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Improvement of rebaudioside A diterpene glycoside content in *Stevia rebaudiana* Bertoni using clone selection

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Abstract: This study, conducted in Antalya, Turkey, between 2014 and 2016, aimed to determine genotypes with a high amount of rebaudioside A (reb A) and high reb A/stevioside (stv) ratio in *Stevia rebaudiana* Bertoni, and reveal superior candidate varieties via the clone selection method. The study started with 200 genotypes in the first year, followed by the stem cuttings of 40 genotypes selected in the second year, in accordance with the obtained data, which were grown in rows (A clones), and the 10 genotypes (B clones) selected from the clones in the third year, which were grown in 3 replications according to a completely randomized block design. In accordance with the criteria for the evaluation of the results, 4 plants with superior characteristics were selected (C clones). Genotype 82 was regarded as a candidate with superior characteristics in terms of the amount of reb A content (7.95%, 7.77%, and 6.37%, respectively) and reb A/stv ratio (1.48, 1.20, and 1.48) over the 3 years. Genotype 109 was noteworthy for stv alone. Genotype 133 was considered as not only high in reb A (8.47%, 6.81%, and 6.40%, respectively), but also as an industrial material suitable for processing and production, as the agronomic properties were above average during the study. Genotype 196 was determined to be high in terms of the amount of reb A + stv (18.31%, 12.54%, and 13.58, respectively) and reb A (7.57%, 6.46%, and 6.2%, respectively). Therefore, these were selected as clonal variety candidates.

Key words: Breeding, clone selection, rebaudioside A, *Stevia rebaudiana*, stevioside

1. Introduction

There is a growing tendency for people to consume foods low in sugar and calories, and this has led to increasing interest in natural sweeteners. Greater use of alternative medicine methods and herbs in treatments has led to increased interest in *Stevia rebaudiana* Bertoni, which is a natural sweetener. *Stevia rebaudiana* Bertoni belongs to the family Asteraceae (Lester, 1999) and *Stevia* leaves produce secondary metabolites known as steviol glycosides (SGs). The most important feature of SGs, particularly stevioside (stv) and rebaudioside A (reb A), is that they are very strong natural sweeteners and insulin is not secreted when they are consumed. Stevia leaves contain more than 35 ent-kaurene-type diterpene glycosides, which are about 200–300 times sweeter than sucrose (Gardana et al., 2010), with reb A and stv as major components (Wölwer-Rieck, 2012). The characteristic sweetness of *Stevia* is due to the presence of stv (4%–13%), reb A (2%–4%), reb C (1%–2%), and reb B, D, and E and dulcoside A (0.4%–0.7%), as well as other less common glycosides, such as steviolmonoside, rubusoside, steviolbioside, and reb F (Lemus-Moncada et al., 2012). The sweetness percentage (%) of reb A ranges from 30% to 40%, which is 180–400 times sweeter than sugar. Liquid extract obtained from this plant is known to regulate blood sugar and has zero calories (Singh and Rao, 2005). In addition to therapeutic features, it has been used for its antihyperglycemic, antihypertensive, antiinflammatory, antitumor, antidiarrheal, and diuretic properties and to prevent dental caries (Bhasker et al., 2015). The aftertaste of these SGs can be perceived as bitter, metallic (Prakash et al., 2012), or licorice-like, which some manufacturers would like to avoid in their products (Vouillamoz et al., 2015). The elimination of the licorice-like and bitter taste profile in SGs is a key challenge for manufacturers (De Roode et al., 2015). Consumers and bioindustry require better quality, higher reb A content, and safe products (Tavarani et al., 2015). Such demands can be met by *Stevia* varieties bred specifically for higher reb A content and quality, which do not have a bitter aftertaste. The native reb A/stv ratio in *Stevia* leaves is usually about 0.5 or less (Yadav et al., 2011). Breeding programs for *Stevia* should aim at improving the total glycoside content and reb A/stv ratio with a higher leaf yield. To date, plant breeding efforts with *Stevia* have been largely focused on

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improving leaf yield and reb A concentration in the leaves (Yadav et al., 2011). With a high level of natural variability due to constant out-crossing, breeders are able to improve the level of sweeteners in the leaves and alter the reb A/stv ratio (Shu, 1989; Huang et al., 1995). *Stevia rebaudiana* is characterized as a cross-pollinated, photoperiod-sensitive crop that produces self-incompatible flowers (Yadav et al., 2014) and seeds that have poor germination rates. Poor seed germination (Megeji et al., 2005) represents a serious obstacle of large-scale cultivation of this crop and limits breeding programs (Martini et al., 2015). Generally, poor germination rates and inadequate cross-pollination and seed propagation result in more heterogeneous plants. Therefore, to obtain homogeneous *Stevia* plants, propagation is usually done via stem cuttings (Turgut et al., 2015). Most breeding programs are based on cross-breeding and selection. Vegetative propagation and cloning have frequently been used to multiply individually selected plants. Some of these selections, although very high-yielding, are self-incompatible and can only be reproduced vegetatively (Lee et al., 1982). A variety of plant-breeding procedures have been used to improve leaf yield and reb A concentration in the leaves (Shizhen, 1995).

The cultivation of *Stevia rebaudiana* in agriculture and the food industry has been undertaken at a very fast rate in many countries, and cultivation studies have been carried out in different parts of the world, from Asia to the Americas (Šic Žlabur et al., 2013), but there is a lack of standardization to produce high-quality plants and end products. It is also important that the amount of reb A and the reb A/stv ratio be high in terms of *Stevia* sweetener quality (Yadav et al., 2011). The EU increased the need for high-quality raw materials by approving *Stevia* for use as a food additive in 2011 (Tavarini and Angelini, 2013). High-quality dry leaf products are known to be in demand in many European countries, especially in Germany, and current production is insufficient to meet this demand. *Stevia* production is influenced by several factors, such as genotype, phenological stage, and growth conditions (Yadav et al., 2011), thereby producing variations in the amount of SGs. It is therefore important to develop varieties that meet the desired quality criteria. For the development of new varieties that have wider adaptability to different climatic conditions, and higher yield and quality, the effect of environmental and genetic factors on the plant must be known, in addition to the provision of agronomy and cultivation conditions for the newly developed varieties. Most growers have taken advantage of that wide variety, as a result of constant out-crossing, to obtain good-quality leaves with a high reb A/stv ratio (Shu, 1989; Huang et al., 1995). Since *Stevia* can naturally grow in tropical and subtropical regions, various studies have been carried out on its adaptation and production. Turkey is a good location for *Stevia* cultivation because of its climate and terrain. Adaptation studies initially began in 2009, in Antalya, in southern Turkey, where there are Mediterranean climate conditions similar to a subtropical climate. Adaptation study results have shown that *Stevia* could be cultivated successfully as a perennial crop in the prevailing conditions of Antalya (Turgut et al., 2015). Cross-pollinated *Stevia* crops have been observed to have high genetic diversity. An important problem with *Stevia* production is that plants produced from seeds are not genetically homogenous. To solve this problem, it is necessary to propagate *Stevia* clonally. Genetic improvement could possibly be achieved by applying clone selection breeding (Demir, 1990). In particular, the development of new varieties with high leaf yield and quality (high reb A/stv ratio) would increase the global competitive power of *Stevia* producers and industrialists.

The aim of this study was to develop new *Stevia rebaudiana* varieties that produce a high amount of reb A and high reb A/stv ratio via clone selection breeding. The production of quality *Stevia* varieties could be a new and alternative source of livelihood for producers, especially in areas such as the Mediterranean region, where growing conditions are suitable, and Turkey could become a leading *Stevia*-producing country with planned sustainable agricultural programs.

2. Materials and methods

2.1. Plant cultivation and experimental design

This study was conducted between 2014 and 2016 in the experimental field of the Faculty of Agriculture at Akdeniz University, Antalya, in the Mediterranean region of Turkey (33 m above sea level; 36°53′N, 30°38′E), which has a Mediterranean climate. The mean annual temperatures in 2014, 2015, and 2016 were 19.8 °C, 19.6 °C, and 20.3 °C, respectively. The weather was warm and there was high humidity throughout the summer. The total rainfall was recorded as 1235.7 mm, 903.6 mm, and 478.6 mm, respectively, for each year of the study. The mean humidity rates were 65.01%, 61.22%, and 61.12%, respectively. The terra-rossa-type soil characteristics of the experimental field were clay loam, high in lime (33.9%), low in salt (0.03%), and alkaline (pH 7.7). The 0–30 cm layer of soil had low concentrations of organic material (1.55%) and a sufficient amount of nitrogen (0.11%). In addition, there was a high amount of available potassium and calcium in the soil of the experimental field.

Stevia rebaudiana Bertoni Criolla was used as the starting plant material. Seeds from open pollinated plants were germinated in a greenhouse at Grow Fide Inc. in Antalya in 2011, and approximately 2000 healthy seedlings were planted in the experimental plot in 2012. A variety of maintenance and drip irrigation procedures were

applied and the seedlings were used as plant material for individual plant selection in 2014. From the 2000 plants, 200 were selected according to phenotypic observations, such as plant height and number of branches and leaves, made during 2014. Next, agronomic features (plant height; fresh herbage, leaf, and stem yields; dry leaf yield; leaf area, periphery, and thickness; leaf/stem ratio; and amount of chlorophyll) and content analysis (stv, reb A, and reb A/stv ratio) were determined. After evaluation of the data, 40 plants were selected, primarily according to the highest content. From each plant (genotype), 10 stem cuttings were rooted under greenhouse conditions in May 2015. Each stem cutting was kept in 500 ppm indole butyric acid for 10 s before planting in a peat + perlite (1:1) volumetric mixture medium. From each clone, at least 40 stem cuttings were taken and planted in the medium (peat + perlite, 1:1), and then those seedlings were taken to the greenhouse under controlled conditions to be rooted. These stem cuttings from the 40 selected clones were rooted under a mist irrigation system in the greenhouse (irrigation every 2 min in week 1, followed by irrigation every 10 min in weeks 2 and 3). After 3 weeks, the seedlings were planted in rows at 60 cm and 40 cm row and intrarow spacing, respectively. Thus, in 2015, a total of 40 rows of 10 plants per row were established in the open field and named A clones. Each row was harvested at the end of 95 days and the same agronomic measurements and analyses were performed.

According to observations and all of the parameters, 10 rows (clones) were selected to establish the B clones. From each row, 300 stem cuttings were rooted and planted in plots in May 2016. The field trial was designed as a completely randomized block design, with 3 replications. Row and intrarow spacing was 60 cm × 40 cm with 100 plants per plot. After collecting data from the plants, each plot was harvested separately at the inflorescence stage in September 2016. After evaluating the agronomic and chemical data, 4 clones were selected as C clones in 2017. The plants were irrigated with drip irrigation, no fertilizers were applied, weeds were controlled manually with garden tools, and plants from each plot were dried at 35 °C in an oven for measurement and analysis during the years of the study. In addition, leaves were stored in the dark until use and they were extracted, and high-performance liquid chromatography (HPLC) analysis was performed using dried samples.

2.2. Extraction of *Stevia rebaudiana* leaves

For the extraction, 1 g of ground dried *Stevia* leaves was homogenized in 10 mL of ultrapure water at 10,000 rpm/min for 10 min with an Ultraturrax IKA T18 (IKA-Werke GmbH & Co. KG). Each extract was centrifuged (15 min, 5000 rpm) and 0.1 mL of aqueous phase was transferred to a 10-mL volumetric flask filled with the mobile phase (acetonitrile/water, 80/20 v/v). The extracts were stored at 4 ± 1 °C in a refrigerator until use. The sample materials were passed through 0.45-μm nylon membrane filters to remove undissolved particles before analysis. This extraction method, which used water as a solvent, was optimized in our laboratory.

2.3. HPLC conditions

The study was carried out in the laboratories of the Field Crops Department of the Faculty of Agriculture. The chromatographic analysis under isocratic conditions was performed on the Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany). The system consisted of a G1311A quaternary pump, G1313A standard automatic sampler, G1316A COLCOM column furnace and cooler, and G1315A diode array detector. To detect stv and reb A, Luna HILIC was used (250 × 4.6 mm, particle size 5 μm, Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of acetonitrile/water (80:20 v/v) set to a flow rate of 1.0 mL/min, kept at 36 °C. The sample injection volume was 20 μL (Wölwer-Rieck et al., 2010).

2.4. Statistical analyses

SAS statistical software (SAS Institute, Cary, NC, USA) was used for the data analysis. Analysis of variance was used to detect differences followed by the Duncan multiple comparison test. $P < 0.05$ was considered statistically significant.

3. Results and discussion

Previous studies have focused on improving new varieties to produce higher diterpene glycoside content and yields. The aim of this study was to select *Stevia rebaudiana* Bertoni genotypes with high reb A content and a high reb A/stv ratio using the clone selection method. The reb A/stv ratio is the accepted measure of sweetness quality; the more reb A is present, the more intense the sweetness. If reb A is present in equal quantities to stv, the aftertaste seems to be eliminated (Yadav et al., 2011).

Tavarini and Angelini (2013) reported that there was a relationship between the time of harvesting and the reb A/stv ratio, and that delaying the harvest until the time of flower formation significantly increased dry leaf weight. Similar to that study, a previous study under Antalya climate conditions determined that the appropriate harvest time for *Stevia* plants that had been adapted in previous years was just before flowering and the glycoside content reached its highest level in that period (Turgut et al., 2015). Therefore, during the 3 years of this experiment, the plants were harvested just before flowering. *Stevia* has shown the best development in areas with an annual mean temperature of 31 °C and a rainfall of 1400 mm. It has been reported that *Stevia* is not resistant to cold and can withstand temperatures close to zero for a very short time (Sing and Rao, 2005). High leaf production is achieved in high light density and high temperature regions. The

climate characteristics of Antalya in general are those of a Mediterranean climate. Summers in the Antalya coastal region are both long and hot. On long summer days, there is close to 15 h of daylight. There is an average of 14.8 h of daylight in June and 12.4 h in September. The average annual rainfall is approximately 1100 mm. When the 3 years of the experiment were evaluated, the mean temperature values of the years did not show great variability, with the average annual temperature in 2016 increasing by approximately 1 °C compared to the other years. The amount of annual rainfall showed great variability over the study years, with the total annual rainfall measured as 1235.70, 903.60, and 478.60 mm in 2014, 2015, and 2016, respectively. Average humidity was recorded as 65% in 2014 and 61% in the following 2 years.

3.1. Variation source

Initially, 200 plants were selected phenologically from a 3-year-old *Stevia* population (2000 plants), which were propagated by seeds of the open-pollinated Criolla cultivar. The open-pollinated population, which revealed great heterogeneity, was used as a source of variation and as the basic population. Therefore, the 1-year-old plants, in which the variation source was established, showed good improvement when evaluated from an agronomic point of view, and they adapted well. In the 3-year-old plants, representing the source of variation, the fresh herbage, leaf, and stem yields and dry leaf yield were almost twice that of the 1-year-old plants. Therefore, in this study, the selection criteria were primarily the reb A, stv, and reb A/stv ratio. Different environmental conditions and varieties caused the performance characteristics of the plant to vary.

In the 200 selected plants, the stv rate was 4.10%–15.96% (mean: 8.34%), reb A rate was 1.23%–13.69% (mean: 8.34%), stv + reb A rate was 8.23%–23.83% (mean: 13.21%), and reb A/stv ratio was 0.46–1.84 (mean: 0.64) (Table 1). Significant variations were observed among single plants. According to the selection criteria (high reb A and stv rate; high reb A/stv ratio; high reb A + stv), 40 plants (genotypes) were selected as A clones.

3.2. A clones

After evaluation of the data obtained from the 200 plants (clones), 40 clones (genotypes) were selected primarily according to the highest SGs contents. In 2015, a total of 40 rows (genotypes) with 10 plants were established in the open field and were named A clones. Since the data for the first year were obtained from 3-year-old plants, the A clones were reduced by almost half in terms of agronomic characteristics when compared to the previous year (data not shown). In the second year of the study (A clones), the stv rate was 4.26%–9.36% (mean: 6.37%), reb A rate was 2.59%–7.77% (mean: 5.24%), stv + reb A rate was 8.54%–14.90% (mean: 11.60%), and reb A/stv ratio was 0.68–1.49 (mean: 0.88) (Table 2). When the A clones were established, there were 9 genotypes with a high amount of stv, 4 genotypes containing approximately the same amounts of reb A and stv, 11 genotypes with a higher reb A/stv ratio, 8 genotypes with a high amount of stv + reb A, 5 genotypes with a high amount of reb A, and 3 genotypes containing only stv (Table 3).

Genotype 82 gave the highest level of reb A (7.77%), followed by genotypes 31 (6.96%), 93 (6.91%), 4 (6.86%), and 133 (6.81%). Genotypes 83 (1.41), 96 (1.51), and 116

Table 1. Some yield and quality characteristics of the variation source.

Parameters	N	Mean	Standard deviation	Min.	Max.
Leaf thickness (mm)	200	0.35	0.06	0.15	0.56
Plant height (cm)	200	71.64	11.19	25.00	100.00
Leaf area (cm^2)	200	24.69	5.98	13.15	42.54
Leaf periphery (cm)	200	16.62	5.53	12.01	23.37
Amount of chlorophyll	200	41.21	3.96	23.10	53.30
Fresh herbage yield per plant (g)	200	260.95	102.06	76.20	655.40
Fresh leaf yield per plant (g)	200	124.22	51.95	30.25	323.56
Fresh stem yield per plant (g)	200	95.55	45.00	25.45	299.10
Dry leaf yield per plant (g)	200	43.15	5.21	13.82	58.27
Reb A (%)	200	4.87	2.31	1.23	13.69
Stv (%)	200	8.34	3.16	4.10	15.96
Reb A + stv (%)	200	13.21	4.13	8.23	23.83
Reb A/stv	200	0.64	0.39	0.46	1.84

Table 2. Some quality characteristics of the A clones.

Parameters	N	Mean	Standard deviation	Min.	Max.
Reb A (%)	40.00	5.24	1.39	2.59	7.77
Stv (%)	40.00	6.37	1.33	4.26	9.36
Reb A + stv (%)	40.00	11.60	1.39	8.54	14.90
Reb A/stv (%)	40.00	0.88	0.33	0.68	1.49

(1.55) gave the highest reb A/stv ratio. The highest level of reb A + stv was obtained from genotypes 4 (14.9%), 196 (12.54%), and 52 (13.36%). Genotypes 28, 109, and 152 were included in the A clones for the stv only (0 reb A), but only genotype 109 continued in the same way with a mean stv of 8.54% in the second year. However, genotypes 28 and 152 produced reb A with a mean of 6.03% and 3.57%, respectively (Table 3). Wood et al. (1955) reported stv as 5%–10% and reb A as 2%–4% of total dry weight.

The studies conducted on the A clones showed that some genotypes retained the characteristics for which they were selected, and some showed great differences. For example, clonal line 4 was selected due to the high amount of stv + reb A contained in the basic population (19.0%), and this reduced to 14.9% in the A clones in the following year. Finally, 10 genotypes (rows) were selected, as they were superior to other genotypes in terms of SG content (Table 3). With aging of the plant, SGs tend to accumulate in tissues at the whole plant level. Thus, older lower leaves have more sweetener than younger upper leaves. After the onset of flowering, the concentration of glycosides in the leaves begins to decrease (Singh and Rao, 2005). It is also necessary to consider whether there was a climatic difference between the 2 years in which the study was conducted. In this regard, there was not a big difference between the temperature and rainfall values in the 2 years, which can cause stress or physiological changes in the plant, and the humidity value (65%–61%) changed only a little.

3.3. B clones

B clones comprising 10 genotypes were established in a completely randomized block design, with 3 replications. A statistical analysis of the normality of the measured major selection properties (reb A, stv, and reb A/stv) was undertaken using SAS statistical software. The data were seen to be normally distributed, with significance of ≥0.05 according to the Shapiro–Wilk test. P-values were calculated as 0.635 (stv), 0.700 (reb A), and 0.345 (reb A/stv). Genotype 82 was selected due to its high reb A/stv ratio in the A clones. When the B clones were evaluated, genotype 82 maintained the same property and gave the highest reb A/stv ratio (1.48), followed by genotypes 185 (1.38), 133 (1.34), and 83 (1.29) (Table 4). The reb A/stv ratio was >1.0 in all genotypes except genotype 196 and the differences between the genotypes were statistically significant.

Genotypes 133, 82, and 191 produced the highest rates of reb A at 6.40%, 6.37%, and 6.24%, respectively, followed by genotypes 196 (6.20%) and 185 (6.11%) (Table 4). The lowest level of reb A was obtained from genotype 111 (4.85%) and genotype 109 gave 0 reb A, as expected. Differences between the genotypes were statistically significant. In terms of the stv content, genotypes 109 (7.59%) and 196 (7.38%) were significantly higher than the others, and genotype 111 (3.84%) was the lowest. Genotype 109 produced only stv without reb A (Table 4). Although the amounts of reb A and stv varied during the years in which the study was conducted, the reb A/stv ratio did not vary greatly.

Genotype 109 contained no reb A at any of the stages and this may be an important finding. Genotype 116 had a high reb A/stv ratio in the first year, and in the following years of the study, the high reb A/stv ratio continued without any major changes. Genotype 133 was selected because of its high reb A content throughout the years of the study. The reb A/stv ratios were also high, depending on the amount of reb A. Genotype 196 was selected because the amount of reb A + stv was high in the basic population and this feature continued in the A and B clones. It is especially important to keep the same characteristics of the properties connected to reb A. Findings have shown that the synthesis of glycosides from *Stevia* is variable. Huang et al. (1995) conducted a survey in China and reported the stv content as 1.48%–6.98% in plant samples. It has been reported that reb A content varies from 4.5% to 12.1% and the total glycoside varies from 10.26% to 19.57%. In another study conducted in China, it was reported that the total SG concentration in some strains reached 20.5% and that a different reb A/stv ratio could be 9/1 (Morita, 1987; Shizhen, 1995). In the current study, a wide variation in the concentration of glycosides in the genotypes was observed over the 3 years.

The C clones were selected by evaluating the first, second, and third year performances of the 10 genotypes

Table 3. Minimum, maximum, and mean values of the SGs contents and selection criteria in the A clones.

	Reb A (%)			Stv (%)			Reb A + stv (%)			Reb A/stv (%)			Selection criteria
Clone no.	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	
1	4.35	5.80	4.83 ± 0.595	5.30	7.30	6.17 ± 0.837	9.80	13.10	11.00 ± 1.335	0.67	0.85	0.7869 ± 0.0729	High reb A + stv
4	5.70	8.20	6.86 ± 0.971	6.75	9.85	8.04 ± 1.428	12.70	18.05	14.90 ± 2.36	0.80	0.93	0.8587 ± 0.0626	High reb A + stv
7	6.40	7.05	6.69 ± 0.256	5.65	6.15	5.92 ± 0.195	12.15	13.05	12.61 ± 0.408	1.09	1.18	1.1303 ± 0.0347	High reb A/stv
14	5.50	7.60	6.72 ± 0.76	4.50	5.80	5.32 ± 0.311	10.00	13.40	12.04 ± 1.248	1.20	1.33	1.2621 ± 0.0542	High reb A/stv
16	4.10	5.10	4.72 ± 0.415	4.90	6.00	5.54 ± 0.472	9.00	11.10	10.26 ± 0.885	0.84	0.87	0.85184 ± 0.0111	Reb A = stv (approximately)
28	5.50	6.75	6.03 ± 0.511	4.90	6.15	5.30 ± 0.496	10.55	12.50	11.33 ± 0.882	1.03	1.27	1.1413 ± 0.094	Only stv
31	6.55	7.40	6.96 ± 0.42	4.65	5.60	5.23 ± 0.51	12.05	12.15	12.09 ± 0.18	1.17	1.59	1.339 ± 0.21	High reb A/stv
44	3.50	4.40	4.19 ± 0.152	5.25	6.50	5.97 ± 0.451	8.75	10.90	9.98 ± 0.813	0.62	0.72	0.6714 ± 0.0366	Reb A = stv (approximately)
51	3.90	5.60	4.00 ± 0.274	5.55	7.05	6.09 ± 0.604	9.65	12.15	11.08 ± 0.997	0.68	0.96	0.8233 ± 0.119	High stv
52	3.30	4.60	4.10 ± 0.218	8.10	10.90	9.36 ± 1.117	11.80	15.50	13.36 ± 1.534	0.39	0.48	0.4283 ± 0.0335	High stv
54	4.60	5.80	4.90 ± 0.43	5.95	7.80	6.81 ± 0.717	7.05	13.60	10.89 ± 2.4	0.48	0.87	0.607 ± 0.345	High reb A+ stv
56	4.30	5.40	4.79 ± 0.452	5.00	7.30	5.97 ± 0.844	9.30	12.20	10.87 ± 1.102	0.67	0.90	0.8291 ± 0.0932	High reb A+ stv
66	4.30	5.45	4.83 ± 0.333	8.00	8.45	8.27 ± 0.104	12.55	13.85	13.06 ± 0.503	0.51	0.65	0.5794 ± 0.0555	High stv
71	4.25	5.10	5.02 ± 0.143	6.15	6.50	6.30 ± 0.1275	10.40	11.60	11.13 ± 0.441	0.69	0.79	0.7662 ± 0.0423	High stv
72	4.45	5.55	4.92 ± 0.466	4.25	6.00	4.91 ± 0.702	8.70	11.55	9.93 ± 1.123	0.93	1.11	1.0289 ± 0.0668	High reb A/stv
78	3.30	4.60	4.00 ± 0.518	5.00	7.30	5.97 ± 0.844	8.75	9.90	9.29 ± 0.467	1.18	1.23	1.1956 ± 0.1075	High stv
82	6.30	11.1	7.77 ± 1.906	5.40	7.00	6.46 ± 0.644	8.50	10.90	9.88 ± 1.027	0.44	0.57	0.492 ± 0.0526	High reb A/stv
83	5.70	6.30	6.11 ± 0.241	3.90	8.00	5.20 ± 1.604	10.20	19.10	12.97 ± 3.51	1.39	1.62	1.4132 ± 0.0844	High reb A/stv
84	2.90	4.15	3.32 ± 0.535	4.25	5.15	4.60 ± 0.348	10.35	10.90	10.71 ± 0.222	1.11	1.44	1.3363 ± 0.1359	High stv
88	3.70	5.70	4.92 ± 0.792	7.20	12.10	8.67 ± 2.191	10.10	16.25	11.99 ± 2.57	0.30	0.49	0.394 ± 0.0751	High reb A
93	6.30	8.00	6.91 ± 0.686	4.30	10.15	6.23 ± 2.3	8.70	14.75	11.15 ± 2.34	0.45	1.26	1.1033 ± 0.295	High reb A
96	4.90	6.90	6.15 ± 0.638	3.00	6.40	4.97 ± 1.238	11.00	12.80	11.88 ± 0.753	1.00	2.67	1.516 ± 0.658	Reb A = stv (approximately)
98	3.20	7.15	4.55 ± 1.547	6.35	9.70	7.53 ± 1.276	12.05	14.60	13.58 ± 0.992	0.51	0.96	0.8404 ± 0.1887	High stv
100	5.05	6.70	6.04 ± 0.616	6.30	8.30	7.21 ± 0.764	9.50	13.85	11.76 ± 1.083	0.49	1.07	0.637 ± 0.242	High stv
105	3.64	5.05	4.668 ± 0.58	5.25	7.05	6.30 ± 0.669	10.30	13.35	12.34 ± 1.093	0.89	1.06	0.9612 ± 0.0714	High reb A + stv
109	-	-	-	8.15	9.05	8.54 ± 0.411	8.15	9.05	8.54 ± 0.411	-	-	-	Only stv
110	4.55	6.10	5.15 ± 0.838	4.90	6.00	5.54 ± 0.472	10.40	11.60	11.13 ± 0.441	0.69	0.79	0.7662 ± 0.0423	High reb A + stv
111	5.00	6.15	5.62 ± 0.493	6.95	8.30	7.95 ± 0.563	11.50	14.40	13.43 ± 1.127	0.65	0.73	0.7081 ± 0.0315	High reb A/stv
116	4.10	6.80	5.72 ± 1.062	3.30	4.10	3.62 ± 0.303	8.30	10.25	9.24 ± 0.777	1.50	1.65	1.5528 ± 0.0581	High reb A/stv
119	2.90	4.55	3.80 ± 0.601	3.70	6.80	4.80 ± 1.286	7.80	13.60	10.52 ± 2.26	1.00	1.43	1.2151 ± 0.1682	High reb A+ stv
129	3.95	6.60	5.17 ± 0.976	6.30	8.30	7.56 ± 0.767	9.20	12.55	11.36 ± 1.029	0.46	0.57	0.5008 ± 0.0414	High reb A/stv
133	5.70	7.35	6.81 ± 0.666	3.60	5.20	4.26 ± 0.647	7.55	11.80	9.43 ± 1.612	1.10	1.27	1.199 ± 0.0695	High reb A
141	4.70	5.10	4.88 ± 0.148	4.50	5.75	4.99 ± 0.531	10.65	13.10	11.80 ± 0.96	1.15	1.60	1.3742 ± 0.1756	High reb A + stv
152	3.30	3.80	3.575 ± 0.20	4.00	5.00	4.41 ± 0.431	8.75	9.90	9.29 ± 0.467	0.98	1.23	1.1146 ± 0.1075	Only stv
159	3.15	5.55	3.77 ± 1.007	4.65	9.20	6.42 ± 1.698	8.45	9.85	9.28 ± 0.52	0.41	0.82	0.51 ± 0.307	High stv
161	5.00	6.40	5.92 ± 0.62	6.70	8.55	7.39 ± 0.711	9.90	14.10	11.16 ± 1.089	0.44	0.65	0.5048 ± 0.0832	High reb A
185	5.70	7.30	6.48 ± 0.593	3.90	10.30	5.46 ± 2.76	9.00	11.35	10.20 ± 0.842	0.73	1.62	1.188 ± 0.657	High reb A/stv
191	6.30	7.10	6.62 ± 0.337	4.00	7.80	5.08 ± 1.648	10.20	13.50	11.56 ± 1.488	0.73	1.63	1.373 ± 0.38	High reb A/stv
196	5.20	7.10	6.46 ± 0.73	5.45	6.65	5.92 ± 0.53	12.00	13.75	12.54 ± 0.712	1.00	1.24	1.1241 ± 0.0991	High reb A
202	3.45	8.05	5.92 ± 1.636	6.70	9.90	7.92 ± 1.203	12.60	17.00	14.38 ± 1.681	0.70	0.99	0.8248 ± 0.1179	Reb A = stv (approximately)

representing the B clones in the last study conducted for 3 years. When making this selection, diterpene glycosides, which were the basic selection criteria, were considered. When assessed for the presence of diterpene glycosides, it was observed that the amount of stv changed more and the amount of reb A appeared to be more stable. During the study, 200 genotypes were selected and the 4 genotypes that emerged came to the forefront in terms of the properties evaluated (Figure). Genotype 82 contained a high amount of reb A (7.95%, 7.77%, and 6.37%, respectively) with the highest reb A/stv ratio (1.48, 1.20, and 1.48, respectively) over the 3 years and was regarded as a superior candidate in terms of the reb A/stv ratio, which maintained its stability throughout the clones. Genotype 109 was noteworthy for stv alone. Enzymatic and chemical reactions have been found to be a good material that can be converted to reb A in the next step in the biosynthesis pathway. Genotype 133 not only had a high amount of reb A (8.47%, 6.81%, and 6.40%, respectively), but was also seen to be an industrial material suitable for production and processing due to the fact that the agonistic properties were also above average throughout the study. The high reb A + stv (18.31%, 12.54%, and 13.58, respectively) and reb A (7.57%, 6.46%, and 6.2%, respectively) contents of genotype 196 showed that the synthesis of secondary metabolites in

Table 4. Some quality characteristics of the B clones.

Clone no.	Stv (%)		Reb A (%)		Reb A /stv		Reb A + stv (%)	
82	4.28	C	6.37	A	1.48	A	10.60	BCD
83	4.43	C	5.69	AB	1.29	AB	9.91	CD
100	4.75	CB	5.52	BC	1.16	BC	10.26	BCD
109	7.59	A	-	-	-	-	7.59	E
111	3.84	D	4.85	C	1.26	B	8.69	E
116	4.90	CB	6.06	B	1.18	BC	10.96	BC
133	4.35	C	6.40	A	1.34	AB	10.75	BC
185	4.40	C	6.11	AB	1.38	AB	10.50	D
191	5.10	B	6.24	A	1.16	BC	11.34	B
196	7.38	A	6.20	AB	0.89	C	13.58	A

Means within a column followed by different letters are statistically significant at $P \leq 0.01$ level.

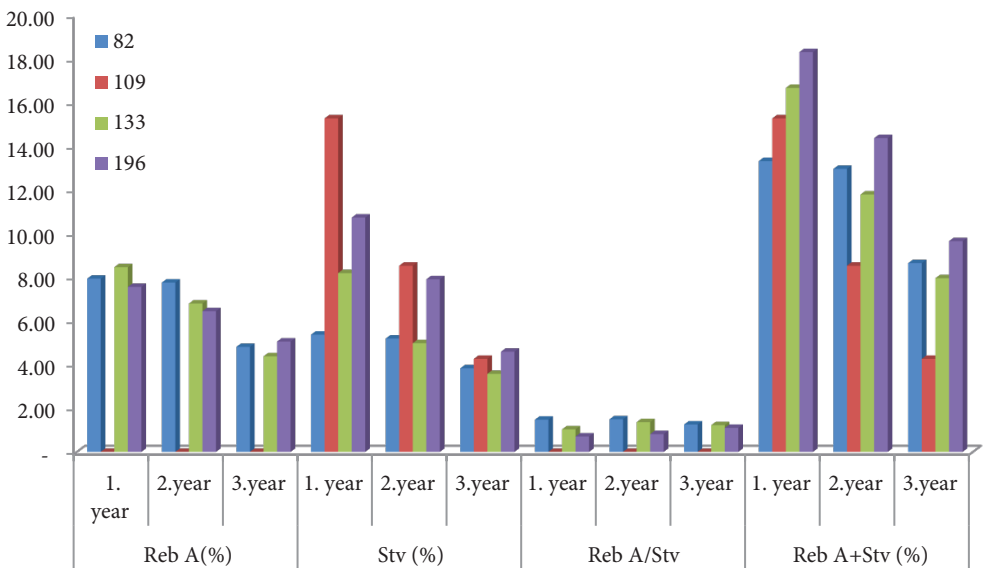

Figure. Steviol glycosides (reb A %, stv %, reb A + stv (%), and reb A/stv) contents in the selected genotypes during the study.

this genotype was intense, and it was therefore selected as a candidate for variety.

While evaluating them for selection, the agronomic performances of the candidate variety were taken into consideration. Applications have been made for the registration of clones 82, 133, and 196 (genotypes) as commercial clonal varieties. Thus, registered genotypes will be propagated clonally either by in vitro or stem cuttings methods.

In conclusion, such diversity in the raw material makes it possible to produce SGs containing a high amount of reb A by traditional extraction methods.

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