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Assessment of initial material for stevia selection (*Stevia rebaudiana* B.)

Ahmed Mehmed*, Stanimir Enchev and Stanislav Bozhimirov

Agricultural Academy, Agricultural Institute, 9700 Shumen, Bulgaria
**Corresponding author:* ahmed_dm82@abv.bg

Abstract

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The leaves of South American plant – Stevia *(Stevia rebaudiana* B.*)* contain low-calorie but highly effective glycosides (stevioside and rebaudiside) that are extracted as a commercial product, which is 300 to 320 times sweeter than sugar and can be safely used by diabetics. The enhanced interest in stevia cultivation *(Stevia rebaudiana* Bertoni) as a source for natural, non-calorific sweeteners requires constant enrichment of the gene pool of initial material for the selection. Propagation of stevia is carried out by seeds, vegetative and *in vitro* methods. Seed germination is usually very poor, and vegetative propagation by cutting the stem requires large stocks of stems, and therefore this method is limited. An alternative approach to successfully replicate and maintain stevia is to use in vitro methods.

The soil and climatic conditions in Bulgaria also allow the successful cultivation of *S. rebaudiana* B.*,* but due to its high sensitivity to low temperatures, stevia in Bulgaria is only grown as an annual plant.

The current study was carried out in 2017 in Agricultural Institute – Shumen (Bulgaria). The used selection material in this study is seedlings of 33 origins, obtained by 3 different methods: from seeds, from stem cuttings and *in vitro* propagation. Around May 10th when the danger of frosts has passed, the plants are planted in the field at 50/30cm distance.

The selection materials Stevia differ in the evaluated morphological and productive traits. From the comparative tests, the most perspective for effective production were the seedlings from in vitro regenerates. A tendency was established for more stable yields in some stabilized populations as a result of prolonged selection. Based on dry mass yield, the R-3 population was most productive – 115 g. The method for obtaining seedlings did not have a significant effect on the height trait of the plants.

An evaluation of the gene pool of Agricultural Institute – Shumen was made and the perspective stevia origins for fresh and dry leaf mass production were selected.

Keywords: stevia; selection; gene pool; productivity

Introduction

In recent years we are observing increasing interest in natural sweeteners, which is complemented by the significant healing properties they possess. Of particular interest to researchers is the South American plant stevia (*Stevia rebaudiana* B.). Stevia gains an increasing economic interest due to the presence of sweet diterpene glycosides that can be safely used by diabetics (Ucar et al., 2018). Stevia is a slow growing healing herb and the biomass yield is low due to the slow growth rate, the smaller number of leaves and the small area of the leaves (Khandaker et al., 2018). The leaves contain low-calorie but highly effective glycosides (stevioside and rebaudiside) that are extracted as a commercial product, which is 300 to 320 times sweeter than sugar.

The propagation of stevia is done by seeds, vegetative and *in vitro.* Because seed germination is usually very poor due to infertility, the need to obtain a large number of identical plants requires the development of an alternative breeding method for large-scale production (Zayova, et al., 2013). An alternative approach for successful reproduction and maintenance of stevia is to use the *in vitro* methods (Bojimirov &

Slavova, 2011). Vegetative reproduction by cutting the stem requires large stem stocks and is therefore limited (Carniero et al., 1997). Based on a literature study for *in vitro* stevia regeneration, over the last decade, studies have focused primarily on direct and/or indirect organogenesis (Ahmad et al., 2011; Das et al., 2011; Preethi et al., 2011; Mathur & Shekhawat, 2013; Khalil et al., 2014; Singh et al., 2014; Gantait et al., 2015). These studies show that the genetic variation of the varieties and different explant sources, cultivation periods and significantly affected the regeneration production. On the other hand, studies on tissue culture have to be combined with field experiments to develop commercial production methods (Yücesan et al., 2016).

There are over 230 varieties of *Stevia rebaudiana* B. in the world as grass, shrub and semi-shrub plants (Cimpeanu et al., 2006; Yadav et al., 2011; Ucar et al., 2016). This variety of genetic material enables the development of new varieties that are adaptable to different agro-climatic conditions (Morita et al., 2009). Stevia selection is directed at obtaining maximum quantities of dry leaf mass from a certain area with relatively high content of sweet substances (Uchkunov et al., 2012).

Soil and climatic conditions in Bulgaria also allow the successful growing of *S. rebaudiana* B. Because of its high sensitivity to low temperatures stevia is grown only as a yearling plant (Kikindonov, 2013). In Bulgarian conditions stevia usually is reproduced with seedlings obtained from rhizome cuttings or from adapted regenerates from *in vitro* maintained branches, but in favorable conditions the obtainment of seeds is also possible.

The enhanced interest in stevia cultivation (*Stevia rebaudiana* Bertoni) as a source for natural non-calorific sweeteners requires constant enrichment of the gene pool of initial material for the selection.

The current study was carried out in 2017 in Agricultural Institute – Shumen. In this study, 33 origins– *in vitro* regenerates stem rhizome cuttings and seed progenies were tested for biological and economic qualities as starting material for selection.

Materials and Methods

The used selection material is seedlings, obtained by 3 different methods: from seeds, from stem cuttings and *in vitro* reproduction.

Seedlings were produced thanks to the mild and warm autumn of 2016 when viable seeds from individual plants were obtained. Early in the spring of 2017 the seeds were sown in a thermostatic room at 20-25°C and 18 h of lighting in pots with diameter 10 cm and in a 2/1/1 mixture of peat, pearlite and sand. The seeds were distributed evenly on the surface of the previously moistened mixture and were pressed tight against it. Optimal moisture was maintained with polyethylene camera during the first 4-7 days, avoiding over-moisturizing (Kikindonov & Enchev, 2012).

Rhizome seedlings were obtained from the rhizomes stored from the previous year. By the end of February, rhizomes were stored in warm and bright rooms. The roots were placed in metal trays, covered with sand and moistened. Once the roots started growing, they were cut at a height of about 10 cm and planted in a 2/1/1 mixture of peat, pearlite and sand for rooting in a thermostatic room at 20-25°C.

Seedlings from in vitro regenerates were obtained at the beginning of March, in a thermostatic room at 20-25°C, and the *in vitro* rooted explants were planted in a 2/1/1 mixture of peat, pearlite and sand.

During April, the rooted and intercepted plants were placed in a greenhouse to adapt to outside conditions for about 3 weeks.

Around May 10th when the danger of frosts has passed, the plants are planted in the field at 50/30 cm distance (Uchkunova & Uchkunov, 2013). To ensure optimal soil moisture, as well as to reduce leaf diseases, drip irrigation was used.

The statistical processing includes evaluating the mean value, mean errors, variation coefficient and experiment accuracy.

Results and Discussion

Productivity qualities of the tested selection materials from *in vitro* regenerates are given in Table 1. The data shows that plants differ significantly in the stem height trait, as the variation was around 65 cm for the K-3 origin; the average mean of this trait for K-5 origin was 78 cm large variations were noted for the fresh leaf mass trait. The lowest values were reported in the K-1 origin – 180 g, and with the highest productivity of fresh leaf mass was K-7 – 360 g. Similar to these values was the K-11 origin – 350 g. The variation coefficients for the dry and fresh leaf mass traits were reasonably high – 18.88% and 20.64%, respectively. The dry leaf mass yield is the main index in stevia production. The average yield from the *in vitro* regenerates is 76.82 g with variations for the separate origins from 55 g (K-1) to 105 g (K-11).

The assessment results for rhizome cuttings are shown in Table 2. The preliminary review found that there was no significant difference between *in vitro* seedlings and seedlings from cuttings in relation to the height indicator of plants. The average height for the tested rhizome origins was 72.36 cm and the R-1 origin reached 85 cm. With almost identical

results was the R-7 origin which reached 82 cm of height. The variation coefficients for the plant height and dry mass content traits are relatively low – between 9 and 10%. This means that these traits were less influenced by the environment al conditions. The number of stems for one plant varied widely – from 5 to 12. This is due to the very high values of the variation coefficient – 35.07%. The least amount of stems was formed by plants of origins R-11 and R-5, and most from R-3, R-6 and R-7. For the yield of fresh leaf mass, a large variation from 35 g (R-8) to 115 g (R-3) was also found. The productivity of the tested stevia selection materials was measured in the highest value by the amount of

Table 1. Biometric and productive indicators for seedlings from *in vitro* regenerates

Origin	Height	Stems	Fresh mass of		Dry mass of		Dry	Output
			Leaves	Stems	Leaves	Stems		
	cm	number	g	g	g	g	%	%
K – 1	80	5	180	120	55	35	30.00	18.30
K – 2	76	5	260	180	70	50	27.30	15.90
K – 3	65	3	240	170	65	50	28.00	15.80
K – 4	82	7	260	185	85	55	31.50	19.10
K – 5	95	7	280	245	70	40	20.90	13.30
K – 6	67	4	270	210	70	60	27.10	14.60
K – 7	80	5	360	280	90	85	27.30	14.10
K – 8	68	5	280	180	100	50	32.60	21.70
K – 9	78	6	230	150	65	40	27.60	17.10
K – 10	90	9	310	280	70	80	25.40	11.90
K – 11	80	5	350	220	105	75	31.60	18.40
Average	78.27	5.55	274.55	201.82	76.82	56.36	28.12	16.38
max	95.00	9.00	360.00	280.00	105.00	85.00	32.60	21.70
min	65.00	3.00	180.00	120.00	55.00	35.00	20.90	11.90
vc	11.81	29.48	18.88	25.32	20.64	29.98	11.76	17.53
s x±x	2.79	0.49	15.63	15.41	4.78	5.09	1.00	0.87
p%	3.56	8.89	5.69	7.63	6.22	9.04	3.55	5.29

Table 2. Biometric and productive indicators for seedlings from rhizome cuttings

Origin	Height	Stems	Fresh mass of		Dry mass of		Dry	Output
			Leaves	Stems	Leaves	Stems		
	cm	number	g	g	g	g	%	%
R – 1	85	7	300	190	90	65	31.60	18.40
R – 2	68	6	220	160	60	45	27.60	15.80
R – 3	70	12	380	200	115	60	30.20	19.80
R – 4	70	11	230	125	60	35	23.90	14.10
R – 5	73	5	325	210	90	70	29.90	16.80
R – 6	75	12	280	170	75	50	27.80	21.40
R – 7	82	12	360	260	90	65	24.20	14.50
R – 8	68	8	150	100	35	25	24.00	14.00
R – 9	65	7	180	85	50	25	28.30	18.90
R – 10	75	6	220	135	60	40	29.90	16.90
R – 11	65	5	170	130	45	35	26.70	15.00
Average	72.36	8.27	255.91	160.45	70.00	46.82	27.65	16.87
max	85.00	12.00	380.00	260.00	115.00	70.00	31.60	21.40
min	65.00	5.00	150.00	85.00	35.00	25.00	23.90	14.00
vc	9.00	35.07	30.55	32.43	34.40	34.86	9.76	14.73
s x±x	1.96	0.87	23.58	15.69	7.26	4.92	0.81	0.75
p%	2.71	10.57	9.21	9.78	10.37	10.51	2.94	4.44

Table 3. Biometric and productive indicators for seedlings from seed progenies

Origin	Height	Stems	Fresh mass of		Dry mass of		Dry	Output
			Leaves	Stems	Leaves	Stems		
	cm	number	g	g	g	g	%	%
S – 1	80	6	170	105	55	35	32.70	20.00
S – 2	94	7	210	150	50	45	26.40	13.90
S – 3	70	7	200	120	50	30	25.00	15.60
S – 4	64	5	150	100	50	25	30.00	20.00
S – 5	76	9	200	110	60	30	29.00	19.40
S – 6	64	5	210	155	65	35	27.40	17.80
S – 7	66	7	190	150	50	45	27.90	14.70
S – 8	70	6	200	135	60	45	31.30	17.90
S – 9	73	5	125	110	60	50	46.80	25.50
S – 10	74	5	180	150	45	30	22.70	13.60
S – 11	60	3	170	105	50	35	30.90	18.20
Average	71.91	5.91	182.27	126.36	54.09	36.82	30.01	17.87
max	94.00	9.00	210.00	155.00	65.00	50.00	46.80	25.50
min	60.00	3.00	125.00	100.00	45.00	25.00	22.70	13.60
vc	13.08	26.71	14.69	17.26	19.56	22.13	20.96	19.30
s x±x	2.84	0.48	8.07	6.57	1.89	2.46	1.90	1.04
p%	3.94	8.05	4.43	5.20	3.49	6.67	6.32	5.82

dry leaf mass. Here we can highlight plants of origin R-3, whose values reach 115 g. The variation factor for dry leaf mass was high – 34.4%. This was indicative for the stronger variability of this trait. The yield ranged between 14-21% and the dry matter content from 23.90% (R – 4) to 31.60% (R – 1).

In Table 3 are presented the obtained results of the seedlings from seed progenies. The large variation in stem numbers from 3 to 9 did not affect the plants' height. The average height for the tested selection materials was 71.91 cm, as the S-2 origin was the highest – 94 cm and the S-11 origin – the lowest – 60 cm. From the information for the ratio of variation coefficient for the tested fresh stems trait it became clear that it reached C=17.26. Dry leaf mass for the tested origins was between 45-46 g with an average of 54.09 g.

Conclusions

The selection materials stevia differ in the evaluated morphological and productive traits.

From the comparative tests, the most perspective for effective production were the seedlings from *in vitro* reentrants, as average values for the fresh mass yield are 275 g and for the dry substance – 77 g from one plant.

A tendency was established for more stable yields in some stabilized populations as a result of prolonged selection. Based on dry mass yield, the R-3 population was most productive – 115 g.

The method for obtaining seedlings did not have a significant effect on the height trait of the plants.

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