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Effects of Acute Gamma Irradiation on the Morphology of *Stevia rebaudiana*

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Abstract. Stevia is a short-day plant with constant day length less than 12 hours in Malaysia cause to generate flower early. The shorter vegetative period, resulting in low content of steviol glycosides compound. Cultivation of stevia in Malaysia is a challenge due to lack of suitable variety. The response to gamma irradiation varies among plant species and affected by the irradiation dose. The method applied was acute gamma irradiation with six doses (50, 100, 250, 300, 400 and 500 Gy) to investigate the effect of radiation. The LD_{50} value was at 182 Gy. The number of days for seed to germinate was varied in all treatments and none of the seedlings survived at 400 Gy and 500 Gy of irradiation. The plant height recorded the highest in treatment 50 Gy (142.0±1.98). Meanwhile, for other parameters such as the number of leaves, the number of branches, leaf width and leaf length were decreased as the dose increases. Overall, our findings suggest that low doses of radiation (below 325 Gy) is the suitable dose to study the improvement of stevia by acute gamma irradiation.

1. Introduction

Several countries around the world are now commercially cultivating stevia plant. All major regulatory agencies have approved or adopted high-purity steviol glycosides as sweeteners, and more than 150 countries have approved or adopted their use in foods and beverages [1]. Stevia was traditionally used in the food industry as well as for medicinal purposes.

Stevia rebaudiana from the Asteraceae family has 950 genera. Stevia is a group of annual and perennial herbs that grows in open forests and mountainous areas. M. S. Bertoni is credited with the first botanical description of the plant. The leaves are the most useful component of this shrub. About 18 species out of 110 tested for sweetness were found to have this sweetness trait [2]. The main sweetener is stevioside, which is present in stevia leaves. In the early 1970s, stevioside was considered a sugar substitute with the goal of commercialization. Stevia leaves contain diterpene glycosides, which are several times sweeter than sugar. They can be used as a synthetic sweetening agents alternative to the consumers that consume healthy lifestyle [3].

Stevia is a short-day plant by nature, with a vital day duration of approximately 13 hours. Stevia will begin flowering once it has developed a minimum of four true leaves [4]. Day length less than 13 h causes stevia to flower early, resulting in a low leaf biomass yield and percentage of sweetener content. Malaysia still lack of suitable variety for delayed flowering when planted in the condition less than 13 hours natural light. To avoid early flowering additional 2 hours artificial light is applied. Therefore, there is need to develop suitable local varieties. Mutation breeding is one of the strategies suitable to be used in the agriculture sector for crop improvement. Mutation breeding has the potential to broaden diversity and isolate desirable economic traits in a shorter period [3]. Among all the physical mutagenic agents, the most frequently used mutagens in mutation breeding are gamma rays [5]. In this study, stevia seeds are treated with various gamma ray doses and effect of various doses on germination, survival, growth and development of the seeds and seedlings are observed.

2. Materials and Methods

2.1 Sample preparation
Seeds of stevia (Accession H1) were obtained from one mother plant for gamma irradiation treatment. The irradiation treatments for the seeds were 50, 100, 250, 325, 400 and 500 Gy from the Biobeam GM 8000 Germany machine at the Agensi Nuklear Malaysia, Bangi, Selangor. The non-irradiated seeds act as a control.

2.2 Seed germination
The seed germination was carried out as following: The apparatus comprised of the planting tray for the seed germination in the peat moss as the sowing medium and two red fluorescent tubes affixed on the light chamber[6]. The apparatus was set up in the Plant Genetic Laboratory at UiTM Jasin.

The data on seed germination was recorded from the emergence of first shoot. The seed germination rate was calculated using formula [7]:

$$\text{Germination (\%)} = \frac{\textit{No.of seeds germinated}}{\textit{No.of seed sown}} \; x\ 100$$

2.3 Survival rate
The survival rate in different treatments was calculated at 40 days after irradiation treatments and the relative survival rate was calculated as follows [7]:

$$\text{Relative survival (\%)} = \frac{\textit{Survival of dose treatment}}{\textit{survival of control}} \; x\ 100$$

The relative survival was used to predict the lethal dose for the stevia accession H1.

2.4 Experimental site and design
The stevia H1 seedlings were transplanted in polybag containing mixture of loam, sand and coco peat. The mixture was mixed according to the ratio of 1:3:3 of soil, sand and coco peat. All portion were mixed thoroughly using soil mixture. In the mixture, organic fertilizer was mixed together to supply nutrients. The soil mixture was filled into the polybags 12” ×12” in size. The experiments were carried out in the greenhouse under the control environment with completely randomized design.

2.5 Evaluation on qualitative and quantitative parameters
The qualitative parameters such as the plant and leaf characteristics that involved shape, tip, base and margin were evaluated. Meanwhile the quantitative parameters were measured on the plant height, number of branches, number of leaves, leaf width and length; (1) Plant height (cm) was measured from the base of the plant until the tip of the plant using a steel meter ruler. (2) Number of leaves (per plant) was determined by counting all leaves in plant including small leaves. (3) Number of branches (per plant) was determined by counting all the branch of the plant. (4) The width and (5) length of leaf was measured using a steel meter ruler in cm and was observed for 12 weeks.

2.6 Data analysis

The data was analyzed using SPSS statistical package version 22.0. The results were expressed as mean ± standard error. The analysis of variance (ANOVA) was carried out to detect the significance differences among the treatment and the value of $p<0.05$ was significant.

3. Results and discussion

3.1 Seed germination rate

Table 1 shows the effects of gamma radiation on the number of days to germinate for stevia accession H1. From the observation, the results were varied between the gamma doses. The stevia seed in treatment 100 Gy showed the highest rate of germination (53%) and irradiated stevia seed at 500Gy showed the lowest germination rate (38%). For the control (0 Gy), the rate of percentage probably lower than treatment because of the viability of the stevia seed itself. The viability of stevia seeds is decrease with time and will affect the seed germination rate. From the results above, we concluded that the rate of germination is treatment-dependent. Different dosage could allow the plant going through certain changes in their metabolic process during the germination occur [8].

Table 1. Seed germination rate (%) among treatments

Gamma rays (Gy)	**Rate of germination (%)**
0	40
50	45
100	53
250	43
325	40
400	40
500	38

3.2 Survival rate of irradiated stevia

The survival rate of irradiated stevia seedlings under different doses was calculated based on the number of germinated seeds survived after 14 days. As shown in Table 2, the survival rate of irradiated seedlings were decreased as the gamma doses increase. The mortality rate was 100% for seedlings in treatment 400 Gy and 500 Gy. Meanwhile, seedlings in treatment 50 Gy and 100 Gy recorded the highest survival rate (60%) and the lowest survival rate was 50% at 325 Gy. Stevia seedlings showed their sensitivity towards gamma radiation exposure. Exposure to higher dose of gamma ray could alter and disturb the growth of cells of the stevia. The results showed that seedling in treatment 400 Gy and 500 Gy showed no changes in growth after 2 weeks transplanted to the seedling tray. The injury in plants occurs either point mutation or changes in plant components that lead to the decreased growth performance [8].

Table 2. Survival rate (%) of irradiated stevia

Gamma rays (Gray)	Survival rate (%)
0	75
50	60
100	60
250	55
325	50
400	0
500	0

Figure 1 shows the survival rate of irradiated seedling. In gamma irradiation study, radiosensitivity test was done to observe the early selection of a variant that had genetic changes [20]. The results shows that lethal dose (LD_{50}) leads to 50% of mortality towards the irradiated samples and were estimated using the published protocol [21].The LD_{50} of irradiated stevia observed in this study was 182.40 Gy, which means that 50% of stevia seedlings probably did not survive and died at this dose.

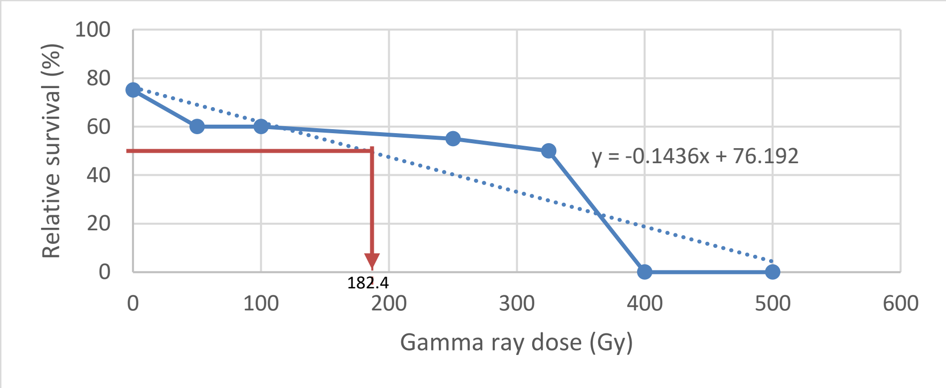

Figure 1. The graph of relative survival (%) against gamma ray dose (Gy) to determine the LD_{50}

3.3 Morphological Characteristics

Morphological characteristics of non-irradiated and irradiated stevia by gamma radiation are shown in Table 3 & Figure 2. In terms of leaf shape, all stevia showed an obovate shape except for treatment 325 Gy, showed a lanceolate shape. For the leaf tip, both treatment 0 Gy and 50 Gy showed acute meanwhile other treatment showed an obtuse tip. Moreover, the leaf base also showed dominant in attenuate base for all treatment except for 325 Gy (rounded) leaf base. Varied type of leaf margin obtained from the observation. Dentate margin leaf appears on treatment 0 Gy, 100 Gy and 250 Gy. For 50 Gy and 325 Gy both are incised and serrated in leaf margin respectively. Variation of shape could appear as different gamma dosage effect on the plant respectively. The gamma mutation disturbed the stevia leaf characteristics at some point of growth stage. The leaf characteristics also showed varies results when treated with different mutation agents [9].

Table 3. Morphological characteristic of Gamma irradiated *Stevia rebaudiana*

Treatment Dose (Gy)	**Plant type**	**Leaf**			
		Shape	Tip	Base	Margin
0	Loose	Obovate	Acute	Attenuate	Dentate
50	Loose	Obovate	Acute	Attenuate	Incised
100	Loose	Obovate	Obtuse	Attenuate	Dentate
250	Loose	Obovate	Obtuse	Attenuate	Dentate
325	Loose	Lanceolate	Obtuse	Rounded	Serrated

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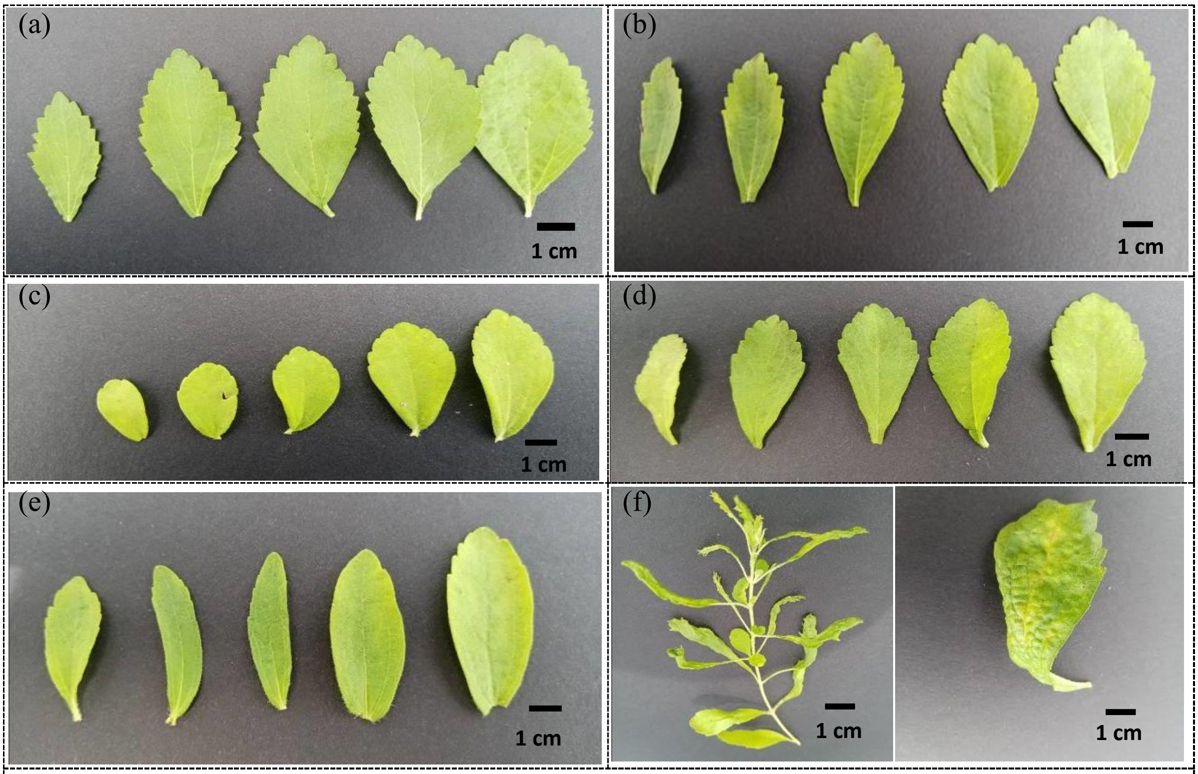

Figure 2. (a) 0 Gy (b) 50 Gy (c) 100 Gy (d) 250 Gy (e) 325 Gy (f) abnormal leaf form.

Most of the irradiated plants showed different morphological characteristics, as an indicator of the effects of gamma ray in all the treatment. Figure 3 shows plants from treatment 50 Gy recorded the highest (142 cm) in plant height followed by treatment 0 Gy (136.13 cm). Meanwhile the lowest treatment in plant height was in treatment 250 Gy (93.38 cm). Thus, the higher the dosage of gamma affect the growth of the stevia plant. From the observation, the difference between the heights of the irradiated stevia could be due to the inhibition of DNA synthesis or other physiological damage after gamma radiation treatment [10]. The differences of plant height in each treatment might be due to the genetic changes that occurred within the cells of the radiated stevia plant. The height of the mutation stevia shows significant difference between treatment 250 Gy and treatment 50 Gy ($p<0.05$). The average seedling height showed unsteady declined trend from the control treatment to maximum dose of gamma irradiation. The differences in the form of plant tissue used and the location of the plant tissue at the time of gamma radiation could be one of the factors that caused this observation [11].

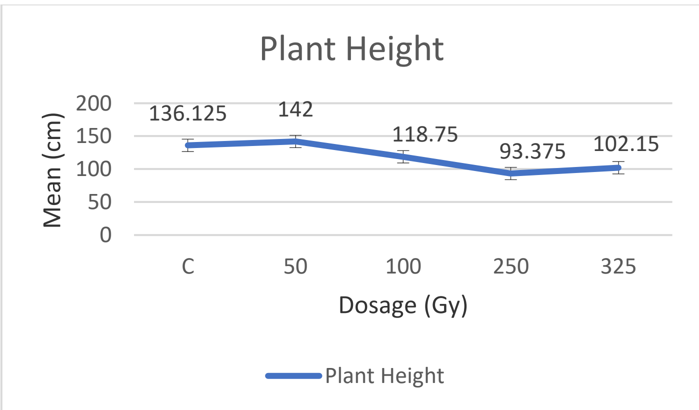

Figure 3. The height of the transplanted stevia mutation after 12 weeks

The number of leaves has been widely used as an index in determining the biological effect of various physical mutagen [12]. Figure 4 shows the number of leaves in control (0 Gy) showed the highest leaves numbers (163.25), while the lowest was observed in treatment 250 Gy (99.75). The genetic mutation interferes the biochemical process in the plant [13]. Besides plant height, number of leaves are also important since most glycoside content are found in the leaf. It is slightly decrease in leaf number as the plant dosage increase. Because leaves are the economically important part of this crop, the application of mutation breeding in stevia can expedite the improvement of plant [14].

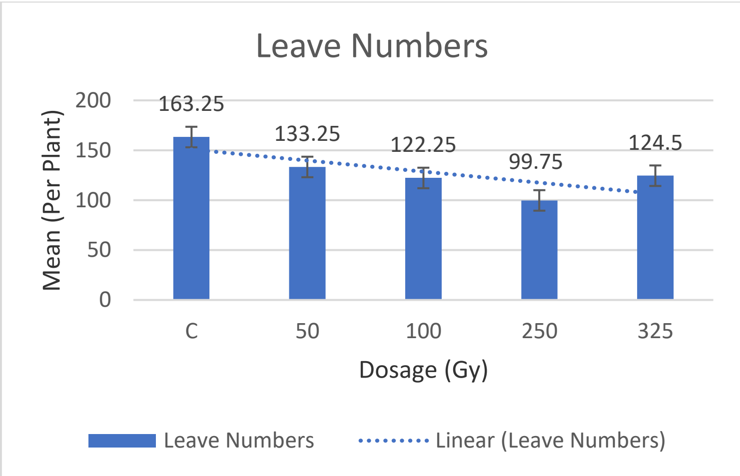

Figure 4. The leaves number of the transplanted stevia mutation after 12 weeks

The accumulation of steviol glycosides (SGs) is a commercially important attribute in Stevia. Increased stevioside content in mutant genotypes with longer vegetative phases can be linked to gamma-irradiation mediated photosynthesis enhancement and carbon fixation rate can be restricted to potential genetic advantage despite altered flowering behaviour. Furthermore, variation in the expression pattern of important pathway genes may also be correlated with variation in Stevioside content accumulation [8].

The more branches present in the plant, the greater number of leaves present. As we know, stevia is popular with the sweetness compound. Figure 5 shows the highest in number of leaves was observed in treatment 325 Gy (32.5 cm) however the result is lower than that in control, 0 Gy (36.75 cm). After 12 weeks, a significant difference ($P < 0.05$) on the number of branches was obtained between the gamma treatments. The number of branches were highly significantly reduced by radiation doses. In general, fluctuate in number of branches was observed in both irradiated and non-rradiated explants. Previous study showed 60CO-γ and ion beam injections had mutagenic impacts on stevia progenies whereby they displayed several characteristics such as lower height, consistent leaf shape, fewer branches, shorter internode length, lodging and were cold-resistant [15].

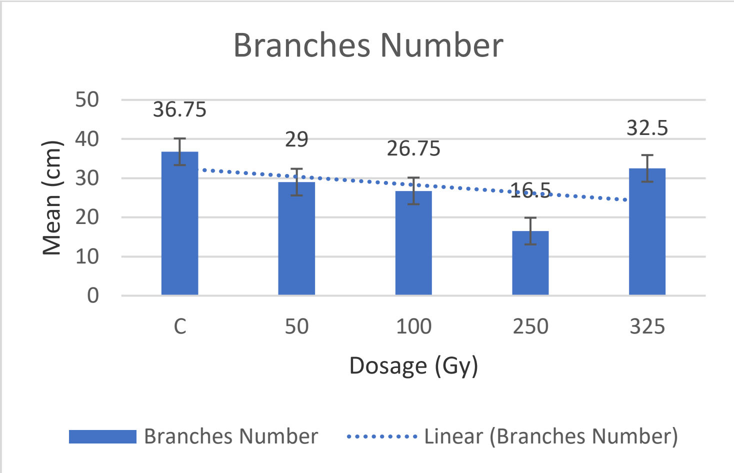

Figure 5. Number of branches of transplanted stevia mutation after 12 weeks

The highest mean of leaf width is higher in 250 Gy followed by 325 Gy, 50 Gy and 100 Gy with 3.4 cm, 3.125 cm, 2.975 cm and 2.9 cm respectively. However the overall results of treated plant is lower from control, 0 Gy, which is 3.55 cm (Figure 6). There is no significant difference for leaf width ($p>0.05$). Leaf width is another good characteristic to indicate larger leaf weight that implies to higher quantity of sweetener content in the leaves. Generally, the leaf is responsible for helping the yield quantity of the stevia plant. In this study, the gamma irradiation did not affect the size of the leaf as the highest leaf ratio is from 0 Gy (control) stevia plant. In other finding, an increased in dosage of gamma radiation may control the plant cells action and slightly alter the morphology of the plant [16].

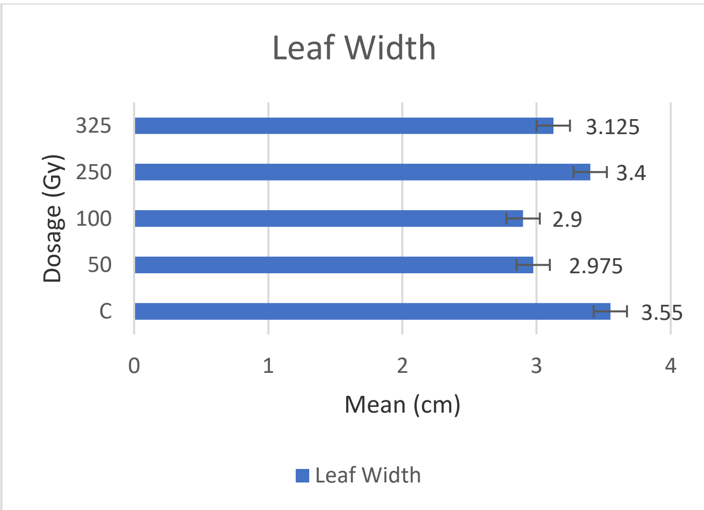

Figure 6. The leaf width of the transplanted stevia mutation after 12 weeks

A significant difference in the leaf length of radiated stevia was observed between all treatments. Treatment 0 Gy (control) showed the highest leaf length meanwhile treatment 100 Gy has the lowest mean of leaf length (Figure 7). This is probably happened due to the alteration of stevia tissue, which cause the leaf for different dosage of gamma exposure effect in overall leaf size. The size of the leaves plays a big role where a large surface area of leaves means that there are many chloroplasts to absorb sunlight. Chloroplasts is the tiny structures in plant cells, which contain chlorophyll, a green pigment that absorbs light energy for photosynthesis [17]. Moreover, in stevia plant the leaf contains chemical compound that make the leaves taste sweet and useful for human [18].

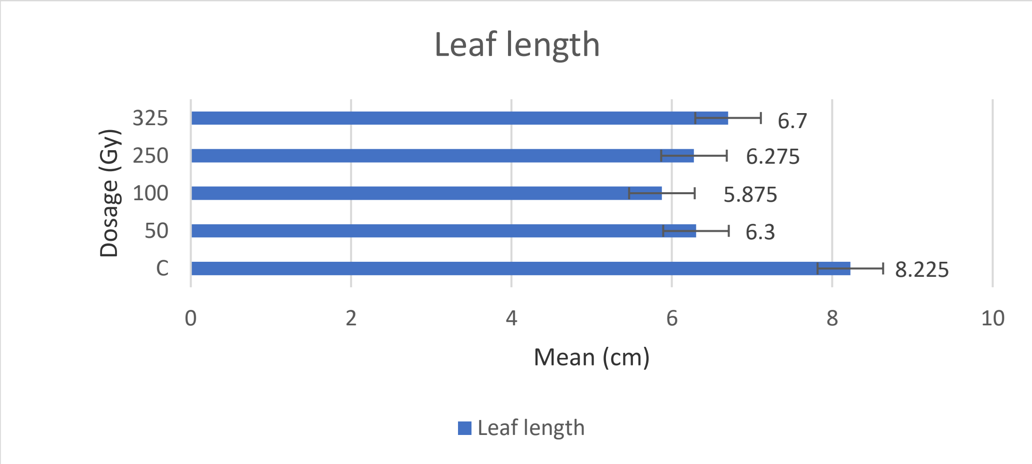

Figure 7. The leaf length of transplanted stevia mutation after 12 weeks

4. Conclusion
In summary, gamma irradiation affected differently on the seed germination, plant height, leave number, branch number, leaf width and leaf length of stevia plant depends on the gamma doses. It is suggested that the suitable dose to generate morphological variability in stevia accession H1 is 182 Gy. Further observation by selection on the potential mutant lines will be carried out to identify the effect on gamma ray on other characteritics such as the production of steviol glycosides compound and flowering to suit the local environment.

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