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EFFECTS OF ACUTE AND CHRONIC GAMMA IRRADIATION ON *IN VITRO* GROWTH OF *Stevia rebaudiana* Bertoni

Norazlina Noordin, Rusli Ibrahim, Nur Hidayah Mohd Sajahan, Salmah Moosa and Sobri Hussein

Agrotechnology and Biosciences Division, Malaysian Nuclear Agency,
Ministry of Science, Technology and Innovation Malaysia (MOSTI),
Bangi, 43000 Kajang, Selangor
Correspondence author: azlina@nuclearmalaysia.gov.my

ABSTRACT

Stevia rebaudiana Bertoni is a perennial herb that belongs to the family of Asteraceae. It is a natural sweetener plant known as sweet leaf, which is estimated to be 300 times sweeter than cane sugar. In this study, micropropagation and in vitro mutagenesis of this natural herb was successfully conducted. It was found that shoot tips on MS medium supplemented with 1 mg/l Kinetin showed the highest shoot induction and multiplication after 3 weeks of culture (5.50±1.95a). Radiosensitivity test was conducted to identify the dose that killed 50% of the irradiated explants (LD_{50}) for in vitro stevia shoots and to select effective doses to be used for the in vitro mutagenesis. Shoot tips were irradiated with acute and chronic gamma radiation at 0, 10, 20, 30, 40, 60, and 80Gy. At 60 Gy and 80 Gy, all treated shoot tips were not survived. In this study, LD_{50} for the stevia was estimated at 29 Gy for acute irradiation and 45 Gy for chronic irradiation. The effective doses were selected at 10, 20, 30 and 40 Gy. These selected doses were applied for the in vitro mutagenesis of the stevia shoots.

Keywords: Doses, *in vitro* mutagenesis, gamma irradiation, micropropagation, *Stevia rebaudiana*

INTRODUCTION

Stevia is a herbaceous perennial shrub originated from the highlands of Paraguay and sections of Argentina and Brazil. Stevia was discovered by Antonio Bertoni, a South American Natural Scientist, in 1887. This plant is a natural sweetener and famously known as “Sweet Weed”, “Sweet Leaf”, “Sweet Herbs” and “Honey Leaf”, which is estimated to be 300 times sweeter than cane sugar. *Stevia rebaudiana* Bert belongs to the family *Asteraceae*, one of 154 members of the genus Stevia, which produces sweet steviol glycosides (Robinson, 1930; Soejarto *et al*., 1982). The leaves of stevia are the source of diterpene glycosides, stevioside and rebaudioside (Yoshida, 1986). Stevioside is regarded as a valuable natural sweetening agent because of its relatively good taste and chemical stability (Yamada et al., 1985). Stevioside is of special interest to diabetic persons with hyperglycemia and the diet conscious (Arpita et al., 2011)). In Japan alone, an estimated 50 tons of stevioside is used annually and in Canada, steviosides sales are valued at $220 million annually (Brandle and Rosa, 1992). Stevia has been introduced as a crop in a number of countries including Brazil, Korea, Japan, Mexico, United States, Indonesia, Tanzania and Canada (Brandle and Rosa, 1992; Fors, 1995; Saxena and Ming, 1988; Shock, 1982) for food and pharmaceutical products. Currently *S. rebaudiana* production is centered in China with major market in Japan (Kinghorn and Soejarto, 1985). The product also can be added to tea and coffee, cooked or baked goods, processed foods, pickles, fruit juices, tobacco products, confectionary goods, jams and jellies, candies, yogurts, pastries, chewing gum and sherbets beverages (Arpita et al., 2011).

Seeds of stevia show a very low germination percentage and vegetative propagation is limited by lower number of individuals (Sakaguchi et al., 1982). Tissue culture is the only rapid process for the mass propagation of stevia and there have been few reports of in vitro growth of stevia (Miyagaya et al., 1986), *in vitro* micropropagation from shoot tip and leaf (Uddin et al., 2006).

Mutation techniques using radiation have been widely applied to improve crop yield, quality and disease and pest resistance. They have produced many germplasm with novel and desired traits (Maluszynski et al., 1995). Mutants of many crops have been successfully released worldwide (Ahloowalia et al., 2004). Gamma rays were reported to be the most efficient ionizing radiation for creating mutants in plants as they can induce high mutation rate. They could also modify physiological characteristics to create new mutants with improved properties that can produce higher amounts of commercially essential metabolites and develop varieties that are agriculturally and economically significant with high productivity potential.

To our knowledge, there is no report on the use of radiation to induce mutation for the improvement of physiological traits and enhancement of secondary metabolites, steviol glycosides in *Stevia rebaudiana*. However, gamma irradiation had been applied on other herbal and medicinal plants for the same purpose. Sina et al. (2011) found that acute gamma rays at doses of 40 and 60 Gy had a positive effect on the stimulation of flavonoid in two accessions of *Centella asiatica (*CA03 and CA23). Biochemical tests revealed that the irradiated accessions displayed higher total flavonoid content than the non-irradiated ones. Moreover, the irradiated plants were found to have the highest flavonoid contents in their eighth week of growth.

The main objectives of this project were to determine the effects of chronic gamma irradiation on growth and multiplication rate of *in vitro* shoots and to evaluate the potential of gamma rays for the stimulation and enhancement of steviol glycosides in *Stevia rebaudiana*. Hopefully with the introduction of a new variety of *Stevia rebaudiana* with enhanced production of steviol glycosides can create an improvement and utilization of this natural sweetener to develop a sustainable Stevia industry in Malaysia.

MATERIALS AND METHODS

Collection of Explants and Surface Sterilization

Young, actively growing shoot tips and nodal segments were collected from stevia seedlings that were maintained in the glasshouse of Malaysian Nuclear Agency. The explants of 2 - 3 cm in length were put under running tap water to remove traces of soil and dirt and later were soaked in systemic fungicide 5% (w/v) for 30 minutes. The following processes were conducted in a laminar air flow which provided a strict aseptic condition. The explants were surface sterilized using 5% (v/v) commercial sodium hypochlorite with a few drops of Tween 20 for 15 min. This step was repeated twice and then rinsed 4 times with sterile distilled water. Excess water adhering to the explants was air-dried and then the explants were ready to be introduced into culture media.

Shoot Induction and Multiplication

Sterilized shoot tips and nodal segments (with a single axillary bud) were cultured onto semi-solid MS Medium (Murashige and Skoog, 1962) supplemented with 6-benzylamino purine (BAP) or 6-furfurylaminopurine (kinetin) at concentrations ranging from 0, 0.5, 1.0 and 1.5 mg/L. These plant

growth regulators (PGRs) were added singly into the MS medium together with 3% (w/v) sucrose and 2.5 g/L gelrite. The pH of the medium was adjusted at 5.8 with 0.1 M NaOH before autoclaving at 121°C for 15 min. The cultures were incubated in the incubation room at 24 ± 2°C with 16-hours photoperiod. Observation on new shoot induction and multiplication was done weekly. The length of plants and number of new shoots formed were monitored weekly for one month. The data was analyzed by SPSS statistical analysis version 19 using one way ANOVA with Duncan test. Table 1 shows different treatment for shoots formation *of Stevia rebaudiana.*

Table 1: Different treatment for shoots formation of *Stevia rebaudiana*

Treatment	Medium Composition (Explants)	Treatment	Medium Composition (Explants)
1	MSO (shoot tip)	8	MS + 1.5mg/L Kin (nodal)
2	MSO (nodal)	9	MS + 0.5 mg/L BAP (shoot tip)
3	MS + 0.5 mg/L Kin (shoot tip)	10	MS + 0.5 mg/L BAP (nodal)
4	MS + 0.5 mg/L Kin (nodal)	11	MS + 1.0 mg/L BAP (shoot tip)
5	MS + 1.0 mg/L Kin (shoot tip)	12	MS + 1.0 mg/L BAP (nodal)
6	MS + 1.0 mg/L Kin (nodal)	13	MS + 1.5 mg/L BAP (shoot tip)
7	MS + 1.5 mg/L Kin (shoot tip)	14	MS + 1.5 mg/L BAP (nodal)

Subcultures were done every 30 days interval. Nodal segments and shoot tips from the proliferated shoots were subcultured for further multiple shoot induction. The regenerated multiple shoots were cut and individual shoots were placed in semi-solid MS without PGR for elongation and root induction.

In Vitro Mutagenesis with Acute and Chronic Gamma Irradiation

The *in vitro* grown and healthy looking stevia shoot tips were selected and cultured onto semi-solid MS Medium supplemented with 1.0 mg/L kinetin. After 7 days of culture, these explants were irradiated with acute gamma radiation at 0, 10, 20, 30, 40, 60, and 80 Gy using Gamma Cell facility and chronic gamma irradiation at 0, 10, 20, 30, 40, 60, and 80 Gy using Gamma Greenhouse facility. The irradiated explants were incubated in the incubation room at 24 ± 2°C with 16-hours photoperiod. The survival rate, length of plant, number of new shoots formed, and number of leaves formed were recorded weekly for a period of 30 days. The data obtained was analyzed by SPSS statistical analysis version 19 using one way ANOVA with Duncan test.

RESULTS AND DISCUSSION

Micropropagation of *Stevia rebaudiana*

The results in Table 2 showed significant difference for the length of plant and number of shoots for each treatment. In this study, explants cultured on MS media without hormone gave the highest length of plantlet but having lowest number of new shoots formed. It was observed that the best treatment for stevia growth and shoot multiplication was in MS media supplemented with low concentration of kinetin (0.5 - 1.0 mg/L) for both shoot tips and nodal explants. After 3 weeks of culture in medium containing kinetin, multiple shoots were formed into complete plantlets with vigor stem, larger, greener and normal looking leaves with no callus formation. Meanwhile, for BAP treatments, the number of new shoot produced was lower and from the observation, stevia

shoots produced in BAP demonstrated curly and abnormal looking phenotype. Callus growth was also observed at the base of the shoots. Comparatively, it was found that MS media supplemented with 1.0 mg/L kinetin is the most suitable and workable treatment to be used for shoot induction and multiplication (5.50 ± 1.95a) and shoot tips showed better potential to be used as explants compared to the nodal. Figure 1 shows the difference in shoot multiplication form in medium containing Kinetin and BAP.

Table 2: Effects of 14 different treatments on *Stevia rebaudiana* growth after 4 weeks of culture

Treatment	Length of Plantlet (cm ± SD)	No of New Shoot Formed (mean ± SD)
1	9.97 ± 3.00a	1.24 ± 0.59f
2	8.06 ± 3.26b	1.70 ± 0.54ef
3	5.89 ± 1.93c	5.32 ± 1.83ab
4	6.18 ± 1.30c	4.92 ± 1.38ab
5	5.95 ± 1.15c	5.50 ± 1.95a
6	5.64 ± 1.32cd	4.70 ± 1.43b
7	1.03 ± 0.54gh	1.40 ± 0.70f
8	0.61 ± 0.44h	1.62 ± 0.70ef
9	5.13 ± 1.18de	3.86 ± 1.39c
10	4.96 ± 2.90e	5.24 ± 2.44ab
11	4.50 ± 0.94ef	2.70 ± 1.75d
12	4.23 ± 0.87f	2.18 ± 1.65de
13	0.92 ± 0.43gh	1.26 ± 2.18f
14	1.53 ± 0.83g	1.62 ± 1.82ef

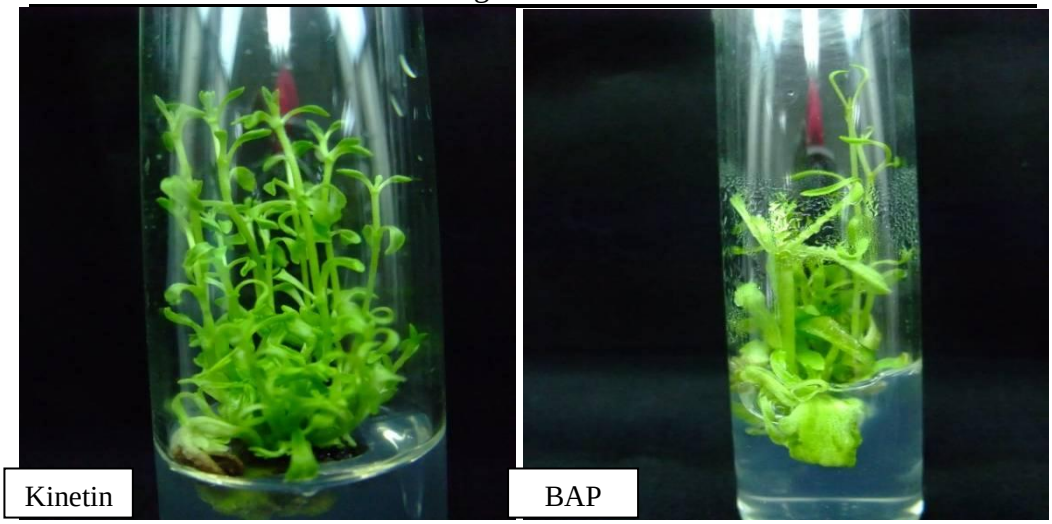

Figure 1: Differences in shoot multiplication form for stevia cultured in medium containing Kinetin and BAP

Effects of Acute and Chronic Gamma Irradiation

Acute Gamma Irradiation

Table 3 shows the effects of acute gamma irradiation on *stevia rebaudiana* growth after 4 weeks of culture. Based on these results, a significant difference in the percentage of the survival amongst

shoots irradiated with lower and higher doses of gamma radiation was observed. All non- irradiated shoots (control / 0 Gy) and shoots irradiated at 10 Gy showed 100% survival rate with the highest number of new shoots formed 5.00 ± 1.98 and 5.06 ± 1.98 respectively. It was observed that, the survival rate of the shoot tips declined with the increasing dose of gamma radiation. At 60 Gy and 80 Gy, the shoot tips demonstrated 0% survival. Figure 2 shows the effects of acute gamma radiation on plantlets growth after 4 weeks of culture in MS + 1 mg/L Kinetin whilst Figure 3 shows the LD_{50} value for *Stevia rebaudiana* that was recorded at week 4 following irradiation treatments with acute gamma.

Table 3: Effects of acute gamma irradiation on *stevia rebaudiana* growth after 4 weeks of culture

Dose (Gy)	**Survival Rate (% ± SD)**	**Length of Plant (cm ± SD)**	**No of New Shoots Formed (Mean ± SD)**	**No of Leaves Formed (Mean ± SD)**
0	100a	5.74 ± 2.43a	5.00 ± 1.98a	26.96 ± 7.92a
10	100a	4.96 ± 1.49b	5.06 ± 1.98a	24.13 ± 9.84a
20	73.33 ± 44.98b	1.35 ± 1.12c	1.40 ± 1.10b	8.37 ± 5.16b
30	46.47 ± 50.74c	1.20 ± 1.49c	0.93 ± 1.23b	4.77 ± 5.78c
40	40.00 ± 49.83c	0.74 ± 1.35cd	0.90 ± 1.63b	4.40 ± 6.99c
60	0	0	0	0
80	0	0	0	0

The data are from 5 replications and were analyze by one way ANOVA statistical analysis. The numbers followed by different letters
shows significant difference at $p < 0.05$ by Duncan test

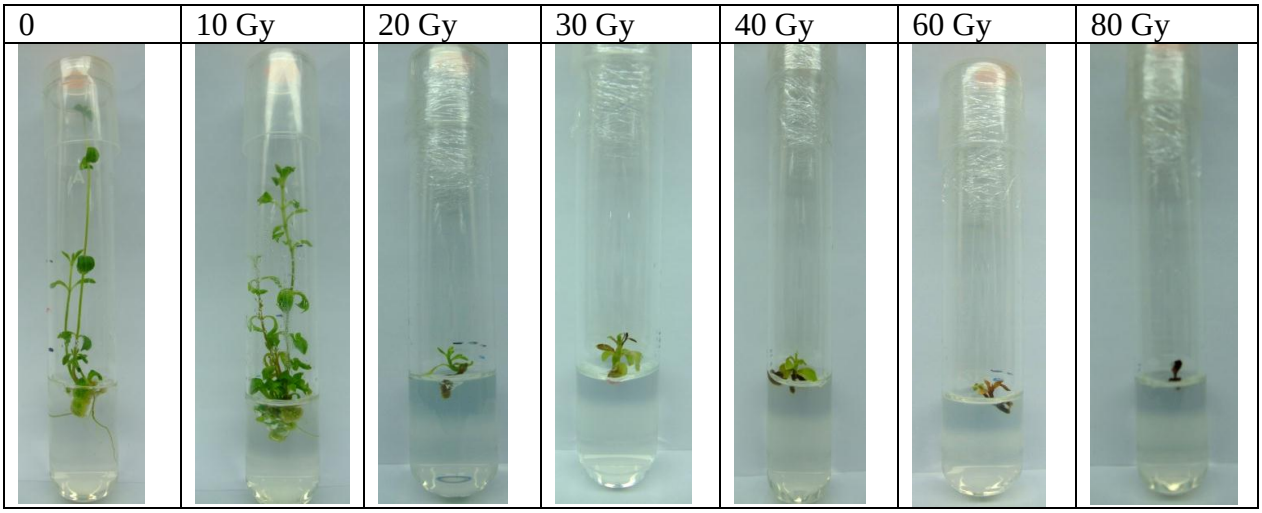

Figure 2: Effects of acute gamma radiation on plantlets growth after 4 weeks of culture in MS + 1 mg/L Kinetin

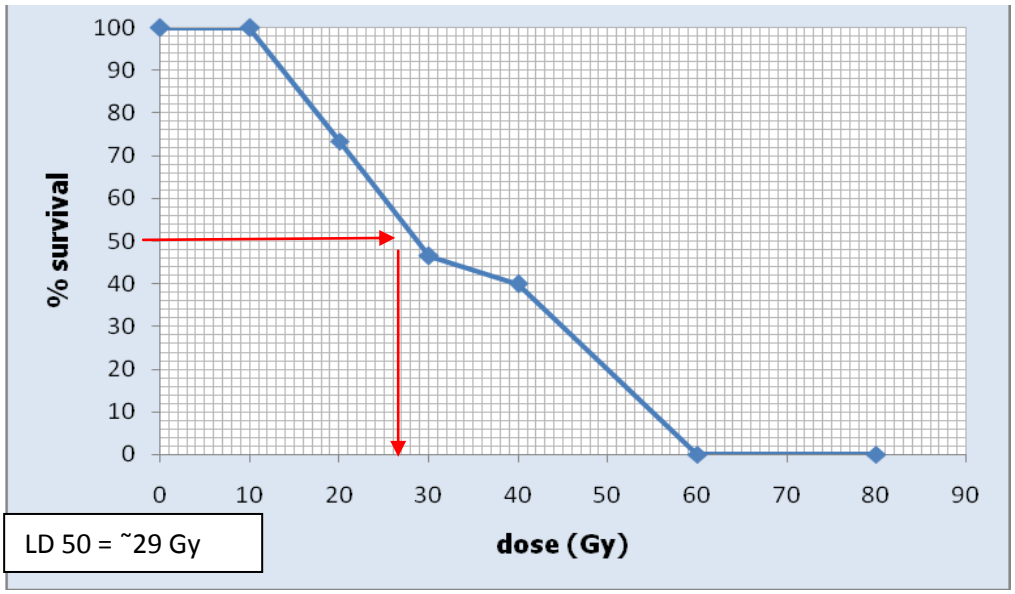

Figure 3: The LD_{50} value for *Stevia rebaudiana* shoot culture after 4 weeks of irradiation with acute gamma

From the LD_{50} results, it was clearly demonstrated that with the increasing gamma dose, the survival rate of stevia shoots showed significant declining in the survival rate. Based on the figure above, LD_{50} for the stevia was at 29 Gy. From this study, the effective doses for acute irradiation were selected at 10, 20 and 30 Gy. These three selected doses were applied for the *in vitro* mutagenesis of the stevia shoots.

Chronic Gamma Irradiation

This radiosensitivity test was conducted as to identify the LD_{50} for *in vitro* stevia shoots and to select effective doses to be used for subsequent *in vitro* mutagenesis. Results from Table 4 showed significant difference in the percentage of the survival amongst shoots irradiated with lower and higher doses of chronic gamma radiation. All non- irradiated shoots (control / 0 Gy) and shoots irradiated at 10 and 20 Gy showed higher survival rate with the highest length of plant, 5.55 ± 2.24, 6.26 ± 2.74 and 6.33 ± 1.84, respectively (Table 4). Non irradiated shoots produced higher number of new shoot as compared to irradiated shoots. It was observed that, the survival rate of the shoot tips declined with the increasing dose of gamma radiation (Figure 4). At 60, and 80 Gy, the shoot tips demonstrated 0% survival and all were killed.

Table 4: Effect of different doses of chronic gamma irradiation on *Stevia rebaudiana* Bertoni growth after 4 week of culture

Dose (Gy)	Survival Rate (% ± SD)	Length of Plant (cm ± SD)	No of New Shoot Form (Mean ± SD)	No of Leave Form (Mean ± SD)
0	100a	5.55 ± 2.24a	4.43 ± 2.09a	25.83 ± 10.10a
10	92.50 ± 26.67a	6.26 ± 2.74a	3.33 ± 1.828b	16.53 ± 7.37b
20	100a	6.33 ± 1.84a	3.00 ± 1.88b	19.18 ± 8.59b
30	80.00 ± 40.50b	4.37 ± 2.89b	1.90 ± 1.70c	12.10 ± 7.53c
40	47.50 ± 50.57c	2.00 ± 2.31c	1.45 ± 1.89d	5.45 ± 6.73d
60	0	0	0	0
80	0	0	0	0

Data was taken from 5 replicates with twenty explants. Significance $p < 0.05$ using Duncan's Multiple Range Test. SD = Standard Deviation

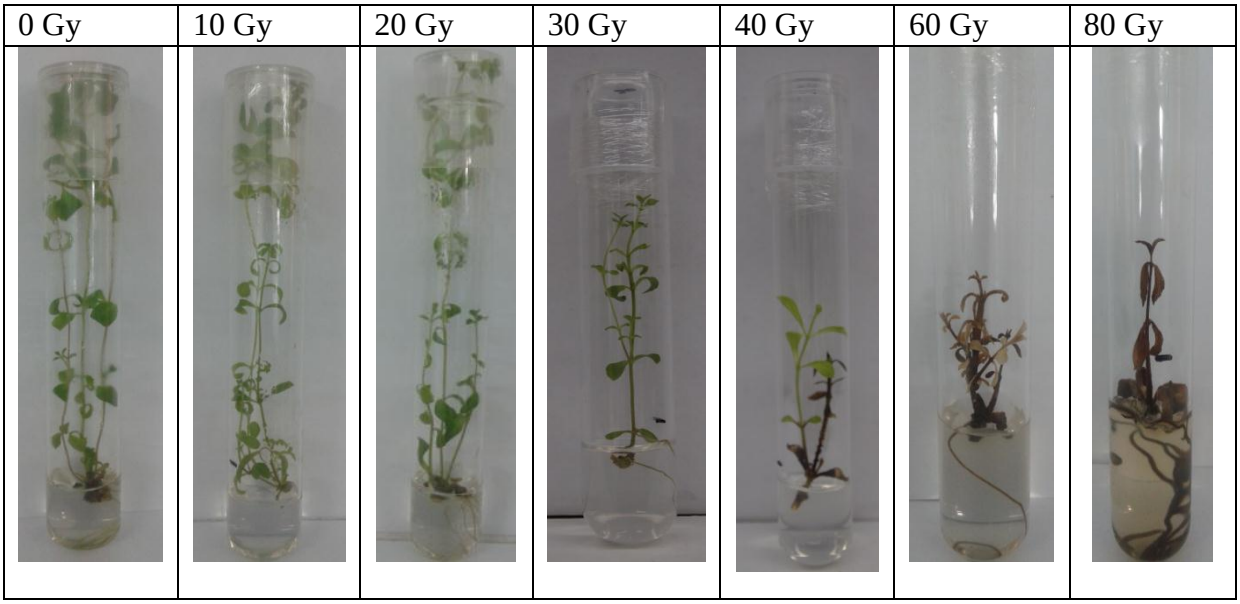

Figure 4: Effect of chronic gamma radiation (Gy) on the growth of plantlets after 4 weeks of culture in MS + 1 mg/L Kinetin

From the LD_{50} results, it was clearly demonstrated that with the increasing gamma dose, the survival rate of stevia shoots showed significant declining. Based on the figure 5, LD_{50} for the stevia was approximately at 45 Gy. From this study, the effective doses were selected at 10, 20, 30 and 40 Gy. These four selected doses were applied for subsequent in vitro mutagenesis of the stevia shoots. Shoot tip explants were irradiated with these doses and regenerated shoots were sub-cultured up to fourth vegetative generation (M_1V_4) to reduce the effect of chimeras.

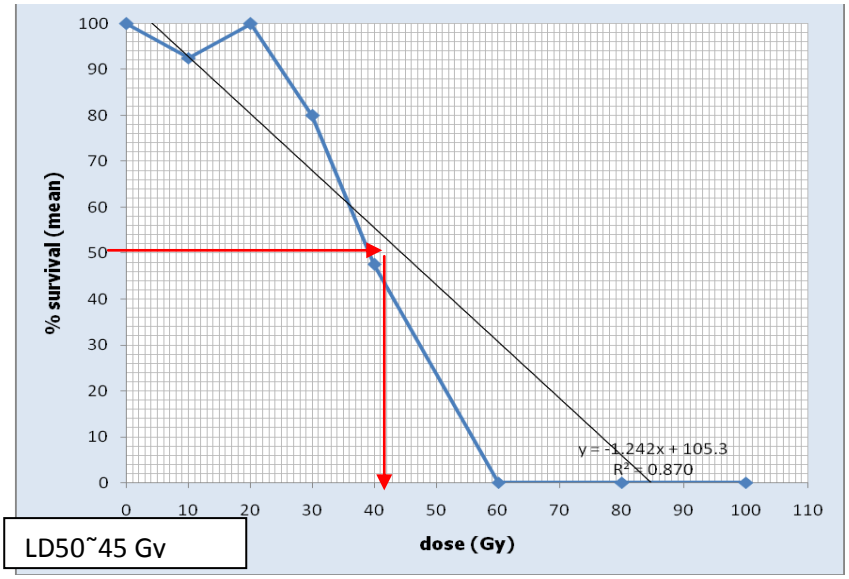

Figure 5: LD_{50} of *Stevia rebaudiana* after 4 weeks of irradiation with chronic gamma

CONCLUSIONS

The micropropagation protocol of *Stevia rebaudiana* Bertoni has been successfully optimized and established. This study suggested that 1.0 mg/L Kinetin is the optimal PGR for stevia shoot multiplication and MS basal medium played a good role in promoting the elongation and root induction of stevia plantlets. The radiosensivity test for *Stevia rebaudiana* Bertoni has been successfully optimized and established for both acute and chronic gamma irradiation. The LD_{50} for stevia shoots was successfully determined and the effective doses were selected at 10, 20, 30 and 40 Gy for chronic and 10, 20, 30 Gy for acute irradiation. These selected doses were applied for the *in vitro* mutagenesis of the stevia shoots and were mass propagated for further screening of desired traits.

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