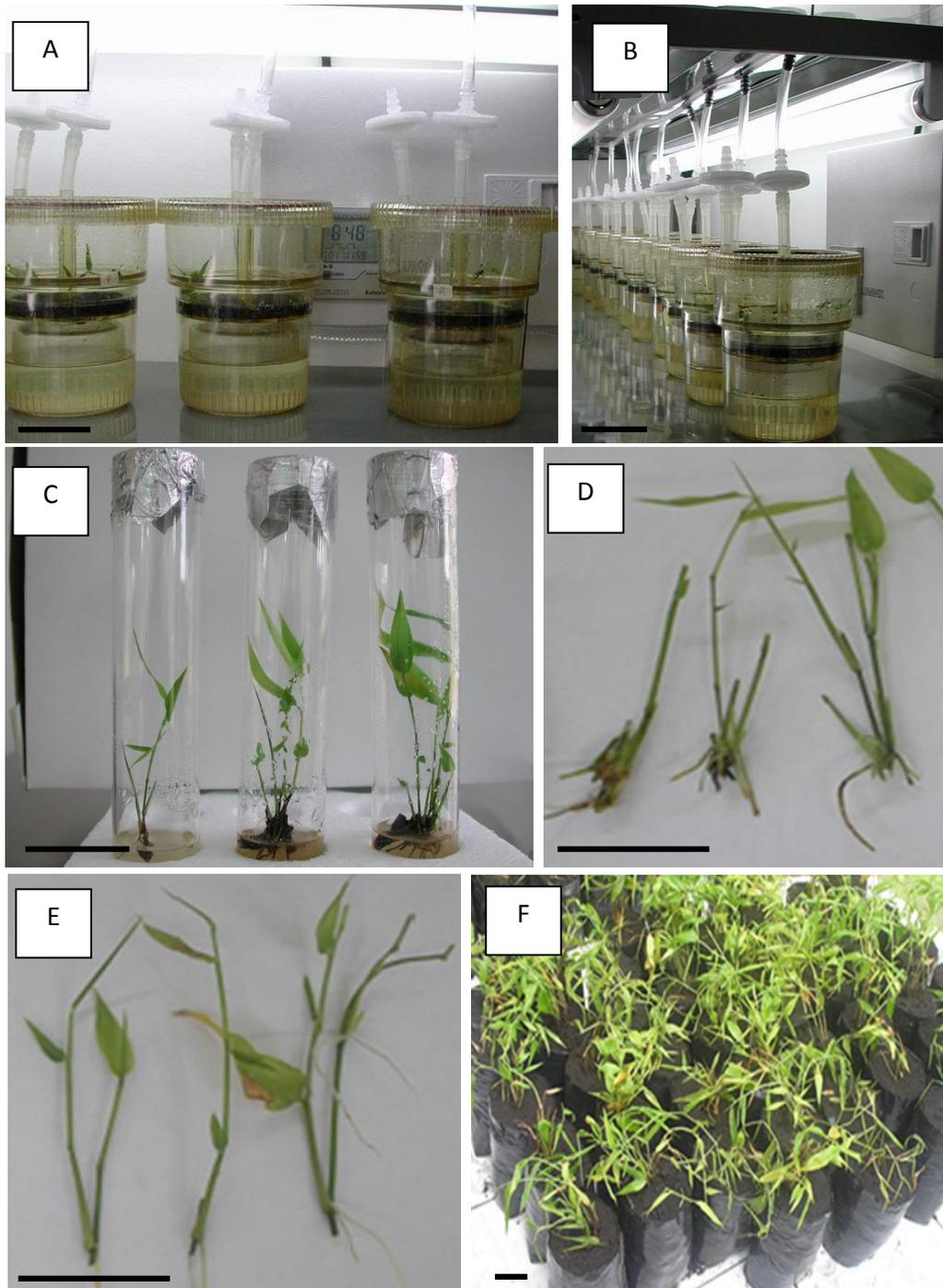


Figure 1. *Guadua angustifolia* shoot multiplication in RITA® (A and B). Shoots of *G. angustifolia* in semi-solid medium (C). Plantlets of *G. angustifolia* obtained from semi-solid medium: non-segmented (presence of rhizome) (D) and segmented (absence of rhizome) (E) and *ex-vitro* acclimatization of plant produced (F). Scale bar, 5 cm.

had been previously established *in vitro*. The study compared the multiplication indexes of *G. angustifolia* plants submitted to TIS with the indexes reached when plants were cultured in semi-solid medium (SSM). Explants, measuring 5 to 10 cm, were inoculated according to the protocol proposed by Marulanda et al. (2005).

Culture conditions

The culture medium, containing MS salts and vitamins, was supplemented with 100 mg L⁻¹ myo-inositol and 30 g L⁻¹ sucrose. Gelrite® was used as gelling agent in the solid medium at 2.5 g L⁻¹,

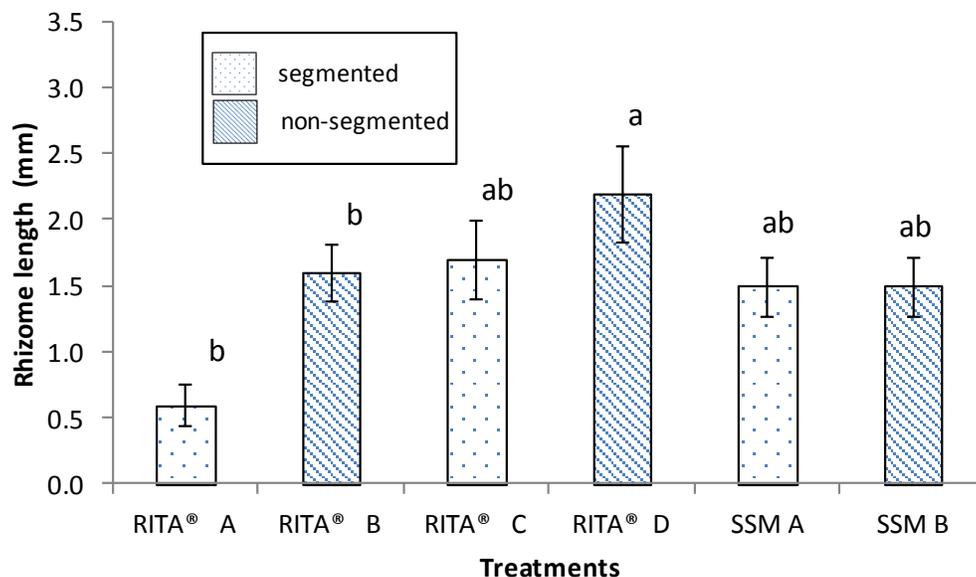


Figure 2. Rhizome size in *Guadua angustifolia* plants depending on culture media and condition of plantlet during four weeks of culture, where RITA® A = 3 cycles, segmented; RITA® B = 3 cycles, non-segmented; RITA® C = 4 cycles, segmented; RITA® D = 4 cycles, non-segmented; SSM A = semi-solid medium, segmented; SSM B = semi-solid medium, non-segmented. Different letters indicate statistically significant differences between groups (mean \pm standard error, $n = 10$, Tukey test, $p < 0.05$).

and BAP was used as growth regulator at a concentration of 3 mg L⁻¹.

A total of 200 ml MS basal medium was added to each culture vessel containing liquid medium (TIS) (Figure 1A and 1B), whereas 50 ml were added to each vessel (200 ml) containing SSM. Vessels of both treatments were autoclaved at 121°C at 15 psi, using RITA® vessels developed by CIRAD (Teisson and Alvard, 1995) (Figure 1A and B), being left for 30 min in the case of TIS and 20 min in the case of SSM.

Five explants were inoculated per TIS vessel, whereas only one explant was inoculated per SSM vessel. All plants were placed in a growth chamber with an average temperature of 24 \pm 2°C and a 12-h artificial photoperiod (lux).

The multiplication index of *G. angustifolia* plants using the TIS, which consisted of three or four 2-min immersions per day (A = 3 cycles, segmented; B = 3 cycles, non-segmented; C = 4 cycles, segmented; D = 4 cycles, non-segmented), was evaluated and compared with the multiplication index of plants cultured in SSM (A = segmented; B = non-segmented). Large 5 to 10 cm clumps of segmented (absence of rhizomes) and non-segmented (presence of rhizomes) of *G. angustifolia* were used in the inoculation. The number of stems was used as basis to evaluate rhizome generation, shoot multiplication index, and increase in plant height over a 4-week period. Rhizome division was based on the following variables: large clumps, either segmented (Figure 1D) or non-segmented (Figure 1E), and number of stems (1, 2 and 3) per inoculation unit.

Statistical analysis

A completely randomized block design (10 blocks) was used, with each block corresponding to one of the established plants (approximately 40 mm long) and a factorial arrangement of two controlled factors: culture medium and conditions of plants at planting.

Data were analyzed using a random block design with 20 replicates (explants). Each replicate corresponded to one explant and each variable depended on the study factors (rhizome size, shoot multiplication index and increase in plant height after 4 weeks of culture). The data obtained were submitted to the Tukey test to compare the means of the applied treatments.

RESULTS AND DISCUSSION

Effect of culture system on rhizome size for segmented and non-segmented *G. angustifolia*

Analysis of variance indicated that highly significant differences exist in rhizome growth for large clumps of segmented and non-segmented *G. angustifolia*, depending on culture medium consistency (TIS or SSM). The Tukey test, designed to make pairwise comparisons among means, indicated that rhizome growth was greater, but non-significant ($p > 0.05$) in plants cultured in the TIS with four 2-min immersions per day as compared with plants cultured in the TIS with three 2-min immersions per day or those cultured in SSM. For RITA®, average rhizome growth was greater in non-segmented large clumps than in segmented large clumps, presenting significant differences ($p < 0.05$) when 3 immersion cycles was used (Figure 2).

Manzur (1989) confirmed that when the rhizome originates in *in vitro* conditions, the established *G. angustifolia* plant is capable of generating a large group of successive plants. Furthermore, Londoño (1991) considers bamboo rhizomes to be segmented axes,

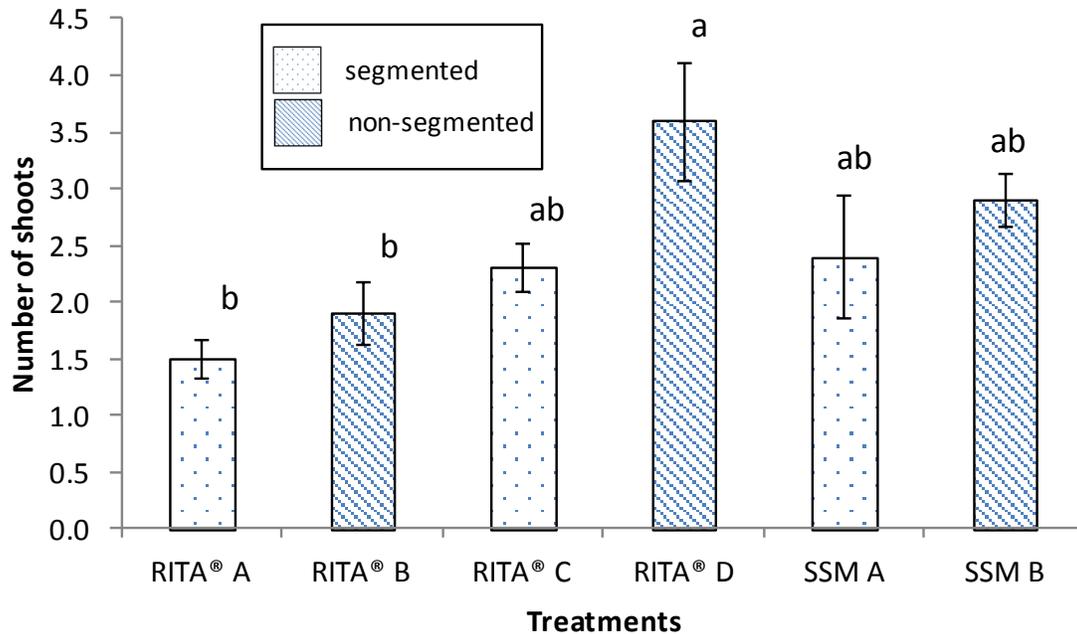


Figure 3. Multiplication of *Guadua angustifolia* shoots depending on culture media and conditions of plantlets during four weeks of culture, where RITA® A = 3 cycles, segmented; RITA® B = 3 cycles, non-segmented; RITA® C = 4 cycles, segmented; RITA® D = 4 cycles, non-segmented; SSM A = semi-solid medium, segmented; SSM B = semi-solid medium, non-segmented. Different letters indicate statistically significant differences between groups (mean \pm standard error, $n = 10$, Tukey test, $p < 0.05$).

characterized by having a diameter larger than that of the stem that it will produce. Mostly single rhizome buds appear on nodes from which new plant structures will emerge. Rhizome size was accordingly larger on bamboo plantlets cultured in the TIS with four 2-min immersions per day than when cultured on SSM (Figure 2).

Effect of culture system on shoot multiplication for segmented and non-segmented *G. angustifolia*

The analysis of variance indicated that there are highly significant differences ($p = 0.0023$) in the number of shoots produced per explant depending on culture medium consistency. Significant differences also occurred between segmented and non-segmented explants. For the Tukey test, plantlets cultured in the TIS (Figure 3), with four 2-min immersion cycles, showed a higher, but non-significant ($p > 0.05$) average number of shoots than plants cultured in the TIS with three 2-min immersion cycles and in SSM. Furthermore, non-segmented plants presented a higher number of shoots than segmented plants (Figure 3). These results agree with those of studies carried out in sugarcane (*Saccharum officinarum*) by Lorenzo et al. (1998) and in *Eucalyptus grandis* by Castro and González (2000), two species that presented a higher increase of biomass in the TIS than in solid and semi-solid culture media.

Effect of culture system on growth of segmented and non-segmented *G. angustifolia*

The analysis of variance revealed that the culture medium consistency (TIS or SSM) significantly affected growth of *G. angustifolia*. Highly significant differences in plant height were also observed when segmented and non-segmented plants were compared in RITA® (Figure 4). Plants cultured in SSM presented greater growth than those segmented and cultured in the TIS (four and three 2-min cycles per day). Overall, non-segmented plants presented higher average growth than segmented plants (Figure 4).

Plant elongation during the multiplication phase in the solid culture medium is similar to that reported by Marulanda et al. (2005) and Jiménez et al. (2006). Plant elongation in the TIS (four 2-min immersions per day) is similar to that obtained using the solid culture medium with non-segmented explants.

In contrast, Castro and González (2000) reported that plant size of *E. grandis* increased in the TIS as compared with plants cultured in solid medium. Lorenzo et al. (1998) also found that sugarcane plants grew taller when cultured in TIS (10.29 cm on average) as compared to solid media (6.22 cm on average).

Fifty seedlings cultured in solid media and 50 cultured in TIS were transferred to the nursery using the hardening protocol developed by Marulanda et al. (2005) (Figure

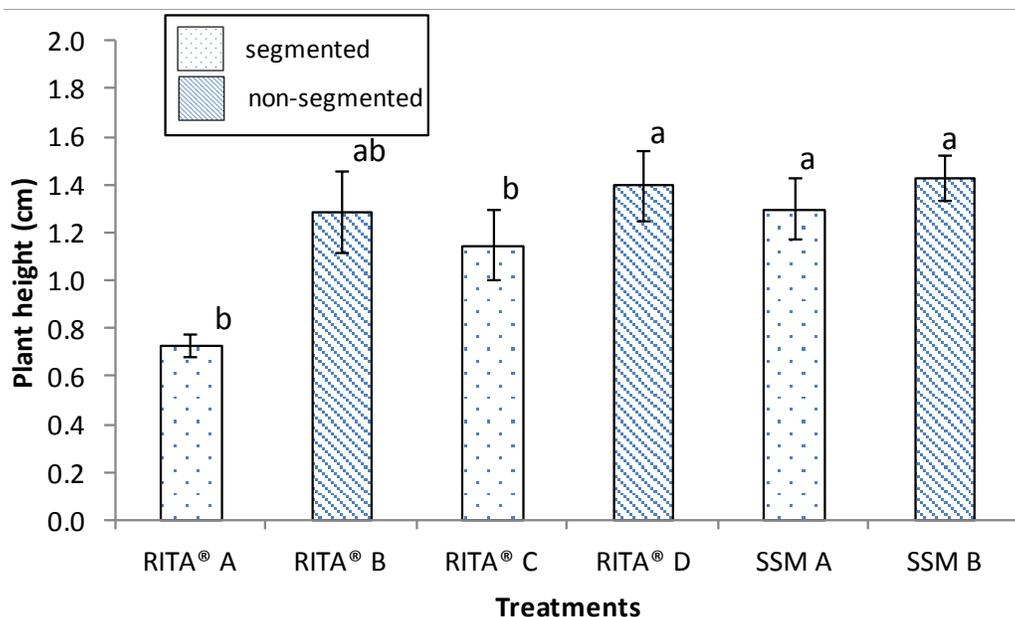


Figure 4. Increase in plant height of *G. angustifolia* plantlets depending on culture media and conditions of plantlets during four weeks of culture, where RITA® A = 3 cycles, segmented; RITA® B = 3 cycles, non-segmented; RITA® C = 4 cycles, segmented; RITA® D = 4 cycles, non-segmented; SSM A = semi-solid medium, segmented; SSM B = semi-solid medium, non-segmented. Different letters indicate statistically significant differences between groups (mean \pm standard error, n = 10, Tukey test, p < 0.05).

1F). No significant differences in plant acclimatization were observed between groups, with a 95% survival rate of plants (data not shown). Plants of *G. angustifolia* from both treatments were then transferred to the field, where plant growth and development are currently being evaluated. Survival rates for other Poaceae in the hardening phase have been reported to be higher than 70% (Gielis and Oprins, 2000; Marulanda et al., 2005; Jiménez et al., 2006).

Effect of culture system on shoot multiplication for segmented and non-segmented *G. angustifolia*

In this study, the average number of shoots produced by plants cultured in the TIS (four 2-min cycles per day) was higher than that of plants cultured in the SSM (Figure 5). The TIS (3 stems) with four 2-min immersions per day produced an average multiplication rate of 3.0 shoots per original explant for non-segmented plants (Figure 5). Marulanda et al. (2005) found an average multiplication rate of 2 shoots per original explant for *G. angustifolia* cultured on solid media, whereas Jiménez et al. (2006) reported an average of 2.5 new individuals (referred to as tillers) per each original plant established. When the averages of all three studies were compared, the higher multiplication efficiency in the TIS reached in this study is clearly demonstrated. In addition, the multiplication rate in

solid culture medium yielded results similar to the aforementioned results, with an average multiplication rate of 2.7 shoots per explant.

Using micropropagation in the liquid system with species of the subfamily Bambusoideae, Das and Pal (2005) with *Bambusa balcooa* and Shirin and Rana (2007) with *Bambusa glaucescens* reported successful work in the *in vitro* multiplication phase. In addition, bamboo species such as *Dendrocalamus latiflorus* (Mongkolsook et al., 2005), *Bambusa ventricosa* and *Dracaena deremensis* (Chaille, 2011) and *Bambusa vulgaris* (Garcia-Ramirez, 2014) have been propagated by TIS, with results similar to those found for *G. angustifolia* in terms of rate of multiplication (3 to 5 shoots per explant).

There are similar reports for other species. According to Lorenzo et al. (1998), the *in vitro* multiplication of sugarcane shoots per plant reached higher levels (average of 8.13 shoots) in the TIS than when a solid culture medium was used (average of 3.96 shoots). Yan et al. (2010) also achieved better results with *Siraitia grosvenorii* when RITA® was used for plant regeneration than with solid culture media.

In this study, the number of shoots emerging from the rhizome was higher for both SSM and RITA when plants were cultured with three stems than when the culture was performed with two and one stems per plant. When cultured in the TIS (four cycles per day), *G. angustifolia*

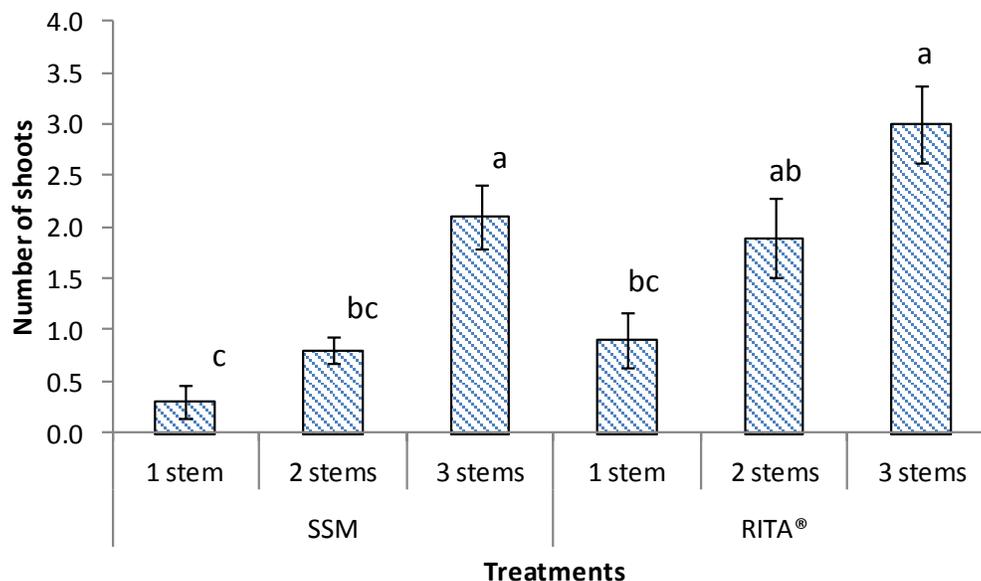


Figure 5. Multiplication rate depending on the number of non-segmented shoots per initial explant inoculated in semi-solid medium and RITA® (four 2-min cycles per day). Different letters indicate statistically significant differences between groups (mean \pm standard error, $n = 10$, Tukey test, $p < 0.05$).

plants with three stems produced the highest number of shoots (Figure 5).

For Ravikumar et al. (1998), the number of shoots generated by bamboo plants differs depending on the species and the inoculated explants. Kane (2000) has also found that the type of explants used in micro-propagation processes, whether nodal segments or shoots, stimulate the generation of axillary buds depending on each species.

Conclusion

Multiplying *G. angustifolia* using TIS highlights the importance of the presence of the rhizome (non-segmented explants) for optimal production of shoots. This study also presented good results with a frequency of three 2-min immersions per day, producing 2.7 shoots per original explant. Further studies should be conducted to develop an application for RITA® for use in the commercial production of *G. angustifolia*.

Conflict of interests

The authors have not declared any conflict of interests.

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Abbreviations

BAP, 6-Benzylaminopurine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **GA**, gibberellic acid; **IAA**, indoleacetic acid; **MS**, Murashige and Skoog (1962); **NAA**, naphthalene acetic acid.

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