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MASS MICROPROGATION OF *Stevia rebaudiana* BERTONI IN TEMPORARY IMMERSION SYSTEMS

Micropropagación masiva de *Stevia rebaudiana* Bertoni en sistemas de inmersión temporal

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ABSTRACT. *Stevia rebaudiana* Bertoni, Asteraceae, is a natural sweetener called "Stevia". Its properties came from the diterpenoid glycosides presented in the leaves: a stevioside and rebaudisioside. The percentage of seed germination of *S. rebaudiana* is very low and the plants produced are heterogeneous, so it is not suitable for mass propagation in the field.The tissue culture in temporary immersion systems, is an effective tool for micropropagation, since it increases the multiplication coefficient and produces the improvement in the quality of *in vitro* regenerated plants. This research determined the efficieness of propagation process using temporary immersion devices for scaling up the mass production of *Stevia rebaudiana*, with the use of liquid media in automated temporary immersion system (RITA®) and twin flasks system (BIT), compared with semisolid culture. Most of the treatments involving temporary immersion systems (BIT y RITA®), produced green plants and vigorous plants, with low levels of hyperhydricity. The highest average of regenerated leaves and shoots was obtained in the RITA® system, with 10 minutes of immersion every 8 hours. It was determined also that 10 minutes on inmersion each 12 hour with the BIT produced vigorous plants, with an increased length, fresh and dry weight mass. The use of *in vitro* culture in temporary immersion bioreactors (RITA® and BIT), offers many advantages in the process of scaling the production of this kind of commercial plant, related to connentional propagation system and encouraging the process of acclimatization.

Key words: sweetener, *in vitro* culture, bioreactors, propagation, *Stevia*

RESUMEN. *Stevia rebaudiana* Bertoni, familia Asteraceae, es conocida como "yerba dulce" por poseer un edulcorante natural. Sus propiedades provienen de la presencia de glicósidos diterpenos denominados esteviósidos y rebaudiósidos en las hojas. El porcentaje de germinación de las semillas de *S. rebaudiana* es muy bajo y las plantas producidas son heterogéneas, por lo que no es conveniente para la propagación masiva en campo. El cultivo en sistemas de inmersión temporal, es una herramienta eficaz para la micropropagación, ya que incrementa el coeficiente de multiplicación y produce el mejoramiento en la calidad del material regenerado *in vitro*. En esta investigación se determinó el proceso de propagación de inmersión temporal más eficiente para el escalamiento de la producción masiva de *Stevia rebaudiana*, con el empleo de medios líquidos en sistemas de inmersión temporal automatizado (RITA®) y vasos gemelos (BIT), en comparación con el cultivo en medio semisólido. La mayoría de los tratamientos en sistemas de inmersión temporal BIT y RITA® produjeron plantas verdes y vigorosas, con bajos niveles de hiperhidricidad. El mayor número promedio de regeneración de hojas y brotes se obtuvo en RITA® con 10 minutos de inmersión cada ocho horas. Se determinó que en el tratamiento que consistió en 10 minutos de inmersión cada 12 horas en BIT, produjo plantas muy vigorosas, con el mayor incremento en longitud, masa fresca y masa seca promedio. El empleo de cultivo *in vitro* en biorreactores de inmersión temporal (RITA® y BIT), ofrece muchas ventajas en el proceso de escalamiento de la producción de esta especie de interés comercial, respecto a los sistemas de cultivo convencional, favoreciendo el proceso de aclimatación.

Palabras clave: edulcorantes, cultivo *in vitro*, biorreactores, propagación, *Stevia*

INTRODUCTION

Stevia rebaudiana Bertoni, from the Asteraceae family, is known as "sweet grass" for having a natural sweetener. It is part of the traditional indigenous

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medicine in Paraguay, for its uses as sweetener and medicinal properties that come from the presence of diterpenoid glycosides known as sterviosides and rebaudisiosides (1, 2).

Stevia rebaudiana accumulates over steviol glycosides at different concentrations, depending on the genotype and crop conditions. The most known glycosides are sterviosides and rebaudisioside A (3). It has been reported that the production of sterviosides vary in a range from 5 to 22 % of the leaf dry weight, and from 25 to 54 % of rebaudisioside and in the improved variety, Morita rebaudisioside production can reach 61,6 % (3, 4, 5, 6).

The seeds of this species are very small, so collecting them is a very slow process that turns difficult because of the little uniform flowering of the crop in the field which influences on their maturity (7). The germination percentage of *S. rebaudiana* seeds is very low. The highest germination percentage is reported at 25,5 % after 9 days of being planted on the soil, but later on it decreased to 6,12 % which is explained by a loss of viability of the seeds if stored for long periods, so it is not recommended for massive propagation in the field (8).

Additionally, sexual reproduction produces heterogeneous populations with a great variability range in the glycosides levels. Vegetative propagation is limited due to the low number of propagules produced with the use of *in vitro* culture techniques (9).

The development of efficient techniques for the massive propagation of this specie is required in order to produce plants free of pathogens that preserve the production rate of secondary metabolytes of interest, glycosides in this case.

The use of culture in bioreactors on liquid media and bioreactors in temporal immersion of twin flasks BIT, have become an effective tool for micropropagation since it increases the multiplication coefficient and brings in quality improvement of the regenerated material *in vitro* (10, 11). The use of these systems influence on the reduction of production costs of the crop which is widely documented for a great quantity of species of commercial value (10, 12). On the other hand, immersion times are controlled and hyperhidricity problems frequent in culture systems on liquid media, are reduced (10, 11, 12).

This research determined the most efficient propagation process of temporal immersion for scaling massive production of *Stevia rebaudiana*, using liquid media on automated temporal immersion systems RITA® and BIT, compared to the culture on semi-solid medium.

MATERIALS AND METHODS

Introduction and *in vitro* establishement

The introduction and *in vitro* establishment was done using micro budsticks from plants grown under greenhouse conditions at the Biotechnology Research Center of the Tecnological Institute of Costa Rica (ITCR), located in the province of Cartago. The material was introduced and multiplied on a semi-solid medium that contained salts and vitamins (13), supplemented with 2 mg L^{-1} of Calcium Pantotenate, 0,5 mg L^{-1} of Gibberellic acid (AG_3), 30 g L^{-1} of sucrose and 3,2 g L^{-1} of Phytgel®, pH 5,7. Explants stayed under diffused light conditions at a temperatrure of 25±2 °C, and a photoperiod of 16 hours light and eight hours darkness.

Vitroplants were subcultured every 30 or 45 days, depending on the growth and development under the same light and temperature conditions already described.

Micropropagation of *S. rebaudiana* in automated temporal immersion culture system RITA® and temporal immersion in twin flasks BIT

Culture conditions were: 25±2 ºC of temperature, diffused light and photoperiod of 16 hours light and 8 hours of darkness. Explants consisted in micro budsticks of 12 mm length taken from vigorous vitroplants of dark green color, with three subcultures and one month since its last multiplication.

System 1. Temporal Immersion in RITA®

Four treatments were evaluated: 5 minutes of immersion every 8 hours (T1) and every 12 hours (T2); 10 minutes of immersion every 8 hours (T3) and every 12 hours (T4). Culture containers had 250 ml of liquid multiplication medium and 25 explants, each, so four RITA® were evaluated for each treatment until completing four repetitions.

System 2. Temporal immersion in BIT

Prototypes of BIT were built using glass flasks of 1 L of capacity with pressure lids, glass pipes for the entry and exit of air, packages, hoses and filters of hydrophobics of 0,22 µm (Figure 1). The system was evaluated through four treatments: 5 minutes of immersion every 8 hours (T5) and every 12 hours (T6); 10 minutes of immersion every 8 hours (T7) and every 12 hours (T8). Each culture container had 500 mL of liquid medium and 50 explants; each treatment consisted of four repetitions.

Figure 1. Temporal Inmersion System of twin flasks (BIT)

Control treatments

The control treatment T9 consisted in culturing 25 explants per culture flask with 250 mL of semi-solid medium, in containers of the temporal immersion system RITA®, with a total of four repetitions. Treatment T10 consisted in culturing 50 explants with four repetitions in flasks BIT with 500 mL of culture medium.

In all systems, a micropropagation medium with M&S salts, supplemented with 2 mg L^{-1} of Calcium Pantotenate and 0,5 mg L^{-1} of AG_3, was used without gel agent, except in the semi-solid medium (control treatments).

Once the three-weeks multiplication period was over, the number of shoots, number of leaves, length, fresh and dry mass, were determined. Average of the variable per repetition in each treatment was calculated. Only the measurement of the dry mass was registered as one data per repetition, it was achieved by placing explants on the heater at 70 °C for 24 hours. Likewise, the chlorophyll content a and b was determined using 15 mg of tissue that later on was frozen with liquid nitrogen, macerated on a mortar and homogenized with 1mL of acetone at 80 %. Extracts were centrifuged at 15000 g (12 500 rpm) for 15 minutes. Finally, the supernatant was taken and readings were made at 646,8 and 663,2 nm in a spectrophotometer. Total chlorophyll concentrations were calculated according to the equations proposed by Lichtenthaler (14).

Qualitative characteristics of color and hyperhidricity per treatment were done through observation and were documented by photographs. The color was evaluated according to a Pantone Color Chart provided by the School of Industrial Design of the Tecnological Institute of Costa Rica.

Statistical analysis

Corresponding averages per repetition were calculated for each of the variables. Results were tabulated in charts that showed the behavior of the variable according to the treatment. Data were analyzed through ANOVA. The comparison of means was made through the Tukey's Test with an honest significant difference of both 1 and 5 %, with the use of the software Minitab®.

RESULTS AND DISCUSSION

On a higher number of species, the production of a higher number of propagules has been reported with the use of liquid medium, since in this system, explants are in direct contact with the culture medium which determines that nutrients uptake is more efficient. On the other hand, waste substances are secreted in liquid medium so they affect the explant to a lesser degree (11, 12, 15).

The *Stevia in vitro* culture in liquid medium on a bioreactor of bubble column increase of fresh mass in the shoots was determined compared to treatments using agitated flasks (16).

This research used the temporal immersion on BIT and RITA® systems. The comparison of means of shoot number, number of leaves, length, fresh and dry mass, was made and the variables reached in all treatments are shown in Table I.

Number sprouts

Treatments T2, T3, T7 and T8 produced a higher average number of shoots, from 12,18 to 14,21, without significant difference among treatments (Table I). The number of average shoots is a determining factor in the multiplication rate so it is relevant in the estimation of the costs of commercial propagation processes.

As to micropropagation using bioreactors information is provided on the facility of production and management of a higher number of seedlings, the stimulation of the growth rate by forced aereation and the suppression of apical dominance that induces a stimule for the growth of lateral sprouts (17). It represents a profit as to the quantity and quality of the *in vitro* material available, which turns even more relevant when it comes to work with difficult-to-manage species in the laboratory making essential the implementation of effective treatments (11, 15, 18, 19, 20).

Table I. Comparison of means for shoot number, number of leaves, length, fresh and dry mass, attained in all temporal immersion treatments through Tukey's Test with an honest significant difference of 1 %

Culture system	Treatments	Variable				
		Δ N° average of shoots	Δ N° average of leaves	Δ Length (mm)	Average fresh mass (mg)	Average dry mass (mg)
Temporal immersion on RITA®	T1	8,14 bc	22,83 bcd	37,91 cd	178,86 bc	23,65 b c
	T2	12,18 ab	30,55 ab	60,87 bc	317,27 bc	40,12 bc
	T3	14,21 a	36,40 a	54,31 bcd	348,05 b	46,20 bc
	T4	7,24 bcd	21,40 bcd	38,70 cd	136,16 bc	20,01 bc
Temporal immersion on BIT	T5	6,03 cd	12,99 de	47,84 bcd	210,46 bc	21,41 bc
	T6	7,31 bcd	16,06 cde	61,49 bc	162,47 bc	14,79 bc
	T7	12,41 ab	26,89 abc	75,91 b	418,35 b	47,42 ab
	T8	13,85 a	23,40 abcd	147,75 a	774,39 a	83,41 a
Control RITA®	T9	7,41 bc	17,64 bcde	44,65 cd	121,76 bc	10,69 c
Control BIT	T10	6,79 bcd	16,02 cde	33,36 cd	103,76 Bc	11,61 bc

Number of leaves

In BIT and RITA® systems, immersion for 10 minutes every 8 hours produced the highest number of average leaves, being higher the average number of leaves with RITA® system. As to the number of leaves regenerated in seedlings, it was determined that the treatment with the highest mean (36,40) was T3 (10 minutes of immersion every 8 hours in RITA®), however, there were not significant differences between this treatment and treatments T2 (10 minutes of immersion every 8 hours, RITA®), with an average of 30,55 leaves, treatment T7 (10 minutes of immersion, every 8 hours, BIT), with 26,89 average leaves and treatment T8 (10 minutes every 12 hours, BIT), with 23,40 average leaves produced.

The average number of leaves is important in *Stevia rebaudiana*, since secondary metabolytes of this specie, the diterpenoid glycosides, endowing them with the sweetening flavor, known as stervioside and rebaudisioside, are accumulated in leaves within a range of 4 to 20 % p/p in commercial crops (15, 21). So it is of interest to produce seedlings with a higher number of leaves and greater foliar area.

Seedling length

The treatment that recorded the highest average length (147,74 mm), consisted in a 10 minutes immersion every 12 hours in the BIT system. It produced plants of length nearly 2,5 times longer that the RITA® treatment, of 5 minutes of immersion every 12 hours (T2). These two treatments, T2 and T8, showed significant differences among themselves. Regenerated seedlings by the BIT system, showed a higher vigorousness in the immersion treatments of 10 minutes every 12 hours, grew up till the edge of the culture container. Probably, the differences recorded on the BIT and RITA® systems, are related to the effective size of the culture container since, though both containers are of 1 liter of capacity, in RITA®, explants are placed on a support that is approximately half a culture flask so plants have 50 % of the available space to grow up, some 7,5 cm, while BIT does not have supports, and containers are 14,8 cm high allowing for a higher length growth of the plants, which could be an advantage of the BIT system if the objective of the process is the production of high-size plants ready for acclimatization. The selection of the immersion system to use depends on the micropropagation strategy to follow, for example, if a higher quantity of shoots is desired, a RITA® (T3) system could be used because it requires less space and produces shorter plants with more shoots; if longer and vigorous plants are desired to be acclimatized or taken directly to the field, the BIT (T8) system can be chosen.

Fresh mass analysis

The highest fresh mass increase attained was 774,39mg in BIT, in treatment T8 (10 minutes every 12 hours). This treatment was statistically different to the rest, with a confidence level of 95 %.

Dry mass analysis

The lowest dry mass values were attained in control treatments, the highest dry mass average of treatments in RITA®, was reached in the treatment that consisted in immersion of 10 minutes every 8 hours (T3). It exceeded over four times the average attained in the control of RITA®. However, there are not significant differences among treatments in RITA® (Table I).

Regarding dry mass results with the BIT system, dry biomass increase was nearly eight times higher than the immersion treatments of 10 minutes every 12 hours (T8), as compared to the respective control treatment. Treatments T8 and T7, that consisted in 10 minutes immersion every 8 hours (Table I) showed significant differences compared to treatments in BIT.

Presence of chlorophyll and coloration

As to chlorophyll content a and b of *in vitro* plants of *Stevia rebaudiana* (Table II), green color is associated to the chlorophyll presence, this measurement is used to determine the photosynthetic status and reactions related to the phytosynthesis process in plants (18).

Colors between 370 and 377 U correspond to different tones of green being 377 U the darkest one, it is present in treatments T1 (5 minutes every 8 hours) and T3 (10 minutes every 8 hours) of RITA®, T6 (5 minutes every 12 hours) and T7 (10 minutes every 8 hours) of BIT. Treatment T4 (10 minutes every 12 hours, RITA®) showed a yellow color that corresponds to the color 618U of the scale and treatment T8 (10 minutes every 12 hours, BIT) showed a green-coffee color (305-1U). These treatments have in common the immersion times of highest duration, ten minutes and the frequency of immersion to greater intervals of 12 hours that correspond to the highest ones in duration and frequency that were evaluated.

The high chlorophyll concentration means more light collecting centers (LHC II) and therefore, it is supposed that there is a high transport of electrons and probably a higher CO_2 fixing rate. The color of immersion treatments for 5 minutes every 8 and 12 hours (T5, T6), were dark green (22). It is reported that this system could induce a photomixotrophic behavior as shown in temporal immersion systems (BIT), in other species as banana (CEMSA), where complete immersion combined with absence periods of culture media can induce metabolic changes in leaves, among them pigmentation, which could lead to a more favorable form of energy in acclimatization (23).

Regenerated plants in temporal immersion systems were characterized by their vigorous appearance with a thicker stem and foliar area in addition to a dark green color. These characteristics were true in the immersion treatments in twin flasks (BIT), T8 (10 minutes every 12 hours) and T7 (10 minutes every 8 hours) and in the RITA® system with treatment T2 (5 minutes every 12 hours).

Hyperhidricity

Hyperhidricity was little frequent, it showed up in 5 % of the explants of RITA® T2 treatment (5 minutes every 12 hours) and in two BIT T5 treatments (5 minutes every 8 hours) and T8 (10 minutes every 12 hours) it was observed 2,5 % and 5 % of hyperhidricity respectively. So in BIT and in RITA®, the presence of this characteristic was 1,56 % of regenerated plants by the BIT and RITA® systems. It does not pose a problem like in plants grown on a bubble bioreactor, treatment included in the preliminary trials not published in this article. Other study reported the presence of hyperhidric plants in the culture of *Stevia rebaudiana* on a rotary roll bioreactor (24). In ferment jars it was determined the non-presence of hyperhidricity, immersed shoots on a liquid medium showed a higher number of leaves, leaf length and a higher multiplication rate compared to explants in semi-solid medium (25).

Table II. Chlorophyll concentration a, b and its respective relationship in *Stevia rebaudiana in vitro* plants

Sistema	Treatments	Chlorophyll (µg mL^{-1})			Coloration
		a	b	a/b	
RITA®	T1	5,71	3,00	1,90	377 U
	T2	8,05	4,11	1,96	376 U
	T3	8,27	4,02	2,06	377 U
	T4	2,11	1,40	1,51	618 U
BIT	T5	4,68	15,83	0,30	376 U y 370 U
	T6	0,56	1,11	0,50	377 U
	T7	7,08	5,37	1,32	377 U
	T8	1,16	2,40	0,48	305-1 U
Control RITA®	T9	0,99	5,63	0,18	376 U y 370 U
Control BIT	T10	3,80	6,10	0,62	376 U y 370 U

Treatments in RITA® T3 (10 minutes of immersion every 8 hours, and BIT T8, (10 minutes of immersion every 12 hours) were the only treatments that showed up significant statistical differences in the average production of shoots compared to the control treatment (16) that used semi-solid medium (Table I). It could be related to immersion periods that were 10 minutes in both treatments. These results differ from those obtained by other authors that recorded a higher sprouting in *Stevia rebaudiana* in all treatments that used liquid culture medium stationary or in agitated flasks.

According to a study carried out in banana (CEMSA), in Temporal Immersion Systems (BIT), an increase in the multiplication coefficient and plant quality compared to other culture systems was determined (23). On the other hand, in another research it was possible a morphological improvement of the banana CEMSA ¾ shoots cultured on temporal immersion systems (26).

Similar results were recorded in this research under BIT systems, with immersion treatments of 10 minutes T8 (every 12 hours) and T7 (every 8 hours) and T2 in RITA®, with immersion times of 5 minutes, every 12 hours, plants of higher morphological quality regenerated, with vigorous appearance, dark green color, greater foliar areas and thickened stems. Likewise, the culture of sugar cane varieties (*Saccharum sp*), in temporal immersion systems stimulated shoot production and growth (27). In other species like *Ananas comosus* the use of temporal immersion increased shoot and dry mass production of the seedlings[A].

Studies on *Caladium xhortulanum*, ornamental aracea, a multiplication coefficient 12 times higher than that of conventional semi-solid media were attained (28). The results of *S. rebaudiana* in this study coincide with the reports of other plant species since the multiplication rate was higher in most of temporal immersion treatments compared to the conventional culture system in semi-solid medium.

Probably, the increased number of shoots recorded in the temporal immersion systems is related to the ventilation system because every time the system injects air to the culture container during the emerged stage, there is a gas exchange, specially CO_2 and ethylene that volatilized. These gasses inhibit the multiplication of shoots in several species. So with a higher number of immersions a day, ventilation and the exchange of gasses like ethylene and CO_2 occurs and their accumulation limits shoots multiplication, a reason for which it is essential to determine the adequate immersion frequency for each specie. Etienne and Berthouly (2002) assure that the immersion stage time is very important since it determines the nutrients uptake rate and controls hyperhidricity. In this research immersion times of 10 minutes, both with RITA® and BIT, induced shoot production and increased the dry and fresh mass of explants (29).

This research attained the mass production of higher-size and weight plants which influenced the quality of regenerated seedlings. Moreover, with the temporal immersion systems, there were an increased number of propagules that can be used in subcultures thus increasing the micropropagation rate. On the other hand, as it is a process that provides uniform culture conditions, the medium can be easily renovated without changing the container. The culture medium can be sterilized by filtration which reduces the use of autoclaves, less containers are required and a higher quantity of explants are sown by culture container.

As proven in this research, the use of second generation *in vitro* culture, with temporal immersion systems provided several advantages to the micropropagation scaling process of *Stevia rebaudiana*, a plant of great commercial interest.

CONCLUSIONS

- Most of the treatments in temporal immersion systems BIT and RITA® produced vigorous plants with low levels of hyperhidricity, a higher number of average leaves and shoots, active growth with an average higher dry mass. The culture system in RITA® T3, with 10 minutes immersion every 8 hours induced the regeneration of plants *de novo* with average number of shoots, average number of leaves, stem length, fresh and dry mass higher than the control (T9). Treatment T8, BIT with 10 minutes immersion every 12 hours, produced the regeneration of seedlings with seven times more average fresh and dry mass compared to the control (T10). It also showed the highest average shoot number. As to length increase, treatment T8 showed a significant difference in relation to the rest of the treatments.
- In this research, the highest explant growth and the increased shoot induction took place in the two temporal immersion systems (RITA® and BIT) in those treatments with immersion times of 10 minutes, which could be a factor to consider in scaling the production of *Stevia rebaudiana* in temporal immersion systems.

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