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Micropropagation of *Stevia rebaudiana* Bertoni Using RITA® Bioreactor

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Abstract. Stevia rebaudiana **Bertoni is used for medicinal and food purposes. The effectiveness of different culture systems in *Stevia* micropropagation was evaluated. Node explants were cultured in semisolid or RITA® bioreactor containing woody plant medium (WPM) without plant growth regulators (PGRs). The effect of three medium volumes (100, 200, and 300 mL) and four immersion frequencies (10 seconds every 1, 4, 6, or 8 hours) were assessed. The shoots and roots developed simultaneously in the RITA® bioreactor treatment containing 100 mL of WPM at 10 seconds/4 hours immersion frequency and the healthiest plantlets were obtained from this RITA® treatment using 5 mL of medium per explant. The root induction rate was 100% in nonhyperhydrated shoots obtained from both semisolid and RITA® bioreactor, and all of them were successfully acclimatized. Thus, an economical and viable protocol was developed by performing the micropropagation in one step, eliminating the use of agar and PGRs, and decreasing the amount of medium used per explant.**

Stevia rebaudiana Bertoni, originating from Paraguay, belongs to the family Asteraceae and is a wild perennial herb. The leaves of this plant synthesize noncaloric, sweet-tasting compounds called steviol glycosides (SGs). Stevioside, rebaudiosides A and C, and dulcoside A are the most important SGs, and their sweetness is 210, 242, 30, and 30 times, respectively, greater than sucrose (Brandle and Rosa, 1992). Because of its nontoxic, low-caloric, natural, and possession of intense, sweetening properties, these SGs can be successfully used as sugar alternatives for diabetic, obese, cardiac, and dental patients (Bayraktar et al., 2015; Bondarev et al., 2001). In addition, *Stevia*-originated natural SGs are of great importance in the food sector and are being used as sugar substitutes in foods and beverages (Singh and Rao, 2005).

The propagation of *Stevia* traditionally is made by seed or vegetative cuttings. Due to self-incompatibility, which results in infertility and a small size, the seed germination rate is very poor. Moreover, the production of *S. rebaudiana* through seeds causes fluctuations in the growth and SGs contents of propagated plants (Singh and Rao, 2005; Tamura et al., 1984a, 1984b). Propagation by stem cuttings requires a high labor input and is restricted by the number of individuals that can be obtained simultaneously from a single plant (Lata et al., 2013; Sivaram and Mukundan, 2003). Therefore, large-scale production of *S. rebaudiana* through seeds or stem cuttings is not productive. The use of plant tissue-culture methods as an alternative propagation of *S. rebaudiana* from a quality genotype can provide large-scale, true-to-type, uniform, simple, and economic production within a short period of time.

In vitro propagation of *S. rebaudiana* has been studied by many researchers to develop an efficient and economic micropropagation protocol in semisolid medium (Ahmed et al., 2007; Giridhar et al., 2010; Hwang, 2006; Lata et al., 2013; Röck-Okuyucu et al., 2016; Sairkar et al., 2009; Sivaram and Mukundan, 2003; Tamura et al., 1984a; Thiyagarajan and Venkatachalam, 2012; Yang et al., 1981). In vitro propagation via semisolid medium involves the use of a large number of small containers, agar products, and making periodical subcultures. Because nutrients in the medium are quickly exhausted and the size of the culture containers limits continuous tissue growth and proliferation, the plant material has to be subcultured at short intervals. Agar products are expensive, making automation difficult and cleaning agar out of small culture containers requires intense labor. Moreover, the filling, cleaning, and handling of a large number of small containers constitute the major part of in vitro production with semisolid culture (Etienne and Berthouly, 2002). Semisolid media provides aeration but the plant material does not have full contact with the medium (Escalona et al., 1999). Liquid medium can reduce plantlet production costs due to the elimination of agar usage and can make automation easy, provide more uniform conditions for plant material, easily be renewed without changing the container, and be easily cleaned from the culture container. In addition, much larger culture containers can be used compared with semisolid culture. Therefore, the use of liquid medium is more advantageous than the use of semisolid medium (Etienne and Berthouly, 2002; Regueira et al., 2017). However, these liquid medium advantages are overshadowed by some disadvantages, like hyperhydricity, shear forces, asphyxia, and the need for complex equipment.

To eliminate these liquid medium disadvantages, temporary immersion systems (TIS) can be used. Temporary immersion culture is the combination of a liquid and semisolid culture. In TIS, plant material can periodically come in contact with the liquid medium and the environment in the systems can be renewed; thus, an efficient nutrient uptake can be made by the plant tissue and disorders like asphyxia and hyperhydricity can be eliminated (Escalona et al., 1999; Etienne and Berthouly, 2002). Bioreactors based on TIS have been successfully used for the micropropagation of many plant species due to their advantages when compared with semisolid and continuous immersion culture (Zhang et al., 2018). These types of bioreactors can reduce consumables and labor costs (McAlister et al., 2005). They minimize energy requirements and are more manually flexible (Zhang et al., 2018). Periodical temporary contact between the liquid medium and the plant material generally increases proliferation rates and plant growth (Albarrán et al., 2005). Furthermore, TIS bioreactors provide ventilation and thus prevent ethylene and carbon dioxide accumulation in the culture tank (Zhang et al., 2018).

Some researchers have focused on the micropropagation of *S. rebaudiana* through a TIS bioreactor (Alvarenga-Venutolo and Salazar-Aguilar, 2015; Ramírez-Mosqueda et al., 2016; Vives et al., 2017). In the study reported by Alvarenga-Venutolo and Salazar-Aguilar (2015), RITA® bioreactor and the twin flasks system were compared with semisolid medium for the mass propagation of *S. rebaudiana*. They used 25 explants and 250 mL of medium per RITA® bioreactor and tested four immersion periods (5 min/8 h, 5 min/12 h, 10 min/8 h, and 10 min/12 h). They reported a ≈2-fold increase in the shoot and leaf number and 3- and 4-fold increase in the fresh and dry weight, respectively, in the RITA® with an immersion frequency of 10 min/8 h compared with semisolid medium. Ramírez-Mosqueda et al. (2016) cultured 10 nodal segments in RITA® bioreactor including 200 mL of Murashige and Skoog (Murashige and Skoog, 1962) medium (MS) containing 1 $mg \cdot L^{-1}$ BA at different immersion frequencies (2 min/4 h, 2 min/8 h, and 2 min/12 h). Compared with the semisolid culture, they obtained an ≈4-fold increase in shoot number from RITA® application with an immersion frequency of 2 min/8 h; however, they did not find significant difference in leaf number.

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They also obtained plants with high genetic stability from micropropagation of *Stevia* via RITA® bioreactor. Vives et al. (2017) cultured 20 node explants in a temporary immersion bioreactor (BIT®) containing 250 mL of MS medium fortified with 100 mg·L^{-1} myo-inositol, 1 mg·L^{-1} thiamine, 0.25 mg·L^{-1} BAP, and 30 g·L^{-1} sucrose. They used one immersion frequency (3 min/6 h). When they compared the semisolid, liquid, and RITA® bioreactor, they observed no differences in the leaf, shoot, and internode number between semisolid and the RITA® bioreactor, and liquid culture produced the lowest results in terms of the parameters examined. Although the benefit of using RITA® bioreactor in the micropropagation of *Stevia* has been shown with these studies, detailed experiments are required to make the process more economic and efficient. The aim of this work was to compare the efficiency of two in vitro culture systems (semisolid and TIS) and evaluate the frequency of immersion and medium volume in terms of the propagation and growth of *S. rebaudiana*. RITA® bioreactor (Récipient à Immersion Temporaire Automatique; VITROPIC, Saint-Mathieu-de-Tréviers, France) was used as bioreactor type. The acclimatization performance of plantlets obtained from semisolid and RITA® bioreactors also was evaluated.

Materials and Methods

Plant material. In vitro–propagated plantlets, which were obtained from single seed descent seedlings possessing the best proliferation rate and SG contents, of *S. rebaudiana* Bertoni in the Bioengineering Department of Ege University, were used as the plant material (Bayraktar et al., 2015). According to our previous study (Bayraktar et al., 2015), to obtain stock plant material for applications, node explants (1 cm) with one axillary bud were initially propagated on WPM (Lloyd and McCown, 1980) including 3% (w/v) sucrose and solidified with 0.65% (w/v) plant agar (Duchefa Biochemie B.V., Haarlem, The Netherlands). The subcultures were carried out on WPM every 4 weeks.

Establishment of different culture systems. To compare the effect of different culture systems on the growth, multiplication, and biomass accumulation of *S. rebaudiana*, two different culture systems (semisolid and TIS) were performed. In a preliminary study by our research group (Röck-Okuyucu et al., 2016), we described a one-step in vitro micropropagation protocol for *S. rebaudiana*. In the PGR-free WPM, shoots and roots were formed simultaneously and well-developed plantlets were obtained. Therefore, in the experiments, PGR-free WPM was used as the culture medium.

Semisolid culture. Node explants (1 cm) with one axillary bud from 4-week-old in vitro–grown shoots were cultured in Sterivent® vessels [107 × 94 × 96 mm (Duchefa Biochemie B.V.)] containing 200 mL of WPM medium supplemented with 3% (w/v) sucrose and 0.65% (w/v) plant agar.

TIS culture. RITA® bioreactors, which are 1-L cylindrical vessels, consist of two compartments: an upper part for culturing the explants and a lower part for the reservoir of liquid medium. Air flow enters by sterilization through sterile 0.22-µm hydrophobic (polytetrafluoroethylene) membrane filters into the lower part of the RITA®. During the immersion period, air pressure from an air compressor pushes the medium from the lower part to the upper part by forming air bubbles to wet the explants completely; thus, the explants take nutrients and the headspace atmosphere inside the RITA® is refreshed at the same time. After the immersion period, the air compressor stops, the excess pressure escapes through an outlet at the upper part of the bioreactor, and the medium flows downward as the result of gravity (Etienne and Berthouly, 2002).

Node explants as specified previously were cultured in the RITA® bioreactor containing liquid WPM supplemented with 3% (w/v) sucrose. A total of three different medium volumes (100, 200, or 300 mL) and four different immersion frequencies (once every 1, 4, 6, and 8 h each, for a duration of 10 s) were tested. An electric timer was used to control the length and frequency of the immersion period.

At the end of all of the culture treatments, the growth parameter data were recorded, and all of the plantlets were harvested and divided into leaves, roots, and stems to identify the fresh biomass weights of each replication. After freeze drying, the leaf, stem, and root dry weights from each application were identified.

Media and culture conditions. All the media pH levels were adjusted to 5.8 with 1 N HCl or 1 N NaOH before the addition of the gelling agent [0.65% (w/v) plant agar]. They were autoclaved at 121 °C at 1.04 kg·cm^{-2} for 15 min. All of the cultures were incubated in a growth room at ≈25 ± 1 °C under a cool white fluorescent light (50 µmol·m^{-2}·s^{-1}) for a light/dark photoperiod of 16:8 h.

Acclimatization. Twenty in vitro–hyperhydrated plantlets (from the RITA® bioreactor) and 40 in vitro nonhyperhydrated plantlets (20 plantlets from semisolid culture and 20 from the RITA® bioreactor) with well-developed roots were acclimated in small pots containing a mixture of perlite and peat (1:4) and covered with perforated transparent bags to maintain humidity. They were maintained in a climate chamber at ≈25 ± 1 °C for a light/dark photoperiod of 16:8 h, with 70% humidity and 50 µmol·m^{-2}·s^{-1} irradiance. They were ventilated once a day for a period of 10 d for 15 min. After this period, the bags were completely removed. The plants were then transferred to greenhouse conditions 3 weeks after the beginning of acclimatization. Data were recorded after 8 weeks.

Statistical analysis. All of the in vitro experiments were conducted in triplicate, with 20 explants used for each replication, and 60 explants being tested in total. The data were recorded 4 weeks after culture initiation. Acclimatization study was conducted in two repeats, with 10 plantlets used for each replication, and 20 plantlets being tested in total. The data were recorded 8 weeks after initiation. The experiments were set up in a completely randomized design. All of the data were presented as mean ± SE and analyzed using standard analysis of variance procedures. The significant differences among the mean values were compared by Tukey's test at $P = 0.05$ using Minitab 17.0 Statistical Software (Minitab Inc., State College, PA). Data presented in percentages (leaf chlorosis and hyperhydricity) for in vitro cultures were subjected to square root transformation (x + 0.5) before analysis.

Results and Discussion

The potential of RITA® bioreactor functioning on the principle of TIS was investigated for the large-scale micropropagation of *S. rebaudiana*. For this purpose, two different culture systems (semisolid and TIS) were compared in terms of growth parameters in *S. rebaudiana*.

The effect of different culture systems on shoot growth. As growth factors; number of shoots, nodes and leaves per explant, shoot and leaf length (cm), root induction (%), root length (cm), hyperhydricity (%), and leaf chlorosis (%) were observed.

The greatest shoot number (8.47 shoots/explant) was obtained from the RITA® bioreactor containing 300 mL of medium and immersion for 10 s/1 h (Table 1). This result was ≈4-fold greater than that of the semisolid culture. Due to the occurrence of bilateral nodes, generally, two shoots emerge from the node culture of *S. rebaudiana* cultured in semisolid WPM without PGRs (Bayraktar et al., 2016; Röck-Okuyucu et al., 2016). In the present study, the node explants cultured in semisolid medium and some RITA® treatments (10 s/4 h-100 mL, 10 s/6 h-200 mL, and 10 s/8 h-200 mL) produced nearly two shoots (Table 1). Immersion frequency and medium volume considerably affect the efficiency of TIS (Berthouly and Etienne, 2005; Pérez-Alonso et al., 2009; Regueira et al., 2017). In the present study, a positive relation was found between the immersion frequency or medium volume and shoot number. By progressively increasing in the immersion frequency from once every 8 h to once every hour or the medium volume from 100 mL to 300 mL, the shoot number per explant increased (Table 1). In the TIS studies carried out by Alvarenga-Venutolo and Salazar-Aguilar (2015), Ramírez-Mosqueda et al. (2016), and Vives et al. (2017) in *S. rebaudiana*, the greatest shoot number per explant was reported as 14.21 shoots, 18.60 shoots, and 5.0 shoots, respectively. This variety among the results may be due to bioreactor type used, immersion frequency, medium volume, explant number and type, basal medium, and supplements added to basal medium like PGRs. Unlike these studies, PGRs-free basal medium (WPM) and less immersion frequency (10 s) were used in the

Table 1. Effect of culture system, immersion frequency (10 s every 1, 4, 6, or 8 h), and medium volume (100, 200, or 300 mL) on in vitro shoot growth of *Stevia rebaudiana*.

Culture system	Immersion frequency (10 s/× h)	Volume of medium (mL)	Number of shoots per explant (± SE)	Shoot length (cm) (± SE)	Number of nodes per explant (± SE)	Number of leaves per explant (± SE)	Leaf length (cm) (± SE)
Semisolid medium	–		2.00 ± 0.00 ef	8.05 ± 0.31 ef	4.93 ± 0.23 f	14.70 ± 0.41 g	0.95 ± 0.02 cdefg
RITA	1	100	2.78 ± 0.20 de	11.99 ± 0.28 a	5.33 ± 0.21 ef	15.08 ± 0.69 g	0.99 ± 0.02 cde
RITA	1	200	3.80 ± 0.24 c	6.95 ± 0.39 fg	6.65 ± 0.33 cde	17.87 ± 0.65 efg	1.07 ± 0.03 bc
RITA	1	300	8.47 ± 0.33 a	5.09 ± 0.14 g	8.88 ± 0.41 b	32.77 ± 1.30 b	1.25 ± 0.05 a
RITA	4	100	2.03 ± 0.03 ef	8.14 ± 0.30 def	5.82 ± 0.20 def	15.77 ± 0.42 g	0.79 ± 0.02 g
RITA	4	200	5.02 ± 0.39 b	6.71 ± 0.44 fg	6.57 ± 0.32 cde	23.10 ± 1.11 cd	0.91 ± 0.03 defg
RITA	4	300	5.83 ± 0.38 b	5.79 ± 0.30 g	7.12 ± 0.27 cd	25.93 ± 1.13 c	0.87 ± 0.02 efg
RITA	6	100	1.68 ± 0.08 f	9.98 ± 0.32 bcd	6.38 ± 0.26 cdef	16.13 ± 0.60 fg	0.83 ± 0.04 fg
RITA	6	200	2.23 ± 0.13 def	11.62 ± 0.82 ab	7.88 ± 0.55 bc	19.97 ± 1.26 def	1.04 ± 0.05 bcd
RITA	6	300	5.22 ± 0.26 b	9.43 ± 0.36 cde	13.07 ± 0.32 a	37.27 ± 1.14 a	1.18 ± 0.02 ab
RITA	8	100	1.87 ± 0.07 ef	9.77 ± 0.37 bcde	5.92 ± 0.23 def	15.57 ± 0.54 g	0.97 ± 0.06 cdef
RITA	8	200	2.00 ± 0.00 ef	10.23 ± 0.48 abc	5.95 ± 0.34 def	15.35 ± 0.74 g	0.86 ± 0.03 efg
RITA	8	300	3.10 ± 0.22 cd	9.29 ± 0.49 cde	7.00 ± 0.39 cd	20.83 ± 1.09 de	0.80 ± 0.03 g

ANOVA summary table

	Number of shoots		Shoot length		Number of nodes		Number of leaves		Leaf length	
Source	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value
Immersion frequency (IF)	80.49	0.000	36.23	0.000	39.23	0.000	28.67	0.000	28.18	0.000
Volume of medium (VM)	281.70	0.000	31.42	0.000	127.27	0.000	251.21	0.000	10.72	0.000
IF × VM	30.95	0.000	16.76	0.000	17.67	0.000	20.76	0.000	11.74	0.000

Data shown are mean values ± SE from measurements performed in three Sterivent® vessels or RITA® bioreactors each containing 20 explants. In columns, different letters indicate significant differences ($P = 0.05$) between treatments according to Tukey's test.
ANOVA = analysis of variance.

Table 2. Effect of culture system, immersion frequency (10 s every 1, 4, 6, or 8 h), and medium volume (100, 200, or 300 mL) on root formation, hyperhydricity, and leaf chlorosis of *Stevia rebaudiana*.

Culture system	Immersion frequency (10 s/× h)	Volume of medium (mL)	Root induction (%) (± SE)	Root length (cm) (± SE)	Hyperhydricity (%) (± SE)	Leaf chlorosis (%) (± SE)
Semisolid medium	–		100.00 ± 0.00	4.79 ± 0.14 e	0.00 ± 0.00 e	0.00 ± 0.00 g
RITA	1	100	100.00 ± 0.00	5.21 ± 0.15 de	78.29 ± 9.93 b	10.00 ± 2.89 ef
RITA	1	200	100.00 ± 0.00	4.51 ± 0.10 e	100.00 ± 0.00 a	11.67 ± 1.67 ef
RITA	1	300	100.00 ± 0.00	4.13 ± 0.14 e	100.00 ± 0.00 a	16.67 ± 4.41 e
RITA	4	100	100.00 ± 0.00	7.48 ± 0.34 cd	0.00 ± 0.00 e	0.00 ± 0.00 g
RITA	4	200	100.00 ± 0.00	6.09 ± 0.33 de	70.86 ± 3.86 bc	5.00 ± 0.00 f
RITA	4	300	100.00 ± 0.00	5.61 ± 0.70 de	100.00 ± 0.00 a	15.00 ± 2.89 e
RITA	6	100	100.00 ± 0.00	8.92 ± 0.30 bc	0.00 ± 0.00 e	53.33 ± 6.01 cd
RITA	6	200	100.00 ± 0.00	8.52 ± 0.28 bc	60.94 ± 2.65 c	40.00 ± 5.00 d
RITA	6	300	100.00 ± 0.00	7.50 ± 0.29 cd	100.00 ± 0.00 a	36.67 ± 3.33 d
RITA	8	100	100.00 ± 0.00	12.00 ± 1.32 a	0.00 ± 0.00 e	100.00 ± 0.00 a
RITA	8	200	100.00 ± 0.00	10.83 ± 0.44 ab	32.62 ± 1.05 d	78.33 ± 1.67 ab
RITA	8	300	100.00 ± 0.00	8.91 ± 0.57 bc	100.00 ± 0.00 a	70.00 ± 2.89 bc

ANOVA summary table

	Root length		Hyperhydricity		Leaf chlorosis	
Source	F value	*P* value	F value	*P* value	F value	*P* value
Immersion frequency (IF)	70.49	0.000	67.95	0.000	302.18	0.000
Volume of medium (VM)	45.43	0.000	603.44	0.000	318.43	0.000
IF × VM	8.61	0.000	59.67	0.000	45.57	0.000

Data shown are mean values ± SE from measurements performed in three Sterivent® vessels or RITA® bioreactors each containing 20 explants. In columns, different letters indicate significant differences ($P = 0.05$) between treatments according to Tukey's test.
ANOVA = analysis of variance.

present study, making propagation more economical for commercial production.

A considerable variation existed among the tested culture types with regard to the mean shoot length. The maximum shoot length (11.99 cm/explant) was observed in the RITA® bioreactor with immersion for 10 s/1 h and 100 mL of medium. The shortest shoots (5.09 cm) formed in the RITA® bioreactor with immersion for 10 s/1 h and 300 mL of medium (Table 1).

In most cases, the mean number of nodes per explant determines the multiplication rate and, therefore, this parameter is significant for micropropagation (Bayraktar et al., 2016; Röck-Okuyucu et al., 2016). The greatest number of nodes (13.07 nodes/explant) and, accordingly, number of leaves (37.27 leaves/explant) per explant was produced by the RITA® bioreactor with immersion for 10 s/6 h and 300 mL of medium, whereas the lowest number of nodes (4.93 nodes/explant) and number of leaves (14.70 leaves/explant) per explant was formed in the semisolid culture (Table 1). Similar results were obtained by Alvarenga-Venutolo and Salazar-Aguilar (2015), who reported the average number of leaves as 36.40 per explant in RITA® bioreactor containing 250 mL of medium and immersion for 10 min/8 h. However, in the present study, these parameters were greater than those reported by Vives et al. (2017) (17 leaves/nodal explant) and Ramírez-Mosqueda et al. (2016) (5.20 leaves/shoot). The longest leaf length (1.25 cm) was achieved in culture established in the RITA® bioreactor containing 300 mL of medium and immersed for 10 s/1 h (Table 1).

Root development in in vitro shoots (previous acclimatization) is necessary for the success of the acclimatization stage. Root induction rate was 100% in all of the

applications. The root length ranged between 12.00 cm (10 s/8 h-100 mL) and 4.13 cm (10 s/1 h-300 mL) (Table 2). In previous studies, PGRs have been used for rooting of in vitro *Stevia* shoots (Ahmed et al., 2007; Hwang, 2006; Sivaram and Mukundan, 2003; Tamura et al., 1984a). In this study, on the PGR-free WPM, development of shoots and roots occurred simultaneously and no additional rooting step was needed. Ramírez-Mosqueda et al. (2016) used an additional stage for rooting of *Stevia* shoots. They cultured in vitro shoots in semisolid medium (PGRs-free MS) to prevent entangling the roots in the pores of the RITA® support mesh. They obtained 100% rooting. In the present study, the roots were entangled in the support mesh and injured during the harvesting of the plantlets from RITA® bioreactor. However, this injury did not affect the acclimatization performance of the plantlets. In addition, to prevent this injury, explants can be cultured by removing the mesh in RITA® and therefore eliminating the need of a separate solid culture stage. In other micropropagation studies of *Stevia* by TIS bioreactor, there is no information provided on rooting (Alvarenga-Venutolo and Salazar-Aguilar, 2015; Vives et al., 2017).

Vitrified or hyperhydric plantlets developed from the RITA® bioreactor showed abnormalities in their morphology and anatomy. Translucent and curled leaves formed. A relationship between the medium volume and hyperhydricity was observed. By progressively increasing the medium volume, the hyperhydric plantlet production increased and reached 100% in all of the 300-mL volume treatments (Table 2). With the exception of the treatment with semisolid medium (Fig. 1A), 10 s/4 h-100 mL (Fig. 1B and C), 10 s/6 h-100 mL, and 10 s/8 h-100 mL, a high percentage of hyperhydricity (32.62% to 100%) occurred with the remaining treatments (Table 2). Until the second week of the culture, no hyperhydricity was observed in any of the RITA® cultures. However, by the fourth week of the culture, the shoots were too long and the upper compartment of the RITA® was shorter than the shoots; the shoots were curled like a ball. Due to the dense leaf and shoot formation, especially in the RITA® bioreactor containing 200 and 300 mL of medium, the medium might permanently remain on the plant material after immersion. Thus, the high rate of hyperhydricity in RITA® cultures may be directly related to this situation. The shoots and roots were developed simultaneously on the PGR-free WPM; therefore, the curling of in vitro plantlets and, consequently, hyperhydricity can be decreased or prevented by reducing the culture period from 4 weeks to 2 to 3 weeks. In addition, hyperhydricity can be controlled by adjusting the immersion frequency (Vidal et al., 2015). In this study, in some of the RITA® treatments (10 s/4 h-100 mL, 10 s/6 h-100 mL, and 10 s/8 h-100 mL), no hyperhydricity formation was observed (Table 2). The occurrence of hyperhydricity in *S. rebaudiana* also was reported by Ramírez-Mosqueda et al. (2016), and they also observed a slight reddish color and abnormalities in regenerated shoots in RITA® cultures. In the present study, the hyperhydricity problem was solved by decreasing the volume of medium. In addition, root formation initiated 3 d after culture and continued to grow until hyperhydricity formation (after the second week of culture) and afterward. Therefore, hyperhydricity formation did not prevent root formation and development.

By increasing the time between the immersion periods from 1 to 8 h led to a high level of leaf chlorosis. As seen in Table 2, except for the treatments with semisolid medium and RITA® culture with immersion for 10 s/4 h and 100 mL of medium, the remaining treatments showed leaf chlorosis and reached 100% (10 s/8 h-100 mL). Specifically, immersion for 6 h and 8 h resulted in high level of chlorosis. The greatest chlorosis formations were observed with immersion for 8 h and ranged between 70.00% and 100%. Because the time between the two immersion periods was so long, this chlorosis and necrosis formation might be related to a nutrient deficiency.

The effect of different culture systems on biomass growth. Biomass also was examined to determine the differences in the plant growth among the applications. The biomass accumulation of different organs (leaf, stem, and root) was determined separately.

The plant tissues are periodically ventilated and come into contact with liquid medium in TIS, which results in increased biomass accumulation (Pérez-Alonso et al., 2009). Similarly, in the present study, all of the RITA® bioreactor applications, with the exception of immersion for 10 s/4 h and 100 mL of medium, increased the biomass accumulation when compared with the semisolid culture. Biomass accumulation was influenced by the volume; as the volume increased, the total shoot weight increased accordingly. Maximum values of the total shoot fresh and dry weights (1560.1 mg and 145.5 mg per explant, respectively) were obtained with immersion for 10 s/6 h and 300 mL of medium (Table 3). This result was much greater than the result reported by Alvarenga-Venutolo and Salazar-Aguilar (2015). However, it was almost the same as the result obtained by Vives et al. (2017).

SGs biosynthesis initiates in the chloroplasts and occurs exclusively in the leaves of *S. rebaudiana* (Brandle et al., 2002; Brandle and Telmer, 2007). Hence, it would be a great

Fig. 1. Micropropagation stages of *S. rebaudiana* in different cultures: Plantlet regeneration in semisolid (**A**) and RITA® bioreactor containing 100 mL of medium and immersion for 10 s/4 h (**B**, **C**) (bar 1.0 cm). In vitro root formation in the RITA® bioreactor (**D**, **E**) and semisolid culture (**F**) (bar 1.0 cm). Acclimatization of nonhyperhydrated plantlets from semisolid culture and from RITA® bioreactors (**G**) and hyperhydrated plantlets from RITA® bioreactors (**H**) (bar 4.0 cm).

Table 3. Effect of culture system, immersion frequency (10 s every 1, 4, 6, or 8 h), and medium volume (100, 200, or 300 mL) on biomass production of *Stevia rebaudiana*.

Culture system	Immersion frequency (10 s/× h)	Volume of medium (mL)	Total wt of leaves (mg) per shoot (± SE)		Total wt of stems (mg) per shoot (± SE)		Total wt of roots (mg) per shoot (± SE)		Total wt (mg) per shoot (± SE)	
			Fresh wt	Dry wt	Fresh wt	Dry wt	Fresh wt	Dry wt	Fresh wt	Dry wt
Semisolid medium	–		55.6 ± 1.4 b	5.5 ± 0.2 e	122.1 ± 13.9 ef	12.3 ± 1.5 e	88.6 ± 4.3 ef	9.0 ± 0.6 c	266.3 ± 16.9 gh	26.78 ± 1.8 fg
RITA	1	100	63.0 ± 3.7 b	12.2 ± 0.2 de	181.5 ± 6.3 ef	23.0 ± 0.8 de	58.5 ± 7.1 ef	6.1 ± 0.7 c	303.0 ± 12.8 gh	41.3 ± 0.9 f
RITA	1	200	74.2 ± 3.9 b	6.0 ± 0.3 e	197.1 ± 10.5 ef	15.9 ± 0.9 e	75.1 ± 8.8 ef	7.8 ± 0.2 c	346.4 ± 21.7 gh	29.8 ± 0.9 fg
RITA	1	300	393.6 ± 18.3 a	39.0 ± 4.6 a	474.4 ± 15.2 c	47.1 ± 7.2 bc	47.7 ± 3.9 f	4.7 ± 0.4 c	915.8 ± 12.9 bc	90.8 ± 6.1 bc
RITA	4	100	65.2 ± 3.3 b	4.3 ± 1.0 e	78.8 ± 6.6 f	6.9 ± 0.7 e	57.0 ± 6.7 ef	5.0 ± 0.6 c	200.9 ± 16.5 h	16.2 ± 1.4 g
RITA	4	200	338.2 ± 12.1 b	18.3 ± 1.9 cd	440.4 ± 14.4 cd	54.8 ± 5.9 b	49.8 ± 5.5 ef	5.5 ± 0.5 c	828.3 ± 23.6 bcd	78.5 ± 8.2 cd
RITA	4	300	375.0 ± 32.2 ab	25.6 ± 2.7 bc	441.9 ± 9.9 cd	54.2 ± 7.9 b	45.0 ± 3.8 f	6.1 ± 0.7 c	861.9 ± 45.4 bcd	85.9 ± 11.2 bcd
RITA	6	100	61.3 ± 13.9 b	8.5 ± 1.5 e	107.7 ± 3.9 f	15.0 ± 0.6 e	251.9 ± 3.0 bc	21.1 ± 3.3 b	420.9 ± 43.4 fgh	44.6 ± 3.9 ef
RITA	6	200	143.9 ± 8.7 b	17.8 ± 1.0 cd	658.9 ± 4.5 b	60.6 ± 3.3 b	223.5 ± 9.9 bc	23.8 ± 3.1 b	1026.3 ± 38.4 b	102.2 ± 1.5 b
RITA	6	300	357.7 ± 16.1 ab	32.5 ± 2.4 ab	1014.9 ± 13.9 a	92.7 ± 1.8 a	187.4 ± 12.5 cd	20.3 ± 0.8 b	1560.1 ± 6.57 a	145.5 ± 0.9 a
RITA	8	100	59.2 ± 6.4 b	9.7 ± 0.6 de	111.8 ± 5.5 f	15.5 ± 0.9 e	339.1 ± 5.1 a	39.9 ± 1.7 a	510.1 ± 50.4 efg	65.4 ± 0.9 de
RITA	8	200	89.9 ± 7.5 b	9.9 ± 0.4 de	289.2 ± 8.1 de	35.2 ± 4.5 cd	302.2 ± 16.7 ab	37.6 ± 1.4 a	681.3 ± 38.8 cde	82.4 ± 5.3 bcd
RITA	8	300	108.8 ± 4.1 b	12.1 ± 1.2 de	389.5 ± 5.1 cd	50.5 ± 3.1 bc	134.9 ± 8.9 de	17.5 ± 1.2 b	633.3 ± 19.1 def	80.0 ± 2.6 bcd

ANOVA summary table

Source	Fresh wt of leaves		Dry wt of leaves		Fresh wt of stems		Dry wt of stems		Fresh wt of roots		Dry wt of roots		Total fresh wt		Total dry wt	
	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value
Immersion frequency (IF)	22.4	0.000	13.8	0.000	52.3	0.000	25.1	0.000	94.7	0.000	192.4	0.000	43.2	0.000	45.3	0.000
Volume of medium (VM)	109.7	0.000	132.5	0.000	198.2	0.000	168.3	0.000	27.2	0.000	46.5	0.000	190.2	0.000	232.4	0.000
IF × VM	13.9	0.000	16.9	0.000	24.3	0.000	15.3	0.000	16.3	0.000	33.9	0.000	22.0	0.000	25.5	0.000

Data shown are mean values ± SE from measurements performed in three Sterivent® vessels or RITA® bioreactors each containing 20 explants. In columns, different letters indicate significant differences ($P = 0.05$) between treatments according to Tukey's test.
ANOVA = analysis of variance.

Table 4. Acclimatization success of *Stevia rebaudiana* plantlets obtained from different culture systems.

Plantlets obtained from different culture systems	Acclimatization success (%) (± SE)	Number of shoots per plantlet (± SE)	Shoot length (cm) (± SE)	Number of nodes per plantlet (± SE)	Number of leaves per plantlet (± SE)	Leaf length (cm) (± SE)	Root length (cm) (± SE)
Semisolid medium	100.0 ± 0.0 a	1.7 ± 0.1 a	27.3 ± 0.6 b	11.9 ± 0.8 ab	27.8 ± 1.6 b	3.9 ± 0.1 b	11.4 ± 0.4 b
RITA (nonhyperhydrated)	100.0 ± 0.0 a	2.1 ± 0.2 a	34.3 ± 0.9 a	15.1 ± 1.4 a	38.4 ± 3.7 a	4.4 ± 0.2 a	17.2 ± 0.6 a
RITA (hyperhydrated)	75.0 ± 5.0 b	1.6 ± 0.1 a	20.2 ± 1.4 c	8.9 ± 0.6 b	21.6 ± 1.2 b	2.9 ± 0.1 c	12.9 ± 0.8 b

ANOVA summary table

Source	Acclimatization success		Number of shoots		Shoot length		Number of nodes		Number of leaves		Leaf length		Root length	
	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value
Type of plantlets	25.00	0.013	2.15	0.126	49.28	0.000	9.47	0.000	12.53	0.000	28.31	0.000	23.80	0.000

Data shown are mean values ± SE from plantlets obtained from different culture systems. In columns, different letters indicate significant differences ($P = 0.05$) between treatments according to Tukey's test.
ANOVA = analysis of variance.

Table 5. Biomass growth of acclimatized *Stevia rebaudiana* plantlets obtained from different culture systems.

Plantlets obtained from different culture systems	Total wt of leaves (g) per plantlet (± SE)		Total wt of stems (g) per plantlet (± SE)		Total wt of roots (g) per plantlet (± SE)		Total wt (g) per plantlet (± SE)	
	Fresh wt	Dry wt	Fresh wt	Dry wt	Fresh wt	Dry wt	Fresh wt	Dry wt
Semisolid	1.1 ± 0.1 b	0.3 ± 0.1 b	0.8 ± 0.2 a	0.7 ± 0.2 a	2.9 ± 0.1 b	1.5 ± 0.1 b	4.9 ± 0.4 b	2.4 ± 0.3 b
RITA (nonhyperhydrated)	1.8 ± 0.0 a	0.5 ± 0.1 a	1.3 ± 0.1 a	1.0 ± 0.1 a	4.4 ± 0.1 a	2.2 ± 0.1 a	7.4 ± 0.1 a	3.6 ± 0.1 a
RITA (hyperhydrated)	0.7 ± 0.1 b	0.2 ± 0.1 b	0.6 ± 0.1 a	0.4 ± 0.1 a	3.3 ± 0.3 b	1.7 ± 0.1 b	4.5 ± 0.4 b	2.3 ± 0.2 b
ANOVA summary table								
	Fresh wt of leaves	Dry wt of leaves	Fresh wt of stems	Dry wt of stems	Fresh wt of roots	Dry wt of roots	Total fresh wt	Total dry wt
Source	F value *P* value	F value *P* value	F value *P* value	F value *P* value	F value *P* value	F value *P* value	F value *P* value	F value *P* value
Type of plantlets	48.73 0.005	48.73 0.005	8.84 0.055	7.62 0.067	33.13 0.009	33.13 0.009	26.11 0.013	18.52 0.021

Data shown are mean values ± SE from plantlets obtained from different culture systems. In columns, different letters indicate significant differences ($P = 0.05$) between treatments according to Tukey's test.
ANOVA = analysis of variance.

advantage to produce more foliar biomass. The greatest leaf fresh (393.6 mg/explant) and dry (39.0 mg/explant) weights were observed with the RITA® bioreactor containing 300 mL of medium and immersion for 10 s/1 h (Table 3). The greatest total stem fresh (1014.9 mg) and dry (92.7 mg) weights per explant were obtained with RITA® treatment containing 300 mL of medium and immersion for 10 s/6 h (Table 3). In parallel with the greatest root length, the greatest root fresh (339.1 mg/explant) and dry (39.9 mg/explant) weights also were observed with the RITA® bioreactor containing 100 mL of medium and immersion for 10 s/8 h (Table 3). Roots produced by the RITA® treatments (Fig. 1D and E) generally appeared to be longer and more branched than those in the semisolid medium (Fig. 1F).

Acclimatization. The success of micropropagation depends on the survival rates of the acclimatized plantlets. Therefore, acclimatization is one of the most important stages of micropropagation. In the present study, shoots developed in RITA® were successfully rooted and acclimatized under glasshouse conditions.

All the nonhyperhydrated plantlets from the semisolid culture and from RITA® bioreactors were acclimatized to a glasshouse (Table 4; Fig. 1G). The greatest number of shoots (2.1), nodes (15.1), and leaves (38.4) and shoot (34.3 cm), leaf (4.4 cm), and root (17.2 cm) length was observed on plantlets acclimatized from nonhyperhydrated plantlets developed in RITA® bioreactor applications. Biomass production was also greatest in nonhyperhydrated plantlets grown in RITA® (Table 5). The reason for the superiority of nonhyperhydrated plantlets developed in RITA® might be the well-developed roots of these plants (Fig. 1D and E). Roots produced by the RITA® treatments generally appeared to be stronger and, therefore, plants were able to adapt to soil quicker. Ramírez-Mosqueda et al. (2016) obtained 90% acclimatization success. However, they used only plantlets obtained from semisolid medium for acclimatization. In other studies, regarding the micropropagation of *Stevia* by TIS bioreactor, there is no information on acclimatization (Alvarenga-Venutolo and Salazar-Aguilar, 2015; Vives et al., 2017). In the present study, the acclimatization performance of plantlets obtained from semisolid and RITA® bioreactors (hyperhydrated and nonhyperhydrated) were evaluated.

It is known that the process of hyperhydricity is generally reversible and new shoots or leaves formed by hyperhydric shoots after transfer to the greenhouse may have a morphology and anatomy approaching those of normal plants (Kevers et al., 2004). The hyperhydrated plantlets developed in RITA® bioreactor applications were acclimatized 75%. The leaves of hyperhydrated plantlets from RITA® bioreactor showed shoot tip necrosis within 3 to 4 d after acclimatization. Due to apical shoot tip necrosis, the effect of apical dominance on lateral buds was removed and new shoots were induced from lateral buds. Although all the leaves of the first acclimatized hyperhydrated plantlets were completely necrosed after 8 to 10 d of acclimatization, these new shoots continued to grow healthily (Fig. 1H). The system developed here enables an economical and less time-consuming method of S. *rebaudiana* production, which may be of interest to commercial producers.

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