Investigation note

In vitro propagation of *Stevia rebaudiana* and preliminary analysis of steviosides

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Abstract

The *Stevia rebaudiana* Bertoni plant has not been evaluated with the scientific approach on its possible *in vitro* propagation in the state of Puebla. For this reason, in June 2016 different concentrations of 6-Benzylaminopurine (0.0, 0.3, 0.5, 0.7 and 1 mg L^{-1}) and three conditions of culture medium were evaluated for its micropropagation. A preliminary analysis was performed on the presence of steviosides in the culture medium after obtaining and multiplying *Stevia rebaudiana* buds from nodal segments submerged therein for eight weeks. For the analysis of the data on number and length of buds *in vitro*, a completely random analysis of variance was performed and the Tukey test (p= 0.05) was used to determine differences between the means of each treatment using Minitab 16, where the concentration of 0.5 mg L^{-1} of 6-Benzylaminopurine was the treatment that originated the highest number of buds *in vitro* (3.6 ±1.4) and as for the type of support, the culture medium without support (liquid) was the best originating up to 21.45 new buds. The preliminary analysis of steviosides was carried out by thin layer chromatography observing the possible presence of said metabolites in the culture medium.

Keywords: chromatography, explant, micropropagation.

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The *Stevia rebaudiana* Bertoni plant is an herbaceous plant belonging to the Compositae family whose importance is the content of natural sweetener that it produces (stevioside) and which is 300 times sweeter than the sugar obtained from sugarcane (Espinal *et al*., 2006). For this reason, its massive propagation has been sought, one way is by seeds, however, it has low germination by rapidly losing its viability as well as presenting a lot of genetic and phenotypic variability since it is an allogamous plant (Tamura *et al*., 1984). An alternative of mass propagation is through the cultivation of plant tissues *in vitro* to conserve the genetic and phenotypic characteristics of a selected plant (Pierik, 1990).

For this purpose, several *in vitro* studies have been carried out with different results, as mentioned by Taware *et al*. (2010); Arpita *et al*. (2011); Suárez and Quintero (2014); Vázquez *et al*. (2014); Martínez *et al.* (2016); Rock-Okuyuku *et al*. (2016) and even subjected to systems of temporary immersion (Rosales *et al*., 2018), a system that offers the advantage of not dispensing with agar as a gelling agent since its use increases production costs under the conventional tissue culture system vegetables.

The presence of steviosides in plants obtained *in vitro* has also been identified (Bondarev *et al*., 2003; Bravo, 2009; Bondarev *et al*., 2001; Vázquez *et al*., 2014; Magangana *et al*., 2018) to ensure quality. of the micropropagated material. However, it has not been investigated that the plant material of *Stevia rebaudiana* can biosynthesize the sweetening metabolites *in vitro* and these can be released into the liquid culture medium as a consequence of being in contact with the plant material (maceration), this would open an opportunity for biosynthesize them *in vitro* on a larger scale directly in bioprocessing systems for micropropagation.

Based on this, the objective of this work was to determine the concentration of 6-Benzylaminopurine and type of support in the culture medium that promotes the formation of buds from nodal segments of *Stevia rebaudiana* Bertoni *in vitro* for the preliminary detection of steviosides in the culture medium.

Vegetal material

The present investigation was carried out with nodal segments of *Stevia rebaudiana* Bertoni from plants provided by producers from the Izucar de Matamoros region, Puebla. The experimental work was carried out in the Laboratory of Biotechnology Area of Cultivation of Cells and Plant Tissues *in vitro* of the Educational Program of Agrobiotechnology of the Technological University of Izucar de Matamoros. The explants were disinfected by a 70% ethanol sequence plus a 15% sodium hypochlorite solution (Cloralex®) for 15 min and by rinsing three times with sterile distilled water.

Effect of 6-Benzylaminopurine on buds formation and length

To evaluate this response, we used the culture medium prepared with the mineral salts MS (Murashige and Skoog, 1962) 100%, supplemented with myo-inositol 100 mg L^{-1}, thiamin-HCl 0.40 mg L^{-1}, 30 g L^{-1} of sucrose, adding different concentrations of 6-Benzylaminopurine (0.0, 0.3, 0.5, 0.7 and 1 mg L^{-1}), plus 7 g L^{-1} of agar and adjusting the final solution of the treatments at a pH of 5.7 ±0.01. Stem segments with two axillary buds were cut to approximately 2 cm to establish a segment in each test tube. *In vitro* cultures were incubated for eight weeks under 24-hour artificial light conditions using white light lamps and at a temperature of 26 ±2 °C in the incubation area.

Support of the culture medium

Based on the treatment that gave the best response for the formation of *in vitro* buds with the help of growth regulator (0.5 mg L^{-1} of 6-Benzylaminopurine), it was decided to compare the effect of said treatment by three conditions: one gelled with agar 7 g L^{-1}, another in liquid form incorporating cotton and finally in liquid form without any support to determine if the type of support in the culture medium had any effect on the formation and length of *in vitro* buds. The cultures were incubated for eight weeks under similar conditions as in the previous experiment.

Preliminary analysis of steviosides

To do this, thin-layer chromatography (CCD) was performed using silica gel plates (TLC Silica gel 60 F254 25 aluminum sheets 20 x 20 cm) of 5 x 3 cm, where a stevioside sample was placed from a Stevia sweetener 100% natural (ebiem®). Another sample was liquid culture medium concentrated by water vapor where *Stevia rebaudiana* Bertoni was micropropagated, being there for eight weeks simulating a maceration, finally a sample from cane sugar to avoid confusion if the revealed bands correspond to steviosides or to this one compound since it was incorporated into the culture medium. After eluting the chromatographic plate with methanol: ethyl acetate (7:3), it was chemically revealed using phosphomolybdic acid (Lenis *et al*., 2007) with the aid of a heating grid for the visualization of the compounds.

Analysis of data

To obtain *in vitro* response data, 10 repetitions per treatment were taken by taking one test tube as an experimental unit with one explant each and recording the observations each week for 8 weeks. The data were subjected to analysis of variance and the Tukey test (p= 0.05) was applied to define the difference between the mean effects of the treatments. The statistical package used was Minitab 16.

Effect of 6-Benzylaminopurine on buds formation and length

The best activation of *in vitro* buds from nodal segments in *Stevia rebaudiana* Bertoni was determined by the concentration of 0.5 mg L^{-1} of 6-Benzylaminopurine (BAP) as a regulator of plant growth in a statistically significant way (Tukey, p= 0.05) (Figure 1A) at 30 days of *in vitro* culture; however, the development of buds in the explants occurred in all the treatments from the first week of culture. In addition, it was observed that the responses for the variables: buds formation and length are strongly correlated (-0.856) inversely according to the concentration of BAP and where from a concentration of 0.3 mg L^{-1} of BAP the length of the buds decrease possibly as a consequence of their effect in the neoformation of buds (Table 1). However, from the 0.7 mg L^{-1} concentration of BAP, both the length and the number of buds formed were affected causing severe damage with appearance burned with 1 mg L^{-1} of BAP and even the death of most of buds after 45 days of *in vitro* culture (Figure 1).

Table 1. *In vitro* organogenic responses from nodal segments in *Stevia rebaudiana* Bertoni in MS 100% medium and in different concentrations of BAP.

Treatments	Variables	
	Number of buds	Bud length (cm)
0 mg L^{-1}	1 ±0 b	7.8 ±1.2 a
0.3 mg L^{-1}	1 ±0.15 b	6.59 ±1.41 ab
0.5 mg L^{-1}	3.6 ±1.4 a	4.52 ±1.08 b
0.7 mg L^{-1}	3.2 ±0.8 a	5.12 ±0.88 b
1 mg L^{-1}	2.4 ±0.46 a	4.42 ±2.18 ab

Nodal segments responses *Stevia rebaudiana* Bertoni per treatment. Treatments that have the same letter are not statistically different (Tukey, p= 0.05).

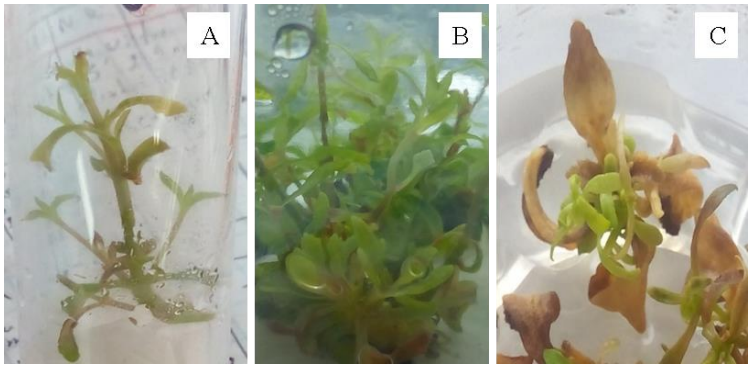

Figure 1. *In vitro* organogenic responses from stem segments of *Stevia rebaudiana* Bertoni. A) buds obtained with 0.3 mg L^{-1} of BAP, B) buds obtained with 0.5 mg L^{-1} of BAP; and C) appearance of buds obtained with 1 mg L^{-1} of PAB after 45 days of *in vitro* culture.

Results slightly lower than those published by Suárez and Quintero (2014), since they report 5.5 new buds from nodal segments with 2 mg L^{-1} and in this investigation 3.6 ±1.4 new buds were obtained per explant with 0.5 mg L^{-1} and as the concentration of BAP increased, the number of buds decreased to 2.4 ±0.46. However, they are superior to the results published by Vázquez *et al*. (2014) who report 2.68 buds per explant using kinetin (1.3 mg L^{-1}) as the best phytohormone compared to 6-Benzyladenine (BA) 4 mg L^{-1} plus IAA 0.3 mg L^{-1} obtaining 2.08 buds per explant, response similar to that obtained in the present research work (2.4 buds per explant) with 1 mg L^{-1} of BAP, indicating that the results may vary according to the genotype of the species or to the type and combination of growth regulators (Vázquez *et al.*, 2014; Rock-Okuyuku *et al*., 2016).

Also, Vázquez *et al*. (2014) report the use of BAP in 0.5 and 0.8 mg L^{-1} obtaining 1.4 and 1.92 buds per explant respectively, responses lower than those achieved in *Stevia rebaudiana* Bertoni with 0.5 and 0.7 mg L^{-1} (Figure 1A). On the other hand, it is important to mention that Suárez and Quintero (2014) used an MS culture medium at 50% of their concentration, which could have been a point in favor to express this response. However, for the variable length of buds the results are similar to what Suárez and Quintero (2014) mention, since they also report to the control treatment or without phytohormones as the statistically superior treatment to BAP treatments for said variable. Similarly, the same authors mention that the higher concentration of BAP the number of

leaves decreased, but for the research discussed here, treatment with 1 mg L^{-1} did not reduce the number of leaves, but caused them a burnt appearance possibly due to the concentration of the MS medium at 100% that was used for this investigation.

In another case, the results obtained in the present work are superior with 0.5 mg L^{-1} of BAP (Table 1) to the results by Martínez *et al*. (2016), who mentioned up to 3 buds with 0.5 mg L^{-1} of BAP + 0.2 mg L^{-1} of AIB from apices, but when they used nodal segments there was a decrease in the number of buds in the treatments with growth regulators. control, which formed 1.93 buds per nodal segment, being statistically superior (LSD $p< 0.05$) to the other treatments, contrary to that obtained in the present work, where the formation of new buds was 1 ±0 in the control treatment (Table 1), which suggests that for each plant tissue culture work, even if it is the same species, its morphogenic behavior should be studied *in vitro* before starting a commercial scaling.

Support of the culture medium

The best treatment to obtain a greater number of buds was the liquid medium without some support with 21.45 buds per nodal explant, this response being statistically significant (Tukey, $p= 0.05$) in comparison with the other two treatments (Table 2). The buds in the gelled culture medium showed the same damage response in leaves and buds after 45 days of *in vitro* culture. In the liquid culture medium with cotton, as a support medium, the damage was specifically in leaves and not in stem, the liquid medium without support being the best treatment for the *in vitro* multiplication of *Stevia rebaudiana* Bertoni buds from nodal segments without causing damage to leaves or stem, on the contrary, the buds showed an intense green color.

Table 2. *In vitro* organogenic responses from nodal segments in *Stevia rebaudiana* Bertoni in MS 100% medium and in different inert supports.

Treatments	Variables	
	Number of buds	Bud length (cm)
Liquid medium	21.45 ±1.55 a	3.88 ±0.62 a
Agar-agar	4.15 ±1.15 c	4.25 ±1.75 a
Cotton	10.68 ±2.32 b	5.58 ±1.42 a

Nodal segments responses *Stevia rebaudiana* Bertoni per treatment. Treatments that have the same letter are not statistically different (Tukey, $p= 0.05$).

When using a liquid culture medium and without any support, the number of buds obtained is higher because the explants are in direct contact with the culture medium and this allows greater effectiveness in the intake of nutrients by the tissues (Watt, 2012). In spite of this, the number of buds obtained (21.45 ±1.55) per nodal segment is still lower than that published by Parvatam *et al*. (2010) who obtained 28 ±1 buds per explant; however, they used 1 mg L^{-1} (4.44 μM) of BA + 0.15 mg L^{-1} (0.8 μM) of ANA in a B5 culture medium. However, Parvatam *et al*. (2010), obtained 6.66 ±0.57 buds when using the same phytohormones and their concentrations in an MS culture medium. This indicates that the number of buds (21.45 ±1.55) per nodal segment of *Stevia rebaudiana* Bertoni can be increased by combining BAP with other phytohormones (auxins) and for this, different concentrations and combinations should be studied to increase the number of buds in MS medium (Martínez *et al*., 2016; Rock-Okuyuku *et al*., 2016).

Preliminary analysis of steviosides

The disclosure of the chromatographic plate shows that it is possible to obtain steviosides by culturing *in vitro* stem segments of *Stevia rebaudiana* as a biotechnological tool. Furthermore, it seems that these metabolites can be released into the liquid culture medium as observed in the chromatographic plate in which bands coincide between the sample obtained from the culture medium where *Stevia rebaudiana* Bertoni was micropropagated with the commercial sample (ebiem®) (Figure 2: Rf= 0.52, Rf= 0.51). Also, you can see the bands in darker color that are those that correspond to cane sugar included in the liquid culture medium, as a source of carbon and energy, but with a lower Rf (Rf= 0.42) to Rf (0.52) of the commercial sample of steviosides (ebiem®) (Figure 2). This confirms that the mark enclosed in a red circle (Figure 2: Rf= 0.51), possibly can be steviosides released in the culture medium.

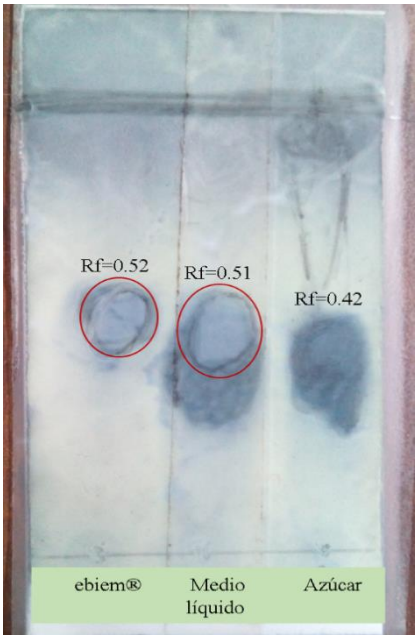

Figure 2. Chromatographic plate (CCD) eluted with methanol:ethyl acetate (7:3).

Therefore, steviosides can be biosynthesized *in vitro* and released to the liquid culture medium in response to some type of stress, since the synthesis of steviosides usually begins at very early stages of plant development and varies its concentration by the conditions environmental (Bondarev *et al*., 2003). Similar results are mentioned by Parvatam *et al*. (2010); however, they analyzed plant leaves obtained *in vitro* and not the culture medium. In the same way, Vázquez *et al.* (2014) report the synthesis of steviosides in *Stevia rebaudiana* Bertoni plants propagated *in vitro*, both in micropropagated and acclimated plants in greenhouse and in plants directly extracted under *in vitro* conditions. Based on this, it may be that this type of project can be scaled at the level of bioreactors to obtain steviosides commercially once the variables that directly intervene in the process have been studied and adjusted, as shown by Magangana *et al*. (2018).

Conclusions

The establishment and *in vitro* multiplication of *Stevia rebaudiana* Bertoni buds from nodal segments was achieved. Also, the best concentration and culture medium that increase buds formation was identified. On the other hand, a preliminary analysis on the presence of steviosides by thin layer chromatography was achieved from the liquid culture medium where buds of *Stevia rebaudiana* Bertoni were micropropagated giving apparently positive results on the presence of steviosides under said conditions.

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