In Vitro Propagation and Mutagenesis of Ruta Graveolens L. Plants

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ABSTRACT

***In vitro* regeneration of *Ruta graveolens* L. was carried out using three different explants (shoot tips, lateral buds and leaves) with three medium protocols to determine the best explants, which was shoot tips, linked with the optimum medium, which was MS+ 0.5 mg/l BAP + 0.3 mg/l NAA, for the high potentiality of shoot formation. To induce mutation, the best explant was treated with four doses of gamma radiation 10,20,30,40 Gy. Radiation levels affected shoot length and leaves number, where shoot length and leaves number increased by 10 Gy dose. Finally, rutin was extracted from shoots of field mother plant, *in vitro* plantlets and irradiated plants and determined using U.V. spectrophotometer. Plantlets irradiated by 10 Gy contained the highest amount of total rutin, (rutin in DW), whereas the lowest amount of total rutin was at 40 Gy. The *in vivo* unirradiated plantlets exhibited total rutin greater than those of 40 Gy irradiated plantlets, thus gamma radiation can affect the plant phenotype and the production of rutin.**

INTRODUCTION

Ruta graveolens (common name: Rue) is an ornamental, culinary, and a strongly scented medicinal aromatic plant which belongs to family Rutaceae that consists of more than 1600 different species of shrubs and small trees that grow mostly in countries of world's temperate zones. They produce a great number of essences, alkaloids and glucosides. *R. graveolens* is native to Europe, especially the Mediterranean region (Anonymous, 2003). It is used as an ornamental plant because of its yellow-cupped beautiful flowers. It is a very popular garden shrub in South America. It is also cultivated for its medicinal purposes and as a charm against evil. The medicinal value is due to the presence of many secondary metabolites, such as furocoumarins, furoquinolines, andacridon alkaloids. All plant material parts have active components, although they have mostly encountered in leaves (especially before blooming).

Rutin, which constitutes about 2% of the leaf extract, is one of the major components in the plant. Rutin and its hydrolysis product "quercetin" were reported to be genotoxic and possess prooxidant activity (Sahu and Gray, 1996; Da Silva *et al*., 2002). Although there are some reports on the toxicity, no data is available on the genotoxic and clastogenic effect.

Rue is a cross-pollinated plant, thus plants obtained would not be genetically identical to parent plant and the genetic makeup may vary with individual plant. This may lead to variations in secondary metabolites production. Besides, the seed-set is low and seeds exhibit dormancy (Faisal *et al*., 2005). Therefore, there is an urgent need to look for alternate means of propagation which could ensure large-scale production of plants to fulfill the growing demands. Tissue culture provides a mean of rapid propagation of large number of uniform plants maintaining their genotype (Arikat *et al*., 2004). Direct shoot bud induction in nodal segments of *R. graveolens* through axillary shoot multiplication has been reported by Faisal *et al.*, 2005. They also reported a rapid regeneration system from leaves, shoot tips and lateral buds callus (indirect organogenesis) and the subsequent transplantation of the plantlets to natural environmental conditions.

Radiation has been successfully used for the development of new flower colour or shape mutants in many ornamental plants. Therefore, induced mutagenesis through irradiation or chemical treatment has become a very important method for plant breeding, including flower colour. By the year of 2005, around 2335 varieties were released through mutagenesis in the world, in which ornamental crops and decorative crops are 552 varieties (IAEA, 2005). So, introduction of valuable variation in *R. graveolens* through tissue culture and mutagens may help in programmes designed to improve the plant characteristics.

The objectives of the present work were:

1- Determining the capability of *R. graveolens* L for plant regeneration using three explant types (shoot tips-lateral buds-leaves)on three media and subsequently, to find out the best explant source linked with the optimum medium for the high potentiality of shoot formation.

2- Inducing mutation in *R. graveolens* L through *in vitro* mutagenesis by treating the explants with four doses of gamma radiation.

3- Determining rutin percentage in mother plant, regenerated plants from the optimum medium and explant and irradiated regenerated plants using UV spectrophotometer.

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MATERIALIS AND METHODS

The present investigation was carried out during the years 2010-2012 at the Tissue Culture Laboratory, Floriculture, Ornamental Horticulture and Garden Design Department, Faculty of Agriculture, El - Shatby, Alexandria University. It consisted of three experiments:

1. *In vitro* regeneration capability determination of *Ruta graveolens* L. using three different explants (shoot tips, lateral buds and leaves) with three medium protocols.
2. Mutation induction in *R. graveolens* L. through *in vitro* mutagenesis by treating shoot tips with four doses of gamma radiation (10, 20, 30, and 40Gy).
3. Extraction and determination of rutin in leaves from field mother plant, *in vitro* shoots and irradiated plants.

Plant material

Cuttings of *R. graveolens* L. were provided by the Faculty of Agriculture Nursery, El Shatby, Alexandria University, and then shoot tips, lateral buds and leaves were collected from these cuttings.

Media Preparation and Sterilization

Murashige and Skoog medium (1962) supplemented with vitamins + 3% sucrose + 0.8% agar was used. Table (1) shows full details of the three media for plant regeneration and root induction in *Ruta graveolens* L.

The pH of all media used was adjusted to 5.8, using 1.0 M of HCL and /or 1.0 M of NaOH. The agar was added before adjusting the pH. The media were then sterilized by autoclaving**.** All equipments were steam-sterilized for 20 min. at 121˚C (15 PSI nominal steam pressure).

Culture Preparation and Conditioning

Shoot tips, lateral buds and leaves were washed with tap water and transferred into a clean (200 ml) jar. In a clean laminar air flow, they were surface sterilized by immersing in 70% ethanol, for 1min followed by soaking in 10% Clorox (NaOCl 5.25%) for 10 min, immersing in 0.1% mercuric chloride for 7 min., then washed with seven changes of sterile distilled water. After finishing the sterilization steps, explants were cut into small pieces (5 mm) and cultured in jars (200 ml) containing 20 ml of culture medium (Table 1). Each jar with one explant was considered as replication. Seven replications were used for each explant /medium protocol. Cultures were incubated in a growth chamber at 25±2 ˚C under 16 hrs illuminations (2000 Lux, daylight fluorescent tubes), and 50 % relative humidity. Jars were checked regularly for growth.

Measurements for *in vitro* culture traits

After six weeks of incubation, five *in vitro* culture traits were determined for each jar.

1. Number of shoots/explant.
2. Shoot length (cm) derived from each shoot tip, lateral bud and leaf explant.
3. Propagules induction (%): determined as a percentage of explants which produced propagules. Propagules (Any of various usually vegetative portions of a plant, such as a bud or other offshoot, that aid in dispersal of the species and from which a new individual may develop).
4. Number of branches /explant.
5. Number of leaves/explant.

Regenerated shoots were transferred to jars containing MS medium with half strength for root induction.

Root induction

Shoots with 6-8 leaves were transferred to the rooting medium described in (Table 1) after six weeks. Root induction (%) was recorded for each treatment after 8 weeks of culturing on rooting medium.

Acclimatization

Regenerated plants (with shoots and roots) were washed with tap water to remove the agar residues and then, transplanted again in small pots filled with peat moss, perlite and sand (1:1:1 v/v/v). The pots were incubated under moist conditions in the greenhouse for acclimatization in the Floriculture Department Nursery, Faculty of Agriculture, El – Shatby, Alexandria.

Table 1. Components of the three medium protocols used for shoot formation and root induction in *R. graveolens* L

Protocol components	Protocol sequence/liter			
	Shoot formation			Root induction
	Protocol (A)	Protocol (B)	Protocol (C)	
MS salts	4.42g/l	4.42g/l	4.42g/l	2.21g/l
BAP	0.5mg/l	0.5mg/l	0.5mg/l	–
NAA	0.1mg/l	0.2mg/l	0.3mg/l	–
Sucrose	30g/l	30g/l	30g/l	30g/l
Agar	8g/l	8g/l	8g/l	8g/l

Statistical analysis

Data were statistically analyzed as a 2- factors experiment (explants and medium protocols) in a completely randomized design with seven replicates. Data in the form of percentage were subjected to arcsine transformation prior to statistical analysis (Steel and Torrie, 1980). Comparison among means was made using the Least Significant Difference (L.S.D.). The data were analyzed, using SAS program (1985).

***In vitro* mutagenesis**

Shoot tips of *R. graveolens* L. were irradiated in a Gamma 60 Cobalt Source at the National Center of Radiation Research and Technology, Nasr City, Cairo, Egypt, with the doses of 10, 20, 30 and 40 Gy. Shoot tips were directly planted in jars (200 ml) contained 20 ml of medium protocol C (Table 1) as the best medium protocol and best explant for shoot formation.

After four weeks of incubation, three *in vitro* culture traits were recorded for each jar:

1. Shoot length (cm) derived from the shoot tip/explant.
2. Number of shoots/ explant.
3. Number of leaves/explant.

Statistical Analysis

Data were, statistically, analyzed as CRD (Complete Randomize Design) with ten replicates. CRD was used also for morphological character analysis. Comparison among means was made using the Least Significant Difference (L.S.D.). The data were analyzed, using SAS program (1985).

Rutin extraction and determination

Ten replicates of shoots were obtained from the mother plants, tissue culture unirradiated and irradiated shoots with doses of 10, 20, 30 and 40 Gy.

KH_2Po_4, absolute methanol and orthophosphoric acid 85% from Merck. Standard rutin CHR was obtained from Fluka, AG, Buchs, and SG.

Phosphate buffer (pH 8.0) was prepared as described by Mohamed and Ibrahim (2011) as follows; 13.609 g KH_2Po_4 dissolved and diluted to 1000 ml with distilled water and 1ml orthophosphoric acid was added and mixed. A mixture of phosphate buffer and methanol was prepared with the ratio 55:45 and used as solvent for standard rutin blank and extractant for plant samples.

Standard rutin was prepared as described by the Association of Official Analytical Chemists A.O.A.C (2000). 4.0 mg rutin was transferred to volumetric flask (10 m) dissolved and diluted with extraction buffer to give stock solution (0.4 mg/l). A working solution was prepared by diluting 5 ml of stock solution to 100 ml with the extraction buffer to give a standard working solution (20 µg /ml).

The working solution of rutin (20µg/ml) was analyzed against blank by scanning the absorbance in the ultraviolet region (200-400 nm) using double beam spectrophotometer (Helios α /Thermo spectronic) in order to estimate the wave length at which maximum absorbance occurred (À).

Different plant samples were air dried for two weeks at laboratory temperature (17- 24 ˚c) and finally in evacuated desiccator over sulphuric acid for 48 hr. The fresh and dried samples were weighed and both moisture content and dry matter were calculated in every sample.

Rutin was extracted from different dried plant samples using the method discussed by Mohamed and Ibrahim (2011). A known weight (0.01 – 0.1 g) was extracted by shaking the pulverized sample with 20 ml of the extraction buffer for 20 min and then transferred to stoppered tube and left overnight at room temperature, then filtered through whatman No.41 filter paper.

Determination of rutin

Rutin was estimated in the plant extracts by measuring the absorbance (A) for each sample at the wave length of maximum absorbance. Rutin concentration in each sample was calculated as follows:

$$\text{mg. rutin /g dry sample} = \frac{A}{\grave{A}} \chi \quad \frac{20}{1000} \chi \quad \frac{d}{w}$$

Where A, À = absorbance of sample and standard rutin respectively.

d = dilution factor. , w = weight of sample (g).

RESULTS AND DISCUSSION

Shoot number

Data in Table (2) indicate that medium protocol (C), which was MS supplemented with 0.5 mg/l BAP and 0.3 mg/l NAA, gave the significantly highest average mean of shoots number (65.6 shoots/ explant). On the other hand, medium protocol (B) gave the significantly lowest average mean of shoots number (43.1 shoots/ explant). Results in Table (2) showed that shoot tip explant significantly gave the highest response to shoot number (67.5 shoots/explant), while leaf explant gave the lowest response to shoot number (40.3 shoots/explant). However, there were no significant differences between shoot tip explant and lateral bud explant and between leaf explant and lateral bud explant.

The effect of medium protocol x explants interaction is significant; it shows that interaction between medium protocol C and shoot tip explant has the highest response for shoot number (107.6 shoot/explant). On the other hand the interaction between medium protocol C and

leaf explant gives the lowest response for shoot number (33.9 shoot/explant). The best explant used with three media protocols is the shoot tip explant then the lateral bud explant, while leaf explant have a weak response for the shoot number (Table 2).

Most of the previously published reports on *R.graveolens* have concentrated on developing adventitious shoot regeneration from nodal segments or shoot tips (Faisal *et al*. 2005, Baskaran *et al.* 2007, and Atta-Alla *et al*. 2008).

In general, the concentration of BA and NAA in the media had a great effect on the shoot number. The synergistic effect of NAA in combination with BA on promotion of *R. graveolens* shoot cultures is in agreement with observations of Atta-Alla *et al.* (2008), who established a new medium protocol for regeneration of *R. graveolens* L. using shoot tips explnts and reported that further increase in the cytokinin/auxin ratio had no effect on the number of proliferate shoots.

These results confirmed that some plant species have enough levels of endogenous hormones and do not require high levels of exogenous growth regulators for plant regeneration (Shabana *et al*., 2001, Wala and Jasrai 2003, Faisal *et al.*, 2005 and Bohidar *et al.*, 2008).

Shoot length

Data in Table (2) indicate that shoot length has high values on medium protocol A (2.8 cm) compared to the other medium protocols B and C (2.5, 2.4 cm).

Though, statistically insignificant means reveal that shoot tip explant has higher values for shoot length (2.8 cm) compared with lateral bud (2.5 cm) and leaf explant (2.4 cm).

The medium protocol A with shoot tip explant gives the highest value of shoot length (2.9 cm), while medium protocol C with leaf explant gives the lowest value of shoot length (2.2 cm).

Propagules induction

Table (2) shows insignificant variation in medium protocols and significant variation in explants. Leaf explants has the highest value of propagules induction (10.2), compared with shoot tips and lateral buds (4.7 and 3.8).

The medium protocol C with leaf explant has the highest value of propagules induction (12.7), while medium protocol C with lateral buds explant gives the lowest value of propagules induction (1.6).

Table 2. Effect of medium protocol x explant interaction on shoots number /explant, shoot length (cm), propagules induction, branches number /explant, leaves number/explant, and root induction (%) of R. graveolens*

Protocol	Explant	Shoot No./explant	Shoot length (cm)	Propagules induction (%)	Branches No. /explant	Leaves No. / explant	Root induction (%)
A	Leaf	43.6 b	2.6	10.87 ab	1.3 bc	3.9	11.5 cd
	Bud	42.6 b	2.7	3.7 c	1.1 c	6.2	17.7 b
	Tip	57.6 b	2.9	4.3 c	1.2 c	5.7	30.1 a
χ A		47.9 ab	2.8 a	6.3 a	1.2 b	5.3 a	19.8 a
B	Leaf	43.4 b	2.5	7.1 b	1.5 ab	3.8	14.8 bc
	Bud	48.7 b	2.4	6.1 b	1.3 bc	5.9	19.3 ab
	Tip	37.3 b	2.6	2.5 c	1.3 bc	5.6	10.1 c
χ B		43.1 b	2.5 ab	5.2 a	1.4 a	5.1 a	14.8 a
C	Leaf	33.9 b	2.2	12.7 a	1.7 a	3.2	0.0 d
	Bud	55.3 b	2.4	1.6 c	1.1 c	6.0	17.3 b
	Tip	107.6 a	2.7	7.2 b	1.2 c	5.4	29.1 ab
χ C		65.6 a	2.4 b	7.2 a	1.4 a	4.9 a	15.5 a
χ leaf explant		40.3 b	2.4 a	10.2 a	1.5 a	3.6 b	8.8 b
χ Bud explant		48.9 ab	2.5 a	3.8 b	1.2 b	6.0 a	18.1 a
χ Shoot tip explant		67.5 a	2.8 a	4.7 b	1.2 b	5.6 a	23.1 a

L.S.D $_{(0.05)}$ for shoot number / explant = 37.2
L.S.D $_{(0.05)}$ for propagules induction = 5.1
L.S.D $_{(0.05)}$ for branches number = 0.26
L.S.D $_{(0.05)}$ for leaves number = 0.42
L.S.D $_{(0.05)}$ for root induction = 11.9
*Means followed by the same letter(s) in each column are not significantly different at 0.05 level of probability.

Branches number

Data in Table (2) show that medium protocol B and medium protocol C have the same and high value for branches number (1.4). However, medium protocol A has the lowest value for branches number (1.2).

Statistically highly significant means reveal that leaf explant has higher value for branches number (1.5) compared with lateral bud and shoot tip explants (1.2).

The medium protocol C with leaf explant gave the highest value for the branches number (1.7), while medium protocol A with lateral bud explant and medium protocol C with bud explant have the lowest values for branches number (1.1).

Leaves number

Data presented in Table (2) indicates that medium protocol A has higher values for leaves number (5.3) compared with medium protocol B (5.1) and medium protocol C (4.9). The means reveals that lateral bud explant and shoot tip explant have higher values for leaves number (6.04 and 5.6) compared with the leaf explant (3.6).

The medium protocol A with lateral bud explant has the highest values of leaves number (6.2), while medium protocol C with leaves explant has the lowest value of leaves number (3.2).

Root induction (%)

Root formation (Fig. 1) was induced when shoots with 4-6 leaves were transferred to rooting medium (Table 1).

Means of the three medium protocols are statistically equal, as shown in Table (2). Explants indicate highly significant variation. Shoot tip explant and lateral bud explant give the highest values in root induction (23.1 and 18.1 %), on the other hand, leaf explant gives the lowest value in root induction (8.8 %).

Table (2) showed highly significant variation in root induction influenced by explant and medium protocol interaction. Medium protocol A and shoot tips explant interaction give the highest value in root induction (30.1%); followed by medium protocol C and shoot tips explant (29.1%).

Subculture

After assessment of shoot multiplication for primary culture, the small shoots were transferred to the same fresh medium (subcultured) and then, the shoots number, shoot length, propagules induction, branches number, leaves number and root induction were recorded after six weeks of incubation on different medium protocols.

Shoots were subcultured on rooting medium .Plants established *ex-vitro*, and then established into soil (Fig 2 and 3), where they kept.

Shoot number

Results in Table (3) show that there are no differences among the means of the medium protocols. The medium protocol C gives the highest value of shoot number (124.9), medium protocol A gives (102.3), and medium protocol B gives the lowest value of shoot number (91.9).The shoot tip explant derived small shoot from the primary culture are the most effective for shoot multiplication. Data presented in Table (3), indicate that the shoot tip explant has the highest response to shoots number (117.9shoots/explant) as compared with lateral bud explant and leaf explant (116.3 and 84.9 shoots/explant). The data also reveal that there are no significant interactions between medium protocols and explant types (Table 3). the medium protocol C and

shoot tips explant interaction gives the highest value of shoot number (186.1shoots/explant), while medium protocol C and leaf explant interaction gives the lowest value of shoot number (71.4 shoots/explant).

Shoot length

Table (3) shows the means of secondary shoot length as affected by the explants and medium protocols. It reveals that medium protocol B gives the highest value for shoot length (2.8 cm), while medium protocol A gives the lowest value for shoot length (2.5cm), followed by medium protocol C which gives (2.6 cm) for shoot length.

Data in Table (3) show that there are differences among explant types. Shoot tips explant has the highest value for shoot length (2.8 cm), while lateral buds explant has the lowest value for shoot length (2.4 cm).

It is evident from Table (3) that the shoot length ranged from 2.9 cm (shoot tips explant x medium protocol C and shoot tips explant x medium protocol B) to 2.2 cm (lateral buds explant x medium protocol A) among explant types. There is no significant difference between explant types x medium protocol interaction for shoot length.

Propagules induction

Data presented in Table (3) indicate that medium protocol C has the highest value for propagules induction (5.7); followed by medium protocol B (5.6), while medium protocol A has the lowest value for propagules induction (3.5). The shoot tips explant significantly gives the highest response for propagules induction (6.4), while leaves explant gives the lowest response for propagules induction (3.5). The effect of

medium protocol x explant type interaction is not significant. The interaction between medium protocol C and shoot tip explant gives the highest response for propagules induction (8.5). On the other hand, the interaction between medium protocol A and leaves explant is the least (2.5).

Branches number

Data in Table (3) indicate that the medium protocol C gives (1.2) branches, while medium protocol A and medium protocol B gives (1.1) branches. There are no differences between medium protocols for the branches number.

The results in Table (3) show that shoot tip explant, lateral bud and leaf explants give the same value for the branches number (1.1). The effect of medium protocols x explants interaction is not significant. Almost all interactions give similar values for branches number (1.1 or 1.2).

Leaves number

Data in Table (3) indicate that there is no significant differences among the medium protocols , the medium protocol B gives the highest value for leaves number (6) compared with medium protocols C and A (5.8 and 5.6), respectively.

Fig. 1. Root induction on ½ MS medium without hormones after eight weeks

Fig . 2.Whole plant regeneration during and after acclimatization

Fig. 3. Whole plant after almost 1 month from acclimatization

Table 3. Effect of secondary medium protocol x explant interaction on shoot number /explant, shoot length (cm), propagules induction, branches number/explant, leaves number/explant and root induction (%) of *R. graveolens**

Protocol	Explant	Shoot No. / explant	Shoot length (cm)	Propagules induction	Branches No. / explant	Leaves No. / explant	Root Induction (%)
	leaf	108	2.5	2.5	1.1	5.5	25.4
A	Bud	105.9	2.2	3	1.1	5.1	15.1
	Tip	93	2.7	4.9	1.1	6.3	23.8
x A		102.3 a	2.5 b	3.5 a	1.1 a	5.6 a	21.4 b
	leaf	75.3	2.6	3.5	1.1	6.1	23.5
B	Bud	125.9	2.7	7.5	1.1	5.7	35.9
	Tip	74.6	2.9	5.9	1.1	6.3	44.8
x B		91.9 a	2.8 a	5.6 a	1.1 a	6 a	34.8 a
	leaf	71.4	2.7	4.6	1.2	5.8	31.1
C	Bud	117.1	2.4	3.9	1.1	5	19.7
	Tip	186.1	2.9	8.5	1.2	6.5	33.1
x C		124.9 a	2.6 ab	5.7 a	1.2 a	5.8 a	27.9 ab
x leaf explant		84.9 a	2.6 ab	3.5 b	1.1 a	5.8 ab	26.7 ab
x Bud explant		116.3 a	2.4 b	4.8 ab	1.1 a	5.3 b	23.6 b
x Shoot tip explant		117.9 a	2.8 a	6.4 a	1.1 a	6.4 a	33.9 a

* Means followed by the same letter(s) in each column are not significantly different at 0.05 level of probability.

Table (3) shows the differences between means of explant types, it reveals that shoot tip explant gives the highest value for leaves number (6.4), while lateral buds explant gives the lowest value (5.3).

The data show that medium protocol C x shoot tip explant interaction gives the highest value for leaves number (6.5), while medium protocol C x lateral bud explant interaction gives the lowest value for leaves number (5).

Root induction (%)

Medium protocol B gives the highest value for the percentage of root induction (34.8 %), and medium protocol A gives the lowest value for percentage of root induction 21.4 % (Table 3).

The data indicate that shoot tip explant gives the highest value for the percentage of root induction (33.9%), while lateral bud explant gives the lowest value for the percentage of root induction (23.6%).

The medium protocol B x shoot tip interaction gives the highest value for the percentage of root induction (44.8%). On the other hand, the medium protocol A and lateral bud gives the lowest value (15.1%).

***In vitro* mutagenesis**

Primary culture

Each shoot tip explant cultured on medium protocol C gave only one shoot for all gamma doses (10, 20, 30 and 40 Gy) Fig 4. Thus, gamma rays irradiation affected the plant ability for shoot formation.

Data in Table (4) indicate that shoot length has the highest value at 10 Gy (7.3cm) and the lowest value at C2 (2.7 cm), shoot length value at radiation level (30, 20 and 40 Gy) are (6.5, 5.3 and 5 cm), respectively.

Leaves number is insignificantly affected by radiation levels and the high value was observed at 10 Gy (6.3) followed by 40Gy (5.6), C2 (5.4) and 30 Gy (5.1), whereas, the lowest value is at 20 Gy radiation level (5.0).

Gamma irradiation induces physiological changes in crops. Although, gamma radiation is a technology with immense applications in agriculture, industry and medicine, its potential exploitation in agriculture is limited mainly because of lack of information awareness on optimal dose of irradiation which differs from one crop to another and from one application to another. Radiation mediated morphological, structural and/or functional changes in a plant are governed by the intensity and duration of the gamma irradiation, (Piri *et al.*, 2011).

These results are in agreement with those of Mashev *et al.* (1995), on wheat, Villavicencio *et al.* (1998), on common bean and mung bean, Chaudhuri (2002), on lentil, and Toker and Cagirgan (2004) on chickpeas and wild cicer species.

Table 4. Means of shoot length (cm), and leaves number/explant, as affected by radiation level*

Gamma ray dose	Shoot length (cm)	Leaves No. /explant
C2**	2.7 c	5.4 a
10	7.3 a	6.3 a
20	5.3 b	5.0 a
30	6.5 ab	5.1 a
40	5 b	5.6 a

*Means followed by the same letter (s) in each column are not significantly different at 0.05 level of probability.
** Control 2: shoots propagated *in vitro* by shoot tip.

Subculture

Shoot formation is affected by gamma-ray doses (Fig 4). All gamma-ray doses affected shoot formation, shoot length, shoot number, branches number and leaf shape and numbers.

The differences between treated and untreated tissue for shoot formation are significant expect for branches number. Data in Table (5) show means of shoot length, branches number and leaves number. Shoot length has the highest value at 10 Gy dose (7.3cm), while the lowest value of shoot length is recorded at control 2.

Branches numbers have almost similar values. The values ranged from (2) at 10 Gy dose to (1) at 40 Gy dose.

The largest leaves number is at 10 Gy doses (13.3), while the lowest one is at 40Gy (3.7).

Cheng *et al*. (1990) reported that lower radiation doses should yield fewer mutations while higher doses will result in decrease in regenerability, leading to loss of prospective variants. Several studies have been conducted on the radio sensitivity of *in vitro* cultures of several crops (Walther and Sauer, 1986 on roses; Shen *et al.*, 1990 on *Chrysanthemum*; Barakat *et al.*, 2010 on *Chrysanthemum morifolium*). Variation induced by the tissue culture process is a common phenomenon in plants; it may be caused by physiological stress, chromosome alteration, or change in gene expression (Evans *et al*., 1984).

Table 5. Means of secondary shoot length (cm), branches number andleaves number/explant as affected by radiation levels*

Gamma ray dose(Gy)	Shoot length (cm)	Branches No. /explant	Leaves No. /explant
C2**	2.9 c	1.2 a	6.5 bc
10	7.3 a	2.0 a	13.3 a
20	5.2 ab	1.7 a	9.0 ab
30	4.1 bc	1.3 a	5.8 bc
40	3.3 bc	1.0 a	3.7 c

*Means followed by the same letter (s) in each column are not significantly different at 0.05 level of probability.
** Control 2: shoots propagated *in vitro* by shoot tip.

Fresh and Dry weights as affected by irradiation

Fresh weight of gamma irradiated variants is between 0.3 g at 10 Gy and 1.4 g at C1, that mean % moisture is 86 and 96.7 % respectively, and dry weight between 0.04 g at C1 and 0.08 g at 40 Gy that mean % dry weight is 3.3and 11.2% respectively, (Table 6).

The sequence of variable data for fresh and dry weights, respectively, exhibits that irradiated plant at low doses (10- 20- 30 Gy) had less fresh and dry weights, whereas irradiated plants at high doses (40 Gy) and non-irradiated plant, mother plant and *in vitro* propagated plant had the highest fresh and dry weights.

C2 (0Gy)

10Gy

20 Gy

30 Gy

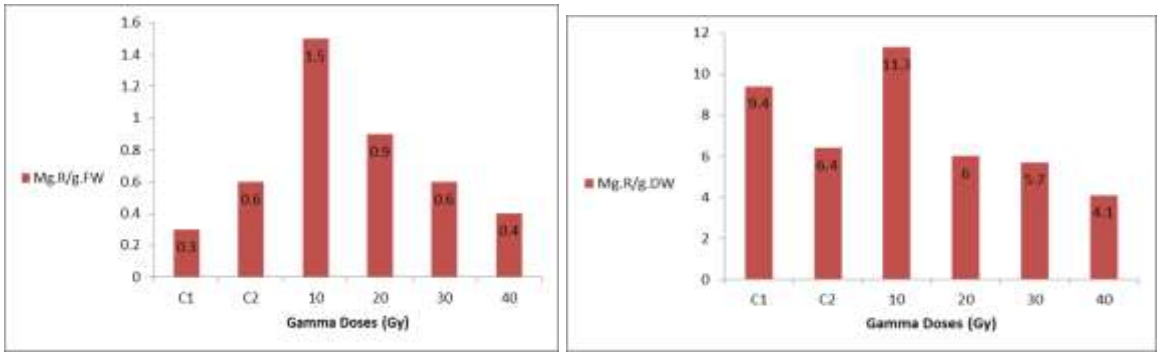
40 Gy

Fig. 4. Effect of gamma – ray doses (0, 10, 20, 30 and 40 Gy) on the shoot formation

Table 6. means of fresh and dry weights and moisture contents of *R.graveolens* as affected by gamma radiation

Treatments	Fresh weight (g)	Dry weight (g)	**Mean moisture (%)	**Mean dry (%)
C1	1.4 a*	0.04 b	96.7	3.3
C2	0.8 b	0.07 ab	90.2	9.8
10Gy	0.3 c	0.05 ab	86	14
20Gy	0.4 c	0.05 ab	85.6	14.4
30Gy	0.6 bc	0.06 ab	89.9	10.2
40Gy	0.8 b	0.08 a	80.8	11.2
L.S.D.	0.4	N.S	-	-

*Means followed by the same letter (s) in each column are not significantly different at 0.05 level of probability.
**mean of ten replicates.
C1: shoots from *in vivo* explant.
C2: shoots from *in vitro* explant (shoot tip).



(a) **(b)**

Fig. 5.Rutin content of fresh(a) and dry (b) weight of *R. graveolens* as affected by different doses of gamma irradiation

Table 7. Means of mg rutin/g. fresh weight, % rutin on fresh weight, mg. /g. dry weight, and % rutin on dry weight as affected by gamma irradiation*

Gamma radiation doses	mg rutin/g fresh weight	rutin % infresh weight	mg.rutin /g dry weight	Rutin % in dry weight
C1	0.3 d	0.0003 b	9.4 a	0.9 a
C2	0.6 bc	0.0006 b	6.4 b	0.6 b
10	1.5 a	0.002 a	11.3 a	1.1 a
20	0.9 b	0.0009 b	6 b	0.6 b
30	0.6 cd	0.0006 b	5.7 b	0.6 b
40	0.4 cd	0.0004 b	4.1 b	0.4 b
L.S.D.	0.3	0.0006	2.8	0.3

*Means followed by the same letter (s) in each column are not significantly different at 0.05 level of probability.

It seems that the response of irradiation stress was induced by high doses, expressed by metabolism enhancement, therefore, biosynthesis of different compounds which totalized (substance and structure) lead to the increase of dry mass (Cretu *et al.*, 2009).

Rutin content as affected by gamma irradiation

Total rutin content of *R. graveolens* showed some differences depending on the gamma irradiated doses. Biochemical (Flavonoids content) differentiation based on total rutin content revealed that plantlet irradiated at 10 Gy contain the highest amount of total rutin, 1.1% rutin on DW (11.3 mg rutin /g DW), whereas only 0.4 % rutin on DW (4.1 mg rutin / g DW) of total rutin was detected in 40 Gy as shown in (Fig 5) and Table (7).

Comparing the total rutin content based on dry weight of control1(*in vivo* plant) plantlets and 40 Gy irradiated plantlets, the C1 plantlets exhibited

significantly greater content than those of 40 Gy irradiated plantlets.

Plantlets irradiated with 40 Gy exhibited a total rutin content of 0.4% rutin on DW (4.1 mg rutin /g DW) lower than that of the non-irradiated plantlets, 0.9 % rutin on DW (9.4 mg rutin / g DW) control 1. However there was no significant differences among the plantlets irradiated with 20, 30 and 40 Gy also non-irradiated plant control 2 (tissue culture plants).

The study also revealed that plantlets irradiated at 20, 30 and 40 Gy recorded total rutin content of 0.6%, 0.6 % and 0.4% rutin on DW(6,5.7,and 4.1 mg rutin /g DW), respectively. There was an irregular distribution of total rutin content in irradiated and non-irradiated plantlets. According to the results obtained in the present study, it was found that plantlets irradiated at high doses (20,30 and 40Gy) contained a lower total rutin as compared to their non- irradiated plantlets (control 2). Conversely, it was observed that plantlets irradiated at relatively low doses 10 Gy and non-irradiated mother plant (control 1) contained more total rutin (Table 7).

Owczarczyk *et al.* (2000) reported that the content of biologically active substances including the essential oil, flavonoids, and glycosides did not change significantly after irradiation.

The biological effect of gamma rays is based on the interaction with atoms or molecules in the cell, particularly water to produce free radicals which can damage different important compounds of plant cell. (Kovacs and Keresztes, 2002).

The most crucial function of plant cell is to respond to gamma stress by developing defenses mechanisms. These defenses are brought about by alteration in the pattern of gene expression. This led to modulation of certain metabolic and defensive pathway. Owing to gene expression altered under gamma stress, qualitative and quantitative changes in total absorbed rutin content was obvious (Ling *et al.*, 2008).

Flavonoids as one of the most diverse and widespread group of natural compounds, are likely to be the most important natural phenolics. Therefore, the content of flavonoids and some of their derivatives (flavonols, flavonones and anthocyanins) are also determined in the methanolic extracts of irradiated cilantro fresh plantlets at dose levels of 0.0, 10.0, 20.0,30.0 and 40.0 Gy. (Aly, 2010). He reported that the increase in anthocyanin may be due to the effect of γ-irradiation which enhances the activities of phenylalanine ammonia-lyase (PAL) and flavonoid glucosyltransferase (GT), the two key enzymes involved in the anthocyanin biosynthesis from phenylalanine, so anthocyanins and flavonoids compounds can be enhanced under γ-irradiation stress. Low doses of irradiation prevented the loss of anthocyanin, while higher doses decreased the content of anthocyanin in grape pomace. Moreover, Beaulieu *et al.* (1999) reported that the activity of catechol oxidase, (an enzyme related to the biosynthesis of anthocyanin monomers) was significantly higher in irradiated mushrooms both at low and high doses than in controls.

Aly (2010) reported that low doses of γ-irradiation increased phenolic compounds, flavonoids, anthocyanins, as well as water soluble vitamins biosynthesis were also enhanced.

Further studies are needed to investigate the possibility of using low doses of γ-irradiation for potential research and development value in the field of pharmaceutical compounds from different medicinal plants.

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الملخص العربي

إكثار واستحداث طفرات لنباتات السذب باستخدام زراعة الأنسجة

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تم زراعة السذب معمليا باستخدام ثلاثة أنواع مختلفة من الأجزاء النباتية وهي القمة النامية والبرعم الجانبي والورقة حيث زرعت علي ثلاثة أنواع مختلفة من البيئات وتم تحديد أفضل جزء نباتي وهو القمة النامية وأفضل بيئة غذائية وهي MS + 0.5mg/l BAP + 0.3 mg/l NAAوذلك للحصول علي أكبر عدد من المجاميع الخضرية,كما تم إعادة زراعة الأفرع الناتجة من المعاملات فى التجربة الاولي ثم تم إجراء التجربة الثانية حيث ثم تعريض النبات لأربع جرعات مختلفة من أشعة جاما وهي 10,20,30,40 Gy ثم زراعة القمة النامية علي أفضل بيئة غذائية والتي تم التوصل إليهم من التجربة الأولي لمحاولة إحداث طفرة في النبات من خلال التاثير عليه بأشعة جاما

تم الحصول علي مجاميع خضرية مختلفة في الشكل المظهري عن النبات الام وعن النبات النامي معمليا والذي لم يعرض لأشعة جاما, ومن هنا تم إجراء التجربة الثالثة وهي تقدير كمية مادة الروتين في المجاميع الخضرية للنبات الاصلي المزروع في المشتل وللنبات النامي معمليا غير المعرض لأشعة جاما وللنبات النامي معمليا والمعرض للجرعات المختلفة لأشعة جاما.

تم التوصل إلي أنه يمكن إنتاج السذب معمليا بأعداد كثيرة وذلك بزراعة القمة النامية علي البيئة الغذائية MS+0.5 mg/l BAP+0.3mg/l NAA

ويمكن لأشعة جاما التاثير علي الشكل المظهري لنبات السذب وعلي إنتاج النبات لمادة الروتين, حيث تم التوصل الي أن الجرعة 10 Gy تزيد من كمية الروتين في النبات.