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Study of Morphogenetic and Physiological Responses of *Stevia rebaudiana* to Colchicine Treatment in An in Vitro Micropropagation System and Ex Vitro Adaptation

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Abstract

This study investigated the dose- and time-dependent effects of colchicine treatment (0.0125-0.1%, 24-48 h) on the morphogenetic characteristics, viability, and subsequent adaptation of *Stevia rebaudiana* Bertoni. Seeds were treated with colchicine, followed by micropropagation, rooting with auxins (IBA, IAA), and acclimatization to a soil substrate. The most favorable morpho-physiological responses, including an increase in leaf area and thickness, enhanced branching, and larger shoot size, were observed after a 24-hour treatment with 0.025% colchicine. However, higher concentrations and longer exposure significantly reduced viability, indicating strong phytotoxicity. The use of auxins (especially IBA) substantially improved rooting efficiency and suppressed callus formation. The developed staged acclimatization protocol ensured high survival, though with a noticeable difference between the control (85%) and experimental groups. A colchicine concentration of 0.025% with a 24-hour exposure was determined to be optimal for inducing beneficial morphological modifications while maintaining acceptable viability losses under ex vitro conditions. Although the observed changes morphologically correspond to polyploid forms, direct cytogenetic confirmation is required. This work lays the groundwork for using colchicine to modify Stevia morphology in biotechnological propagation systems.

Keywords: *Stevia rebaudiana*; Colchicine; Morphogenesis; Micropropagation; Auxins.
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1. Introduction

Stevia rebaudiana Bertoni (family Asteraceae) is a valuable perennial plant, globally recognized as a natural source of high-intensity, non-caloric sweeteners (stevioside and rebaudioside) widely used in the food and pharmaceutical industries.[1-3] The growing global demand for sugar substitutes has positioned *S. rebaudiana* as a crop of significant economic and biotechnological value. However, its large-scale cultivation is hampered by limitations in conventional propagation: natural propagation is limited by low and unstable seed germination, while traditional vegetative methods (cuttings) have a low multiplication rate and risk pathogen transmission.[4-7] To overcome these challenges, advanced biotechnological approaches, particularly in vitro micropropagation, are crucial for producing genetically uniform, pathogen-free planting material on a commercial scale to meet industrial demands.[4-6,8-10]

One approach to improving the agronomic and morpho-physiological traits of plants is the induction of polyploidy. Colchicine, an antimitotic alkaloid, is a widely used and effective agent for this purpose as it inhibits spindle formation during cell division.[11,12] This technique is a cornerstone of modern plant breeding, known to enhance biomass, produce larger organs (the "gigas" effect), and increase the concentration of valuable secondary metabolites.[13] It has been successfully applied to improve the productivity and chemical profiles of various industrial crops and medicinal plants like *Digitalis lanata*.[12,14] In addition to inducing polyploidy, colchicine can also have direct morphogenetic effects,

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influencing cell division and elongation, which can lead to beneficial phenotypic changes even without a confirmed change in ploidy.[11-14]

For *S. rebaudiana*, systematic studies on the dose-dependent effects of colchicine on key micropropagation parameters remain fragmented. While some studies have explored polyploidy induction a comprehensive investigation that integrates the initial treatment with its downstream consequences on rhizogenesis and, crucially, the final ex vitro adaptation success is still lacking.[15-17] Furthermore, the interaction of colchicine pretreatment with auxin application during the rooting stage has not been sufficiently studied.

The aim of the present study was to comprehensively evaluate the dose-dependent effect of colchicine on the morphogenetic parameters (survival, shoot growth, rhizogenesis), morphometric characteristics, and adaptive potential of *Stevia rebaudiana* during in vitro micropropagation and subsequent acclimatization to ex vitro conditions.

2. Materials and Methods

2.1 Plant Material

Seeds of *Stevia rebaudiana Bertoni* were used in this study. The seeds were collected from mother plants adapted to the agro-climatic conditions of the southeastern region of the Republic of Kazakhstan, grown at the Agro-biological Station of Al-Farabi Kazakh National University in the Almaty region (Fig. 1).

Fig. 1: Seeds of *Stevia rebaudiana* Bertoni grown at the Agro-biological Station of KazNU.

2.2 Seed Sterilization and Polyploidy Induction

Seed sterilization was performed in two stages. First, seeds were immersed in 70% ethanol for 30 s with constant stirring. Subsequently, they were transferred to a 0.1% mercuric chloride ($HgCl_2$) solution containing two drops of Tween-60 surfactant and agitated for 5-7 min. Finally, the seeds were rinsed three times with sterile distilled water, with each wash lasting 5 min. For polyploidy induction, sterilized seeds were incubated in aqueous colchicine solutions at various concentrations (0.0125%, 0.025%, 0.05%, and 0.1%) for two exposure durations (24 and 48 h). Control seeds were incubated in sterile distilled water under the same conditions. After treatment, the seeds were sown on a hormone-free 1/10 MS medium containing 3% sucrose and 0.8% agar (Difco-Bacto). The pH was adjusted to 5.8 before autoclaving. Cultures were initially maintained in complete darkness for 2-3 days at 23 ± 2 °C and then transferred to a light chamber with a 16-hour photoperiod.

2.3 Microclonal Propagation and Rooting

Following germination, seedlings were used for microclonal propagation. Apical and axillary shoots were excised and used as microcuttings. To stimulate rhizogenesis, the basal parts of the microcuttings (approx. 0.5 cm) were pre-treated by dipping them in aqueous solutions of either indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA) at a concentration of 50 mg/L for two hours. Control microcuttings were treated with sterile water. After treatment, the microcuttings were cultured on a ½ MS nutrient medium with a reduced sucrose concentration (2%). Cultures were maintained at 23 ± 2 °C under a 16-hour photoperiod and a relative air humidity of 65-70%.

2.4 Acclimatization to Ex Vitro Conditions

Well-rooted plantlets were carefully removed from the culture vessels, and their root systems were gently washed with sterile water to remove any residual agar medium. To increase survival rates during acclimatization, a pre-transplant treatment was applied: the aerial parts were sprayed with a 10% aqueous glycerin solution, and the root system was immersed in a 0.001% IAA solution for 7-10 minutes.

The plantlets were then transferred to plastic pots containing a sterilized substrate mixture of soil, sand, and perlite (1:1:1, v/v/v) with a neutral pH (7.0). The soil mixture had the following characteristics: macronutrient content – potassium (270-320 mg/L), phosphorus (220-270 mg/L), nitrogen (20-250 mg/L); and trace amounts of micronutrients. A 3-5 cm layer of expanded clay was placed at the bottom of the pots for drainage. The pots were covered with transparent lids to maintain high humidity and were kept in a climatic chamber for three weeks at 23±2 °C, 60-70% relative humidity, and a 16-hour photoperiod. The lids were gradually opened for increasing durations each day to acclimatize the plants to lower humidity. Upon completion of the three-week adaptation stage, the plants were transferred to greenhouse conditions for 1.5 months before being planted in the open ground on the experimental plots of the Agro-biological Station.

2.5 Data Collection and Statistical Analysis

Morphogenetic parameters, including explant survival rate (%), primary shoot length (cm), number of internodes, leaf area (cm^2), number of main roots, main root length (cm), and adventitious root formation (%), were recorded after 21 days of in vitro culture. During the ex vitro stage, shoot length, branching, leaf number, leaf area, leaf thickness (µm), and survival rate (%) were measured after three weeks of acclimatization. All experiments were performed in a completely randomized design with three replicates. The data are presented as mean ± standard error (SE). The statistical significance of the differences between means was determined by one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test for multiple comparisons, using STATISTICA 10.0 software. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

Our study revealed a clear dose- and time-dependent effect of colchicine on the seed germination and subsequent morphogenesis of *Stevia rebaudiana*. The strong inhibitory effect observed at higher concentrations (0.05-0.1%) is consistent with colchicine's well-established role as a potent antimitotic agent.[11,12] This pronounced cytotoxicity, leading to a germination rate as low as 13% at 0.1% concentration (Fig. 2), is a documented phenomenon in numerous plant species, as high doses of colchicine disrupt microtubule polymerization, leading to mitotic arrest and ultimately, cell death.[13,16,18,19] The LD_{50} (lethal dose, 50%) in our experiments was found to be around 0.05% for a 24-hour exposure, a value comparable to those reported for other herbaceous species, though sensitivity can vary significantly depending on the genotype and seed coat permeability.[14,15] The morphological differences between treatments are visually evident in Fig. 3. The control seedlings (Fig. 3A) exhibit normal, vigorous development. In contrast, treatment with 0.1% colchicine (Fig. 3C) resulted in severely stunted growth, thickened and shortened hypocotyls, and poor root development, all classic signs of acute cytotoxicity. The optimal 0.025% concentration (Fig. 3B) presents an intermediate phenotype, showing robust seedlings that are morphologically comparable to the control but are known from our data (Table 1) to possess altered characteristics, indicating successful induction of morphogenetic changes without lethal toxicity.

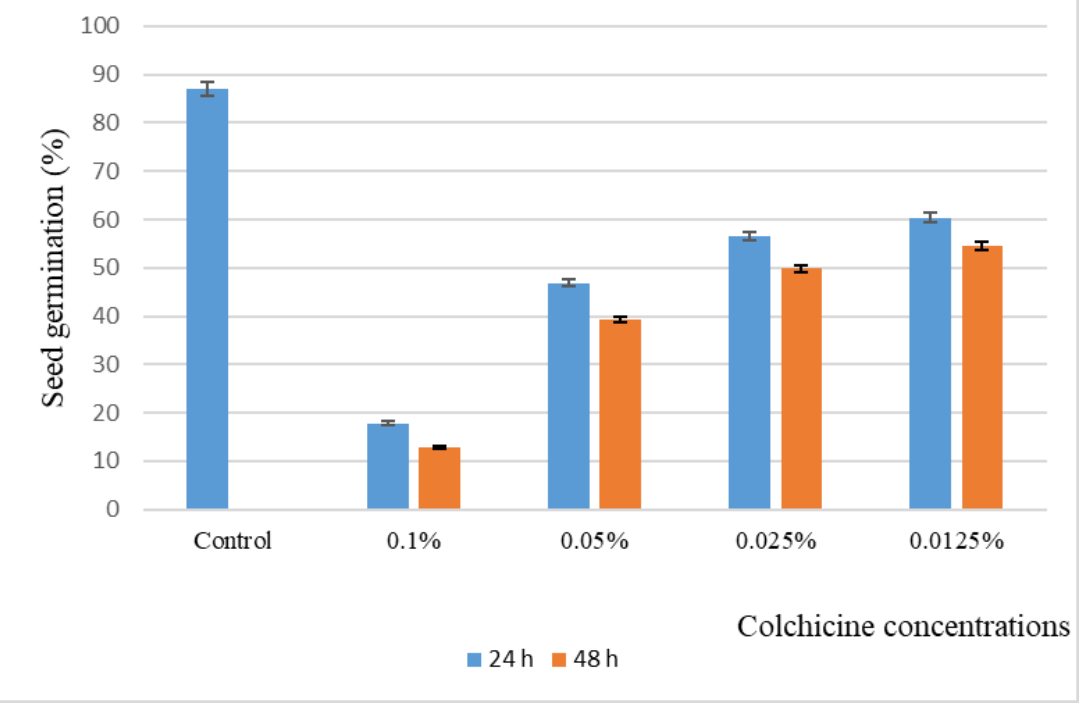

Fig. 2: The effect of colchicine on stevia seed germination.

Notably, the biphasic response, where lower concentrations of colchicine (0.0125-0.025%) exhibited a stimulatory or less inhibitory effect on certain morphometric parameters after the initial toxic shock. This phenomenon can be interpreted as a manifestation of hormesis, where low doses of a toxic substance induce a beneficial stress response.[20,21] The optimal results obtained with 0.025% colchicine for 24 hours, which yielded the best balance of morphometric parameters (Table 1) and viable seedlings (Fig. 3), suggest a delicate balance between inducing desirable morphogenetic changes and maintaining plant viability. Similar optimal concentration ranges have been identified for other medicinal

Fig. 3: Morphology of *Stevia rebaudiana* seedlings after 24-hour colchicine treatment: (A) control (untreated); (B) 0.025% colchicine; (C) 0.1% colchicine.

Table 1: The effect of colchicine on the survival and morphogenetic parameters of *Stevia rebaudiana* plants under in vitro conditions (21 days of cultivation).

Colchicine concentration, %	Explant survival rate, %	Primary shoot length, cm	Number of internodes	Leaf area, cm²	Number of main roots	Main root length, cm	Adventitious root formation, %
Control	65±1.7	16.7±0.4	5±0.08	1.2±0.03	6±0.15	4.5±0.12	40±0.9
				24 hour			
0.1%	0	3.5±0.06***	2±0.04	0.5±0.01	1±0.025	0.6±0.02	0
0.05%	19±0.5***	6.5±0.16**	5±0.12	1±0.023	2±0.03	1.0±0.02	5±0.09***
0.025%	32±0.7***	10±0.26**	5±0.1	1.5±0.03	4±0.07	5.8±0.15	12±0.28***
0.0125%	43±1.0***	16±0.3	6±0.15	1.8±0.04	7±0.17	7±0.15*	18±0.35***
				48 hour			
0.1%	0	2.7±0.06***	2±0.05**	0.3±0.007	1±0.02***	0.3±0.006	0
0.05%	10±0.26***	4.6±0.1***	2±0.04**	0.6±0.012	1±0.025***	0.7±0.01	3±0.07***
0.025%	26±0.5***	8±0.2***	3±0.07*	1.3±0.03	2±0.05***	5.2±0.09	10±0.25***
0.0125%	32±0.8***	12±0.3*	4±0.1	1.5±0.035	5±0.08	4.8±0.1	15±0.4***

Note: 1. * - at P>0.95**,** 2. at P>0.99***, 3. - at P>0.999, 4. K – control.

plants, such as *Digitalis lanata*, highlighting a common principle in chemical mutagenesis.[14] The observed increase in leaf size and shoot branching in this treatment group can be attributed to the so-called "gigas" effect, a typical morphological marker of successful polyploidy induction, which results from larger cell sizes and altered hormonal balances.[12,22-24]

The synergistic effect observed between colchicine

pretreatment and subsequent auxin-induced rhizogenesis is a key finding of this study. The superiority of IBA over IAA in promoting a highly branched root system is significant (Table 2), a finding consistent with other *in vitro* studies of *Stevia*.[25-27] IBA is known to be more stable than IAA and is converted slowly into active auxin within the plant tissues, providing a sustained stimulus for root initiation that also modulates expansin expression, affecting root length.[28,29]

The complete suppression of callus formation is particularly noteworthy. This effect can be attributed to the downregulation of key pluripotency genes like *WUSCHEL* and *SHOOTMERISTEMLESS*, which are inhibited by auxin treatment, thus preventing undifferentiated cell proliferation at the shoot base.[30,31] Furthermore, colchicine pretreatment appears to sensitize the pericycle cells to this auxin signal, potentially by modulating the expression of auxin receptors like TIR1/AFB and transcription factors such as ARF7/19, as suggested by recent molecular studies.[32,33]

Fig. 4 visually substantiates the pronounced efficacy of this synergistic protocol. Control microcuttings (Fig. 4A), rooted

Table 2: Morphogenetic parameters of *Stevia rebaudiana* microcuttings under the influence of auxins (IBA, IAA) after colchicine induction (in vitro, 21 days of cultivation).

Treat-ment	Colchi-cine concen-tration, %	Root initiation period, days	Number of roots	Root length, cm	Adventitious roots			Callus formation at shoot base, %	Number of aerial roots
					Number	length, cm	Frequency, %		
Control		7-12	6±0.2	3.0±0.1	-	-		57±2.3	9±0.4
IBA	0.05%	5-7	10±0.1***	3.8±0.01	5±0.1	0.7±0.01	15±0.3	0	2±0.05***
	0.025%	3-5	17±0.9***	4.7±0.2*	10±0.3	1.3±0.03	33±2	0	7±0.23*
	0.0125%	3-5	13±0.6***	7.3±0.3***	15±0.3	1.8±0.04	45±1.2	0	3±0.06***
IAA	0.05%	6-8	7±0.2	3.5±0.01	3±0.1	0.5±0.01	9±0.3	0	-
	0.025%	6-8	8±0.3	4.1±0.2	6±0.2	0.8±0.02	13±0.6	0	-
	0.0125%	6-8	10±0.1***	4.5±0.2	11±0.4	1.5±0.03	37±1.2	0	-

Note: 1. * - for P>0.95**, 2. - for P>0.99 ***, 3. - for P>0.999, 4. K – control.

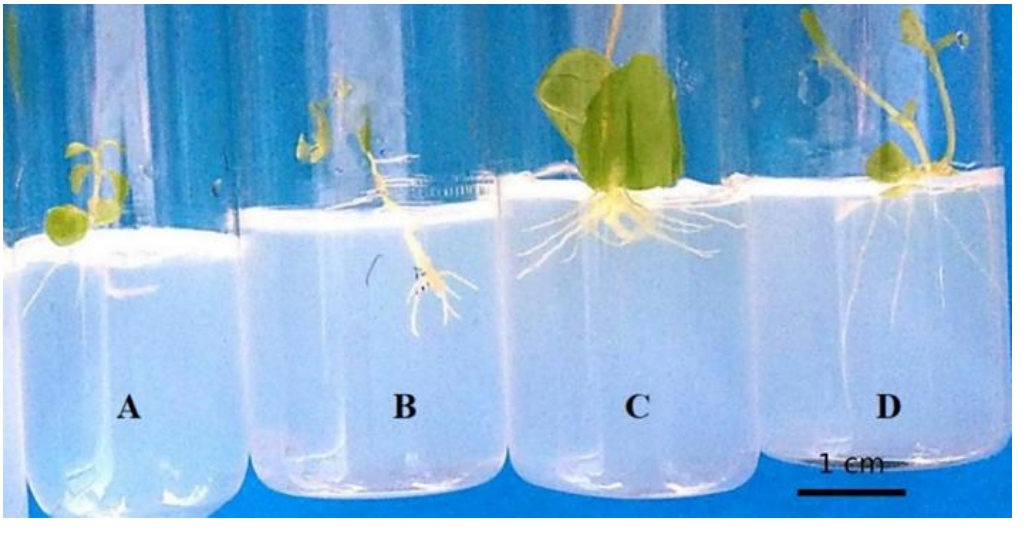

Fig. 4: The effect of IBA on the rooting of *Stevia rebaudiana* microcuttings under in vitro conditions. (A) Control; (B – D) variants with colchicine pretreatment: (B) 0.05%, (C) 0.025%, and (D) 0.0125%.

without IBA, displayed a poor root system dominated by callus at the base. Treatment with a high, suboptimal colchicine concentration (0.05%) followed by IBA application (Fig. 4B) shows some improvement but still exhibits limited root growth. In stark contrast, microcuttings pre-treated with the optimal (0.025%, Fig. 4C) and low (0.0125%, Fig. 4D) colchicine concentrations develop a dense, highly branched, and architecturally superior root system. This robust root

structure is ideal for nutrient uptake and is a key factor for successful ex vitro acclimatization, directly demonstrating the practical benefit of our combined treatment.

Furthermore, our protocol effectively eliminates the common morphogenetic anomalies that hinder *Stevia* micropropagation, as illustrated in Fig. 5. Untreated microcuttings frequently develop undesirable traits such as intensive basal callus (Fig. 5A), the formation of aerial roots in the nodes (Fig. 5B), or a combination of both (Fig. 5C). These abnormalities, often linked to hormonal imbalances within the culture system,[34] compromise the quality and viability of the plantlets. The complete absence of these traits in our IBA-treated plants (as seen in Fig. 4C and 4D) underscores the effectiveness of the protocol in normalizing development and producing high-quality, uniform regenerants ready for acclimatization.

Fig. 5: Morphogenetic anomalies in untreated *Stevia rebaudiana* microcuttings in vitro. (A) Intensive callus formation in the basal shoot zone; (B) Formation of aerial roots in the nodes; (C) Combined abnormalities (callus and aerial roots).

This represents a significant practical improvement of the protocol, leading to the formation of architecturally sound plantlets with higher adaptive potential (Fig. 4) and the complete suppression of common issues in *Stevia* micropropagation, such as callus and aerial roots (Fig. 5).[34]

A critical aspect highlighted by our results is the significantly lower ex vitro survival rate of colchicine-treated plants (31–37%) compared to the control group (85%) (Table 3). This observation is crucial for the practical application of this method.

The reduced viability is likely a consequence of several factors. Firstly, residual phytotoxicity of colchicine may persist, leading to long-term physiological stress that compromises the plant's ability to adapt to a non-sterile

Table 3: The effect of colchicine pretreatment on the morphometric parameters and survival of *Stevia rebaudiana* plants after acclimatization.

Colchicine concentration, %	Shoot length, cm	Branching (no. of shoots)	Leaves			Survival rate, %
			Number	area, cm²	thickness, µm	
Control	25 ± 0.6	6 ± 0.15	28±0.7	24.8±0.6	22.2±0.5	85±2.1
0.05%	25.9 ± 0.7	7 ± 0.8	32±0.8**	24.2±0.5	24.9±0.6	25±0.5***
0.025%	28.3 ± 0.7	9 ± 0.22**	36±0.9***	29.5±0.7***	26.8±0.7**	31±0.8***
0.0125%	26.9 ± 0.7	8 ± 0.19*	34±0.8***	26.3±0.6**	25.7±0.4*	37±0.9***

Note: 1. * - at P>0.95, 2. ** - at P>0.99***, 3. - at P>0.999, 4. K – control.

environment.[35,36] Secondly, while colchicine-induced changes like larger leaves and thicker stems are desirable, they may also lead to anatomical or physiological imbalances, such as altered stomatal function or a less efficient root system, making the plantlets more vulnerable to transplantation shock and pathogens.[37,38]

Therefore, developing strategies to improve survival is essential for translating these findings into practice, a common challenge in micropropagation.[37,39] Future optimization could involve several approaches: (1) implementing a more gradual, extended acclimatization period with finer control over humidity and light intensity;[40] (2) incorporating a "recovery" phase on a hormone-free medium after rooting but before acclimatization to alleviate residual stress; and (3) enriching the soil substrate with biostimulants or inoculating it with beneficial soil microbes, such as plant growth-promoting rhizobacteria (PGPR), to enhance root health and nutrient uptake.[37,41,42] Addressing this challenge is a key priority for developing a robust protocol for the large-scale propagation of modified *Stevia* lines.

Despite the lower survival rates, it is crucial to note that the plants which successfully acclimatized exhibited the desired positive morphological changes (Table 3). The optimal treatment (0.025% colchicine) led to a significant increase in key biomass indicators, including leaf area (19%), leaf blade thickness (20%), and shoot branching (50%). These phenotypic enhancements are consistent with the "gigas" effect often associated with induced polyploidy and are of high practical importance, as greater leaf biomass is directly linked to a higher potential for stevioside accumulation.[11,12,16] These promising morphological findings, coupled with the established protocol improvements, provide a strong basis for the subsequent cytogenetic and biochemical validation outlined in our conclusion.[40]

4. Conclusion and perspectives

This study successfully established an effective protocol for inducing significant morpho-physiological modifications in *Stevia rebaudiana* using colchicine within an in vitro propagation system. We identified that a 24-hour treatment with 0.025% colchicine represents an optimal balance, inducing beneficial traits such as increased leaf area (19%), leaf thickness (20%), and shoot branching (50%), while maintaining an acceptable, albeit reduced, ex vitro survival rate (31%). Furthermore, the developed protocol, which integrates colchicine pretreatment with IBA-stimulated rooting, significantly improves rhizogenesis and eliminates common morphogenetic anomalies, representing a valuable refinement for *Stevia* micropropagation.

A primary limitation of the current study is the absence of direct cytogenetic verification (*e.g.*, flow cytometry or chromosome counting) to confirm changes in ploidy level. Therefore, the observed morphological changes should be interpreted as characteristics of presumptive polyploids. Another limitation is the lack of biochemical analysis to quantify the steviol glycoside content in the modified plants, which is the ultimate goal of such breeding efforts.

Consequently, this work lays a critical foundation for future research perspectives. The immediate priorities are to (1) perform flow cytometry on the most promising lines to confirm their ploidy status and (2) conduct a quantitative analysis of key steviol glycosides (stevioside and rebaudioside A) to determine if the observed morphological enhancements correlate with increased sweetener yield. Successfully validated polyploid lines with superior biochemical profiles can then be advanced to field trials to assess their agronomic performance and stability, opening new perspectives for the industrial cultivation of high-yielding *Stevia* varieties.

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Conflict of Interest

The authors declare no conflict of interests.

Supporting Information

Not applicable.

CRediT Statment

Saltanat Asrandina: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - Original draft preparation, Writing - Review and editing, Visualization, Supervision, Project administration, Funding acquisition. **Zhanar Chunetova:** Software, Validation, Formal analysis, Investigation, Writing - Review and editing. **Saule Atabayeva:** Methodology, Investigation, Data curation. **Yerlan Kirshibayev:** Software, Investigation. **Saule Kenzhebayeva:** Investigation, Data curation. **Nurgul Mamytova:** Formal analysis, Investigation. **Sabina Shoinbekova:** Investigation, Data curation. All authors have read and agreed to the published version of the manuscript.

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