# Rapid *in vitro* propagation of *Stevia rebaudiana* Bertoni and evaluation of its stevioside content

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Received: 11 May 2016; Accepted: 5 Apr 2017.

**Abstract**

An efficient protocol was established for rapid *in vitro* propagation of *Stevia rebaudiana* Bertoni, using explants from mature plants. The effect of 1/2MS, MS and B5 culture media, also 27 different combinations of auxins and cytokinins was studied on *in vitro* growth and development of *Stevia rebaudiana* Bertoni. The best culture medium for shoot and root formation was 1/2MS, producing 1.30 shoots/explant, 10 leaves/explant, 6.68 roots/explant and 100% rooting. Shoot and root formation increased by using plant growth regulators. The best shoot formation (2.83 shoots/explant and 12.35 leaves) was obtained in the MS basal medium supplemented with 2.21 µM BAP and 1.42 µM IAA. The best root formation (100% rooting, 7.2 roots/explant and 2.19 cm root length) were obtained in the MS basal medium supplemented with 1.42 µM IAA. *S. rebaudiana* Bertoni plantlets were adapted and grown well in the growing medium containing perlite and vermiculite (1:3). After transplantation to the field. HPLC results demonstrated that stevioside content was similar between the regenerated (19.48 mg $g^{-1}$ dry weight) and the mother plants (20.45 mg $g^{-1}$ dry weight).

***Key words:*** HPLC, MS medium, Plant growth regulator, Plant sweetener, Sugarleaf.

## INTRODUCTION

*Stevia rebaudiana* Bertoni is a small perennial herb belonging to Asteraceae (Compositae) family, and characterized by a very limited range of natural habitats. *S. rebaudiana* is native to the regions between 22–24 ºS and 53–56 ºW in Paraguay and Brazil. Leaves of *S. rebaudiana* are the source of diterpene steviol glycosides, which are estimated to be 300–400 times sweeter than sucrose at 4% (w/v) concentration (Geuns, 2003). These glycosides are non-toxic, non-mutagenic, low caloric and have heat stability up to 100 ºC. They feature a lengthy shelf life compared to the traditional sugar substitutes such as xylitol, sorbitol and aspartame. In addition, steviol glycosides do not have unpleasant side effects (Matsui *et al.*, 1996). These compounds do not cause tooth decay and could be successfully used as a sugar substitute for the patients suffering from diabetes and other diseases related to the disturbance in carbohydrate metabolism (Rajasekaran *et al.,* 2007). The major sweetening compound isolated from the leaves of *S. rebaudiana* is called stevioside. (Rieck, 2012). Stevioside usually includes 1-3% of dry weight of *S. rebaudiana* leaves (Serfaty *et al.*, 2013; Ferrazzano *et al.*, 2015). However, the yield of this compound in leaf tissues can vary according to the method of propagation, day length and agronomic practices (Lemus-Mondaca *et al.*, 2016). Only few attempts have been made to compare the stevioside content between *in vitro* derived plants and field grown plants of stevia. Hwang (2006) reported 12.01 mg $g^{-1}$ dry weight of stevioside in leaves of mother plants and 10.68 mg $g^{-1}$ dry weight stevioside in leaves of *in vitro* regenerated plants of *S. rebaudiana*. In contrast, Singh *et al.* (2014) reported a 2% increase in stevioside content in the leaves of *in vitro* regenerants compared to the leaves of field grown plants.

Since seed germination rate of *S. rebaudiana* is

**Table 1.** Effect of culture medium on *in vitro* growth and development of *S. rebaudiana* cultured after 4 weeks from culturing.

| Culture medium | Average shoot number | Average shoot length (cm) | Average leaf number | Rooting (%) | Root number | Root length (cm) |
|---|---|---|---|---|---|---|
| 1/2MS | 1.30 a | 7.17 a | 10.00 a | 100.00 a | 6.86 a | 2.75 b |
| MS | 1.00 b | 5.83 b | 6.68. b | 66.67 b | 6.00 a | 1.23 c |
| B5 | 1.00 b | 1.00 c | 5.00 b | 33.33 c | 3.83 b | 6.17 a |

Means sharing the same letter do not differ significantly at P≤0.05 (Duncan's test).

very low, this approach for propagation does not allow the production of a homogeneous population. Furthermore, success in its vegetative propagation is also limited by the low number of individuals that can be obtained simultaneously from a single plant (Hellwig *et al.*, 2004). Therefore, tissue culture techniques hold the great promise for the micropropagation of *S. rebaudiana*. This plant can also be used in experiments involving mutant selection, protoplast isolation, gene transfer, and in cellular traits studies (Bondarev *et al.*, 2003; Chan *et al.*, 2005). The objective of the present investigation was to establish a system for rapid *in vitro* propagation of *S. rebaudiana* and subsequently, establish plantlets in the field. Also compare stevioside content between mother plants and *in vitro* regenerated plantlets.

## MATERIALS AND METHODS

### Plant material and surface sterilization

Young shoots of pot grown *S. rebaudiana* plants were collected in April 2014 from Medical Plants Research Institute, Alborz, Iran. The nodal segments were used as the source of explant. Nodal explants were surface sterilized with 70% (v/v) ethanol for 1 minute and 0.15% (v/v) $HgCl_2$ solution for 10 minutes, then rinsed 3 times by sterile distilled water. All subsequent operations were performed under aseptic conditions.

### Nutrient medium and culture conditions

To determine the effect of different culture media and plant growth regulators on *in vitro* growth and development of *S. rebaudiana,* the nodal segments were cultured on most popular plant tissue culture media, 1/2MS, MS (Murashige and Skoog, 1962) and B5 (Gamborg *et al*., 1968) without plant growth regulators(Table 1). In separate experiments MS medium was supplemented with 27 different combinations of auxins and cytokinins (Table 2). Each culture medium was supplemented with 3% (w/v) sucrose. The pH of all the culture media was adjusted to 5.8 ± 0.1, before autoclaving. Culture media were solidified with 0.6% (w/v) agar (Sigma-Aldrich, USA) and sterilized under 121 ºC and 1.1 kg/cm$^2$ pressure for 20 minutes. The medium was aseptically dispensed into sterile 200 ml plant tissue culture vessels and four nodal explants were cultured per vessels. Cultures were incubated in a growth room at 24 ± 1 ºC under a 16-h photoperiod (3000 lux).

### Transferring the rooted plantlets in to the soil

The rooted plantlets were removed from their culture vessels, washed and transplanted into plastic pots containing a mixture of perlite and vermiculite (1:3). After 3 weeks of acclimatization in a mist tunnel without additional lighting, they were transferred to the field.

### Stevioside extraction and HPLC analysis

The extraction and analysis of stevioside was carried out by following the method of Ahmed *et al.* (1980). For each sample, 1 g of leaf tissue was powdered and extracted with 25 ml of boiling deionized water, with occasional shaking. The extracts were then filtered, pooled, and their volume was adjusted to 100 ml with deionized water. A portion of this extract was diluted 10 times and cleaned via solid-phase extraction. Stevioside was purchased from Sigma-Aldrich, USA. The 10 µl of extract of samples or standard samples were injected into a C18 column for reversed-phase high-performance liquid chromatography (RP-HPLC) analysis and run at isocratic conditions, using a solvent mixture of acetone trill water (3:2) with a flow rate of 0.5 ml/min and the wavelength was set at 258 nm. Quantitative measuring of stevioside was carried out based on the peak area of specific concentrations of the sample and the standard.

### Measured parameters and statistical analysis

The following parameters were measured: number of shoots per explant, shoots length, number of leaves, rooting percentage, number of roots and roots length. Observations were made 4 weeks after the culturing, in 12 replicates. Data were statistically analyzed by Factorial Randomized Complete Design using SAS program. The least significant dif-

**Table 2.** Effect of plant growth regulators on *in vitro* growth and development of *S. rebaudiana* after 4 weeks from culturing.

| Plant growth regulators (µM) | | | Average shoot number | Average shoot length (cm) | Average leaf number | Rooting (%) | Root number | Root length (cm) |
|---|---|---|---|---|---|---|---|---|
| Control | | | 1.00 h | 5.83 b | 6.68 i | 66.67 c | 6.00 b | 1.23 b |
| BAP | IAA | NAA | | | | | | |
| 2.21 | | | 2.00 b | 0.33 h | 6.33 i | 0 | 0 | 0 |
| 4.43 | | | 1.33 e | 0.20 i | 4.00 k | 0 | 0 | 0 |
| 11.09 | | | 1.13 g | 0.30 h | 0 | 0 | 0 | 0 |
| | 0.57 | | 1.33 e | 5.67 b | 11.17 d | 66.67 c | 5.00 c | 1.42 b |
| | 1.42 | | 1.42 de | 7.26 a | 15.83 a | 100.00 a | 7.2 a | 2.19 a |
| | 2.85 | | 1.83 bc | 2.50 d | 10.75 e | 50.00 d | 3.5 d | 0.47 c |
| | | 0.53 | 1.50 d | 4.12 c | 13.33 b | 91.67 b | 6.2 b | 1.42 b |
| | | 1.34 | 1.33 e | 3.97 c | 11.25 d | 50.00 d | 5.4 c | 0.40 c |
| | | 2.68 | 1.23 f | 2.08 d | 5.67 j | 58.33 c | 2.8 d | 0.40 c |
| 2.21 | 0.57 | | 1.73 c | 0.90 f | 10.67 e | 0 | 0 | 0 |
| 2.21 | 1.42 | | 2.83 a | 1.43 e | 12.35 c | 0 | 0 | 0 |
| 2.21 | 2.85 | | 1.67 c | 0.63 f | 7.47 h | 0 | 0 | 0 |
| 4.43 | 0.57 | | 2.00 b | 0.35 h | 2.33 m | 0 | 0 | 0 |
| 4.43 | 1.42 | | 1.77 c | 0.30 h | 3.33 l | 0 | 0 | 0 |
| 4.43 | 2.85 | | 1.30 e | 0.30 h | 3.00 m | 0 | 0 | 0 |
| 11.09 | 0.57 | | 1.67 c | 0.50 g | 2.83 m | 0 | 0 | 0 |
| 11.09 | 1.42 | | 1.33 e | 0.43 g | 4.47 k | 0 | 0 | 0 |
| 11.09 | 2.85 | | 1.58 d | 0.50 g | 3.48 l | 0 | 0 | 0 |
| 2.21 | | 0.53 | 1.67 c | 1.23 e | 9.70 f | 0 | 0 | 0 |
| 2.21 | | 1.34 | 1.50 d | 0.93 f | 8.00 g | 0 | 0 | 0 |
| 2.21 | | 2.68 | 1.00 h | 1.07 f | 8.30 g | 0 | 0 | 0 |
| 4.43 | | 0.53 | 1.33 e | 0.30 g | 4.33 k | 0 | 0 | 0 |
| 4.43 | | 1.34 | 1.00 h | 0.20 g | 3.00 m | 0 | 0 | 0 |
| 4.43 | | 2.68 | 1.00 h | 0.46 g | 3.00 m | 0 | 0 | 0 |
| 11.09 | | 0.53 | 1.33 e | 0.48 g | 3.33 l | 0 | 0 | 0 |
| 11.09 | | 1.34 | 1.67 c | 0.43 g | 3.67 kl | 0 | 0 | 0 |
| 11.09 | | 2.68 | 1.33 e | 0.95 f | 3.33 l | 0 | 0 | 0 |

Means sharing the same letter do not differ significantly at P≤0.05 (Duncan's test).

ference among levels of each treatment was compared using Duncan's test at 5% level.

## RESULTS AND DISCUSSION

To determine the best type of basal medium for culturing *S. rebaudiana*, nodal segments were cultured on three commonly used media, 1/2MS, MS, and B5 without plant growth regulators. The best results of shoot number (1.3 shoots per explant), shoot length (7.17 cm), leaf number (10 leaves per explant), rooting (100%) and root number (6 roots per explant) was obtained in 1/2MS basal medium. However, the best result of root length (6/17 cm) was obtained with B5 basal medium with significant differences ($p<0.05$) amongst the treatments (Table 1 and Figure 1). Similar results were reported in *S. rebaudiana* (Hwang, 2006) and in other plant species (Beike *et al.*, 2015; Harris *et al.*, 2016). The variations of *in vitro* growth and development of *S. rebaudiana* on different media may be due to the differences of nitrate/ammonium ratio, an important factor on nitrogen uptake and pH regulation during plant tissue culture (Fracago and Echeverrigaray, 2001).

Plant growth regulators are organic compounds naturally synthesized in plants, which influence growth and development. They are usually active at different sites in plant, from where they are produced and they are only present and active in very small quantities. In plant *in vitro* culture, growth regulators, especially auxins and cytokinins, are very important. It can be said that *in vitro* culture is often impossible without plant growth regulators (Pierik, 1987).

In the present study, 27 combinations of auxins and


**Figure 1.** Effect of different culture media on growth and development of *S. rebaudiana*, cultured *in vitro* after 4 weeks. **A:** 1/2MS, **B:** MS, **C:** B5.

cytokinins (Table 2) in MS medium were tested on *in vitro* growth and development of *S. rebaudiana*. Using BAP (2.21 µM) singly gradually increased the number of shoots, but these shoots were very thin and vitrified and were not suitable for micropropagation (Figure 2). Using auxins (IAA or NAA) singly or in combination with BAP produced normal plants suitable for root formation and acclimatization. These results are in agreement with those of Ibrahim *et al.* (2008) and Yücesan *et al.* (2016), who mentioned that hyperhydricity or vitrification is a physiological disorder affecting *S. rebaudiana* micropropagation and this phenomen will increase upon using cytokinins. The best values for shoot formation parameters (2.83 Shoot number, 1.43 cm shoot length and 12.35 leaf number) were obtained using 2.21 µM BAP and 1.42 µM IAA (Table 2). These results are in agreement with the findings of Anbazhagan *et al.* (2010) and Thiyagarajan and Venkatachalam (2012). They reported that the combination of BAP and IAA was best for shoot formation from nodal explants of *S. rebaudiana*.

Rooting and the transfer of plantlets to the field is the most important but difficult task in micropropagation processes (Murashige, 1974). In the current study, the auxins concentration significantly affected the success of root formation (Table 2). IAA was significantly more effective for rooting than NAA in all used concentrations. The maximum root formation parameters (100% rooting, 7.2 roots/explant and 2.19 cm root length) were obtained on the MS medium supplemented with 1.42 µM IAA. Similar observations with IAA and NAA, at different concentrations were reported earlier on the root formation of *S. rebaudiana* (Alhady, 2011; Yücesan *et al.*, 2016). The balance between the two sorts of growth regulators is usually required to initiate growth and differentiation in tissue cultures. The requisite concentration of each type of plant growth regulator differs greatly according to the kind of plant being cultured and the cultural conditions. Our results suggests that *S. rebaudiana* can be produced via tissue culture techniques without plant growth regulators or using 2.21 µM BAP and 1.42 µM IAA for shoot and using 1.42 µM IAA for root formation.

*In vitro* plants need to be hardened before transplantation to the field. All the transplanted plantlets survived in pots containing perlite and vermiculite (1:3) after 8 months, and no significant morphological differences were observed between the acclimatized plants and the field-grown mother plants. This transfer from *in vitro* culturing conditions to the external environment must be conducted carefully, because their leaves lack epicuticular waxes and it can enhance water loss when they are placed in to the soil (George and Sherrington, 1984). Hwang (2006) reported a 98% survival rate for rooted shoots of *S. rebaudiana* following transplantation. In this present study, the acclimatization and hardening processes resulted in 100% survival on the field.

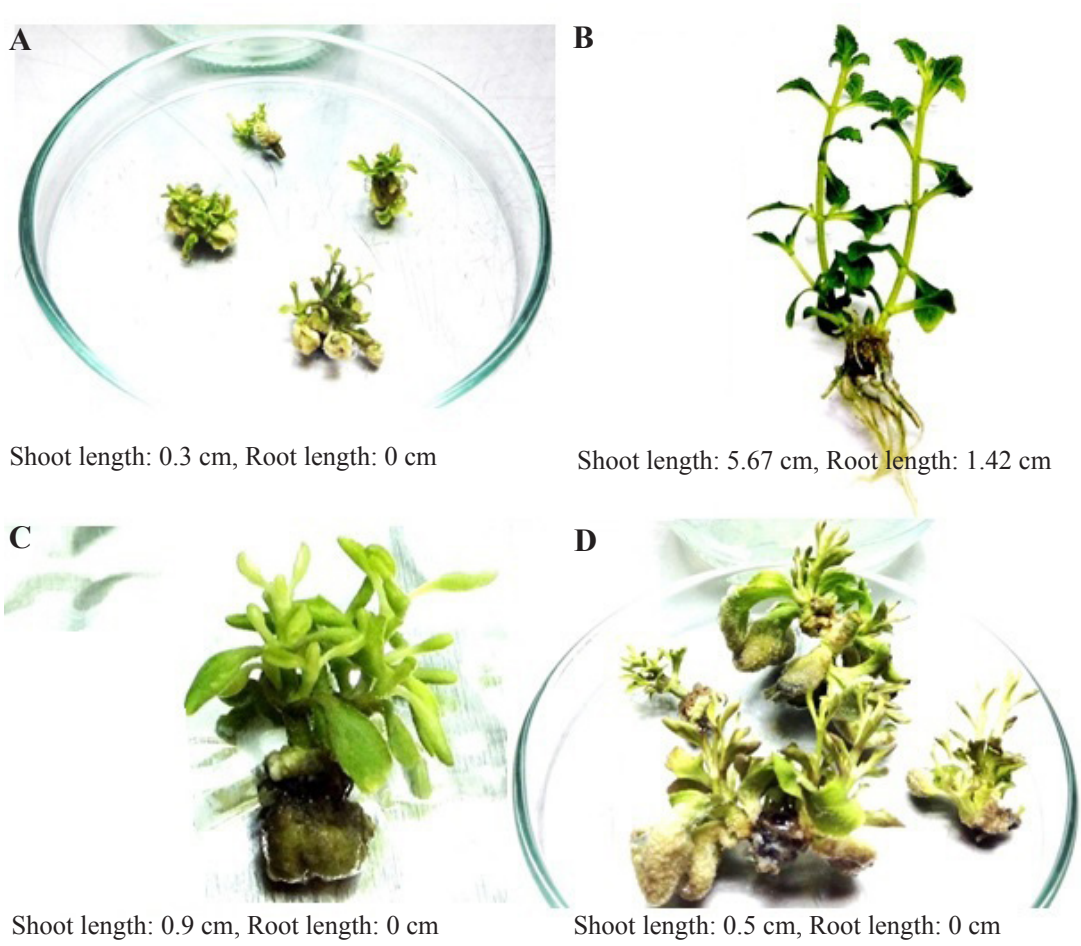


**Figure 2.** Effect of plant growth regulators on growth and development of *S. rebaudiana*, cultured *in vitro* after 4 weeks. **A:** MS basal medium supplemented with 2.21 µM BAP, **B:** 0.57 µM IAA, **C:** 2.21 µM BAP and 0.57 µM IAA, **D:** 11.09 µM BAP and 0.57 µM IAA.

The amounts of stevioside in leaves of *in vitro* regenerated plantlets were quantified when they were transferred to the soil. The synthesis of this glycoside (19.48 mg $g^{-1}$ dry weight) was similar to those measured in the mother plants (20.45 mg $g^{-1}$ dry weight, Table 3). Similar results have been reported by earlier authors (Hwang 2006).

**Table 3.** Stevioside content in leaves of *in vitro* regenerated plants versus leaves of mother plants of *S. rebaudiana*.

| Sample | Stevioside content (mg $g^{-1}$ dry weight) |
|---|---|
| *in vitro* regenerated plants | 19.48 |
| mother plants | 20.45 |

In conclusion, the present investigation demonstrated that by optimizing the level of plant growth regulators and the choice of suitable culture medium, one can achieve in large scales, the rapid propagation of *S. rebaudiana.* Also, the HPLC results demonstrated that culture conditions did not affect the amounts of stevioside in leaves of *S. rebaudiana*. This shows that this finding can be used effectively for commercial production of stevioside in bioreactors. Further studies are required to investigate the effect of precursor feeding, elicitation, biotransformation techniques and media manipulation as tools for increasing the production of stevioside on *in vitro* culture of *S. rebaudiana.*

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