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Field performance and characterization of two stable stevia mutants with increased biomass and rebaudioside - 'A' content

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ABSTRACT

Stevia is a perennial herbaceous medicinal plant that has received commercial attention from the pharma industry because of its sweetening properties. In the present study, two Bidhan *Stevia rebaudiana* mutants ('BSRV1' and 'BSRV2') were selected (Source development). All major vegetative growth traits, steviol glycoside contents, and flow cytometry analyses were carried out for 'BSRV1' and 'BSRV2' along with the control (non-irradiated). The *in vitro*-regenerated mutants along with the control plantlets were initially *ex vitro* acclimatized successfully. The plantlets of 'BSRV1' exhibited exceptional field growth performance, followed by 'BSRV2'. The 'BSRV1' plants after three months of field transfer produced the maximum (63.47 cm) plant height, number of shoots (6.40), number of branches (10.40), number of leaves (129.40), leaf area (13.84 cm^2), fresh leaf yield (2.05 t ha^{-1}) per single cutting, and dry leaf yield (0.38 t ha^{-1}) per single cutting followed by 'BSRV2' and the control. The stability of the ploidy level of both mutants against the control was ascertained *via* flow cytometry. The results of the high-performance liquid chromatography analysis of rebaudioside-A were the highest for 'BSRV1' (95.45 mg g^{-1} DW), followed by 'BSRV2' (89.93 mg g^{-1} DW) and the control (65.30 mg g^{-1} DW). Stable mutants with distinct morphological traits and ameliorated rebaudioside-A content can be exploited as new genotypes by the growers and pharmaceutical industry.

Keywords: Gamma irradiation, stable mutants, HPLC, steviol glycosides, flow cytometry.

INTRODUCTION

Stevia rebaudiana Bert. (Asteraceae family) is a perennial, self-incompatible, and industrially important herbaceous medicinal plant that originated from the Paraguay region in South America and is now widely cultivated across the world, particularly North America, Europe, and Asia (Lewis, 11). Most stevia breeding activities worldwide have focused on increasing the concentration of key steviol glycosides (stevioside and rebaudioside-A) (Hajihashemi *et al*., 5). Owing to the high demand for natural sweeteners and antioxidant compounds, stevia cultivation and domestication is necessary. This crop has undergone recent domestication and has been performed by small- and medium-sized farmers because of the lack of appropriate adapted and accessible varieties (Cosson *et al*., 2). The present area under stevia is 32,000 ha worldwide, while the requirement of stevia has increased from 5,100 t in 2014 to 8,507 t in 2020 (Singh *et al*., 13). Furthermore, as of 2023, no stevia varieties have been documented with enhanced steviol glycoside content by mutation, although approximately 3,433 varieties have been recorded in the IAEA Mutant Variety Database (MVD) in 23 other different crops (IAEA, 6). Hence, the demand for this crop is expected to increase in the near future, which would necessitate new stevia genotypes with higher steviol glycoside contents. Our research group has developed two mutants of stevia *via in vitro* mutagenesis *via* gamma irradiation (Subrahmanyeswari *et al*., 14).

The extent of the morphological divergence and stability of the ploidy status of the mutants is an imperative step in mutagenesis studies. In this context, flow cytometry analysis is one of the primary approaches used to validate the comparative genetic status of the mutants and the control. The present study aims to characterize two gamma radiation-induced *in vitro*-regenerated mutants of stevia by analyzing their growth and field performance under *ex vitro* conditions to quantify the content of a major steviol glycoside (rebaudioside-A) *via* high-performance liquid chromatography (HPLC) and to determine the ploidy status *via* flow cytometry analyses to determine how these induced mutants are distinguishable from control plants.

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MATERIALS AND METHODS

The experiment was undertaken during 2022-24. The well-rooted *in vitro*-regenerated mutant plantlets ('BSRV1' and 'BSRV2') and the control plantlets (Subrahmanyeswari *et al*., 13) were removed from the culture vessels and cleaned with tap water, followed by treatment with Bavistin® (Carbendazim 50% WP) fungicide solution for 2–3 mins, after which the roots were trimmed to ~2–3 cm in size. The uniform cuttings were treated with 250 mg/ L IBA solution. The cuttings were then transferred to sand as a substratum in acclimatization trays (with light-emitting diodes and humidifiers) for primary acclimatization to ensure the required aeration and favorable relative humidity (Fig. 1a). Intermittent spraying was performed with water at regular intervals to maintain the moisture content at the optimum levels. The primary acclimatized plantlets were shifted for secondary acclimatization to pots containing sand and soil (1:1; *w/w*) to assess their *ex-vitro* survival at Mondouri Farm, Bidhan Chandra Krishi Viswavidyalaya, West Bengal (22°56′42.88″N 88°32′0.86″E and altitude of 9.75 m), in the alluvial plains of the Indian subcontinent with a subtropical climate. The plants were raised under shade net (50%). An average temperature of 26°±2°C with 80% relative humidity and 200 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density was maintained during the acclimatization procedure for adequate growth and development of the mutants. The plants were maintained following the standard package of practices.

The *ex-vitro* performance of the mutants was compared against that of the control at three months after their field transfer (during November-February in the years 2023 and 2024). The primary acclimatized plantlets in sand were shifted for secondary acclimatization to pots containing sand and soil (1:1; *w/w*) to assess their *ex-vitro* field growth performance. All the primary field growth traits were recorded to evaluate the differences in morphology and growth between the mutants and the control. Data regarding the plant height (cm), total number of shoots, number of branches, number of leaves, leaf area (cm^2), and leaves were collected from the basal portion above the 3rd node for fresh leaf yield (t ha^{-1}) per single cutting, and dry leaf yield (t ha^{-1}) per single cutting (dried in an oven at 60°C for 24 h) were recorded during the three-month growth period.

Assessment of ploidy levels in mutants, as well as control plants, was carried out via the improved Cystatin UV ploidy Partec method. Fine nuclei suspensions were made by slicing 2.0–2.5 cm^2 stevia leaf tissues into small pieces *via* a sharp razor blade with the addition of 5 mL of freshly prepared modified Galbraith's extraction buffer (Galbraith *et al*., 3) (pH = 7), which included 200 mM Tris (hydroxymethyl) aminomethane, 45 mM magnesium chloride, and 0.5% (*v/v*) Triton X-100.

Rebaudioside-A in the leaf samples (1.5 g DW) of the mutants and the control plants was quantified (three months after field transfer) using HPLC analysis (Waters™, Milford, USA). Leaves from the mutants and the control were uniformly collected, washed with running tap water, and then dried under blotting paper in a hot-air oven at 60°C for 48 h. Approximately 1.5 g of finely oven-dried powdered samples were taken for Soxhlet extraction with 100 mL of HPLC-grade methanol. The extract was air-dried, and 5 mg of Soxhlet extract from each sample was diluted in 1 mL of HPLC-grade methanol and vortexed for 2–3 min. The obtained samples were centrifuged for 5 min at 5000 rpm at room temperature. The supernatant was filtered through a syringe filter (0.22 µm) prior to HPLC analysis. A rebaudioside-A (Sigma-Aldrich, USA) standard was prepared according to Subrahmanyeswari *et al*. (14). The amount of rebaudioside-A was measured via a Waters Breeze™ 2 HPLC system at 210 nm with a photodiode-array detector (PDA) and a C18 column. The mobile phase consisted of HPLC-grade water and acetonitrile at a 20:80 (*v/v*) ratio. The run time was kept constant, *i.e.*, 20 min for both the standard and the mutants, including the control, while the injection volume and the flow rate were maintained at 20 mL and 1 mL min^{-1}, respectively. The peak areas of the samples were analyzed, and the quantity of rebaudioside-A was measured on a mg g^{-1} dry weight (DW) basis.

All the experiments were carried out in a randomized block design, wherein each experiment consisted of 15 replications with 100 plantlets to minimize errors in the experiment. The two-year pooled data were collected and evaluated via SPSS (ver. 20.0, SPSS Inc., Chicago, USA) software. The significant differences between the mutants (mean and standard error values) were computed *via* Tukey's test at the $P = 0.05$ level.

RESULTS AND DISCUSSION

Notable differences in all the growth traits considered during initial acclimatization were observed among the *in vitro*-regenerated mutants ('BSRV1' and 'BSRV2') and the control plantlets. The pre-sterilized sand substratum was successfully used to acclimate the plantlets because of its excellent aeration, drainage properties and water retention ability and it ensured the necessary anchorage, which plays a crucial role in allowing the *in vitro* plantlets to survive under *ex vitro* growth conditions and healthy root growth and development (Fig. 1a, b). The rooting of roots improved the formation of

uniform roots. New leaves emerged at the top portion of the plantlets after successful acclimatization, after which the leaves tilted toward the sand (Fig. 1b). Initially, the plantlets were acclimatized in the sand and subsequently transferred to pots containing sand and soil (1:1; *w/w*), which was effective at providing good aeration and a favorable balance between moisture retention and drainage for the further shoot–root growth and development. During the initial stages of acclimatization, the mutants adjusted themselves for photoautotrophic growth, whereas during secondary acclimatization, they were exposed to field conditions (Lata *et al*., 10). This gradual acclimatization procedure helps the mutants adapt from the laboratory to external environmental conditions with 100 percent survival.

The shoot growth traits of the mutants significantly differed under *ex vitro* conditions. Compared with those of the control, the morphology of the mutants ('BSRV1' and 'BSRV2') significantly differed at 1, 2 and 3 months after field transfer (Fig. 1c-j). Mutant 'BSRV1' produced the maximum plant height (63.47 cm), number of shoots (6.40) and number of branches (10.40), followed by 'BSRV2' and the control after three months of field transfer (Table 1). Compared with the control, 'BSRV1' resulted in profuse branching and biomass, followed by 'BSRV2'. The control plants presented the minimum plant height (31.20 cm), number of shoots (2.87) and number of branches (4.67) after three months of field transfer. In the case of the control, the plants were erect with an upright branching pattern, whereas the mutants exhibited a spreading and profuse branching pattern (Fig. 1h-j).

Leaf biomass is one of the most significant characteristics that indirectly affect the amount of steviol glycoside. Steviol glycosides accumulate in greater quantities in leaves than in other tissues, such as roots and stems. This was most likely caused by a change in stevia tissue, which affected leaf size

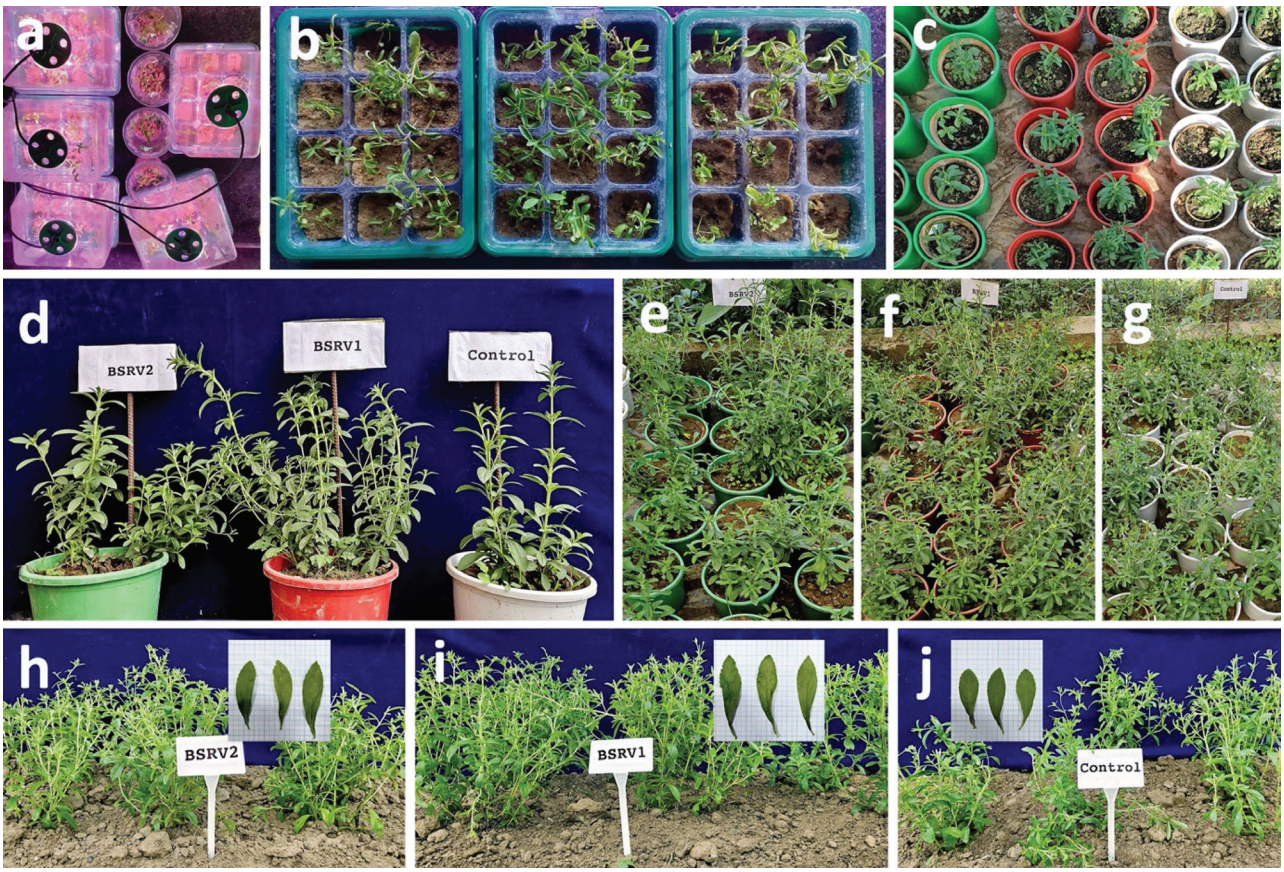

Fig. 1. Acclimatization and comparative field performance of *Stevia rebaudiana* Bert. mutants and control plants. a. Acclimatization, b. primary acclimatized plantlets, c. growth comparison among 'BSRV2', 'BSRV1', and Control (after one month of field transfer), d. growth comparison among 'BSRV2', 'BSRV1', and Control (after two months of transfer), growth comparison among e. 'BSRV2', f. 'BSRV1', and g. Control (after two months of transfer), growth comparison among h. 'BSRV2', i. 'BSRV1', and j. Control (inset, leaf size variation) (after three months of transfer).

and might have been caused by gamma radiation. Compared with the control plants (Table 1), the mutants ('BSRV1' and 'BSRV2') presented superior *ex vitro* growth performance and leaf biomass after three months of field transfer. The maximum number of leaves (129.40), leaf area (13.84 cm^2), fresh leaf yield (2.05 t ha^{-1}, and dry leaf yield (0.38 t ha^{-1}) were recorded for 'BSRV1', followed by 'BSRV2', where the control plants produced the minimum number of leaves (70.40), leaf area (6.14 cm^2), fresh leaf yield (1.12 t ha^{-1}), and dry leaf yield (0.20 t ha^{-1}) per plant after three months of field transfer (Fig. 1h-j). Compared with the control plants, the mutants ('BSRV1' and 'BSRV2') presented greater leaf areas after three months of field transfer. To date, no such detailed comparative studies have been performed.

HPLC evaluation of methanolic stevia leaf extracts revealed a significant variation in the rebaudioside-A concentration in the mutants compared with the control (Table 1). Comparative HPLC chromatograms of the representative rebaudioside-A standard along with mutants ('BSRV1' and 'BSRV2') and the control stevia (three months after field transfer) are shown (Fig. 2). The maximum concentration of rebaudioside-A (95.45 mg g^{-1} DW) was recorded in 'BSRV'1, followed by 'BSRV2' (89.93 mg g^{-1}), whereas the control plants produced lower amounts (65.30 mg g^{-1} DW). The 'BSRV1' mutant, followed by 'BSRV2', outperformed the control in terms of *ex vitro* field performance and rebaudioside-A

Table 1: Comparative field performance of the mutants and control plants of *Stevia rebaudiana* Bert. (three months after field transfer).

Mutant	Plant height (cm)	No. of shoots	No. of branches	No. of leaves	Leaf area (cm^2)	Fresh leaf yield (t ha^{-1}) single cutting	Dry leaf yield (t ha^{-1}) single cutting	Reb-A (mg g^{-1}) DW
Control	31.20 ± 0.73c	2.87 ± 0.19c	4.67 ± 0.21c	70.40 ± 1.17c	6.14 ± 0.28c	1.12 ± 0.29c	0.20 ± 0.04c	65.30 ± 1.15c
'BSRV1'	63.47 ± 1.29a	6.40 ± 0.34a	10.40 ± 0.59a	129.40 ± 1.73a	13.84 ± 0.21a	2.05 ± 0.41a	0.38 ± 0.14a	95.45 ± 0.46a
'BSRV2'	41.27 ± 0.93b	5.00 ± 0.29b	8.13 ± 0.43b	93.47 ± 2.12b	11.52 ± 0.28b	1.45 ± 0.45b	0.26 ± 0.06b	89.93 ± 0.58b

The data for each *column* indicated with different *letters* are significantly different according to Tukey's test at $P = 0.05$.

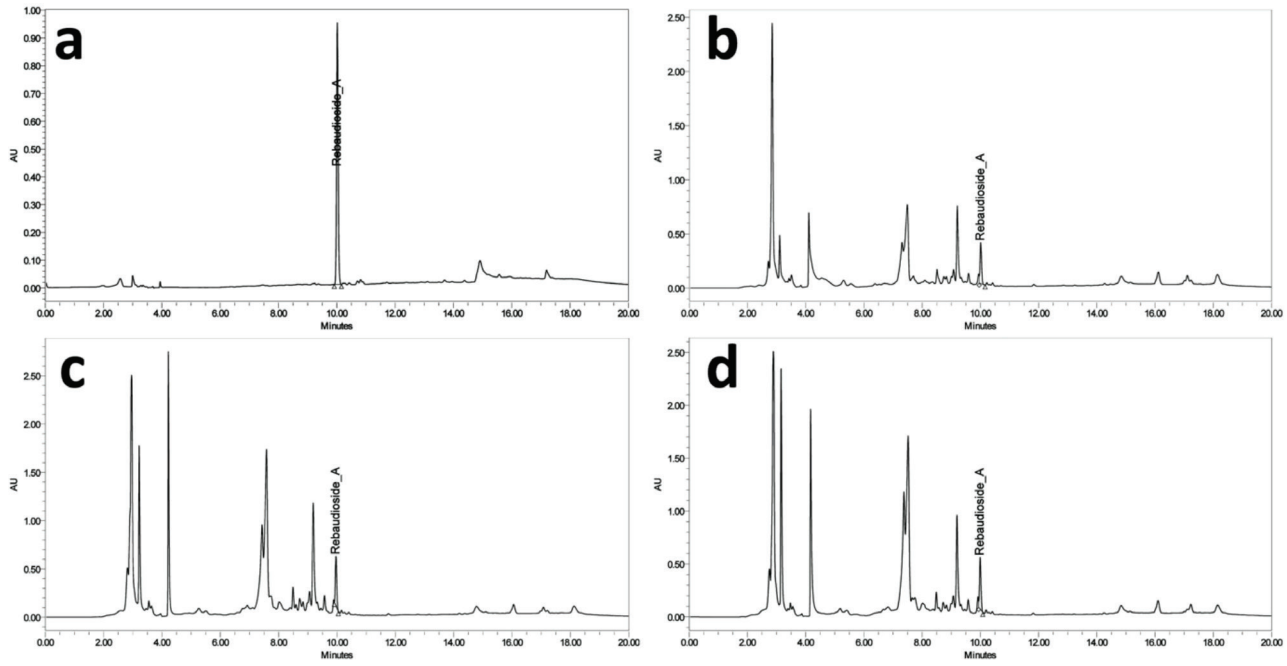

Fig. 2. Comparative HPLC chromatograms of the mutants and control *Stevia rebaudiana* Bert. (three months after field transfer) a. Rebaudioside-A standard, b. Control, c. 'BSRV1', and d. 'BSRV2'.

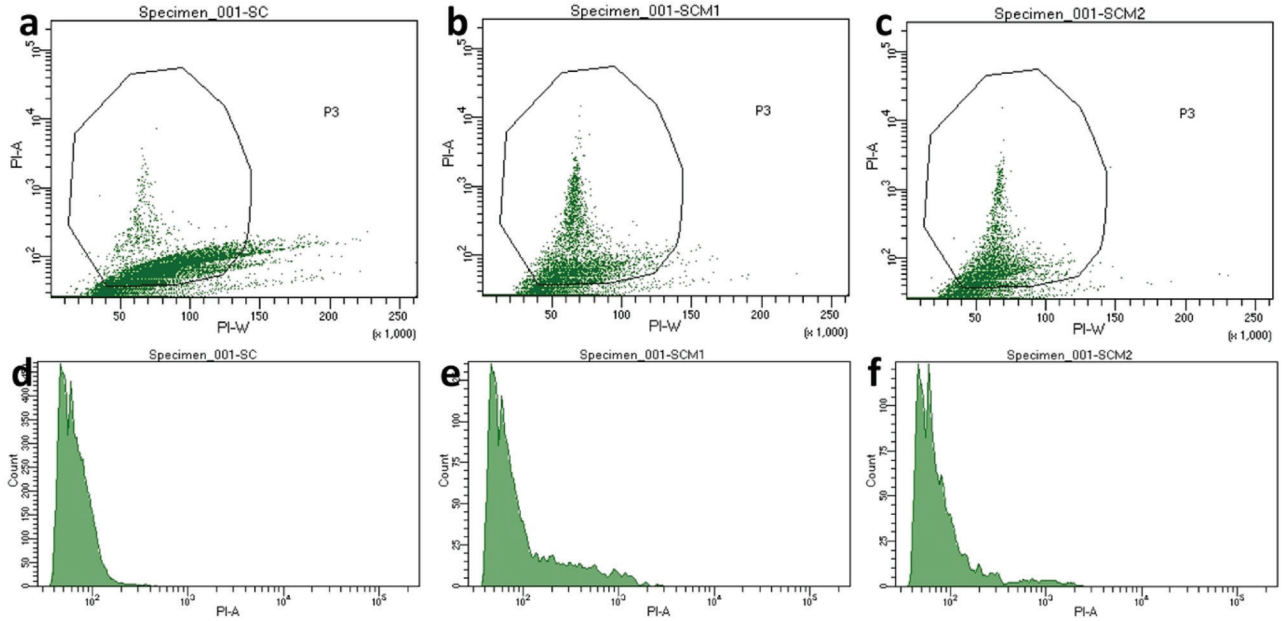

Fig. 3. Flow cytometry analysis of *Stevia rebaudiana* Bert. showing their ploidy levels. Dot plots of a. Control, b. 'BSRV1', and c. 'BSRV2'; histograms (relative nuclear DNA content) of log-transformed fluorescence intensities; d. Control; e. 'BSRV1', and f. 'BSRV2'.

accumulation. In stevia, among the many steviol glycosides, rebaudioside-A is known to accumulate in larger amounts (Vallejo and Warner, 15) and is said to be 240-fold sweeter than sucrose. Rebaudioside-A includes one unit of additional glucose at the 13th carbon position, which might result in a sweeter and somewhat bitter taste after consumption (Basharat *et al*., 1; Yücesan and Altuğ, 16; Rezvankhah *et al*., 12). The gamma radiation-induced acclimated stevia plants (three months after field transplantation) presented a 2-fold greater rebaudioside-A content (62 mg g^{-1} DW) than did the control plants (33 mg g^{-1} DW) (Khan *et al*., 8). Under stress, plants can generate stress-relieving compounds such as flavonoids and phenolics that mitigate stress, thereby increasing the steviol glycoside content (Khalil *et al*., 7). The interaction effect of gamma rays and free radicals in plant cells stimulates signaling molecules that engage in the defense system, ultimately increasing secondary metabolite content.

The mutant DNA peak in the histograms (relative log-transformed fluorescence intensities) was compared with that of the control, and no shift in the DNA peak was detected (Fig. 3). The ploidy status of the mutants ('BSRV1' and 'BSRV2') remained unaffected, implying that the genetic stability of the mutants was conserved. Validating the ploidy stability of mutants among themselves along with the control plants (non-irradiated) is a critical prerequisite and this analysis generates valid outcomes (Gantait and Mukherjee, 4). The documented flow cytometry data revealed that, in comparison to control plants, the mutants presented increased rebaudioside-A content without any variation in their nuclear DNA content. Earlier, Kumar *et al*. (9) estimated the genomic DNA content of different gamma radiation doses exposed to stevia to analyze their impact under *ex vitro* conditions in the M_2 generation. A review of a handful of reports revealed that, to date, no such detailed research has analyzed the nuclear DNA content of acclimatized *in vitro*-regenerated gamma radiation-induced mutants in stevia.

The present research investigation offers insights into characterized mutants with distinctive morphological and phytochemical properties that can be of extensive potential for release in various varieties in the future.

AUTHORS' CONTRIBUTION

Conceptualization of research (SG, SNK, SS, SB); Designing of the experiments (SG, SNK, SS, SB); Contribution of experimental materials (TS, SG, SNK); Execution of field/lab experiments and data collection (TS, SG, JMS); Analysis of data and interpretation (TS, SG, JMS, SNK); Preparation of the manuscript (TS, SG, JMS, SNK, SS, SB).

DECLARATION

The authors declare that they have no conflict of interest.

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