

***In vitro* propagation in Temporary Immersion System of sugarcane plants variety 'RB 872552' derived from somatic embryos**

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ABSTRACT

In this study, we used a temporary immersion system (TIS) to multiply sugarcane (*Saccharum* spp.) plants obtained by somatic embryogenesis (SE). SE was induced from immature leaf segments that were grown in culture medium supplemented with 2,4-D and BAP. Embryo formation occurred in 81% of the inoculated explants and 254 plants were regenerated. Ninety plants were transferred to TIS and cultured in medium supplemented with BAP. After three subcultures, 60 000 plantlets were obtained and transferred to rooting media. After 30 days of acclimatization period plantlets were well developed and exhibited a 96% survival. The results demonstrate the feasibility of the combined use of two important techniques of *in vitro* culture (SE and shoot multiplication in TIS) to sugarcane *in vitro* propagation.

Key words: acclimatization, 6-benzylaminopurine, *Saccharum* spp.

Propagación *in vitro* en Sistemas de Inmersión Temporal de plantas de caña de azúcar variedad 'RB 872552' obtenidas de embriones somáticos

RESUMEN

En este estudio se utilizaron sistemas de inmersión temporal (SIT) para la multiplicación de plantas de caña de azúcar (*Saccharum* spp.) obtenidas por embriogénesis somática (ES). La ES fue inducida en segmentos de hojas inmaduras cultivadas en medio de cultivo con 2,4-D y BAP. La formación de embriones ocurrió en el 81% de los explantes inoculados y se regeneraron 254 plantas. A los SIT se transfirieron 90 plantas que fueron cultivadas en medio de cultivo con BAP. Después de tres subcultivos, se obtuvieron 60 000 plantas que fueron transferidas a medio de cultivo de enraizamiento. Las plantas mostraron un buen desarrollo, con una supervivencia del 96% después de 30 días de aclimatización. Los resultados demostraron la viabilidad del uso combinado de dos técnicas de cultivo *in vitro* (ES y la multiplicación de brotes en SIT) en la propagación *in vitro* de caña de azúcar.

Palabras clave: aclimatización, 6-bencilaminopurina, *Saccharum* spp.

INTRODUCTION

Somatic embryogenesis (SE) is the primary method used for *Saccharum* spp. improvement through genetic engineering (Lakshmanan *et al.*, 2005). SE has proven to be more efficient for the generation of transgenic plants than organogenesis (Desai *et al.*, 2004; Singh *et al.*, 2013). However, for commercial micropropagation of sugarcane, plants are typically regenerated through organogenesis from shoot tips in conventional culture (static) and in temporary immersion system (TIS) (Lemos, 2009).

The advantages of TIS for growth and development of plants have been demonstrated in different species of commercial importance, such as *Musa* spp. (Roels *et al.*, 2005), *Phalaenopsis* and *Cymbidium* orchids (Tirado *et al.*, 2005; Gao *et al.*, 2014) and *Ananas comosus* (Silva *et al.*, 2007), besides, the shoot multiplication of sugarcane (Lorenzo *et al.*, 1998; Mordoco *et al.*, 2009).

In micropropagation protocols, the multiplication phase, which includes numerous subculturing steps, consumes the

greatest amount of time and resources in the laboratory. The acclimatization stage should be performed carefully, taking into account the high commercial value of plantlets. Furthermore, micropropagated plantlets should be handled in order to ensure their survival and full development under environmental conditions of nurseries and greenhouses, which differ extensively from the *in vitro* conditions (Chandra *et al.*, 2010).

This study describes the multiplication in TIS and the acclimatization of sugarcane plants, variety 'RB 872552', generated by SE.

MATERIALS AND METHODS

Plant material

Plants of sugarcane (variety 'RB 872552') previously established *in vitro* were selected and transferred to MS (Murashige and Skoog, 1962) culture medium without growth regulators. After 40 days, the plants were used to induce SE.

Somatic embryogenesis

Somatic embryos were obtained from immature leaf fragments grown in MS culture medium supplemented with 3% (w/v) sucrose, 13.6 μM 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1.1 μM 6-benzylaminopurine (BAP), 6 g l^{-1} agar, and pH 5.8. The explants were kept in a growth room at 25 ± 2 °C with a photoperiod of 16 h and a luminous intensity of 47 $\mu\text{mol m}^{-2} \text{s}^{-1}$, during 40 days (Silva *et al.*, 2014).

After the conversion phase, which was accomplished through the suppression of growth regulators in the culture medium, the number of regenerated plants was quantified.

Multiplication in TIS

Ninety regenerated plants (> 2 cm, measured with a ruler from the base of stem until the last expanded leaf) were transferred to three TIS (30 plants per TIS), each with a 5 liters capacity, and containing MS medium (pH 5.8) supplemented with 3% sucrose and 0.9 μM BAP. The TIS were incubated under a 25 ± 2 °C, photon flux density of 47 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16 h photoperiod and immersion frequency of 5 min every 3h. After 8 weeks, the

secondary shoots (tillers) generated were subcultured to a new TIS (50 tillers per TIS), which were maintained under the culture conditions described above. Two other subcultures were performed every 4 weeks, and the number of plants formed and multiplication rate were recorded.

Rooting and acclimatization

After the multiplication stage the tillers were transferred to TIS containing rooting medium composed of MS salts with 53.7 μM α -naphthaleneacetic acid (NAA) and were maintained during 15 days under same culture conditions described above. Afterward, plants with an average height of 9.5 cm, fresh mass of 0.326 g, average of four leaves per plant and presence of roots, were transferred to a greenhouse (30 °C and 56-80% relative humidity). The substrate was composed of sugarcane bagasse, sand and clay (1:1:1 ratio), and irrigation was performed three times a day (morning, afternoon and night) to the point of drainage. The plants were analyzed every 15 days for stem and root length, and basal stem diameter using a ruler and a digital caliper, respectively, and the number of leaves was counted. The fresh biomass of aerial and root parts were weighed, and then the parts were dried in a drying oven (60°C) to a constant mass. The survival rate and tillering were assessed after 30 days of acclimatization stage.

The experiment was conducted in a completely randomized design. Sixty replicates were used for ES induction, and 50 plants were evaluated every 15 days (until 30 days) during acclimatization. The data were analyzed by descriptive analysis.

RESULTS AND DISCUSSION

The initial formation of somatic embryos occurred on the 8th day. After 40 days, 81% of inoculated explants showed a positive response with somatic embryos developed directly from leaf tissue (Fig. 1a). At the end of conversion stage, within 90 days after inoculation, 254 plants were regenerated (Fig. 1b, c).

After subcultures in multiplication medium, 150 TIS (Fig. 1d) containing approximately 400 plants each, totaling 60 000 plants, were

obtained (Table 1). In the last subculture, it was obtained a multiplication rate of 1:12.34, demonstrating the effectiveness of the TIS use. Yadav *et al.* (2014), studying the effect of trimming of propagules on the rate of shoot multiplication of sugarcane, obtained 1:6.0 as the higher average, using shoot clumps with 3.0 cm of length and conventional culture in flasks.

At the end of the multiplication and rooting phases, which lasted approximately 130 days, the plants showed adequate developed (size, number of leaves, coloration, and root system) and were acclimatized (Fig. 1e). Because of increased automation, reduced costs, enhanced production rate and a high quality of plant material, TIS offers many advantages over conventional micropropagation procedures (Lima *et al.*,

2012). The increased of multiplication rate and the marked improvements in the quality of the plants generated in TIS are due, in part, to the regular supply of nutrients and oxygen (Watt, 2012).

After 30 days of acclimatization, the plants had a 96% survival rate and exhibited an increase in all of the growth parameters assessed (Table 1). The rate of tillering was 1:2.88. Thus, from the 5 400 plants that were planted in greenhouse, 15 552 plants suitable for planting in the field were obtained. Plantlets quality is extremely important because it influences survival and growth rates. Therefore, to obtain fully-grown, well-developed healthy plants, the control of limiting factors, such as light, temperature and humidity, are basic conditions that are necessary for successful acclimatization (Souza *et al.*, 2006).

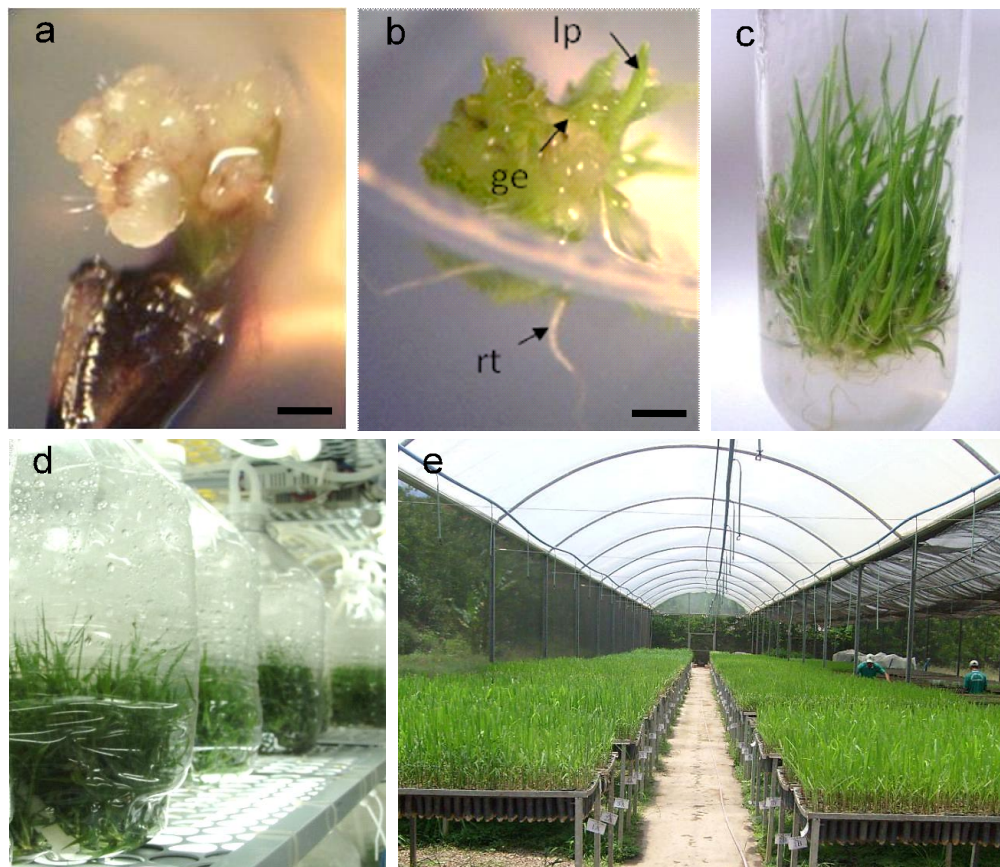


Figure 1. Sugarcane plants variety 'RB 872552', derived from direct somatic embryogenesis and multiplied in TIS. (a) Somatic embryos formed in explant (*bar* = 1 mm). (b) Beginning of the conversion of somatic embryos into plants (*bar* = 2 mm). (c) Plants regenerated from somatic embryos. (d) Multiplication of plants in TIS. (e) Acclimatization of plants multiply in TIS. *lp* leaf primordia; *ge* globular embryo; *rt* root.

Table 1. Multiplication in TIS of sugarcane plants, variety 'RB 872552', obtained from somatic embryogenesis, and growth parameters assessed during acclimatization.

MULTIPLICATION					
Multiplication phase in TIS	Cultivation time (weeks)	Number of TIS	Initial number of plants	Final number of plants	Multiplication rate
1st subculture	8	3	90	803	1:8.9
2nd subculture	4	16	803	4 683	1:5.83
3rd subculture	4	150	4 863	60 000	1:12.34
ACCLIMATIZATION					
Growth parameters	Days				
	0	15	30		
Survival rate (%)	-	-	96		
Tillering	-	-	2.88		
Aerial part length (cm)	9.50	16.14	37.37		
Root length (cm)	0.50	5.80	13.50		
Stem diameter (mm)	0.95	2.40	4.27		
Aerial part fresh biomass (mg)	326.0	1 200.0	3 910.0		
Aerial part dry biomass (mg)	64.20	358.0	762.0		
Root fresh biomass (mg)	3.20	736.0	1 680.0		
Root dry biomass (mg)	0.50	128.0	307.0		
Total dry biomass (mg)	64.70	486.0	1 069.0		
Number of leaves	4	5	7		

n=50

This study demonstrates that the induction of direct SE in sugarcane 'RB 872552' combined with the use of TIS is a promising alternative for large-scale multiplication of plants regenerated from SE. The successful application of these propagation techniques was corroborated by high survival rate of plants after the acclimatization stage, implying that this method can be used with positive effect in other varieties or species of commercial interest.

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