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Effect of Low Dose Gamma Irradiation on Some Phytochemicals and Scavenger Ability of *in Vitro* Culantro (*Eryngium foetidum* L.) Plantlets

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ABSTRACT

This research work aimed to enhance the concentration of some bioactive compounds in culantro (*Eryngium foetidum* L.) plantlets through gamma irradiation (0.0, 10.0, 20.0 and 40.0 Gy range) treatments. Gamma irradiation at 40.0 Gy significantly stimulated total phenolic, flavonoids, tannin and saponin contents. The effect of irradiation on antioxidant properties of culantro extracts was investigated by radical-scavenging effect on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals and the content of thiobarbituric acid-reactive substances (TBARS). Some significant changes were observed in thiobarbituric acid-reactive substances between non-irradiated and irradiated samples. Irradiation at 40 Gy affected the DPPH radical-scavenging activity significantly. Total soluble sugars increased from 2.68 g/100 g dw at 0.0 Gy to 6.08 g/100 g dw at 10.0 Gy. Gamma radiation enhanced the level of ascorbic acid accumulation compared to control (non-irradiated) plantlets. Total chlorophyll decreased significantly under all irradiation treatments. These results suggested that both low γ-irradiation doses and tissue culture techniques could be used to produce culantro plantlets with high quantities of certain metabolites.

Keywords: DPPH radical-scavenging activity, flavonoid, saponin, thiobarbituric acid reactive substance

INTRODUCTION

Research on the interaction of radiation with biological systems has contributed to human society through applications in agriculture, pharmaceutical uses, and other technological developments. In agricultural science, relatively low-dose ionizing radiation on plants are manifested as accelerated cell proliferation, germination rate, cell growth, enzymes activity, stress resistance, and enhancing the production of secondary metabolites (Chung *et al*. 2006; Maity *et al.* 2009).

Over the centuries numerous plants have been exploited as an excellent source of biologically nature active compounds, with multivarious functions such as nutritious as well as medicinal value. Culantro (*Eryngium foetidum* L., Apiaceae) was reported as a medicinal herb used in traditional medicine for common ailments such as fevers, vomiting and diarrhea (Honeychurch 1980). It is also acclaimed as a health food due to its high content of calcium, iron, carotene, riboflavin, proteins and vitamins (Bautista *et al.* 1988). Besides, phytochemical investigation of the aerial parts of *E. foetidum* resulted in the isolation of novel pentacyclic triterpenoid glycosides that are of wide interest due to their beneficial effects as antiinflammatory and analgesic properties (Anam 2002). However, there was compelling evidence to show that the *Eryngium* genus contained flavonoids (Hohmann *et al.* 1997) and saponins (Kartal *et al*. 2006) as the major bioactive constituents. In the search for alternative production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue culture, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra Rao 2000). On the other hand, γ-irradiation, as a phytosanitary treatment of food and herbal materials, was successfully used for promoting some phytochemicals such as ascorbate, photosynthetic pigments and some antioxidant enzymes activity in red pepper (*capsicum annuum*) when treated with gamma rays ranged from 2-16 Gy (Kim *et al.* 2005). Moreover, the combined effect of both γ-irradiation and *in vitro* techniques could be considered as a good tool to improve the physiological and biochemical processes in plants. There is no information available in the literature concerning the effect of low doses ionizing radiation on culantro. However, for other plant materials, such as *Lithospermum erythrorhizon* cells diverse effects of relatively low to high doses γ-irradiation (2-32 Gy) on some important ingredients (shikonin) have been reported (Chung *et al*. 2006). In addition, low dose of γ-irradiation has been applied to enhance phenolic compounds at 4-12.7 KGy as a potential antioxidant in almond (*Prinus amygdalus*) skins extract (Harrison and Were 2007). Meanwhile, Fan *et al.* (2003) reported that free radicals generated during γ-irradiation treatment at 0.5, 1, and 2 kGy may act as stress signals and thus leads to a rapid increase of stress-response compounds such as phenols, flavonoids, and other antioxidant compounds in fresh-cut iceberg lettuce (*Lactuca sativa*). Additionally, low doses of γ-irradiation (0.1 KGy) have been reported to stimulate the accumulation of reducing and non-reducing sugars in onion (*Allium cepa*) and potato (*Solanum tuberosum*) plants due to the degradation of oligosaccharides (Nouri and Toofanian 2001). Concerning the positive effects of γ-irradiation, Aly and Mohamed (2005) declared that the use of γ-irradiation at low doses (50, 100 and 150 Gy) enhanced total phenolic, sinapin, glutathione, ascorbic acid and α-tocopherol contents in maize (*Zea mays* L.) callus tissue. Irradiation by using γ-rays at 0.05, 0.1, 0.15 and 0.2 KGy markedly stimulated biosynthesis of ascorbic acid and riboflavin in germinating soybean (*Glycine max* L.) plants (Sattar *et al.* 1992). Nevertheless, questions focusing on nutrient loss, free radicals and changes in antioxidant properties during irradiation are still being debated in the scientific field. The present study was undertaken to investigate the effect of low doses of γ-irradiation on some important plant metabo-

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lites, and to provide valuable information on the utilization of beneficial effects of γ-radiation on culantro plantlets that were induced *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Folin-Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and aluminum chloride were purchased from Sigma Chemical Co., Ltd (St. Louis, MO, USA). All other reagents were of analytical grade.

Plant materials

Culantro (*Eryngium foetidum* L.) plantlets were kindly provided by Prof. Mohamed-Yasseen, Genetic Engineering and Biotechnology Research Institute at Sadat City -Minufia University, Egypt.

Nodal segments of culantro were sterilized. Explant sterilization was accomplished firstly by rinsing in tap water for 5 min. Then the explants were treated with 70% ethanol plus 2 drops of Tween 20 for 5 min followed by 3 washes with autoclaved distilled water. Finally the explants were treated with NaOCl (10%) for 5 min as described previously by Mohamed-Yasseen (2003). Explants were cultivated on MS (Murashige and Skoog 1962) basal medium without plant growth regulators. Explants were cultured at 5 segments/200 ml glass jar containing 50 ml of solidified basal MS medium. The pH of the media was adjusted to 5.7 using 0.1 N HCl and 0.1 N NaOH and finally agar-agar (8.0%, w/v) was added as the gelling agent. Media were autoclaved at 121°C and at a pressure of 15 lb/in^2 and kept in the laminar air flow hood for 1-2 days before inoculations. The explants were excised asepically and cultured on the above-mentioned media (apical cut section in an upward orientation). Cultures were incubated in a 16 h light/8 h dark cycle at 25 ± 2°C in a plant tissue culture chamber. The cultures were sub-cultured every 15-20 days on the same media for 2 months.

Irradiation treatments

After 6 weeks from subculture, the glass jars containing five plantlets per jar were exposed to γ-rays at four doses (0.0, 10.0, 20.0 and 40.0 Gy) with a ^{60}Co-source (Gamma cell 200, MDS Nordion) at an average dose rate of 0.7 Gy/min at a source surface distance of 70 cm. Immediately after irradiation, plantlets were aseptically transferred into sterile fresh MS medium, then placed in a growth chamber at 26 ± 2°C with a 16-hr photoperiod. Subculture was repeated four times after irradiation. Young leaves from earlier developed axillary shoots were taken after 6 weeks from the last subculture and used as follows.

Monthly subcultures

Monthly subcultures were carried out by separating shoot clusters developed from explants into individual shoots which were then trimmed down to shoot tips and re-cultured under the same conditions. This process was repeated for four months after which well formed shoots were subsequently transferred into a rooting medium.

Preparation of plant extract

Leaves were dried for two days at room temperature and ground in a mortar. Pulverized samples (5.0 g) were extracted with 50 ml of 80% methanol using an orbital shaker (Heidolph Uni Max 2010) for 12 h at room temperature. The extract was separated from the solids by filtration with Whatman No. 1 filter paper. The remaining solids were extracted twice with the same solvent and extracts were combined. The extracts were concentrated under reduced pressure at 45°C in a rotary evaporator (Buchi, Switzerland). Concentrated extracts were stored in a refrigerator until use.

Total phenolic content

The amount of total phenolics (TPs) was assayed using the Folin–Ciocalteu reagent procedure as described by Chaovanalikit and Wrolstad (2004). Absorbance at 755 nm was measured using a spectrophotometer (UNICAM UV300). TPs were calculated using a calibration curve of gallic acid (Sigma , Germany). Total phenol values were expressed in terms of gallic acid (GAL) equivalent per gram dry weight (mg/g of dw) which is a common reference phenolic compound.

Total flavonoid content

Aluminum chloride colorimetric method was used for flavonoid determination (Chang *et al*. 2002). The absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared from a series of quercetin (QU) solutions at 20 to 100 g/ml in methanol. The results were expressed as mg of quercetin equivalents per g of dry extract (mg QU equivalent/g dw).

Tannin content

Tannins were assayed colorimetrically according to a modified vanillin method (Price *et al.* 1978). Pure tannic acid was used for standard curve preparation and the amount of total tannin expressed as mg standard tannic acid/gram dry plant extract (mg/g dw).

Saponin content

Total saponin content was determined using the spectrophotometric method of Chen *et al.* (2007) in which the mixture reaction were recorded at 550 nm using vanillin acetic acid reagent. The results were expressed as mg/100 g dw.

Free radical-scavenging assay with DPPH

Antioxidant activity was determined based on the radical scavenging ability in reacting with a stable DPPH (2, 2-diphenyl-1-picryl-hydrazyl) free radical according to Yamasaki *et al.* (1994). About 100 µl of plant extract (from 50 to 250 µg/ml) was added to 1.9 ml of DPPH in methanol solution (150 µM) and shaken vigorously. After incubation at 37°C for 35 min in the dark, the absorbance of each solution was determined at 517 nm. The percentage of DPPH scavenging activity was calculated.

Carbohydrate content

Total carbohydrates, total soluble sugars and reducing sugars were determined using potassium ferricyanide reagent according to A.O.A.C. (1990), Ackerson (1981) and Schales and Schales (1945), respectively.

Ascorbic acid analysis

Ascorbic acid (AA) was extracted from the plant materials with metaphosphoric acid (2%) and determined spectrophotometrically using 2,6 di-chlorophenol indophenol dye according to Augustin *et al.* (1985). The amount of AA was calculated from a standard curve that was prepared by using standard AA.

Lipid peroxidation

The lipid peroxidation level was determined in terms of malondialdehyde (MDA) content using 2-thiobarbituric acid (TBA) reaction as described by Haraguchi *et al.* (1995). The absorbance of pink color was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated based on $A_{532} - A_{600}$ (Σ = 155 mM/cm).

Protein contents

Total soluble protein content was measured using Bradford's method (Bradford 1976).

Pigment determinations

Chlorophyll a (chl *a*), chlorophyll b (chl *b*) total chl and carotenoid contents were determined after extraction by 80% acetone. The absorbance of the extract was measured using spectrophotometer at 663, 646 and 472 nm according to Lichtenthaler (1987).

Statistical analyses

The experiment was conducted using a Completely Randomized Design (CRD) with five replications. The results were reported as mean ± standard deviation (SD). The significance of differences among different γ-radiation treatments was determined by one-way analysis of variance (ANOVA). Least significant difference (LSD) at $P = 0.05$ level was analyzed using M-STAT statistical package (Nissen 1990).

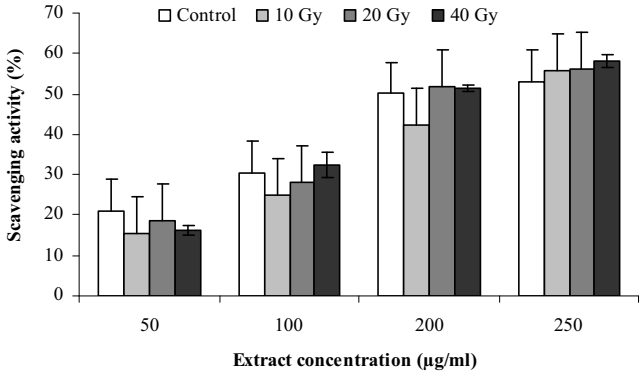

Fig. 1 Free radical scavenging activity of methanolic extract (50-250 µg/ml) of culantro plantlets at different γ-irradiation doses.

RESULTS AND DISCUSSION

Radiation effects on phenolic, flavonoid, tannins, and saponin contents

The influence of γ-irradiation doses (0.0, 10.0, 20.0 and 40.0 Gy) on total phenolic, flavonoid, tannins, and saponin of culantro plantlet were analyzed. Generally, significant increases in total phenolic, flavonoid, tannins and saponin were observed as γ-irradiation dose increased (**Table 1**). The maximum increase was observed at 40 Gy, and the values were 61.66 mg/g dw for total phenolic, 45.62 mg/g dw for flavonoid, 76.20 mg/g dw for tannin and 166.07 mg/100 g dw for saponin. Accumulation of phenolic compounds in cells can be explained by the release of phenolic compounds from glycosidic components and the degradation of larger phenolic compounds into smaller ones by γ-irradiation (Štajner *et al.* 2007). Variation in flavonoid and tannins content at different doses of irradiation treatment may be because γ-radiation induces oxidative stress and *de novo* synthesis of flavonoids by increased phenylalanine ammonia-lyase (PAL) activity which is a crucial enzyme for the biosynthesis of flavonoid and tannins (Oufedjikh *et al.* 2000). Saponins were enhanced gradually in all treated samples. The highest occurrence of saponin was found at 40 Gy, but much more work needs to be done on the effect of γ-rays on saponin in plant tissues, especially given their possible interactions with both condensed and hydrolysable tannins in influencing the potency of these secondary compounds.

Radiation effects on antioxidant activity

Irradiations doses at 10.0, 20.0 and 40.0 Gy affected the reactive scavenging capacity (RSC) of irradiated extracts (**Fig. 1**). Irradiation resulted in a significant tendency to increase the DPPH radical-scavenging activity of culantro methanolic extracts, mainly at the maximum level of irradiation. The data reveled that the antioxidant activity increased significantly ($P < 0.05$) when increasing the extract concentrations (50 to 250 µg/ml). At 250 µg/ml, the DPPH-scavenging activity was 55.8, 56.1 and 58.1% for 10.0, 20.0 and 40.0 Gy, respectively. γ-irradiation doses up to 40.0 Gy enhanced reactive scavenging capacity of soybean extracts (Variyar *et al.* 2004). Higher levels of DPPH antiradical activity were correlated with polyphenols (Brand-Williams *et al.* 1995). High antioxidant activity of plant extracts could be attributed to its content of various phenolic compounds. Theses phenolic compounds, are commonly contain at least one hydroxyl substituted aromatic ring system, that can easily oxidized, as well as serving as important units for donating electrons. Plant extracts which show the potent proton-donating ability on DPPH$^{\cdot}$ to produce DPPHH, considered as an important mechanism of antioxidants. In addition, low dose irradiation results in the intracellular generation of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) in plant tissues, which may alter the phytochemical antioxidant content (Kovacs and Keresztes 2002). In terms of exposure to radiation sources, this depends on the dose applied (usually low and medium doses have insignificant effects on antioxidants), the sensitivity of the antioxidant or the phytochemicals towards irradiation, and the effect of irradiation itself on other food constituents that might be responsible for the production and/or the accumulation of phytochemicals/antioxidants in the plant. In a study on the antioxidant properties of seven dessert spices (anise, cinnamon, ginger, licorice, mint, nutmeg, and vanilla), Murcia *et al.* (2004) revealed that irradiation up to 10 kGy did not have any affect on the antioxidant properties. This was attributed to the low water content, which limited the possibility of free radicals being formed.

Radiation effects on total carbohydrates and vitamin C content

Total carbohydrate, total soluble sugars and reducing sugars contents (**Table 2**) showed high values at 10.0 Gy. The values were 7.14, 6.08 and 5.34 g/100 g dw compared to non irradiated samples (3.65, 2.68 and 1.77 g/100 g dw) for total carbohydrate, total soluble sugars, and reducing sugars, respectively. A similar trend was detected for ascorbic acid (vitamin C). Ascorbic acid of all the plantlets irradiated with γ-rays was noted to increase in a dose-dependent manner. Irradiation treatment with all the doses generally exhibited acceleration of ascorbic acid synthesis as compared to control and the maximum accumulation of vitamin C was detected at 40 Gy (30.24 mg/100 g) compared to control (25.02 mg/100 g). Low dose of γ-radiation catalyzing enzymes or hormones that accelerated the metabolic activity of ascorbate during soybean (*Glycine max* L.) germination (Sattar *et al.* 1992). Culantro as a leafy green herb is a rich

Table 1 Effect of gamma irradiation on total phenolic, flavonoid, tannin and saponin contents of culantro plantlet extract.

Irradiation doses (Gy)	Phenolic (mg/g dw)	Flavonoid (mg/g dw)	Phenolic/ flavonoid	Tannin (mg/g dw)	Saponin (mg/100 g dw)	Tannin/ saponin
Control	36.64 ± 0.05 d	35.59 ± 0.14 d	1.03 ± 0.02 c	58.90 ± 0.15 d	120.08 ± 0.94 a	0.49 ± 0.12 a
10	45.63 ± 1.33 c	39.45 ± 0.04 c	1.16 ± 0.03 b	61.40 ± 0.51 c	139.95 ± 0.75 b	0.43 ± 0.11 b
20	52.70 ± 3.01 b	44.35 ± 0.22 b	1.19 ± 0.02 b	72.90 ± 0.14 b	143.95 ± 1.35 c	0.51 ± 2.10 c
40	61.66 ± 1.26 a	45.62 ± 0.35 a	1.35 ± 0.05 a	76.20 ± 0.32 a	166.07 ± 1.28 d	0.46 ± 0.92 d
L.S.D. $P < 0.05$	0.066	2.48	0.063	0.497	2.49	0.06

Means within same column followed by different letters were significantly different at $P < 0.05$.
Values are means of three replicates (±SE).

Table 2 The content of total carbohydrates, total soluble sugars, reducing sugars, ascorbic acid and lipid peroxidation of culantro plantlets grown under γ-irradiation stress.

Irradiation doses (Gy)	Total carbohydrates	Total soluble sugars	Reducing sugars	Ascorbic acid	Lipid peroxidation
Control	3.56 ± 0.11 d	2.68 ± 0.51 d	1.77 ± 0.22 d	25.02 ± 5.0 d	2.9 ± 1.1 d
10	7.14 ± 0.21 a	6.08 ± 0.72 a	5.34 ± 0.41 a	27.01 ± 2.0 c	3.2 ± 1.0 c
20	6.52 ± 0.09 b	4.89 ± 0.11 b	2.53 ± 0.81 b	28.86 ± 3.6 b	3.9 ± 1.2 a
40	6.25 ± 0.85 c	4.64 ± 0.44 c	1.92 ± 0.33 c	30.24 ± 1.6 a	4.1 ± 0.8 a
L.S.D. $P < 0.05$	0.063	0.09	0.02	2	0.8

The content of total carbohydrates, total soluble sugars and reducing sugars expressed as g/100 g dw
Ascorbic acid expressed as: mg/100 g fw
Lipid peroxidation expressed as: µmol MDA/mg Pro.
Means within same column followed by different letters were significantly different at $P < 0.05$.Values are means of three replicates (±SE)

Table 3 Chlorophyll and carotenoid contents of culantro plantlets grown under γ-irradiation stress.

Irradiation doses (Gy)	Chlorophyll and carotenoid contents (mg/100 g fw)						
	Chl *a*	*%	Chl *b*	*%	Total chl	Carotenoids	*%
Control	24.60 ± 0.33 c	100	14.50 ± 0.01 d	100	39.1 ± 0.20 d	8.20 ± 0. 55 d	100
10	15.60 ± 2.3 b	63.41	7.10 ± 0.21 c	48.96	22.60 ± 0.35 c	6.50 ± 1.20 c	79.27
20	13.50 ± 1.20 a	54.87	4.50 ± 0.34 b	31.03	18.0 ± 1.09 b	5.50 ± 0.85 b	67.07
40	11.17 ± 0.60 a	45.4	3.5 ± 0.37 a	24.14	14.67 ± 0.74 a	3.50 ± 0.99 a	42.68
L.S.D. $P < 0.05$	2.43		0.32		2.2	0.53	

*% relative to control
Means within same column followed by different letters are significantly different at $P < 0.05$. Values are means of three replicates (±SE).

source of bioactive phytochemical constituents such as vitamin C, riboflavin, folic acid, carotenoids (lycopene and β-carotene), iron, and potassium. These constituents may serve as chemopreventive agents (Bautista *et al.* 1988). The observed increase in reducing and non-reducing sugars in onion (*Allium cepa*) and potato (*Solanum tuberosum*) plants due to exposure to ionizing radiation (0.1 kGy) is in agreement with the finding of Nouri and Toofanian (2001), who documented an increase in reducing and non-reducing sugars content by γ-irradiation. Additionally, Inayatullah *et al.* (1987) stated that when soybean (*Glycine max*) plant exposed to low doses of γ-rays (0.25–5 kGy) carbohydrate constituents were highly increased. Biosynthesis of vitamins in seeds takes place during plant growth or germination (Kutsky 1973) which can be accelerated at low doses of γ-irradiation (Sax 1963). There has been an interest in the ascorbate ability as an antioxidant to prevent the formation of carcinogenic substances from dietary material. Biosynthesis of AA and riboflavin in irradiated soybean (*Glycine max* L.) plant was higher than un-irradiated control plants (Sattar *et al.* 1992). It has been recognized that radiation-induced acceleration of reactions involved in the biosynthesis provides reasonable basis for their higher levels in treated than untreated samples.

Radiation effects on lipid peroxidation

Lipid peroxidation (LP) gradually increased with increasing irradiation doses (**Table 2**). The increase of MDA content as an indicator of membrane lipid peroxidation under γ-radiation stress is related to unsaturated fatty acids content. According to the obtained results, the lipid peroxidation detected in irradiated leaves at 40.0 Gy was noted to increase nearly 2-fold compared to non-irradiated sample (4.1 µmol MDA/mg Pro. at 40.0 Gy) compared to 2.9 µmol MDA/mg Pro. in the control. This may be related to a temporary adaptation to γ-radiation-stimulated protective responses, such as increases in free radical scavenging system. The lipid oxidation may be attributed to the combination of free radicals with O_2 to form hydroperoxides. As biological systems consist of 55-80% water, the main interaction of radiation is with water molecules. Similarly a prolonged irradiation of wheat (*Triticum aestivum* L.) seeds with UV light (for 1-6 hr) led to an increase in the level of LP (Rogozhin *et al.* 2000). This suggested a breakdown of acylglycerols during radiation processing, resulting in the release of free fatty acids. In the present study biochemical parameters like MDA content may be helpful in early assessment of effectiveness and superiority of the irradiation dose.

Radiation effects on pigment contents

Stimulation of chl breakdown as a primary response to radiation exposure in all irradiated treatments (**Table 3**) reflects metabolic boosting of culantro to counter radiation stress. Generally, the results showed that γ-irradiation caused a slight reduction of chl *a* and *b* contents. A gradual decrease in chl *a* and *b* contents was observed at 20.0 and 40.0 Gy, respectively compared to untreated plantlets. In addition, there was irradiation-induced breakdown of carotenoid content in comparison to control treatment. The carotenoid content was significantly decreased in all the γ-exposed treatments, and the maximum reduction was noted at 40.0 Gy. Reduction of chl content has a negative effect on plant photosynthetic efficiency. Carotenoids serve as a protective function against UV-B (Rau *et al.* 1991) and UV-C (Campos *et al.* 1991) radiation. The efficiency of carotenoids in protecting the photo systems is likely due to their function as efficient quenchers of high energy short wave radiation. The Food and Drug Administration (FDA) has established a list of foods and food ingredients that may be treated with ionizing radiation and the maximum overall average absorbed dose shall not exceed than 30 kGy for dried aromatic herbs, spices and vegetable seasonings (Code of Federal Regulation 2004).

CONCLUSION

γ-Irradiation at 10.0, 20.0 and 40.0 Gy enhanced bioactive constituents in culantro such as phenolic compounds, flavonoids, tannins and saponins and enhancing the reactive scavenging capacity. The obtained results suggest that both low doses of γ-irradiation and tissue culture could be used to produce plantlets with high amounts of certain metabolites. More studies are needed to establish the using of low γ-radiation as a tool to enhance the production of some metabolites in a large-scale system.

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