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Effect of Gamma Radiation on Ephedrine and Pseudoephedrine in Callus and Suspension Cultures of *Ephedra alata*

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Abstract: Ephedra is the most widely and largest widespread genus in the Gnetopsida, a subgroup of the gymnosperms. This research was done to find out how gamma radiation affected the *in vitro* growth of callus and suspension cultures. In addition, the effect of gamma rays (γ-rays) on the ephedrine and pseudoephedrine concentrations was evaluated through HPLC analysis. The cell suspension and callus cultures were formed on Murashige and Skoog's basal medium (MS) supplemented with 0.5 mg/l kinetin (Kn), and 3 mg/l of 2, 4-dichlorophenoxy acetic acid (2,4-D) and exposed to variable gamma radiation doses (0, 5, 10, 15, 20, 25 and 30 Gray). Highly significant differences in both fresh and dry callus weights were recorded due to the effect of gamma rays (γ-rays). The findings demonstrated that in the cell suspension and callus cultures, 15 Gray achieved the highest fresh and dry weights when compared to the control. Similarly, the highest concentrations of ephedrine and pseudoephedrine were found in suspension and callus cultures compared to control at 15 Gray.

1 Introduction

The natural plant species *Ephedra alata* L. is primarily found in Eastern Mediterranean, Sinai Desert, and coastal areas (Boulos 2009). It is a medicinal plant that is a member of the Ephedraceae family. It has a variety of qualities of gymnosperms and is well recognized for having therapeutic qualities (Chebouat et al 2014, Al- Snafi 2017).

They are a collection of ancient shrubs that grow in different types of soil, dry, rocky and sandy in arid or desert regions. The ephedra plant has a potent scent and a harsh flavor.

The dried stem is the used part of the plant. *Ephedra alata* is a significant medicinal plant that has numerous uses in the pharmaceutical sector (Hegazi et al 2020). *Ephedra alata* is a small, evergreen, nearly leafless shrub that reaches heights of 60 to 90 cm. The stems are 1.5 mm in diameter, green in color, thin, upright or reclining, small ribbed and grooved, and typically culminate in a sharp point. The stem nodes have little triangular leaves that are spaced 4 to 6 cm apart. The nodes have a distinctive reddish-brown color. The base of the stems typically has branches. They produce tiny, yellow-green blooms and fruits (Al-Snafi 2017).

Ephedra plants are typically used for allergy, bronchial asthma, colds, cough, headache, fever, edema, chills, and flu in traditional medicine.

Through phytochemical investigations, tannins, phenolics, cardiac glycosides, reducing sugars, alkaloids, and flavonoids were discovered in *E. alata* (Jaradat et al 2015). Ephedrine makes up 30–90% of the total alkaloids in the stem, which contains 1-3% alkaloids. This plant also possessed antibacterial and antioxidant activities (Parsaeimehr et al 2010, Soltan and Zaki 2009). Even though ephedrine was discovered in 1887, the Chinese were aware of around 5000 years ago. Pseudoephedrine makes up the majority of commercial pharmaceuticals derived from ephedra extracts (Hoffman et al 2009, Wang et al 2009).
Secondary metabolites are a varied range of chemical compounds that aid plants in building defenses and interacting with abiotic and biotic surroundings (Murthy et al 2014). Secondary metabolites include phenolic compounds, terpenoids, and alkaloids (Vardhan and Shukla 2017).

Gamma-ray is ionizing radiation, the most intense type of this electromagnetic radiation is an example of an abiotic elicitor that alters plant metabolism by triggering their defensive mechanisms and enhancing the manufacture of bioactive substances. As a result, they penetrate more deeply than other radiation types like beta and alpha rays. Gamma rays interact with molecules or atoms in cells, especially water, to create free radicals, which has significant biological effects (Ludovici et al 2020).

Plant cell cultures can be made from a staggering array of plant species, including those producing secondary metabolites. For specific biological tasks, researchers have repeatedly reported the critical importance of callus formation in secondary metabolite production. Some of the key factors influencing callus formation and development are genetic makeup, physiological state, tissue, chemical composition, the physical state of the culture media, and culture conditions (Cheynier et al 2013).

In plant tissue culture systems, secondary metabolites are frequently produced in response to abiotic and biotic stressors (Akula and Ravishankar 2011). Nowadays, for both molecular biologists and bioinformaticians as well as organic chemists, the production of secondary metabolites is a main research area. Therefore, this work aimed to straighten the effect of gamma radiation on growth parameters and the synthesis of bioactive compounds (ephedrine and pseudoephedrine) in the *Ephedra alata in vitro* culture.

2 Material and Methods

2.1 Callus production

2.1.1 Collection of plant material

Young shoots of *Ephedra alata* Decne. obtained from a mature plant grown in EL. Zohria Botanical Garden, Cairo. Explants (nodal segments) were excised from healthy and juvenile shoots and were wrapped with wet paper and transferred in an icebox to the lab of the Genetic Resources Department's Tissue Culture Unit at the Desert Research Center in Cairo, Egypt.

2.1.2 Disinfection method

Only the nodal regions of the *Ephedra alata* are used as explants for the callus establishment because the plant doesn't have a leaf. They were properly cleaned for 30 minutes with tap water. The nodal explants (2 cm segment) were sterilized under a laminar airflow bench by sodium hypochlorite 1.75% for 30 min and cleaned with sterile distilled water three times

2.1.3 Callus induction

Explants produced from aseptic culture are transferred to the medium for starting stage. The wound terminal ends were trimmed. The MS medium was used in this study containing 30 g/l sucrose and growth regulators; 2 mg/l for 2,4-D as auxin and 0.5 mg/l kin as cytokinin. The media were solidified with phytagel 2.5 g/l and pH was adjusted to 5.7 ± 0.1 using 0.1N HCl or NaOH then autoclaved for 15 minutes at 121ºC and 1.06 kg.cm^{-2} pressure. The cultures were kept at 25°C with a 16-hour photoperiod while being exposed to fluorescent light (2500-3000 lux) (Hegazi and El-Lamey 2011).

2.2 Gamma irradiation experiment

The callus cultures were treated with gamma irradiation at the Chemical Warfare of the Armed Forces (Egypt). Gamma irradiation was conducted using a cesium (Cs137) gamma source.

2.2.1 Callus cultures

Callus (5.0 g) of *ephedra* was cultured on MS media exposed to gamma rays at different doses (0, 5, 10, 15, 20, 25 and 30 Gy). Developing callus was repeated three times by subculturing at the end of four weeks intervals on the same media treatments.

2.2.2 Cell Suspension Culture

The acquired 2.0 g of *E. alata* fresh friable callus that had been subjected to various radiation doses was put into 250 ml conical shake flasks with 100 ml MS liquid medium that included 3.0 mg/l 2, 4-D and 0.5 mg/l kin, as well as 30 g of sucrose without phytagel. Before autoclaving, the pH of the medium was adjusted to 5.7–5.8. Under the conditions of the culture room, the suspension culture was placed in a rotary shaker at 80 rpm.

With six replicates (jars) each containing three pieces of callus and five replicates (flasks) each containing two grams of cells for cell suspension, all tests were carried out in a fully randomized fashion.

2.2.3 Extraction and determination of ephedrine and pseudoephedrine concentrations

Using a grinder and Whatman No. 1 filter paper, ephedrine and pseudoephedrine were extracted from 0.5 g dried callus and cells from a suspension culture of *Ephedra alata* in 5 ml ethanol (HPLC grade). Acidic chemicals were taken away from the filtrate by adding aliquots of plant extracts to Accell Plus QMA Sep-Pak cartridges (Waters) after dilution with an equal volume of water.

Using HPLC (Agilent 1100 series) along with the UV-Vis detectors G1322A and G1315B DEGASSER, the amount of ephedrine and pseudoephedrine in the aqueous eluent was measured (Barkan et al 1981). On a ZORBAX-EclipseXDB-C18 column, samples were chromatographed using 1% acetonitrile in 0.05 mol monobasic sodium phosphate at 1.0 ml /min (4.6 250 mm, particle size 5 m). To determine what was in the peaks at 210 nm, retention times and UV spectra were compared to those of actual chemicals.

2.3 Experimental design and statistical analysis

The one-way analysis of variance (ANOVA) program was used to calculate the variance analysis of the data. Using Duncan's multiple range test A B (Duncan 1955), the significance of the differences between the mean values for the treatments was examined at the 5% level (Snedecor and Cochran 1990).

3 Results and Discussion

The current investigation aimed to determine how different gamma radiation exposure levels affected the synthesis and accumulation of ephedrine and pseudoephedrine in *E. alata* callus and suspension cultures. As the first step in the suspending process cultivars for the synthesis of active substances in tissue culture, callus induction is necessary (Bhatia 2015).

It has been demonstrated that the stationary growth phase is the best time to promote secondary metabolite synthesis. As a result, it is crucial to set up a two-stage culture where callus biomass first develops in an environment that is suitable to the synthesis of biomass before cells are transferred from the callus to an ideal production setting that promotes the synthesis of secondary chemicals (Ramirez-Estrada et al 2016).

3.1 Gamma radiation and *in Vitro* culture

In the current study, low gamma doses encouraged callus growth, whereas cultures exposed to doses of 10, 15, and 20 Gy showed a considerable increase in biomass growth. Evaluation of callus growth is divided into 4 measurements, mean of fresh and dry weights of callus (g), callus colors and callus quality. At various gamma dosages, all variables displayed significant variability. The outcomes represent the average values of all the variables, the data for which were gathered after three months. In *E. alata* after gamma irradiation, two different trends towards positive and negative growth with respect to control were evident.

With low gamma irradiation doses (10, 15, and 20 Gy), mean fresh and dry weights of callus (g) were significantly higher than control. These doses were stimulatory and led to positive growth in callus and cell suspension cultures, while higher doses (25 and 30 Gy) caused a significant reduction in growth. These doses exhibited an inhibitory effect on *E. alata* callus and cell suspension cultures.

Within lower dose groups, as the dose increased, there was a gradual increase in the growth. In plant callus culture, the maximum weight was found at 15 Gy with an increase of 62.2 and 52.2% for mean fresh and dry weights, respectively as compared to those of the control (**Table 1**). Also, in suspension culture, the maximum weight was observed at 15 Gy with 8.800 g (mean fresh weight) and 0.972 g (mean dry weight) as compared to those of the control (**Table 2**).

The most notable aspect of radiation treatment, however, was a sharp decline in growth indices with a slight dose increase from 25 to 30 Gy. In callus culture, the mean dry weight (from 0.385 to 0.117 g) and mean fresh weight (from 4.500 to 1.343 g) both significantly decreased **(Table 1, 2)**. Likewise, growth parameters in cell suspension culture followed the same trend the dose increased from 25 to 30 Gy, and there was a pronounced decrease in mean fresh weight (from 3.850 to 1.450 g) and mean dry weight (from 0.278 to 0.405 g).

Moreover, a severe reduction in fresh weight could be seen in a higher dose (30 Gy) in callus and suspension cultures which was 1.343 and 1.450 g respectively, as compared to control (4.520 and 4.933 g). The changes in fresh weight at larger doses are significant.

In the lower dose (5Gy) the fresh weight was obtained (4.767 and 5.333 g) as compared to control (4.520 and 4.933 g) in both callus and cell suspension cultures. The dry weight of the callus of *E. alata* was obtained in 5Gy (0.448 and 0.407 g) as compared to control (0.392 and 0.389 g) in both callus and suspension cultures.

In addition, in the higher doses, the color change was observed from green to brownish-black as compared to the lower doses. This alteration was accompanied by morphological anomalies, a rapid rise in brown pigmentation in the calli tissue, and other symptoms (**Figs 1**, **2**).

(Patil et al 2018) found that, in contrast to non-irradiated cultures, gamma irradiation at a dose of 5 Gy greatly increased the growth of *Artemisia annua* callus culture and in higher doses severely inhibited calli growth. Although these indicators considerably decreased in comparison to the control with increasing radiation exposures. In addition, At the 90 and 100 Gy treatments, morphological abnormalities and a significant increase in brown pigmentation in calli tissue were associated with a significant decrease in callus growth in the third generation compared to the control.

Other researchers have also provided information based on comparable observations (Azeez et al 2017, Jan et al 2011, Khalil et al 2015). Numerous theories have been proposed by academics, despite the fact that the mechanism dictating how gamma rays affect plant growth and development is not entirely understood. Gamma radiation at low doses typically below 20 Gy can enhance plant growth by altering hormone signaling in cells, boosting amino acid production, enhancing fundamental metabolic processes, changing the intake of minerals and nutrients, and changing enzyme activity (Gudkov et al 2019, Le et al 2019, Singh et al 2012).

Plants exposed to large levels of gamma radiation may develop less rapidly as a result of hormonal imbalances in the body (Hasbullah et al 2012). High neutron radiation doses dramatically reduced the expression of auxin-activated genes involved in IAA production and auxin response factors in *Arabidopsis thaliana*, according to a study by Fortunati et al (2010).

In comparison to the control, the contents of IAA and Zeatin in *Hordeum vulgare* seedlings treated by gamma irradiation at doses more than 50 Gy also dramatically decreased (Bitarishvili et al 2018). Furthermore, several studies recorded that exposure to ionizing radiation increases the amounts of ROS and free radicals in plant cells, where these substances induced the endogenous breakdown of IAA (Gudkov et al 2019, Ahuja et al 2014, Esnault et al 2010).

Cell and organ culture growth and development depend heavily on cytokinins and auxins. These phytohormones must be present in an adequate quantity and proportion *in vitro* cultures (Bednarek and Orowska 2020, Phillips and Garda 2019). Therefore, it can be assumed that the reduction in *E. alata* callus and cell suspension cultures' fresh and dry weight observed in the current investigation was related to a change in the endogenous hormones' levels. The failure of RNA and protein synthesis, which reduced the content of soluble protein in plant cells and hindered cell growth, has also been theorized to be a result of strong gamma-ray exposures (Mariadoss et al 2020).

3.2 Effect of gamma radiation on ephedrine and pseudoephedrine content

For many researchers, the production of secondary metabolites using a cell culture technique of well-known medicinal plants has been a difficult problem. Ephedrine and pseudoephedrine are significant and beneficial secondary metabolites that *E. alata* produces in large quantities. As a result, callus and cell suspension cultures from irradiated and non-irradiated

sources were subsequently gathered in order to estimate the contents of ephedrine and pseudoephedrine.

Using **Fig 2** as a guide callus irradiated at 30 Gy showed the lowest ephedrine concentration, 2.33 mg/g DW, and was 5.07 mg/g DW lower than that of the suspension cultures irradiated at 30 Gy (7.4 mg/g DW). The highest ephedrine concentration was recorded at 15 Gy, 8.68 mg/g DW and was 3.22 mg/g DW lower than that of the suspension cultures irradiated at 15 Gy (11.9 mg/g DW). In addition, ephedrine concentrations of calli which were subjected to irradiation at 5, 10, 20 and 25 Gy were 4.38, 4.42, 8.53 and 2.62 mg/g DW, respectively (**Fig 3**). Also, Ephedrine concentrations of cell suspension cultures that were subjected to irradiation at 5, 10, 20 and 25 Gy were 8.04, 9.8, 11.3 and 10.4 mg/g DW respectively (**Fig 3**).

Using **Fig 3** as a guide callus irradiated at 30 Gy showed the lowest pseudoephedrine concentration, 6.08 mg/g DW, and was 1.09 mg/g DW lower than that of the suspension cultures irradiated at 30 Gy (7.17 mg/g DW). The highest pseudoephedrine concentration was recorded at 15 Gy, 9 mg/g DW and was 4.09 mg/g DW lower than that of the suspension cultures irradiated at 15 Gy (13.09 mg/g DW). In addition, pseudoephedrine concentrations of calli which were subjected to irradiation at 5, 10, 20, and 25 Gy were 7.63, 7.35, 8.62, and 7.86 mg/g DW respectively **(Table 1)**. Also, pseudoephedrine concentrations of suspension cultures that were subjected to irradiation at 5, 10, 20, and 25 Gy were 7.66, 8, 8.93 and 7.76 mg/g DW respectively **(Table 2)**.

Table 1. Effect of different gamma rays doses on Mean fresh and dry weights (g), callus color, callus quality, Ephedrine (mg\g DW) and Pseudoephedrine (mg\g DW) of *Ephedra alata* callus cultures

Radiation dose (Gy)	Mean fresh weight of callus (g)	Mean dry weight of callus (g)	Callus color	Callus quality	Ephedrine (mg\g DW)	Pseudoephedrine (mg\g DW)
Control	4.5200^{b}	0.39200^{b}	Yellow	Friable	2.89	7.24
5 (Gy)	4.7667^{b}	0.44833^{b}	Yellow	Friable	4.38	7.35
10 (Gy)	5.0167^{b}	0.42000^{b}	Green, Brown	Compact, Friable	4.42	7.63
15 (Gy)	7.3333^{a}	0.59667^{a}	Green, Brown	Compact, Friable	8.68	9
20 (Gy)	6.6000^{a}	0.52667^{ab}	Yellow, Brown	Compact, Friable	8.53	8.62
25 (Gy)	4.5000^{b}	0.38500^{b}	Brown	Compact	2.62	7.86
30 (Gy)	1.3429^{c}	0.11714^{c}	Brown	Compact	2.33	6.08

Values in a column that are immediately followed by the similar superscripted letter do not significantly differ, according to Duncan's multiple range test (P > 0.05).

Table 2. Effect of different gamma rays doses on Mean fresh and dry weights (g), callus color callus quality, Ephedrine (mg\g DW) and Pseudoephedrine (mg\g DW) of *Ephedra alata* cell suspension cultures

Radiation dose (Gy)	Mean fresh weight of callus (g)	Mean dry weight of callus (g)	Callus color	Callus quality	Ephedrine (mg\g DW)	Pseudoephedrine (mg\g DW)
Control	4.9333^{c}	0.3883^{ab}	Yellow	Friable	6.4	7.16
5 (Gy)	5.3333^{bc}	0.4067^{ab}	Yellow	Friable	8.04	7.66
10 (Gy)	5.2833^{bc}	0.6333^{ab}	Brown, Yellow	Compact, Friable	9.8	8
15 (Gy)	8.8000^{a}	0.9717^{a}	Brown, Yellow	Compact, Friable	11.9	13.09
20 (Gy)	6.0833^{b}	0.4867^{ab}	Yellow, Brown	Compact, Friable	11.3	8.93
25 (Gy)	3.8500^{d}	0.2783^{b}	Brown	Compact, Friable	10.4	7.76
30 (Gy)	1.4500 ^{e}	0.4050^{ab}	Brown	Compact, Friable	7.4	7.17

Values in a column that are immediately followed by the similar superscripted letter do not significantly differ, according to Duncan's multiple range test (P > 0.05).

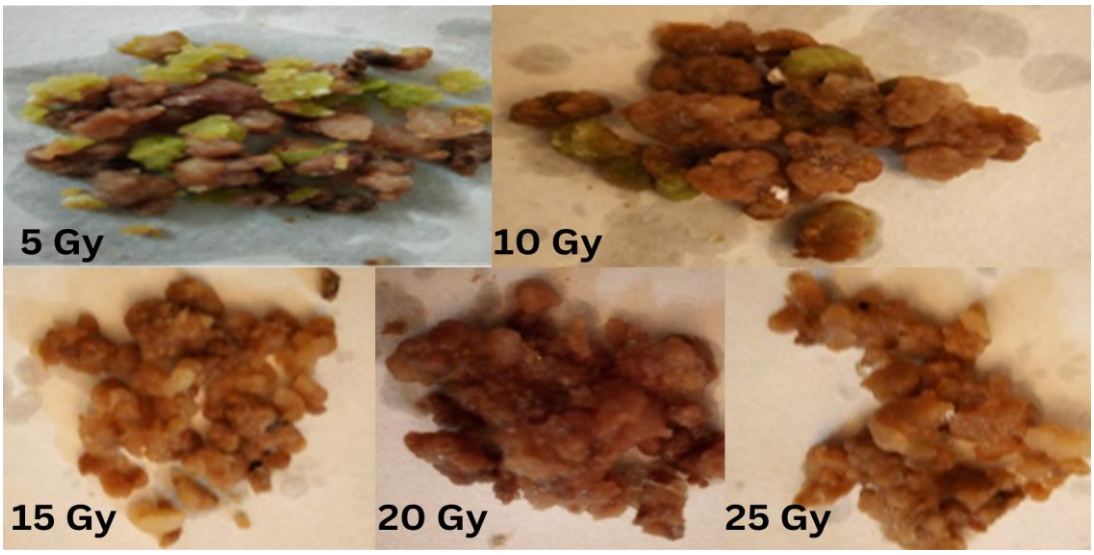

Fig 1. Effect of different gamma rays' doses (0, 5, 10, 20, 25 and 30 Gy) on callus culture of *Ephedra alata*

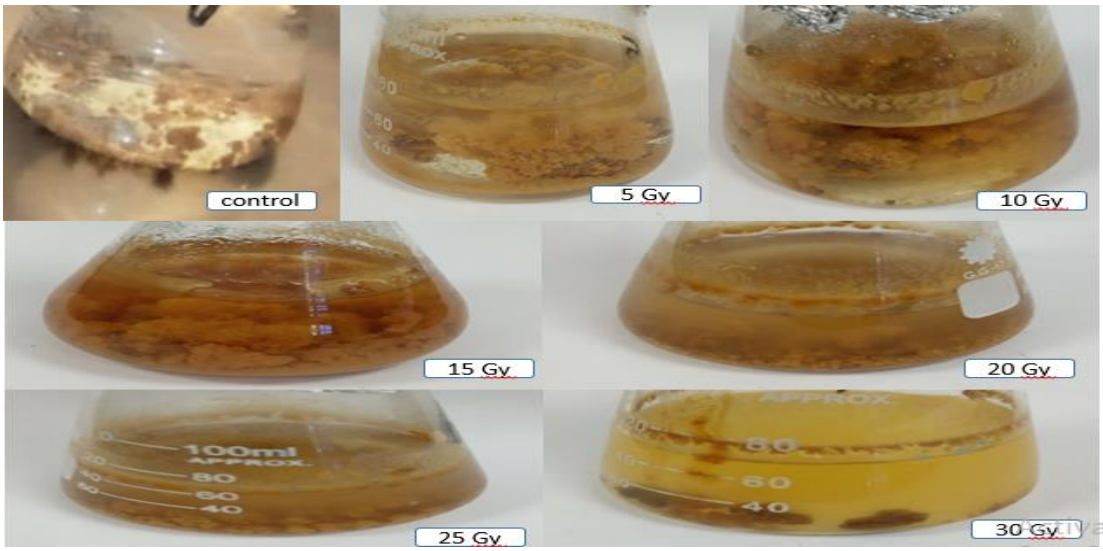

Fig 2. Effect of different gamma rays' doses (0, 5, 10, 20, 25 and 30 Gy) on cell suspension cultures of *Ephedra alata*

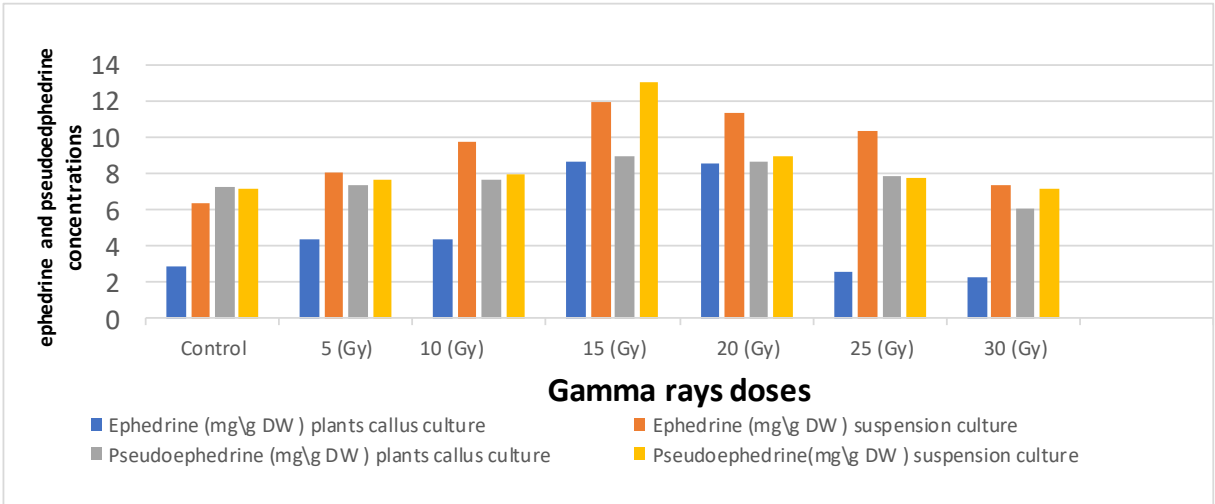

Fig 3. Effect of various gamma radiation doses on the levels of ephedrine and pseudoephedrine (mg/g DW) in *Ephedra alata* callus and cell suspension cultures

The impact of different gamma radiation doses (5, 10, 15, 20, 25, and 30 Gy) on the generation of camptothecin for *Nothapodytes foetida* callus cultures that are 3 weeks old (Fulzele et al 2015). By 20 Gy, the total production of camptothecin was up to 20 times higher. The amount of camptothecin increased most when calli were treated to 15 and 20 Gy treatments. In terms of 9-methoxy-camptotheicin, 20 Gy led to the most increase whereas 15 Gy had a little impact. However, the high gamma irradiation (25 Gy) had a negative impact on the levels of output and growth. A modified monoterpenoid indole alkaloid known as a quinoline alkaloid, camptothecin is one of these substances. Our results are in agreement with those of Chung et al (2006), who found that in *Lithospermum erythrorhizon* cell suspension cultures with radiation exposure, gamma radiation significantly elevated the cells' ability to produce shikonin, increasing the overall yields of the compound by 400% at 16 Gy. Similar to this, El Betagi et al (2013) showed that 20 Gy of gamma irradiation caused total phenol and total flavonoid accumulation in *Rosmarinus officinalis* callus culture.

4 Conclusion

In the present study, gamma irradiation treatments induced physiological and morphological variations in *Ephedra alata*. *In vitro* plantlets grew more successfully during the callus multiplication stage when exposed to a lower amount of gamma radiation than when exposed to a larger dose, which resulted in a higher mortality rate and reduced growth in the callus. According to the findings of the current study, low doses of gamma radiation may have a positive impact on the development of putative mutants that have high amounts of ephedrine and pseudoephedrine. The highest accumulation of ephedrine and pseudoephedrine concentration was observed at a 15Gy dose. Our findings offer some evidence for the development of high-yield callus culture cell lines that could be scaled up in bioreactors.

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