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SURVIVAL AND QUALITIES OF STEVIA SEEDLINGS DEPENDING ON THE METHOD OF REPRODUCTION

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Abstract

In the conditions of Bulgaria, Stevia is propagated by planting seedlings. Seedlings are produced by cuttings from stored rhizomes, from seeds and from in vitro micropropagation and rooting techniques. In the laboratory of tissue cultures of the Agricultural Institute - Shumen, methods have been developed for efficient preservation of elite clones and micropropagation. In the 2020-2023 study, the influence of genotype on the proportion of rooting in vitro was evaluated. A comparative analysis of the survival of rooted in vitro regenerants during their adaptation to external conditions was performed, compared to that of cuttings from rhizomes and propagation from seeds. Seedlings are qualified according to biometric parameters - length and mass of the stem and roots. In vitro techniques remain the safest method for preserving and propagating elite material of Stevia for Bulgaria`s conditions.

Key words*: Stevia, reproduction, micropropagation.*

INTRODUCTION

Stevia (*Stevia rebadudiana* Bertoni) is a perennial, cross-pollinated plant of the Asteraceae family. It originates from the Paraguayan and Brazilian mountains (Lewis, 1992). In recent years, there has been a heighten interest in its cultivation as a source of natural, non-caloric sweeteners. They are of the diterpene glucosides such as rebaudioside A, B, C, E and Stevioside and are up to 300 times sweeter than sucrose (Geuns, 2004).

Due to its sensitivity to low temperatures in countries with temperate climates, it can be grown as an annual, but it also can be maintained by storing the rhizomes (Lankes and Pude, 2008). Propagation by rooting cuttings from rhizomes stored in winter is widely used. An alternative method is propagation and maintenance by in vitro methods, in which the initial genotype is also preserved (Krumov et al., 1984).

In natural conditions, it reproduces by seeds. Stevia is a short-day plant and a certain light and temperature regime is required for insemination (Zaidan et al., 1980). As a cross-pollinated crop, self-incompatibility mechanisms prevent self-pollination and the production of homozygous offspring (Nakamura et al., 1985). Flowering occurs non-simultaneously and is prolonged.

As early as the 1980s, research was conducted at the Sugar Beet Institute in Shumen on the introduction and cultivation of Stevia, establishing the optimal technological conditions and developing methods for *in vitro* propagation (Vurbanov et al., 1996). With the renewed interest, Agricultural Institute - Shumen is working on the selection of new forms of Stevia. In 2017, the first Bulgarian variety with high economic qualities and adapted to the conditions of Bulgaria was recognized (Uchkunov, 2006).

Some aspects of obtaining and growing seeds in vitro and under outdoor conditions have been studied (Bozhimirov and Slavova, 2011; Kikindonov and Enchev, 2012). Under our conditions, from propagated *in vitro* and after prolonged adaptation in spring in greenhouse conditions, individual plants begin to bloom at the end of July. Mass flowering is from September until the rhizomes are harvested before the formation of autumn frosts (Bozhimirov and Slavova, 2011).

The study presents the results of the adaptation to outdoor conditions of germinated plants from seeds, rhizome cuttings and from *in vitro* regenerants to obtain seedlings for cultivation under field conditions in Bulgaria.

MATERIALS AND METHODS

The data for the study were obtained from research in the period 2021-2023 at Agricultural Institute Shumen, Bulgaria.

The study used selection material from the gene pool of Agricultural Institute - Shumen. The sources are from Japan, which are smaller-leaved, densely overgrown, and from America with larger and thicker leaf petioles. After many years of work on individual, family and clonal selection and adaptation to our conditions, elite clones have been formed, as well as origins - a product of somaclonal and gametoclonal variation in *in vitro* culture of meristems, embryos, seeds.

Perennial rhizomes from 15 to 30 elite clones, annual rhizomes from clones grown from seedlings in the previous year and new seedlings from *in vitro* micropropagation of supported clones, cuttings from rhizomes and from seeds are stored and planted annually.

In adaptation rooms with controlled temperature, studies are carried out on seed germination, adaptation of rooted *in vitro* regenerants to external conditions, rooting of rhizome cuttings and young plants, picking and growing seedlings suitable for planting in field conditions.

The cleaned seeds are sown in a soil substrate of peat, sand and perlite in a ratio of 4/1/1 in pots, by evenly distributing them on the surface with pressing without backfilling. A moist chamber is provided with a polyethylene cover tightly for 4-6 days and with gradual opening up to the 14th day.

Under the same conditions, the rooted in vitro regenerants and cuttings with 3-5 leaf nodes from the tips of the rhizome buds, cleaned from the nutrient medium, are planted. For the first 20 days until the development of the root system, they can be planted more densely, since some of them do not develop roots or rot.

To obtain suitable seedlings, 45 to 60 days of cultivation are required, and for seed offspring this period is extended to 75 days. Then they are taken outdoors for 14-20 days for hardening before planting in field conditions.

Figure 1. Stevia reproduction

RESULTS AND DISCUSSIONS

Adaptation of germinated plants from seeds to external conditions for obtaining seedlings for field cultivation

The trend for a longer and warmer autumn in Bulgaria in recent years has made it possible to cover the requirements of the Stevia plant for combined temperature and short day at the end of the growing season, to obtain viable seeds. In our study, whole plants are harvested from the field at the end of the season and dried. Then the seeds are collected by threshing, without separating the empty from the full seeds. The seeds collected by threshing of dried whole plants have an average germination rate of

21.8%, with a coefficient of variation of 22.6%. This allows for a more widespread use of seeds for propagation in practice.

In order to increase the volume of Stevia seedlings, the obtained seeds from 2023 were sown in series every 15-20 days in pots with a soil mixture of 100 grams of origin. On the 14th day, the germinated seeds were counted. After this period, some of the germinated plants did not survive and 45-50 days after germination, the surviving plants were counted and dived to obtain adapted seedlings for planting in the field (Table 1).

Previous studies have established the mass levels of 1000 seeds of 400 mg or 250 seeds are sown with 100 g. This significantly facilitates the technology for accelerated propagation.

The germination in the soil mixture and the survival of seeds from the 2023 harvest is relatively high, with a high degree of variation depending on both the genotype and the type of seedlings. Most viable are seeds from one-year-old seedlings. The genotype is a determining factor for seed reproduction within a single harvest. For Bulgaria, the specific agroclimatic conditions of the year are the limiting factor.

Table 1. Seed germination and survival to external conditions for obtaining Stevia seedlings, 2023

Origin of donor plants	One-year-old seedlings		Two-year-old rhizomes		Perennial rhizomes	
Origin	Sprouted seeds	Survival rate	Sprouted seeds	Survival rate	Sprouted seeds	Survival rate
	amount	%	amount	%	amount	%
1	9	11.1	10	90.0	34	94.1
2	127	78.0	9	66.7	95	84.2
3	6	16.7	4	25.0	5	40.0
4	123	69.9	85	63.5	6	33.3
5	92	91.3	89	78.7	11	9.1
6	80	80.0	66	81.8	6	33.3
7	110	72.7	123	79.7	74	67.6
8	61	90.2	65	86.2	77	80.5
9	95	86.3	91	87.9	93	36.6
10	124	95.2	5	20.0	34	38.2
x	82.7	69.1	54.7	67.9	43.5	51.7
vc	54.1	43.7	80.4	37.4	86.3	53.8
s x±x	14.1	9.57	13.9	8.05	11.8	8.80
p%	17.1	13.8	25.4	11.8	27.2	17.0

Rooting cuttings from rhizomes

To establish the parameters for effective rooting of cuttings in 2022, 7 genotypes were selected, with plants from each being planted on two different dates - one on 25.03 and the other on 25.05. The aim was to determine whether the planting dates had any effect on the rooting percentage of the plants. From Table 2 it can be clearly seen that the percentage of rooted plants in March was 79.76%, while the percentage in May was 67.38%. The only deviation from the average values was observed in two of the origins A10 and E7, which is most likely due to genetic factors.

Table 2. Rooting cuttings from winter-stored rhizomes of elite Stevia plants, 2022

Variants	25.03			25.05			Total	Total rooted
Genotypes	Studied	Rooted	Rooted %	Studied	Rooted	Rooted %	amount	%
75 ШЗ	48	38	79.2	48	26	54.2	96	66.67
210/9	30	27	90.0	30	18	60.0	60	75.00
214/2	30	28	93.3	30	26	86.7	60	90.00
7 E	30	22	73.3	30	24	80.0	60	76.67
9 E	30	23	76.7	30	11	36.7	60	56.67
A 10	48	26	54.2	48	38	79.2	96	66.67
CP 28	48	44	91.7	48	36	75.0	96	83.33
X	**37.7**	**29.**	**79.7**	**37.7**	**25.5**	**67.3**	**75.4**	**73.5**
Sx	**3.64**	**3.09**	**5.20**	**3.64**	**3.57**	**6.73**	**7.27**	**4.25**
CV	**25.5**	**27.**	**17.2**	**25.5**	**36.9**	**26.4**	**25.5**	**15.2**
P %	**9.64**	**10.4**	**6.52**	**9.64**	**13.9**	**9.98**	**9.64**	**5.77**

In 2023, this experiment was expanded and a total of 15 origins were used to monitor effective rooting of cuttings, with the cuttings being planted in three groups in different months – January, February and March, preceding the dates of the previous study. In addition to the 7 origins planted in the first experiment, 8 more origins were added. From Table 3, it can be clearly seen that the percentage of rooted plants in January is 98.54%, decreasing to 81.96% in the following month and to 72.95% in March. Once again, a deviation was observed in some of the origins – 7E, 9E and A10, where in February there was less or the same percentage of rooted plants compared to March. This confirms the claim that hereditary factors are important for the rooting of plants. It is also impressive that the percentage of rooted plants in March 2022 and 2023 is approximately the same for the origins used in both experiments. From the conducted observations, it can be concluded that for the most successful rooting of the plants, the cuttings should be planted as early as possible in the year.

Table 3. Rooting cuttings from winter-stored rhizomes of elite Stevia plants, 2023

Origin	26-30.01		28-30.02		26-30.03		Total amount	Total rooted %
	Studied	Rooted %	Studied	Rooted %	Studied	Rooted %		
3	30	100.0	45	97.8	20	85.0	95	95.79
4	28	100.0	35	60.0	25	48.0	88	69.32
5	18	100.0	30	80.0	15	60.0	63	80.95
1-1	25	92.0	30	86.7	15	66.7	70	84.29
10 ШЗ	31	100.0	40	85.0	25	80.0	96	88.54
75 ШЗ	20	95.0	15	93.3	20	80.0	55	89.09
203/2	15	100.0	15	86.7	15	73.3	45	86.67
210/9	36	97.2	45	91.1	22	77.3	103	90.29
214/2	43	97.7	15	93.3	15	86.7	73	94.52
7 E	11	100.0	15	73.3	15	80.0	41	82.93
9 E	17	100.0	15	60.0	15	73.3	47	78.72
A 10	40	100.0	45	62.2	25	60.0	110	75.45
CP 27	26	96.2	30	76.7	15	60.0	71	80.28
CP 28	15	100.0	30	90.0	20	80.0	65	89.23
CP 162	13	100.0	30	93.3	25	84.0	68	91.18
x	**24.5**	**98.5**	**29.0**	**81.9**	**19.1**	**72.9**	**72.6**	**85.1**
Sx	**2.60**	**.64**	**3.02**	**3.31**	**1.13**	**2.96**	**5.57**	**1.88**
CV	**40.9**	**2.50**	**40.2**	**15.6**	**22.8**	**15.7**	**29.6**	**8.55**
P %	**10.5**	**0.64**	**10.4**	**4.04**	**5.90**	**4.06**	**7.67**	**2.21**

Adaptation of rooted in vitro regenerants

Between 2021 and 2023, an assessment of the rooting under outdoor conditions of regenerants obtained from in vitro cultures was carried out (Table 4). Fifteen origins were used, and the percentage of rooted plants was measured for each origin. The results are presented by year as well as in total for the three years. An increase in rooting was observed for each subsequent year, which may be due to environmental factors. On average, 236 regenerants were used for each of the origins, with the total number for individual genotypes varying between 172 and 338 individuals. The average rooting percentage for the three years is 87.8%. It is interesting to note that for some of the genotypes (20% of the genotypes) the rooting is 100%. As 60% of the origins achieve rooting, in total for all three years, more than 90%.

Table 4. Rooting of *in vitro* regenerants in outdoor conditions, 2021-2023

Year	2021		2022		2023		Total for the period	
Genotypes	Total amount	With root %	Total amount	With root %	Total amount	With root %	Total amount	With root %
3	120	100.0	90	100.0	100	86.0	310	95.5
4	74	94.6	155	54.8	100	90.0	329	74.5
5	105	36.2	57	96.5	120	95.0	282	73.4
1-1	100	37.0	120	90.0	100	88.0	320	72.8
10 ШЗ	100	100.0	100	85.0	75	93.3	275	92.7
75 ШЗ	120	89.2	70	100.0	185	100.0	375	96.5
203/2	105	100.0	90	85.6	100	90.0	295	92.2
210/9	100	67.0	100	91.0	100	85.0	300	81.0
214/2	30	86.7	145	97.9	95	87.4	270	93.0
7 E	143	93.0	120	90.8	80	95.0	343	92.7
9 E	174	89.1	120	85.8	100	89.0	394	88.1
A 10	150	59.3	150	90.0	95	92.6	395	79.0
CP 27	120	70.8	70	100.0	105	100.0	295	88.1
CP 28	60	100.0	100	96.0	112	98.2	272	97.8
CP 162	75	74.7	175	92.6	163	96.3	413	90.8
x		**79.8**		**90.4**		**92.3**	**324.5**	**87.2**
Sx		**5.64**		**2.88**		**1.28**	**12.5**	**2.25**
CV		**27.3**		**12.3**		**5.35**	**14.9**	**10.0**
P %		**7.06**		**3.19**		**1.38**	**3.87**	**2.58**

Evaluation of adapted seedlings from in vitro regenerants and from rhizome cuttings

Table 5 displays the evaluation of adapted seedlings from *in vitro* regenerants and from rhizome cuttings. From the data on the evaluation parameters of the two groups it is noticeable that, plants obtained from in vitro regenerants have higher indicators than those obtained from cuttings. Parameters such as stem height, number of leaves, root length, as well as the weight of dry matter (root, leaf, stem) were compared. The observed difference in terms of height, number of leaves and root is significant and is in favor of plants obtained from *in vitro* regenerants. The total dry matter of plants obtained from cuttings is also less than that of plants obtained from *in vitro* regenerants. When analyzing the different parts of the plants, it was found that the weight of dried roots and stems in plants obtained from in vitro is less compared to the same weight of plants obtained from cuttings. Conversely, the dry weight of leaves in plants obtained from cuttings is greater than that of leaves in *in vitro* regenerants. This applies not only to the average leaf weight, but also to each plant compared individually.

Table 5. Evaluation of adapted seedlings obtained from *in vitro* regenerants and from rhizome cuttings, 2023

Variants	Stem height, cm	Amount of leaves	Root lenght	Weight (in g) of:			
				Root	Leaves	Stem	Total dry mass
Cuttings							
1.	13.5	10.0	6.0	0.48	0.44	0.28	0.24
2.	11.0	13.1	5.2	0.23	0.34	0.13	0.14
3.	10.5	10.7	3.5	0.17	0.16	0.27	0.12
4.	10.9	10.0	9.9	0.47	0.29	0.14	0.18
5.	14.5	12.0	7.0	1.04	0.44	0.21	0.34
6.	21.0	12.3	6.1	0.40	0.63	0.30	0.26
7.	15.2	12.0	5.1	0.48	0.44	0.17	0.22
8.	16.2	10.6	5.4	0.41	0.32	0.18	0.13
9.	14.1	10.0	9.4	0.47	0.38	0.17	0.21
10.	11.4	10.6	7.4	0.41	0.34	0.15	0.18
11.	17.2	14.0	8.4	0.33	0.59	0.28	0.24
12.	8.0	10.0	5.6	0.19	0.23	0.18	0.18
Average	**13.6**	**11.3**	**6.6**	**0.42**	**0.38**	**0.20**	**0.20**

Regenerants							
1.	23.5	16.0	12.1	0.40	0.25	0.30	0.26
2.	19.5	16.3	9.5	0.49	0.26	0.43	0.31
3.	21.0	14.0	11.0	0.61	0.30	0.48	0.38
4.	23.4	18.0	13.0	1.07	0.25	0.47	0.48
5.	18.2	16.3	11.0	0.60	0.27	0.38	0.34
6.	18.0	16.0	9.8	0.67	0.23	0.41	0.35
7.	17.0	12.0	9.0	0.77	0.22	0.42	0.38
8.	16.7	12.3	8.5	0.38	0.23	0.31	0.25
9.	17.2	14.0	11.0	0.90	0.30	0.48	0.45
10.	18.0	14.0	13.0	0.74	0.23	0.52	0.40
11.	17.5	16.3	15.0	0.91	0.31	0.52	0.49
12.	12.5	12.0	11.0	0.28	0.12	0.29	0.49
Average	**18.5**	**14.8**	**11.2**	**0.65**	**0.24**	**0.42**	**0.35**

CONCLUSIONS

The assessment of adaptation to external conditions and the development of seedlings under field conditions allows us to draw certain conclusions important for the practical implementation of Stevia.

Germination in soil mixture and seed survival is relatively high, with a high degree of variation depending on both the genotype and the duration of use of donor plants. The most viable are seeds from one-year-old seedlings. The genotype is a determining factor for seed reproduction within one harvest.

The average rooting rate of in vitro regenerants under external conditions for the three years in total is 87.8%. It is interesting to note that for 20% of genotypes, rooting is 100%, and 60% of origins achieve rooting of more than 90%.

Research has shown that the timing of cuttings is critical for successful rooting of plants. The highest rooting rates are observed for cuttings planted early in the year, with January showing the best results followed by February and March. This highlights the importance of early planting for optimal rooting. Hereditary factors also play a role, with some genotypes showing deviations from the average values.

Evaluation of rooting of regenerants from in vitro cultures indicates that these plants have better morphological and economic indicators compared to plants obtained from cuttings.

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