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Determination of Clone Lines Having a High Content of Stevioside and Rebaudioside A for In Vitro Commercial Production of Stevia rebaudiana

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ABSTRACT: ***Stevia rebaudiana*** *Bertoni, which is an important plant for both the health and the food sectors, contains non calorie sweetener compounds and there is a continuous increase in the demand for these stevia-based non-synthetic sweeteners. The aim of the present work was to determine clones that have high stevioside and rebaudioside A contents for* ***in vitro*** *commercial production of* ***Stevia rebaudiana****. For this purpose, the seeds of* ***Stevia*** *were cultured* ***in vitro*** *and the clone lines that showed the highest multiplication rate and highest stevioside and rebaudioside A contents were selected. Two clone lines obtained were adapted by a commercial producer for the commercial production of* ***Stevia****.*

Keywords: Stevia rebaudiana*,* ***in vitro*** *seed germination,* ***in vitro*** *commercial production, HPLC, stevioside, rebaudioside A.*

Stevia rebaudiana'nın In Vitro Ticari Üretimi Amacıyla Yüksek Düzeyde Stevioside ve Rebaudioside A İçeren Klonların Belirlenmesi

ÖZ: *Hem sağlık hem de gıda sektörü için önemli bir bitki olan* ***Stevia rebaudiana*** *Bertoni, kalorisi olmayan tatlandırıcı bileşikler içermektedir ve stevia-kaynaklı sentetik olmayan bu tatlandırıcılar için talepte sürekli bir artış söz konusudur. Mevcut çalışmanın amacı,* ***S. rebaudiana****'nın* ***in vitro*** *ticari üretimi için yüksek steviosid ve rebaudiosid A içeriğine sahip klonların belirlenmesidir. Bu amaçla,* ***Stevia*** *tohumları* ***in vitro*** *koşullarda kültüre alınmış ve yüksek çoğaltım oranı ve yüksek steviosid ve rebaudiosid A içeriği gösteren klon hatları seçilmiştir. Elde edilen iki klon hattı ticari bir üretici tarafından* ***Stevia****'nın ticari üretimi için adapte edilmiştir.*

Anahtar Kelimeler: Stevia rebaudiana*,* ***in vitro*** *tohum çimlenmesi,* ***in vitro*** *ticari üretim, steviosid, rebaudiosid A.*

INTRODUCTION

Stevia rebaudiana Bertoni, which originated from Paraguay and Southern Brazil, is a wild perennial herb and belonging to the Asteraceae family (Bondarev et al., 2001; Reis et al., 2011). The leaves of *Stevia* synthesize sweet-tasting steviol glycosides (SGs) derived from the tetracyclic diterpenesteviol (Soejarto et al., 1983; Richman et al., 1999; Ladygin et al., 2008). The most important diterpenoid SGs are stevioside and rebaudiosides A and C, and these SGs are approximately 300 times sweeter than sucrose at

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their concentration of 4% (w/v) (Bondarev et al., 2001). Due to some of their crucial features, such as being nontoxic, non-mutagenic, and low-caloric, these glycosides may be successfully used as sugar alternatives for patients suffering from diabetes, other diseases related to a disturbance in carbohydrate metabolism (Bondarev et al., 2001; Rajasekaran et al., 2007), obesity, heart disease, and dental maladies (Reis et al., 2011). Aside from the aforementioned beneficial effects on human health, *S. rebaudiana* has commercial value and is currently being used as a sweetening agent in food and beverages (Soejarto et al., 1982; Koyama et al., 2003; Singh and Rao, 2005; Tadhani et al., 2007; Modi et al., 2014).

All of these features have focused attention on *Stevia*. The development of *Stevia* lines with high and homogenous SGs contents and the propagation of sufficient planting material that show this quality characteristic are of great importance. *Stevia* is traditionally produced by seed or vegetative cuttings (Brandle et al., 1998; Singh and Rao, 2005). As the seeds are very small in size and infertile, the seed germination rate is very poor and therefore large scale production of *Stevia* via seeds is not fruitful. No viable seed formation occurs due to high self-incompatibility (Singh and Rao, 2005; Ali et al., 2010). In addition, the seeds show a wide variation in their SGs contents and morphological characters (shape and color) of leaves (Tamura et al., 1984a, b). The usual propagation method for stevia is by stem cuttings, which require a high labor input but root easily (Sivaram and Mukundam, 2003; Lemus-Mondaca et al., 2012). The number of individuals that can be obtained simultaneously from a single plant limits the vegetative propagation due to the accumulation of pathogens in the tissue (Ahmed et al., 2007; Lata et al., 2013). Propagating a genetically homogeneous population from a selected plant with chosen characters proves necessary to prevent segregation and improve SGs yield. Hence, the best alternative for fast mass propagation of *S. rebaudiana* would be *in vitro* clonal propagation (Tamura et al., 1984a, b; Sivaram and Mukundam, 2003; Lemus-Mondaca et al., 2012). This method makes it possible to rapidly produce valuable and endangered true-to-type plant species that could not quickly or easily be produced using more traditional methods in limited area and ensures the production of high-yielding and premium quality planting material (Ali et al., 2010; Bayraktar et al., 2015).

The objective of the present study was to determine clone lines that have high stevioside and rebaudioside A contents, and a rapid multiplication rate for *in vitro* commercial propagation of *S. rebaudiana*. For this purpose, the clone lines obtained from *in vitro*-germinated seeds were evaluated in terms of their highest multiplication rate, and highest stevioside and rebaudioside A contents. Subsequently, 2 clones selected according to these properties were adapted for *in vitro* commercial production of *S. rebaudiana.*

MATERIALS AND METHODS

Decontamination

The seeds of *S. rebaudiana* (Bertoni) used in this study were obtained from Paraguay. Because the seeds are very small, they were sterilized in the package made of filter paper. First, they were subjected to decontamination treatments. The seeds were surface-sterilized by 70% ethanol (v/v) for 1 min; 1% (v/v) sodium hypochlorite (Merck) with a drop of Tween 20 (Merck) for 5 min, 10 min, or 15 min, with continuous stirring; and then rinsed 3 times with sterile distilled water. A total 3 experiments were conducted in triplicate with 18 explants in each replication. In total, 54 explants were tested per treatment.

In vitro germination

To study the effect of seed color and Gibberellic acid (GA_3) on *in vitro* germination, the seeds were first categorized into 2 groups according to their color, as pale and dark, and subsequently, half of each group was pretreated with 100 ppm GA_3 for 24 h prior to surface sterilization.

To maximize seed germination, 2 different culture systems were tested: A two-layer, in which an agar-solidified medium was topped with a thin

layer of liquid medium of the same composition and as a control agar-solidified medium. The pale and dark seeds pretreated with or without GA_3 were transferred onto these culture systems. As a germination medium, we used half-strength Murashige and Skoog (MS; Murashige and Skoog, 1962) basal medium containing 1.5% (w/v) sucrose without any plant growth regulator. Media were prepared with or without 0.6% (w/v) plant agar (Duchefa®, Haarlem, Netherlands) resulting in semi-solid medium or liquid medium, correspondingly. Half-strength MS medium was transferred in 210-mL glass culture jars (30 mL media) as the basal medium fraction. After cooling the media, 10 mL volumes of cool, liquid half-strength MS media (without agar) were decanted into the culture vessels in a laminar air-flow cabinet.

The effect of dark and light incubation on the *in vitro* seed germination of *S. rebaudiana* (Bertoni) was also evaluated. The seeds, having been divided into 2 colors, were pretreated with or without GA_3, and placed in 2-layer or semi-solid medium, were incubated in a controlled room at 25 ± 1 °C in under continuous darkness or in cool white fluorescent light (50 µmol m^{-2} s^{-1}) for a light/dark photoperiod of 16:08.A total of 16 experiments (Table 1) were conducted in triplicate with 20 explants in each replication. Sixty explants were tested in total per treatment.

After 7 days of *in vitro* germination, the tiny seedlings grown on 2-layer medium and/or under continuous darkness were aseptically taken out and transferred to semi-solid MS medium containing 3.0% (w/v) sucrose, without any plant growth regulator, and to the illuminated conditions described above.

Selection of clone lines

The selection of best clone lines was based on their vitro multiplication rate and stevioside and rebaudioside A contents. For this purpose, the following was observed;

1) Clone lines, each obtained from 1 *in vitro*-germinated seed, were subcultured. For subculture, shoot tips and node explants (1.0–1.5 cm in length) were transferred into glass tubes (23/24×140 mm, Lab Associates b.v., Oudenbosch, The Netherlands) containing a 10 mL MS medium, 3% (w/v) sucrose and solidified with 0.6% (w/v) agar monthly for 3 months.

2) After 3 subcultures, 5 clone lines were selected according to the multiplication rate.

3) After 3 subcultures, a decrease was observed in the multiplication rate and necrosis and chlorosis were found in the selected clone lines. To determine a more efficient basal medium for the multiplication of selected clone lines, the node explants of 1 clone line (clone 18), which have an appropriate explant number, were cultured in glass tubes, each containing 10 mL of MS, ½ MS, Woody plant medium (WPM; Lloyd and McCown, 1980), or Gamborg medium (B5; Gamborg et al., 1968), 3.0% (w/v) sucrose, and 0.6% (w/v) plant agar. The experiments were conducted in triplicate with 15 explants in each replication. In total, 45 explants were tested per treatment. The data were recorded 4 weeks after culture initiation. The cultures were incubated in a growth room at 25 ± 1°C and under cool white fluorescent light (50 µmol $m^{-2}s^{-1}$) for a light/dark photoperiod of 16:08.

4) The remaining clone lines (clones 1, 13, 14, and 15) were multiplied on WPM.

5) The stevioside and rebaudioside A contents in the leaves of these 5 clone lines, grown *in vitro* and under field conditions, were analyzed using high performance liquid chromatography (HPLC).

6) Clone lines showing the highest multiplication rate, and stevioside and rebaudioside A contents were selected.

Large scale propagation of the two selected clone lines

To compare plant growth, nodal explants obtained from 2 clone lines (0.5–1.0 cm in length) were transferred into culture tubes and Vitro Vents® (115 × 90 × 96 mm) (Duchefa Biochemie B.V., The Netherlands). To maintain the explant/nutrient medium proportion, 1 explant was cultured in a culture tube containing 10 mL WPM and 15

explants were cultured *in vitro* Vents® containing 150 mL WPM. The experiments were conducted in triplicate and 15 explants were used for each replication. In total, 45 explants were tested per treatment. The data were recorded 4 weeks after culture initiation.

Media conditions

The pH of all of the media was adjusted to 5.8 with 1 N HCl or 1 N NaOH prior to the addition of the gelling agent, and autoclaved at 121 °C at 1.04 kg cm^{-2} for 15 minutes.

Acclimatization

Four-week-old plantlets with well-developed roots were transferred into small pots containing a 1:3 peat to loam mixture and covered with perforated transparent bags to retain humidity. These plantlets were then kept in a climate chamber at 25 ± 1 °C for a light/dark photoperiod of 16:08 at 70% humidity and 50 µmol $m^{-2}s^{-1}$ irradiance. They were ventilated once a day for a period of 1 week. After 1 week, the bags were completely removed. The plants were then transferred to greenhouse conditions 3 weeks after the beginning of acclimatization and maintained there for 6 weeks. Completely acclimatized plants were transferred to field conditions.

Stevioside and rebaudioside A analysis

The stevioside and rebaudioside A contents in the leaves of 4-week-old *S. rebaudiana* shoots, grown *in vitro* and under field conditions, were analyzed using HPLC.

Chemicals

HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Ultrapure water, which was used for the analysis, was obtained from an in-house ultrapure water system (Sartorius Arium 611, Sartorius Stedim Biotech, Göttingen, Germany). Stevioside and rebaudioside A were isolated from the leaves of *S. rebaudiana* at a purity of + 90%.

HPLC sample preparation

Samples were prepared according to the method of Erkucuk et al. (2009), with slight modifications; 50 mg of leaves were sonicated four 4 times with 5 mL methanol. The clear extracts were combined and diluted with methanol to 30 mL. Prior to HPLC analysis, the samples were filtered with a 0.45 µm PTFE filter (Sartorius AG, Göttingen, Germany) to remove non dissolved particles.

HPLC-ELSD Conditions

A Thermo Surveyor Plus HPLC (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a quaternary pump, an autosampler, and a SofTA 300S ELS Detector (SofTA Corporation, Westminster, CO, USA) were used to perform the HPLC-ELSD analyses. An apHera NH_2 column (150 mm × 2 mm, particle size 5 µm, Sigma Aldrich, Germany) was used as an analytical column. An acetonitrile/water mixture (75:25 v/v) served as the isocratic mobile phase. The total run time was 10 min at a flow rate of 0.8 mL/min. A sample of 10 µL was injected (partial loop injection) each run. The spray chamber temperature was set to 40 °C, the drift tube temperature was 105 °C, the gas pressure was 50 psi, and the filter of the ELSD was 6. The retention time was 4.22 min for stevioside and 6.2 min for rebaudioside A.

Calibration

Methanol was used to prepare the standard stock solutions (1000 µg/mL stevioside and rebaudioside A, respectively). From each standard stock solution, 5 additional lower concentration standard solutions (500, 250, 100, 50, and 10 µg/mL) were prepared by dilution with methanol. The solutions were stored at 4 °C. Concentrations and peak areas were log transformed and the calibration curve was prepared using these values. Regression coefficients were 0.9989 for stevioside and 0.9980 for rebaudioside A, respectively.

Statistical analysis

The data were recorded 4 weeks after culture initiation. The experiments were set up in a completely randomized design and all factors/ treatments were done in triplicate. Data were analyzed using standard ANOVA procedures. Significant differences among the mean values were compared using Duncan's multiple range test

(DMRT) at P = 0.05 using SPSS Version 16.0 (SPSS Inc., Chicago, USA).

Results and discussion

In our study, we aimed to find clone lines that have high SGs contents and multiplication rate for commercial production of *S. rebaudiana*. For this purpose, seeds were used as the starting material since the sterilization of seeds is easier than that of other parts of the plant and the seeds have variability in SGs content. This variability provided us the selection of clone lines containing a high SGs content.

Decontamination

The effect of 3 exposure times of sodium hypochlorite on seed decontamination was examined. It was observed that the exposure times (5, 10, and 15 min.) had no statistical effect on the percentage of decontamination (66.6%, 70.37%, and 81.48%, respectively). According to the results obtained, the seeds used for *in vitro* seed germination studies were surface sterilized with 1% (v/v) sodium hypochlorite for 15 min. A total of 1800 sterile seeds were obtained and 2 clone lines (clones 1 and 2) were obtained from these seeds.

***In vitro* germination**

For *in vitro* seed germination studies, 16 experiments were carried out and 960 seeds were cultured. These 16 experiments were observed to have no effect on *in vitro* germination. The mean percentage of *in vitro* germination ranged between 0% and 5% without significant differences among the experiments (Table 1). In general, the seeds of *Stevia* show quite a low germination rate (Tamura et al., 1984a). During pollination, growing conditions affect seed viability and yield, and both seed yield and germination can be affected by excessive rainfall. Although it is best to store seed at 0 °C, even at this temperature, germination will decline by 50% over 3 years (Madan et al., 2010). It was reported that *Stevia* seeds quickly lose viability after collection and most of the seeds cannot germinate due to the presence of immature embryos. The seeds cannot be stored for long period and therefore it is better to use them with in the first week of collection. Khalil et al., (2014) used fresh seeds and obtained a 25.51% germination rate *in vitro*. They also irradiated the seeds with different radiation doses (2.5, 5.0, 7.5, and 10 Gy) for improving the germination percentage, but did not find a significant change in the germination rate. In the present study, the low germination rate, ranging between 0% and 5%, may be due to the use of old seed material.

In total, 21 seeds were germinated from the culturing of 960 seeds. *In vitro* seedlings obtained from the germinated seeds were evaluated as separate clone lines. Each seedling clone was given numerical label. Thus, 2 clone lines from the decontamination experiments and 21 clone lines from the *in vitro* germination experiments (a total of 23 clone lines) were obtained. The germination time for each seed is given in Table 1. Accordingly, the seeds germinated at the earliest in 6 days and at the latest in 56 days.

Selection of clone lines

In previous studies on the micropropagation of *S. rebaudiana*, MS was mostly used as basal medium (Bondarev et al., 2003; Hwang, 2006; Ahmed et al., 2007; Ibrahim et al., 2008; Ladygin et al., 2008;Ali et al., 2010; Khalil et al., 2014). Based on these studies, the seedlings (clone lines) obtained from the germination of seeds were subcultured in glass tubes containing 10 mL of MS containing 3% (w/v) sucrose and solidified with 0.6% (w/v) agar 3 weeks after germination.

Although 7 of the clones (3, 6, 7, 8, 16, 19, and 23) obtained from the different experiments showed germination, they did not grow due to deterioration of the cotyledons (Fig. 1) even though they were transferred onto fresh medium (Table 1). The seedlings developed from clones 4 and 5 were lost due to week and abnormal growth. The remaining clones (1, 2, 9, 10, 11, 12, 13, 14, 15, 17, 18, 20, 21, and 22) showed further growth (Table 1) (Fig. 1). They were subcultured on fresh MS medium at 4-week intervals to increase the clone line shoot numbers. After subculture 1, clones 17, 20, 21, and 22 showed weak and abnormal growth, and necrosis

Table 1. Effects of different treatments on *in vitro* germination of *S. rebaudiana* and description of the clone lines obtained.

Number of experiments	Light conditions	Seed color	GA_3 pretreatment	Medium type	Total number of seeds	Number of germinated seeds	Germination percentage(%)	Clone line number, description of clone lines, and time of germination (day)
1	Continuous darkness	Dark	-	One-layer	60	3	5.00	Clone 14: Germination and further development (day 14) Clone 19: Seed germinated but no growth, deterioration of cotyledons (day 26) Clone 20: Germination and further development (day 26)
2				Two-layer	60	1	1.67	Clone 15: Germination and further development (day 14)
3			+	One-layer	60	1	1.67	Clone 11: Germination and further development (day 6)
4				Two-layer	60	2	3.33	Clone 10: Germination and further development (day 6) Clone 21: Germination and further development (day26)
5		Pale	-	One-layer	60	1	1.67	Clone 12: Germination and further development (day 6)
6				Two-layer	60	3	5.00	Clone 9: Germination and further development (day 6) Clone 16: Seed germinated but no growth, deterioration of cotyledons (day 14) Clone 17: Germination and further development (day 14)
7			+	One-layer	60	0	0	
8				Two-layer	60	0	0	
9	16-hour photoperiod	Dark	-	One-layer	60	2	3.33	Clone 3: Seed germinated but no growth, deterioration of cotyledons (day 6) Clone 4: Weak and abnormal growth (day 6)
10				Two-layer	60	2	3.33	Clone 18: Germination and further development (day 18) Clone 22: Germination and further development (day 56)
11			+	One-layer	60	0	0	
12				Two-layer	60	2	3.33	Clone 7: Seed germinated but no growth, deterioration of cotyledons (day 6) Clone 13: Germination and further development (day 14)
13		Pale	-	One-layer	60	1	1.67	Clone 5: Weak and abnormal growth (day 6)
14				Two-layer	60	0	0	
15			+	One-layer	60	1	1.67	Clone 6: Seed germinated but no growth, deterioration of cotyledons (day 6)
16				Two-layer	60	2	3.33	Clone 8: Seed germinated but no growth, deterioration of cotyledons (day 6) Clone 23: Seed germinated but no growth, deterioration of cotyledons (day 56)

and chlorosis. Therefore, these clones were lost. Since the total number of some clone lines (2, 9, 10, 11, and 12) did not increase enough for the further stages of the clone selection during subcultures 1–3, they were eliminated (Table 2). Although the total number of clones 1, 13, 14, 15, and 18 increased during subcultures 1 and 2, the multiplication rate and shoot length decreased substantially, and necrosis and chlorosis were observed in subculture 3 (Table 2). Therefore, after this stage, we focused on the finding best basal medium for the multiplication of clones. Based on the results, clone 18 alone was chosen for the basal medium experiments because of its shoot explants availability (Table 2). The nodal segments (approximately 1.0–1.5 cm long with 1axillary bud) of the 4-week-old shoots were transferred into glass tubes containing 10 ml of MS, ½ MS, B5, or WPM supplemented with 3% (w/v) sucrose and solidified with 0.6% (w/v) plant agar.

Table 2. Effects of different basal media (MS and WPM) and subculture on the *in vitro* multiplication of *S. rebaudiana* clone lines obtained from *in vitro*-germinated seeds.

Clone No.	Subculture and medium	Number of shoots according to shoot length (cm)							Total number of shoots
		0–0.5	0.5–1	1–2	2–4	4–8	8–12	12–24	
1	1. MS	-	-	-	3	1	-	-	4
	2. MS	9	10	4	9	2	-	-	34
	3. MS	48	22	9	6	5	-	-	90
	4. WPM	-	-	13	10	34	32	55	144
2	1. MS	-	-	1	-	1	-	-	2
	2. MS	1	2	2	1	-	-	-	6
	3. MS	6	2	-	2	-	-	-	10
9	1. MS	-	-	1	2	1	-	-	4
	2. MS	6	2	1	2	-	-	-	11
	3. MS	9	2	5	-	-	-	-	16
10	1. MS	-	-	-	2	1	-	-	3
	2. MS	8	-	-	-	1	-	-	9
	3. MS	12	4	5	-	-	-	-	21
11	1. MS	-	-	-	1	1	-	-	2
	2. MS	6	-	1	-	-	-	-	7
	3. MS	12	1	1	-	-	-	-	14
12	1. MS	-	-	2	1	-	-	-	3
	2. MS	6	-	1	1	1	-	-	9
	3. MS	10	9	4	-	-	-	-	23
13	1. MS	-	-	-	2	1	1	-	5
	2. MS	10	-	1	5	1	2	-	19
	3. MS	32	10	1	8	3	7	-	61
	4. WPM	-	-	15	12	38	37	65	167
14	1. MS	-	-	2	2	-	2	-	6
	2. MS	3	8	3	4	7	3	-	28
	3. MS	69	14	14	12	6	-	-	115
	4. WPM	-	-	18	44	14	46	77	199
15	1. MS	-	-	-	-	3	2	-	5
	2. MS	20	4	7	3	3	-	-	37
	3. MS	57	10	7	4	7	2	-	87
	4. WPM	-	-	11	15	37	63	35	161
17	1.	4	1	-	-	-	-	-	5
18	1. MS	-	-	-	1	3	4	-	8
	2. MS	20	5	16	10	5	1	-	57
	3. MS	36	50	37	21	12	15	-	171
	4. WPM	-	-	31	40	89	112	175	447
20	1.MS	4	2	-	-	-	-	-	6
21	1.MS	6	2	-	-	-	1	-	9
22	1. MS	-	1	1	1	-	-	-	3

Although the highest number of shoots was observed on MS, B5, and WPM equally (2.0, 1.98, and 2.0/nodal explant, respectively), the mean shoot length per explant formed on WPM (7.88 cm) was higher compared to that on the other basal media. Among the 4 basal media, the highest number of nodes and leaves were obtained in explants cultured on B5 and WPM (Table 3). In contrast to the other basal media, the development of the leaves was very good on WPM (Fig. 2) and the highest leaf length per explant was obtained as 1.10 cm.

In the experiment made for the determination of the best basal medium, all of the basal media formed also roots ranging between 27.76% in B5 medium and 94.45% in WPM. The first roots began to emerge in the explants cultured on WPM after 7 days of culturing. The highest number of roots (14.03/rooted shoot) and root length (4.71 cm/rooted shoot) were also observed in shoots grown on WPM (Table 4).

Based on our results, WPM basal medium, which supported shoot and root development simultaneously, was selected as the best basal medium for the multiplication of clone lines, and clone lines 1, 13, 14, 15, and 18 were multiplied on this medium. After subculture 4 on WPM, the total number of shoots, the shoot lengths, and the shoot development of clones were improved (Table 2).

Aside from the abovementioned parameters, the effect of the basal medium on the stevioside and rebaudioside A contents of clone line 18 was also studied. It was observed that the basal medium affected the stevioside and rebaudioside A contents differently. Specifically, WPM led to considerably higher total SGs contents (42.0 mg/g-DW) in the leaves compared to the content in the leaves of shoots grown on the other basal media (Table 5). A remarkable finding of the present study was that the shoots grown on WPM showed the best development and SGs contents. The highest rebaudioside A (21.6 mg/g-DW) and stevioside contents (29.4 mg/g-DW) were obtained on ½ MS and WPM, respectively. Bondarev et al., (2003) reported that the production of SGs in Stevia tissues may be significantly modified by composition changes in the nutrient medium, which would, in turn, cause a physiological regulation of this process. In their study, it was shown that 2-fold elevation of the mineral salt concentration stimulated growth of the shoots considerably, while there was a decrease in the SGs content in their leaves of about order of magnitude. Their explanation of this situation was that the effect was probably due to inorganic phosphate, which is well documented in the literature with regards to its negative influence of increased concentrations of the given anion on the biosynthesis of many secondary compounds in plant cell cultures *in vitro*. A similar result was also observed in the present study. The stevioside and rebaudioside A contents showed changes according to basal medium used. However, in contrast to the results of Bondarev et al. (2003), in the present study, the SGs content increased in direct proportion to the growth of the *in vitro* shoots. This contrast could be explained as follows: *Stevia* plantlets cultured on WPM grew better than the plantlets transferred onto other media. After 3 weeks of culturing, plantlets cultured on WPM may have slowed their growth processes and begun the biosynthesis of SGs. The chloroplasts found in the mesophyll of Stevia leaves, which have well-developed grana and thylakoids, are most likely responsible for the production of SGs (Bondarev et al., 2001). A positive correlation between the development and activity of the photosynthetic apparatus in Stevia leaves and the biosynthesis of diterpenoid SGs was reported by Ladygin et al. (2008). It has also been reported that the biosynthesis of SGs initiates in the chloroplasts (Brandle and Telmer, 2007; Ladygin et al., 2008). The shoots grown on WPM showed a larger leaf area than those grown on other basal media. Due to good leaf development, the SGs content could be higher in plantlets grown on WPM than in plantlets grown on other basal media.

For clone line selection, the stevioside and rebaudioside A contents as well as the multiplication efficiency of clone lines were also taken into consideration. For this purpose, after determination of the best basal medium, the clone

lines were subcultured on WPM. After subculture 1 of 5clone lines on WPM, they were compared in terms of the stevioside and rebaudioside A contents. Among the 5 clone lines, the stevioside content reached its maximum value (29.4 mg/g DW) in clone line 18, and the rebaudioside A content reached its maximum value (34.2 mg/g DW) in clone 15 (Table 6). When the total SGs content and rebaudioside A/stevioside ratio were compared, clone lines15and 13 were found to have the highest total SGs content (50.0 mg/g DW) and rebaudioside A/stevioside ratio (2.5), respectively.

Table 3. Effects of different basal media (½ MS, MS, B5, or WPM) on the multiplication of *S. rebaudiana* clone line 18 after 4 weeks of culturing.

Medium	Mean number of shoots / explant ± SE	Mean shoot lengths (cm) / explant ± SE	Mean number of nodes / explant ± SE	Mean number of leaves / explant ± SE	Mean leaf lengths (cm) / explant ± SE
½ MS	1.78 ± 0.02 b	6.24 ± 0.04 b	3.18 ± 0.03 b	8.36 ± 0.06 b	0.93 ± 0.01 a
MS	2.00 ± 0.00 a	1.14 ± 0.03 d	1.60 ± 0.03 c	5.20 ± 0.05 c	0.41 ± 0.01 b
B5	1.98 ± 0.01 a	3.53 ± 0.04 c	7.36 ± 0.06 a	16.71 ± 0.12 a	0.31 ± 0.00 b
WPM	2.00 ± 0.00 a	7.88 ± 0.06 a	7.87 ± 0.05 a	17.73 ± 0.09 a	1.10 ± 0,00 a

Means within a column and main effect followed by the same letter are not significantly different based on Duncan's multiple range test, where P = 0.05.

Table 4. Effects of different basal media (½ MS, MS, B5 or WPM) on the root formation of *S. rebaudiana* clone line 18 after 4 weeks of culturing.

Medium	Percentage of shoots rooted ± SE	Mean number of roots/ rooted shoot ± SE	Mean root lengths (cm)/ rooted shoot ± SE
½ MS	75.56 ± 1.15 b	5.95 ± 0.07 b	3.81 ± 0.07 b
MS	31.11 ± 1.03 c	1.28 ± 0.06 c	1.55 ± 0.05 c
B5	27.76 ± 1.25 c	1.18 ± 0.02 c	0.93 ± 0.03 d
WPM	94.45 ± 1.03 a	14.03 ± 0.05 a	4.71 ± 0.06 a

Means within a column and main effect followed by the same letter are not significantly different based on Duncan's multiple range test, where P = 0,05.

Table 5. Effects of different basal media (½MS, MS, B5, or WPM) on stevioside and rebaudioside A production in leaves of *in vitro*-grown plantlets of *S. rebaudiana* clone line 18 after 4 weeks of culturing.

Medium	Stevioside (mg/g dry weight)	Rebaudioside A (mg/g dry weight)	Total (mg/g dry weight)	Rebaudioside A /Stevioside
½ MS	11.3	21.6	32.9	1.9
MS	7.3	7.6	14.9	1.0
WPM	29.4	12.6	42.0	0.4
B5	11.5	7.0	18.5	0.6

Table 6. Stevioside and rebaudioside A content in leaves of *in vitro* and field grown plants of *S. rebaudiana* clone lines.

Clone No	Stevioside (mg/g dry weight)	Rebaudioside A (mg/g dry weight)	Total (mg/g dry weight)	Rebaudioside A / Stevioside
In vitro				
1	13.3	13.7	27.0	1.0
13	10.4	25.5	35.9	2.5
14	13.5	7.7	21.2	0.6
15	15.8	34.2	50.0	2.2
18	29.4	12.6	42.0	0.4
Ex vitro (field)				
1	20.5	10.0	30.5	0.5
13	28.5	4.3	32.8	0.2
14	17.6	10.0	27.6	0.6
15	56.2	34.6	90.8	0.6
18	25.2	16.0	41.2	0.6

The stevioside and rebaudioside A contents in the leaves of 5clone lines grown under field conditions were also followed and analyzed using HPLC. The stevioside (56.2 mg/g DW), rebaudioside A (34.6 mg/g DW), and total SGs (90.8 mg/g DW) contents were highest in clone line 15. While any significant changes in the SGs content in the other clone lines grown in the field were not observed, the total SGs content in clone line 15 grown in the field appeared to be about 2 times higher than that in clone line 15 grown *in vitro* (Table 6). It was reported that *Stevia* plants propagated *in vitro* are very uniform in terms of growth and chemical quality (Tamura et al., 1984b). Hwang (2006) compared the amount of stevioside between *in vitro*-regenerated plantlets and mother plants grown under field conditions. *In vitro*-regenerated shoots accumulated stevioside at a rate of 10.69 mg/g-DW and when those plantlets were transferred to the field, they showed stevioside contents similar to those measured in the mother plants (12.1 mg/g-DW). In the present study, the total SGs content remained almost the same. Only clone line 15 showed a significant increase in the SGs content.

Large-scale propagation of the two selected clone lines

For the 2 clone lines, culture tubes and Vitro Vent® were observed to have no statistical effect on the mean number of shoots per explant, mean shoot lengths (cm) per explant, mean number of nodes per explants, mean number of leaves per explant, mean leaf lengths (cm) per explant, percentage of explants forming roots, mean number of roots per rooted explant, and mean root lengths (cm) per rooted explant. In both culture types, the shoots showed good growth with normal morphology (Fig. 2d and 3a, b).The type of culture vessels (tubes or Vitro Vent) did not create important effects on the growth of the plantlets.

It has been reported that the use of large culture vessels for commercial-scale propagation can;

1) Reduce production costs significantly by cutting the labor cost to nearly half that of the conventional system,

2) Improve the quality of transplants and ex vitro survival,

3) Shorten the production period, and

4) Simplify the propagation process (Zobayed et al., 2004).

Based these results, hundreds of uniform plantlets can be produced from nodal and shoot tip explants cultured in alarge culture vessel containing WPM without plant growth regulators with continuous monthly subculturing. The multiplication efficiency of our results was not higher than that of other micropropagation studies of *S. rebaudiana* (Tamura et al., 1984a; Hwang, 2006; Sairkar et al., 2009; Giridhar et al., 2010; Satpathy and Das, 2010; Lata et al., 2013; Khalil et al., 2014). However, in the present study, plant growth regulators were not used and the development of shoots and roots was achieved simultaneously on basal medium.

The 4-week old plantlets (10–12 cm long with 8–10 leaves and well-developed roots) (Fig. 3b) were transferred to soil and completely acclimatized plants were transferred to field conditions at the end of 9 weeks (Fig. 3c, d).

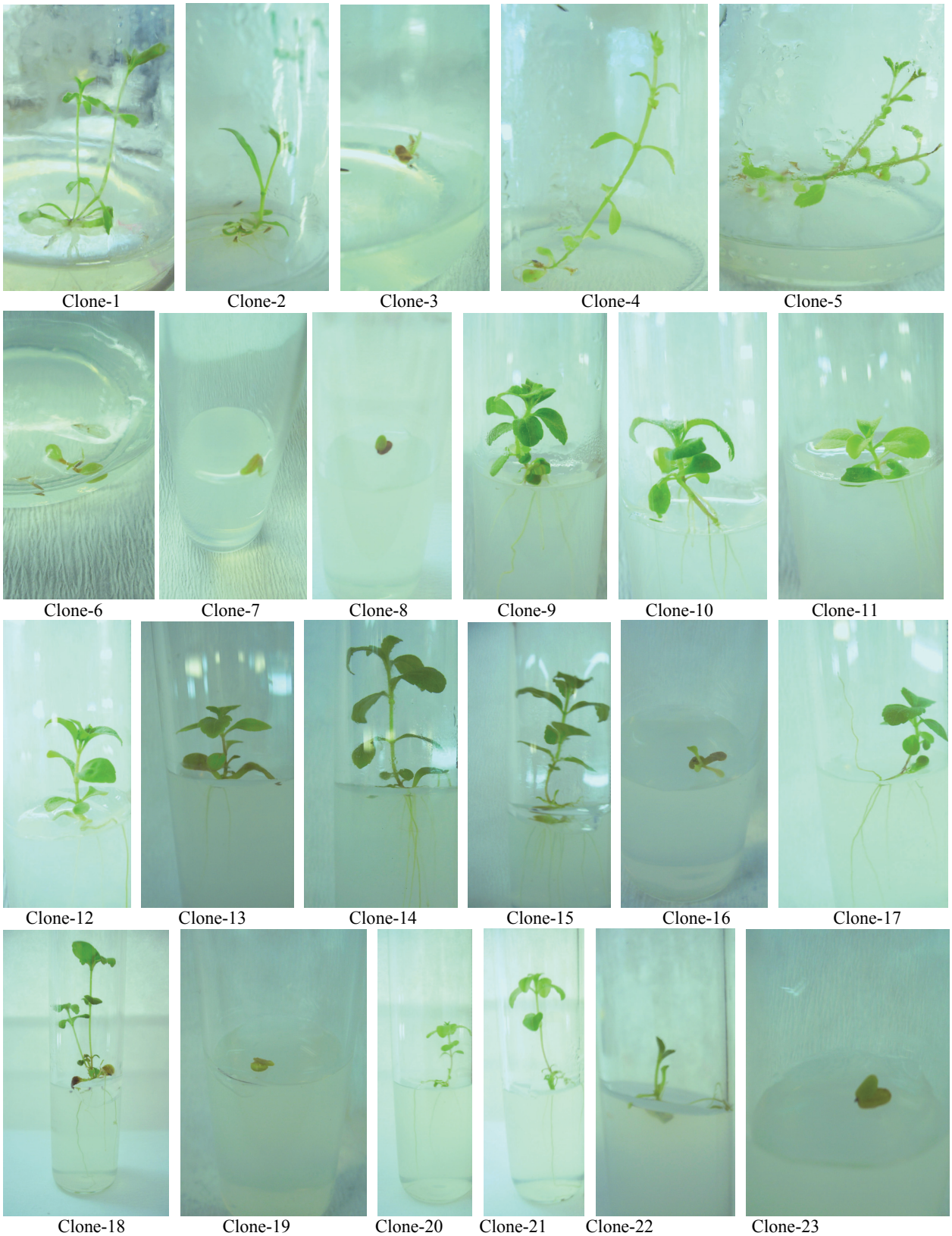

Figure 1. The development of clone lines obtained from *in vitro* germination of *S. rebaudiana* seeds after 20 days of culture.

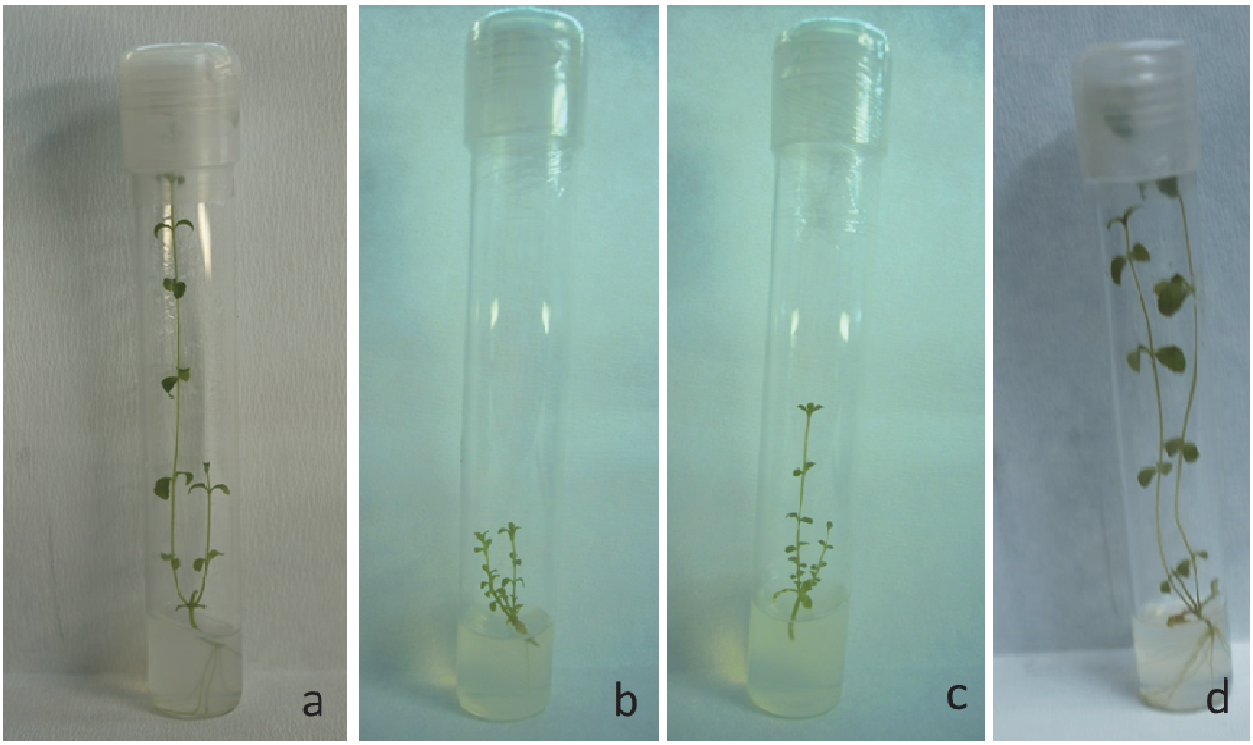

Figure 2. *In vitro* shoots development from node explants cultured on ½ MS (a), MS (b), B5 (c), and WPM (d) after 4 weeks of culture.

Figure 3. Acclimatization of *S. rebaudiana* plantlets: rooted plantlets on WPM (a), acclimatizated plants in the greenhouse (b), and hardened plants in the field (c).

CONCLUSIONS

In conclusion, in the present study, we obtained 2 *S. rebaudiana* clone lines, which have a high multiplication rate and stevioside and rebaudioside A contents, from *in vitro*-germinated seeds. These clone lines were successfully transferred to field conditions. The *in vitro*-grown plantlets showed stevioside and rebaudioside A contents similar to those measured in field plants. After this stage, the 2 clone lines (clones 15 and 18) obtained were delivered to a commercial tissue culture laboratory for the *in vitro* propagation of *S. rebaudiana*.

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