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Clonal propagation of Stevia (*Stevia rebaudiana* Bertoni) advanced lines in a Temporary Immersion Bioreactor System

Propagación clonal de líneas avanzadas de Stevia (*Stevia rebaudiana* Bertoni) en un sistema de Biorreactores de Inmersión Temporal

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Abstract

Stevia (*Stevia rebaudiana A* Bertoni), is a native species from Paraguay and Brazil, which is used as a natural sweetener with medicinal value in the preparation of beverages, sweets and others. Its propagation through sexual seeds is limited by their poor quality, for this reason its asexual propagation is promoted through different *in vitro* techniques. In the present investigation, the response of three genotypes of stevia to propagation in Temporary Immersion Bioreactor (TIB) System was studied with four different media as a treatments. The results showed that the treatment M2 (MS + 1 mg/L BAP + 0.1mg/L NAA + 30 g Sucrose) gave the best results for all evaluated characteristics with the exception of the number of rootlets/seedling and being the treatment M1 (MS without growth regulators + 30 g of Sucrose) achieved the highest number of roots and good values for the other characteristics. High quality plants were achieved and the genotype that responded best was IBT-1, and in the acclimatization phase a high survival rate was obtained.

Keywords: *sweetener, in vitro, bioreactor, genotype, Stevia.*

Resumen

La Stevia (*Stevia rebaudiana* Bertoni), es una especie originaria de Paraguay y Brasil, la cual se utiliza como edulcorante natural con valor medicinal en la preparación de bebidas, dulces y otros. Su propagación a través de semillas sexuales está limitada por la mala calidad de las mismas, por lo que se promueve su propagación asexual mediante diferentes técnicas in vitro. En la presente investigación se estudió la respuesta de tres líneas avanzadas de stevia a la propagación en Biorreactores de Inmersión Temporal con cuatro tratamientos diferentes. El tratamiento M2 (MS + 1 mg/L BAP + 0.1 mg/L NAA + 30 g Sacarosa) dio los mejores resultados para todas las características evaluadas con excepción del número de raicillas/plántula y siendo el tratamiento M1 (MS sin reguladores de crecimiento + 30 g de Sacarosa) con la que se logró el mayor número, de raíces y buenos valores para las demás características. Se lograron plantas de alta calidad y el genotipo que mejor respondió fue IBT-1 y en la fase de aclimatación se obtuvo una alta tasa de supervivencia.

Palabras clave: *edulcorante, in vitro, biorreactor, genotipo, Stevia.*

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Introduction

Stevia (*Stevia rebaudiana* Bertoni), is a specie used as a natural sweetener in the preparation of beverages. It has medicinal value due to its ability to reduce glycemia and hypertension problems. Hossain et al. (2017), points out the value in human health of steviol, which is a substance synthesized by the plant and is the one that provides benefits such as regulation of blood pressure, control of blood glucose levels, as well as inducing the pancreas to insulin production, also has antibacterial and antifungal effect.

Yadav et al. (2011), mentions that stevia is a species belonging to the Asteraceae family. It can be propagated by cuttings, seeds, or tissue culture, through the use of explants such as leaves, axillary shoots, root shoots, or internodal explants.

The use of Temporary Immersion Bioreactors (TIB) is an alternative to achieve numerous, uniforms, vigorous plants, with larger size and numerous leaves and a better root system. The use of TIB in Stevia micropropagation was reported by Akita et al. (1994) and acquired greater development of shoots with a total mass of 64.6 kg. Likewise, Alvarenga & Salazar (2015), indicated a greater number of vigorous shoots with better morphological development of stevia in bioreactors in a liquid medium, much higher than that obtained in semisolid media. Plants under temporary immersion show better development at the level of buds, leaves and stems, due to the conditions provided by the Temporary Immersion Bioreactor System (Vives et al., 2017). On the other hand, Rosales et al. (2018) points out that temporary immersion media are more effective for micropropagation, because they provide more space for seedling development . In addition, Melviana et al. (2021), indicates that the availability of seedlings to absorb nutrients is related to the immersion period, which is favorable for plant growth.

Although there are studies of the favorable response of *Stevia* to propagation in bioreactors, it is important to evaluate the response of new advanced lines of this species in a TIB system. The present work aims to study the response of new advanced lines of stevia to *in vitro* culture in a TIB system.

Materials and methods

Experiment location

This research work was carried out in the *in vitro* Tissue Culture Laboratory of the Institute of Biotechnology - IBT, at the La Molina National Agrarian University, located in La Molina, Lima province, Lima department. Peru.

Vegetal material

Three genotypes were used. Advanced lines, IBT-1, IBT-2 and IBT-3.

IBT – 1: variety Morita II, was developed by Toyoshigue Morita.

IBT – 2: variety Miskibamba, developed from Morita II by mass selection.

IBT – 3: clone derived from variety Miskibamba by gamma radiation.

Culture media

It was used following four culture media

M1: (MS without growth regulators + 30 g of Sucrose)

M2: (MS +1 mg/L Benzyl amino purine (BAP) + 0.1 mg/L Naphthaleneacetic acid (NAA) + 30 g Sucrose)

M3: (MS+ 1.5 mg/L Benzyl amino purine (BAP) + 0.1 mg/L Naphthaleneacetic acid (NAA) + 30 g of Sucrose)

M4: (MS+ 2.5 mg/L Benzyl amino purine (BAP) + 0 Naphthaleneacetic acid (NAA) + 30 g Sucrose)

Methodology

1.- Explants (internode with four buds) come from mother plants of advanced lines IBT1, IBT2 and IBT3 cultivated *in vitro*, which are free of diseases.

2.- Preparation and sterilization of four liquid culture media.

3.- For the bioreactors, two twin 500 ml transparent Erlenmeyer flasks were used.

4.- 20 cuttings with four buds were planted in one Erlenmeyer. In other Erlenmeyer, the liquid culture medium was placed. Both were interconnected by silicone hoses and hermetically sealed with rubber lids. A five-minute immersion was used in intervals of three hours, regulated by the timer (MICRO PC-MOLLER R) that at the same time controls the photoperiod. The protocol established by Escalona et al. (1999) was used in this experiment (Figure 1A).

5.- After 30 days, the plants were removed, incubated at a temperature of 22 °C and 16 hours of light and 8 hours of darkness (Figure 1B) .

Evaluations

Height Plant, number of stems, number of leaves, fresh weight and number of roots per seedling were measured following established procedure for this type of studies.

The experiments were organized in a completely randomized design due to the use controlled parameter in the incubation chamber.

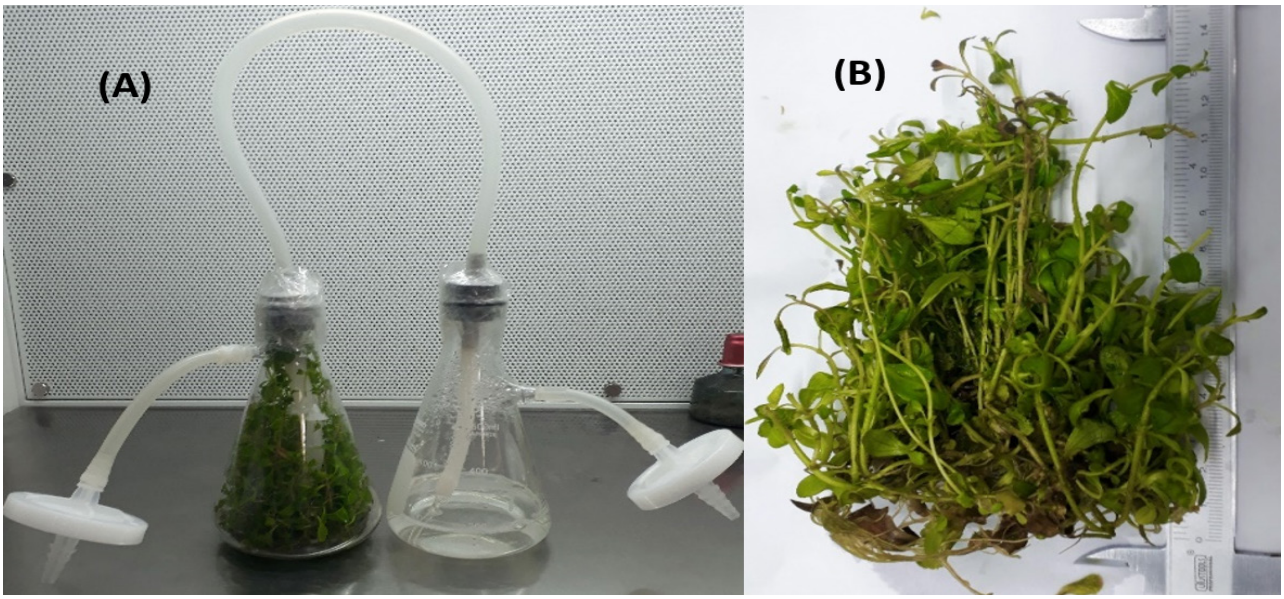

Table 1. Clonal micropropagation of stevia. (A) TIB system showing both erlenmeyers with plantlet and culture media, (B) Plantlets removed after 30 days incubation.

Table 1. Mean squares of the ANVA of seedling height, number of stems/seedlings, number of leaves/seedlings, weight (g) and number of roots/seedlings of Stevia (*Stevia rebaudiNAA* Bertoni) propagated in Temporary Immersion Bioreactors in four different culture media. La Molina 2021.

Source of variation	Degree free	Seedling height	Number of stems/seedling	Number of leaves/ seedling	Weight	Number of roots/seedling
Treatment	3	55.84 ***	265.22 ***	1966.2 ***	2.811 ***	40.18 ***
Residuals	12	0.64	1.02	14.1	0.028	0.10
C.V (%)		11.652	14.501	12.417	12.059	15.983
Media		6.858	6.967	30.213	1.373	2.017

Note. "***" α = 0.001, there are highly significant differences between treatments

The data obtained in the evaluations were processed using the statistical program R study version 3.6.3.

Results and discussion

Determination of the effect of different culture media

The results of the Analysis of variance are presented in Table 1 and highly significant differences can be seen in treatments for all the variables evaluated. The coefficient of variation for seedling height was 11.66 %, number of stems per seedling 14.50 %, number of leaves/seedling 12.41 %, fresh weight/seedling 12.05 %, while for the number of rootlets/seedling it was equal to 15.98 %. A general mean of 6.85 cm for seedling height, 6.96 stems/seedling, 30.21 leaves/seedling, 1.37 g fresh weight/seedling and 2.07 rootlets/seedling were observed.

Table 2 shows the mean values for seedling height, number of stems per seedling, number of leaves/seedlings, fresh weight/seedling and number of rootlets/seedlings. Significant differences (Tukey significance test α= 0.05) were observed for all the characteristics. A range of 1.90 cm to 9.88 cm for seedling height were founded, and it can be seen that the lowest value was obtained with the M4 medium (MS + 2.5 mg/L BAP + 0 NAA +30 g Sucrose) and the highest in the M2 medium (MS +1 mg/L BAP + 0.1 mg/L NAA + 30 g Sucrose). For the number of stems/seedlings, the range was of 1 to 19 stems, the lowest value was found in the medium M4 and the highest with the M2. For the number of leaves/seedlings, the observed values ranged from 7 to 59.46 leaves, with the lowest value in medium M4 and the highest in medium M2. For fresh weight/seedling the range varied from 0.27 g to 2.27 g and the lowest value was founded in Medium M4 and the highest in medium M2. For number of rootlets/seedlings the observed values ranged from 0 to 6.66 rootlets and no rootlets were formed in the M3 (MS + 1.5 mg/L BAP + 0.1 mg/L NAA+ 30 g of Sucrose) and M4 media and the highest number of rootlets was detected in the M1 media (MS without growth regulators + 30 g of Sucrose) (Table 2).

In the present research, the best values were observed in the M2 medium (MS + 1 mg/L BAP + 0.1 mg/L NAA + 30 g Sucrose) and the lowest in the M4 medium (MS + 2.5 mg/L BAP + 0 NAA + 30 g Sucrose) for all the characteristics evaluated with the exception of rootlets formation.

Alvarenga & Salazar (2015), using Stevia micropropagation with Temporary Immersion Bioreactors, observed seedlings with 23.40 leaves on average and a height range of 3.7 cm to 6.00 cm and absence of roots. Villamarin et al. (2020) points out that the addition of IBA (0.37 mg. L^{-1}) promoted the development of more shoots, larger size, more leaves, and root formation, in the TIB system. According to Aguilar et al. (2019), the high concentration of 6-Benzyl amino purine can act negatively on the development of stevia plants, suggesting the use of low concentrations, such as 0.5 mg. L^{-1}, complement with auxins or use culture media without growth regulators.

Alexander et al. (2016) pointed out that the addition of 2 ppm of kinetin to the culture medium generated a greater number of shoots. On the other hand, exposure to red light promotes a greater development of shoots; also stimulating the accumulation of steviosides.

Table 2. Mean values of seedling height, number of stems/seedlings, number of leaves/seedlings, weight (g) and number of roots/seedlings of Stevia (*Stevia rebaudiNAA* Bertoni) in Temporary Immersion Bioreactors in four different culture media. La Molina 2021.

Treatment	Seedling height (cm)	Number of stems/ seedling	Number of leaves/ seedling	Weight (g)	Number of roots/ seedling
M1	9.600 a	4.167 b	32.750 b	1.675 b	6.667 a
M2	9.880 a	19.000 a	59.467 a	2.270 a	1.400 b
M3	6.053 b	3.700 b	21.633 c	1.270 c	0.000 c
M4	1.900 c	1.000 c	7.000 d	0.277 d	0.000 c

Note. Means with the same letter are not significantly different, according to Tukey test (α = 0.05)

Evaluation of the performance of three genotypes of stevia in TIB System

The results of the Analysis of variance are presented in Table 3, and highly significant differences can be seen in treatments for all the variables evaluated. A coefficient of variation was found for seedling height, number of stems per seedling, number of leaves/seedlings, fresh weight/seedling and number of rootlets/seedling equal to 11.02 %, 10.72 %, 12.62 %, 8.01 % and 9.846 %, respectively. A general mean of 9.10 cm for seedling height, 3.93 stems/seedling, 29.94 leaves/seedling, 1.65 g fresh weight/seedling and 6.74 rootlets/seedling was found.

Table 4 shows the mean values for seedling height, number of stems per seedling, number of leaves/seedlings, fresh weight/seedling and number of rootlets/seedling and the Tukey significance test (α= 0.05) and significant differences are observed for seedling height and number of leaves/seedlings. A range of 8.13 cm to 9.90 cm were observed for seedlings height, and the lowest seedling height corresponded to the IBT3 genotype and the highest value to IBT1 genotype. The number of stems/seedlings had a range of 3.80 to 4.17 stems and the lowest value corresponded to the IBT3 genotype and the highest value to IBT1 genotype. For number of leaves/seedlings the corresponded values ranged from 21.50 to 36.50 leaves and the lowest value was found in the IBT3 genotype and the highest value in IBT1 genotype. For fresh weight/seedling, the observed range varied from 1.52 g to 1.73 g, with the lowest value corresponding to the IBT3 genotype and the highest to IBT1 genotype. For the number of rootlets/seedlings, the range was 6.26 to 6.80 rootlets and the lowest number was found in IBT3 genotype and the highest in IBT1 genotype and IBT2 genotype.

Among the genotypes studied, IBT1 line stands out, presenting the highest values of seedling height, number of stems per seedling, number of leaves per seedling, fresh weight per seedling and number of rootlets per seedling. In some characters slightly higher values than IBT2 genotype were observed in IBT1; however, these two genotypes presented values higher than IBT3 genotype for the characters evaluated.

Oviedo (2017), who worked with progenies 7 and 3, reports that there are significant differences between progenies in plant size, number of leaves and shoots. This coincides with the results obtained with the IBT1 and IBT3 genotypes which responses were also different in the condition of this experiment.

Bayraktar (2019) and Melviana et al. (2021b) indicate that immersion periods of more than 10 seconds are detrimental to seedlings, which become chlorotic. In the present work, the immersion was 4 seconds and the seedlings did not present negative effects.

Vives et al. (2017) and Ramírez et al. (2016), reported that in micropropagation in Temporary Immersion Bioreactors, seedlings with better development at the level of shoots, leaves and stems are achieved, due to continuous gas exchange which favors better plant nutrition. In the present research work, in similar way, seedling height of 9.90 and 36.50 number of leaves in the IBT1 genotype were founded.

Table 3. Mean squares of ANVA of seedling height, number of stems, number of leaves/seedlings, fresh weight/seedling and number of roots/seedlings of three genotypes of Stevia (*Svevia rebaudiNAA* Bertoni) propagated in Temporary Immersion Bioreactors. La Molina 2021.

Source of variation	Degree free	Seedling height	Number of stems/ seedling	Number of leaves/seedling	Weight	Number of roots/ seedling
Treatment	2	4.053 *	0.208	283.52 ***	0.070 *	0.505
Residuals	12	1.006	0.178	13.76	0.018	0.4406
C.V (%)		11.02	10.72	12.62	8.01	9.85
Media		9.10	3.93	29.34	1.65	6.74

Note. "***" α = 0.001, there are highly significant differences between treatments

Table 4. Mean values of seedling height (cm), number of stems/seedlings, number of leaves/seedlings, fresh weight/seedling (g) and number of roots/seedlings of three genotypes of Stevia (*Stevia rebaudiNAA* Bertoni) propagated in Temporary Immersion Bioreactors. La Molina 2021.

Treatment	Seedling height (cm)	Number of stems/seedling	Number of leaves/seedling	Weight (g)	Number of roots/seedling
IBT 1	9.90 a	4.17 a	36.50 a	1.73 a	6.80 a
IBT 2	9.28 ab	3.83 a	30.17 b	1.71 a	6.80 a
IBT 3	8.13 b	3.80 a	21.50 c	1.52 a	6.26 a

Note. Means with the same letter are not significantly different, according to Tukey test (α = 0.05)

Conclusions

Good quality *in vitro* plants were obtained in the three advanced Stevia lines, in the M2 culture medium (MS + 1 mg/L BAP + 0.1 mg/L NAA + 30 g Sucrose) in Temporary Immersion Bioreactors system. Among the genotypes, IBT1 stands out with the best performance and the least response in the treatments studied was observed in IBT3 genotype.

Author contributions

Elaboration and execution, development of methodology, conception and design; editing of articles and supervision of the study have involved all authors.

Conflicts of interest

The signing authors of this research work declare that they have no potential conflict of personal or economic interest with other people or organizations that could unduly influence this manuscript.

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