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In vitro Organogenesis of *Stevia rebaudiana* Bert. with Different Explants and Growth Regulators

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ABSTRACT

Objective: To evaluate various explants and growth regulators in order to improve *in vitro* propagation of *Stevia rebaudiana* through organogenesis.

Design/Methodology/Approach: Explants and growth regulators in two different concentrations were evaluated. The explants were nodal segment, axillary bud, and apical meristem; while the growth regulators were benzylaminopurine (BAP) at 1.125 mg L^{-1} and 3.0 mg L^{-1}, naphthaleneacetic acid (NAA) at 1.5 mg L^{-1} and 3.0 mg L^{-1}, and CIDEF-4 brassinosteroids (BRs) at 1.0 mg L^{-1} and 1.5 mg L^{-1}. In total 18 treatments with seven repetitions. Contamination, oxidation, and survival were recorded during induction; while leaf number, regrowth height, and root presence were recorded during multiplication.

Results: At the induction stage there was a differential response between explants according to their ontogenetic age; during multiplication, the morphological components showed differences between concentrations of growth regulators and explants, with higher effectiveness when adding BAP to apical meristems.

Study Limitations/Implications: Both the origin and the age of explants can induce differential growth while interacting with growth regulators.

Findings/Conclusions: Apical meristem explants showed better advantages for *in vitro* reproduction of *S. rebaudiana* since they present less contamination and higher survival at the induction stage, even when exhibiting the highest oxidation among explants, which did not influence the decrease in their survival. At the multiplication stage with apical meristem, height, leaf number, and root presence were increased. Values were high when interacting with BAP.

Keywords: stevia, micropropagation, brassinosteroids.

INTRODUCTION

Stevia rebaudiana Bert. is a native bush from Paraguay, known as sweet leaf given the presence of diterpene glycosides, such as stevioside and rebaudioside A (Oviedo *et al*., 2015). The leaves of the plant have traditionally been used as a sweetener (Pande and Gupta, 2013). It is non-caloric and sweeter than sucrose or sugar cane (Jagatheeswari and Ranganathan, 2012). The natural sweeteners in *S. rebaudiana* are appropriate for people who should control the concentration of sugar in their

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blood (Deshmukh and Ade, 2012; Jagatheeswari and Ranganathan, 2012). In addition, it is non-carcinogenic (Ramya *et al*., 2014), an attribute distinguishing it from artificial sweeteners. This fact has increased its demand in the national and international markets, and in Mexico an increase in its crop surface has been suggested. It is considered that *S. rebaudiana* Bert. is likely to have good productivity in the Pacific Slope and some regions of the Gulf of Mexico (Ramírez *et al*., 2011).

Conventionally, the plant is propagated through cuttings, and this traditional method only allows for the reproduction of few plants. Obtaining the plant via sexual reproduction is also limited due to the scarce seed production of *S. rebaudiana* and its low percentage of germination (Jagatheeswari and Ranganathan, 2012). Faced with this situation, *in vitro* reproduction poses an alternative in order to increase multiplication rates (Das, *et al*., 2011).

Different studies on *S. rebaudiana* micropropagation have been carried out with various explants, such as leaves (Karimi *et al*. 2014), internodal segments (Singh *et al*., 2014), and apexes (Das *et al*., 2011), and different growth regulators have been tested as well (Singh *et al*., 2014). However, changes in the cells can be induced with some regulators (Izquierdo *et al*., 2012), such as cytokinins in high concentrations (Orbovic *et al*., 2008). Through in vitro reproduction, the absence or concentration of both the medium's compounds and the growth regulators trigger several responses in explant development. Brassinosteroid or homobrassinolide (BR) analogues, which are non-traditional growth regulators, can be used as substitutes for auxins and cytokinins in biotechnological processes (Izquierdo *et al*., 2012). These are metabolites that are capable of stimulating plant growth through cell division and elongation of shoots and roots (Sirhindi *et al*., 2011; Vleesschauwer *et al*., 2012) in segments of different organs and explants (Salgado *et al*., 2008). In addition, their application positively influences against biotic and abiotic stress during development of the plants (Bajguz, 2010). With that aim, various explants and growth regulators were evaluated in other to improve *in vitro* propagation of *Stevia rebaudiana* through organogenesis.

MATERIALS AND METHODS

The experiment was carried out at the Biotechnology Laboratory of the School of Agricultural Sciences, Campus IV, at the Autonomous University of Chiapas (Universidad Autónoma de Chiapas, UNACH), located in the municipality of Huehuetán, Chiapas (15° 00' 25.02'' N and 92° 23' 59.06'' W, at 44 meters in altitude). Two stages were considered: the first was induction, 11 weeks in duration, the second multiplication, which lasted for 17 weeks.

Biological material was obtained from *S. rebaudiana* var. Morita 2 established in the greenhouse of the Experimental Field at the School of Agricultural Sciences (UNACH). The explants –axillary bud, apical meristem, and nodal segment– were obtained after a two-month growth period. At the greenhouse, the plant was sprayed with Amistar® (azoxystrobin 1.5 g L^{-1}) fungicide during three days before getting the explants. Once removed from the plant, the explants were taken to the laboratory and washed with soap and water. Then, they were kept in agitation for 10 minutes in an azoxystrobin (1.5 g L^{-1}) solution, to which a drop of Tween® 20 (polyoxyethylene sorbitan monolaurate) was added; likewise, sodium hypochlorite (NaClO) solution at 2.0% was added for 15 minutes in the laminar flow chamber. Subsequently, the explants were rinsed three times with sterile distilled water and 1.5 cm-long nodal segments, axillary buds between 2.0 and 3.0 mm in diameter, and 1.5 cm-long apical meristems were obtained from the plants' branches. Before planting, the explants were placed in an antioxidant solution, composed of citric acid (0.1 g), ascorbic acid (0.15 g), and sucrose (30 g).

The Yasuda *et al*. (1985) semisolid medium was employed, modified by adding vitamins and the following growth regulators: BAP (6-benzylaminopurine), NAA (naphthaleneacetic acid) and Br (brassinosteroid), each one in two different concentrations. Brassinosteroid (BR) CIDEF-4 is a homobrassinolide, produced in Mexico by the Natura del Desierto, S.A. de C.V., company which has 80% steroidal content and 10% active with a non-toxic soluble form presentation, compatible with fertilizers, insecticides, and fungicides. The medium was sterilized by autoclave at 15 PSI and 120 °C for 20 minutes.

The explants were placed in test tubes with 10 mL medium. Once the planting was carried out in each treatment, they were placed in the incubation room at temperature of 26±1 °C, 60% relative humidity, and 45 mE m^2 s^{-1} light intensity for a period of 16 h of darkness and 8 h of light.

The treatments were generated with the combination of these factors: a) explants (nodal segments, axillary buds, and apical meristems), and b) growth regulators in two concentrations: BAP (1.125 mg L^{-1} and 3 mg L^{-1}), NAA (1.5 mg L^{-1} and 3 mg L^{-1}), and BR (1 mg L^{-1} and 1.5 mg L^{-1}). The complete factorial (3×3×2) produced 18 treatments with seven repetitions, distributed in a completely random experimental design. Each explant represented one experimental unit.

After evaluating the induction stage, regrowths from the explants were transplanted for their multiplication and activated carbon (1 g) was added to the cultivation medium in order to stimulate rhyzogenesis, and medium changes were carried out every 20 days.

The response variables during the induction stage resulted from the average of contamination, oxidation, and survival percentages, evaluated each week. At the multiplication stage, regrowth height, leaf number in each regrowth, and percentage of roots were evaluated. The mean results of the variables from the induction stage were graphed using Sigma Plot (V. 11.0) software by Jandel Scientific. Data of the variables from the multiplication stage were analyzed with SAS for Windows Ver. 8.1 (1999-2000) software, and the means comparison between treatments was performed with Tukey's test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Induction Stage; Contamination, Oxidation and Survival

The explants showed lower survival percentages as medium contamination increased. During week 11 of evaluation, average contamination was 65% in nodal segments, 27% in axillary buds, and 20% in apical meristems (Figure 1). In general, contamination in apical meristems decreased when interacting with the lowest doses of all growth regulators. When adding the lowest concentration of NAA (1.5 mg L^{-1}) to the medium, the same explant did not show contamination, but when concentration increased to 3.0 mg L^{-1}, 10% showed contamination (Figure 1).

Higher contamination was recorded in nodal segment and axillary bud explants, which have older ontogenetic ages, when interacting with growth regulators. With regards to NAA application, a contracting effect was present in contamination; that is, contamination increased in nodal segments with the highest concentration (3.0 mg L^{-1}), while an increase in contamination occurred in axillary buds with the lowest dose (1.5 mg L^{-1}). When adding BAP to the cultivation medium, contamination increased in both nodal segments and axillary buds with the lowest dose (1.125 mg L^{-1}). When using Br in the medium, no differences in contamination figures of axillary buds were detected in the two concentrations evaluated; however, contamination was higher with the highest doses in both nodal segment and apical meristem explants (Figure 1).

The increase in contamination in explants that are older is described by López-Gómez *et al*. (2010) in *Coffea* spp. leaves, while Martínez *et al*. (2016) add that explants do not always respond to the procedures employed in the initial decontamination of the material, especially fungi and bacteria. It is worth mentioning that some plants have various particular morphological attributes that hinder the elimination of contaminants, such as presence of epicuticular waxes, and increase in trichome type, form, and density; or else when a plant is exposed longer to the environment, favoring fungal invasion of the stomatic complex.

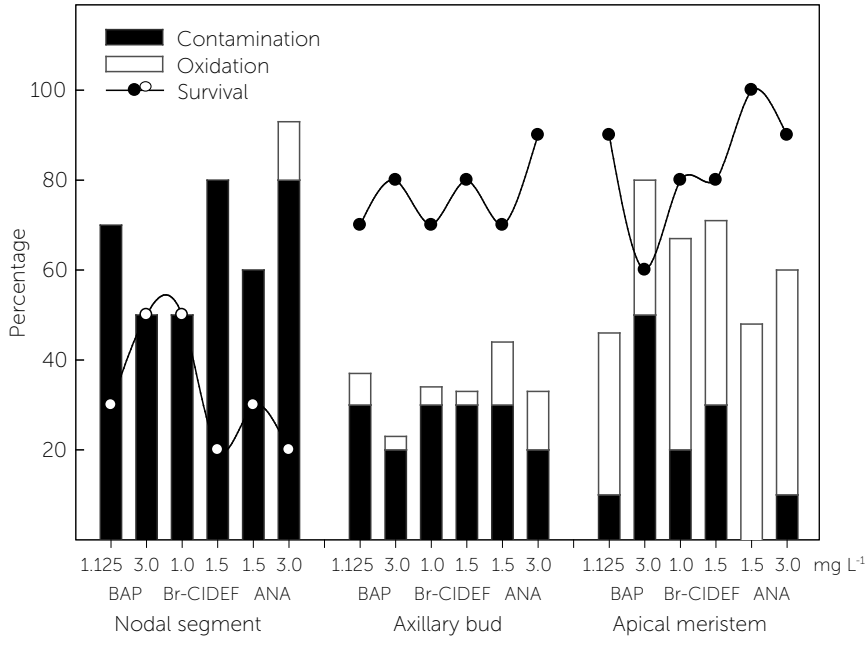

Figure 1. Contamination, Oxidation and Survival of *Stevia rebaudiana* explants in Yasuda medium with different concentrations of growth regulators. Values are average of seven repetitions.

Concerning oxidation or tissue darkening, it seems to be more closely related to the explant's age. Apical meristem, which is the young tissue, showed the highest oxidation average (42%), and decreased to 7% in axillary buds and to 2% in nodal segments, which are older tissues. The increase in oxidation in apical meristem was found when NAA was added to the medium. In this regard, the increase in oxidation of young tissue can be caused by the tissue's oxidative stress, resulting from deficiencies in antioxidant defenses (Turrens, 2003), or else by the presence of phenols in the tissues as a result of their reactions to polyphenol oxidases (Scherer *et al*. 2006), which are exuded into the medium through wounds in the tissue. This problem tends to stop or decrease when the explant starts growing, as was observed in *Gossypium hirsutum* L (Ozyigit *et al*. 2007). Oxidation in some species has resolved using polyvinylpyrrolidone (PVP) (Méndez-Alvarez and Abdelnour-Esquivel, 2014). However, Aguirre-Medina *et al*. (2018) used PVP combined with growth regulators, such as BAP, NAA, and IAA, in *S. rebaudiana* explants of leaves, longitudinally cut stems, and apical meristem, and they indicate that there was no influence in the percentages for oxidation averages.

Among explants, the highest survival of seedlings with different concentrations of growth regulators was observed in apical meristem (82%) and axillary bud (75%), while the lowest survival corresponds to the nodal segment (33%). In *Heliconia bihai* (L.) cv. Lobster Salmón, Marulanda-Angel *et al*. (2011) account for lower survival on the basis of floral meristems treated with BAP (2 mg L^{-1}). In our case, survival was high in apical meristem with 1.25 mg L^{-1} concentration, and it decreased in axillary bud and nodal segments when increasing concentration to 3.0 mg L^{-1}.

Mutiplication Stage: Regrowth Height and Leaf Number

The higher average increase in regrowth height among the explants was found in apical meristems (2.26 cm), and it amounted to 10 and 36% more growth in relation to nodal segments and axillary buds. Apical meristems (3.45) also presented the highest number of leaves per regrowth on average, and it amounted to 22 and 66% more leaves when compared to nodal segments and axillary buds. Likewise, it was statistically different ($P \leq 0.05$) (Table 1).

The regrowth height and the leaf number in explants with growth regulators showed the highest increase when adding BAP in the highest concentration (3.0 mg L^{-1}) and were also statistically different ($P \leq 0.05$) from other regulator concentrations. In this respect, Hassanem and Khalil (2013) mention the same response when applying higher concentrations of BAP, but to MS medium. On the contrary, lower height appeared with Br at a 1.5 mg L^{-1} concentration, and represented 5% less when compared to a Br concentration of 1.0 mg L^{-1}, although the leaf number increased by 4% with the highest Br concentration when compared to the lowest one (Table 1). This outcome is probably linked to its increase in the plant's apexes. The Br is not subjected to active transport inside the plant (Hategan *et al*., 2013), since its metabolism interacts with different enzymes in the cell's organelles and is capable of exhibiting one of the many physiological activities of Br, such as cell division and elongation (Sirhindi, 2013). Induction in plant growth depends on Br concentration, treatment duration, and age when the treatment was introduced, as happened when applying different concentrations of 24-epibrassinolide and 28-homoethylcastasterone to *Vigna irradian* L. and *Brassica juncea* L., which improved photosynthetic rate, total chlorophyll, chlorophylls a and b, carotenoids, shoot length, and fresh and dry weight (Fariduddin *et al*., 2011; Sirhindi *et al*., 2011).

Regarding the interaction between concentrations of growth regulator and explants, the greatest increase in height occurred for apical meristems and nodal

Table 1. Comparisons of explant and growth regulator factors in *Stevia rebaudiana* Bert.

Explant	Height (cm regrowth^{-1})	Number of leaves regrowth^{-1}
Nodal segment	2.03 b*	2.66 b
Apical meristem	2.26 a	3.45 a
Axillary bud	1.43 c	1.14 c
Growth regulators (mgL^{-1})		
BAP 1.125	1.91 c	2.71 b
BAP 3.0	2.01 a	3.14 a
Br 1.0	1.86 e	2.42 d
Br 1.5	1.77 f	2.52 c
ANA 1.5	1.98 b	1.42 f
ANA 3.0	1.91 d	2.28 e

*Different letter values, within columns and factor, are statistically different (Tukey, $p \leq 0.05$). BAP (6-benzyl amino purine), Br (Brassinosteroid), ANA (Naphthaleneacetic Acid).

segments. The first increased with the two concentrations of NAA, plus BAP at 3.0 (mg L^{-1}), and in nodal segments all treatments were included within the first statistical group.

With regards to the interaction of both factors, explants and growth regulators, the number of leaves per regrowth increased by 5.0 in apical meristems and the lowest BAP dose (1.125 mg L^{-1}), and was statistically superior to the rest of the treatments ($P \leq 0.05$), followed by the induction of 4.4 leaves with BAP at 3.0 mg L^{-1} (Table 2). This result agrees with what was reported by Jagatheeswari and Ranganathan (2012), who mention that high BAP doses delay growth in *S. rebaudiana*. Other authors recount similar results when adding BAP to MS medium in the same species (Khalil *et al.*, 2014; Jagatheeswari and Ranganathan, 2012).

The highest leaf number per regrowth (5.0) was achieved in epical meristems interacting with BAP at 1.125 mg L^{-1}, and in nodal segments (4.0) with inclusion of BAP. In this respect, the addition of BAP to MS medium has conveyed this same result in Stevia (Abd-Alhady, 2011; Rangappa and Aind, 2013). In relation to concentrations, there is evidence that adding 3.0 mg L^{-1} fosters regrowth (Abd-Alhady, 2011; Fatima and Khan, 2011; Hassanen and Khalil, 2013), but also the 1.0 mg L^{-1} dose (Thiyagarajan and Venkatachalam, 2012) in MS medium. Intermediate values of 2.7 leaves per regrowth were obtained with Br in nodal segments. Br (28-homoethylcastasterone) was applied to *Mallus prunifolia* (Willd.) Borkh. apex, which increased the number of shoots when benzyladenine was added to the medium (Schaefer, 2002).

In axillary buds, BAP and NAA induced lower regrowth, one regrowth on average, and just two regrowths when adding Br in its lowest concentration (1.0 mg L1). Other authors have combined BAP and naphthaleneacetic acid (in a 2 to 1 proportion) in *Stevia* axillary bud and MS medium, in order to increase the number of regrowths (Rangappa and Aind, 2013). In other results for nodal segment and axillary bud, it was found that there was better regrowth induction when adding low concentrations, BAP from 1.0 to 1.5 mg L^{-1} plus kinetin in more than 0.5 mg L^{-1}, to MS medium (Aamir *et al.*, 2010; Fatima and Khan, 2011). On the contrary, Khalil *et al.* (2014) indicate that adding 2,4-D to the medium, in combination with BAP and NAA or indolebutyric acid, on its own or combined with NAA, significantly inhibits the number of stems per explant in *S. rebaudiana*.

The presence of roots was apparent in the apical meristem explant while interacting with the three growth regulators added to the medium. This result suggests the importance of both the explant and its age in order to favor organ development in plants under *in vitro* conditions. Several authors report greater radical growth when applying NAA to S. rebaudiana explants (Shatnawi *et al.*, 2011; Sikdar *et al.*, 2012).

CONCLUSIONS

The apical meristem explant shows better advantages for *in vitro* reproduction of *Stevia rebaudiana* given that it shows less contamination and higher survival at the induction stage. Even when it did show the highest

Table 2. Morphological components in explants of *Stevia rebaudiana* Bert. in interaction with different concentrations of growth regulators during the multiplication stage.

Explant	Treatment (mgL^{-1})	Height (cm regrowth^{-1})	Number of leaves regrowth^{-1}	Roots (%)
Nodal Segment	BAP 1.125	1.80 bcde*	2.28 de	20
	BAP 3.0	2.21 ab	4.00 bc	0
	Br 1.0	2.11 abc	2.71 de	0
	Br 1.5	1.42 de	2.71 de	10
	ANA 1.5	2.42 a	1.14 fg	0
	ANA 3.0	2.21 ab	3.14 cd	0
Axillary bud	BAP 1.125	1.50 cde	1.00 g	0
	BAP 3.0	1.44 de	1.00 g	0
	Br 1.0	1.54 cde	2.00 ef	0
	Br 1.5	1.50 cde	1.00 g	0
	ANA 1.5	1.34 de	1.00 g	0
	ANA 3.0	1.25 e	1.00 g	0
Apical meristem	BAP 1.125	2.45 a	5.00 a	20
	BAP 3.0	2.31 ab	4.42 ab	20
	Br 1.0	1.92 abcd	2.57 de	20
	Br 1.5	2.40 ab	3.85 bc	10
	ANA 1.5	2.18 ab	2.14 e	20
	ANA 3.0	2.25 ab	2.71 de	10

* Different letter values, within columns and factor, are statistically different (Tukey, $p \leq 0.05$). BAP (6-benzyl amino purine), Br (Brassinosteroid), ANA (Naphthaleneacetic Acid).

oxidation among explants, this did not influence on the decrease of their survival. Height, leaf number, and root presence were increased at the multiplication stage with apical meristem. Values were outstanding when interacting with BAP.

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