



Establishment of a Suitable Protocol for Clonal Propagation of Stevia (*Stevia rebaudiana*) and Creating its Advanced Lines using Gamma-Ray

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Abstract

A suitable protocol was developed for *in vitro* clonal propagation of *Stevia rebaudiana* Bertoni from shoots tip and nodal explant. Experiments were conducted to standardize the culture media composition with plant hormone for multiple shoot proliferation and rooting for obtaining plantlets with uniform characteristics like the mother plant in terms of growth and habits. The best response towards multiple shoot regeneration was observed from nodal explants on Murashige and Skoog (MS) basal media fortified with 3.0 mg/L Benzyl amino purine (BAP). In this combination, an average of 14 shoots were regenerated from the shoot tip and 16 shoots were regenerated from nodal explant. Multiple shoots were increased up to two to three times higher when they were sub-culture in the same media combination. Plantlets produced profuse rooting within 10 to 16 days after transfer to MS supplemented with different concentrations of Indole-3-butyric acid (IBA), Alpha naphthyl acetic acid (NAA) and Indole-3-acetic acid (IAA) (0.5-2.5 mg/l). Best root development was obtained in MS containing 1.5 mg/l IBA. Rooted plantlets were transferred to soil for hardening. About 90% of plantlets were successfully established to the field condition. For the induction of the mutation, radio sensitivity tests were performed by determining lethal dose-50 (LD⁵⁰) after irradiating the explants by gamma ray. The LD⁵⁰ was 45 gray (Gy) for shoot tip explants whereas 55 gray (Gy) for nodal explants. Some morphological changes occurred in the treated explants which is significant for induction of mutation.

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Introduction

The *Stevia rebaudiana* Bertoni, widely known as sweet leaf, is a perennial herbal species of the Asteraceae family, which is widely used as a natural sweetener in many countries around the world due to the great demand for its steviol glycosides (SVgly) and rebaudioside A (Reb. A) (Taweel *et al.*, 2021). Initially, this plant was cultivated in the hilly area of Paraguay. This plant was first noted by MS Bertoni in 1887. The *S. rebaudiana* Bertoni is indigenous to

the elated terrain of north eastern Paraguay near its borders with Brazil (Soejarto *et al.*, 2021).

Stevia is becoming a major crop for the nontoxic, nonnutritive, high-potency manufacturing of sweeteners in both developed and developing countries. The *S. rebaudiana* is considered an important plant due to the presence of active compounds steviol glycosides in the leaves. The purified form of steviol glycoside is known as Stevioside which is 300 times sweeter than commercially available sucrose (Hwang *et al.*,

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2006). Stevia species are specially used for the treatment of diabetic patients. The natural Steviosides cannot enter into the blood stream due to the absence of receptor for absorbance. Still today there are no reports that a single patient has completely recovered from diabetes after using different synthetic drugs (Thiyagarajan and Venkatachalam 2012).

The *S. rebaudiana* plants are conventionally propagated through cuttings, but the traditional method is unable to produce a large number of plants. The seeds of this species are smaller in size and the germination percentage is very low due to decreased viability (Singh and Rao, 2005). Propagation by seed does not produce homogenous populations, resulting in great variability in some important features, *e.g.* sweetening level and composition (Tamura, 1984). Moreover, when large scale propagation is necessary for commercial purpose, the conventional way of production is not adequate to fulfill the demand. Therefore, modern propagation techniques, such as *in vitro* regeneration or tissue culture are needed to enhance the production. For these reasons tissue culture techniques are widely used to produce maximum mass from a single plant in a short span of time and also provide opportunities for germplasm conservation (Sivaram and Mukundan, 2003; Anbajhagan *et al.*, 2010; Taware *et al.*, 2010; Jagatheeswari and Ranganathan 2012; Sabah and Rasha 2013).

The plant *S. rebaudiana* has a critical day duration of roughly 13 hours, making it a restricted short-day plant. As for stevia, it takes a few short days for it to flower. According to certain research, certain plants that flower sooner when cultivated in short days would flower later when grown in long days (Abdullateef and Osman, 2011). The demand for a lengthy day duration for stevia to grow has become an important aspect for its production as the awareness of stevia use grows quickly. This is because certain countries did not satisfy the requirements for the ideal stevia yield due to a critical length. To combat stevia's early blossoming, genetic advancement is therefore essential. Researchers from all around the world are paying close attention to the genetic enhancement of stevia

through mutant breeding, particularly when exposed to gamma irradiation (Raina *et al.*, 2016).

Radiation has been shown to be an effective physical agent for inducing mutation. In the recent years scientists around the world were successful to developed new improved commercially important plant varieties which are resistant to different biotic and abiotic factors (Novak and Brunner, 1992). Therefore, based on these studies, attempts will be made to create enhanced stevia varieties that can be grown commercially in the country's climate-changed regions. The soils type and climate of Bangladesh make it ideal for the commercial propagation of this significant natural sweetener plant. Given its significance and in order to address the issue of its propagation through traditional means, the current study was conducted in order to create a straightforward, quick, and affordable protocol for the large-scale production of plantlets and to create advanced and improved mutant varieties of stevia through the use of radiation breeding techniques.

Materials and Methods

In Vitro Organogenesis

Young *Stevia rebaudiana* Bertoni plants were collected from local nursery and maintained in the experimental garden for obtaining fresh explants. Shoot tips and nodal segments were used as initial explants. After collection of these explants from experimental garden, they were washed thoroughly under running tap water and afterwards rinsed several times with distilled water. These were surface sterilized for 2 minutes in 70% ethanol, and 10 minutes in 1.5% sodium hypochlorite in which 0.01% Tween-20 was added as surface sterilizer. Explants were then treated with 0.1% mercuric chloride (HgCl₂) solution for 8 to 10 minutes followed by five washes with sterilized distilled water. Explants were then cultured onto Murashige and Skoog (MS) basal medium added with different concentrations of Benzyl amino purine (BAP), Kinetin (Kn), Indole-3-acetic acid (IAA) singly or in combination with 2-Isopentenyl adenine (2ip) and Alpha naphthyl acetic acid (NAA) combinations after cutting into convenient sizes. For root induction, regenerated shoots were excised and cultured onto a half-strength MS medium supplemented with

different concentrations of Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA). For all experiments, the pH of the medium was adjusted to 5.8 solidified with 0.4% phytigel and dispensed into culture vessels. The culture vessels containing the medium were sterilized at 121°C and 1.05 kgcm⁻² pressure for 20 minutes. Following inoculation, all the cultures were kept in a growth room with a 16 hours photoperiod having a light intensity of 2000 lux (approx.) from white florescent tube light and at the temperature of 26±1°C. When required, subcultures were carried out at an interval of four weeks. Data on multiple shoot regeneration from shoot tip and nodal segment, percentage of explants regenerated, number of shoots per culture, mean shoot length, days to rooting, percentage of plantlets rooted, number of roots per shoot and average length of roots were recorded after four weeks of culture.

Radio Sensitivity Test

Radio sensitivity test is crucial for successful and significant induced mutation in plant species by irradiation. Radio sensitivity ensures plant mutation successfully by different rays, viz. alpha, beta, gamma, cosmic etc. One of the most important steps in mutagenesis experiments is to determine the appropriate dose. Theoretically, lethal dose-50 (LD⁵⁰) would cause the highest frequency of mutations. In the present study shoot tips and nodal segments were irradiated with five level of doses from 25 to 65 gray (Gy) of a cobalt⁶⁰ gamma irradiator of 1850 Terabecquerel (50 kilo curie) at a dose rate of 20 Gy/min. Radiation sensitivity and post irradiation recovery were assessed by

measuring survival rate, propagation rate, shoot height, and fresh weight.

Results and Discussion

Multiple Shoot Regeneration

In the present investigation shoot tips and nodal segments were used for direct organogenesis. MS medium containing different concentrations of BAP, Kn and IAA singly and combined treatment of 2ip and NAA were utilized for multiple shoot induction. Among the various hormonal supplements used, the best response towards multiple shoot regeneration was observed from both the shoot tips and nodal segments on MS medium fortified with 3.0 mg/L BAP (Table 1). In this combination an average of 14 shoot buds were regenerated from the shoot tips (Figure 1a), whereas 16 shoot buds were regenerated from the nodal segments (Figure 1b). The regenerated multiple shoots were routinely sub-cultured for further multiplication in the same medium (Figure 1c). The number of shoot buds increased 3 to 4 folds and also got elongation when sub-cultured on the same medium (Figure 1d). The effects of BAP singly for the regeneration of multiple shoots in the different species of stevia were also reported by several investigators (Jairkar *et al.*, 2009; Mirniam *et al.*, 2013; Bhingradiya *et al.*, 2016; Jadid *et al.*, 2024). Nodal segments produced a comparatively higher number of multiple shoots in the present investigation rather than shoot tips explant when cultured on different BAP concentrations (Table 1). BAP has been considered to be most effective for the induction of multiple shoots in *Coleus* spp. and *Eclipta* spp. (Asamenew and Nrayanaswamy 2000; Baskaran and Jayabalan 2005).

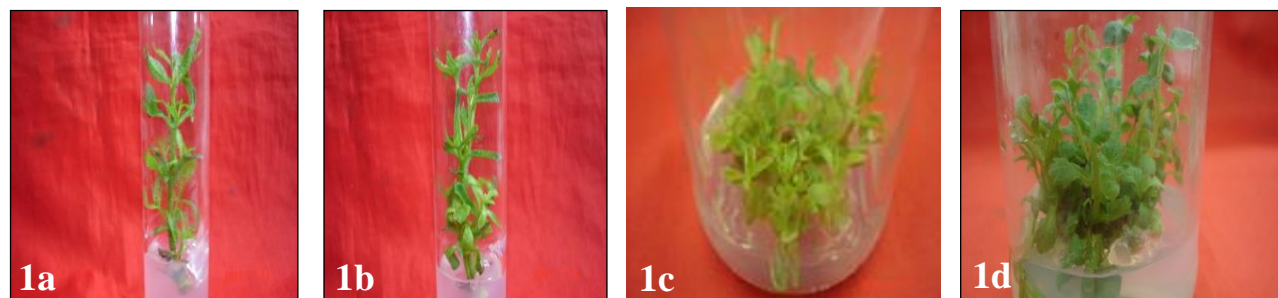


Figure 1(a-d). *In vitro* shoot development in *Stevia rebaudiana*. **1a.** Induction of shoots from shoot tips and nodal segments when they were inoculated on MS fortified with 3.0 mg/L BAP. **1b.** Development of multiple shoots when nodal segments were sub-cultured in the same medium mentioned in 1a. **1c.** Multiple shoot regeneration from nodal segments at the same media compositions. **1d.** Multiple shoots after sub-culture.

Table 1. Effects of different concentrations and combinations of auxins and cytokinin on multiple shoot regeneration from shoot tips and nodal segments of stevia

Growth regulators (mg/L)	Shoot Tips			Nodal Segments		
	Explants Regenerated (%)	No. of shoots/explant	Shoot Length (cm)	Explants Regenerated (%)	No. of shoots/explant	Shoot Length (cm)
BAP						
0.5	70	6	4.2	75	6	4.3
1.0	80	8	4.4	80	9	4.5
2.0	92	10	4.6	94	12	4.7
3.0	100	14	5.1	100	16	5.2
4.0	90	10	4.8	95	12	4.9
Kn						
0.5	60	6	4.0	65	7	4.2
1.0	72	7	4.2	75	7	4.2
2.0	85	10	4.4	90	10	4.3
3.0	95	12	4.7	95	12	4.6
4.0	88	09	4.5	92	11	4.5
IAA						
0.5	65	6	4.0	70	8	4.2
1.0	74	8	4.3	78	11	4.3
2.0	84	10	4.4	90	11	4.4
3.0	90	12	4.5	92	12	4.5
4.0	85	9	4.3	89	10	4.3
2iP+NAA						
0.5+0.5	60	7	4.0	65	8	4.2
1.0+1.0	80	10	4.2	82	10	4.4
2.0+1.5	85	12	4.5	90	12	4.6
3.0+2.0	80	10	4.1	80	12	4.2

Thirty explants were taken for each treatment and data were recorded after five weeks of culture.

Aamir *et al.* (2008) also reported best shoot formation on MS supplemented with 4.0 mg/L BAP in carnation, on the other hand, Nagaraju and Parthasarathy (1995) obtained a maximum number of shoots on a medium containing 0.75 mg/L BAP in Gladiolus. Several investigators earlier reported multiple shoot induction using different concentration of BAP. They reported the best response in case of multiple shoot regeneration from *in vitro* micro-cutting of *S. rebaudiana* using shoot tip and nodal segments (Das *et al.*, 2010; Khalil *et al.*, 2014). In the present study, 3.0 mg/L BAP was found suitable for large-scale shoot initiation and further multiplication. This difference may be due to the difference in the physiological condition of the explant sources.

Determination of LD⁵⁰ for Induced Mutation

Now a days, for the development of improved varieties radiation breeding technology is very useful and it was efficiently used in the production of mutant lines in different crop plants, *viz.* Gerbera, Wheat, Soybean etc. (Laneri *et al.*, 1990; Borzouei *et al.*, 2010; Hanafiah *et al.*, 2010). The most commonly used mutagens so far are physical mutagens such as gamma rays (Laneri *et al.*, 1990). One of the most important steps in mutagenesis experiments is to determine the appropriate dose. Theoretically, LD⁵⁰ would cause the highest frequency of mutations (Akter *et al.*, 2011).

In the present study shoot tips and nodal segments excised from the axenic culture of different stevia plant species were used for mutagenesis. The

explants were irradiated with five levels of dose from 25 to 65 Gy of a cobalt⁶⁰ source at a dose rate of 20 Gy/min. Radiation sensitivity and post-irradiation recovery were assessed by measuring survival rate, propagation rate, shoot height, and fresh weight. The time at which the cells are irradiated is very critical. The optimal timing for irradiation is considered to be 4 to 6 days after subculture because at this time the majority of cells are in the G₁ phase. LD⁵⁰ was determined for different explants of stevia, viz. shoot tips and nodal segments. 45 Gy were effective for shoot tip, on the other hand 55 Gy were effective to nodal segment (Figure 2). Several investigators obtained positive response for developing advanced varieties of different plant species using cobalt⁶⁰ gamma irradiators (Maluszynski *et al.*, 1995; Stove 2002; Çiftçi *et al.*, 2006; Borzouei *et al.*, 2010; Hanafiah *et al.*, 2010). The results of the current investigation concur with those of the earlier researchers.

Phenotypic Study of Irradiated Plants

For ensuring induced mutation through gamma irradiation in plant species physiological as well as molecular observation is necessary. In the recent years few cytological studies also proved induction of mutation in plants successfully. In the present investigation some morphological studies viz. plant height and root numbers of irradiation induced stevia plants were examined. They all were survived in LD⁵⁰.

Plant Height

A common metric for assessing the biological impact of physical mutagens is plant height. In the current study, the control population's average plant height was found to be higher (Figure 3a), and gamma rays were found to lower this average height (Figure 3b). The average plant height decreased at all of the doses (25 Gy to 65 Gy) employed in this investigation, and the average plant height also demonstrated inverse proportionality to radiation intensity.

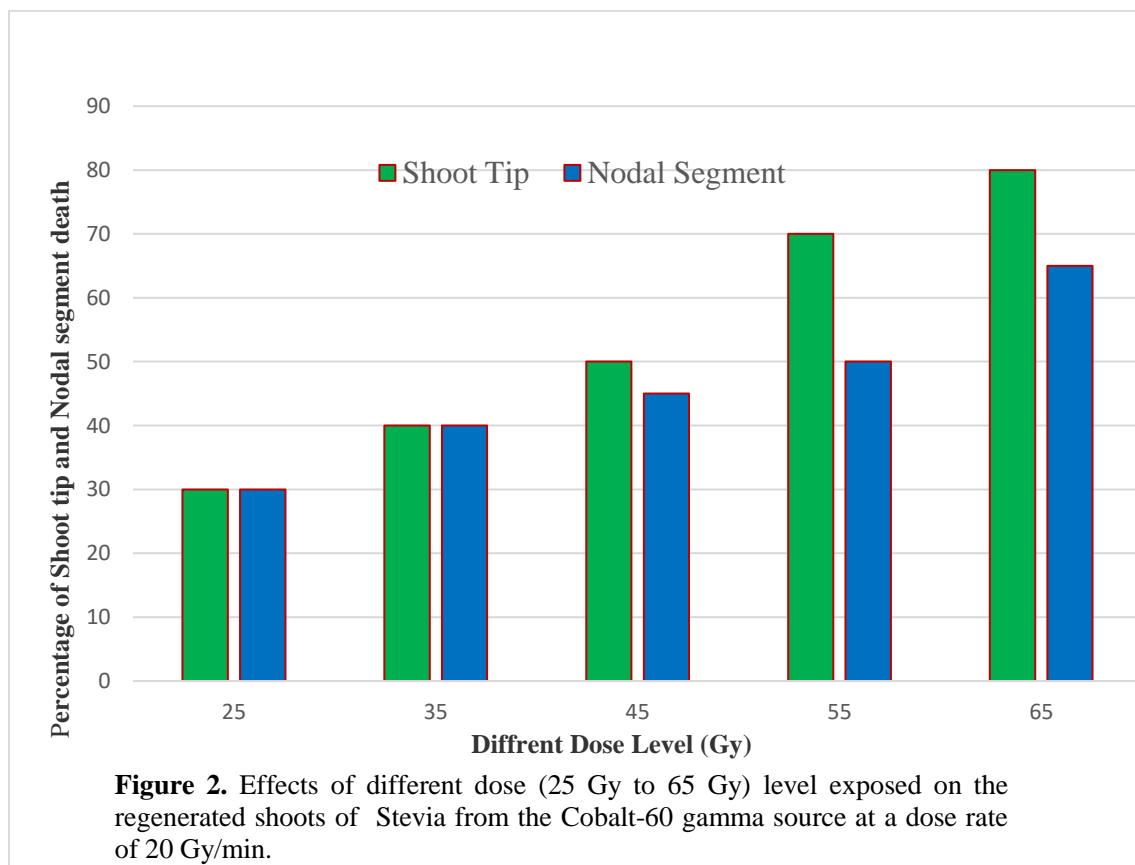




Figure 3a. Vigorous growth of multiple shoots without irradiation (Control)



Figure 3b. Stunted growth of multiple shoots after exposed to irradiation



Figure 4a. Developing of adequate roots without irradiation (Control)

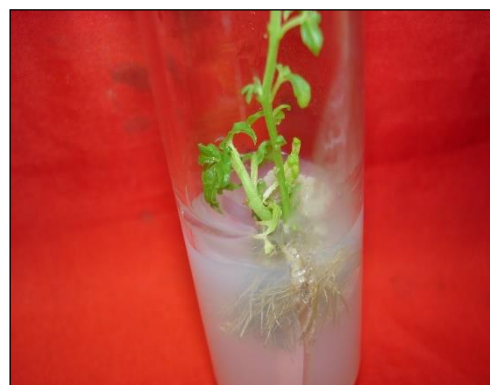


Figure 4b. Reduced development of roots after radiation treatment

The lowest plant height recorded was in 65 Gy treatment. This result showed that increasing gamma irradiation caused slow height increment. Previous study has recorded the negative effects of high dosage of gamma irradiation (Pande *et al.*, 2011).

Roots Number

Highest results were observed for the number of roots in treatment 0 Gy (control) to 35 Gy (Figure 4a) with the average 26.4 roots grown in each case. The lowest number of roots recorded was in treatment 65 Gy (20.6 roots).

There is no significant difference in root number between the control treatment and treatment 35 Gy but there is significant difference with treatment 45 to 65 Gy (Figure 4b). These results showed after gamma irradiation exposure with low dose of mutagen treatment can stimulate the growth and

development of roots. This is consistent with earlier research conducted by various researchers, which found that low doses of gamma irradiation were appropriate for plant development (Fusconi *et al.*, 2006; Majeed *et al.*, 2018). On the other hand, high doses of gamma would cause growth abnormalities, impede the emergence of the plants, and ultimately cause the plants to die (Fusconi *et al.*, 2006; Majeed *et al.*, 2018).

Rooting In Vitro

Multiple shoots growing *in vitro* conditions is devoid of roots. Root development in the regenerated shoots is especially important for establishing tissue cultured derived plants in the natural condition. Regenerated shoots did not produce root spontaneously. So, for root formation, *in vitro* raised shoots were separated from the clamp of multiple shoots and they were cultured onto half strength of

MS fortified with different concentrations of IBA, NAA and IAA. In this experiment the best root development was obtained in the rooting medium containing half strength MS medium with 1.5 mg/L IBA (Figure 5a and Table 2). It took around ten to twelve days to obtain a good root formation. It

has been reported that IBA, is a suitable auxin for adventitious root induction and it was also found superior to NAA or IAA for its more stable nature (Huthchinson, 1981; Litz and Jaiswal, 1990). The current results concurred with the researchers who were mentioned.



Figure 5(a-c). *In vitro* rooting and acclimatization in *Stevia rebaudiana* Bertoni. **5a.** Rooted shoots on half-strength MS supplemented with 1.5 mg/L IBA. **5b.** Rooted plant after shifted in poly bag. **5c.** Well hardened plant in earthen tub.

Table 2. Effect of different concentrations of IBA, NAA and IAA on root induction from micro cutting in *Stevia rebaudiana*

Supplements (mg/L)	Number of shoots inoculated	Average number of roots from each shoot	Days to initiation of roots	Average root length (cm)
IBA				
0.5	30	6.0	10–12	4.2
1.0	30	8.2	10–12	4.4
1.5	30	10.5	10–12	4.8
2.0	30	8.4	10–12	4.4
2.5	30	5.6	10–12	4.0
NAA				
0.5	30	5.6	14–16	3.8
1.0	30	7.4	14–16	4.0
1.5	30	8.4	14–16	4.3
2.0	30	6.8	14–16	4.1
2.5	30	5.4	14–16	3.9
IAA				
0.5	30	5.4	12–14	4.0
1.0	30	8.4	12–14	4.1
1.5	30	8.8	12–14	4.5
2.0	30	7.2	12–14	4.1
2.5	30	6.4	12–14	4.0

Data were recorded after four weeks of culture.

After development of sufficient healthy roots, plantlets were taken out from the culture tubes and thoroughly washed under tap water to remove the gelling substances. Then the plantlets were transferred to sterilized soil containing poly bags (Figure 5a) for acclimatization. Finally, the plantlets were transferred to small pots containing 1:1 non-sterilized garden soil and coco-peat and gradually acclimatized to out-door conditions (Figure 5b) and ultimately transferred to soil. About 80% of the plantlets were able to survive in natural conditions. The protocol thus established can be exploited for commercial propagation of this world wide demanding natural sweetener plant in Bangladesh.

Conclusions

The result of the present investigation demonstrated the establishment of an *in vitro* plant regenerations protocol for *Stevia rebaudiana* and this technique appears to be commercially suitable for large scale clonal propagation of this natural sweetener in the country. Apart from this, the study expressed how stevia response to gamma radiation. Lower doses of gamma irradiation (25 Gy to 65 Gy) induced positive morphological changes in stevia, while higher doses (above 100 Gy) negatively affect plant growth and survival due to injurious effects such as chromosomal damages, which reduce the stability of the plant genome.

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Declaration

The authors declare no conflict of interest.

Authors' Contributions

MRI conceptualized the idea; MRI also supervised resourced and validated the project; MRI, MTJ and MAI are responsible for data acquisition,

methodology, investigation and writing of original draft; MRI and MTJ analyzed the data. The completed manuscript has been read and approved by all authors.

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