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EFFECT OF DIFFERENT PROPAGATION TECHNIQUES AND GAMMA IRRADIATION ON MAJOR STEVIOL GLYCOSIDE'S CONTENT IN *STEVIA REBAUDIANA*

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ABSTRACT

In this study, the effect of different propagation techniques (*in vitro* and *in vivo*) and gamma irradiation has been investigated on Steviosides (Stev) and Rebaudioside (Reb) A content in *Stevia rebaudiana* Bertoni. Stev and Reb-A are the major active compounds of *Stevia rebaudiana* that is non-genotoxic and frequently used in many food items as sugar alternative due to its extremely sweet taste. After ingestion these compounds cannot enter into the blood stream and useful for diabetic patients. Seeds and calli were irradiated with different doses of gamma rays and LD_{50} (8.75 Gy) and GR_{50} value (13.33 Gy) were calculated on the basis of germination percentage and growth reduction. Higher germination (20%) was observed in 2.5 Gy irradiated seeds as compared to control (23.31%). Plantlets were obtained from both seeds and callus cultures. The leaves from these mutant plants were subjected to High Performance Liquid Chromatography (HPLC) for Stev and Reb-A contents. Stev and Reb-A content were also determined in different *in vitro* (callus, shoots, plantlets and acclimatized plants) and *in vivo* (seeds, floral parts, stem parts and roots) grown tissues and organs. Higher Stev content (2.597±1.40 mg/g-DW) was recorded in the leaves of MV_1 generation of callus derived plants as compared to control (1.733±0.63 mg/g-DW). Reb-A content was found lower than Stev content. Similarly *in vitro* shoots showed higher biosynthesis of Stev content (2.808±0.70 mg/g-DW) than other *in vitro* and *in vivo* grown tissues and organs. These studies are reported for the first time from Pakistan which can be used for different commercial applications. It is concluded from the current study that *in vitro* shoots (2.808±0.70 mg/g-DW) obtained through tissue culture serve the best organs for Stev and Reb-A content accumulation than other *in vivo* and irradiated tissues.

Key words: *Stevia rebaudiana*; seeds; callus culture; gamma radiation; HPLC; Steviol Glycosides; Stevioside; Rebaudioside-A.

INTRODUCTION

Stevia rebaudiana (Bert.) in the family Asteraceae is cultivated throughout the world for its Stev and Reb A content (Steviol Glycosides) (SGs) which are 300 times sweeter than commercial sugar (Ahmad *et al*., 2011; Reis *et al*., 2011; Mathur and shekhawat, 2012). These sweet compounds are low calorie, non toxic, non mutagenic and approved by FDA and recently used as food supplement in Canada, China, Indonesia, Japan, Korea, Mexico, South America, UK and United States. The Stevia products are useful for obetic persons and diabetic patients (Dey *et al*., 2013; Bondarev *et al*., 2003). Beside sweet taste, Stevia has well reputed antioxidant activity and its bioactive compounds are anti-cancerous, anti-hyperglycemic, anti-hypersensitive and prevent dental caries (Kim *et al*., 2011; Dey *et al*., 2013). The major part of SGs in the leaves is Stev and Reb A content. Other components present but in lower quantities are Steviol-bioside 2, Reb C-F and dulcoside A. Some evidence exists that Steviol-bioside and Reb B are not genuine constituents of *S. rebaudiana* but rather are formed by partial hydrolysis during the extraction process (Aman *et al*., 2013; Dey *et al*., 2013; Woelwer-Riek *et al*., 2010).

The aim of the present study was to investigate the effect of gamma irradiation and different *in vitro* and *in vivo* propagation techniques on main SGs (Stev and Reb A) production. There are enough reports on the composition of steviol glycosides in the leaves of Stevia but it is the first report in Pakistan. Different studies showed that seed germination and stem cutting are not suitable approaches for healthy biomass and Steviosides production. Because Stevia seeds lost viability during storage while, stem cutting need higher input stock and laborious. In the present study we have compared micropropagated, irradiated and *in vivo* grown plants for SGs content. Such studies will provide an opportunity for further selection of suitable tissues for SGs accumulation, which can be used for different commercial applications.

MATERIALS AND METHODS

Seeds collection and irradiation: Healthy and mature seeds were collected from field grown plants of *S. rebaudiana* at the Nuclear Institute for Food and

Agriculture (NIFA), Peshawar. Black coated seeds (1500) containing mature embryos were exposed to 2.5, 5.0, 7.5 and 10 Gy doses of gamma rays from Co^{60} gamma cell source. Hundred seeds without irradiation were used as control. Moisture content of the seed at the time of radiation was kept at 10-12%. The treated seeds along with control were sown in soil for LD_{50} value determination.

Irradiated seeds germination on three different media: The effect of different doses of gamma rays on germination of Stevia seed was investigated. Seeds were germinated on 3 different media for comparison including petri plates, MS-medium and soil. The irradiated seeds were surface sterilized by using recent method of Ahmad *et al*. (2014). The sterilized seeds (400) along with control (100) were germinated in petri plates. Similarly, 400 seeds along with control (100) were placed on MS-medium in Randomized Complete Block Design with 3 replications. Seeds (400) without surface sterilization were sown in soil. To germinate irradiated seeds in soil, 100 Pots were taken and filled with a mixture of sand, silt and clay. 20 pots were used for each dose (2.5, 5.0, 7.5 and 10 Gy) along with control and 5 treated seeds from each dose were taken and sown in each pot. Data regarding seed germination was taken after 28 days of sowing. The LD_{50} was calculated (8.75 Gy) for the applied gamma doses on the basis of germination percentage (Figure-1).

Creation of genetic variability for higher yield and Steviosides content through gamma irradiation in Stevia: For induction of genetic variability to enhance biomass and Steviosides content, accurately 1200 seeds were exposed to gamma irradiation (8.75 Gy). These seeds were then sown in soil to rise M_1 generation. After maturation of M_1, seeds were collected and sown in field to get M_2 generation. In M_2 generation selection were made on the basis of phenotypic characters such as stunted growth, profuse leaves, short internode length and higher biomass production. The leaves from these plants were subjected to HPLC for analysis. Plants with vigorous growth, maximum number of leaves and with uniform suitable height and higher Steviosides content were selected for further propagation.

Selection from *in vitro* mutagenized callus cultures: Calli were irradiated with 05, 10, 15 and 20 Gy doses to create genetic variability and development of somaclonal variants. For each dose 27 flasks containing calli were replicated three times along with control. Murashige and Skoog (1992) basal medium was selected for callus growth with 3% sucrose and solidified with 8 g l^{-1} agar. All callus cultures were placed in growth room at 25±1°C under a 16/8hrs photoperiod. The data on callus growth was recorded after 30-days of culture. The GR_{50} value was calculated on the basis of percent (%) growth reduction in callus for all doses (Figure-2). After GR_{50} calculation, 13.33 Gy dose was selected for creation of genetic variability in calli and raising of MV_1 generation.

The Irradiated calli were shifted to MS- medium containing combination of BA (1.0 mg l^{-1}), NAA (0.3 mg l^{-1}), IBA (0.3 mg l^{-1}) and GA_3 (0.3 mg l^{-1}) for callus multiplication. After multiplication the calli were shifted to shoot regeneration medium containing 1.0 and 2.0 mg l^{-1} of BA, respectively. After successful shoots regeneration, the *in vitro* shoots were shifted to rooting medium containing half strength MS-medium with IBA (0.5 mg l^{-1}) and NAA (0.5 mg l^{-1}) respectively. After 4-5 weeks of culturing, the plantlets of 4-5 cm height were removed from the medium, washed thoroughly with sterile distilled water and shifted to pots for acclimatization. Combination of soil, sand and manure (1:1:1) was used for establishment of plants in pots. After complete establishment and weaning, plantlets were shifted to field for selection and Steviosides content.

Selection of plant materials for steviosides contents: Callus, *in vitro* shoots and plantlets were collected from the laboratory cultures while *in vivo* grown tissues (Leaves from different plants, seeds, floral parts, stem parts, roots) were collected from the field grown plants at Nuclear Institute for Food and Agriculture (NIFA), Peshawar, Pakistan.

Extract preparation: The collected tissues both *in vitro* and *in vivo* grown were dried in an electric oven at 50 ºC for 24 hr. The dried tissues were well grounded to get fine powder as shown in Figure-3. The powdered material were soaked in distilled H_2O for 24hrs and then filtered through Whatman filter paper No.1 (Whatman Ltd., England). The filtrate was dried by using a freeze dryer and stored at 4ºC in airtight bottles and the extractive values were recorded as given in Table-1. The extracts obtained from each part were dissolved independently in HPLC grade water for analysis.

Steviosides analysis: Extraction of Steviol glycosides (SG) from freeze dried powdered was determined according to the recent method of Dey *et al*. (2013) with slight modification. Dried extract (50 mg) was independently dissolved in HPLC grade water (20 ml) for analysis and stock solution preparation. HPLC analysis was performed by using a Perkin-Elmer HPLC system (USA) set with quaternary pump, solvent vacuum degasser, C18 column (ODS; 150 × 4.6 mm), 5µm particle size, a variable wavelength detector, and an auto sampler with a 10 µl injection loop. Mobile phase was prepared from HPLC grade water (25%; A) and acetonitrile (75%; B), respectively. The flow rate was 1.0 ml/min and the injection volume was 10 µl. SGs standard was purchased from Sigma (USA). The Standard was run on HPLC system and retention time for Stev and Reb A was determined. Identification of Stev and Reb A in

different *in vitro* and *in vivo* grown tissues was calculated by comparing the retention time of samples with standard. The results obtained for each sample was expressed in mg/g of dry weight.

Statistical Analysis: All the analysis was repeated three times, and the design of the experiment was Randomized Complete Block Design (RCBD). Statistical analysis of mean values, standard deviation (±), and least significant difference (LSD) were carried out by using Statistix software (8.1 version).

RESULTS AND DISCUSSION

Seed germination: In petri plates experiment, higher seed germination of 20% was observed in seeds irradiated with 2.5 Gy dose as compared to control (23.31%) as shown in Figure-4. Similarly higher seed germination of 13.44% in soil was observed when seeds were irradiated with 2.5 Gy dose as compared to control (41.90%). The least germination (9.43%) was observed on MS-medium with 2.5 Gy dose as compared to control (24.83%). The LD_{50} value was determined for all applied doses.

Effect of gamma irradiation on SGs content in seed derived plants: A total of 1200 seeds were irradiated with 8.75 Gy dose and sown in field to rise M_1 generation. After flower maturation, seeds were collected and sown in soil to raise M_2 generation. A total population of 800 plants was obtained successfully in field. In M_2 generation mutant plants with best growth parameters were selected for Steviosides contents. Leaves from M_2 mutant Stevia obtained from irradiated seeds (MSIS) were analyzed through HPLC for Stev and Reb-A content (Figure-5). In this study, Higher Stev content (1.937±0.72 mg/g-DW) was observed in the leaves of M_2 generation than control (1.73±0.63 mg/g-DW). Similarly, plants obtained from treated seeds showed higher Reb-A content than control. Maximum of 2.19±0.79 mg/g-DW was observed in leaves obtained from irradiated seed derived plants before flowering. In the literature cited there are no reports on the effect of gamma irradiation on Steviosides biosynthesis. However, the effect of radiation on other active compounds biosynthesis is widely reported. Suk *et al*. (2005) reported that gamma irradiation increases 8% antioxidant compounds in *Raphanus sativus* L. young seedlings than control. Costa de Camargo *et al*. (2012) reported that gamma radiation change total phenolic content, condensed tannins, flavonoid content in peanut skin. Harrison and Were (2007) also observed that gamma irradiation enhanced the yield of total phenolic content as well as enhanced antioxidant compounds in *Prunus amygdalus* skins extracts. Pérez et al. (2005) reported 35% higher phenolic content in water extracts after gamma irradiation in *Rosmarinus officinalis* L.

Effect of gamma irradiation on SGs content in callus derived plants: Stevia calli that were irradiated with 05, 10, 15 and 20 Gy gamma rays were cultured on MS-medium containing different combination of auxin and BA. After 4-5 weeks of callus culturing, the GR_{50} value (13.33 Gy) was determined on the bases of growth reduction. Fresh callus cultures were irradiated with 13.33 Gy for creation of genetic variability. The irradiated callus was shifted to shoot regeneration medium. Optimum callus growth (90%) was observed on MS-medium containing combination of BA (1.0 mg l^{-1}), NAA (0.3 mg l^{-1}), IBA (0.3 mg l^{-1}) and GA_3 (0.3 mg l^{-1}). Best shoots regeneration (85%) and multiplication (88%) was recorded on MS-medium containing 1.0 and 2.0 mg $l^{-1}$of BA, respectively. The elongated shoots were transferred to rooting medium containing ½ strength MS + IBA in combination with NAA (0.5 mg l^{-1}) showed 95% rooting response. These rooted-shoots were successfully acclimatized (80%) in pots (Figure-6). Before transferring of plants, the pots were sprinkled with water to retained humidity. The plants were shifted to pots and immediately covered with plastic bags to arrest maximum humidity. The plastic bags were periodically removed from the pots after 23 days of plantation. These mutant plants were then shifted to field conditions. After three months of calli clone acclimatization, leaves were collected from MV_1 plants before and after flowering for Steviosides analysis. Higher Steviosides content (2.597±1.40 mg/g-DW) was observed in the leaves of MV_1-callus derived plants than control (1.73±0.63 mg/g-DW). However, the leaves taken from MV_1-callus derived plants showed lower Reb-A content (0.436±0.24 mg/g-DW) than control (0.896±0.35 mg/g-DW) as shown in Figure-5.

Effect of *in vitro* conditions on Major SG's content: In this study *in vitro* shoots showed higher Stev content (2.8 mg/g-DW) than callus (0.23 mg/g-DW) and *in vitro* plantlets respectively (0.35 mg/g-DW; Figure-7). The higher Stev content in shoots may be due to the addition of different plant growth regulators (PGRs) to the medium. Recently Dey *et al*. (2013) reported that addition of IBA in combination with chlorocholine chloride increased the Stev content than other PGRs. Tissue cultured plants were then transferred to the field conditions and after 8 weeks of establishment, cuttings were taken from these plants. After acclimatization, the Stev content increases (1.45 mg/g-DW) in leaves than *in vitro* plantlets but decreases in the leaves taken from cuttings of tissue cultured plants (1.41 mg/g-DW). Ladygin *et al*. (2008) reported lower Stev content in callus (0.06 mg/g-DM), *in vitro* shoots (0.39 mg/g-DM) but found higher in *in vitro* plantlets (3.3 mg/g-DM). The difference in data may be due growth conditions and solvent used for extraction. Steven *et al*. (1992) reported similar Stev content in callus, *in vitro* shoots and rooted

shoots respectively. Hwang (2006) reported 0.14 mg/g-DW Stev content in callus and higher amount in regenerated plants (10.68 mg/g-DW). The difference in data is due to age of plantlets, a solvent used for extraction and HPLC conditions especially mobile phase. Similarly, Reb A content was found higher in shoots (5.52 mg/g-DW) than calli (1.64 mg/g-DW), *in vitro* plantlets (1.08 mg/g-DW) and acclimated plantlets (4.9 mg/g-DW). The current data are in agreement with the results of Steven *et al*. (1992). Ladygin *et al*. (2008) reported that callus, *in vitro* shoots and *in vitro* plantlets accumulate lower Reb A content than field grown plants. However, the leaves taken from cutting of tissue cultured plant showed the lower amount of Reb A content (0.82 mg/g-DW).

Effect of *in vivo* conditions on Major SG's content: Significantly, less amount of Stev content was observed in seeds (0.44 mg/g-DW), floral parts (0.87 mg/g-DW) and stem parts (0.28 mg/g-DW) as compared to *in vitro* shoots (2.8 mg/g-DW) and *in vivo* leaves. Bondarev *et al*. (2003) obtained similar results that flower, seeds and stem containing lowest amount of Stev content than leaves. However, the roots taken from *in vivo* grown plants showed higher content (1.25 mg/g-DW) than seeds, stem and floral parts (Figure-8). The current data are in agreement with the results of Bondarev *et al*. (2003). Similar results were also reported by different worker that leaves containing higher Stev content than other parts (Bondarev *et al*., 2003; Ladygin *et al*., 2008; Hwang, 2006; Woelwer-Rieck *et al*., 2010). The floral parts, stem parts, seeds and root aqueous extract showed less than 1 mg/g-DW Reb A content. A similar observation was also reported by Bondarev *et al*. (2003) that roots, stem, flower and seeds contained lower amount of Reb A content than leaves. The current data showed that Stev and Reb A content are not equally distributed in different organs but showed variation. In conclusion, different *in vitro*, *in vivo* grown tissues and leaves from irradiated callus and seed derived plants were analyzed for SGs accumulation through HPLC. *In vitro* shoots obtained through tissue culture serve the best organs for Stev and Reb A content accumulation than other *in vivo* and irradiated tissues. This study will be highly useful for further work on selecting and extraction of SGs from different *in vitro* and *in vivo* grown tissues and can be used for commercial applications.

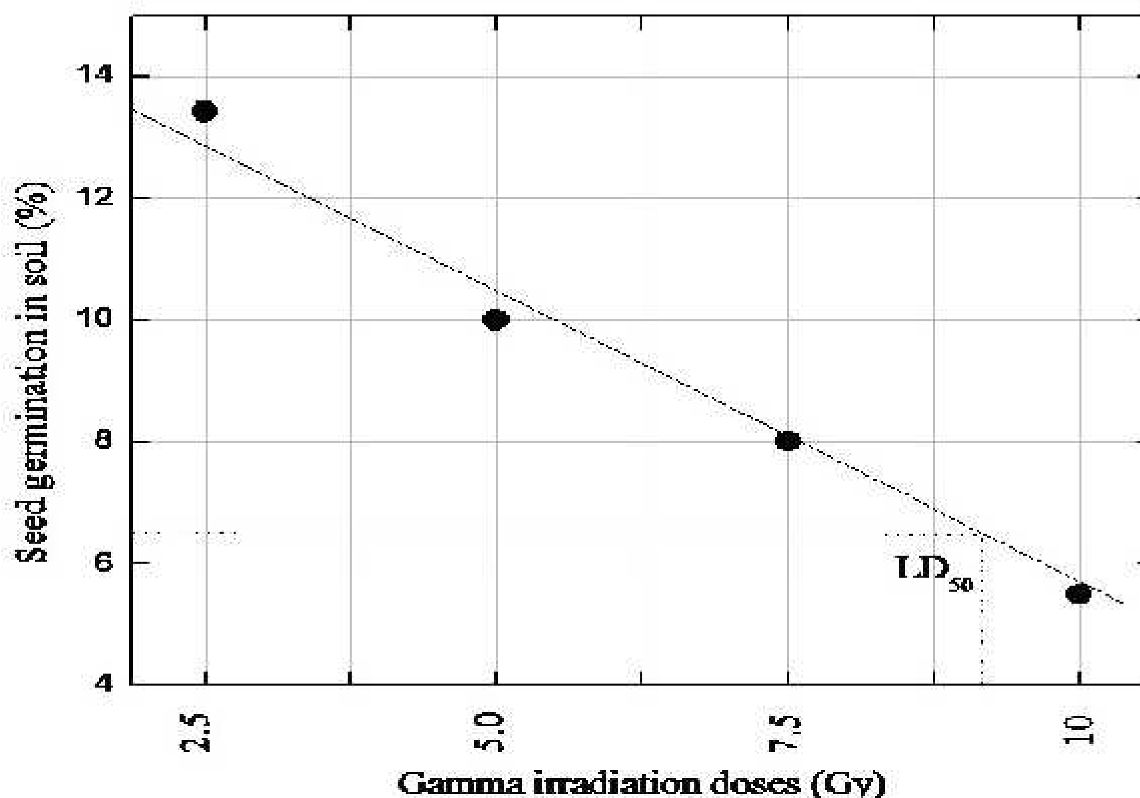

Figure-1: Determination of LD_{50} value for Stevia irradiated seeds germinated in soil media

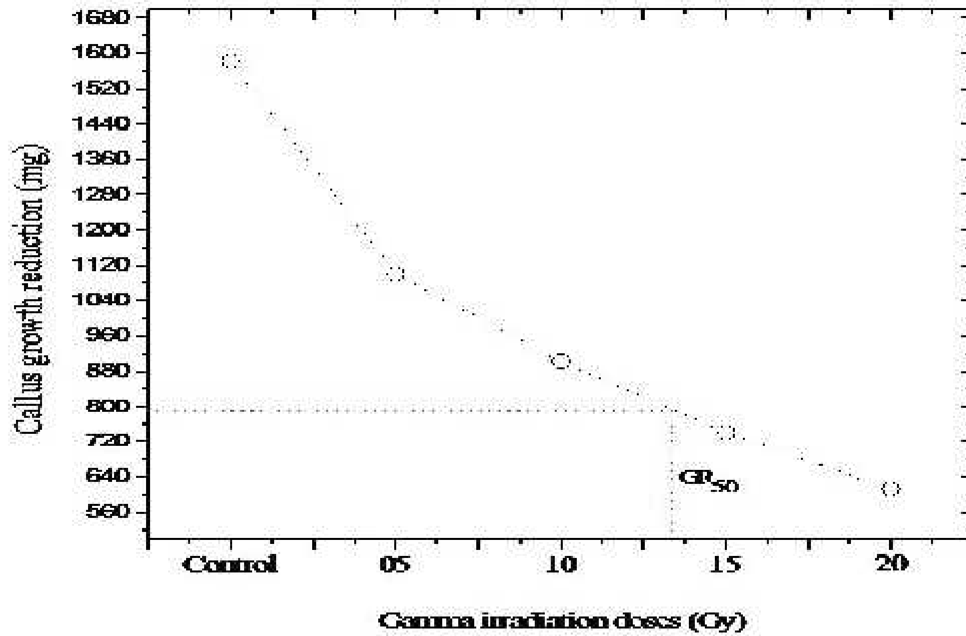

Figure-2: LD_{50} value determination in irradiated calli based on growth reduction

Figure-3: Powdered materials of *in vitro*, *in vivo* and leaves obtained from irradiated callus and seed derived plants of *S. rebaudiana* Bert. (1a) Callus (1b) *In vitro* shoots (1c) *In vitro* plantlets (2a) Leaves from acclimated plants (2b) Leaves taken from cuttings of T.C plants (2c) Seeds (3a) Floral parts (3b) Stem parts (3c) Roots (4a) Leaves from mutant Stevia from irradiated callus (MSIC) MV_1 (4b) Leaves from mutant Stevia from irradiated seeds (MSIS) M_2 generation.

Table 1: Fresh weight, dry weight and aqueous extractive values of different tissues and organs of Stevia

Source	Fresh weight (g)	Dry weight (g)	Extractive values in H_2O (g-DW^{-1})
Callus	26.57	2.57	0.35
In vitro shoots	35.83	2.05	0.75
In vitro plantlets	11.41	1.10	0.39
Acclimated plant leaves	07.83	1.74	1.30
Leaves from tissue culture plant cuttings	0.35	0.94	0.35
Seeds	07.18	6.13	0.26
Floral parts	12.69	4.62	0.43
Stem parts	31.69	14.3	0.53
Roots	04.97	1.84	0.92
Leaves from irradiated callus derived plants (MV_1)	25.97	6.66	0.94
Leaves from irradiated seed derived plants (M_2)	05.24	1.43	0.53
Control	20.19	1.73	0.67

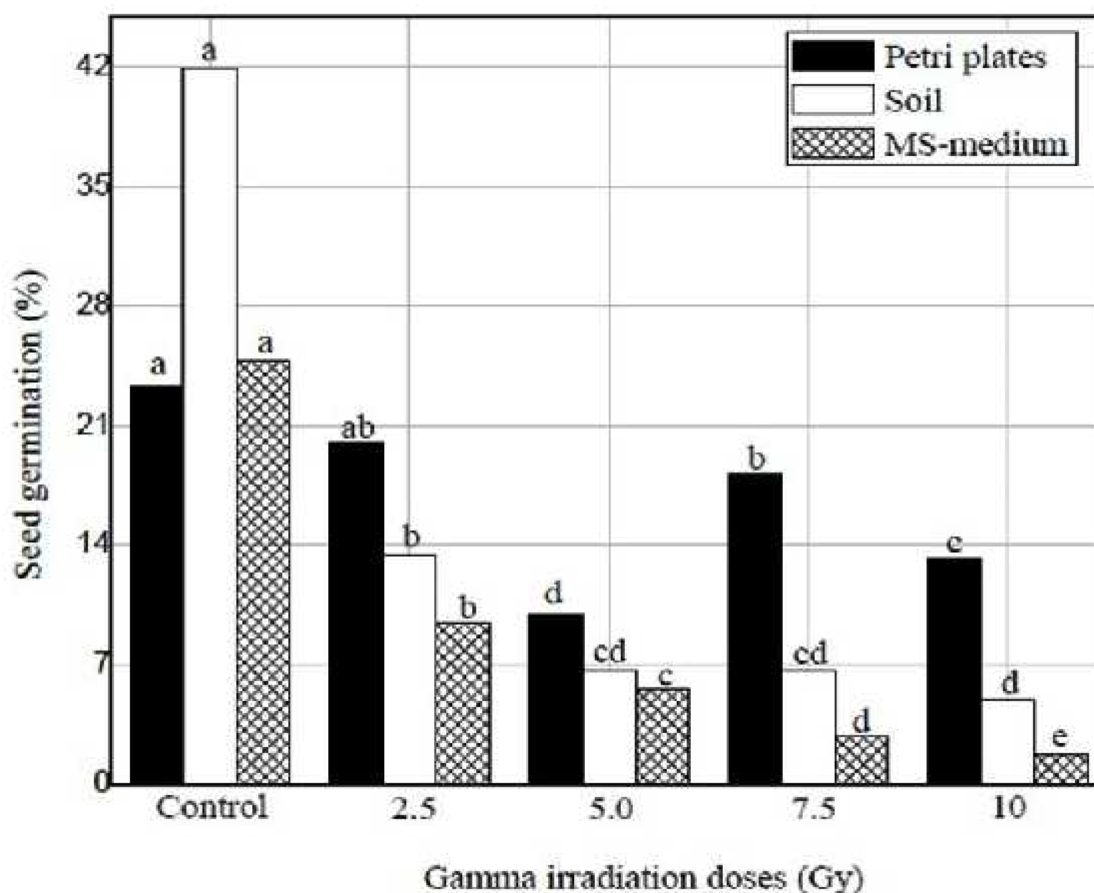

Figure-4: Comparison of irradiated seed germination in petri plates, soil and MS-medium. Data were collected from triplicate experiments. Mean values are significantly different at *P*<0.05

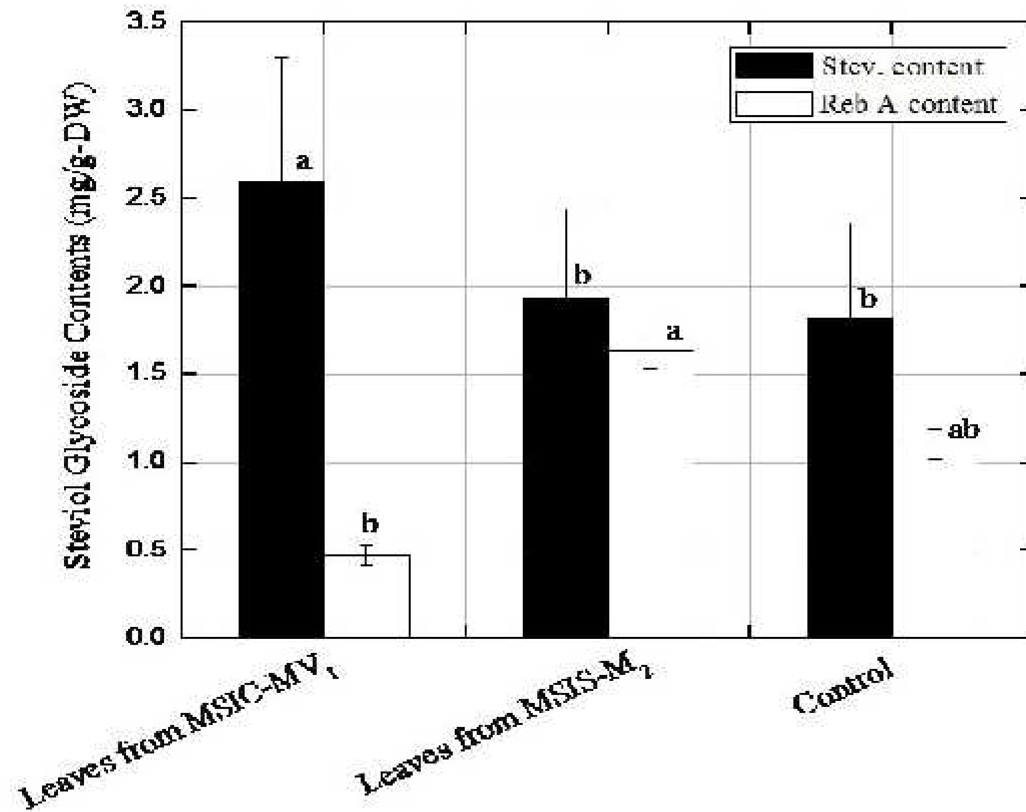

Figure-5: Steviosides (Stev) and Rebaudioside-A (Reb-A) content in leaves obtained from irradiated callus and irradiated seed derived plants. Stev and Reb A content was expressed in milligram per gram dry weight. Data (mean ± SD + LSD) were collected from three independent experiments. Mean values are not significantly different at *P* < 0.01

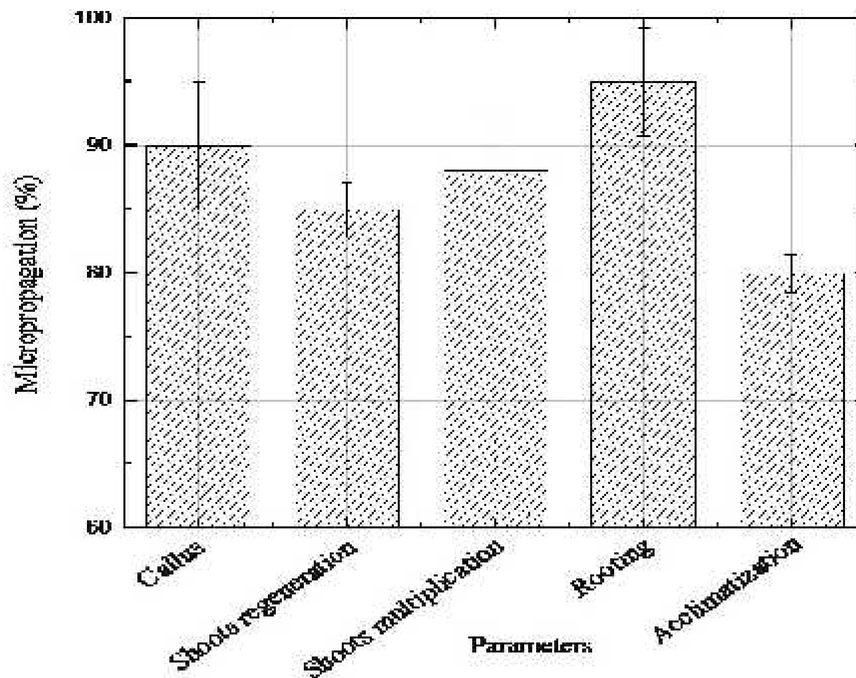

Figure-6: Subculture of callus into fresh medium. Shoot regeneration and multiplication from callus culture. Rooted shoots and acclimatization of *in vitro* mutant plantlets

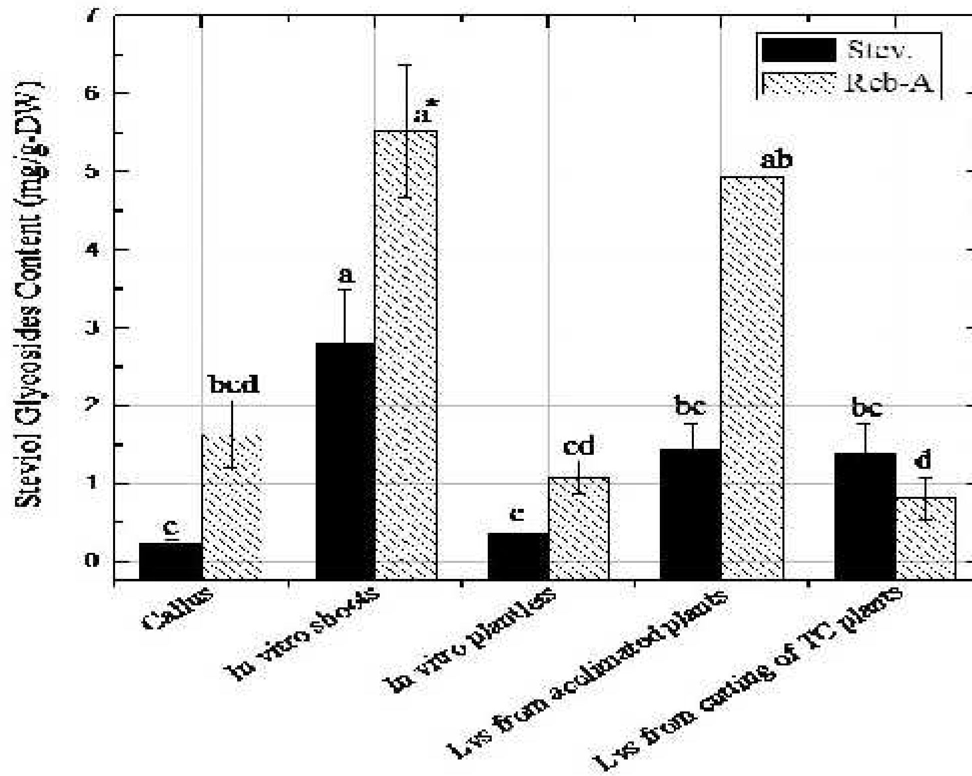

Figure-7: Steviosides (Stev) and Rebaudioside (Reb-A) content in different *in vitro* grown tissues. Stev and Reb A content was expressed in milligram per gram dry weight. Data (mean ± SD + LSD) were collected from three independent experiments. Mean values are not significantly different at *P* < 0.01

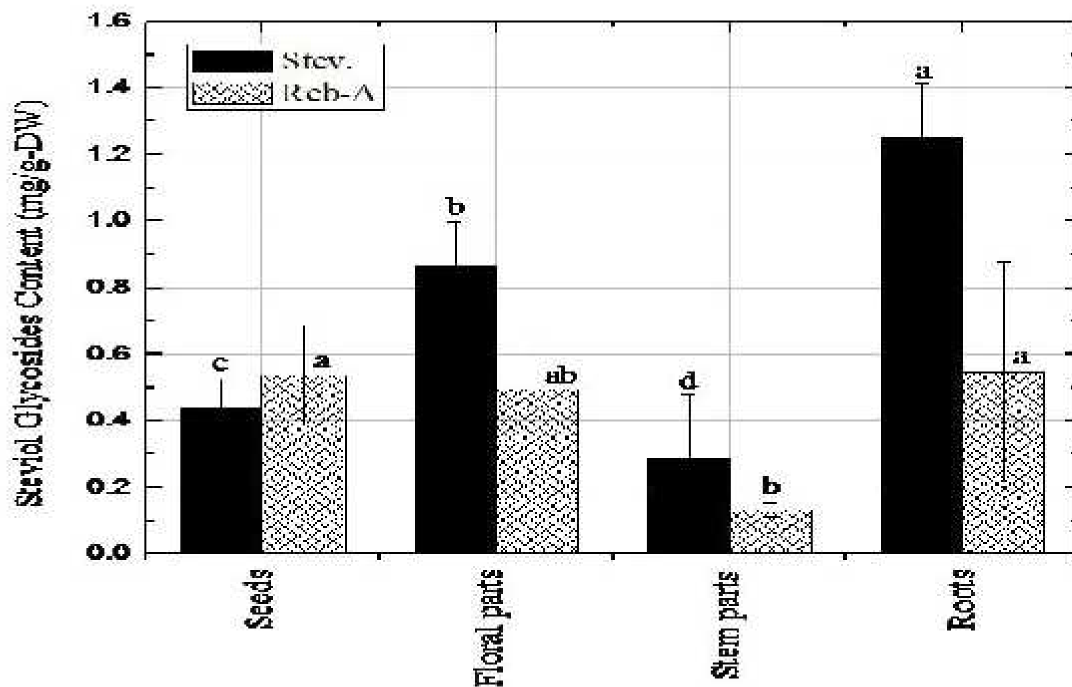

Figure-8: Steviosides (Stev) and Rebaudioside-A (Reb-A) content in different *in vivo* grown tissues. Stev and Reb A content was expressed in milligram per gram dry weight. Data (mean ± SD + LSD) were collected from three independent experiments. Mean values are not significantly different at *P* < 0.01

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