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RESEARCH ARTICLE

Radio-Sensitivity Assessment of In Vitro Tissues of Stevia (*Stevia rebaudiana* Bert.) for Induced Mutagenesis

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Abstract

In vitro mutagenesis approach using gamma irradiation has its proven advantages over conventional breeding methods, since the same exhibits more potential for mutation induction (with desirable traits) with minimal negative effects, within a short time-span, in various plant species. Present study reports an assessment of sensitivity of stevia to gamma irradiation in order to optimize the irradiation doses [median lethal dose (LD_{50}) and median growth reduction dose (GR_{50}) along with LD_{25}, LD_{75}, GR_{25}, and GR_{75}] for induced mutagenesis. Nodal segments from in vitro-regenerated stevia shoots were exposed at six different doses of gamma irradiations (5, 10, 15, 20, 25, and 30 Gy). The irradiated nodal segments were then cultured on Murashige and Skoog basal medium supplemented with 1.5 mg/L *meta*-Topolin and 1 mg/L indole-3-butyric acid along with the control (non-treated) explants for 3 weeks to assess the effect of irradiation on multiple shoot–root formation. Upon exposure to different gamma ray doses from 5 to 30 Gy, a gradual and morphological trait-specific differential decline of in vitro growth was detected. The individual as well as the cumulative impact of gamma irradiation doses on the growth and development traits were assessed using hierarchical clustering heat map (based on ward distance matrix) and principal component analyses. In addition, based on the probit analysis on trends of gamma irradiation effect, the $LD_{25, 50, 75}$ values were calculated to be 9.1, 18.2, and > 30 Gy, respectively. On the other hand, $GR_{25, 50, 75}$ values were calculated to be in between 7.1–12.6 Gy, 15.8–21.3 Gy, and 25.5 to way beyond 30 Gy, respectively. On studying the response of all the in vitro growth traits, it was deduced that in order to induce desirable mutations and also to develop novel mutants with adequate survival rate, the optimum irradiation dose (from $LD_{25, 50, 75}$ or $GR_{25, 50, 75}$ values) should be calculated based on LD_{50}/GR_{50}, which was determined to be 15–20 Gy in stevia. Hence, this optimum dose can be utilized to produce a higher percentage of beneficial mutations, resulting in maximal desirable genetic diversity in M_1V_2 and its subsequent generations.

Keywords Gamma irradiation · $GR_{25, 50, 75}$ · $LD_{25, 50, 75}$ · Nodal segments · Probit analysis · Survival rate

Abbreviations

GR Growth reduction
Gy Gray
IBA Indole-3-butyric acid
LD Lethal dose
MS Murashige and Skoog
*m*T *meta*-Topolin

Introduction

Stevia (*Stevia rebaudiana* Bert.) is a perennial, highly valued medicinally and industrially important short-day plant belonging to Asteraceae family, with diploid chromosome number $2n = 2x = 22$, and is frequently termed as "Sugar leaf", "Sweet leaf", "Candy leaf", "Honey leaf", or "Sweet herb of Paraguay". It is well-known as a non-caloric natural sweetener (due to presence of steviol glycosides). It was believed to be originated in North Paraguay region and later spreaded to several other countries in the world (Lewis et al. 1992). Stevia has gained significant commercial attention

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recently, owing to its flavour and several other medicinal properties associated with its leaves that makes it one of the most valuable plants around the globe (Gantait et al. 2015, 2018; Srivastava and Chaturvedi 2022; Subrahmanyeswari et al. 2023a). The demand for this plant is increasing rapidly day by day in various pharmaceutical, industrial and food sectors, in several well-developed as well as developing nations (Ahmad et al. 2021). Recently, the main concern of the plant breeders' remains the development of new varieties with improved traits including enhanced steviol glycosides content. However, researchers are continuously exploring the ways to increase the glycosides content of stevia via different biotechnological strategies (Libik-Konieczny et al. 2021; Laha et al. 2023).

Spontaneous mutations usually result in heritable variations. However, the frequency of spontaneous mutations is quite low in higher plants, ranging from 10^{-5} to 10^{-8} per loci (Jiang and Ramachandran 2010). Hence, there is a need to induce mutations with the aid of physical and chemical mutagens that generate novel genetic variations, and the mutants thus generated with desired characteristics can be identified and selected (Mba et al. 2010; Wolabu and Tadege 2016). Conventional propagation of stevia brings several limitations with it such as poor germination, inadequate setting, and incompatibility of seeds due to less pollinators and deficient rooting from cuttings, resulting in limited production of plant propagules (Gantait et al. 2015). Hence, in vitro mutagenesis has proven to be a feasible alternative to traditional breeding strategies for inducing desired changes in several traits, owing to its easy-handling of large sample size in short time-span and limited space. This breeding strategy enables the screening and selection of the desired mutants with better agro-economic features in plants (Khursheed et al. 2015, 2016; Laskar et al. 2018). Selection of an appropriate mutagen is a crucial step during in vitro mutagenesis programme. The chemical mutagens are more likely to cause point mutations, whereas, physical mutagens are likely to promote chromosomal aberrations and rearrangement. The chemical mutagens have been considered to be extremely toxic. On the other hand, physical mutagens have far fewer negative impacts on health (Guha Mallick et al. 2022). According to Mba et al. (2010), gamma ray acts as an efficient electromagnetic radiation since it penetrates deeper into target plant tissue and it is also less detrimental, non-particulate in nature, highly accessible, reproducible and causes high rate of mutations.

There are numerous reports published on in vitro mass propagation of stevia by various researchers to date, but there are only a few reports available on development of new variant via in vitro mutagenesis. Most of the available reports found to be dealt with the irradiation of seeds, calli and their performance under ex vitro conditions (Pande and Khetmalas 2011; Khalil et al. 2015; Ali et al. 2019; Abdullah et al. 2021; Abdullateef et al. 2023). During a gamma rays dose-dependent study, Khan et al. (2016) exposed the leaf explants to different doses of radiation (9.5, 19, 28.5, 37.5, and 47 Gy), among them 9.5 Gy was found to be effective resulting in high-frequency mutagenesis whereas, higher doses caused tissue browning and death of the explants. Interestingly, there is merely one report available on the irradiation of in vitro-regenerated shoot tips and nodal segments of stevia to different gamma ray doses (10–80 Gy) in which post irradiation growth response of explants was observed via a few growth parameters such as survival rate, shoot length, number of shoots and leaves per explant. The median lethal dose (LD_{50}) that was obtained during that study was 29 Gy (Nurhidayah et al. 2014). Chiew et al. (2019) studied the impact of gamma radiation on shoot tip explants of AKH L1 variety and found that the LD_{50} was around 23 Gy.

Prior to initiation of any large-scale mutation breeding experiment, LD_{50} and median growth reduction (GR_{50}) need to be determined and standardized (Layek et al. 2022). In that context, the optimum dose of mutagens must be chosen, since the responses of each gene differs depending on the type and rate of mutagen (Laskar et al. 2015). These doses are recommended to be precisely determined because they increase the likelihood of achieving higher rates of mutations in the desired (positive) direction (Khalil et al. 2014). Lower gamma irradiation doses have a negligible influence on the genome, and higher doses cause undesired changes, making it difficult to fulfil the desired mutagenesis (Álvarez-Holguín et al. 2019). An interesting method for achieving the expected mutations is via optimization of GR_{50}, that determines 50% reduction in the plant growth parameters when exposed to any source of irradiation. Due to the known fact that GR_{50} does not induce plant mortality, it may be utilized to successfully produce desired mutant plants. However, induction of mutagenesis and optimization of LD_{50} and GR_{50} was found to be species-, variety- and even genotype-dependent (Guha Mallick et al. 2022; Hazra et al. 2022). Rate of success of induced mutagenesis mostly depends on the use of optimal dose of mutagenic agents (Hazra et al. 2021; Layek et al. 2022), irrespective of the crop species involved. Nonetheless, recent studies showed that when utilizing gamma irradiation, the dose range below and beyond the LD_{50} (LD_{25} and LD_{75}) and GR_{50} (GR_{25} and GR_{75}) was crucial for determining the appropriate dose for high frequency mutagenesis (Muhammad et al. 2021; Ghasemi-Soloklui et al. 2023).

To date, there is no report available on in vitro mutagenesis via radio-sensitivity assessment and optimization of $LD_{25,\ 50,\ 75}$ and $GR_{25,\ 50,\ 75}$ doses in stevia, to the best of our knowledge. Therefore, it is very crucial to ascertain the optimum effective dose of mutagen that can be pertained to induce desirable alterations with minimal unwanted effects eventually to ensure the success of mutational induction.

There is lack of comprehensive data regarding the impact of in vitro mutagenesis on the various growth parameters of stevia, using the in vitro-regenerated nodal segments as explant. Keeping all of these in view, the main objective of the present report is optimization of dose of gamma irradiations for induction of mutations in stevia using nodal segments as explant source.

Materials and Methods

Plant Material and In Vitro Culture Establishment

Approximately, two-week-old shoot tip were excised from a year-old full-grown mother plant (BSR-2021–01), a genotype that was obtained via vegetative propagation and was procured from the germplasm collection of All India Coordinated Research Project on Medicinal and Aromatic Plants & Betelvine, Bidhan Chandra Krishi Viswavidyalaya, West Bengal, India. Actively growing shoot tips of 3–4 cm size were chosen for culture establishment. The excised shoot tip explants were washed with double-distilled water for three times. The explants were then immersed in 0.2% (w/v) Bavistin® solution for about 1 h to mitigate the fungal contamination and washed thoroughly thrice with autoclaved double-distilled water (following Subrahmanyeswari et al. 2023b). The next phase of disinfection procedure was completed under the laminar air-flow cabinet. The explants underwent treatment with different disinfectants, starting with 10% (v/v) Tween-20 for 5 min, 20% (v/v) sodium hypochlorite for 5 min, 1% (w/v) cetrimide for 5 min, 70% (v/v) ethyl alcohol for 30 s, and 0.1% (w/v) mercuric chloride (Merck Life Science Pvt. Ltd., India) for 5 min. After each treatment with disinfecting agents, the explants were rinsed thoroughly twice with sterile double-distilled water. The disinfected explants were dried on sterile blotting papers to remove excess water and trimmed down to ~ 1.5 cm, prior to the inoculation. The explants were inoculated in multiple shoot-root regeneration medium i.e. full-strength Murashige and Skoog (MS) (Murashige and Skoog 1962) basal medium (HiMedia Laboratories Pvt. Ltd., India) comprising 3% (w/v) sucrose, 0.7% (w/v) agar, 0.02% (w/v) myo-inositol, along with 1.5 mg/L *meta*-Topolin (*m*T) and 1 mg/L indole-3-butyric acid (IBA) for obtaining multiple shoot–root formation (following Subrahmanyeswari et al. 2023b). Prior to autoclaving the prepared medium at 1.1 kg/cm^2 pressure at 121 °C temperature for about 20 min, the pH of the medium was maintained at 5.7 (often by adding 0.1 N NaOH or 0.1 N HCl). All the in vitro experimental cultures were incubated in the growth chamber under aseptic environment by maintaining 23 ± 2 °C temperature, 60% relative humidity, 16-h photoperiod illuminating with white light emitting diodes under 50 µmol/m^2/s photosynthetic photon flux density.

Exposure to Gamma Irradiation

The in vitro-regenerated nodal segments obtained from multiple shoot-root regeneration medium at their two-week growth stage were exposed to different doses of gamma rays (5, 10, 15, 20, 25 and 30 Gy), using ^{60}Co based Gamma cell 2000 (GC-2000) blood irradiator as a source at Bhabha Atomic Research Center (BARC), Mumbai, India; and the non-irradiated nodal segments (0 Gy) served as a control. The effective dose rate at the time of irradiation was about 6.16 Gy/min. Based on this, the explants were exposed to different doses. After irradiation, the samples were kept in dark overnight since sudden exposure of irradiated samples to light would enhance the production of reactive oxygen species and might develop irradiation sickness in the exposed plants.

Revival of Irradiated Nodal Segments via Multiple Shoot-Root Formation and Proliferation

The irradiated nodal segments were incubated in same regeneration medium for assessing the effect of irradiation on multiple shoot–root induction and proliferation. In order to optimize the LD_{25}, LD_{50}, and LD_{75}, as well as GR_{25}, GR_{50}, and GR_{75} doses, the cultures were closely monitored for 3 weeks; and days to de novo shoots initiation, number of shoots, length of shoots (mm), number of leaves, number of days for root initiation, number of roots, and length of roots (mm) were recorded during their growth period.

Collection of Data and Statistical Analysis

The experiments were laid out in a completely randomized design (CRD) and was performed in 10 replicates with 70 samples (nodal segment explants) per treatment in order to minimize experimental error. The collected data were subjected to one-way analysis of variance (ANOVA), using SPSS (version26.0, SPSS Inc., Chicago, IL, USA) software. The significant variation between the treatments data (mean ± standard error) was computed applying Tukey's test at $P = 0.05$ level. Furthermore, in order to assess individual as well as the cumulative impact of gamma irradiation doses on the growth and development traits, the hierarchical clustering heat map and principal component analyses (PCA) were carried out using Metaboanalyst and PAST4.02 software package (Hammer et al. 2001). Probit analysis was carried out to

estimate the LD_{25}, LD_{50}, and LD_{75}, as well as GR_{25}, GR_{50}, and GR_{75} doses and these were calculated on the basis of equation given by Busvine (1971) using STAR software (Statistical Tool for Agricultural Research, R-packages, ver. 1.5 STAR 2.0.1, International Rice Research Institute, Los Banos, Philippines).

Results and Discussion

Impact of Gamma Irradiation on Growth Parameters

Shoot Initiation, Multiplication and Elongation

The earliest fresh shoot initiation was recorded in the control (non-irradiated) (3.9 days). Shoot initiation was recorded in 5.1 days from the explants that were exposed to 5 Gy, and in 18 days from the 30 Gy-irradiated explants (Table 1). It was observed that increase in irradiation dose prolonged the fresh shoot initiation in irradiated explants. On an average, the maximum number (8.1) of shoots was noticed in the control (Fig. 1a, b) followed by 5 Gy (6.1 shoots) (Fig. 1c, d), 10 Gy (5.9 shoots) (Fig. 1e, f) and 15 Gy (5 shoots) (Fig. 1g, h). Induction of the minimum number (1.7) of shoots were observed when the explants were exposed to 30 Gy (1.7 shoots) followed by 25 Gy (2.1 shoots) (Fig. 1k, l) and 20 Gy (4.1 shoots) (Fig. 1i, j). Similar trend was observed by Chiew et al. (2019) who reported that there was no new shoot formation from the stevia shoot tip explants when exposed to 40 and 50 Gy doses, in addition, a gradual decline in shoot number was observed from 10 to 30 Gy. In the present study, the maximum shoot elongation (48.6 mm) was recorded in control, followed by 5 Gy (38.9 mm). With an increase in gamma irradiation doses (from 5 to 30 Gy), a significant decline in the shoot length (from 38.9 to 18.1 mm) was observed as well (Fig. 1; Table 1). Such variation in the shoot length of the irradiated stevia plants may be the result of physiological and genetic damage/changes brought on by the gamma irradiation as suggested by Khalida et al. (2022).

Leaf Growth and Proliferation

The maximum number (19.4) of leaves per shoot clump was recorded in control and the minimum number was recorded (4.8) in 30 Gy dose. With the rise in gamma irradiation doses (from 5 to 30 Gy), the number of leaves produced per shoot clump decreased gradually from 17 to 4.8 number per shoot clump (Fig. 1; Table 1). Drying and yellowing of leaves were observed after 1 week of exposure in 30 Gy- followed by 25 Gy-irradiated explants, whereas after 15 days, partial yellowing of leaves was noticed in 20 Gy- and 15 Gy-irradiated explants. The minimal drying of leaves was observed in 10 Gy- as well as 5 Gy-irradiated explants in the 3rd week. Chiew et al. (2019) reported similar results wherein inadequate development of the regenerated leaves and necrosis of the explants were noticed, upon exposure to higher doses of gamma irradiation (50 Gy).

Root Formation, Multiplication and Elongation

The earliest (7.4 days) root induction was observed in control whereas, the most delayed root formation (18.5 days) was recorded in 25 Gy dose. With an increase in gamma irradiation doses from 5 to 25 Gy, a gradual delay in root formation was observed (from 8.4 to 18.5 days). There was no root formation when the explants were exposed to the highest dose of gamma irradiation (30 Gy) (Fig. 1m, n). The maximum number (6.1) of roots were induced in control whereas, only single root was induced in each plant exposed to 25 Gy. Likewise, the most elongated roots (20.8 mm) were noticed in control and the minimum root elongation

Table 1 Influence of gamma irradiation doses on in vitro multiple shoot–root initiation and proliferation of stevia (*Stevia rebaudiana* Bert.) (at 3-week growth stage)

Gamma irradiation (Gy)	Days to de novo shoot initiation	No. of shoots	Length of shoots (mm)	No. of leaves (per shoot clump)	Days to root initiation	No. of roots	Length of roots (mm)
0 (Control)	3.9 ± 0.3 e	8.1 ± 0.7 a	48.6 ± 4.9 a	19.4 ± 1.6 a	7.4 ± 0.5 e	6.1 ± 0.5 a	20.8 ± 1.3 a
5	5.1 ± 0.5 de	6.1 ± 0.5 ab	38.9 ± 3.9 ab	17.0 ± 2.8 a	8.4 ± 0.5 de	4.8 ± 0.8 ab	20.3 ± 2.1 a
10	7.2 ± 0.5 d	5.9 ± 0.5 b	34.0 ± 3.3 a-c	16.3 ± 2.0 ab	10.1 ± 0.4 c	4.1 ± 0.7 ab	17.3 ± 1.8 ab
15	9.9 ± 0.5 c	5.0 ± 0.5 b	33.6 ± 2.9 a-d	15.7 ± 1.9 ab	12.1 ± 0.7 b	4.0 ± 0.8 ab	13.2 ± 1.3 bc
20	13.1 ± 0.5 b	4.1 ± 0.8 bc	24.1 ± 4.8 b-d	9.2 ± 1.1 cd	17.6 ± 0.4 a	3.2 ± 0.7 bc	10.7 ± 1.7 bc
25	17.3 ± 0.5 a	2.1 ± 0.5 cd	21.7 ± 2.4 cd	8.6 ± 1.4 cd	18.5 ± 0.3 a	1.1 ± 0.1 cd	8.8 ± 1.7 c
30	18.0 ± 0.8 a	1.7 ± 0.4 d	18.1 ± 2.2 d	4.8 ± 0.8 d	–	–	–

Data represent mean of 10 replicates with 70 samples (nodal segment explants) per treatment

Data (mean ± standard error) for each column with different alphabets are significantly different according to Tukey's test at $P = 0.05$

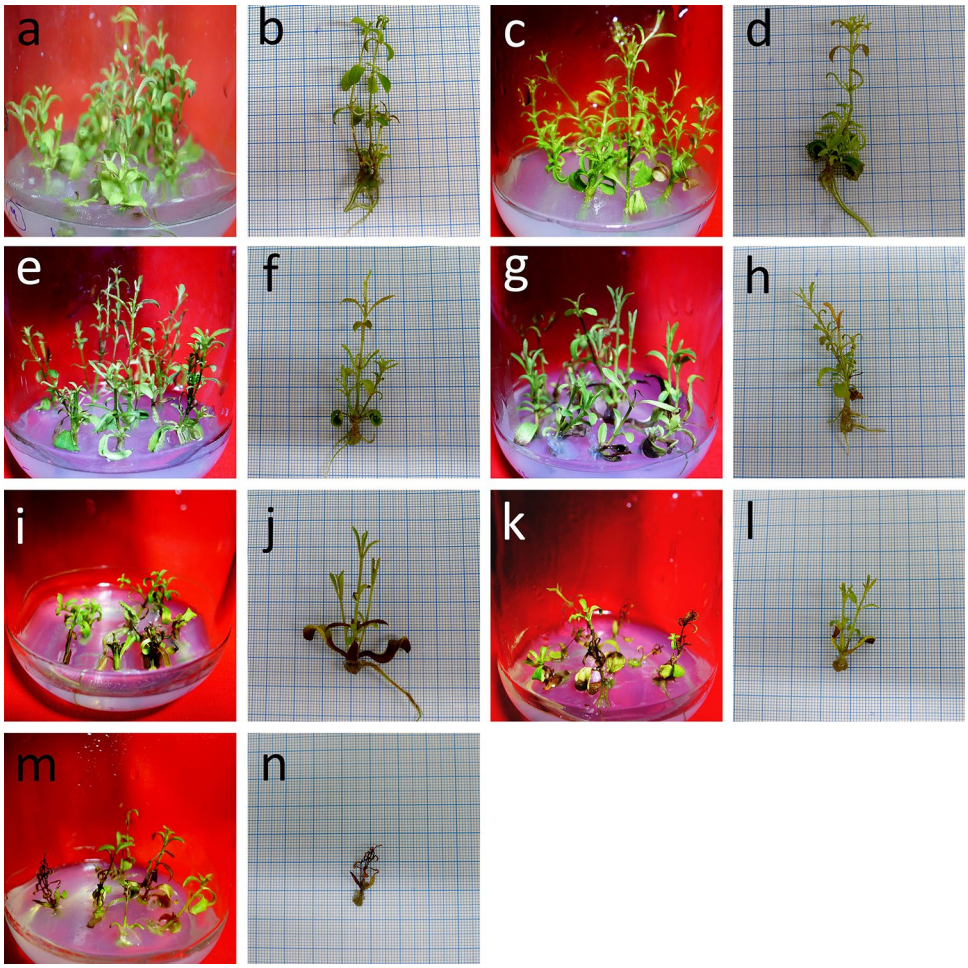

Fig. 1 Influence of different doses of gamma irradiations on degree of in vitro de novo multiple shoot–root growth of stevia (*Stevia rebaudiana* Bert.) in MS medium supplemented with 1.5 mg/L *m*T plus 1 mg/L IBA (3-weeks growth). **a, b** 0 Gy (control), **c, d** 5 Gy, **e, f** 10 Gy, **g, h** 15 Gy, **i, j** 20 Gy, **k, l** 25 Gy, **m, n** 30 Gy

(8.8 mm) was recorded in 25 Gy-irradiated explants (Fig. 1; Table 1). Eventually, with a rise in gamma irradiation doses from 5 to 25 Gy, the root elongation declined steadily (from 20.3 to 8.8 mm).

Higher doses of gamma irradiation proved to be detrimental for growth and development of the irradiated-plants (Guha Mallick et al. 2022). Generally, the plants exposed to ionising radiation typically show retardation of morphological and growth characteristics. Dose-dependent inhibition of various growth parameters is typically seen with rising doses of gamma irradiation and occasionally results in complete death of irradiated propagules, as well as reduces the possibility of production of novel mutants (Layek et al. 2022). On the same context, Hazra et al. (2022) reported that there was linear decline in seed germination and in shoot elongation, with an increase in the gamma irradiation doses in five genotypes of tomato. Similar finding was reported by Ghasemi-Soloklui et al. (2023), wherein, with increase in gamma irradiation doses (from 10 to 50 Gy) a decrease in the plant height and root number of ex vitro-grown grape vines was observed. Our results are consistent with the previous findings of Nurhidayah et al. (2014) and Chiew et al. (2019) which establish that upon exposure to gamma irradiations from 10 to 50 Gy and from 10 to 80 Gy, respectively, the in vitro growth and developmental traits of stevia declined gradually. However, both the reports did not give any information about the rooting characteristic as well as GR_{50} for the individual traits and they studied merely the impact of gamma doses on stevia shoot tips.

Cumulative Impact of Gamma Irradiations on Different In Vitro Growth Traits

Hierarchical Clustering Heat Map

Such heat map, which is based on ward distance matrix, explained how the different gamma irradiation doses were clustered into groups based on their similarity in performance in one way. In the another way it explained, based on the individual response to different irradiation doses how different morphological traits were clustered (Fig. 2). In this heat map, different growth parameters were represented on the Y-axis while the gamma irradiation doses were plotted on the X-axis. The lowest value in the heat map is indicated

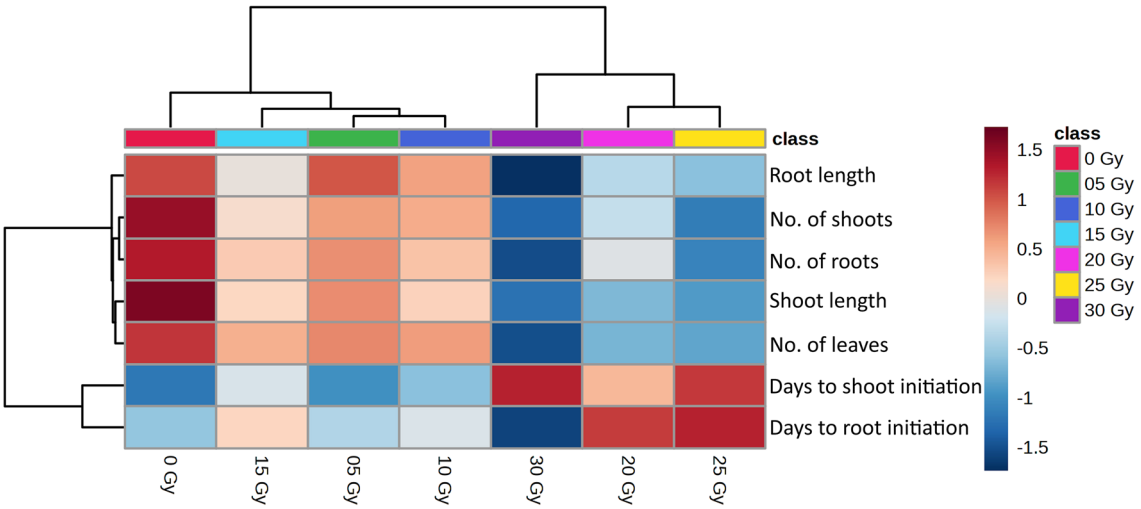

Fig. 2 Hierarchical clustering heat map based on ward distance matrix of the gamma irradiation-affected in vitro de novo shoot–root growth traits of stevia (*Stevia rebaudiana* Bert.) in MS medium supplemented with 1.5 mg/L *m*T plus 1 mg/L IBA (3-weeks growth)

by dark blue, the highest value by brilliant red, and the mid-range values by their relevant transition gradients. From the hierarchical clustering heat map, it was observed that there were two main groups. In the first main group there were two sub-groups. The control (0 Gy) was classified under the first sub group whereas, the second sub-group consisted of 5, 10, and 15 Gy doses. In the second sub-group, the performance of 5 Gy was better than the higher doses (10 and 15 Gy) in respect of all the growth and developmental traits. Similarly, in the second main group, two sub-groups were observed wherein, 20 and 25 Gy doses were grouped under the first sub-group and the 30 Gy dose alone comprised the second sub-group. By considering all the growth traits, it was observed that the performance of control was superior and 30 Gy dose was inferior in comparison to the all other doses. On the other hand, it was observed that the days to shoot initiation and days to root initiation traits were categorized under first main group, which meant that upon exposure to gamma irradiation doses, the performance of plants in respect to these traits were almost similar. In the second main group, three sub-groups were formed. In the first sub-group, shoot length and number of leaves were clustered, then in the second sub-group, number of shoots and number of roots were clustered. Finally, root length trait was assigned under the third sub-group (Fig. 2). From two-way clustering pattern, it can be deduced that the control was superior than other treatments in terms of all the concerned growth traits. It also explained the reducing effect of higher doses on the growth related traits. Similar to the present study, Ghasemi-Soloklui et al. (2023) explained the impact of different gamma irradiation doses on various growth traits of grape cuttings grown under ex vitro conditions using cluster analysis wherein, they clustered the five different gamma irradiation doses along with the control, and stated that the control was superior than the other treatments in terms of all the growth and developmental traits.

Principal Component Analysis

PCA or dimensionality reduction method is a technique that minimizes the complexity of large data sets, improving data interpretability without reducing information thereby, allowing the visualization of complex data sets. In the present study, PCA depicted the information regarding the association and the influence of gamma irradiation doses on multiple shoot–root initiation and proliferation traits of stevia (Fig. 3). The analysis retrieved two main component axes, component 1 and component 2 accounted with 82.3% and 15.7%, respectively (out of total 100% variation). The length of shoots, length of roots, and number of leaves were found to be the largest contributor to component 1 based on the scree plot and loading values. Similarly, for component 2, the number of days to root initiation and length of roots was observed to be the factor with the greater significance. It was observed that 20 and 25 Gy doses were the maximum influencers and were positively correlated for the days to de novo shoot initiation trait. On the other hand, 15 Gy was found to be the maximum influencer for the number and length of roots. Similarly, 10 Gy was the maximum influencer for the number of leaves; whereas, 5 Gy and 0 Gy (control) were the maximum influencers for the length of shoots. Out of all six gamma irradiation doses, 30 Gy was positioned far away from all the growth parameters indicating that for the above-mentioned growth traits this dose was negatively

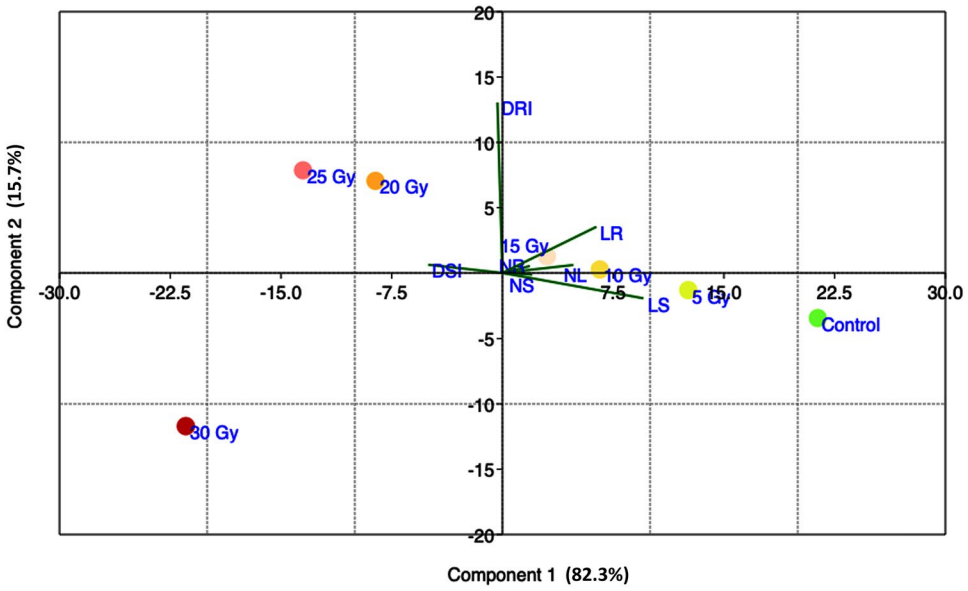

Fig. 3 Principal component analysis (scattered plot/bi-plot) explaining the association of gamma irradiation doses (in connection to their individual influence) with individual in vitro de novo shoot–root growth traits of stevia (*Stevia rebaudiana* Bert.) in MS medium supplemented with 1.5 mg/L *m*T plus 1 mg/L IBA (3-weeks growth) [DSI days to fresh shoot initiation, NS number of shoots, LS length of shoots (mm), NL number of leaves, DRI days to root initiation, NR number of roots, LR length of roots (mm)]

correlated and displayed the maximum negative influence. It was quite interesting to note that the hierarchical clustering heat map based on ward distance matrix results confirmed the PCA as well. Similar kind of PCA was carried out to study the influence of gamma irradiations in case of grapes cv. Yaghouti wherein the PCA results confirmed the cluster analysis in terms of grouping the different gamma irradiation doses (Ghasemi-Soloklui et al. 2023).

Table 2 Probit analysis on effect of gamma irradiation on in vitro survival %, days to shoot initiation, number of shoots, shoot length, number of leaves, days to root initiation, number of roots and root length in M_1V_1 generation of stevia (*Stevia rebaudiana* Bert.) grown in MS medium supplemented with 1.5 mg/L *m*T plus 1 mg/L IBA (3-weeks growth)

Dose (Gy)	log10 of doses	Survival		Days to shoot initiation		No. of shoots		Shoot length	
		Corrected mortality %	Working probit	Corrected reduction %	Working probit	Corrected reduction %	Working probit	Corrected reduction %	Working probit
Control	–	–	–	–	–	–	–	–	–
5	0.69	12	3.84	7.45	3.68	24.69	4.35	19.95	4.16
10	1	26	4.36	20.49	4.18	27.16	4.39	30.04	4.47
15	1.17	30	4.48	37.26	4.67	38.27	4.70	30.86	4.50
20	1.3	48	4.94	57.14	5.17	49.38	4.98	50.41	5.01
25	1.39	60	5.25	83.22	5.93	74.07	5.63	55.34	5.13
30	1.47	79	5.78	87.57	6.12	79.01	5.79	62.75	5.32
Dose (Gy)	log10 of doses	No. of leaves		Days to root initiation		No. of roots		Root length	
		Corrected reduction %	Working probit	Corrected reduction %	Working probit	Corrected reduction %	Working probit	Corrected reduction %	Working probit
Control	–	–	–	–	–	–	–	–	–
5	0.69	12.37	3.93	7.93	3.67	21.31	4.22	2.40	3.02
10	1	15.97	4.02	21.42	4.22	32.78	4.55	16.82	4.05
15	1.17	19.07	4.17	37.30	4.68	34.42	4.60	36.53	4.65
20	1.3	52.57	5.06	80.95	5.83	47.54	4.93	48.55	4.96
25	1.39	55.67	5.14	88.09	6.14	81.96	5.84	57.69	5.19
30	1.47	75.25	5.65	–	–	–	–	–	–

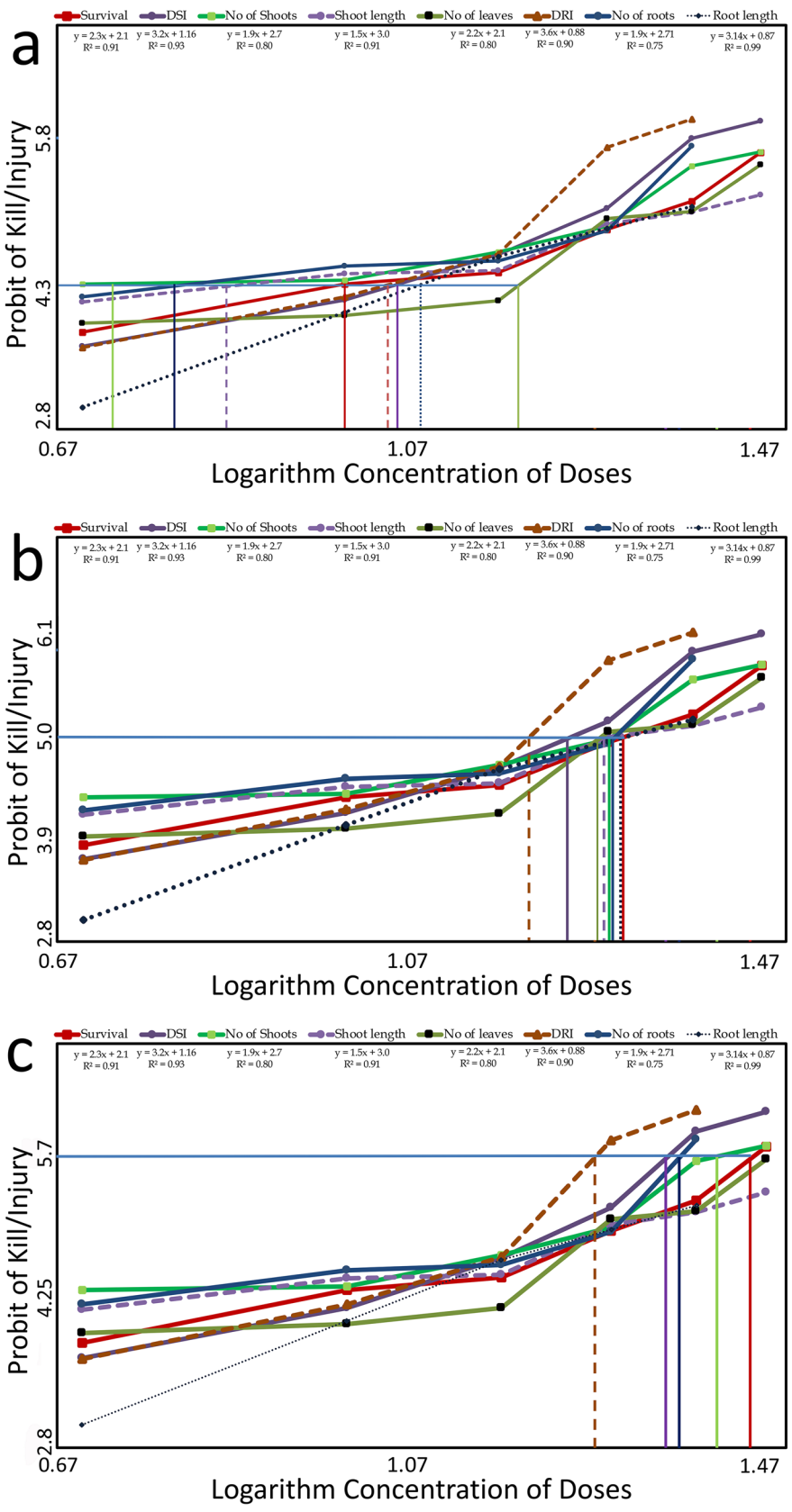

Fig. 4 Probit analysis on effect of gamma irradiation on in vitro survival %, days to shoot initiation (DSI), number and length of shoots, number of leaves, days to root initiation (DRI), number and length of roots in M_1V_1 generation of stevia (*Stevia rebaudiana* Bert.) in MS medium supplemented with 1.5 mg/L *m*T plus 1 mg/L IBA (3-weeks growth). The point of intersection of horizontal and vertical lines indicate the logarithmic value of the **a** LD_{25} and GR_{25}, **b** LD_{50} and GR_{50}, **c** LD_{75} and GR_{75} doses, and by taking the antilogs of those values the effective $LD_{25, 50, 75}$ and $GR_{25, 50, 75}$ doses were deduced

Probit Analysis

In order to optimize gamma irradiation doses, the probit analysis on effect of gamma irradiation on several growth traits in M_1V_1 generation of stevia with their corresponding corrected reduction % and working probit was presented in Table 2. According to Dowlath et al. (2021), ionising radiation may trigger the generation of peroxyl radicals in the cell membranes, disrupting ion cross-membrane transport and creates radiation sickness in the exposed plants. Ionising radiation disrupts the cellular proteins, resulting in reduction or entire loss of protein functioning that leads to imbalance of numerous critical metabolic and biochemical pathways. Elevated oxidants generation disrupts the redox state of the cells, accelerates the cell ageing and, in some cases, death. Such findings conceptualized the necessity of calculation of optimal gamma irradiation doses.

All the irradiated explants subjected to different doses exhibited varied mortality % (corrected), with the lowest being 12% when exposed to 5 Gy. The mortality (corrected) rate found to have increased (12–79%) with an increase in the gamma irradiation doses (5–30 Gy) (Table 2). However, all the control explants did not show any mortality. The recent findings of Chiew et al. (2019) is in support to our report wherein the survival rate of stevia was inversely related to the irradiation doses. The impact of gamma irradiation on several growth traits in M_1V_1 generation of stevia was depicted using probit analysis graphs (Fig. 4a, b, c) as well. In these graphs, the X-axis represents the logarithm concentration of doses whereas, the Y-axis indicates the probit of kill/injury. Here, the intersection of horizontal and vertical lines represents the logarithmic value of the LD_{25} and GR_{25} (Fig. 4a), LD_{50} and GR_{50} (Fig. 4b), as well as LD_{75} and GR_{75} (Fig. 4c) doses, and the $LD_{25,\ 50,\ 75}$ and $GR_{25,\ 50,\ 75}$ were computed by considering the antilog of those values. Probit analysis revealed a linear decline in the above mentioned in vitro growth and developmental traits of stevia with increasing gamma irradiation doses (Fig. 4a, b, c) and it was supported by the R^2 coefficient values. Likewise, in a recent report, probit analysis for the length of shoots and roots along with seedling emergence traits exhibited a progressive decline with increase in gamma irradiation doses in case of okra under laboratory conditions (Hazra et al. 2021).

In the present study, $LD_{25,\ 50,\ 75}$ values were found to be 9.1, 18.2, and > 30 Gy, respectively. In terms of GR assessments, GR_{25} was found to be in between 7.1–12.6 Gy for all the traits; likewise, GR_{50} was found to be in between 15.8–21.3 Gy. However, GR_{75} ranged from 25.5 to way beyond 30 Gy. From this observation, it can be suggested that LD_{75}/GR_{75} was inflicting more damage that might increase the frequency of mutation but the isolation of desired utilizable mutants will decrease; on the other hand, LD_{25}/GR_{25} might prove to be a suboptimal dose that would fail to create phenotypic variation (Hazra et al. 2021). Hence, from the present study, it can be concluded that dose optimization using gamma irradiation should be carried out via determination of LD_{50}/GR_{50}. After considering all the responding traits towards gamma irradiation, the LD_{50}/GR_{50} values were calculated to be in between 15–20 Gy for the in vitro culture of stevia. Our results were found to be nearly comparable to the previous report of Chiew et al. (2019) wherein they stated that the LD_{50} was obtained at 23 Gy in AKH L1 variety of stevia.

The magnitude of dose-dependent growth retardation, however, varied substantially, based on the traits as well. On that context, it was found that the $GR_{25,\ 50,\ 75}$ values for the different growth traits significantly varied indicating that there was differential mutagenic response of different growth parameters upon exposure to different doses of gamma irradiation. Such differential mutation rates among the plantlets subjected to varied amounts of ionising radiation brought in differences in plant growth and development when compared with non-radiated propagules (Guha Mallick et al. 2022). On the same context, Alphonse et al. (2022) studied the influence of gamma irradiation on in vitro-regenerated shoots in *Gentiana kurroo* Royle and reported enhanced production of its key secondary metabolite (gentiopicroside) via an optimum gamma ray exposure (10 Gy) in the successive generations (subcultures) in comparison to their non-irradiated counterparts.

Conclusion

The current research found that exposure of in vitro-regenerated nodal segments of stevia to varied doses of gamma irradiation resulted in differential response in terms of the morphology and growth traits. Upon exposure to lower dose of gamma irradiation (5 Gy), the growth and developmental pattern of stevia shoots was almost similar to the control. However, the highest gamma dose (30 Gy) had a negative effect (yellowing, drying of leaves and complete inhibition of root development) on morphological growth traits and recorded maximum death rate in comparison to other intermediate doses. With gradual increase in the irradiation doses, a progressive decline in the growth and developmental traits were observed. The individual as well as the cumulative impact of gamma irradiation doses on the growth and development traits were evaluated via the hierarchical clustering heat map based on ward distance matrix and principal component analyses. Taking into account all of the in vitro stevia growth attributes, the optimum irradiation dose fell in between 15 and 20 Gy. Hence, this optimum dose

determined in the present study can be useful in producing the desired mutations (including improvement of steviol glycosides content) and to create novel mutants with optimum survival rate with a little detrimental impact in M_1V_2 and subsequent in vitro generations of stevia.

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Declarations

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References

Abdullah, S., N.Y.M. Fauzi, A.K. Khalid, and M. Osman. 2021. Effect of gamma rays on seed germination survival rate and morphology of *Stevia rebaudiana* hybrid. *Malaysian Journal of Fundamental and Applied Sciences* 17: 543–549. https://doi.org/10.11113/mjfas.v17n5.2157.

Abdullateef, R.A., Z.B. Zainuddin, and M.B. Osman. 2023. Impacts of gama irradiations on the development new mutants of *Stevia rebaudiana* Bertoni. *EAS Journal of Biotechnology and Genetics* 5: 20–33. https://doi.org/10.36349/easjbg.2023.v05i02.001.

Ahmad, N., A. Rab, M. Sajid, N. Ahmad, H. Fazal, M. Ali, and U. Egertsdotter. 2021. Sucrose-dependent production of biomass and low-caloric steviol glycosides in adventitious root cultures of *Stevia rebaudiana* (Bert.). *Industrial Crops and Products* 164: 113382. https://doi.org/10.1016/j.indcrop.2021.113382.

Ali, A., A.N. El-Banna, A. Ahmed, and E.E. El-Dabaawy. 2019. Assessment of genetic divergence, stevioside and rebaudioside a contents and the effects of gamma irradiation on the performance of stevia (*Stevia rebaudiana* Bertoni) genotypes. *Egyptian Journal of Genetics and Cytology* 48: 295–315.

Alphonse, M., K. Thiagarajan, D.P. Fulzele, M. Pillay, R.K. Satdive, S.N. Kamble, R. Raina, S. Ramamoorthy, and R. Chandrasekaran. 2022. Effect of gamma radiation on gentiopicroside production in *Gentiana kurroo* Royle in vitro cultures. *Industrial Crops and Products* 176: 114392. https://doi.org/10.1016/j.indcrop.2021.114392.

Álvarez-Holguín, A., C.R. Morales-Nieto, C.H. Avendaño-Arrazate, R. Corrales-Lerma, F. Villarreal-Guerrero, E. Santellano-Estrada, and Y. Gómez-Simuta. 2019. Dosis letal media (DL_{50}) y reducción de crecimiento (GR_{50}) por irradiación gamma en pasto garrapata (*Eragrostis superba*). *Revista Mexicana De Ciencias Pecuarias* 10: 227–238. https://doi.org/10.22319/rmcp.v10i1.4327.

Busvine, J.R. 1971. *A critical review of the techniques for testing insecticides*, 2nd ed. Slough: Commonwealth Agricultural Bureaux.

Chiew, M.S., K.S. Lai, S. Hussein, and J.O. Abdullah. 2019. Acute gamma irradiated *Stevia rebaudiana* Bertoni enhanced particular types of steviol glycosides. *Asia Pacific Journal of Molecular Biology and Biotechnology* 27: 56–65.

Dowlath, M.J.H., S.K. Karuppannan, P. Sinha, N.S. Dowlath, K.D. Arunachalam, B. Ravindran, S.W. Chang, P. Nguyen-Tri, and D.D. Nguyen. 2021. Effects of radiation and role of plants in radioprotection: A critical review. *Science of the Total Environment* 779: 146431. https://doi.org/10.1016/j.scitotenv.2021.146431.

Gantait, S., A. Das, and J. Banerjee. 2018. Geographical distribution, botanical description and self-incompatibility mechanism of genus *Stevia*. *Sugar Tech* 20: 1–10. https://doi.org/10.1007/s12355-017-0563-1.

Gantait, S., A. Das, and N. Mandal. 2015. *Stevia*: A comprehensive review on ethnopharmacological properties and in vitro regeneration. *Sugar Tech* 17: 95–106. https://doi.org/10.1007/s12355-014-0316-3.

Ghasemi-Soloklui, A.A., M. Kordrostami, and R. Karimi. 2023. Determination of optimum dose based of biological responses of lethal dose ($LD_{25,\ 50,\ 75}$) and growth reduction ($GR_{25,\ 50,\ 75}$) in 'Yaghouti' grape due to gamma radiation. *Scientific Reports* 13: 2713. https://doi.org/10.1038/s41598-023-29896-z.

Guha Mallick, R., S. Pramanik, M.K. Pandit, A.K. Gupta, S. Roy, S. Jambhulkar, A. Sarker, R. Nath, and S. Bhattacharyya. 2022. Radiosensitivity of seedling traits to varying gamma doses, optimum dose determination and variation in determined doses due to different time of sowings after irradiation and methods of irradiation in faba bean genotypes. *International Journal of Radiation Biology* 99: 534–550. https://doi.org/10.1080/09553002.2022.2107723.

Hammer, Ø., D.A.T. Harper, and P.D. Ryan. 2001. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 4: 9.

Hazra, S., S. Gorai, S. Bhattacharya, S. Bose, P. Hazra, A. Chattopadhyay, and A. Maji. 2022. Radio-sensitivity of diverse tomato genotypes with respect to optimization of gamma irradiation dose. *Brazilian Journal of Botany* 45: 917–927. https://doi.org/10.1007/s40415-022-00823-2.

Hazra, S., S. Gorai, V. Umesh Kumar, S. Bhattacharya, A. Maji, S. Jambhulkar, N. Ali, and A. Chattopadhyay. 2021. Optimization of gamma radiation dose for induction of mutations in okra. *International Journal of Vegetable Science* 27: 574–584. https://doi.org/10.1080/19315260.2021.1894626.

Jiang, S.Y., and S. Ramachandran. 2010. Natural and artificial mutants as valuable resources for functional genomics and molecular breeding. *International Journal of Biological Sciences* 6: 228–251. https://doi.org/10.7150/ijbs.6.228.

Khalida, H.A., M. Azhar, Y.N. Azma, and A. Shamsiah. 2022. Effects of acute gamma irradiation on the morphology of *Stevia rebaudiana*. In *IOP Conference Series: Earth and Environmental Science* 1114: 012029. IOP Publishing. https://doi.org/10.1088/1755-1315/1114/1/012029.

Khalil, S.A., N. Ahmad, and R. Zamir. 2015. Gamma radiation induced variation in growth characteristics and production of bioactive compounds during callogenesis in *Stevia rebaudiana* (Bert.). *New Negatives in Plant Science* 1–2: 1–5. https://doi.org/10.1016/j.neps.2015.06.002.

Khalil, S.A., R. Zamir, and N. Ahmad. 2014. Effect of different propagation techniques and gamma irradiation on major steviol glycoside's content in *Stevia rebaudiana*. *Journal of Animal & Plant Sciences* 24: 1743–1751.

Khan, S.A., L.U. Rahaman, R. Verma, and K. Shanker. 2016. Physical and chemical mutagenesis in *Stevia rebaudiana*: variant generation with higher UGT expression and glycosidic profile but with low photosynthetic capabilities. *Acta Physiologiae Plantarum* 38: 4. https://doi.org/10.1007/s11738-015-2003-8.

Khursheed, S., A. Raina, and S. Khan. 2016. Improvement of yield and mineral content in two cultivars of *Vicia faba* L. through physical and chemical mutagenesis and their character association analysis. *Archives of Current Research International* 4: 1–7. https://doi.org/10.9734/ACRI/2016/24802.

Khursheed, S., R.A. Laskar, A. Raina, R. Amin, and S. Khan. 2015. Comparative analysis of cytological abnormalities induced in *Vicia faba* L. genotypes using physical and chemical mutagenesis. *Chromosome Science* 18: 47–51. https://doi.org/10.11352/scr.18.47.

Laha, S., T. Subrahmanyeswari, S.K. Verma, S.N. Kamble, S. Singh, S. Bhattacharyya, and S. Gantait. 2023. Biogenic synthesis, characterization and application of silver nanoparticles as biostimulator for growth and rebaudioside-A production in genetically stable stevia (*Stevia rebaudiana* Bert.) under in vitro conditions. *Industrial Crops and Products* 197: 116520. https://doi.org/10.1016/j.indcrop.2023.116520.

Laskar, R.A., A.A. Laskar, A. Raina, S. Khan, and H. Younus. 2018. Induced mutation analysis with biochemical and molecular characterization of high yielding lentil mutant lines. *International Journal of Biological Macromolecules* 109: 167–179. https://doi.org/10.1016/j.ijbiomac.2017.12.067.

Laskar, R.A., S. Khan, S. Khursheed, A. Raina, and R. Amin. 2015. Quantitative analysis of induced phenotypic diversity in chickpea using physical and chemical mutagenesis. *Journal of Agronomy* 14: 102–111. https://doi.org/10.3923/ja.2015.102.111.

Layek, S., S. Pramanik, A. Das, A.K. Gupta, A. Bhunia, and M.K. Pandit. 2022. Effect of gamma radiation on seed germination and seedling growth of snake gourd (*Trichosanthes anguina* L.). *South African Journal of Botany* 145: 320–322. https://doi.org/10.1016/j.sajb.2021.07.039.

Lewis, W.H., A.S. Rawat, A.S. Pharswan, M.C. Nautiyal, and A.J.G.H. Kostermans. 1992. Notes on economic plants. *Economic Botany* 46: 336–337. https://doi.org/10.1007/BF02866633.

Libik-Konieczny, M., E. Capecka, M. Tuleja, and R. Konieczny. 2021. Synthesis and production of steviol glycosides: Recent research trends and perspectives. *Applied Microbiology and Biotechnology* 105: 3883–3900. https://doi.org/10.1007/s00253-021-11306-x.

Mba, C., R. Afza, S. Bado, and S.M. Jain. 2010. Induced mutagenesis in plants using physical and chemical agents. In Plant Cell Culture: Essential methods, ed. Davey M.R., and P. Anthony, 20: 111–130. Chichester: John Wiley & Sons. https://doi.org/10.1002/9780470686522.ch7.

Muhammad, I., M.Y. Rafii, M.H. Nazli, S.I. Ramlee, A.R. Harun, and Y. Oladosu. 2021. Determination of lethal (LD) and growth reduction (GR) doses on acute and chronic gamma-irradiated Bambara groundnut [*Vigna subterranea* (L.) Verdc.] varieties. *Journal of Radiation Research and Applied Sciences* 14: 133–145. https://doi.org/10.1080/16878507.2021.1883320.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologiae Plantarum* 15: 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Nurhidayah, S., N. Norazlina, and I. Rusli. 2014. Effect of acute gamma irradiation on in vitro growth of *Stevia rebaudiana* Bertoni. *Innovative Plant Productivity and Quality* 22: 214–217.

Pande, S., and M. Khetmalas. 2011. Biological effect of gamma irradiation on in vitro culture of *Stevia rebaudiana*. *Indian Journal of Applied Research* 1: 11–12.

Srivastava, V., and R. Chaturvedi. 2022. An interdisciplinary approach towards sustainable and higher steviol glycoside production from in vitro cultures of *Stevia rebaudiana*. *Journal of Biotechnology* 358: 76–91. https://doi.org/10.1016/j.jbiotec.2022.08.018.

Subrahmanyeswari, T., S. Laha, S.N. Kamble, S. Singh, S. Bhattacharyya, and S. Gantait. 2023a. Alginate encapsulation of shoot tips and their regeneration for enhanced mass propagation and germplasm exchange of genetically stable *Stevia rebaudiana* Bert. *Sugar Tech* 25: 542–551. https://doi.org/10.1007/s12355-022-01194-4.

Subrahmanyeswari, T., S. Gantait, S.N. Kamble, S. Singh, and S. Bhattacharyya. 2023b. *meta*-Topolin-induced regeneration and ameliorated rebaudioside-A production in genetically uniform candyleaf plantlets (*Stevia rebaudiana* Bert.). *South African Journal of Botany* 159: 405–418. https://doi.org/10.1016/j.sajb.2023.05.045.

Wolabu, T.W., and M. Tadege. 2016. Photoperiod response and floral transition in sorghum. *Plant Signaling & Behavior* 11: e1261232.