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Short Communication

Gamma radiation induced variation in growth characteristics and production of bioactive compounds during callogenesis in *Stevia rebaudiana* (Bert.)

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ABSTRACT

Background: Stevia rebaudiana Bertoni is an important anti-diabetic medicinal herb containing non-caloric sweet compounds. In this study, the effect of gamma irradiation on growth kinetics and accumulation of various bioactive compounds were investigated during callogenesis.

Results: Callus was developed from leaf pieces inoculated on Murashige and Skoog (MS) medium containing combination of 6-benzyladenine (BA; 1.0 mg l^{-1}), α-naphthalene acetic acid (NAA), indole butyric acid (IBA) and gibberellic acid (GA_3; 0.3 mg l^{-1}). After 30-days, vigorous calli were transferred to fresh medium and exposed to various gamma irradiations (5.0, 10, 15 and 20 Gy). It has been observed that the increasing doses of gamma rays inhibited callus proliferation (88.61–79.16%) as compared to control (95.83%). Similarly, 10, 15 and 20 Gy doses induced friable, granular and spongy callus as compared to control (compact). Furthermore, 5.0, 10 and 20 Gy doses significantly reduced the fresh callus biomass (FCB), however, 15 Gy dose enhanced FCB (1660 mg) and dry callus biomass (DCB; 159.36 mg) than control (1520; 145.92 mg). The chromatographic data revealed that 15 Gy dose slightly enhanced stevioside content (0.251 mg/g-DCB) than control (0.232 mg/g-DW), while other doses showed a negative effect on stevioside content. Higher antioxidant activity (88.73%) was observed in 20 Gy treated callus cultures. However, higher total phenolic content (TPC; 43.90 mg/g DCB) and total flavonoids content (TFC; 6.87 mg/g DCB) were observed in 15 Gy treated callus cultures.

Conclusions: The application of gamma irradiation did not show major variation in biomass and bioactive compounds production in callus cultures of *S. rebaudiana*.

1. Introduction

Stevia rebaudiana (Bert.) is a traditional medicinal plant that has recently gained global importance due the presence of commercially important stevioside content and frequently used in many countries for controlling obesity and diabetes [1–3]. Stevioside, rebaudioside-A and dulcoside-A are the major steviol glycosides present in the leaves of *S. rebaudiana* [1]. Among different steviol glycosides, stevioside is one of the important active compound that have been applied in many food products in Canada, China, Indonesia, Japan, Korea, Mexico, South America, UK and United States [4].

Stevia species are conventionally propagated through seed and stem cuttings. But seeds loose viability shortly after collection and stem cutting requiring higher initial stock and more labor [1,2]. To avoid these issues, plant cell, tissue and organ cultures are the most suitable techniques for biomass and bioactive compound production [5–7]. Among different culture systems, callus culture is one of the initial biotechnological steps for large scale production of shoots, and exploited for the establishment of cell suspension culture and active compound production [6,8,9].

Addition of plant growth regulators (PGRs), additives, application of elicitors and radiation are some of the important steps that facilitate and enhance biomass and bioactive compounds [8,10]. Gamma rays, X-rays, visible light and ultra violet are all electromagnetic (EM) radiation that initiate or inhibit the growth

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and differentiation of plant cells and organs [11]. Gamma rays interact with cell internal components and release free radicals. These free radicals either damage or modify the differentiation process, morphology, physiology and bioactive components depending on applied dose [12]. The effect of gamma irradiation on callus cultures of *S. rebaudiana* has not been reported yet. But the effect of irradiation on callus cultures is widely reported in other elite plant species [13–16]. Lower doses of gamma rays (04 and 05 kRs) enhanced the growth of callus in *Cucumis melo* while higher doses (15 and 20 kRs) drastically reduced it [13]. The gamma irradiation significantly stimulated the shikonin biosynthesis in *Lithospermum erythrorhizon* cell cultures [14]. Moreover, gamma irradiation remarkably increased the cerium perhydroxide in different tissues of pumpkin [15]. Similarly, gamma irradiation enhanced the production of antioxidant defense enzymes while reduced ascorbic acid, total soluble protein, amino acids and sugars in callus cultures of *Rosmarinus officinalis* L. [16].

Therefore, the overall objective of the present study was to investigate the effect of gamma irradiation on callus biomass and biochemical parameters, which can be helpful for the establishment of cell suspension culture, and production of bioactive compounds in bioreactors.

2. Materials and methods

2.1. Explant collection and callus induction

In vitro regenerated shoots were taken according to the procedure by Aman et al. [1] and leaves were excised for culture development. Callus culture was developed from leaf pieces of *in vitro* regenerated shoots. Suitable leaf pieces were incubated on Murashige and Skoog (MS) [17] medium supplemented with a combination of 6-benzyle-adenine (BA; 1.0 mg l^{-1}), naphthalene acetic acid (NAA), indole butyric acid (IBA) and gibberellic acid (GA_3; 0.3 mg l^{-1}), 30 g l^{-1} sucrose and finally solidified with 8 g l^{-1} agar (Oxoid; England). The pH was adjusted to 5.8 and all the cultured media were maintained in a growth chamber at 25 ± 2 °C under 16/8 h photoperiod for 30 days period.

2.2. Gamma irradiation

After 30 days of callus formation, green callus was shifted to new medium containing similar PGRs and irradiated with different gray (Gy) doses (5.0, 10, 15 and 20 Gy) of gamma rays according to the method of Ahmad et al. [10] through ^{60}Co gamma rays unit at Nuclear Institute for Food and Agriculture (NIFA), Peshawar, Pakistan. Callus proliferation, callus morphology and callus color was observed visually after 30 days of sub-culturing and irradiation.

2.3. Irradiated callus biomass

>Fresh callus biomass (FCB) was investigated after consecutive 3 days intervals for a 30 day period. Solid media particles and surface water were carefully removed by using autoclaved filter paper (Whatman Ltd., England) and finally weighed (Sortorious digital balance CP 32025; Germany). Similarly, for investigation of dry callus biomass (DCB), fresh calli were dried in an oven at 60 °C (Thermo Scientific; Germany) and stored in air tight bottles before grinding.

2.4. Extract preparation for high performance liquid chromatography (HPLC) analysis

The dried calli from each sample were grinded in a mortar and pestle for extract preparation. The stevioside content in each treated callus culture was determined by using the recent methods of Aman et al. [1] and Dey et al. [4] with little modification. The extract was prepared in HPLC grade ethanol (Merk; Germany). 20 mg of a dried ethanolic extract of each sample was independently dissolved in 10 ml of HPLC grade ethanol. A Shimadzu HPLC system (LC-8A; Japan) was used for stevioside quantification. The system was adjusted with C-18 column (150 mm × 4.6 mm), variable length detector, binary pump, solvent vacuum degasser and 10 μl injection loop. HPLC grade methanol (70%; A), and water (30%; B) was used as the mobile phase with a flow rate of 1.5 ml min^{-1}. Stevioside standard (Sigma; USA) was prepared in HPLC grade water (200 μg ml^{-1}). 10 μl standard was run on HPLC system and the retention time of each sample was compared with standard retention time. The results obtained for each sample was expressed in milligram/gram (mg/g) of dry callus biomass.

2.5. Determination of total phenolics and flavonoids content

Total phenolics content (TPC) was determined using the method of the Singleton and Rossi [18] and flavonoids content following the method of Kosar et al. [19]. From the prepared extract, accurately, 200 μl of each sample was mixed with 10 ml of Folin–Ciocalteu reagent (1:10). The mixture was further mixed with 7 ml of sodium carbonate (0.115 mg/ml) and incubated fro 120 min in dark. The absorbance of each sample was taken at 765 nm. Gallic acid (1.0–10 mg/ml) was used for the calibration curve. Results were expressed as Gallic acid equivalent (GAE) mg/g of dried concentration. Flavonoids content were expressed as rutin equivalent (RE) mg/g-DW of extracts. Calli extract in methanol (1 ml) and $AlCl_3$ in ethanol were mixed and diluted to 25 ml with pure ethanol. Blank samples contained other chemicals without calli extract with a single drop of acetic acid (CH_3COOH) and diluted to 25 ml. Rutin (1.0–10 mg/ml) was used for the calibration curve. The absorbance of these samples was checked at 20 °C after an incubation period of 40 min.

3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

DPPH-radical scavenging activity (DRSA) was determined according to the recent method of Ahmad et al. [20]. Briefly, ethanolic extract (1.0 ml) of each calli sample (5 mg/20 ml) was mixed with 2.0 ml of DPPH free radical solution (0.25 mg/20 ml ×4). The mixture was incubated in the dark for approximately 30 min. The absorbance of the mixture was measured at 517 nm at room temperature by UV–visible spectrophotometer (Shimadzu-1650PC, Japan). Finally the radical scavenging activity was calculated as the percentage of DPPH discoloration using the following equation;

$$\text{DRSA } (\%) = 100 \times (1 - A_C/A_D)$$

where A_C represents absorbance of calli extract at 517 nm and A_D is the absorbance of the DPPH solution without calli extract.

3.1. Statistical analysis

Analysis of triplicate mean values, standard errors (±), and least significant difference (LSD) were carried out by using Statistix software (8.1 versions) and Origin Lab (8.1) software was used for graphical presentation.

4. Results and discussion

4.1. Effect of gamma irradiation on callus proliferation

In the present investigation, maximum callus proliferation (95.83 ± 4.17%) was observed on MS-medium containing BA

Table 1
Effect of different doses of gamma irradiation on callus induction, morphology, color and stevioside content.

Radiation doses	MS + PGRs (mg l^{-1})	Callus proliferation (%)	Callus morphology	Callus color	Steviosides content (mg/g-DW)
05 Gy	1.0 BA + NAA + IBA + GA_3 (0.3)	88.61 ± 2.737 b*	Compact	Yellowish green	0.173
10 Gy	1.0 BA + NAA + IBA + GA_3 (0.3)	83.33 ± 9.624 b	Granular	Yellowish green	0.216
15 Gy	1.0 BA + NAA + IBA + GA_3 (0.3)	82.49 ± 3.157 b	Friable	White & green	0.251
20 Gy	1.0 BA + NAA + IBA + GA_3 (0.3)	79.16 ± 6.367 c	Spongy	Half white & green	0.219
Control	1.0 BA + NAA + IBA + GA_3 (0.3)	95.83 ± 4.167 a	Compact	Green	0.232

* Data were taken from triplicate experiments. Mean data in each column with common letters and standard errors (SE) are not significantly different at $P < 0.05$.

(1.0 mg l^{-1}) in combination with NAA, IBA and GA_3 (0.3 mg l^{-1}) without irradiation. Different doses (5.0, 10, 15 and 20 Gy) of gamma irradiation significantly reduced callus proliferation (88.61 ± 2.74, 83.33 ± 9.62, 82.49 ± 3.16 and 79.16 ± 6.37%) as compared to untreated culture (Table 1). Venkateshwarlu [13] reported that lower irradiation doses stimulate callus growth but higher doses drastically reduce callus formation in *Cucumis melo* cv. Bathasa. Similarly, Patade et al. [21] also reported that an untreated embryogenic callus culture of *Saccharum officinarum* L. showed better regeneration potential than irradiated callus cultures. However, Moallem et al. [22] did not observe any change in callus induction of *Rosa canina* after irradiation. However, the current results are in agreement with those reported by Hasbullah et al. [11].

4.2. Effect of gamma irradiations on callus morphology

Higher doses of gamma irradiation significantly changed callus color and morphology (Table 1). Lower irradiation dose (05 Gy) slightly showed inhibitory effects on callus color (yellowish green) and morphology (compact; Fig. 1a) as compared to untreated callus culture (green and compact; Fig. 1e). Visual variation in callus color and morphology was clearly observed after 10, 15 and 20 Gy doses (Fig. 1b–d). Granular and yellowish green callus was observed in response to 10 Gy dose. White and green friable callus was observed in response to 15 Gy dose. Moreover, 20 Gy dose produced half white and green spongy callus. Such variation in calli is helpful for optimization of cell cultures in bioreactors for enhanced production of medicinally important metabolites.

4.3. Effect of gamma irradiation on callus biomass and growth kinetics

The FCB and DCB of treated and untreated callus cultures were documented after 3 days interval for a period of 30 days (Fig. 2). Higher FCB of 1660 mg was observed in callus cultures irradiated with 15 Gy dose as compared to untreated cultures (1520 mg) after 30 days of incubation on MS-medium (Fig. 2c). However, 05, 10 and 20 Gy doses significantly reduced FCB (980, 1170 and 1150 mg; Fig. 2a, b, d). Hasbullah et al. [11] also observed that higher irradiation doses (20 Gy) inhibit the accumulation of FCB (76.4 mg) in *Gerbera jamesonii* as compared to the control (89.7 mg). The possible reason in gradual decline in FCB may be due to the effect of radiation on endogenous growth regulators that stimulate cell division. Bajaj et al. [23] observed significant reduction in callus growth of *Gerbera jamesonii* when exposed to 20–50 Gy doses. These results are consistent with those reported by Omar et al. [24]. Similarly, higher DCB (159.36 mg) was observed after 30 days of inoculation in callus cultures irradiated with 15 Gy doses as compared to the control (145.92 mg; Fig. 2e). Lower DCB (94.08, 112.32 and 110.4 mg) was observed in cultures irradiated with 05, 10 and 20 Gy doses. These results suggest that 15 Gy dose is slightly effective for accumulation of FCB and DCB as compared to the control (Fig. 2e).

4.4. Effect of gamma irradiations on biochemical parameters

In this study, we observed that 15 Gy dose slightly enhanced stevioside content (0.251 mg/g-DW) over the control (0.232 mg/g-DW). However, other doses (05, 10 and 20 Gy) showed a negative effect (0.173, 0.216 and 0.219 mg/g-DW) on steviosides biosynthesis (Table 1). On the contrary, maximum TPC (43.90 mg/g DCB) and TFC (6.87 mg/g DCB) were observed in 15 Gy treated callus cultures (Fig. 3). However, higher antioxidant activity (88.73%) was observed in 20 Gy treated callus cultures (Fig. 4). Chung et al. [14] reported that lower doses of gamma irradiation significantly influence the production of shikonin derivatives in callus cultures of *Lithospermum erythrorhizon* but higher doses than 32 Gy did not enhance the shikonin derivatives. Similarly, El-Beltagi et al. [16] observed that 15 and 20 Gy doses enhances the production of phenolic and flavonoid contents in callus cultures of *Rosmarinus officinalis* L. Plant either produces stress enzymes or stress alleviating compounds like phenolics and flavonoids to combat with stress environment. Moreover, elicitors of biological and non-biological origin stimulate the production of secondary metabolites. Therefore, in the present study, gamma irradiation plays the role of elicitor to enhance the production of phenolics and flavonoids content. The higher antioxidant activity is also due to stress conditions.

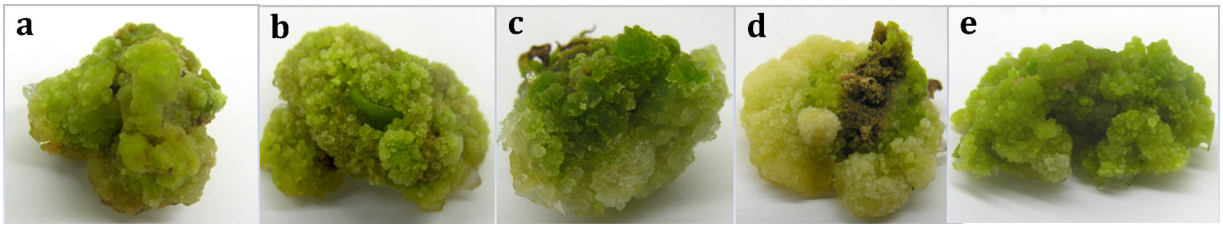

Fig. 1. Callus cultures of *S. rebaudiana* (Bert.). Effect of gamma irradiation (5.0, 10, 15 and 20 Gy doses) on callus color and morphology. (a) Compact and yellowish green callus obtained after 5.0 Gy gamma dose; (b) granular and yellowish green callus on 10 Gy dose; (c) friable, white and green callus on 15 Gy dose; (d) spongy, half white and green callus on 20 Gy dose and (e) compact and green callus without gamma irradiation (control).

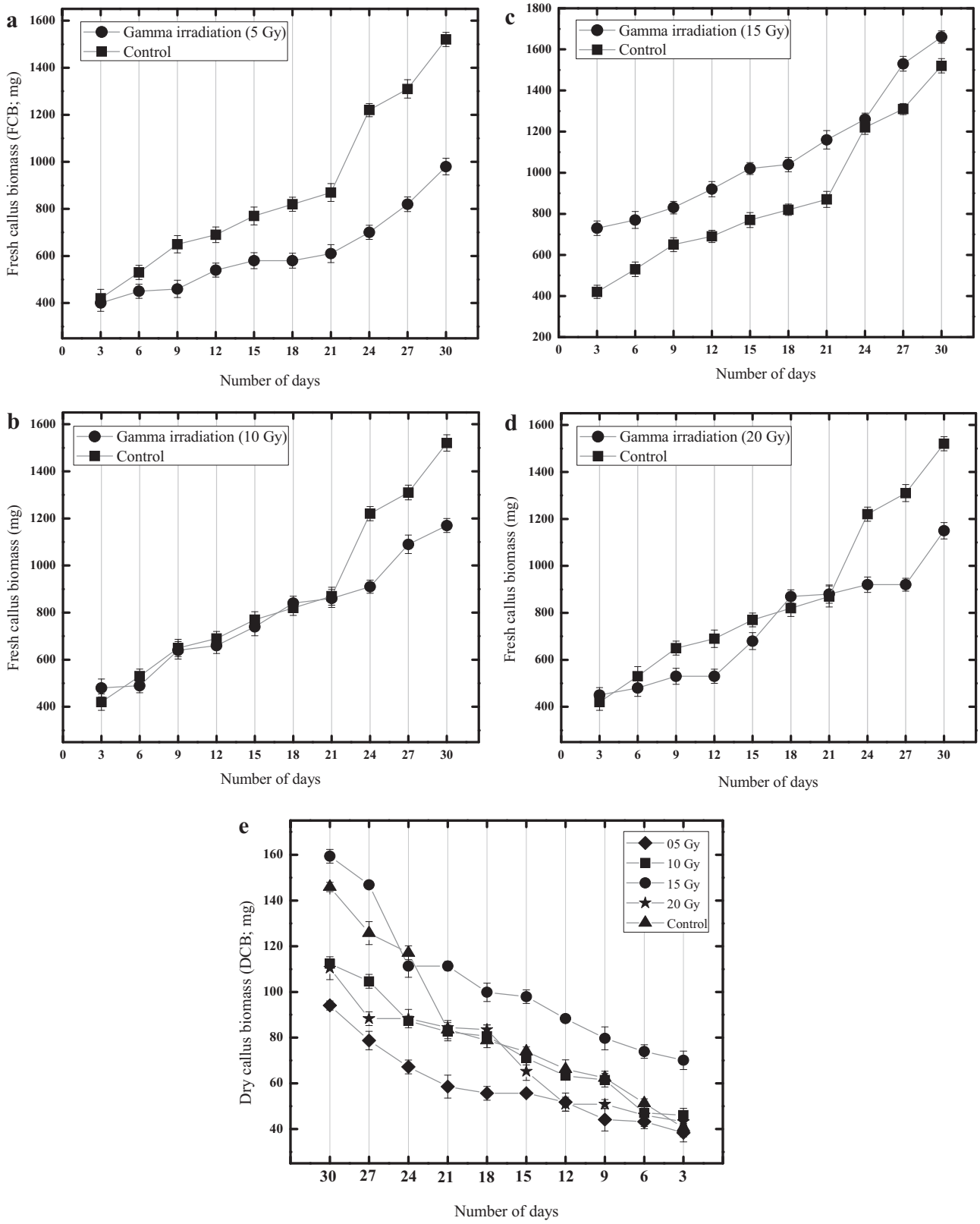

Fig. 2. FCB and DCB in *S. rebaudiana* (Bertoni), (a) FCB of 5.0 irradiated cultures and control, (b) FCB of 10 Gy treated cultures, (c) 15 Gy treated cultures, (d) 20 Gy treated cultures and (e) DCB of 5.0–20 Gy treated cultures along with untreated cultures. Data were collected from three independent experiments. Mean values with standard errors (±) are significantly different at $P < 0.05$.

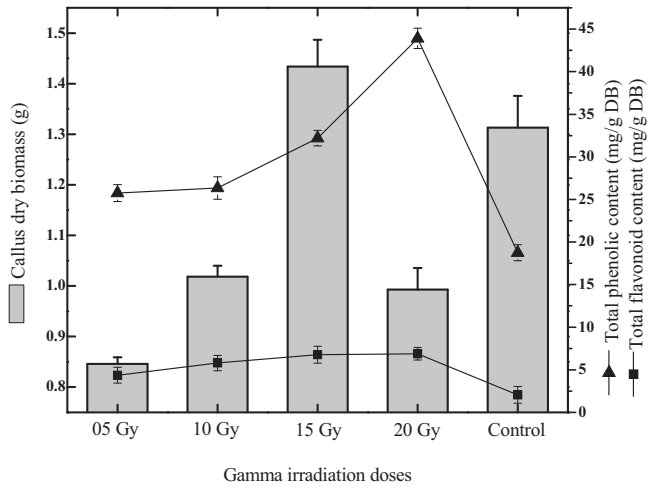

Fig. 3. Correlation of callus dry biomass with total phenolics and flavonoids content in callus cultures of *S. rebaudiana* in response to different doses of γ-irradiation. Mean values with standard errors (±) are significantly different at $P < 0.05$.

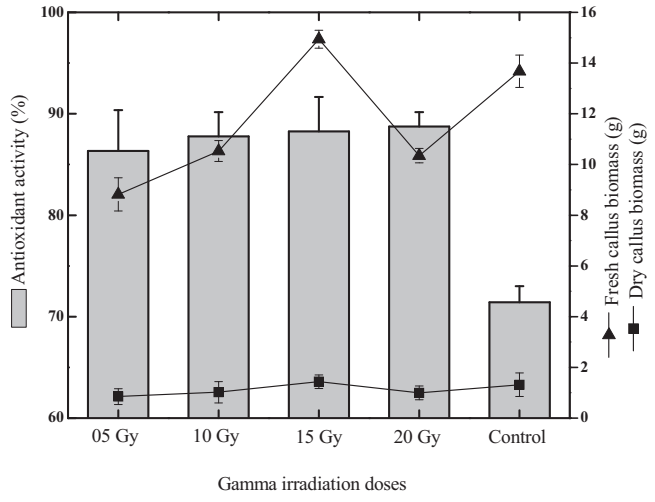

Fig. 4. Correlation of antioxidant activity with FCB and DCB in callus cultures of *S. rebaudiana* in response to different doses of γ-irradiation. Mean values with standard errors (±) are significantly different at $P < 0.05$.

5. Conclusions

In conclusion, the current results suggest that most of the irradiation doses are less effective in biomass accumulation and production of bioactive compounds, however, 15 Gy dose slightly enhances biomass and bioactive compounds. Mostly the irradiation doses showed negative effects on biomass accumulation and production of metabolites during Stevia callus proliferation. However, the effect of radiation is not restricted to callogenesis alone; radiation might be effective for seed germination and organogenesis to enhance metabolites of interest in *S. rebaudiana*.

Author's contribution

Shahid Akbar Khalil designed the experiments for *in vitro* callogenesis from leaf explants and wrote the initial draft of the manuscript. Nisar Ahmad established callus cultures and determined total phenolics, flavonoids and antioxidant activity and performed statistical analysis. Roshan Zamir provides lab facilities for culture development. Nisar Ahmad and Shahid Akbar Khalil will submit this manuscript as corresponding authors.

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