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A fast and effective protocol for obtaining genetically diverse stevia (*Stevia rebaudiana* Bertoni) regenerants through indirect organogenesis

Szybki i efektywny protokół otrzymywania zróżnicowanych genetycznie regenerantów stewii (*Stevia rebaudiana* Bertoni) drogą pośredniej organogenezy

Summary. Plant regeneration through indirect organogenesis allows obtaining genetic variability that can be used in the creation of new cultivars. The study presents a fast and effective protocol of one-step preparation of stevia (*Stevia rebaudiana* Bertoni) regenerants. To obtain callus tissue and shoot regeneration, leaves and nodal segments were used as primary explants, which were placed on MS (Murashige and Skoog) medium supplemented with plant growth regulators (PGRs): NAA (1-naphthaleneacetic acid – 2.0 mg·dm^{-3}, BA (6-benzylaminopurine – 4.0 mg·dm^{-3}), 2,4-D (2,4-dichlorophenoxyacetic – 2.0 mg·dm^{-3}). Callus tissue was formed on both types of explants, however, only those derived from nodal segments were proliferating. An average of 3.92 shoots per explant were obtained from leaf explants on the applied medium after 6 weeks of culture. The analysis of the morphogenetic capacity of the obtained regenerants was carried out on MS medium supplemented with PGRs – kinetin (0.25 mg·dm^{-3}), BA (0.5 mg·dm^{-3}). The evaluation of the mean number of shoots, mean shoot length (cm), and the mean number of nodes per shoot indicated phenotypic variability of regenerants. The use of RAPD (randomly amplified polymorphic DNA) markers confirmed the differences also at the DNA level. The proposed one-step indirect organogenesis regeneration protocol induced somaclonal variation of *Stevia rebaudiana* Bertoni and the obtained regenerants, after selection, could be used in the breeding of this species.

Key words: micropropagation, molecular markers, RAPD, shoot regeneration, somaclonal variation

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a perennial plant native to Paraguay and northern Brazil. Its current range of natural distribution spans from the southeastern United States to central Argentina [Chacón-Morales et al. 2013]. It belongs to the genus

Stevia, family Asteraceae (Compositae) alongside over 200 other species [Gantait et al. 2018]. For over 1500 years, it has been used by the Guarani people as a medicine for ailments related to blood pressure, digestion and to heal wounds and skin lesions. At present, it is primarily known for its sweet taste. Thanks to the content of steviol glycosides, which is about 250–300 times sweeter, and according to some sources even 450 times sweeter than sucrose, it has gained the name of a "sweet leaf" [Gantait et al. 2015]. In addition to steviol glycosides, stevia also contains compounds from the group of sterols, flavonoids, essential oils, alkaloids, tannins, saponins, and anthraquinones [Ferrazzano et al. 2016]. Stevia also supports the functioning of the immune system and has anti-inflammatory and antioxidant properties [Samuel et al. 2018]. Therefore, it is a plant with significant scientific potential and growing economic importance, which is associated with an increasing demand for good-quality raw materials. Stevia glycoside profile is subject to significant variations depending on the genotype, geographical area, plant maturity, environmental conditions, harvest date, and processing method. It is necessary to control the quality of the raw material to ensure the effectiveness and safety of the use of this species in food and medicine [Dyduch-Siemińska et al. 2020]. Growing stevia from seeds is not practiced on professional plantations, because as a cross-pollinated plant, it is characterized by a large diversity of populations produced from seeds and this reproduction method is used only when it is not possible to deliver selected seedlings to a given region. It can be reproduced using *in vitro* techniques [Al-Taweel et al. 2021].

In vitro techniques are important tools in modern plant improvement programs. When used in combination with classical breeding methods, an efficient *in vitro* plant proliferation and regeneration system accelerates the breeding programs of many cultivars. The ability to regenerate plants is crucial for the successful application of *in vitro* methods [Cao and Hammerschlag 2000]. Not all regenerated plants are the same in *in vitro* cultures – some differences in the genotype are visible even to the naked eye in the form of changes in plant morphology [Krishna et al. 2016]. The first documented reports of this phenomenon in plant breeding programs were published by Heinz and Mee in 1971 [Heinz and Mee 1971, Skirvin et al. 1993]. The phenomenon has been given the name of somaclonal variation, sometimes also called tissue culture-induced variation [Krishna et al. 2016]. Because of the initial aim of *in vitro* plant propagation, i.e. to obtain clones, genetically homogeneous material, somaclonal variation was a problem to be solved, but over time it was also recognized as a potential source of useful new genotypes [Jain 2001, Bairu et al. 2011, Krishna et al. 2016]. Somaclonal variation is a group of different types of mutations that arise *in vitro* between regenerants and their corresponding donor plants. [Predieri 2001, Bairu et al. 2011]. It includes changes such as point mutations, chromosomal rearrangements, and recombinations within homologous chromosomes, DNA methylation, changes in the copy number of certain sequences, transposon rearrangements [Jain 2001, Bairu et al. 2011, Sarmah et al. 2017]. Genetic diversity of regenerants derived from *in vitro* cultures may result from four main groups of factors: genotype (a mutation present in some explant cells before the culture was established), the origin of explants, the reaction of explants to culture conditions, such as medium composition, environmental

parameters or long culture duration [Jain 2001, Bairu et al. 2011, Krishna et al. 2016]. These changes include hereditary as well as epigenetic mutations [Jain 2001, Miguel and Marum 2011].

The aim of this study was to present a methodology for the preparation of stevia regenerants by indirect organogenesis, characterized by somaclonal variation, in a short time, in one step, without the need to change the medium. Moreover, the study assessed the genetic diversity of the obtained regenerants using DNA markers based on polymerase chain reaction – RAPD (randomly amplified polymorphic DNA). Such an assessment is the basis for the selection of plants with new genotypes and their further utilization in breeding programs of the species.

MATERIALS AND METHODS

Plant material

Stevia (*Stevia rebaudiana* Bertoni) plants grown at the Experimental Farm of the Department of Vegetable and Medicinal Plants of the University of Life Sciences in Lublin (51°14'53"N, 2°34'13"E) were used in the investigation. Shoot samples with three nodes were collected after 120 days of vegetation in the field, i.e. on August 20th, 2018. Immediately after harvesting, the explants were transported to the *in vitro* culture laboratory at the Institute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences in Lublin. Explant sterilization and establishment of *in vitro* culture through nodal explant was conducted in accordance with the protocol proposed by Mubarak et al. [2008]. The shoots obtained in that procedure (*Stevia rebaudiana* Bertoni) were defined as donor plants (DP) and used in the subsequent stages of the experiment. The use of nodal segments ensures the stability of the obtained plants.

Callus induction and shoot regeneration

Shoots were collected from disinfected stevia plants cultivated *in vitro* from which leaf explants, about 0.5 cm × 0.5 cm in size, and 0.8 cm-long internodes were excised. The explants were inoculated on Petri dishes with callus induction medium containing Murashige and Skoog Basal Medium (MS) [Murashige and Skoog 1962] supplemented with sucrose (30.0 $g \cdot dm^{-3}$), thiamine (0.4 $mg \cdot dm^{-3}$), pyridoxine (0.5 $mg \cdot dm^{-3}$), nicotinic acid (0.5 $mg \cdot dm^{-3}$), inositol (100 $mg \cdot dm^{-3}$), plant growth regulators (PGRs) – NAA (1-naphthaleneacetic acid – 2.0 $mg \cdot dm^{-3}$), BA (6-benzylaminopurine – $mg \cdot dm^{-3}$), 2,4-D (2,4-dichlorophenoxyacetic acid – 2.0 $mg \cdot dm^{-3}$) and agar-agar (Sigma-Aldrich – 8.0 $g \cdot dm^{-3}$). For the experiment, pH of the medium was adjusted to 5.8 with 1 M NaOH and 1 M HCl before autoclaving at 121°C for 20 min. The temperature in a growth chamber was (21°C ±2°C). After initiation, the explants were cultured under 40 µmol m^{-2} s^{-1} light provided by cool white fluorescent tubes at a photoperiod of 16 h. The experiment was carried out in three replicates. One replicate consisted of 10 Petri dishes with 10 explants on each. Observations of callus tissue formation were carried out during the culture. Its structure and color were assessed after 14 days. The morphogenetic capacity

of callus tissue was assessed after 6 weeks. Shoots regenerated on callus tissue (regenerants) were subjected to i) assessment of multiplication efficiency ii) and analysis of genetic diversity at the DNA level using RAPD markers.

Shoot multiplication of regenerants

Shoots regenerated on callus tissue were cut into nodal fragments which were used as explants. From all obtained regenerants, 30 were randomly selected and used in the multiplication stage and DNA analysis. Three nodal explants were collected from each regenerant for multiplication. Nodes were placed on MS medium supplemented with sucrose (30.0 $g \cdot dm^{-3}$), thiamine (0.4 $mg \cdot dm^{-3}$), pyridoxine (0.5 $mg \cdot dm^{-3}$), nicotinic acid (0.5 $mg \cdot dm^{-3}$), inositol (100 $mg \cdot dm^{-3}$), PGRs – kinetin (0.25 $mg \cdot dm^{-3}$), BA (0.5 $mg \cdot dm^{-3}$) and agar-agar (8.0 $g \cdot dm^{-3}$). The culture condition, pH of the medium, and the sterilization temperature were the same as in the section 'Callus induction and shoot regeneration'. The number of propagated shoots, their average length, and the average number of nodes were assessed after 8 weeks of culture. To compare the results obtained at that stage of culture with the donor plants (DP), the morphogenetic potential of DP was analogously assessed.

Molecular assays

DNA extraction

The material for DNA isolation was collected after shoot multiplication of all regenerants. DNA was extracted following the CTAB method described by Doyle and Doyle [1987]. DNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific). Test samples were diluted to a final concentration of 20 $ng \cdot \mu l^{-1}$.

RAPD analysis

Based on the results of the multiplication stage, the following regenerants were selected for RAPD analyses: 1, 5, 6, 16, 21, 22, 24, 25, 30, and DP. Ten 10-base primers selected from 40 arbitrary primers were used for PCR amplification. DNA amplification of RAPD markers was carried out in a final volume of 15 µl containing 0.5 U of Taq DNA Polymerase (Fermentas), 0.3 µl of oligonucleotide primer (10 µM), 200 µM dNTPs, 1 × PCR Buffer with $MgCl_2$, and 40 ng of genomic DNA as templates. The amplification was performed in a gradient thermal cycler (Biometra GmbH) with reaction conditions programmed as follows: initial predenaturation at 94°C for 4 min, followed by 44 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min. A final extension was carried out for 7 min at 72°C with a hold temperature of 4°C. PCR products were electrophoresed in 1.5% agarose gels stained with ethidium bromide at constant voltage (3 $V \cdot cm^{-1}$ of gel) until bromophenol blue/loading dye migrated to the other end of the gel. The gel was visualized on a UV--transilluminator and photographed using GeneSnap ver. 7.09 (SynGene) gel documentation system. GeneRuler 100 bp DNA Ladder Plus was used to establish molecular weight of the products. RAPD products (clearly identifiable and repetitive) were scored as present (1) or absent (0) based on the photographs. As a polymorphic profiles considered bands detected in specified genotypes, whereas specific bands was restricted to a particular individual.

Statistical analysis

Statistical analysis for shoot multiplication of regenerants was determined using ANOVA and the significance of differences between mean values was calculated using Duncan's multiple range tests performed at $p < 0.05$.

In RAPD analysis – the similarity coefficient between the studied genotypes was evaluated according to Jaccard's formula [Jaccard 1908]. Cluster analysis was conducted using the UPGMA (unweighted pair-group method with arithmetic mean) distance method implemented in the PAST software [Hammer et al. 2001].

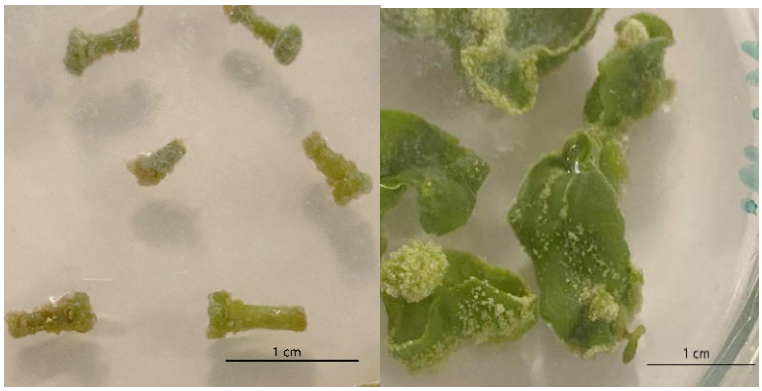
RESULTS AND DISCUSSION

Callus induction and shoot regeneration

The induction of callus tissue in *in vitro* cultures occur especially in the cuts made during explant isolation or by exposing the surface of the explant to contact with a proper medium. Appropriate growth regulators are applied for callus induction, usually mutual proportions of auxins and cytokinins [Bairu et al. 2011, Grafi and Barak 2014, Shah et al. 2015]. According to Singh et al. [2017] and Preethi et al. [2011], callus tissue is a good source of generating genetic variability. Currently, many micropropagation protocols are developed for stevia for various purposes and from different types of explants, such as leaves, nodes, and shoot tips. Somaclonal variation was observed relatively often in different species among regenerants in the case of procedures involving indirect organogenesis [Singh et al. 2017]. In the presented experiment, callus tissue began to appear on both leaf explants and internodes 10 days after culture initiation. Callus was formed mainly at the cut site and gradually covered entire explants. Leaves and internodal explants were completely covered with callus tissue after approximately 4 weeks. Both the structure and color of the callus tissue varied depending on the used explant (Tab. 1, Figs 1–3). Callus on internodal segments was hard, dry, light yellow-green, while the callus on leaf explants was moist, granular, crumbly, and green in color. The whole callus occurring in internodal segments was non-morphogenic. That callus proliferated but did not produce regenerants on the applied medium. Morphogenic callus was obtained from 30% of leaf explants, and it also allowed to obtain regenerants. Singh et al. [2017] and Sikdar et al. [2012] indicated leaf explants as a good source for the production of callus tissue, and the rate of its formation, weight, color and structure depended largely on the combination of various PGRs in medium composition. In turn, El-Zaidy et al. [2014] pointed out that it could also be dependent on the studied genotype. Gunasena and Senarath [2019] observed callus formation on leaf explants and internodes, similarly to our research, and the rate of its formation was also similar. In contrast, the proportion of explants giving response in case of leaf explants obtained by Patel and Shan [2009], Janarthanam et al. [2009], Sharma et al. [2015], Majumder and Rahman [2016], and Masri et al. [2019] ranged from 30% to 100%. Moreover, callus that formed directly on leaf explants on the medium supplemented with BA (1.0–3.0 $mg \cdot dm^{-3}$) and NAA (0.2–2.0 $mg \cdot dm^{-3}$) in the study of Patel and Shan [2009] was not able to regenerate shoots, the process occurred only after passaging the callus to a medium with 2.0 $mg \cdot dm^{-3}$ BA and 0.2 $mg \cdot dm^{-3}$ NAA. For that reason, indirect organogenesis

Table 1. Characterization and evaluation of the morphogenetic potential of callus tissue

Explant source	Structure	Color	Percentage of explant giving response	Number of morphogenic/ non-morphogenic callus	Mean number of shoots per explant
Internodal segments	hard, dry	light green-yellow	100	0/100	0
Leaves	moist, granular, crumbly	green	100	30/70	3.92

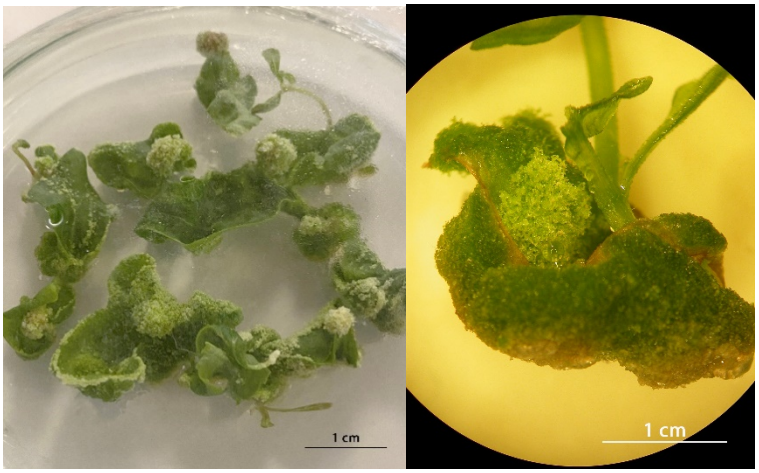


(a) (b)

Fig. 1. Callus on internodal segments (a) and leaves explants (b)

in the *Stevia rebaudiana* Bertoni species involves obtaining callus tissue what lasts 4–5 weeks, followed by passaging onto medium supplemented with an appropriate combination of growth regulators for shoot regeneration, which requires further 4 weeks of culture [Janarthanam et al. 2009, Khalil et al. 2014]. However, in the current experiment, regenerants began to appear on the callus formed on leaf explants after about 2 weeks from the start of the culture. The average number of regenerants obtained in the experiment ranged from 1 to 8, and it was 3.9 on average. Thus, the applied procedure allowed to obtain regenerants in one step. It should be noted that the number of regenerants obtained in the one-step procedure can be comparable to the two-step method, in which the yield obtained by Patel and Shan [2009] ranged from 2.25 to 4.65, 2.4–8.5 in the study of Preethi et al. [2011] and 2.94–8.24 in the work of Masri et al. [2019]. The above values of the analyzed trait were obtained on MS medium supplemented with BA (2.0 mg·dm^{-3}) and one of the auxins (NAA, IAA, 2,4-D). According to Taware et al. [2010], variations in the results may be due to the endogenous growth regulators contents in primary explants, their uptake, type of PGRs used, and their mode of action.

Asthana et al. [2011], Esmaeili et al. [2016], and Shah et al. [2015] reported that BA showed specific callus-inducing properties and somaclonal variation development in regenerants obtained in many species. On the one hand, the use of high concentrations of that growth regulators might reduce the rate and efficiency of multiplication, and on the other hand, it could cause genetic instability of regenerants, especially if they were obtained by indirect regeneration. Thus, BA applied at a high concentration (4 mg·dm^{-3}) may show this direction of action in the proposed one-step regeneration protocol.



(a) (b)

Fig. 2. Callus formation and indirect organogenesis on stevia leaves after 2 weeks of the culture (a), regenerated shoot coming from the callus (b)

Fig. 3. Callus with the highest number of regenerated shoots

Shoot multiplication of regenerants

In that stage explants with nodes were used, which guaranteed genetic stability of the regenerated plants and ensured reproducibility of the results. Since each analyzed regenerant was obtained through callogenesis, it could constitute a separate, unique genotype, and thus show a morphogenetic reaction different from the others. Therefore, a large variation in the number of regenerating shoots, their length and the number of nodes could be expected between regenerants. The data given in Table 2 demonstrated that the highest variation, expressed by the coefficient of variation, was associated with the mean shoot length and it was two times higher as compared to the mean number of nodes per shoot. A high variation of 40% was also observed for mean shoot number. The high value of the coefficient of variation for the number of shoots per explant resulted from a large variability range of the observed values, i.e. from 1.0 to 11.3 shoots per explant, and thus it reflected the genetic diversity of regenerants obtained by indirect organogenesis. Therefore, it could be assumed that if the micropropagation of the established genotype is carried out with the use of nodal segments, the range of variability of the analyzed properties (mean number of shoots per explant, mean shoot length, number of nodes per shoot) should be lower. The analysis of the literature data confirmed that assumption. The range of such a variation in the study of Soliman et al. [2014] was from 3.42 to 9.48, depending on mutual proportions between BA and kinetin applied to the same genotype. Taleie et al. [2012], by testing 24 MS media with different PGR concentrations, obtained from 0.4 to 4.27 shoots per explant. Razak et al. [2014] tested 12 various concentrations of PGRs on multiple shoot induction from nodal segments and obtained values from 2.10 to 7.82. Doliński and Jabłońska [2015] analyzed the effect of BA on the number of shoots and obtained numbers in the range from 4.71 to 11.36 with a coefficient of variation from 23% to 28%, shoot length in the range from 1.44 to 1.99 with a coefficient of variation of 21–28%. On the other hand, on the medium with indole-3-butyric acid (IBA) addition, those authors obtained shoots with a length of 4.19 to 5.41 cm with a coefficient of variation of 24–30%. The number of nodes estimated for that medium ranged from 3.36 to 4.60 with a coefficient of variation of 15–19%. Therefore, the range of variation presented by the above authors resulted from different concentrations of PGRs applied in the media always affecting the same genotype. In our research, the variability of morphogenetic capacity within the analyzed regenerants was caused by the genetic background shaping somaclonal variation. That was confirmed by the comparison of the values obtained for DP and the tested regenerants (Tab. 2, Fig. 4). The average number of shoots obtained for DP was 5.0. Of 30 analyzed regenerants, 17 produced a lower number of shoots than DP, and 9 regenerants had a higher morphogenetic potential. On the other hand, there are examples of high sensitivity of the same genotype to the growth regulators used in different concentrations. Hassanen and Khalil [2013] reported that they obtained 43.9 shoots/explants on the medium with 2 $mg \cdot dm^{-3}$ BA, and only 9.8 shoots/explants on the medium with 0.5 $mg \cdot dm^{-3}$ kinetin, while studying the same genotype. In the presented experiment, the average length of shoots produced by the regenerants was 4.26 cm and the average number of nodes was 6.37 per regenerated shoot. Javed et al. [2018] obtained similar results for shoots length in the range of 2.6–5.5 cm and slightly lower for the mean number of nodes per shoot, in the range of 2.3–4.6. When assessing the morphogenetic capacity of regenerants based on

literature data, one should carefully consider whether they relate to the assessment of the effect of different concentrations of PGRs on the same genotype or the evaluation of different genotypes at a constant concentration of PGRs, or the analysis of the reactions of various genotypes to the applied PGRs and their concentrations.

Table 2. Assessment of the morphogenetic capacity of the obtained regenerants

Number of regenerants	Mean number of shoot	Mean shoot length (cm)	Mean number of nodes per shoot
1	11.3 a*	4.2 ab	5.3 abc
2	6.0 c–f	6.3 a	6.8 abc
3	4.0e–h	6.5 a	7.3 abc
4	7.7a–d	4.7 ab	4.9 abc
5	9.3a–c	6.9 a	6.9 abc
6	5.7 c–f	7.4 a	7.2 abc
7	3.0 f–h	4.2 ab	7.3 abc
8	1.0 h	3.5 ab	5.0 abc
9	9.7 a–b	2.9 ab	5.4 abc
10	5.3 d–g	5.5 ab	7.0 abc
11	1.0 h	7.0 a	9.0 a
12	7.3 b–e	4.9 ab	7.0 abc
13	3.3 f-h	2.3 ab	5.0 abc
14	5.3 d–g	4.0 ab	6.4 abc
15	1.0 h	0.5 b	4.0 c
16	2.7 f–h	2.7 ab	8.3 ab
17	2.0 h	3.5 ab	7.5 abc
18	2.7 f-h	4.2 ab	5.7 abc
19	4.0 f–h	2.5 ab	6.0 abc
20	3.0 f–h	1.8 ab	5.0 abc
21	1.0 h	2.0 ab	5.0 abc
22	2.0 h	4.7 ab	8.0 abc
23	4.3 e–h	6.4 a	7.5 abc
24	8.0 a–d	3.1 ab	6.6 abc
25	9.7 a–b	4.5 ab	5.5 abc
26	3.3 f–h	2.5 ab	4.3 bc
27	3.0 f–h	3.7 ab	5.7 abc
28	4.7 d–g	6.2 a	7.6 abc
29	5.0 d–g	4.0 ab	6.2 abc
30	3.7 e–h	4.6 ab	7.7 abc
DP	5.0 d–g	4.9 ab	6.5 abc
Mean	4.67	4.26	6.37
Coefficient of variation (%)	61.0	40.0	19.5

* Means following the same letter within columns are not significantly different according to Duncan's test ($p < 0.05$)

Fig. 4. Shoot multiplication of regenerants after 8 weeks of the culture
Molecular assays

RAPD analysis

Numerous authors used RAPD marker systems and demonstrated their usefulness in the assessment of genetic variability both *in vivo* and *in vitro* [Chester et al. 2013, Thiyagarajan and Venkatachalum 2015, Sharma et al. 2016]. In turn, Moktaduzzaman and Mahbubur-Rahman [2009], Hassanen and Khalil [2013], and Khan et al. [2016] confirmed genetic stability of plants regenerated using direct organogenesis *in vitro*, even after several passages either for shoot tips, nodal segments, or axillary buds used as explants. For RAPD analysis, DP and the following regenerants were selected: 1, 5, 6, 16, 21, 22, 24, 25, and 30, representing the entire variability of the studied population in terms of the multiplication capacity.

The primers with the sequence listed in Table 3 amplified varying banding patterns. The amplification products after electrophoretic separation for primer RAPD9 are shown in Figure 5. The total number of amplification products was 61, giving a mean score of 6.1 bands per primer. Polymorphic bands were present for all primers and a total of 42 were detected, an average of 4.2 bands per primer. For four primers (Starter1, Starter2, Starter5, Starter9), 12 specific bands were detected. The size of the obtained products ranged between 600 and 4500 bp. The percentage of polymorphism detected with the applied primers was in the range of 37.5–87.5%, giving an average of 70.33% polymorphic sites for all the above-mentioned primers. Polymorphism of stevia regenerants obtained by Sharma et al. [2015] from leaf fragments via callus was higher and amounted to 89.39%.

Table 3. Molecular polymorphism analyzed by RAPD markers

No.	5′-3′ primer sequence	Number of products			Percentage of poly-morphism	Size range (bp)
		total	polymorphic	specific		
RAPD1	CGATTGGACG	8	4	3	50.0	700–2500
RAPD 2	ATGCCGCGAT	6	5	2	83.3	1450–4500
RAPD 3	TAGCGCCAAT	8	3	0	37.5	900–3000
RAPD 4	TTAAGGCCT	4	3	0	75.0	800–2500
RAPD 5	CACCCGATGA	5	4	3	80.0	1500–3000
RAPD 6	ATGTGCCGTA	6	5	0	83.3	600–3000
RAPD 7	TGGCGCAATA	6	4	0	66.7	800–3000
RAPD 8	ACAACGCCTC	5	3	0	60.0	700–3000
RAPD 9	GACCGCTTTG	8	7	4	87.5	1000–3350
RAPD 10	CCTCCTCATC	5	4	0	80.0	1200–2500
Average/primer		6.1	4.2	1,2	–	–
Total		61	42	12	70.33	600–4500

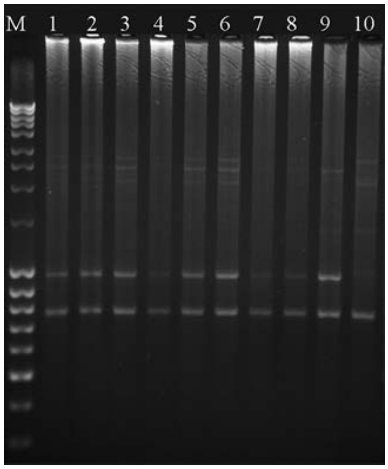


Fig. 5. RAPD profile of DP-1 and regenerants 2-10 (1, 5, 6, 16, 21, 22, 24, 25, 30 respectively) for primer RAPD 9

RAPD markers are often used in plant genetic diversity studies, including those obtained *in vitro*, both to confirm the genetic homogeneity of regenerants and to detect their polymorphisms. Repetitive band patterns were obtained in the case of regenerants obtained from nodal segments [Thiyagarajan and Venkatachalam 2012, Khan et al. 2016, Singh et al. 2017] and shoot tips [Modi et al. 2012], confirming their genetic uniformity. RAPD analysis invariably showed genetic fidelity when stevia cultures were derived

from explants containing primary meristematic tissues and when callus-limiting culture conditions were applied, while regenerants from indirect organogenesis exhibited variability visible in at least part of RAPD primers [Moktaduzzaman and Mahbubur-Rahman 2009, Sharma et al. 2015]. In our study, regenerants selected for molecular analysis, characterized by different morphogenetic potential, showed similarity ranging from 0.767 to 0.297 when 10 RAPD primers were used (Tab. 4, Fig. 6). Genetic similarity between the regenerated plants and the DP based on RAPD markers indicated that all regenerants showed a varying degree of genetic variation from DP. For regenerants 5, 22, 24, 25, 30, the genetic similarity coefficient was less than 50%, for regenerant 1, it was 74% and it slightly exceeded 50% for the remaining ones (6, 16, 21). Regenerants obtained in our experiment were more differentiated from DP than those obtained by Moktaduzzaman and Mahbubur-Rahman [2009], who reported, on the basis of the estimation of genetic similarity coefficient and RAPD band-sharing data analysis that regenerated plants were 100% similar to DP and the other were in 71%, 57% and 14% similar. In both cases, the regenerants were obtained through the callus stage, which, according to Miguel and Marum [2011], is considered subject to very intensive cell divisions, which poses a risk of changes in the genome. A broad spectrum of species regenerated via the callus stage and assessed using the RAPD method was presented in the study of Bairu et al. [2011], who indicated that the *in vitro* plant regeneration process could be influenced by such factors as a type of starting material, regeneration method, genotype, type and concentration of PGRs.

Table 4. Genetic similarity coefficient of 9 regenerated plants and DP, based on RAPD markers calculated using the Jaccard coefficient

Genotype	DP	1	5	6	16	21	22	24	25	30
DP	1.000	0.742	0.457	0.515	0.555	0.514	0.454	0.405	0.333	0.333
1		1.000	0.485	0.600	0.588	0.500	0.437	0.470	0.389	0.350
5			1.000	0.552	0.545	0.600	0.483	0.424	0.516	0.417
6				1.000	0.667	0.516	0.615	0.533	0.437	0.316
16					1.000	0.767	0.454	0.625	0.529	0.474
21						1.000	0.452	0.531	0.581	0.472
22							1.000	0.467	0.375	0.297
24								1.000	0.500	0.444
25									1.000	0.486
30										1.000

The results of the presented work indicated the presence of polymorphisms between the studied stevia regenerants. Regenerants were obtained from the established DP genotype, therefore their genetic variability was caused by somaclonal variation.

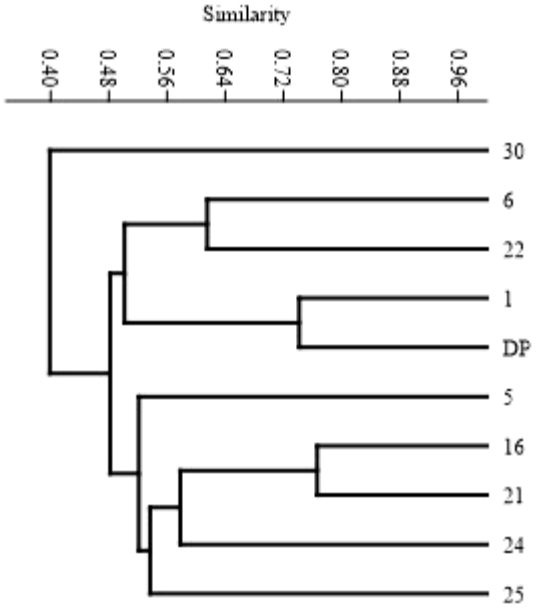


Fig. 6. UPGMA dendrogram of DP and regenerants (1, 5, 6, 16, 21, 22, 24, 25, 30) based on RAPD marker analysis

The applied set of primers revealed genetic distinctiveness of regenerant 30 (Fig. 6) to the other studied genotypes, which formed two separate clusters. The first one within which the smallest variation was observed between regenerants 16 and 21, and to which regenerants 24, 25 and 5 were sequentially added. Two subclusters were observed within the second cluster. The first one comprised DP and regenerant 1 and the second one included regenerants 6 and 22. Therefore, RAPD analysis confirmed that the plant regeneration protocol proposed in the study allowed to obtain genetic variation between the obtained regenerants, which, after further analysis at the phenotype level, could become a potential source of new commercial cultivars.

CONCLUSIONS

1. The proposed *in vitro* regeneration protocol by indirect organogenesis induces somaclonal variation manifested by variability observed at the genotypic and phenotypic levels in the regenerants.

2. Obtaining a genetically diversified population of regenerants creates an opportunity for effective selection of new genotypes with the possibility of their application as future commercial cultivars.

3. The application of a nutrient medium on which simultaneously callus tissue formation and plant regeneration occurs saves time and lowers the cost of breeding.

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