ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Hortorum Cultus, 18(6) 2019, 47–56

https://czasopisma.up.lublin.pl/index.php/asphc ISSN 1644-0692 e-ISSN 2545-1405 DOI: 10.24326/asphc.2019.6.5

ORIGINAL PAPER

Accepted: 15.03.2019

SUSTAINABLE MICROPROPAGATION OF SELECTED *Stevia rebaudiana* Bertoni GENOTYPES

Begum Kaplan[1], Selda Durakliogl[2], Kenan Turgut[1]✉

[1]Department of Field Crops, Faculty of Agriculture, Akdeniz University, 07058 Antalya, Turkey

[2] Grow Fide Inc., Pinarli District, 07112 Aksu, Antalya, Turkey

ABSTRACT

Stevia rebaudiana Bertoni is a perennial plant belonging to Asteraceae family and its leaves contain steviol glycosides (SGs) that are 150 to 300 times sweeter than sucrose. The sweeteners obtained from *S. rebaudiana* can be safely used by diabetics as insulin secretion is not required during digestion of this sweetener. As it has zero calories, it is also used in diet products. Adaptation studies for *Stevia* conducted in Antalya, Turkey have shown that the stevia plant could easily be cultivated as a perennial. However, the lack of a sustainable vegetative propagation method creates a significant problem for stevia production. In the generatively populations, homogeneity and therefore quality are decreased because of cross-pollination. Stevia, as a self-incompatible and cross-pollinated species, has been shown to have very high genetic diversity. Therefore, development of a sustainable *in vitro* propagation method to prevent genetic heterogeneity of selected varieties is crucial for stevia cultivation. The aim of this study was to evaluate 2 different gelling agents (plant agar and Gelrite) and 20 different growth regulators combinations. The results demonstrated an approximately 200-fold multiplication rate obtained within 13 weeks using MS medium supplemented with 0.5 mg·dm^{-3} BAP and 0.25 mg·dm^{-3} kinetin and solidified with Gelrite. Average stevioside and rebaudioside A contents in *in vitro* propagated plant samples were found to be 8.1% and 8.6%, respectively.

Key words: sweet herb, *in vitro* propagation, plant growth regulators, Gelrite

INTRODUCTION

Stevia rebaudiana Bertoni, also known as "sweet herb", is a subtropical plant which belongs to the Asteraceae family and because of the steviol glycoside content, is used as a non-calorific natural sweetener. In past decades, *Stevia* has become a more widely-known plant with the increased interest in natural sweetener sources. Stevia's diterpenoid steviol glycosides that are sweeter than sucrose are produced in stevia leaves and it is used in many countries around the world. The main sweetening compounds in *Stevia* are stevioside and rebaudioside A, which taste from 150 to 300 times sweeter than sucrose [Brandle et al. 1998, Yadav et al. 2011]. In addition to the taste of *Stevia* it also has antihypertensive, anti-inflammatory, antihyperglycemic, diuretic, antitumoral, antidiarrheal and antioxidant effects [Chatsudthipong and Muanprasat 2009]. Due to these features, *Stevia* has been mainly used in the food and pharmaceutical industries and it has great economic potential because of growing consumer interest in natural sweeteners [Pinheiro et al. 2005]. *Stevia rebaudiana* is characterised by a cross-pollinated, photoperiod-sensitive crop and produces self-incompatible flowers [Yadav et al., 2014] and seeds that have poor germination rates. *Stevia* is a self-incompatible

✉ kturgut@akdeniz.edu.tr

plant and it is difficult to protect characteristic features in a stable state [Yadav et al. 2011]. Furthermore, the seeds are very small, with 1000 seeds weighing 0.3–1.0 g and germination rates are much lower than 50% [Colombus 1997, Brandle et al. 1998]. The results of the adaptation studies showed that *Stevia* could be cultivated successfully as a perennial crop in the prevailing conditions of Antalya [Kaplan and Turgut 2019]. It has been observed that the genetic diversity is very high in *Stevia* crops that are cross-pollinated. An important problem with *Stevia* production is that the plants produced from seeds are not genetically homogenous [Kaplan and Turgut 2019]. Therefore, clonal propagation seems to be inevitable for the protection of good quality *Stevia* varieties. It is possible to obtain high rates of multiple shoot regenerations and thereby to use *in vitro* methods in the mass production of plantlets with micropropagation of the *Stevia* plant [Jain et al. 2009, Gridhar et al. 2010, Andlib et al. 2011, Thiyagarajan and Venkatachalam 2012, Rangappa and Aind 2013, Abdul Razak et al. 2014]. However in some studies, adaptation of *in vitro* propagated plantlets to outside conditions was found be problematic [Gridhar et al. 2010, Andlib et al. 2011, Thiyagarajan and Venkatachalam 2012] and also stevioside and rebaudioside A rates of these plants were found to be very low [Gridhar et al. 2010]. For applicable and sustainable stevia plantlet production, *in vitro* propagation methods must be optimised. The aim of the study was to develop and optimise a sustainable *in vitro* propagation method in order to prevent genetic heterogeneity of selected varieties of *Stevia.*

MATERIAL AND METHODS

Plant material and culture conditions. A selected *S. rebaudiana* genotype was used as plant material in the experiment [Kaplan and Turgut 2019]. All micropropagation studies were carried out in the tissue culture laboratories of Grow Fide seedling firm in Antalya, Turkey. For shoot bud induction, *S. rebaudiana* nodal explants were collected from 2-month old plants grown in the greenhouse and they were washed in running tap water. Then, explants were surface sterilized in 10% (v/v) commercial bleach for 15 minutes, and rinsed with sterile tap water. The sterilized nodal explants were used for *in vitro* studies as described below.

MS [Murashige and Skoog, 1962] medium was used as basal medium and supplemented with plant growth regulators (PGRs). In the experiment, BAP (0.5, 1.0, 1.5, 2.0 mg·dm^{-3}), Kinetin (KIN) (0.25 mg·dm^{-3}), NAA (0.10, 0.15, 0.20, 0.25 mg·dm^{-3}) and IAA (0.5 mg·dm^{3}) were examined (Tab. 1). The pH was adjusted to 5.7 before adding gelling agents and autoclaving. The medium was solidified with plant agar (0.7%) or Gelrite (3%) and sterilized by autoclaving at 121°C for 20 min. Nodal explants were plated on semi-solid medium containing 30 g/l sucrose and supplemented with various hormones at a density of 5 explants per jar (60 ml). Cultures were maintained in a culture room at 25°C with a 16 h photoperiod and light intensity of 4000 lux. The experiment design used in the study is shown in Table 1.

Two different gelling agents (plant agar and Gelrite) and 20 different PGRs combinations were examined as initiation media. In total, 40 treatments were established. Each treatment consisted of 10 replications (jars) giving a total of 50 explants per treatment. After 4 weeks in initiation media, *in vitro* shoot segments were cultured onto the same media for multiplication. After 3 weeks, multiple shoot buds were cultured onto 0.5 BAP + 0.25 KIN medium solidified with Gelrite due to its better performance over other treatments. The fourth and final culture was also carried out in the same medium. When the final culture was completed, multiple shoots were transferred to the MS0 medium for rooting. *In vitro* rooted plantlets were transferred to the seedling trays containing peat substrate (fine) + perlite (3 : 1) for acclimatization in a moist chamber at 25°C with a 16 h photoperiod and light intensity of 40 µmol m^{-2}·s^{-1}. After 2 weeks in the acclimatization room, they were transferred to the greenhouse and were kept there until being transplanted in the open field.

Extraction of *Stevia* leaves. 1 g of ground dried *Stevia* leaves were homogenized in 10 ml ultra-pure water at 10000 rpm/min for 10 min with ultraturrax IKA T18. Each extract was centrifuged (15 min, 5000 rpm) and 0.1 mL aqueous phases were transferred to a 10 mL volumetric flask filled with the mobile phase (asetonitrile/water – 80/20 v/v). The extracts were stored at 4 ±1°C in a refrigerator until use. The sample materials were passed through 0.45 µm nylon membrane filters to remove non-dissolved particles before analysis.

Table 1. MS media supplemented with various concentrations and combinations of different PGRs and gelling agents used in the experiment

Growth regulator concentrations ($mg \cdot dm^{-3}$)	Gelling agent (3.0%)	Gelling agent (0.7%)
MS0 (control)	Gelrite	agar
BAP 0.5	Gelrite	agar
BAP 1.0	Gelrite	agar
BAP 1.5	Gelrite	agar
BAP 2.0	Gelrite	agar
BAP 0.5 + IAA 0.5	Gelrite	agar
BAP 1.0 + IAA 0.5	Gelrite	agar
BAP 1.5 + IAA 0.5	Gelrite	agar
BAP 2.0 + IAA 0.5	Gelrite	agar
IAA 0.5	Gelrite	agar
BAP 0.5 + KIN 0.25	Gelrite	agar
BAP 1.0 + KIN 0.25	Gelrite	agar
BAP 1.5 + KIN 0.25	Gelrite	agar
BAP 2.0 + KIN 0.25	Gelrite	agar
KIN 0.25	Gelrite	agar
BAP 1.0 + NAA 0.10	Gelrite	agar
BAP 1.0 + NAA 0.15	Gelrite	agar
BAP 1.0 + NAA 0.20	Gelrite	agar
BAP 1.0 + NAA 0.25	Gelrite	agar
BAP 0.5 + KIN 0.25 + IAA 0.5	Gelrite	agar

HPLC conditions. The study was carried out in the laboratories of Field Crops Department of the Faculty of Agriculture. The chromatographic analysis under isocratic conditions was performed on Agilent 1100 systems. The system consists of G1311A Quaternary Pump, G1313A Standard automatic sampler, G1316A COLCOM column furnace and cooler and G1315A Diode Array Detector (DAD) parts.

Statistical analysis. Total number of shoots in each treatment were counted and divided by the total number of explants after the initiation culture and three subcultures, and shoot lengths were measured in each treatment after the initiation culture. The multiple number of shoots from second masses for each treatment were also considered. SAS statistical software (SAS Institute, Cary, NC, USA) was used for the data analysis. Analysis of variance was used to detect differences followed by Duncan's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Stevia breeding programs are mostly based on crossing and selection. The most important problem in Stevia breeding programs is cross-pollination within the populations due to self-incompatibility of *Stevia* [Yadav et al. 2011, Rangappa and Aind 2013, Pande and Gupta 2013]. Cross-pollination causes high genetic variations in offspring. Therefore, successfully selected genotypes can only be reproduced vegetatively without any genetic variation [Lee et al. 1982, Yadav

et al. 2011, Pande and Gupta 2013]. The single problem within clonal propagation is that costs limit the general applicability of large-scale production of the *Stevia* plant [Yadav et al. 2011].

The clonal propagation method is often used for the propagation of individually selected plants. Vegetative production such as propagation with cuttings and tissue culture has been reported to be a more appropriate method of propagation in *Stevia* than seeds [Schnelle 2010, Kaplan and Turgut 2019]. One of the most effective methods which could be of benefit to be able to produce large quantities of stable varieties, in a short time, is micropropagation. As *Stevia* seeds have a low germination rate and tissue culture is the single and most rapid process for mass propagation of *Stevia*, it is important in respect to the production of the plant. In this study, experiments were conducted in tissue culture conditions to observe the frequency of the plantlet formation for production potential of the *Stevia* plant, without having selected any genotype. The most appropriate development medium was found for *Stevia* from different media with different PGRs and concentrations. Also, effects of agar and Gelrite on *in vitro* propagation of *Stevia* were evaluated first time.

The effect of different PGRs combinations on shoot initiation in Gelrite solidified media. In the experiment, nodal explants from mature *S. rebaudiana* plants were placed on MS medium supplemented with different concentrations of BAP (0.5, 1.0, 1.5, 2.0 mg·dm^{-3}), KIN (0.25 mg·dm^{-3}), NAA (0.10, 0.15, 0.20, 0.25 mg·dm^{-3}) and IAA (0.5 mg·dm^{-3}) and solidified with plant agar (0.7%) or Gelrite (3%) for shoot bud initiation. Two gelling agents (plant agar and Gelrite) were evaluated separately as shown in Tables 2 and 3. Nodal explants were used as most preferred explants for *in vitro* propagation of sweet herb [Jain et al. 2014].

In the Gelrite experiment, the percentage of shoot induction was found to be very high (mean 96.5%) and the mean number of shoots per explant and mean shoot length were found to be 2.46 and 10.24 cm respectively (Tab. 2). The differences between PGRs combinations were determined to be statistically significant. The highest number of initial shoots were obtained from 1.0 BAP + 0.25 KIN (3.18 shoots/explant), followed by 0.5 BAP + 0.25 KIN (3.00 shoots/explant), 1.0 BAP + 0.25 NAA (2.96 shoots/explant), 1.0 BAP + 0.15 NAA (2.92 shoots/explant), 1.0 BAP + 0.10 NAA (2.82 shoots/explant) and 2.0 BAP (2.70 shoots/explant) (Tab. 2). In one of the first studies, MS medium with 0.6 mg·dm^{-3} BAP gave the maximum number of shoots [Tathani et al. 2006]. However, Taware et al. [2010] reported optimum shoot regeneration from nodal explants in MS nutrient medium containing 0.3 mg·dm^{-3} KIN. Andlip et al. [2011] evaluated effects of two cytokinins (BAP and KIN) at different concentrations on *in vitro* shoot proliferation of *Stevia*. They found that involvement of cytokinins, particularly BAP improved shoot production regardless of concentration than KIN and the highest number of shoots per explant was observed on MS medium containing 1.25 mg·dm^{-3} BAP and solidified by agar. Thiyagarajan and Venkatachalam [2012] examined MS medium supplemented with different concentrations of BAP (0.5–3.0 mg·dm^{-3}) and KIN (0.5–3.0 mg·dm^{-3}) for shoot bud initiation from nodal explants of *Stevia* plants. After all, they concluded that the presence of cytokinins in the medium was essential to induce bud break and shoot proliferation from nodal explants, and BAP was more effective than KIN for shoot initiation from nodal explants. Rangappa and Aind [2013] reported that combination of BAP and NAA (ratio of 2 : 1) was the most suitable for *in vitro* shoot regeneration from the nodal segments of *Stevia*. Similar to present study, Abdul Razak et al. [2014] obtained the highest shoot production from MS medium containing 0.5 mg·dm^{-3} BAP and 0.25 mg·dm^{-3} KIN solidified with agar. On the other hand, MS0 medium gave the lowest shoot induction (1.26 shoots/explant) with some root formation, followed by 0.5 IAA (1.52 shoots/explant) and 0.25 KIN (2.12 shoots/explant) (Tab. 2). In the present study, BAP + KIN combination was appeared to be better than BAP + NAA and BAP + IAA combinations. When 0.5 BAP, 1.0 BAP, 1.5 BAP and 2.0 BAP were used alone, 2.58, 2.50, 2.62 and 2.70 shoots/explant were obtained (Tab. 2). The addition of 0.25 KIN to 0.5 BAP and 1.0 BAP increased the number of shoots significantly and resulted in 3.00 and 3.18 shoots/explant respectively under Gelrite condition. However, the addition of 0.25 KIN to 1.5 BAP and 2.0 BAP did not increase the shoot numbers and caused vitrification, which might have been due to the high amount of cytokinin [Andlip et al. 2011, Thiyagarajan and Venkatachalam 2012, Abdul

Razak et al. 2014]. On the contrary, Rafiq et al. [2007] found no specific increase in multiple shoot formation occurred when the explants were cultured on KIN based media. Instead of 0.25 KIN, a supplement of 0.5 IAA to the same media resulted in poor shoot initiation (Tab. 2). Nonetheless, Shatnawi et al. [2011] achieved the highest shoot bud proliferation in MS medium supplemented with 1.5 mg·dm^{-3} BAP and 0.2 mg·dm^{-3} IAA. Although low levels of NAA with 1.0 BAP increased the shoot number slightly, most of the shoots appeared to be vitrified (Tab. 2). Consequently, presence of BAP was appeared to be prerequisite for successful shoot bud proliferation and also BAP + KIN combinations were found to be superior to other combinations in respect of shoot number per explant (Tab. 2). These findings are consistent with other studies [Jain et al. 2009, Andlip et al. 2011, Thiyagarajan and Venkatachalam 2012, Abdul Razak et al. 2014]. Unlike other *in vitro* propagation studies, Giridhar et al. [2010] examined BAP, IBA and NAA growth reg-

Table 2. Evaluation of shoot induction frequency, number of shoots per explant, shoot lengths (cm) in initiation culture and multiple number of shoots after second culture in MS media supplemented with 20 different combinations with 4 different PGRs, solidified by Gelrite (3%)

Growth regulator concentrations (mg·dm^{-3})	Frequency of shoot induction (%)	No. of shoots per explant in initiation culture	Shoot lengths in initiation culture (cm)	Multiple No. of shoots after 2nd culture
MS0 (control)	100	1.26f	16.40a	5.34f
BAP 0.5	100	2.58bcde	13.06abc	12.80ab
BAP 1.0	100	2.50bcde	10.73cde	12.62ab
BAP 1.5	90	2.62bcde	7.89ef	11.31abc
BAP 2.0	100	2.70abcd	7.72ef	10.16abc
BAP 0.5 + IAA 0.5	90	2.32de	12.16bcd	13.38a
BAP 1.0 + IAA 0.5	100	2.46bcde	10.38cde	10.84abc
BAP 1.5 + IAA 0.5	100	2.30de	10.74cde	12.36ab
BAP 2.0 + IAA 0.5	100	2.08e	8.68def	8.64cdef
IAA 0.5	100	1.52f	14.46ab	5.86f
BAP 0.5 + KIN 0.25	100	3.00ab	10.36cde	12.42ab
BAP 1.0 + KIN 0.25	90	3.18a	9.30cdef	11.46abc
BAP 1.5 + KIN 0.25	80	2.48bcde	5.92f	6.32ef
BAP 2.0 + KIN 0.25	100	2.50bcde	5.78f	6.56def
KIN 0.25	100	2.12e	15.08ab	8.50cdef
BAP 1.0 + NAA 0.10	100	2.82abcd	9.18def	11.19abc
BAP 1.0 + NAA 0.15	90	2.92abc	9.48cdef	9.84abcd
BAP 1.0 + NAA 0.20	90	2.38cde	9.58cdef	9.70bcde
BAP 1.0 + NAA 0.25	100	2.96ab	8.78def	11.20abc
BAP 0.5 + KIN 0.25 + IAA 0.5	100	2.40cde	9.18def	8.46cdef
Mean	96.5	2.46	10.24	9.95

Means within a column followed by different letters are different at the $P \leq 0.05$ level

ulators combinations in MS and B5 medium using shoot tip and nodal explants and, they obtained better shoot proliferation from shoot tip culture using B5 medium supplemented with BAP and NAA.

When shoot length data were examined, significant differences were found between the treatments (Tab. 2). The longest shoot length was obtained from MS0 medium (16.40 cm) and followed by 0.25 KIN (15.08 cm), 0.5 IAA (14.46 cm), 0.5 BAP (13.06). On the other hand, 2.0 BAP + 0.25 KIN (5.78 cm) and 1.5 BAP + 0.25 KIN (5.92 cm) treatments gave the shortest shoot lengths (Tab. 2). As occurred in the number of shoots, higher BAP levels reduced shoot lengths. Our results are consistent with other reports [Andlip et al. 2011, Thiyagarajan and Venkatachalam 2012]. Abdul Razak et al. [2014] obtained the highest shoot lengths from MS medium containing 1.0 mg·dm^{-3} KIN (5.05 cm) and 0.5 mg·dm^{-3} KIN (4.67 cm) solidified with agar. However, they observed the lowest shoot lengths from 1.5 mg·dm^{3} BAP + 0.25 mg·dm^{-3} KIN and 2.0 mg·dm^{-3} BAP + 0.25 mg·dm^{-3} KIN.

In order to develop shoot multiplication, *in vitro* regenerated shoot buds from nodal explants were cultured on MS medium supplemented with same PGRs, and then the mass number of multiple shoots were calculated (Tab. 2). The results revealed that the number of multiple shoots was affected significantly by different PGRs combinations. The highest mass number of multiple shoots after the second culture was obtained from 0.5 BAP + 0.5 IAA medium (13.38 shoots/explant), followed by 0.5 BAP (12.80 shoots/explant), 1.0 BAP (12.62 shoots/explant), 0.5 BAP + 0.25 KIN (12.42 shoots/explant) 1.5 BAP + 0.5 IAA (12.36 shoots/explant), 1.5 BAP (11.31 shoots/explant), 1.0 BA + 0.25 NAA (11.20 shoots/explant), 1.0 BAP + 0.10 NAA (11.19 shoots/explant), 1.0 BAP + 0.5 IAA (10.84 shoots/explant) and 2.0 BAP (10.16 shoots/explant) media. As similar to our results, Thiyagarajan and Venkatachalam [2012] achieved 15.69 and 11.22 shoots/explant in an MS medium containing 1.0 mg·dm^{-3} BAP and 1.5 mg·dm^{-3} BAP respectively in the second culture. MS0 (5.34 shoots/explant) and 0.5 IAA (5.86 shoots/explant) treatments gave the lowest values followed by 1.5 BAP + 0.25 KIN (6.32 shoots/explant) and 2.0 BAP + 0.25 KIN (6.56 shoots/explant) (Tab. 2). Although 0.5 BAP + 0.5 IAA medium gave the highest number of multiple shoots after second culture, the shoots turned brownish in the early stage. On the other hand, the shoots grown in the 0.5 BAP and 1.0 BAP media appeared to be rather weak. Therefore, MS medium containing 0.5 BAP + 0.25 KIN was preferred for subsequent cultures since it produced higher and better quality shoots than the others. The quality of the shoots is very important for the acclimatization and transplanting stages.

The effect of different PGRs combinations on shoot initiation in plant agar solidified media. When the media was solidified with agar (0.7%), the percentage of shoot induction was found to be high (mean 92%) and 1.0 BAP + 0.10 NAA medium gave the lowest rate at 70% (Tab. 3). The mean number of shoots per explant and mean shoot length were found to be 1.82 shoots/explant and 2.62 cm respectively for all combinations in plant agar media (Tab. 3). In the statistical evaluation made in respect to the number of shoots, a statistically significant differences were determined. MS medium containing 0.25 KIN gave the highest value at 2.64 shoots/explant and followed by 1.0 BAP + 0.10 NAA (2.40 shoots/explant), 1.0 BAP + 0.25 NAA (2.22 shoots/explant), 0.5 BAP + 0.25 KIN + 0.5 IAA (2.18 shoots/explant) and 1.0 BAP + 0.15 NAA (2.12 shoots/explant) (Tab. 3). Similarly, Rangappa and Aind [2013] revealed that combination of BAP and NAA (ratio of 2 : 1) in MS medium was the most suitable for *in vitro* shoot induction from the nodal explants of *Stevia*. On the other hand, Abdul Razak et al. [2014] achieved the highest shoot proliferation from MS medium containing 0.5 mg·dm^{-3} BAP and 0.25 mg·dm^{-3} KIN solidified with agar. In all combinations including BAP, higher BAP concentrations had retardant effect on shoot proliferation and so 0.5 and 1.0 mg·dm^{-3} BAP were found to be the most appropriate levels. Unlike Gelrite treatments, BAP + KIN combinations were failed in terms of *in vitro* shoot initiation from nodal explants. Furthermore, the main problem observed in the agar solidified media was vitrification in all PGR combinations.

When shoot length data was examined, significant differences were found between the treatments with the mean shoot length of 2.62 cm (Tab. 3). The highest shoots were obtained from MS0 medium (9.76 cm) followed by 0.25 KIN (4.52 cm) and 0.5 IAA (3.54 cm), but the shortest shoot length was

observed in presence of 2.0 BAP + 0.5 IAA (1.5 cm) and 2.0 BAP (1.54 cm), with the other media determined to be statistically similar (Tab. 3). Similarly, Abdul Razak et al. [2014] obtained the highest shoot lengths from MS medium containing 1.0 mg·dm^{-3} KIN (5.05 cm) and 0.5 mg·dm^{-3} KIN (4.67 cm) solidified with agar. However, they observed the lowest shoot lengths from 1.5 mg·dm^{-3} BAP + 0.25 mg·dm^{3} KIN and 2.0 mg·dm^{-3} BAP + 0.25 mg·dm^{-3} KIN. In general, higher BAP concentrations reduced the shoot length (Tab. 3) and similar results were obtained from other studies [Giridhar et al. 2010, Thiyagarajan and Venkatachalam 2012].

Primary shoots were cultured onto the same media and then the number of multiple shoots after the second culture were determined to be significantly affected by different PGRs combinations (Tab. 3). The mean number multiple shoots were appeared to be very low (2.92) after second culture. The highest multiple shoot numbers after the second culture were obtained from 0.25 KIN medium (5.90 shoots/explant), followed by MS0 (5.04 shoots/explant). On the

Table 3. Evaluation of shoot induction frequency, number of shoots per explant, shoot lengths (cm) in initiation culture and multiple number of shoots after second culture in MS media supplemented with 20 different combinations with 4 different PGRs, solidified by plant agar (0.7%)

Growth regulator concentrations (mg·dm^{-3})	Frequency of shoot induction (%)	No. of shoots per explant in initiation culture	Shoot lengths in initiation culture (cm)	Multiple No. of shoots after 2nd culture
MS0 (control)	100	1.48fg	9.76a	5.04b
BAP 0.5	100	1.98cde	2.14cd	3.10cde
BAP 1.0	100	1.92cde	1.94cd	2.72cdef
BAP 1.5	100	1.62defg	1.76cd	1.90fg
BAP 2.0	80	1.44fg	1.54d	1.94fg
BAP 0.5 + IAA 0.5	90	1.86cdef	2.42cd	3.0cde
BAP 1.0 + IAA 0.5	90	1.56efg	2.34cd	2.48efg
BAP 1.5 + IAA 0.5	100	1.46fg	2.0cd	2.0fg
BAP 2.0 + IAA 0.5	90	1.4g	1.5d	1.72g
IAA 0.5	80	1.44fg	3.54bc	3.46cd
BAP 0.5 + KIN 0.25	80	2.02bcd	2.14cd	2.54defg
BAP 1.0 + KIN 0.25	90	1.56efg	2.02cd	2.56defg
BAP 1.5 + KIN 0.25	90	1.62defg	1.84cd	2.28efg
BAP 2.0 + KIN 0.25	100	1.46fg	1.90cd	1.92fg
KIN 0.25	100	2.64a	4.52b	5.90a
BAP 1.0 + NAA 0.10	70	2.40ab	2.36cd	3.66c
BAP 1.0 + NAA 0.15	100	2.12bc	2.18cd	3.02cde
BAP 1.0 + NAA 0.20	90	1.98cde	2.0cd	2.52defg
BAP 1.0 + NAA 0.25	100	2.22bc	2.10cd	3.60c
BAP 0.5 + KIN 0.25 + IAA 0.5	90	2.18bc	2.42cd	3.04cde
Mean	92	1.82	2.62	2.92

Means within a column followed by different letters are different at the $P \leq 0.05$ level

other hand, 2.0 BAP + 0.5 IAA (1.72 shoots/explant) gave the lowest multiple shoot numbers followed by 1.5 BAP (1.90 shoots/explant), 2.0 BAP + 0.25 KIN (1.92 shoots/explant), 2.0 BAP (1.94 shoots/explant) and 1.5 BAP + 0.5 IAA (2.0 shoots/explant) (Tab. 3). Unlike our result, Thiyagarajan and Venkatachalam [2012] achieved 15.69 and 11.22 shoots per explant in an MS medium containing 1.0 mg·dm^{-3} BAP and 1.5 mg·dm^{-3} BAP respectively in the second culture. MS0 (5.34 shoots/explant) and 0.5 IAA (5.86 shoots/explant) treatments gave the lowest values followed by 1.5 BAP + 0.25 KIN (6.32 shoots/explant) and 2.0 BAP + 0.25 KIN (6.56 shoots/explant) (Tab. 2).

When the results of both gelling agents were compared without statistical evaluation, Gelrite appeared to be superior to plant agar in terms of shoot initiation number, shoot length, number of multiple shoots and shoot hardiness. The number of shoots/explant, shoot length and multiple shoot numbers were found to be much higher in Gelrite than in plant agar (Tabs 2 and 3). While the mean number of initial shoots per explant was 1.82 in agar medium, Gelrite medium produced 2.46 initial shoots/explant. In respect of shoot length, a great difference was seen between Gelrite (10.24 cm) and agar (2.62 cm) media. The mean multiple shoot numbers after the second culture were found to be 3.4 times higher in Gelrite than agar. Gelrite and agar media produced a mean number of 9.95 and 2.92 shoots, respectively from a single explant. Similar results were obtained from different plant species [Lim et al. 2012, Ismail et al. 2016]. Lim et al. [2012] found the highest shoot induction and number of shoots per explant on Gelrite medium in chrysanthemum. Vitrification of the shoots was a significant problem in agar media, but not common in Gelrite media. In conclusion, Gelrite appeared to be a much better gelling agent in terms of shoot number, shoot length and quality of the shoots when compared to plant agar. This is the first report on comparison of agar and Gelrite for *in vitro* propagation of *Stevia* within our knowledge.

Subsequent cultures for shoot multiplication. MS medium containing 0.5 BAP + 0.25 KIN and solidified with Gelrite was used for subsequent cultures since it produced higher shoot numbers without vitrification and other abnormalities. The shoots obtained from plant agar solidified media were not subcultured due to low quality properties. Therefore, only the shoots initiated in Gelrite media were cultured for further multiplication. After three weeks in the second culture, all the shoots from various PGRs combinations with Gelrite were transferred to MS medium containing 0.5 BAP + 0.25 KIN and solidified with Gelrite. In the third culture, 52.16 multiple shoots per explant with a mean length of 14.23 cm were obtained and in the fourth culture in the same medium, 3.84 shoots/explant with 15.25 cm length. After the fourth culture, 200.29 shoots per explant with multiplication rate of 66.76 were produced within 13 weeks in MS medium containing 0.5 BAP + 0.25 KIN and solidified with Gelrite. This result was very promising for commercial plantlet production. Thiyagarajan and Venkatachalam [2012], took cultures of axillary buds in an MS medium containing 1.0 mg·dm^{-3} BAP and obtained 94.5% regeneration frequency and 15.69 shoots/explant and reached 123 shoots/explant after 3 sub cultures. However, at the stage of adaptation to outside conditions, only 65.8% of the *in vitro* plants could survive. In that study no information was given about the steviol glycoside content of the plants from *in vitro* conditions.

Rooting and acclimatization. MS0 medium solidified with Gelrite was used for *in vitro* rooting of the shoots. Rooting was observed within a period of approximately one week and occurred at a rate of 97%. After mass propagation, 30000 plantlets were rooted successfully. In the previous studies in *Stevia*, the researchers achieved best rooting frequency on ½ MS or MS medium containing auxin (IBA and NAA). Taware et al. [2010] optimised *in vitro* root induction on MS medium fortified with 2.0 mg·dm^{-3} IBA. Andlip et al. [2011] achieved 100% rooting on ½ MS medium supplemented with mg·dm^{-3} IBA. Thiyagarajan and Venkatachalam [2012] obtained the highest frequency of rooting (96%) on ½ MS medium containing with 0.4 mg·dm^{-3} NAA. Abdul Razak et al. [2014] obtained up to 100% rooting on MS medium fortified with 1.0 mg·dm^{-3} IBA.

The *in vitro* rooted plantlets were transferred to the seedling trays containing peat substrate + perlite for acclimatization in a moist chamber. After 2 weeks in the acclimatization room, they were transferred to the greenhouse with 95% recovery and were grown for 6 weeks. Finally, 28000 healthy seedlings were transplanted to the soil for cultivation. In *in vitro* propagation studies, despite generally obtaining a high rate

and number of shoots, there have been significant losses in the adaptation to outside conditions [Gridhar et al. 2010, Andlib et al. 2011, Thiyagarajan and Venkatachalam 2012]. Andlib et al. [2011] obtained a high rate of shoots (100% frequency, 73.2 shoots/explant) in an MS medium in *in vitro* conditions using axillary buds of the *Stevia* plant. In contrast, only 70% of the *in vitro* plants could survive in field conditions and the steviol glycoside content of the plants in question were not examined.

Steviol glycosides contents. The plants were harvested in September 2018 and analysed for Stevioside and Rebaudioside A content after drying. No differences were determined between *in vitro* propagated and stem cutting propagated plants in terms of leaf yield and amount of SGs (data not given). Average stevioside and rebaudioside A contents in *in vitro* propagated plant samples were found be 8.1% and 8.6% respectively. However, in the majority of *in vitro* propagation studies, the agronomic performance and steviol glycoside rates were not examined. In those where steviol glycoside analysis was applied, the stevioside and rebaudioside A rates were found to be very low [Bondarev et al. 2001, Gridhar et al. 2010].

CONCLUSIONS

The aim of the study was to develop a sustainable *in vitro* propagation method for the of selected *Stevia* genotypes and provide good quality plantlets for the farmers. At the end of the study, a multiplication rate of approximately 200-fold was obtained within 13 weeks using MS + 0.5 BAP + 0.25 KIN and solidified with Gelrite. After that, high frequency of rooting and acclimatization were achieved and plantlets were transplanted to the field successfully. More importantly, *in vitro* propagated plants produced high quality of SGs similar to plants which were propagated by stem cuttings. Therefore, this method is used commercially for reliable and sustainable propagation of *Stevia* cultivars in Turkey.

ACKNOWLEDGMENT

This study was supported by the Scientific and Technological Research Council of Turkey (project no. 0862.STZ.2015). All propagation studies were carried out in the research facilities of Grow Fide Inc.

REFERENCES

Abdul Razak, U.N.A., Ong, B.B., Yu, T.S., Lau, L.K. (2014). *In vitro* micropropagation of *Stevia rebaudiana* Bertoni in Malaysia. Braz. Arch. Biol. Techn., 57 (1), 23–28.

Andlib, A., Verma, R.N., Saxena, S., Batra, A. (2011). High-frequency induction of multiple shoots of *Stevia rebaudiana* bertoni through *in vitro* Micropropagation techniques. Elixir Hor. Sig., 34C, 2465–2468.

Bondarev, N., Reshetnyak, O., Nosov, A. (2001). Peculiarities of diterpenoid steviol glycoside production in *in vitro* cultures of *Stevia rebaudiana* Bertoni. Plant Sci., 161(1), 155–163.

Brandle, J.E., Starratt, A.N., Gijzen, M. (1998). *Stevia rebaudiana*: its agicultural, biological and chemical properties. Can. J. Plant Sci., 78, 527–536.

Chatsudthipong, V., Muanprasat, C. (2009). Stevioside and related compounds: therapeutic benefits beyond sweetness. Pharmacol. Ther., 121, 41–54.

Colombus, M. (1997). The cultivation of stevia, "nature's sweetener". Ontario Ministry of Agriculture. Food and Rural Affairs, Toronto, ON. p. 4.

Crammer, B., Ikan, R. (1987). Sweet glycosides from the stevia plant. Chem. Britain, 22, 915–916.

Giridhar, P., Sowmya, K.S., Ramakrishna, A., Ravishankar, G.A. (2010). Rapid clonal propogation and stevioside profiles of *Stevia rebaudiana* Bertoni. Int. J. Plant Dev. Biol., 4(1), 47–52.

Ismail, H., Muniandi, S.K., Yusoff, A.M., Hassan, N.H., Shukor, N.A. (2016). *In vitro* micropropagation of *Acacia auriculiformis* from selected juvenile sources. Dendrobiology, 75, 157–165.

Jain, P., Kachhwaha, S., Kothari, S.L. (2009). Improved micropropagation protocol and enhancement in biomass and chlorophyll content in *Stevia rebaudiana* Bertoni by using high copper levels in the culture medium. Sci. Hortic., 119, 315–319.

Jain, P., Kachhwaha, S., Kothari, S.L. (2014). Biotechnology and metabolic engineering of *Stevia rebaudiana* (Bert.) Bertoni: perspective and possibilities. Int. J. Life Sci. Biotechnol. Pharma Res., 3(3), 15–37.

Kaplan, B., Turgut, K. (2019). Improvement of rebaudioside A diterpene glycoside content in *Stevia rebaudiana* Bertoni using clone selection. Turk J. Agric. For. (Unpublished).

Lee, J.I., Kang, K. H., Park, H.W. (1982). New high rebaudiosid A stevia variety 'Suweon 11'. Res. Rept. ORD, 24, 186–188. [in Korean, English summary].

Lim, K.-B., Kwon, S.J., Lee, S.I., Hwang, Y.-J., Naing, A.H. (2012). Influence of genotype, explant source, and gelling agent on *in vitro* shoot regeneration of chrysanthemum. Hortic. Environ. Biotechnol., 53(4), 329–335.

Metivier, J., Viana, A.M. (1979). The effect of long and short day length upon the gowth of whole plants and the level of soluble proteins, sugars and steviosid in leaves of *Stevia rebaudiana* Bert. J. Exp. Bot., 30, 1211–1222.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol., 15, 473–497.

Pande, S.S., Gupta P. (2013). Plant tissue culture of *Stevia rebaudiana* (Bertoni): A review. J. Pharmacog. Phytother., 5(1), 26–33.

Pinheiro, M.V.S., Oliveria, M.N., Penna, A.L.B., Tamime, A.Y. (2005). The effect of different sweeteners in low-calorie yogurts, a review. Int. J. Dairy Technol., 58, 193–199.

Rafiq, M., Dahot, M.U., Mangrio, S.M., Naqri, H.A., Qarshi, I.A. (2007). *In vitro* clonal propagation and biochemical analysis of field established *Stevia rebaudiana* Bertoni. Pak. J. Bot., 39(7), 2467–2474.

Rangappa, K., Aind, D.S. (2013). High frequency multiplication of shoots using axillary buds for production of elite lines of *Stevia rebaudiana.* Adv. Biosci. Biotechnol., 4, 781–784.

Schnelle, R. (2010). Stevia Agriculture & Natural Resources. U.K. Cooperative extension service. University of Kentucky – College of Agriculture.

Shatnawi, A.M., Shibli, R.A., Abu-Romman, S.M. (2011). Clonal propagation and cryogenic storage of the medicinal plant *Stevia rebaudiana*. Span. J. Agric. Res., 9, 213–220.

Tadhani, M.B., Jadeja, R.P., Rena, S. (2006). Micropopagation of *Stevia rebaudiana* Bertoni using multiple shoot culture. J. Cell Tissue Res., 6, 545–548.

Taware, A.S., Mukadam, D.S., Chavan, A.M., Taware, S.D. (2010). Comparative studies of *in vitro* and *in vivo* grown plants and callus of *Stevia rebaudiana* (Bertoni). Int. J. Integr. Biol., 9(1), 10–15.

Thiyagarajan, M., Venkatachalam, P. (2012). Large scale *in vitro* propagation of *Stevia rebaudiana* (bert) for commercial application: pharmaceutically important and anti-diabetic medicinal herb. Ind. Crops Prod., 37, 111–117.

Yadav, A.K., Singh, S., Dhyani, D., Ahuja, P.S. (2011). A review on the improvement of Stevia [*Stevia rebaudiana* (Bertoni)]. Can. J. Plant Sci., 91, 1–27.