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Research Article

Effects of gamma irradiation on the alkaloid content in seeds of *Datura stramonium* and the radiosensitivity of derived seedlings

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Abstract

Tropane alkaloids are a group of secondary metabolites occurring naturally in Solanaceae family as *Atropa belladona*, *Datura stramonium*, *Mandragora officinalis*, and *Hyoscyamus niger*. These molecules have valuable therapeutic applications, for example, atropine and hyoscyamine are utilized as antimuscarinic besides being stomach and intestinal diseases drugs. Plants of the Solanaceae family can provide a natural yet less expensive source of these compounds. Hitherto, in order to emphasize these metabolites biosynthesis, *D. stramonium* seeds were irradiated using a cobalt-60 source of gamma rays of 5 to 80 Gy and germinated *in vitro* on MS medium in growth controlled chamber. Mutagenesis of *D. stramonium* seeds was attempted aiming at obtaining plants from *in vitro* source that are genetically variable for enhancing the biosynthesis of secondary metabolites, namely alkaloids. Results indicated that *D. stramonium* seeds exhibited a good radiosensitivity and the mutagen damage index GR (30-50) for *D. stramonium* was determined at 80 Gy. The Characterization of alkaloids (Atropine and hyoscyamine) was done by infrared spectroscopy which showed that alkaloids content of the irradiated seeds is altered by irradiation as the reference bands were not found with all doses used. In addition, seedlings grown from irradiated *in vitro* seeds exhibited remarkable morphological variations that varied based on the employed dose of gamma rays. These findings permitted the selection of the optimal irradiation dose (80 Gy) to induce mutations that are likely to prompt changes at genetic and metabolic level of the targeted alkaloids.

Keywords: Alkaloids; *Datura*; *In vitro* culture; Mutation; Radiosensitivity

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Introduction

Datura, a member of the Solanaceae family, produces various alkaloids such as atropine, scopolamine and hyoscyamine (1, 2). These secondary metabolites induce in humans and animals various physiological responses by interfering with the neurotransmitters. In the case of atropine for example, muscles activity are regulated by the parasympatic nervous system. This occurs because atropine is a competitive antagonist of the muscarinic acetylcholine receptors. Therefore, it may cause swallowing difficulties, reduced salivation and bronchial excretions (3, 4). Most of the alkaloids are toxic in high doses, but have important therapeutic properties in low doses (5). Tropane alkaloids or their derivatives are commonly used as muscle relaxants, analgesics, tranquilizers or even as psychotropic drugs. They also act as antispasmodic, sedative, anticholinergic and mydriatic substances (6, 7).

In fact, the chemical synthesis of these alkaloids is more expensive than their extraction from plants. Interestingly, through biotechnological tools, the enhancement of secondary metabolite production from plants *in vitro* became recently feasible. Indeed, various strategies have been adopted for improving the production of secondary metabolites in plants. More recently, emergence of DNA recombination technology has opened a new avenue for altering gene expression regulations related to biosynthesis of metabolites (8, 9). *In vitro* culture with growth regulators offers a promising alternative in comparison with open field culture or chemical synthesis (10). It was reported higher alkaloid contents in cell suspensions of *Datura innoxia* Mill. compared to that in whole plants (11).

Importantly, hairy roots have been extensively investigated in many medicinal plant species (12-20) as well as other applications in plant molecular pharming and phytoremediation (14, 21-23). Hairy root culture, in comparison to cell suspension cultures, provide a good source for continuous production of secondary metabolites and is characterized by its genetic stability and ability to produce a variety of chemical compounds (8). These transformed roots represent an interesting system to produce many secondary metabolites as Indole and Tropane Alkaloids (24-26).

Additionally, other studies have demonstrated an enhanced production of tropane alkaloids in transgenic hairy root cultures via *Agrobacterium rhizogenes* (17, 27). Factors that can increase alkaloids production by hairy roots involve optimization of culture medium components, elicitor-mediated stimulation of secondary metabolites and the efficient use of competent tissues (28). Induction of mutagenesis could be an alternative strategy to improve alkaloid production. Gamma irradiation causes mutations in plants by changing DNA structure and function and consequently can enhance metabolites biosynthetic pathways (29). According to our best knowledge; mutagenesis has never been investigated in *Datura* plants for improving tropane alkaloid contents. This work aimed at studying tropane alkaloids profile in irradiated *Datura stramonium* using IR spectrometer. Thus, the effects of irradiation doses on *in vitro* seed germination and the growth of resulting seedlings were assessed. This report provides bases to estimate radiosensitivity of *Datura stramonium* seeds, and to determine the optimal dose to be applied for optimizing the optimal mutations induction rate and effects on the biosynthesis of molecules of interest.

Materials and Methods

Plant material

Seeds of *Datura stramonium* L were harvested in the locality of El Harrach, Algiers, then scarified and disinfected as described by (30). Seeds were dipped in ethanol at 70°C for 30 seconds, followed by passing through sodium hypochlorite at 12°C for 10 minutes and then rinsed three times in sterile distilled water. Finally, seeds were dried on sterile blotting paper. Disinfected seeds were planted in test tubes containing 10 ml of MS culture medium (31). The tubes were then placed in a growth chamber at 26±1°C with a photoperiod of 16 hours.

Irradiation of seeds

The seeds of *Datura* were divided into homogenous lots of 50 seeds in glass tubes and gamma irradiated at different doses (0, 5, 10, 20, 40, 60 and 80 Gy) (32). These doses were determined following the recommendations of (26). The irradiation of *Datura stramonium* seeds was carried out at the nuclear application division of the Algiers nuclear research center at a rate of 0.623 Gy/mn. During irradiation, a cobalt-60 gamma radiation source was used. The pilot irradiation of the Algerian Nuclear research center is composed of three cylindrical cobalt-60 sources. They are arranged vertically in a source holder of stainless steel. The dimension of the sources is 384 mm in height and 26.6 mm in diameter. They form an active height of 1152 mm.

Hyoscyamine and scopolamine analysis by infrared spectrometry

The control and irradiated seed samples were finely ground and dried for 24 h at 45°C. A quantity of 0.1 g of kBr (potassium bromide) was added to each sample of the seed powder followed by drying in an oven at 50°C. The pellets were subjected to IR spectrometer (Chromatography Nicolet 380 FT-IR) for infrared chromatography detection of the two alkaloids targets (hyoscyamine and scopolamine).

Radiosensitivity study

Few criteria have been taken into account to study the radiosensitivity (33). These characteristics such as germination percentage, stem and root length, and rate of increase or decrease in the average length of stems and roots of seedlings derived from *in vitro* culture of irradiated (exposed to different irradiation doses) and no-treated seeds were determined 90 days after culture on a synthetic culture medium (31). To determine matugen damage index, Analysis of Variance (ANOVA) was performed for data concerning average stem and root length of seedlings derived from irradiated and non-treated seeds and means were compared at 0.05 and 0.01 significance levels.

Results

Characterization of irradiated seeds alkaloids by infrared spectrometry

The spectra for alkaloids as shown in Fig. 1 were obtained by infrared spectrometry from control and irradiated *Datura stramonium* seeds (at different irradiation doses). Formation of amines was detected in the range of 3300 to 3500 vibrations cm^{-1}. The lack of bands in this region indicated most likely the presence of a tertiary amine. The OH bond (O-H) formation is evident by the stretching mode located at 3600 cm^{-1}, whereas the absorption frequency of the C-N bond appears at 1300 cm^{-1}.

Scopolamine

Analysis of the spectra obtained for alkaloids by infrared spectrometry in the control and irradiated seeds of *Datura stramonium* at different irradiation doses is presented in Table 1. It is shown for scopolamine that the main bands contained in the standard spectrum indicated by (34) are not fully recovered in the spectra from the control and irradiated seeds except for the band 1730 cm^{-1} which is found at all doses (Table 1a). The standard band 853 cm^{-1} (C -H, aromatic) is found only in D5. The 1166 cm^{-1} band (C- N, aliphatic amines) exists at all doses except D2, D4 and D5. The 736 cm^{-1} band (C- H, aromatic) appears only at D0 and D6. The 705 cm^{-1} band (C- H, aromatics) existed neither in the D0 nor in the other doses. Finally, the 1047 cm^{-1} band (C- 0, alcohol, carboxylic acid) was found only in the control (D0) (34) (Table 1a, Fig. 1).

Hyoscyamine

The 1720 cm^{-1} band (C=0, saturated aliphatic aldehydes) is found in all doses tested (Table 1b). The bands: 1035 cm^{-1}, 1153 cm^{-1} and 1063 cm^{-1} (C - N, aliphatic amines) were not found in any doses. The doses D2, D4 and D5 did not produce 1163 cm^{-1} band (C-H, Alide Alkyl). Only dose D3, among the tested doses of gamma irradiation, has exhibited the spectral band at 1204 cm^{-1}(C-N, aliphatic amines) (Table 1b, Fig. 1).

This investigation reported the production of the secondary metabolites scopolamine and hyoscyamine in *Datura stramonium* seeds that were exposed to gamma irradiation at doses ranging from 5 to 80 GY. The identification of alkaloids in control and irradiated material following the specification of (35), with regard to wavenumber (cm^{-1}) indicated the presence of the functional groups (OH located at 3600 cm^{-1}, C-N located at 1300 cm^{-1}) and amines (most likely tertiary amines) at 3300 to 3500 cm^{-1} (Fig. 1). Furthermore, Comparison with scopolamine and hyoscyamine standard bands indicated the presence of C=O (1732 cm^{-1}), C-O (1170 cm^{-1}) and C-O-C (858 cm^{-1}) functional groups (Table 1a, b) (36).

Discussion

Infrared spectroscopy shows that alkaloids contained in seeds are altered by irradiation because reference bands, except 1730 cm^{-1} band of scopolamine and 1720 cm^{-1} of hyoscyamine are not found in response to all doses. The absence or presence of infrared bands (Table 1a, b), may be explained by the changes at the molecular level induced by the radiation energy applied to seeds. Plant genes react differently to radiation as certain genes would be more expressive than others. Moreover, genes were shown to be up-regulated and down-regulated by gamma-irradiation as reported in *Arabidopsis thaliana* (37). This could be due to variations in repair of DNA sequence damage or the presence of redundant genes or changes in gene structure and function features (38, 39). Indeed, Gamma rays interact with the atoms and molecules for creating free radicals that alter the major components of plant cells. These radicals affect morphogenesis, plant biochemistry and physiology according to applied doses of gamma (40, 41).

Effects of irradiation on seed radiosensitivity

Fig. 2 shows that seed germination percentage varied with radiation dose. Germination % was the highest (96.07) in D2 treated seeds, and the lowest (85.24) with D5. In general, the percentage of seed germination was enhanced by all doses except D5 that exhibited a slight decline in germination capacity.

Variance analysis confirms a highly significant dose effect on average stem and root length. Fig. 3 shows that the highest average stem length is recorded at D2 (8.42 cm), while the lowest average length is recorded at D6 (4.93 cm). LSD (Least significant difference) showed four homogeneous groups that were quite often overlapping. The variation coefficient with regard to stem length ranges from 57 to 82%, which indicates the existence of a high variability in the response of treated seeds. Variance analysis for root length also indicated a highly significant effect of radiation dose (Fig. 3). LSD test revealed two groups, group A being represented by D6 and group B by the other doses. The coefficient of variation (about 71.48) indicated the existence of a wide variability in root length of established

Table 1: Comparison of the infrared main vibrational modes (cm $^{-1}$) for scopolamine (A) and hyocyamine (B) standards with those of control and irradiated *Datura stramonium* seeds

(A) References scopolamine Band **(cm^{-1})**	Radiations doses of *Datura stramonium* seeds - Obtained bands						
	D0	D1	D2	D3	D4	D5	D6
1730 (1)	3009.0	2926.5	2925.7	2926.6	3733.6	3786.2	3289.8
853 (2)	2926.1	2855.0	1746.4	2855.0	3675.5	3625.8	2926.3
1166 (3)	2854.7	1742.9	1460.2	1743.0	2925.7	2926.6	2854.8
736 (4)	2360.1	1462.9	599.0	1164.5	2854.6	2855.0	1743.4
705 (5)	1746.2	1166.6	546.1	1101.8	2361.0	1743.5	1661.6
1047 (6)	1650.1		440.1		2939.3	1456.2	1549.5
	1540.1				1746.3	1258.1	1463.0
	1460.5					845.6	1164.8
	1163.0					623.9	723.6
	722.1					547.1	
	486.7						

(B) References hyoscyamine Band **(cm^{-1})**	Radiations doses of *Datura stramonium* seeds Obtained bands						
	D0	D1	D2	D3	D4	D5	D6
1720 (7)	3009.0	2926.5	2926.7	2926.6	3852.2	2926.6	2926.3
1035 (8)	2926.1	2855.0	2854.5	2855.0	3840.1	1743.5	2854.8
1153 (9)	2854.7	1742.9	1746.4	1743.0	3615.9	1456.2	1743.4
1163 (10)	2360.1	1462.9	1460.2	1463.1	3567.1	1258.1	1164.8
1063 (11)	1746.2	1166.6	599.0	1164.5	2925.7	845.6	
1204 (12)	1650.1		546.1	1101.8	2854.6	623.9	
	1540.1		440.1	471.5	2364.0	547.1	
	1460.5				2339.3	442.2	
	1163.0				1746.3		
	722.1						
	486.7						

(1) Corresponds to the 1730 to 1715 cm $^{-1}$band, C = O, α, β -unsaturated ester. (2) (4) and (5) correspond to the interval of the 900-675 bands, CH Aromatic. (3) Represents the interval of 1250-1020 bands, CN, aliphatic amines. (6) Corresponds to the interval of 1320-1000, C -0, alcohol, carboxylic acid, ester and ether. (7) Corresponds to the interval of 1740-1720, C = 0, saturated aliphatic aldehydes. (8, 9, 11, 12) Corresponds to the interval of 1250-1020, CN, aliphatic amines. (10) Corresponds to the interval of 1300-1150, CH Alide alkyl.

seedlings in response to irradiation of seeds (Figs. 3, 4). In fact increase in stem length was evident at (D2, D3 and D4), whereas reduction occurred at other doses (Fig. 4a). The highest rate of decrease in stem length was (-30.03) at D6. On the other hand, reduction in root length was observed in all radiation doses tested and recorded the maximum at D6 (- 46.76%) (Fig. 4b).

Interestingly different studies have shown that parameters as seeds germination and plant growth rate are affected also by gamma irradiations and chemical mutagens. In the present study, germination percentage is stimulated with all doses except D5. Therefore, Irradiation of *Datura stramonium* seeds could be used in the future to improve their germination Ability. The obtained results are in agreement with Kouchebagh and Mirshekari (42) on *D. stamonium* fesor effects evaluation of some physical seeds material on germination. The results revealed that the highest germination rate was due to seeds treated by gamma irradiation. These results are in partial agreement with the finding of Thapa on *Pinus* spp., and that of Melki and Dahmani on wheat (43, 44). In fact, according to the above mentioned studies low doses of gamma irradiation lead to an increase in the germination rate. Enhanced germination can be attributed to effects of irradiation on the elimination of some pests that are thought to affect seed germination (45), parallel increase in the DNA and protein amounts (46), and / or synthesis or activation of growth hormones (47). This agrees with the generalization drawn by (48) that plants exposed to low doses of gamma rays develop normally or may evince growth stimulation, whereas high doses (like 50Gy) can inhibit their growth. The effect of radiation on the average length of stems and roots was significant. Stem length showed an increase while root length remained unchanged at relatively lower doses, but both stem and root showed a significant decrease at higher doses (D6) (Figs. 3, 5). The rate of reduction/ increase in the plant axis (shoots and roots) did not follow per say a regular pattern and was not dose-dependent (49).The significant increase in shoot length of *D. stramonium* at low doses (D2, D3 and D4) could be due to stimulated cell division and /or cell elongation (49). However, at higher doses a reduced growth rate was recorded, possibly because of alteration of metabolic processes resulting from nucleic – acid disruption, which in turn, disrupted the hormonal system. It can be

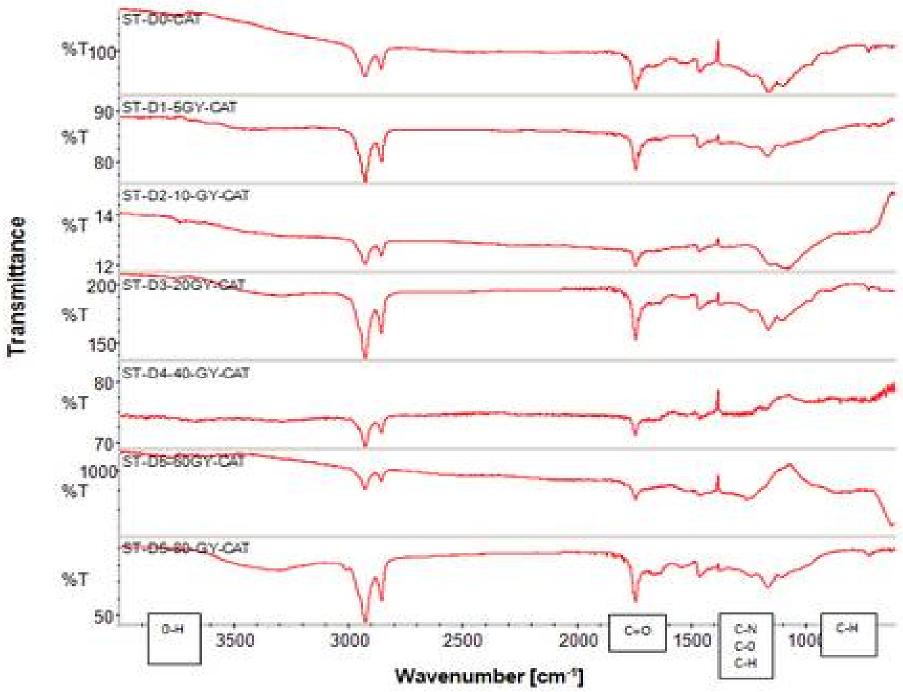

Fig.1. Infrared spectra general appearance of the control and irradiated *Datura stramonium* seeds at different doses of irradiation

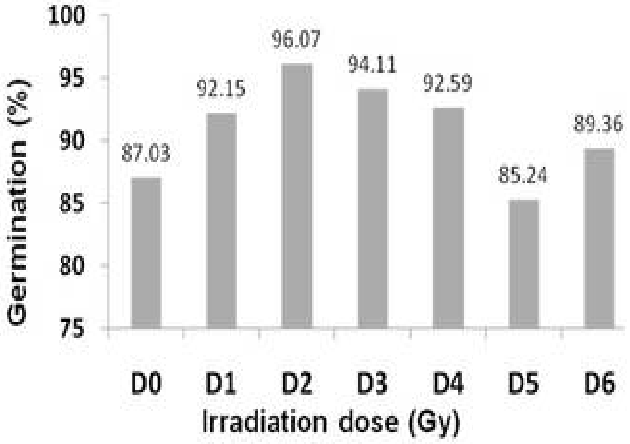

Fig. 2. Effect of different gamma irradiation doses on the *Datura stramonium* seed germination

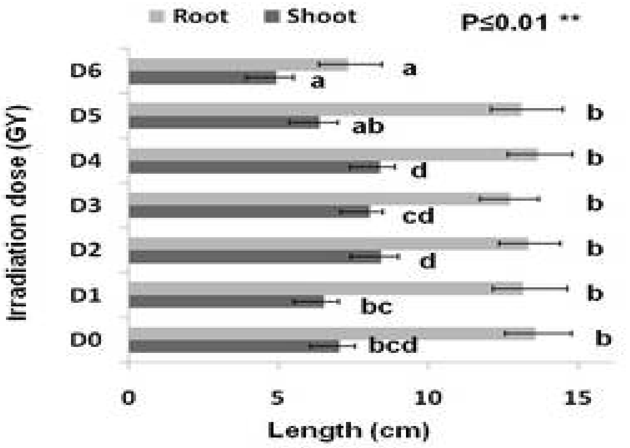

Fig. 3. Variation in average length of *Datura stramonium* stems (A) and root (B) in response to doses of irradiation employed.** The effect was significant at (*P<0.01*)

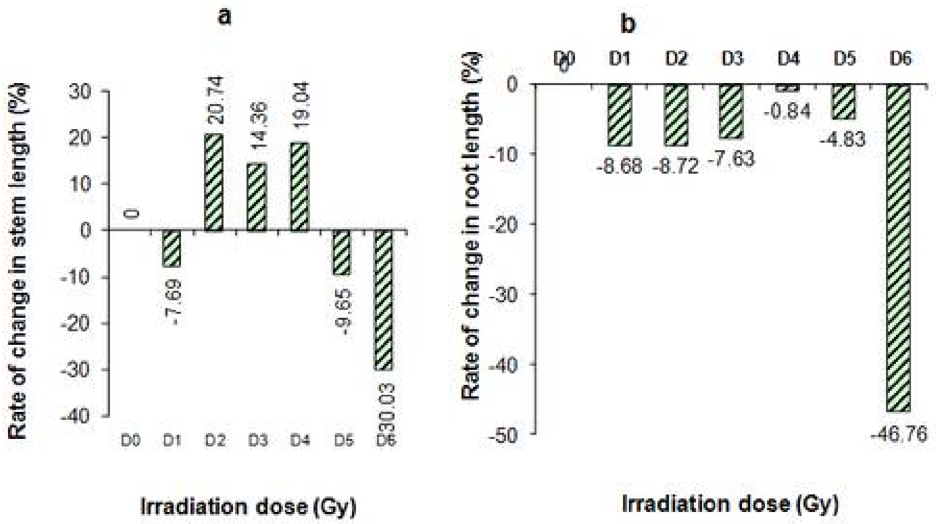

Fig. 4. Rate of change (reduction/ increase) in (A) stem and (B) root length in response to irradiation intensity

related to reduction in mitotic cell division in the apical meristem (50, 51).

On the other hand, adverse effects of increase in gamma irradiation dose on seed germination and subsequent seedling growth in other plants are well documented (41, 52, 53). The increased frequency of chromosomal damage associated with higher doses may be responsible for reduced germination and seedlings survival rate (46). High doses of irradiation can additionally cause an inhibition of mitotic divisions (53), resulting from the alteration of certain substances (such as alkaloids) in the seeds.

To determine the suitable irradiation dose for future breeding of plants GR 30-50 should be determined. GR 30-50 could be defined as the dose that cause 30- 50% inhibited germination of cultured seeds. However, in the present study, seed germination exceeded 50% whatever dose was applied and was approximately equal or higher compared to the control. The GR50 was defined as dosage that causes 30-50% decrease in growth (54, 55) used another criterion as the dosage that caused considerable physiological damage where leaf count and primordial damage was given weight in his case.

According to (56, 57) reduction in seedling height of 30-50% is generally assumed to produce high incidence mutation rate, and therefore, was adopted for *Datura stramonium* in the present study. The reduction rate of the stem and root length of *Datura stramonium* in the present investigation was (-30.03% and -46.76%, respectively) at 80 Gy. This indicates that *D. stramonium* seeds are radiosensible at this dose where a physiological damage to shoot and root growth in response to irradiation is in range of (30-50%) in comparison to control (Figs. 3, 5). Reduction of the stem and root lengths with increasing doses of radiation has been observed in several species like peanuts, Okra and maize (58).

Conclusion

Radiosensitivity study of seeds is often applied in plant improvement and breeding programs. This study explored the effect of different doses of cobalt -60 gamma rays on the seed germination and stems/ roots growth of *D. stramonium*. It is clear, from the results obtained on seeds irradiation for the parameters studied *in vitro* that GR50 is reached at 80 Gy. The selection of optimal dosage was based on 30% to 50% reduction rate in stem and root length in comparison to the control. This dose will be selected for seeds' irradiation of *Datura stramonium* with the purpose of producing mutant lines capable of producing more enhanced tropane alkaloids profile. Further research should be taken that aims at quantitatively studying tropane alkaloid production in mutant plants of *Datura stramonium*. Additionally, it is important to reveal the effects of radiation by studying the genes involved in tropane alkaloids biosynthesis pathway, their epigenetic regulation and transcriptional activation mechanisms at different levels.

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Authors' contributions

All authors have done the experiments and prepared the article content.

Competing Interests

The authors declared that they have no conflict of interest.

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