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FAST DIRECT REGENERATION OF PLANTS FROM NODAL EXPLANTS OF *Stevia rebaudiana* Bert.

Romuald Doliński, Krzysztof Kowalczyk✉

Insitute of Plant Genetics, Breeding and Biotechnology, University of Life Sciences in Lublin, Akademicka 15, 20-950 Lublin, Poland

ABSTRACT

In the preceding research, stevia has been typically cloned *in vitro* using two media, on which the shoots were formed (3–6 weeks), and on the other they were rooted (3–5 weeks). This study aimed at finding the possibility for rapid stevia propagation from large nodal explants using the MS basal medium [Murashige and Skoog 1962], with low auxin concentrations (0.5, 1 and 2 mg·dm^{-3}). The plants were obtained as soon as after three weeks. The best results were obtained from media with various concentrations of the indole-3-acetic acid (IAA) and the highest concentration of phenylacetic acid (PAA). Plants were formed by 83.9–86.0% of explants, they had high weight (234–253 mg), two shoots measuring 2.07–2.37 cm and 5.8–8.3 roots measuring 1.00–1.24 cm. Mean plant weight was the lowest on media with indole-3-butyric acid (IBA) (185–192 mg). Both explant buds formed single shoots, but their development was typically uneven. The differences in the length and weight of shoots were the lowest on media with IAA and at lower PAA concentrations. Plants from the media with IAA and the control medium were distinguished by higher number of nodes. The percentage share of shoots in the total plant weight was the highest on media with PAA (62.1–62.7%), and the lowest at higher concentrations of α-naphthaleneacetic acid (NAA) (47.9 and 48.9%). Parts of explants immersed in media developed callus, and the highest amounts of this tissue were found in the media with NAA. 92.3% of plants survived the acclimatization. The applied procedure may be used for rapid *in vitro* cloning of selected stevia genotypes. The use of one medium enables reduction of seedling production costs. Moreover, cyclical cloning and extending the production scale is possible.

Key words: one-step micropropagation, nodal explants, low concentrations auxins

INTRODUCTION

Stevia rebaudiana is a perennial plant of *Asteraceae* family, native to Paraguay. Guarani Indians have been using it for centuries to sweeten herbal infusions and chewing. Its world-wide career began at the end of the 19th century when the Swiss botanist Bertoni described its botanical properties and gave the scientific name [Soejarto 2002]. Plenty of studies has already been done on it. It was found that the sweet taste was due to specific glycosides, which may represent more than 10% of leaves dry mass [Brandle et al. 1998, Soejarto 2002]. The dry leaves are 20–30 times sweeter than sucrose, and the mixture of crystalline glycosides as much as 300 times. It is important that sweet glycosides stimulate insulin release in humans, which lowers blood pressure. They do not provide energy because the digestive tract does not digest them. They are decomposed by large intestine bacteria, but they consume the released glucose [Genus et al. 2007]. Gastronomy and food industry use stevia leaves, extracts and pure glycosides instead of sucrose [Sawi-

✉ krzysztof.kowalczyk@up.lublin.pl

ta et al. 2004, Lemus-Mondaca et al. 2012]. Markets around the world increasingly often sell stevia sweeteners, calorie-free beverages and ready-made low calorie foods. From the standpoint of medicine, stevia is a good source of sweets for obese, diabetic and hypertensive people. It can be used in case of tooth decay, kidney diseases and inflammatory bowel disease [Grenby 1991, Huxtable 2002].

Stevia is a thermophilic plant of the short day, but can be cultivated under different conditions. In tropical and subtropical climates, it is a perennial, and in a moderate climate it is an annual plant [Brandle et al. 1998, Ramesh et al. 2006]. The demand for stevia leaves grows fast but there are great difficulties in setting up a plantation. Traditionally, it is propagated from seeds and stem cuttings. Seeds germinate poorly [Carneiro et al. 1997]. The highly variable plantations are established, which inter alia concerns plant sweetness and glycosides composition [Tateo et al. 1998]. Vegetative reproduction is laborious and inefficient. In the study by [Khalil et al. 2014], 11.1% stem cuttings were rooted in the soil without hormones, and 33.3% in case of IBA application at 1000 ppm.

Efficient cloning of selected stevia plants is possible in *in vitro* cultures. This plants can be regenerated on larger scale by embryogenesis or organogenesis. The best way to produce seedlings is the use of explants with buds, the development of shoots from the meristems does not generate somaclonal variability. Studies demonstrated that stevia can be multiplied *in vitro* from apical [e.g. Anbazhagan et al. 2010, Das et al. 2011] and nodal explants [e.g. Verma et al. 2011, Razak et al. 2014]. Numerous studies have been found in the available literature concerning two-stage micropropagation of stevia, which lasted for 6–10 weeks and required the use of two media [e.g. Razak et al. 2014, Yücesan et al. 2016]. However, only little information could be found on the studies in which plants were developed on a single medium, in the period of 3−4 weeks. In such studies, the explants had low weight and small assimilation surface, various media and growth regulator were used. Taleje et al. [2012] placed small nodal explants on MS medium with agar (0.8%), supplemented with sucrose (30 g·dm^{-3}) and two concentrations (1 and 2 mg·dm^{-3}) of 6- benzylaminopurine (BAP) and kinetin (KIN) and IAA, IBA and NAA auxins (0.5 and 1 mg·dm^{-3}). Peixe et al. [2015] used 3 media: MS, WPM [Lloyd and McCown 1980] and DKW [Driver and Kuniyuki 1984], enriched them with sucrose (20 g·dm^{-3}), IBA at concentrations 0.1 and 0.5 mg·dm^{-3} and anti-gibberellin paclobutrazol (PBZ) at concentration 0.5 mg·dm^{-3}, solidified with agar (0.7%). Rosales et al. [2018] used liquid, semi solid MS medium enriched with 30 g·dm^{-3} sucrose, 2 mg·dm^{-3} calcium pantothenate (CaPa) and 0.5 mg·dm^{-3} gibberellic acid (GA_3). They compared the action of 3 temporary immersion systems, differing in the shape and capacity of the vessels. All the explant had 10 ml of medium and the same immersion time (2 min).

The main objective of the study has been to assess rapid *in vitro* stevia cloning using large nodal explants and agar solidified MS medium with low concentrations of four auxins.

MATERIAL AND METHODS

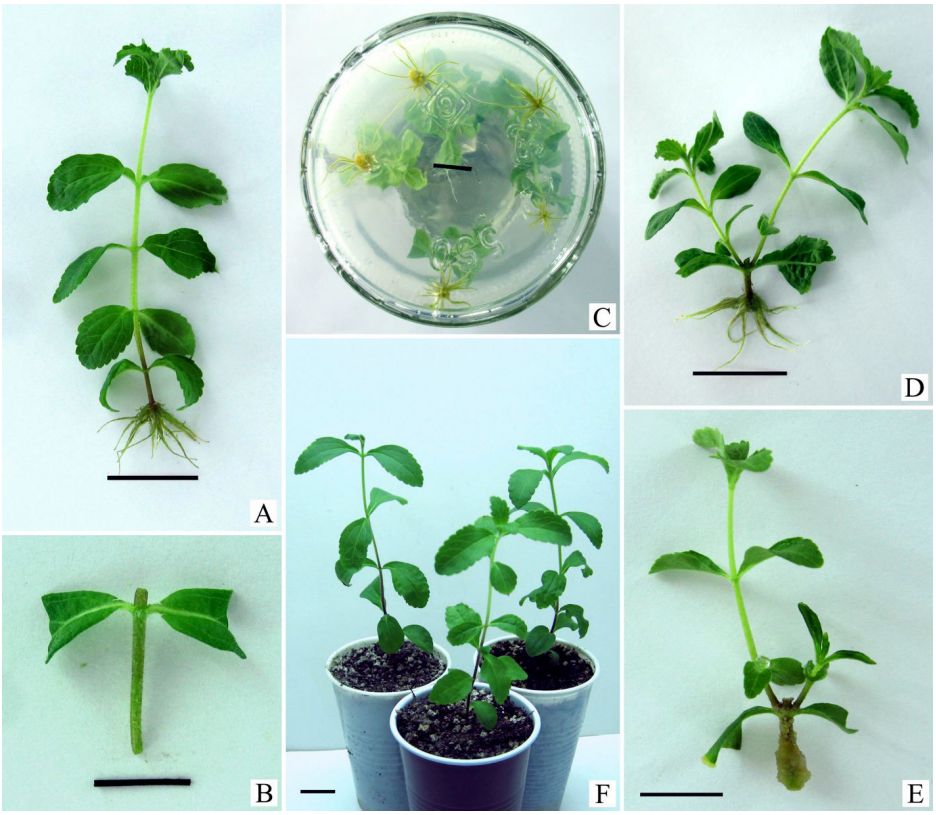
The study was carried out on one stevia genotype. The basic stevia plant was selected from population obtained from seeds purchased in a gardening shop, originating from Vilmorin Garden, a commercial company (Poland). The cultures were established in glass jars of 0.4 dm^3 capacity, MS basal medium enriched with 30 g·dm^{-3} sucrose and supplemented with auxins: IAA, IBA, NAA and PAA, was used. The vessels contained 25 ml of agar-solidified media (0.8%), five explants were perpendicularly placed in each of them. They were sealed with aluminum foil and transferred to a growth room with a temperature of 25°C, white light at 30–40 μ mol·s^{-1}m^{-2} and photoperiod of 16/8 h. Apical explants of a length of 1.5–2 cm were collected from *in vitro* produced plants. They were placed in MS medium supplemented with 0.5 mg·dm^{-3} IAA. The plants were obtained after 4 weeks, and large apical and nodal explants were isolated from them (Phot. 1A and 1B). The nodal explant consisted of a shoot node composed of two leaves and angular buds, and two sections of the stem (1.5–2.0 and 0.3–0.5 cm). The surface of leaf blades was reduced by about 50% so that the cutting edges did not come into contact with the media. Apical explants were placed in separated vessels and use for production material to start a next experiment. The control medium did not contain hormones, while the auxins at concentrations of

0.5, 1 and 2 mg·dm^{-3} were added to the others. The experimental combination consisted of 15 vessels. They were randomly placed in the growth room (on three shelf). After three weeks, plants and shoots without roots were counted for each medium. The qantity of callus, number and length of roots, shoot length and number of nodes, as well as mass of plants, shoots and explants were evaluated for 30 consecutive plants. Part of the plants (13 × 5) was planted to a sterile substrate consisting of garden soil, sand and perlite (3 : 1 : 1). The plants were covered with plastic containers and left in a growth room for 7 days. Then the covers were removed and pots were transferred to an unheated greenhouse. Hardening results were evaluated after three weeks.

The subsequent stages of stevia micropropagation were documented photographically. All experimental data were arranged in complete randomized design and analysis of variance was performed using STATISTICA program, version 13.1. [DELL]. The significance of the differences between the mean values was estimated using Tukey's test.

RESULTS

In this study, shoots and roots developed directly from the explants. The beginning of shoots development was observed after a few days, the roots appeared 3–4 days later. The explants cut from upper part of shoots started organogenesis faster than those from lower parts. During the evaluation of the cloning results, the explants with shoots without roots were found in all media (Phot. 1E). The percentage of explants forming plants was the highest in IAA and



Phot. 1. Micropropagation of stevia: A – sterile plant, B – node explant, C – roots of regenerated plants, D – plant developed from node explant, E – node explant with shoots without roots, F – well-rooted, hardened stevia cuttings. Scale = 1 cm for B and C, 2 cm for D, E and F

Table 1. Estimation of *Stevia rebaudiana* plants regenerated from nodal explants

PGR mg·dm^{-3}	Shoot with roots %	Plant weight		Explant weight		Shoots weight		Roots weight		Shoot lenght		Roots number		Root length	
		ȳ mg	V %	ȳ mg	V %	ȳ mg	V %	ȳ mg	V %	ȳ mg	V %	ȳ Nu.	V %	ȳ cm	V %
0	62.5	178	17.8	39.2	20.9	96.6	23.8	42.2	34.9	2.05	21.6	5.10	16.6	1.18	20.0
IAA															
0.5	86.0	253	17.9	58.7	24.7	151.6	23.8	42.5	23.1	2.22	18.9	5.82	34.9	1.15	12.2
1	85.1	248	20.4	55.7	20.1	146.7	27.8	46.0	19.0	2.37	24.6	7.43	24.9	1.05	15.6
2	84.2	244	21.3	54.1	27.2	143.3	23.5	45.1	30.6	2.28	24.4	8.30	27.6	1.00	22.6
IBA															
0.5	85.7	192	21.6	41.8	23.0	106.8	28.6	43.1	13.4	1.70	26.0	5.37	37.1	1.04	13.4
1	84.4	187	17.8	41.7	21.1	103.2	25.7	42.5	22.1	1.83	21.2	7.30	24.4	0.87	14.6
2	84.0	185	16.8	42.1	15.6	102.6	23.3	40.5	29.3	1.69	18.3	8.40	30.4	0.86	20.7
NAA															
0.5	78.0	278	20.8	67.6	26.7	147.2	34.9	63.1	31.4	2.48	18.6	8.50	29.4	1.16	21.2
1	73.6	279	17.2	62.1	27.9	135.8	21.5	80.7	27.0	2.54	22.6	9.53	31.1	1.10	16.0
2	74.2	275	21.7	64.4	28.9	132.0	28.0	79.0	33.2	2.36	17.8	10.20	26.7	1.19	18.4
PAA															
0.5	80.0	197	17.4	47.0	15.5	122.4	25.7	27.7	18.4	2.03	20.7	4.93	22.5	1.10	11.3
1	82.5	212	20.1	50.0	20.7	132.3	28.9	28.7	33.6	2.03	30.7	5.70	23.6	1.25	13.7
2	83.9	234	19.2	47.9	17.6	146.5	21.6	39.1	27.6	2.07	25.3	5.80	21.4	1.24	14.5
$LSD_{0.05}$	–	24	–	6.0	–	18.1	–	7.5	–	0.27	–	1.03	–	0.08	–

PGR – plant growth regulator; ȳ – mean; V – variability coefficient

Table 2. Characteristic of shoots regenerated from nodal explants and callus quantity

PGR mg·dm^{-3}	Shoot weight				Shoot length				Node number					Callus quantity	
	ȳ		V		ȳ		V		ȳ		V		ȳ	ȳ	V
	mg		%		cm		%		Nu.		%		Nu.	1–5°	%
	A	B	A	B	A	B	A	B	A	B	A	B	A + B		
0	62.0	34 .6	32.0	37.1	2.44	1.66	27.9	32.0	3.53	2.93	16.2	19.9	6.47	0.87	98.9
IAA															
0.5	94.5	57.1	26.6	43.2	2.64	1.86	25.4	40.5	3.60	2.87	13.8	28.6	6.47	2.23	38.5
1	96.9	49.8	26.8	46.6	2.80	1.93	20.3	42.3	3.53	2.93	14.4	25.2	6.46	2.07	33.2
2	101.6	41.7	27.7	70.2	3.15	1.41	18.8	75.9	3.73	2.30	12.0	42.9	6.03	2.10	32.0
IBA															
0.5	78.0	28.8	31.2	72.9	2.32	1.05	20.6	79.9	3.33	1.97	14.4	55.8	5.30	1.20	55.4
1	78.0	25.2	36.8	86.6	2.60	1.01	25.3	80.2	3.37	1.90	16.5	59.2	5.27	1.60	35.2
2	73.1	29.5	29.5	68.6	2.43	0.96	27.8	64.7	3.53	1.87	14.3	50.2	5.40	2.37	34.1
NAA															
0.5	108.5	38.7	32.5	80.2	3.64	1.29	23.9	79.2	3.50	1.77	14.5	46.2	5.27	3.40	23.9
1	104.0	31.8	32.3	64.2	3.66	1.44	25.3	65.4	3.63	1.93	13.5	44.9	5.56	3.63	19.8
2	101.6	30.4	32.2	86.0	3.56	1.15	29.2	85.9	3.57	1.80	14.3	44.3	5.37	4.10	18.5
PAA															
0.5	72.1	50.3	30.4	28.9	2.28	1.76	25.5	24.0	3.07	2.83	11.9	23.4	5.90	1.07	77.3
1	82.4	50.4	41.2	55.8	2.48	1.60	30.4	46.6	3.27	2.57	19.6	31.8	5.84	1.63	36.8
2	107.0	39.5	34.7	55.5	2.88	1.31	25.3	57.0	3.33	2.13	14.4	40.3	5.46	2.07	34.4
$LSD_{0.05}$	14.1	12.0	–	–	0.34	0.41	–	–	0.21	0.43	–	–	0.44	0.38	–
$LSD_{0.05}$	13.1		–		0.38		–		0.35		–		–	–	–

IBA media (84.0–86.0), the smallest (62.5) in the control. In PAA media, the share of explants forming plant increased with hormone concentrations, while other auxins were most active at the lowest concentration. The highest average weight (253–279 mg) was found for plants obtained in NAA media and at 0.5 mg·dm^{-3} IAA. The plant weight was low (178–197 mg) on IBA, control medium and 0.5 mg·dm^{-3} PAA medium. In combinations with PAA, plant weight increased with auxin concentrations (Tab. 1). The phenotypic variability of this feature ranged from 16.8 to 21.7%. The plants consisted of mother explant, roots and two shoots (Phot. 1D). The explants "recovered" from plants obtained from IBA and control media had an average weight similar to that used for cultures establishing (41.2 mg). In other media, the mass of these plant parts increased, most strongly 62.1–67.6 mg) in case of NAA application. Two shoots had the lowest weight (96.6–106.8 mg) in IBA and control media, the highest (143.3–151.6 mg) in IAA and 0.5 mg·dm^{-3} NAA. The longest shoots were found for plants obtained on NAA medium (2.36–2.54 cm), the shortest in IBA combinations (1.69–1.83 cm). Large differences were found in root systems structure. The highest average number (9.53–10.2) and weight (79.0–80.7 mg) of roots were noted for the plants obtained with 1 and 2 mg·dm^{-3} NAA. The plants with a small number (4.93–5.80) and weight (27.7–39.1 mg) of roots were obtained on PAA media. The number of roots increased with auksins concentrations. The shortest roots developed at higher concentrations of IBA, the longest at low levels of PAA. The variability was higher for such features as weight and length of shoots and number of roots (16.6–34.9%) than for explants weight and roots length (11.3–28.9%).

In all media, both buds of the explants formed single shoots, but usually one of them developed faster and dominated after three weeks (Phot. 1D, Tab. 2). Large shoots had the highest average weight (94.5–108.5 mg) in combinations with NAA and IBA and at 2 mg·dm^{-3} PAA, the lowest in the control (62.0 mg). The length of these shoots was the highest (3.56–3.66 cm) in NAA media, the lowest (2.28–2.60 cm) in combinations with IBA, control and at 0.5 and 1 mg·dm^{-3} PAA. Smaller shoots developed the best (41.7–57.1 mg) on IAA media and at 0.5 and 1 mg·dm^{-3} PAA, the poorest in combinations with IBA and NAA. They had the highest length (1.60–1.83 cm) in the control medium and at 0.5 and 1 mg·dm^{-3} IAA and PAA. Larger shoots had the highest number of nodes (3.73) at 2 mg·dm^{-3} IAA, the least (3.07) at 0.5 mg·dm^{-3} PAA. Smaller shoots had fewer nodes in IBA and NAA media than in IAA, PAA and control combinations. The variability of the features was lower in dominant shoots and highest for smaller shots. The average number of nodes per plant was the highest in the control combination and at 0.5 and 1 mg·dm^{-3} IAA (6.46–6.47) (Tab. 2).

Callus developed on the parts of stems immersed in media (Tab. 2). It was the least abundant in the control combinations (0.84°), the highest in case of NAA (3.4–4.1°). In the control medium, small amounts of callus emerged only on the explants not forming the roots. The variability of that feature was lowest for NAA media (18.5–23.9%), high at low concentrations of IBA and PAA (54.4–77.3%) and the highest in control medium (98.9%).

In all media, plants with relatively high shoot weight and small roots weight were developed (Fig. 1). The highest proportion of shoots (62.1–62.7%) was observed in case of plants from media containing PAA, the lowest (47.9 and 48.9%) obtained at higher concentrations of NAA. Small shoots in plants from the media containing PAA constituted much higher biomass share than in those developing in the presence of NAA.

In this study, 92.3% of plants survived the hardening. The plant mortality was occurred the first acclimatization stage, in growth room.

DISCUSSION

In the preceding study, the effects of *in vitro* stevia cloning were assayed typically using the percentage share of explants forming shoots and roots, and the average number and length of these organs. Certain papers provided mean value of these traits calculated for explants placed onto media [e.g. Abnbazhagan et al. 2010, Taleje et al. 2012, Razak et al. 2014]. Some authors provided the weight of shoot and the number of internodes [Taleje et al. 2012].

In the discussed study, in the period of 3 weeks large explants obtained from one mother plant formed 3–4 two-shoot plants on the MS medium with low

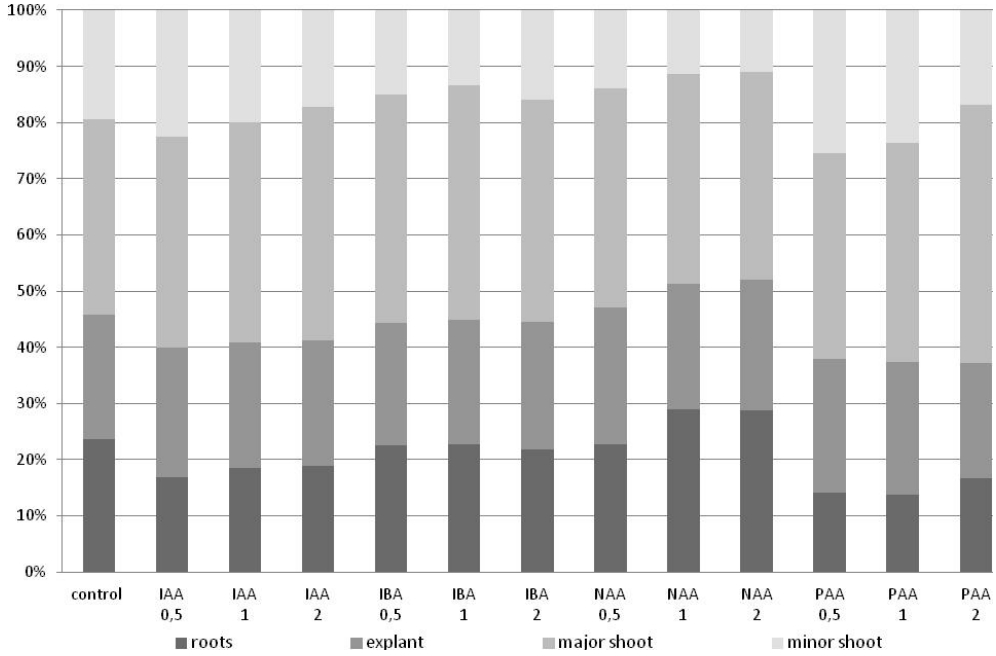


Fig. 1. Percentage of respective parts (organs) in biomas of *Stevia rebaudiana*

auxin concentrations, and from the top of the shoot a single-shoot plant was formed, constituting the material for the subsequent cloning cycle. The best results were obtained on the medium containing 0.5 mg·dm^{-3} IAA. 86% of explants formed plants with 2 shoots each with mean length of 2.22 cm and 5.82 roots measuring 1.15 cm. The plant weight was 253 mg. Good results were also obtained for other media with IAA and at the highest PAA concentration.

In the preceding studies, nodal explants with lower weight and small assimilation surface were used for the one-stage *in vitro* stevia cloning. In the study of Taleje et al. [2012], among 24 combinations of two BAP and KIN concentrations with two concentrations of IAA, IBA and NAA (1–2 and 0.5–1 mg·dm^{-3}), only two were suitable for rapid cloning. On the MS medium with BAP and IAA at concentrations of 2 and 1 mg·dm^{-3}, in the period of 3 weeks, the explant formed 4.27 shoots measuring 8.51 cm and fresh weight of 620 mg, and 13.25 roots measuring 4.04 cm. Similar results were produced by the medium with lower BAP concentration. In the study of Peixe et al. [2015] roots did not develop on the MS, DKW and WPM media without IBA. The best effect was demonstrated by the DKW medium with IBA and PBZ in concentrations 0.5 mg·dm^{-3} where in the period of 30 days 90% explants produced plants. PBZ enhanced rooting, reduced shoot and internode length, but it did not influence their numbers. In the study of Rosales et al. [2018], the effect of liquid, half strength MS medium with 2 mg·dm^{-3} CaPa and 0.5 mg·dm^{-3} GA_3, depended on the immersion system and on the stevia cultivar. The longest shoots were obtained in the BIT system vessels, the shortest in the RITA system (Morita II 11.65 and 2.85 cm, Silvestre 13.50 and 5.50 cm). In the period of 30 days, Morita II developed seedlings possessing 8 shoot in the BIT system, while in the SETIS system they possessed 3 shoots. The Silvestre explant produced 2 shoots in both systems. All seedlings were characterized by weak shoots and small roots.

In two-stage cloning nodal explants were placed onto MS medium solidified with agar, cytokinins were used separately, jointly and together with auxins. In the study of Rafiq et al. [2007], the best effect was obtained by the medium with BAP at 2 mg·dm^{-3} concentration, in the period of 3 weeks 82% of explants

produced shoots, with the average number of 5.25 and length 3.34 cm. Verma et al. [2011] determined the optimum action of the MS medium with BAP and KIN at the concentration of 0.5 mg·dm^{-3}. In the period of 4 weeks explant developed 17.5 shoots measuring 2.5 cm. In the experiment of Mathur and Begum [2015] the best effect was obtained by the MS medium with BAP and KIN in concentrations 2 and 0.5 mg·dm^{-3}. Within the period of 3 weeks 90% of explants produced shoots, the mean number of shoots was 3.42 and the length 5.7 cm. Lata et al. [2013] obtained high numbers of shoots using low thidiazuron (TDZ) concentrations. In the period of 4 weeks on MS medium with 1 μM TDZ, shoots were developed by 96% explants, with the mean number of 60.31 and length 6.24 cm.

In the second stage, media with low auxin concentrations were used. Larger shoots were usually rooted, rejecting the small ones. For instance, Ahmed et al. [2007] rooted shoots longer than 2 cm, while Lata et al. [2013] used the shoots longer than 5 cm. Some researchers rooted all shoots [e.g. Anbazhagan et al. 2010; Verma et al. 2011]. Verma at al. [2011] placed small stevia shoots in MS medium with low concentrations of GA_3. They grew in size in three weeks and were suitable for rapid rooting. In the study of Rafiq et al. [2007] NAA had better effect than IBA. In the period of 4 weeks on MS medium with NAA at concentration of 0.5 mg·dm^{-3}, 81% of shoots rooted, and the plants possessed 6.2 roots. In the research of Anbazhagan et al. [2010], the best effect was demonstrated by the N_6 medium containing 1 mg·dm^{-3} IAA, in the period of 5 weeks 87% of shoots rooted, plant had 11.8 roots on average measuring 4.8 cm. Yücesan et al. [2016] determined stronger effect of IAA than IBA and NAA. In the period of 3 weeks on MS medium with IAA at concentration of 0.25 mg·dm^{-3}, 100% of shoots rooted, and the plants possessed 8.1 roots. Lata et al. [2013] demonstrated that application of very low auxin concentrations should be avoided (0.5–2.5 μM), as better results were produced by MS medium without hormones.

In this study, both buds of explants formed single shoots, but in most cases they had uneven development. After three weeks, differences in the size and weight of two shoots were lowest in the media containing IAA and PAA. In the preliminary study, plants with equally sized shoots developed more rapidly and constituted better seedlings after acclimatization.

The obtained stevia plants differed in terms of the number of nodes on the shoots, it was highest on the control medium and media with IAA. This was not important in single-stage *in vitro* cloning, as only 1–3 large explants could be obtained from a single plant. In the case of rapid stevia cloning, plants developed from the shoot apexes must constitute the source of explants for the establishment of subsequent cloning cycles. Such explants developed single shoot plants in the period of three weeks, which possessed longer internodes (Phot. 1A).

In the preceding study utilizing two-stage cloning, single shoot plants were obtained from the rooted shoots, while with single-stage cloning the number of shoots was higher. The published studies provided mean numbers and lengths of shoots, however, their evenness has not been considered. On the best medium, Taleje et al. [2012] obtained stevia plants possessed 4.27 shoots, capable of providing 12.25 small explants fit for establishment of the subsequent cloning cycle. In the experiment of Peixe et al. [2016], two-shoot plants from the DKW medium supplemented with IBA were capable of producing 3–4 nodal explants. By using immersion systems, Rosales et al. [2018] obtained plants with a greater number of shoots, yet with thin stems and small leaves, unfit for explant isolation.

During the present study, I have considered the possibilities of expanding the scale of seedling production, thus attempts to harden off plants devoid of the shoot apex. Their results were positive, as plants deprived of one of two shoot apexes acclimatized very well. Development of the callus on the fragments of explants immersed in the media constituted an unfavorable phenomenon of the discussed study. In the period of 3 weeks its highest amounts were formed on media with NAA, with the lowest in the presence of PAA.

In the study of Taleje et al. [2012], the callus developed in the media with KIN. At the KIN concentration of 2 mg·dm^{-3} plants incapable of rapid acclimatization were formed, with low number of short shoots and roots. In the study of Rafiq et al. [2007] its highest amounts were found when BAP was used at concentrations of 1 and 2 mg·dm^{-3}. Peixe et al. [2015] were unable to determine the development of callus in WPM and DKW media, but it developed in MS medium. Its amount increased with the use of PBZ. In two-stage cloning, callus accompanied shoot prop-

agation, and it appeared less frequently during their rooting stage. Yücesan et al. [2016] determined intensive development of this tissue in higher concentrations of KIN and BAP (1–2 mg·dm^{-3}). Ahmed et al. [2007] observed of callus development at the shoot rooting stage. The lowest amount of the tissue formed in MS medium with IAA, the highest in higher NAA concentrations (0.5 and 1 mg·dm^{-3}).

In this study, within the period of 3 weeks, the majority of media developed plants with high share of shoots in the total biomass. It was assumed that this is a favorable phenomenon in rapid stevia cloning, as the greater assimilation potential enables a more rapid root development. The highest share of shoots in the total biomass distinguished plants from media with PAA (62.1–62.7%), the lowest from media with NAA (47.9–48.9%) – Fig. 1.

92.3% of plants survived the four-week acclimatization. This result was influenced by a variety of factors. The hardened off plants had relatively high assimilation potential. The roots were relatively short and weakly branched, which increased their resistance to damage during transplanting the plants to the substrate. The soil mix retained water well. Prior to transfer to the greenhouse, the plants grew in the growth chamber for 7 days.

In the previous studies carried out on stevia, the survival rate of hardened off plants typically ranged from 70–90% [e.g. Ahmed et al. 2007, Das et al. 2011, Mathur and Begun 2015]. Some studies described far superior results [e.g. Verma et al. 2012, Taleie et al. 2012] or decidedly inferior [e.g. Razak et al. 2014, Rosales et al. 2018]. In the study of Verma et al. [2011] 94.8% of plants survived hardening off. According to those authors, the use of two-stage acclimatization had a positive impact on the result. The plants were transferred from media to vessels with composted peat moisturized with dissolved MS medium (1/4) and were maintained for 2 weeks in a growth chamber. Subsequently, they were transplanted to a mixture of sand, soil and vermiculite (2 : 1 : 1) and were hardened off for the period of 6 weeks in a greenhouse. The highest percentage of plants (97%) survived hardening off in the study of Taleie et al. [2012], and according to these authors this fact was primarily determined by the large amount of carbohydrates. The plants were characterized by high shoot weight and well developed roots. In the study of Rosales et al. [2018], hardening off lasting for one month was survived by 44% of plants of the Morita II cultivar produced in the BIT immersion system and 48% of plants originating from the SETIS system, and the plants of the Silvestre cultivar hardened off poorer still (only 10 and 15% survived). All acclimatized plants had weak shoots and small, inefficient roots, yet Morita II produced the highest number of shoots. The study of Razak et al. [2014] demonstrated the high impact of soil mixes physical properties on the course of hardening off. A 9-week acclimatization was survived by 83.3% of plants placed into a substrate with the best water retention and good aeration. All plants died in the substrate containing high amount of sand and with low water retention. 33.3% of plants survived in the soil mixture obtained from two substrates (1 : 1).

CONCLUSIONS

The study has shown that stevia can be rapidly cloned *in vitro* by using large nodal explants and MS medium solidified with agar and supplemented with low auxin concentrations. Two-shoot plants were obtained as soon as after three weeks. The best results were obtained from media with various concentrations of IAA and highest concentration of PAA. Plants were formed by 83.9–86.0% of explants, they had mean weight 234−253 mg, two shoots with mean length of 2.07–2.37 cm and 5.8–8.3 roots measuring 1.00–1.24 cm. The use of one medium for seedling production can reduce cloning costs. Single-shoot plants produced from apical explants should constitute the source of large nodal explants. Seedling production can be carried out in a cyclical manner. The scale of production can be expanded by producing additional output material from apical explants cut off from better developed plant shoots of plants used for acclimatization.

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