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Micropropagation of *Stevia rebaudiana* Bert. in temporary immersion systems and evaluation of genetic fidelity

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ABSTRACT

Stevia rebaudiana Bertoni (*Asteraceae*) contains in its leaves sweeteners such as stevioside and rebaudioside, for which it is commercially valuable. Inefficient sexual and asexual propagation methods do not meet the current demand of propagules required for commercial cultivation. Therefore, *in vitro* propagation is a promising alternative to solve this issue. However, the protocols currently available involve low numbers of micropropagated shoots and high production costs. Therefore, this study proposes to establish a commercial micropropagation protocol for *S. rebaudiana* using Recipient for Automated Temporary Immersion (RITA®) aimed at implementing this mass-propagation process at a low cost and in an automated modality. Our findings revealed that the highest number of shoots (18.37) was obtained using 1 mg l^{-1} benzyladenine (BA) at an immersion frequency of 2 min every 8 h in 20 ml medium per explant. 100% rooting of shoots was achieved by using MS medium with no plant growth regulators (PGR). 90% of regenerated plantlets were successfully acclimatized under greenhouse conditions. The regenerated plants showed a low percentage of genetic variation (10.4%). This protocol can be applied in the commercial micropropagation of this species.

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1. Introduction

Stevia rebaudiana (Asteraceae) is a perennial herb native of northern Paraguay and South America that grows in tropical and subtropical climates (Soejarto et al., 1983). The leaves of *Stevia rebaudiana* contain glycosides such as steviosides and rebaudiosides, which are 200 to 300 times sweeter than sucrose or cane sugar (Geuns, 2003).

Conventional propagation of stevia is restricted by the low viability of its seeds due to self-incompatibility, which causes the production of sterile seeds (Ramírez-Mosqueda and Iglesias-Andreu, 2016a). Vegetative propagation is limited to a low number of individuals that may be obtained from a single plant and successfully adapted to the soil. *In vitro* micropropagation techniques are an attractive option to propagate large numbers of plants in a short time (Ramírez-Mosqueda and Iglesias-Andreu, 2016b).

There are different reports of micropropagation of *S. rebaudiana* (Ibrahim et al., 2008; Ali et al., 2010; Das et al., 2011; Modi et al., 2012; Nower, 2014; Ramírez-Mosqueda and Iglesias-Andreu, 2016a; Ramírez-Mosqueda et al., 2016). However, they have reported low propagation and reproducibility rates, as well as long culture times. Prolonged culture times may lead to multiple malformations in propagated *in vitro* plantlets (Ibrahim et al., 2008; Jiménez, 2011).

These issues can be overcome through the use of temporary immersion systems (TIS). These systems allow intermittent and short-lasting contact of the explant with liquid culture medium, which renews the atmosphere and ensures a proper supply of nutrients (Etienne and Berthouly, 2002; Welandera et al., 2014). RITA® (Recipient for Automated Temporary Immersion) was reported as the most efficient system (Alvard et al., 1993). It has been used successfully in different plant species, including vanilla (*Vanilla planifolia*) (Ramos-Castellá et al., 2014; Ramírez-Mosqueda and Iglesias-Andreu, 2016b), pistachio (*Pistacia vera*) (Akdemir et al., 2014), African yam (*Dioscorea rotundata* cayenensis) (Polzin et al., 2014) and oak (*Quercus suber*) (Pérez et al., 2013).

The use of RITA® in *S. rebaudiana* was reported by different authors (Jiménez, 2011; Noordin et al., 2012; Alvarenga and Salazar, 2015; Oviedo-Pereira et al., 2015; Sacco et al., 2015). However, these studies did not consider some key parameters, such as immersion frequency and duration, or volume of medium per explant. In addition to not evaluate the genetic fidelity of the generated plants.

The ISSRs (Inter-Simple Sequence Repeat) are simple, faster and reproducible method to detect polymorphic loci present in nuclear DNA and organellar DNA (Pradeep et al., 2002). Determinate by

Abbreviations: BA, benzyladenine; ISSR, inter simple sequence repeat; MS, Murashige and Skoog medium; PGR, plant growth regulators; RITA®, recipient for automated temporary immersion.

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addition or deletion of repeat units or by point mutation so it can allow evaluating the somaclonal variation occurring in the tissue cultured plant at the genetic level (Jarne and Lagoda, 1996).

This study provides a protocol for micropropagation of *S. rebaudiana* using RITA® and assessment of somaclonal variation of the regenerated plants.

2. Materials and methods

2.1. Plant material

This investigation used *in vitro* plantlets of *S. rebaudiana* variety Morita II established in the Plant Tissue Culture laboratory of *Instituto de Biotecnología y Ecología Aplicada* (INBIOTECA) at the *Universidad Veracruzana* (Ramírez-Mosqueda and Iglesias-Andreu, 2016a). The *in vitro* plantlets were cultivated in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1 mg l^{-1} of benzyladenine (BA), 30 g l^{-1} sucrose and 2.2 g l^{-1} Gelrite™ (Sigma-Aldrich, St. Louis, MO). The pH was adjusted to 5.8 before autoclaving at 1.2 kg cm^{-2} for 15 min at 120 °C. All cultures were incubated at a temperature of 25 ± 2 °C, with a 16/8-h light/dark photoperiod, under a light intensity of 50 μ mol m^{-2} s^{-1} using white-light lamps. After two subcultures of 30 d each, the cultivated explants were used for different assessments.

2.2. Effect of different culture methods and BA concentrations

Explants (nodal segments, 1–2 cm in length) were cultivated in two different culture systems: semi-solid and temporary immersion system (RITA® 1000 ml, 150 × 130 mm) (VITROPIC, Saint-Mathieu-de-Tréviers). Both systems used MS medium supplemented with 30 g l^{-1} sucrose and 2.2 g l^{-1} Gelrite™ (Sigma-Aldrich, St. Louis, MO) as gelling agent (except in TIS). Twenty milliliters of medium was dosed into each "G" container in the semi-solid system, and 200 ml in the bioreactor. The effect of various concentrations of BA (0, 1, 2 and 3 mg l^{-1}) was evaluated in both systems. The pH of the media was adjusted to 5.8 ± 0.2. Culture containers were autoclaved at 1.5 kg cm^{-2} and 121 °C for 15 min. For TIS, the frequency of immersion was 2 min every 4 h.

All cultures were incubated at 25 ± 2 °C under a 16/8-h light/dark photoperiod and a light intensity of 50 μmol m^{-2} s^{-1} using white-light lamps. A total of 50 explants were used per treatment. In the semi-solid system, 10 containers with 50 explants each were used per treatment. For RITA®, 5 containers with 10 explants each were used per treatment. After 4 wk. of culture, the number and length of roots and the number of leaves were determined.

2.3. Effect of different immersion frequencies in RITA®

Three immersion frequencies were assessed: 4, 8 and 12 h (2 min per immersion). Cultures were incubated under the same conditions described above. A total of 50 explants were used per treatment, using five containers with 10 explants each. After 4 wk. of culture, the number and length of roots and the number of leaves were determined.

2.4. Effect of the volume of medium per explant in RITA®

Three different volumes of medium per explant were evaluated: 20, 28.5 and 50 ml (five, seven and 10 explants per RITA®, respectively). In all cases we used MS medium supplemented with 1 mg l^{-1} of BA along with an immersion frequency of 2 min every 8 h. Cultures were incubated under the same conditions described above. After 4 wk. of culture, the number and length of roots and the number of leaves were determined.

2.5. Rooting and acclimatization

The rooting of shoots in semi-solid medium was required, because *S. rebaudiana* roots were entangled in the pores of the RITA® support mesh. To induce rooting, 2-cm length shoots were transferred to MS medium with no plant growth regulators (PGR). The percentage of rooted shoots was determined after three weeks of culture.

Individual shoots (10–15 cm), previously rooted *in vitro*, were rinsed with tap water and sown under greenhouse conditions in separate pots (5 × 5 × 8 cm) containing a sterile substrate consisting in a 1:1 mixture of peat moss™ and agrolite™. Plantlets were maintained under 50% shaded conditions. Irrigation was performed manually by spraying tap water three times per week to maintain a relative humidity between 80 and 95%.

2.6. Evaluation of somaclonal variation

2.6.1. Genomic DNA extraction

Total DNA was extracted by duplicate from 0.2 g of foliar tissue from two mother plants and 28 micropropagated plants in TIS following the method by Stewart and Via (1993). The quality of genomic DNA was examined using agarose gel electrophoresis (1%) and was quantified by spectrophotometry at an OD ratio of 260/280 using an Invitrogen® (Qubit 2.0) fluorometer.

2.6.2. ISSR analysis

12 ISSR primers (UBC, UBC, Biotechnology Laboratory, University of British Columbia) were tested. ISSR amplification reactions by PCR were performed in a total volume of 25 μl containing 0.4 mM dNTPs, 2.5 mM $MgCl_2$, 25 pM primer, 0.5 U Taq polymerase (Bioline®) and 25 ng DNA templates. DNA amplification was performed in an AXIGEN® (Applied Biosystem, CA, USA) thermocycler.

PCR conditions were: an initial denaturation at 94 °C for 1 min, subsequent denaturation at 94 °C for 30 s, annealing at Tm °C for 45 s, extension at 72 °C for 1 min, 35 cycles, and the final extension at 72 °C for 10 min.

The amplification products were separated by electrophoresis in 1.5% agarose gel (*w*/*v*) using 19 TAE buffer (40 mM Tris, 40 mM acetate, 1 mM EDTA and pH 8.4), at 100 V for 1.5 h. Subsequently, gels were stained with 1 mg m l^{-1} ethidium bromide. Gene Ruler DNA Plus™ 100 bp (Fermenteas®) was used as DNA marker. Bands were visualized in a gel documentation system (GelDoc-It® Imager, UVP).

All the reactions for each ISSR primer were repeated twice only reproducible bands between replicates were used to establish a binary base, scoring 1 for presence and 0, absence. On these bases, the total number of bands, mean number of bands per primer, percentage of monomorphic loci and percentage of polymorphic loci were calculated.

2.7. Statistical analyzes

All experiments were run using a randomized design with 5 replicates each. Fifty explants were used per treatment. The data obtained were processed statistically with IBM SPSS Statistics (version 21) software. An analysis of variance (ANOVA) followed by Tukey's test ($p \leq 0.05$) was performed.

3. Results

3.1. Effect of different culture methods and BA concentrations

Significant differences were observed between the different culture systems and BA concentrations evaluated at 4 wk of culture (Table 1). In RITA® higher multiplication rate relative to the semi-solid medium was observed (Table 1). However, shoots obtained in RITA® showed hyperhydricity and a slight reddish color (Fig. 1E–H). However, these abnormalities were not evident in the semi-solid medium (Fig. 1A–D).

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