Acute gamma irradiated *Stevia rebaudiana* Bertoni enhanced particular types of steviol glycosides

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Abstract. *Stevia rebaudiana* Bertoni from Asteraceae family is commercially valuable for its steviol glycosides (SGs) contents, which is 300 times sweeter than commercial sugar. The bottleneck in Malaysia is the lack of suitable stevia varieties that are able to thrive well under her climatic conditions and still produce high SGs. Mutation induction including gamma irradiation is effective in generating genetic variations and developing new plant varieties with desired traits. This study was aimed to determine the effects of acute gamma irradiation on phenotypic changes and enhancement of SGs contents of *Stevia rebaudiana* Bertoni variety AKH L1 (herein after will be designated as AKH L1). *In vitro* shoot tip explants of AKH L1 were subjected to a gamma doses regime of 10Gy to 50Gy, following which phenotypic changes of the irradiated explants and subsequent regenerated plantlets were observed. All irradiated explants exhibited different survival rates, with the lowest at $9.33 \pm 8.33\%$ when subjected to 50Gy, while all the control (non-irradiated explants) survived. The LD₅₀ was found to be at 23Gy. Subsequent irradiation of 900 shoot tip explants at 23Gy, produced 468 surviving shoot tips, which were all capable to develop and successfully sub-cultured until the fourth generation, M₄. These M₄ *in vitro* mutant plantlets exhibited significant increase in the numbers of leaf (16.07 ± 5.19) and average leaf size (1.12 ± 0.26 cm x 0.54 ± 0.15 cm). HPLC analysis performed in parallel further revealed the mutant plants contained higher concentrations of stevioside (387.04ppm), rebaudioside A (670.18ppm) and rebaudioside D (106.26ppm) compared to the non-irradiated plantlets, which exhibited 96.87, 194.42 and 28.25ppm, respectively.

Keywords: stevioside, rebaudioside A, rebaudioside D, HPLC, LD₅₀ irradiation

INTRODUCTION

Stevia rebaudiana Bertoni, a perennial sweet herb belongs to the family Asteraceae, is one of the 154 members of the genus *Stevia*. This plant is the most important source of non-caloric natural sweeteners, and is mainly known as “Sweet Weed”, “Sweet Leaf”, “Sweet Herbs” and “Honey Leaf”. The property of the species that called attention to the plant is the intense sweet taste of the leaves and aqueous extracts (Uddin *et al.*,

2006). The extract of the stevia plant contains mixture of various sweet steviol glycosides, designated as SGs, such as stevioside, rebaudioside A, B, C, D, E and F, dulcoside A, steviolbioside, steviolmonoside, and rubusoside, which accumulate in the leaves and is 300 times sweeter than sugar (Ahmad *et al.*, 2011; Reis *et al.*, 2011; Mathur & Shekhawat, 2012). The compounds that made up the majority

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proportions of SGs are stevioside and rebaudioside A, and they have been extensively used in the food and beverage industry. Stevioside traditionally makes up the majority of the sweetener which comprises 60-70% of the total glycosides content and has been evaluated as being 110-270 times sweeter than sugar (Dubois, 2000). It is believed to give a somewhat bitter aftertaste, or reported as a “licorice” taste or lingering effect (Carakostas *et al.*, 2008). Rebaudioside A (30-40% of total sweet content) is of particular interest as it is 180-400 times sweeter than sugar and has more pleasant sweet taste and no bitter aftertaste (Carakostas *et al.*, 2008). Therefore, a higher ratio of rebaudioside A to stevioside indicates a better sweetness quality, and is hence, preferred as reported by Yadav *et al.* (2011).

Jain (2010) proposed that mutation breeding was preferred over traditional breeding methods and genetically modified organisms (GMO) in the past few years. This is because multiple traits mutants can be isolated in mutation induction, but only single trait can be introduced into the crop in a transgenic way. Also, mutation induction can help to establish mutant lines range and determine trait specific genes in order to create molecular gene database, for molecular and functional genomics study and improve bioinformatics for future plant varieties development (Jain, 2010). Radiation treatment of plants had been reported as one of the most familiar methods for induction of plant mutations (Oladosu *et al.*, 2016). The newly developed mutant varieties were useful in developing new plant varieties as well as for functional studies of genes (Hase *et al.*, 2000; Tanaka *et al.*, 2002; Shikazono *et al.*, 2003).

In Malaysia, stevia is not recognized as a commercial crop due to lack of suitable variety when it was first introduced in the mid-1970s (Armizatul *et al.*, 2009). Stevia is a short day plant with a critical day length of at least about 13 hours (Lester, 1999). This is in line with Armizatul *et al.* (2009) report that stevia plant exhibited reduced vegetative growth and lower steviosides content when the day length in Malaysia was less than the critical 13 hours. Several improvements in crop quality and bioactive metabolites production through induced mutagenesis have been reported such as rice with low amylase and protein contents for diabetes people (Chen *et al.*, 2006), maize with

better protein contents (Tomlekova, 2010), and cassava with increased amylase content (Ceballos *et al.*, 2008). Khalil *et al.* (2014) reported higher stevioside contents were obtained from *in vitro* shoots from irradiated seeds of stevia. With these promising results, induced mutagenesis has potential to be applied on stevia to develop new mutant varieties that are suitable for growing under Malaysia's climatic condition and increases its potential as a viable crop plant. SGs content is the key selling point for stevia, and hence, increasing or improving the yield of SGs is crucial. There is still limited report regarding research on the effect of gamma irradiation on the accumulation of SGs contents of stevia in Malaysia. Hence, the main objective of this study was to create new varieties of stevia with high particular types of sweet glycosides via acute gamma irradiation suitable for growing in Malaysia.

MATERIALS AND METHODS

Plant materials preparation. One-month-old potted *Stevia rebaudiana* Bertoni AKH L1 propagated from stem cutting were bought from nursery located at Denai Alam, Selangor (3.153028, 101.520538). The sterilization methods employed for *in vitro* plants initiation using potted stevia plants were slightly modified from Nurhidayah *et al.* (2014). Young, actively growing shoots were collected from one-month-old potted AKH L1 plants. Nodes measuring 2 cm were chosen as explants where each was cut and placed under running tap water for 20 minutes to remove traces of soil and dirt. Prior to treatments, the explants were shaken with 2 mg/L fungicide (Ridomil) for 5 minutes and rinsed thrice with distilled water. Under aseptic condition, the explants were surfaced sterilized with 70% (v/v) ethanol for 30 seconds, followed by 5% (v/v) commercial sodium hypochlorite for 15 minutes. Then, the explants were rinsed with sterile distilled water for four times and dried with sterile blotting paper.

Shoot induction and multiplication. Shoots induction was initiated using surface sterilized nodal explants cultured on full strength

Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) containing 30 g/L sucrose and solidified with 7.5 g/L of agar (Gelrite) supplemented with 1 mg/L 6-benzylaminopurine (BAP). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 minutes. The cultures were incubated at 24±2°C with a 16 hours photoperiod and allowed for regeneration and multiplication for two weeks. Two types of explants (shoot tip and nodal segment) were used to determine which explants was more suitable for micropropagation. All cultures were examined after one month of incubation for the following parameters: shooting percentage, initiation time (day), number of shoot, shoot length and number of leaf. The regenerated shoots were cut, and shoot tips with four leaflets were placed on MS medium supplemented with 1 mg/L BAP. All experiments were repeated twice.

Acute gamma irradiation. One-week-old AKH L1 shoot tips with four leaflets were irradiated with gamma rays at different doses of 0, 10, 20, 30, 40, and 50Gy using the Malaysia Nuclear Agency (MNA) Gamma Cell Facility, Bangi, Selangor, Malaysia at different doses of the source of gamma rays was Caesium¹³⁷. Each experiment constituted 5 replicates per dose, with 5 explants per replicate. The whole experiment was repeated twice. The irradiated explants were cultured on basal MS medium and incubated at 24±2°C with a 16 hours photoperiod. After 4 weeks of culture, data were taken based on percentage of survival, length of explants, number of new shoots formed and number of leaves. The lethal dose, LD₅₀ was calculated based on the percentage of survival, whereby further acute irradiation was carried out at the selected LD₅₀ dose. The newly regenerated shoots were subcultured up to M₄ generation for selection of steviol glycosides content.

Plant leaf extract preparation. The steviol glycosides content were analyzed for both M₄ generation of irradiated and non-irradiated plantlets. The plant extracts preparation methods used were slightly modified from Abou-Arab *et al.* (2010). The leaves were randomly picked and dried under room temperature ranging from 25±2°C on the bench-top in the laboratory for three days. All the dried irradiated and non-irradiated stevia leaves were, separately, grounded

into powder form by using mortar pestle. Approximately 0.4g of powdered leaves was extracted in 10 ml of distilled H₂O in tubes placed in a beaker of water and allowed to boil for 15 minutes. The crude extracts containing steviol glycosides were cooled to room temperature for 5 minutes, and then filtered through Whatman filter paper No. 1. The filtrates were freeze-dried using the VirTis® BenchTop™ “K” Series Freeze Dryers (SP Industries, USA) for two days and stored at 4°C in airtight bottles.

Steviol glycosides analysis. Dried leaf extracts were subjected to High Performance Liquid Chromatography (HPLC) analysis. The HPLC system used was an Agilent 1260 Infinity (Agilent Technologies, CA, USA) equipped with an auto sampler, quaternary pump and a diode array detector. The HPLC method was performed using an Agilent Poroshell 120 C18 column (3.0 × 50 mm, particle size 2.7 µm) and a UV detector set at 210 nm. Separation was done with a ratio of ultrafiltration 80/20 H₂O:acetonitrile, set to a flow rate of 0.8 mL/min. The standards consisted of stevioside, rebaudioside A and rebaudioside D were run on the HPLC system and the retention time for each was recorded. Identification of stevioside, rebaudioside A and rebaudioside D in the plant leaf extracts were calculated by comparing the retention time of the samples with the standards. The results obtained for each sample were expressed in *parts per million* (ppm).

Data analysis. Each experiment was repeated twice and the data represented the means of three experiments. Statistical analyses of mean values, standard deviation (±) and significant difference were carried out by using IBM-SPSS software Window version 20. The data were subjected to One-Sample T Test or One Way Analysis of Variance (ANOVA) analysis and the means were compared by using Duncan's Multiple Range Test (DMRT) at significance $p < 0.05$.

RESULTS AND DISCUSSION

Establishment of *in vitro* stevia plantlets.

Stevia rebaudiana Bertoni variety AKH L1 can be propagated by stem cutting, seed propagation and micropropagation (tissue culture method). It had been reported that seed propagation was not effective due to low fertility (Tadhani *et al.*, 2006) and self-incompatibility of its flowers, while propagation through stem cutting has limitation on account of the low number of new plants (Miyagawa *et al.*, 1986). Thus, by considering future needs of plant materials, micropropagation was chosen for rapid multiplication of stevia in this experiment. *In vitro* stevia plantlets were established from nodal explants excised from young, actively growing shoot tips of glasshouse grown, one-month-old potted AKH L1. The explants were sodium hypochlorite-ethanol sterilized prior culturing on MS medium supplemented with 1 mg/L BAP. Past findings by Thiagarajan and Venkatachalam (2012) found that MS medium supplemented with 1 mg/L BAP was suitable for shoots multiplication of stevia. Thus this concentration of BAP was used in this study to mass propagate the AKH L1 plantlets. Shoots regenerated from nodal explants were observed after 7 days of culture, and newly regenerated plantlets were formed after one month. Shoot tips were cut and sub-cultured on fresh medium monthly to allow for regeneration (Figure 1).

Shoot tips were chosen as explants for mass propagation of stevia because it showed significantly ($p < 0.05$) higher shooting percentage (97.78%) and shorter initiation time (3.33 days) compared to nodal explants. Figure 2 clearly showed that shoot tip explants were better than nodal explants with respect to the number of shoots formed, shoot length increment and number of leaves produced. The tallest shoots (10.50 ± 2 cm) were induced from shoot tip explants which produced significantly greater numbers of leaf (20.67 ± 3.06) as summarized in Table 1. These observations were similar to El-Motaleb *et al.* (2013) data whereby shoot tip explants also proved to perform better than nodal explants in terms of shooting percentage, shoot length achievement and shoot initiation time. Both Patil *et al.* (1996) and Mubarak *et al.* (2008) also reported that shoot tip explants gave the highest number of plantlets than nodal explants in micropropagation of stevia. This could probably be due to lower endogenous phytohormonal level in the nodes compared to the shoot tips. Introduction of BAP in the medium was found to elevate phytohormone concentration which enhanced shoots proliferation, and cutting off the shoot tips promoted lateral buds formation and rapid regeneration (El-Motaleb *et al.*, 2013).

Figure 1. Initiation of *in vitro* culture of stevia variety AKH L1. (A) Approximately 2 cm long nodal explants obtained from young, actively growing shoot tips of one-month-old stevia potted plant. (Bar=3cm). (B) Shoots multiplication was initiated on MS supplemented with 1mg/L BAP for one month. (Bar=1cm). (C) Newly regenerated plantlets were sub-cultured to new medium monthly. (Bar=1cm).

Figure 2. *In vitro* shoots initiation for plantlets production using different explant types on MS medium supplemented with 1 mg/L BAP after one month. (A) shoot tip; (B) node (Bar=1cm).

Table 1. Growth profiles of different explant types of stevia variety AKH L1 after one month.

Explant types	Shooting percentage	Initiation time (day)	No. of shoots	Shoot length (cm $\pm$ SD)	No. of leaves
Shoot tip	97.78 $\pm$ 3.85 ^a	3.33 $\pm$ 0.58 ^a	6.33 $\pm$ 1.53 ^a	10.50 $\pm$ 2.00 ^a	20.67 $\pm$ 3.06 ^a
Node	71.11 $\pm$ 7.69 ^b	7.33 $\pm$ 0.58 ^b	2.33 $\pm$ 0.58 ^b	7.17 $\pm$ 1.26 ^b	14.33 $\pm$ 2.52 ^b

Data was taken from five replicates (with five explants per replicate) and the experiment was repeated twice. Superscripts within the means of each column (a-b) indicate significant difference among means ($p < 0.05$, using One-Sample T Test). SD = standard deviation.

Optimization of subculture cycle is crucial in order to obtain higher plantlets biomass with regenerative potency. However, information on the effects of repeated sub-culturing cycles on the rate of shoots multiplication and regeneration of *in vitro* stevia plantlets is scanty. In this study, it was observed that repeated subculturing cycles resulted in stunted growth of the regenerated plantlets (Figure 3). Gradual yellowing in some leaves were observed after repeated subculturing for 12 cycles. This indicated that the shoot cultures reduced their regeneration capacity (Ramanand, 2006). Somaclonal variations have been long known to be induced during dedifferentiation and regeneration of plants in tissue cultures (Filipecki & Malepszy, 2006). Rapid multiplication and prolonged culturing may affect genetic stability and thus increased the frequency of somaclonal variation in *in vitro* culture (Petolino *et al.*, 2003). The result paralleled Nakasha *et al.* (2016) findings whereby reduction in regenerative

capacity was observed in *Chlorophytum borivillianum* after repeated subculture cycles of their callus cultures. Thus, frequent subculturing does seem to affect the stevia plantlets growth, hence is not recommended as it may compromise the quality of the *in vitro* cultures.

Acute irradiation of *in vitro* stevia plantlets. In any mutational induction studies, it is critical to determine that the dose of mutagen applied is able to induce desirable alterations with minimal unintended effects. The effective dose of mutagen used will ensure the success of mutational induction for desirable outcomes. Therefore, in order to obtain the effective dose of mutagen used, fixation of LD₅₀ doses was deemed crucial before massive irradiation of similar materials was performed (Rajarajan *et al.*, 2016). Lethal dose, LD₅₀, was referred to a dosage at which greatest frequency of mutation occurred with half of the planting materials survived, while half died

(Kangarasu *et al.*, 2014). This LD₅₀ value varies according to plants, genera and cultivars (Tah, 2006). In this study, the LD₅₀ of *in vitro* shoot tips of stevia plantlets was determined to be 23Gy (Figure 4). There were significant differences ($p < 0.05$) in the survival rates amongst the stevia shoots subjected to lower and higher doses of acute gamma irradiation. The survival rate was inversely proportional to the gamma irradiation dose. All the non-irradiated explants (control) survived while explants treated with 50Gy exhibited the lowest survival rate ($9.33 \pm 8.33\%$).

Figure 3. *In vitro* culture of stevia variety AKH L1. (A) 1st generation plantlet; (B) 12th generation clump of plantlets. (Bar=1cm).

Figure 4. LD₅₀ of stevia variety AKH L1 shoot tips after one month of culture. Data was taken from five replicates and the experiment was repeated twice. The data was the average of three experiments.

Table 2 depicts the mutagenic effects of different doses of gamma rays on *in vitro* growth of AKH L1 shoots and their survival rates. The data highlights that the gamma rays imposed significant effect on the regenerated shoots' length. The control exhibited the highest length (2.53 ± 0.36 cm) while not much difference in shoot length was seen for explants exposed to 10 Gy (2.44 ± 0.67 cm). Nevertheless, gamma doses

greater than 30Gy did significantly reduce the length of the regenerated shoots compared to the control. It was more pronounced at 40Gy and 50Gy which could be due to higher sensitiveness of stevia to higher doses (Figure 5). This observation paralleled Jamie's (2002) data which showed that the mutation rate increased when the amount of exposure to irradiation and intensities of gamma rays used were increased. Inhibition of seeds germination, seedlings growth and other biological responses were frequent occurrences observed in plants subjected to high gamma doses (Wi *et al.*, 2007). Preussa and Britta (2003) attributed such inhibitive effects to be caused by cell cycle arrest at G2/M phase during somatic cell division and/or various damages in the entire genome.

Likewise, in this study, the highest number of regenerated shoots with mean value of 1.84 was observed in the control and no new shoots were formed at 40 and 50Gy. Unfortunately, the number of leaves was negatively affected by the gamma doses. The non-irradiated regenerated shoots (control) showed greater number of leaves at 16.52 ± 5.05 . At 50Gy, necrosis of explants and poor development of regenerated leaves (2.81 ± 2.47) were observed. Increasing gamma irradiation dosage resulted in reduced survival rate, length of regenerated shoots, number of new shoots formed and the number of leaves formed. The radiosensitivity test for AKH L1 was successfully performed with a LD₅₀ value of 23Gy.

Table 3 summarized the explants establishment percentage and the growth of control and irradiated stevia plantlets after four generations. It is recommended and a common practice for irradiated plantlets to be subjected to at least four generations/cycles of sub-culturings in order to screen off potential chimeras and to select for stable mutants. Therefore, M₄ generation was chosen to maximize the probability of obtaining stable mutants. Each sub-culturing cycle was counted as one generation as the excised explants regenerated into new plantlets. Irradiated stevia *in vitro* plantlets exhibited significantly ($p < 0.05$) longer shoot length of 10.16 ± 1.85 cm, higher number of leaves (16.07 ± 5.19) and greater leaf size (1.12 ± 0.26 cm x 0.54 ± 0.15 cm) compared to non-irradiated *in vitro* plantlets. It can be said that the growth of

stevia plantlets were significantly improved when exposed to gamma irradiation of 23Gy (LD₅₀). Similar observations were reported by Yadav (2016) where there was significant increase in leaf numbers of *Canscora decurrens* Dalz plants exposed to low irradiation doses than the control plants. Such “low dose-high growth” correlation was also observed in several plants such as *Molluccella lavis* (Minisi *et al.*, 2013), *Cucurma longa* (Ilyas & Naz,

2014), *Hibiscus sabdariffa* (Sherif & Sarwat, 2005), and *Dracaena serculosa* (Sakr, 2005). A broad range of negative activities at plant physiological levels such as water exchange, leaf gas exchange, enzymatic and hormonal imbalance, and alteration in protein synthesis were triggered at higher doses of gamma rays (Hameed *et al.*, 2008).

Table 2. Effect of different doses of acute gamma irradiation on growth of regenerated stevia variety AKH L1 shoots after one month.

Dose (Gy)	Survival rate (%±SD)	Length of regenerated shoot (mean±SD)	No. of new shoots formed (mean±SD)	No. of leaf (mean±SD)
0	100±0.00 ^a	2.53±0.36 ^a	1.84±0.63 ^a	16.52±5.05 ^a
10	93.33±8.33 ^a	2.44±0.67 ^a	1.72±0.38 ^a	15.77±5.66 ^a
20	58.67±12.86 ^b	1.76±0.78 ^{ab}	0.73±0.37 ^b	13.35±4.86 ^{ab}
30	37.33±16.65 ^c	1.40±0.55 ^{bc}	0.31±0.19 ^{bc}	8.80±3.56 ^{abc}
40	14.67±4.62 ^d	1.05±0.13 ^{bc}	0.00	6.89±1.92 ^{bc}
50	9.33±8.33 ^d	0.47±0.28 ^c	0.00	2.81±2.47 ^c

Data was taken from five replicates (with five explants per replicate) and the experiment was carried out three times. Superscripts within the means of each column (a-d) indicate significant difference among means ($p < 0.05$, using Duncan's Multiple Range Test). SD = standard deviation.

Figure 5. Effects of acute gamma irradiation on growth of regenerated stevia variety AKH L1 shoots after one month of culture on MS medium supplemented with 1 mg/L 6-benzylaminopurine (BAP). Bar= 1 cm.

Table 3. Comparison of control and irradiated stevia variety AKH L1 plantlets at LD₅₀ (23Gy) after four generations.

Parameter	Control	Irradiated
No. of explant survived (%)	95.83 ^a	87.18 ^b
No. of shoot / explants	6.20±2.39 ^a	2.60±0.90 ^b
Length of shoot / explant (cm)	8.61±3.40 ^a	10.16±1.85 ^b
No. of leaf / explants	13.33±5.42 ^a	16.07±5.19 ^b
Average leaf size,	0.81±0.22	1.12±0.26
Length x Width (cm)	x 0.44±0.14 ^a	x 0.54±0.15 ^b

The data presented was mean±SD of n=300, and the experiment was repeated twice. Superscripts within the means (a-b) indicate significant difference among means (p<0.05 using One-Sample T Test).

Determination of steviol glycosides (SGs) using high performance liquid chromatography (HPLC) analysis. An isocratic HPLC method was validated to determine the concentration of stevioside, rebaudioside A and rebaudioside D as described by Nishiyama *et al.* (1992). The retention times of the stevioside, rebaudioside A and rebaudioside D standards were recorded at 3.98 mins, 3.96 mins, and 3.56 mins, respectively (Table 4).

A standard curve was used to determine the concentration of each steviol glycoside in the irradiated and non-irradiated stevia leaf extracts. Based on Figure 6, the concentration of each steviol glycoside tested was higher (about three folds increment) in irradiated plantlets compared to the control leaf extract. From the HPLC results, a new stevia variety of AKH L1 was characterized with higher stevioside (387.04 ppm), rebaudioside A (670.18 ppm) and rebaudioside D (106.26 ppm).

Rebaudioside A and stevioside made up the majority proportion of the total steviol glycosides contents whereby the ratio of rebaudioside A to stevioside determined the sweetness quality of stevia (Yadav *et al.*, 2011). Similarly, Khalil *et al.* (2014) reported that gamma irradiation at 15Gy produced slightly enhanced stevioside contents in their irradiated plants. Other reports, however, claimed that increasing gamma ray doses induced higher mutation rates while at the same time led to significant reduction in stevioside contents in the stevia plants (Predieri, 2010; Ali *et al.*, 2015). Such conflicting findings probably could be due

to different varieties of stevia used in the experiments, and the different environment and physical conditions where the experiments were conducted.

Table 4. Retention time of standard steviol glycosides: Stevioside, Rebaudioside A, and Rebaudioside D, all done under separation of acetonitrile/water as the elution solvent at flow rate of 0.8 mL/min, and the detection wavelength set at 210nm.

Steviol glycosides	Retention time (min)
Stevioside	3.98
Rebaudioside A	3.96
Rebaudioside D	3.56

Figure 6. Steviol glycosides content in irradiated and non-irradiated (control) stevia leaf samples. Stev = stevioside; Reb A= rebaudioside A; and Reb D = rebaudioside D.

CONCLUSION

Acute gamma irradiation was found to be a clean and effective method to induce mutations in *Stevia rebaudiana* varieties. All irradiated *in vitro* AKH L1 plantlets exhibited different survival rates, with the lowest at $9.33 \pm 8.33\%$ subjected to 50Gy. The high irradiation dose produced plants exhibiting poor growth and development (necrotic leaves, reduced shoot length and number of leaves). Nevertheless, determination of the LD₅₀ provided an effective dose at 23Gy whereby irradiated plantlets showed significant improvement in phenotypic characteristics and steviol glycosides contents, especially stevioside and rebaudioside A. The data obtained can be further applied to develop new stevia plants with other improved features using acute gamma irradiation.

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REFERENCES

- Abou-Arab, A. E., Abou-Arab, A. A., & Abu-Salem, M. F. 2010. Physico-chemical assessment of natural sweeteners steviosides produced from *Stevia rebaudiana* Bertoni plant. *African Journal of Food Science* 4: 269-281.
- Ahmad, N., Fazal, H., Zamir, R., Khalil, S. A., & Abbasi, B. H. 2011. Callogenesis and shoot organogenesis from flowers of *Stevia rebaudiana* (Bert.). *Sugar Technology* 13: 174-177.
- Ali, A. A., Aboshosha, A. A., Kassem, M. K., EL-Dabaawy, E. I., & EL-Banna, A. N. 2015. Salinity tolerance and stevioside improvement of *in vitro* selected stevia (*Stevia rebaudiana*) mutants. *International Journal of Current Research in Bioscience and Plant Biology* 2: 11-20.
- Armizatul, S. A. H., Azhar, K., & Nazrul, R. 2009. Influence of lengthening day on stevia vegetative growth. *Transaction of the Malaysian Society of Plant Physiology* 18: 17-18.
- Carakostas, M. C., Curry, L. L., Boileau, A. C., & Brusick, D. J. 2008. Overview: The history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages. *Food and Chemical Toxicology* 46: 1-10.
- Ceballos, H., Sánchez, T., Denyer, K., Tofiño, A. P., Rosero, E. A., Dufour, D., Smith, A., Morante, N., Pérez, J. C., & Fahy, B. 2008. Induction and identification of a small-granule, high-amylose mutant in cassava (*Manihot esculenta* Crantz). *Journal of Agricultural and Food Chemistry* 56: 7215-7222.
- Chen, X., Liu, X., Wu, D., & Shu, Q. Y. 2006. Recent progress of rice mutation breeding and germplasm enhancement in China. *Plant Mutation Reports* 1: 4-6.
- Dubois, G. E. (2000). Sweeteners: non-nutritive. In: Francis FJ (ed.) *Encyclopedia of Food Science and Technology*. John Wiley and Sons, Inc., New York, NY. pp 2245-2265.
- El-Motaleb, M. A., El-Hady, M. A. S., El-Kholy, M. A., & Badr, A. 2013. *In vitro* propagation of *Stevia rebaudiana* Bertoni in Egypt. *Journal of Applied Science Research* 9: 4597-4605.
- Filipecki, M., & Malepszy, S. 2006. Unintended consequences of plant transformation: a molecular insight. *Journal of Applied Genetics* 47: 277-286.
- Hameed, A., Shah, T. M., Atta, B. M., Haq, M. A., & Sayed, A. 2008. Gamma irradiation effects on seed germination and growth, protein content, peroxidase and protease activity, lipid peroxidation in desi and kabuli chickpea. *Pakistan Journal of Botany* 40: 1033-1041.
- Hase, Y., Tanaka, A., Baba, T., & Watanabe, H. 2000. FRL1 is required for petal and sepal development in *Arabidopsis*. *The Plant Journal* 24: 21-32.
- Hase, Y., Tanaka, A., Baba, T., & Watanabe, H. 2000. FRL1 is required for petal and sepal development in *Arabidopsis*. *The Plant Journal* 24: 21-32.
- Ilyas, S., & Naz, S. 2014. Effect of irradiation on morphology characteristics and isolation of curcuminoids and oleoresoids of *Cucurbita longia* L. *The Journal of Animal & Plant Sciences* 24: 1396-1404.
- Jain, S. M. 2010. Mutagenesis in crop improvement under the climate change. *Romanian Biotechnological Letters* 15: 88-106.
- Jamie, H. 2002. *Induced Mutations: Increasing Agricultural Quality and Yield of Tomato*. Athena High Schools. Science Research Program, Greece, pp 132-145.
- Kangarasu, S., Ganeshram, S., & Joel, A. J. 2014. Determination of lethal dose for gamma rays and ethyl methane sulphonate induced mutagenesis in cassava (*Manihot esculenta* Crantz.). *International Journal of Scientific Research* 3: 3-6.
- Khalil, S. A., Zamir, R., & Ahmad, N. 2014. Effect of different propagation techniques and gamma irradiation on major steviol glycoside's content in *Stevia rebaudiana*. *Journal of Animal and Plant Science* 24: 1743-1751.
- Khalil, S. A., Zamir, R., & Ahmad, N. 2014. Effect of different propagation techniques and gamma irradiation on major steviol glycoside's content in *Stevia rebaudiana*. *Journal of Animal and Plant Science* 24: 1743-1751.
- Lester, T. 1999. *Stevia rebaudiana* sweet leaf. *The Australian New Crops Newsletter* 11: 1.
- Mathur, S., & Shekhawat, G.S. 2012. Establishment and characterization of *Stevia rebaudiana* (Bertoni) cell suspension culture: An *in vitro* approach for production of steviosides. *Acta Physiologiae Plantarum* 35: 931-939.
- Minisi, F. A., El-mahrouk, M. E., El-Din, M. Rida., & Nasr, F. N. 2013. Effects of gamma radiation on germination, growth characteristics and morphological variations of *Moluccella laevis* L. *American-Eurasian Journal of Agricultural and Environment Science* 13: 696-704.
- Miyagawa, H., Fujioka, N., Kohda, H., Yamasaki, K., Taniguchi, K., & Tanaka, R. 1986. Studies on the tissue culture of *Stevia rebaudiana* and its components (II) Induction of shoot primordia. *Planta Medica* 45: 321-322.
- Mubarak, M. H., Belal, A. H., EL-Geddawy, I. H., EmanI. EL-Sarag & Nasr, M. I. 2008. Micropropagation of *Stevia rebaudiana* *in vitro*. Meeting the challenges of sugar crops and integrated industries in developing countries. *Journal of Applied Science Research* 9: 293-298.

- Muradoglu, F., Balta, F., & Battal, P. 2010. Endogenous hormone levels in bearing and non-bearing shoots of walnut (*Juglans regia* L.) and their mutual relationships. *Acta Physiologiae Plantarum* 32: 53.
- Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiology Plant* 15: 473-497.
- Nakasha, J. J., Sinniah, U. R., Kemat, N., & Mallappa, K. S. 2016. Induction, subculture cycle, and regeneration of callus in safed musli (*Chlorophytum borivilianum*) using different types of phytohormones. *Pharmacognosy Magazine* 12: 460.
- Nishiyama, P., Alvarez, M., & Vieira, L. G. 1992. Quantitative analysis of stevioside in the leaves of *Stevia rebaudiana* by near infrared reflectance spectroscopy. *Journal of the Science of Food and Agriculture* 59: 277-281.
- Nurhidayah, S., Norazlina, N., & Rusli, I. 2014. Effect of Acute Gamma Irradiation on *in vitro* Growth of *Stevia rebaudiana* Bertoni. *Innovative Plant Productivity and Quality* 22: 214-217.
- Oladosu, Y., Rafii, M. Y., Abdullah, N., Hussin, G., Ramli, A., Rahim, H. A., & Usman, M. 2016. Principle and application of plant mutagenesis in crop improvement: a review. *Biotechnology and Biotechnological Equipment* 30: 1-16.
- Patil, V., Ashwini, K. S., Reddy, P. C., Purushotham, M. G., Prasad, T. G. & Udayakumar, M. 1996. *In vitro* multiplication of *Stevia rebaudiana*. *Current Science* 70: 960.
- Petolino, J. F., Roberts, J. L., Jayakumar, P. 2003. Plant cell culture: a critical tool for agricultural biotechnology. In: *Vinci VA, Parekh SR (eds.) Handbook of industrial cell culture: mammalian, microbial and plant cells*. New Jersey : Humana Press, p243-258.
- Predieri, S. 2001. Mutation induction and tissue culture in improving fruits. *Plant Cell, Tissue and Organ Culture* 64: 185-210.
- Preussa, S. B., & Britta, A. B. 2003. A DNA-damage-induced cell cycle checkpoint in Arabidopsis. *Genetics* 164: 323-334.
- Rajarajan, D., Saraswathi, R., & Sassikumar, D. 2016. Determination of lethal dose and effect of gamma ray on germination percentage and seedling parameters in ADT (R) 47 Rice. *International Journal of Advanced Biological Research* 6: 328-332.
- Ramanand, M. L. 2004. An efficient protocol for *in vitro* micropropagation of sugarcane. *Sugar Tech* 6: 85-87.
- Reis, R. V., Borges, A. P. P. L., Chierrito, T. P. C., Souto, E. R., Souza, L. M., Lacomini, M., Oliveira, A. J. B., & Goncalves, R. A. C. 2011. Establishment of adventitious root culture of *Stevia rebaudiana* Bertoni in a roller bottle system. *Plant Cell, Tissue and Organ Culture* 28: 151-157.
- Sakr, K. 2005. Effect of high temperature or fire in heavy weight concrete properties. *Cement and Concrete Research* 35: 590-596.
- Sherif, M. H., & Sarwat, M. I. 2005. Physiological and chemical variations in producing rosette plant (*Hibiscus sabdariffa* L.) by using some organic farmyard manure. *World Journal of Agricultural Sciences* 3: 609-616.
- Shikazono, N., Yokota, Y., Kitamura, S., Suzuki, C., Watanabe, H., Tano, S., & Tanaka, A. 2003. Mutation rate and novel the Mutants of *Arabidopsis thaliana* induced by carbon ions. *Genetics* 163: 1449-1455.
- Tadhani, M. B., Jadeja, R. P., & Rena, S. 2006. Micropropagation of *Stevia rebaudiana* Bertoni using multiple shoot culture. *Journal of Cell and Tissue Research*, 6, 545-548.
- Tah, P. R. 2006. Studies on gamma ray induced mutation in mungbean (*Vigna radiate* (L.) Wilezek). *Asian Journal of Plant Sciences* 5: 61-70.
- Tanaka, A., Sakamoto, A., Ishigaki, Y., Nikaido, O., Sun, G., Hase, Y., Shikazono, N., Tano, S., & Watanabe, H. 2002. An ultraviolet-B resistant mutant with enhanced DNA repair in *Arabidopsis* Plant Physiology 129: 64-71.
- Thiyagarajan, M., & Venkatachalam, P. 2012. Large scale *in vitro* propagation of *Stevia rebaudiana* (bert) for commercial application: Pharmaceutically important and antidiabetic medicinal herb. *Industrial Crops and Products* 37: 111-117.
- Tomlekova, N. B. 2010. Induced mutagenesis for crop improvement in Bulgaria. *Plant Mutation Reports* 2: 4-27.
- Uddin, M. S., Chowdhury, M. S. H., Khan, M. M. M. H., Uddin, M. B., Ahmed, R., & Baten, M. A. 2006. *In vitro* propagation of *Stevia rebaudiana* Bert in Bangladesh. *African Journal of Biotechnology* 5: 1238-1240.
- Wi, S. G., Chung, B. Y., Kim, J. S., Kim, J. H., Baek, M. H., Lee, J. W., & Kim, Y. S. 2007. Effects of gamma irradiation on morphological changes and biological responses in plants. *Micron* 38: 553-564.
- Yadav, A. K., Singh, S., Dhyani, D., & Ahuja, P. S. 2011. A review on the improvement of stevia [*Stevia rebaudiana* (Bertoni)]. *Canadian Journal of Plant Science* 91: 1-27.
- Yadav, V. 2016. Effect of gamma radiation on various growth parameters and biomass of *Canscora decurrens* Dalz. *International Journal of Herbal Medicine* 4: 109-115.