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Effect of Gamma Irradiation on *Brunfelsia pauciflora* Plant *in vitro*

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ABSTRACT

In this study on *Brunfelsia pauciflora* plant shoot tips were exposed to gamma irradiation at 0, 5 and 10 Gy as gave 100% of survival percentage during *in vitro* multiplication. The highest values for number of shoots, number of leaves and the longest shoot were recorded after multiplication stage with exposure to 10Gy. The shoots exposed to gamma rays at the dose of 10 Gy gave the maximum values for number of roots, root length, number of leaves and the longest shoot at *in vitro* rooting. Application of gamma rays at 10 Gy gave vigorous growth with healthy appearance at acclimatization stage. Moreover it resulted in the highest content of chlorophyll (a and b) and the lowest content of carotenoids. Stem thickness exhibited an increase due to both doses of 5 and 10 Gy and reached maximum values over the control. However, the thickness of both upper and lower epidermis of leaves were only increased at the rate of 5 Gy over control. The true to tip between mother plant and the micropropagated plants only (sample number 1: control) had been confirmed. Between mother plant and irradiated plants by gamma rays at the doses of (5, 10 and 20 Gy) resulted in the DNA polymorphism in the collection of genotypes analyzed, and generated many polymorphic markers ensuring a good coverage of the genome.

Key words: *Brunfelsia pauciflora*, Micropropagation, *in vitro*, Tissue culture, irradiation, gamma rays and ISSR analysis.

Introduction

Brunfelsia is a genus of flowering plants belonging to the family Solanaceae. There are about 50 species described of shrubs and small trees native to Brazil. The leaves are alternately arranged, simple, and usually oval in shape. The large, tubular flowers have five broad petals (Filipowicz and Renner, 2012). *Brunfelsia* also has horticultural importance, with species being sold under the names ‘‘lady of the night’’ or ‘‘yesterday–today–tomorrow’’ for their nocturnally scented and color-changing flowers, which during anthesis turn from dark purple over mauve to white (Filipowicz and Renner 2012). The typical habitat for these plants is light woodland and thickets. Several Brunfelsia species contain medicinal and toxic alkaloids. For example, *B. grandiflora* is the source of the most important native remedies employed against rheumatism, arthritis and snake bites in the upper Amazon region. The phytochemical analysis revealed the presence of steroids, flavonoids, tannins and saponins in the methanolic leaf extract, while alkaloids, terpenoids, anthraquinones and cardiac glycosides were absent. DPPH radical scavenging activity of extract and ascorbic acid standard was found. The reducing power assay confirms radical scavenging activity. The study revealed high level of antioxidant activity in this plant. Other Brunfelsia species, among them *B. calycina*, have been cultivated as ornamentals. *B. calycina* has become a popular garden and pot plant due to its large blue flowers and pleasant fragrance (Liberman *et al*., 2010 and Raj and Radhamany, 2010). Stem of *Brunfelsia latifolia* culture on 1/2 MS medium supplemented with 0.01 mg/l NAA + 3.0 mg/l BA + 2.0 mg/l kinetin was the best medium for adventitious shoot induction (Wang *et al*., 2008). The isolated shoots of *Solanum trilobatum* were transferred to MS basal medium supplemented with different concentrations of IBA and NAA ranging from 0.5 - 2.5 micro M/l for root induction (Subbaiya *et al*., 2015). *in vitro* plantlets of *Solanum trilobatum* were transferred to pots containing a mixture of vermiculite and red soil (1:1) for acclimatization. The transplantation survival rate was 85-90% (Raja *et al*., 2015).

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The treatment on *Agave fourcroydes* studied of irradiation dose rate (10, 20, 30, 40 and 50 Gy) using a Co-60 source of gamma, and they found that irradiated explants showed a decrease in fresh weight and a greater mortality percentage as the dose of radiation increased, as LD50 was 30 Gy and a 50% of irradiated explants died. Also, with increasing irradiation dose, the number of explants decreased (Oramas *et al*., 2007). Irradiation of *Etlingera elatior* buds with gamma rays ranging from 10 to 140 Gy showed decreasing survival of the explants with increasing radiation doses (Yunus *et al*., 2013). Irradiation at 20 Gy decreased the ratio of chlorophylls a/b (0.74 + 0.104) compared with the mother plant (1.18 + 0.0665) (Palamine *et al*., 2005).

The effect of different doses (control, 5, 10, 15, 20 and 25 Kr) of gamma irradiation on seed germination, flowering, fruit and seed traits of *Jatropha curcas* was studied by Dhakshanamoorthy *et al*. (2010) as they concluded that DNA polymorphism was detected by RAPD analysis and offered a useful molecular marker for the identification of mutants in gamma radiation treated plants. The RAPD technique has successfully differentiated *Gypsophila paniculata* variants obtained through *in vitro* mutagenesis from their parent (Barakat and El-Sammak, 2011).

Therefore, the aims of this study were to investigate the effect of gamma irradiation on shooting behavior, rooting, acclimatization and genetic fidelity of *Brunfelsia pauciflora* shoot.

Materials and Methods

Plant material:

The mother plants are growing in greenhouse condition at some nurseries that were imported from Holland. Shoot tips were used at 0.5 cm as explants.

Incubation conditions:

Explants of Brunfelsia were incubated under16h photoperiod at a constant temperature of 25°C (Liberman *et al*., 2010). All cultures of the different experiments were incubated at 26±2 °C and 16h day/ 8h dark photoperiod at the light intensity of 2000 lux from cooling white florescent lamps in a growth room (Sakr *et al*., 2013).

Surface sterilization of shoot tips:

The shoot tips (0.5 cm) were excised from the mother plants and then washed by a soapy water for 10 min followed by two h under a running tap-water. They were sterilized by immersion in a clorox solution (commercial bleach, 5.25% sodium hypochlorite) at 25% for 15 min plus 2-3 drops of Tween-20 then immersed in mercuric chloride (Hg_2Cl) at 0.2 g/l for 5 min. The shoot tips were washed then 5 times with a sterile distilled water.

In vitro mass propagation and gamma irradiation:

The gamma irradiation treatments were carried out at the Middle Eastern Regional Radioisotope Centre for the Arab Countries, Giza Governorate (Dokki). Gamma irradiation was conducted using Co^{60} gamma source.

Multiplication stage:

Shoots of *Brunfelsia pauciflora* were cultured on Murashige and Skoog (1962) basal medium (MS). Developing shoots were repeated three times by subculturing at the end of four weeks interval on the same media treatments. Shoot tips (7-8 leaves, 5-6 cm shoot length) were exposed to gamma rays at different doses (0, 5, 10, 20 and 30 Gy), the irradiated shoots were sub-cultured on MS medium supplemented with 1mg/l BA and 2 mg/l kin for *in vitro* propagation. Each treatment of irradiation dose consisted of three jars containing three shoots in each jar. The collected data were recorded monthly for three times by subculturing: Number of shoots, number of leaves, shoot length

(cm) and callus formation.
The following parameters were recorded at the end of the experiments:

Anatomical study:

Microtechnique practices for the anatomical studies of *Brunfelsia pauciflora* plants, as samples of plantlets were carried out at the laboratory of Agriculture Botanical Department, Faculty of Agriculture, Cairo University. Samples of stems and leaf were taken (µm). Materials were killed and fixed for at least 48 hrs. In F.A.A. (10 ml. formalin, 5 ml glacial acetic acid, 85 ml ethyl alcohol 70% and dehydrated in a normal butyl alcohol series before being embedded in paraffin wax at melting point 56 °C (Sass, 1951). Sections which were cut on a rotary microtome at a thickness of 15-20 microns were stained with safranin and light green before mounting in Canada balsam, according to Nassar and El-Sahar (1998). Slides were examined microscopically and photomicrographed.

Chemical analysis:

Chlorophyll a, b and total carotenoids were colourmetrically determined in leaf samples (mg/ 100g fresh matter) according to Saric *et al*., (1976). The determination was conducted using acetone (85% v/v as a blank at wavelength of 662, 644 and 440 nm, respectively elicitation).

Total genomic DNA extraction

Total genomic DNA was extracted from mother plant of *Brunfelsia pauciflora* and exposed to gamma rays at different doses (0, 5, 10 and 20 Gy), which were taken at the end of acclimatization and grinded into a fine powder in liquid nitrogen using a pestle and mortar following the steps of CTAB (hexade cyltrimethyl ammonium bromide) protocol (Porebski *et al.*, 1997).

ISSR-PCR analysis

ISSR scorable primers were designed and screened for PCR amplification. PCR was performed in 25 µl reaction volume containing 1X PCR buffer, 1.75 mM $MgCl_2$, 5 mM of each dNTPs, 40 µM oligonucleotide primer from each of the ISSR primers (Operon) Table 1, 25ng genomic DNA and 1 U of Taq DNA polymerase. A high stringency touchdown and hot start thermo cycling profile was used as follows: An initial denaturation step for 5 min at 94ºC followed by ten touch down cycles (94ºC/30 sec, 65-55ºC/45 sec, 72ºC/1 min). This was followed by 35 cycles. The PCR products were separated by electrophoresis in a 2.0% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 90volts. Gel was photographed under UV light with Tracktel GDS-2 gel documentation system. The size of the amplification products was estimated from 100 bp DNA ladder.

Table 1: The ISSR primers, names and sequences, used for ISSR analysis.

No.	Primers name	Primers sequences 5´———3´
1	HB-8	5`GAG AGA GAG AGA GG 3`
2	HB-11	5` GTG TGT GTG TGT TGT CC 3`
3	HB-12	5` CAC CAC CAC GC 3`

Rooting stage:

Shoots of *Brunfelsia pauciflora* (4 cm in length with 4-5 leaves) were exposed to gamma rays at different doses (0, 5, 10, 20 and 30 Gy) then cultured *in vitro* on MS medium supplemented with 2.0 mg/l IBA for rooting and 3.0 g/l activated charcoal to improve roots formation. Number of roots, root length (cm), shoot length (cm), number of leaves and callus formation were recorded after 6 weeks.

Acclimatization stage:

The obtained rooted plantlets were pricked out separately into 10 cm plastic pots filled with a mixture from peatmoss and perlite at the ratio of 2: 1 (v/v). To maintain cultures at high humidity, pots were covered with clear transparent plastic sheets for three weeks. The plastic covers were then gradually removed to reduce humidity and to adapt plantlets to greenhouse conditions, data on shoot length (cm) and number of leaves were recorded after three months.

Experimental design and statistical analysis:

A factorial experiment in a complete randomized design was employed in all of the experiments. Analysis of variance was used to compare statistical differences between the means using the L.S.D. at 5% probability level (Snedecor and Cochran, 1989).

Results and Discussion

Morphological characterization on *in vitro* mass propagation and irradiation:

Effect of gamma irradiation treatments on in vitro the survival percentage of Brunfelsia pauciflora:

Concerning the exposure of *Brunfelsia pauciflora* shoots to gamma irradiation at 0, 5, 10, 20 or 30 Gy, data presented in Table (2) and illustrated in Fig.(1) show that gamma irradiation at 0, 5 and 10 Gy resulted in 100% survival. High levels of gamma irradiation (20 and 30 Gy) gave the lowest survival percentages (63 and 43 %, respectively). The obtained results agree with that obtained by Abu El-Leil (2008) on black cumin and cumin, who found that gamma irradiation at 30 Gy gave the highest mortality percentage 57%. He concluded that, low doses of gamma ray stimulated survival (%), whereas raising irradiation doses decreased it. Oramas *et al*. (2007) found that irradiated plants of *Agave fourcroydes* showed the highest mortality percentage as the LD50 dose increased to 30 Gy and a 50% irradiated explants died.

Table 2: Effect of different doses of gamma rays on survival percentage (%) and mortality percentage (%) after multiplication stage of *Brunfelsia pauciflora* shoots.

Radiation dose (Gy)	Survival (%)	Mortality (%)
0	100	0
5	100	0
10	100	0
20	63	37
30	43	57
$LSD_{at\ 0.05}$	7	7

Effect of gamma irradiation treatments and number of subcultures on shoot formation of Brunfelsia pauciflora during multiplication stage:

Data presented in Table (3) and illustrated in Fig.(1) indicated that the highest values for number of shoots (51.7), the longest shoot (6.8 cm) and number of leaves (27.7) were recorded in the 3rd subculture for that irradiated shoot with 10Gy. However, the greatest value for callus formation (4.0) was recorded at 30 Gy in the 3rd subculture. These results are in harmony with those reported by Kovacs and Keresztes (2002) who concluded that the lethality of explants at high doses of gamma rays had been occurred by breakdown of meristimatic cells and cell nuclear damage.

Effect of gamma irradiation treatments on Brunfelsia pauciflora shootlets formation during rooting stage:

Data presented in Table (4) and illustrated in Fig.(2) showed that exposure of the explants (shoot tips) to gamma irradiation at the dose of 10 Gy resulted in the greatest number of leaves (15.3)

and the longest shoot (10.7 cm), the highest values for number of roots (10.3) and root length (17.3 cm). The obtained results are in agreement with those obtained by El-Shakhs *et al*. (2007) on Canary palm and Washingtonia palm. They demonstrated that the low dose of gamma rays was the most positively effective on subsequent growth.

Table 3: Effect of different doses of gamma rays on shoot length (cm), number of shoots, number of leaves and callus formation after multiplication stage of *Brunfelsia pauciflora*.

Sub / Radiation dose (Gy)	No. of shoots				Shoot length (cm)				No. of leaves				Callus formation (as scores)			
	Sub (1)	Sub (2)	Sub (3)	Mean (A)	Sub (1)	Sub (2)	Sub (3)	Mean (A)	Sub (1)	Sub (2)	Sub (3)	Mean (A)	Sub (1)	Sub (2)	Sub (3)	Mean (A)
0	15.7	19.7	43.0	26.1	3.5	3.8	4.8	4.1	8.3	10.3	13.3	10.7	0.7	1.3	2.7	1.6
5	17.3	21.7	45.7	28.2	3.8	4.7	5.7	4.7	14.7	19.7	21.3	18.6	0.0	0.7	1.3	0.7
10	19.3	24.3	51.7	31.8	4.5	5.8	6.8	5.7	20.7	25.7	27.7	24.7	0.0	1.3	1.5	0.9
20	1.0	1.0	1.0	1.0	2.3	3.0	3.5	2.9	8.3	8.7	10.7	9.2	1.3	1.7	2.7	1.9
30	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.0	8.0	5.0	2.7	5.2	2.3	3.3	4.0	3.2
Mean (B)	10.9	13.5	28.5		3.2	3.9	4.6		12.0	13.9	15.1		0.9	1.6	2.5	
$LSD_{0.05}$ for																
Sub (A)	0.5				0.2				0.5				0.4			
Gy (B)	0.4				0.2				0.4				0.3			
(A×B)	0.9				0.4				0.9				0.7			

Footnote: Results for callus formation/explant were calculated visually as scores (according to Pottino, 1981) Negative (-) = 1, Below average (+) = 2, Average (++) = 3, Good (+++) = 4.

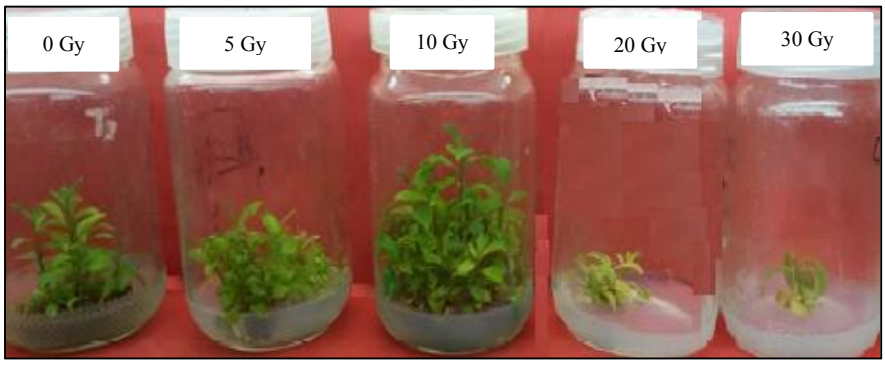

Fig. 1: Effect of different gamma rays doses (0, 5, 10, 20 and 30 Gy) on multiplication stage of *Brunfelsia pauciflora*.

Table 4: Effect of different doses of gamma rays on shoot length, number of leaves, callus formation, number of roots and root length during rooting stage of Brunfelsia pauciflora.

Radiation dose (Gy)	Shoot length (cm)	No. of leaves	Number of roots	Root length (cm)	Callus formation (as scores)
0	5. 7	7. 7	3. 7	12. 7	0.0
5	7. 3	10.3	4.3	13.3	0.0
10	10. 7	15.3	10.3	17.3	0.0
20	4. 7	7. 7	0.0	0.0	1.3
30	4.0	4. 7	0.0	0.0	2.3
$LSD_{at\ 0.05}$	1.1	1.1	0.9	0.8	0.8

Footnote: Results for callus formation/explant were calculated visually as scores (according to Pottino, 1981) Negative (-) =1, Below average (+) =2, Average (++) =3, Good (+++) = 4.

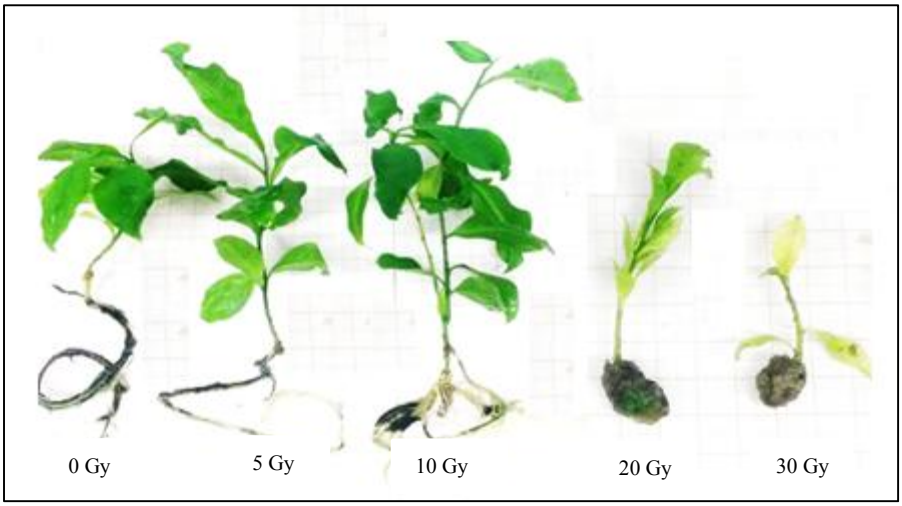

Fig. 2: Effect of different gamma rays doses (0, 5, 10, 20 and 30 Gy) during rooting stage of *Brunfelsia pauciflora*.

Effect of gamma irradiation treatments during adaptation stage of *Brunfelsia pauciflora* plantlets:

Data presented in Table (5) and illustrated in Fig.(3) showed that the treatments of gamma rays at 0, 5, 10, 20 and 30 Gy caused a significant influence on acclimatization by giving vigorously and had healthy appearance of the plantlets grown in peatmoss : perlite (2:1 v/v). The application of gamma rays at the 10 Gy resulted in the longest plantlets (11.7) cm and the greatest number of leaves (16.3).

Effect of gamma irradiation on pigments content:

The application of gamma rays at 10 Gy (Table, 6) increased the content of chlorophyll (a and b) as mg/g f.w, but decreased the carotenoids content to its lowest value. However, the lowest values of chlorophyll a and b contents and the highest content of carotenoids were obtained as a result of using 30 Gy treatment. The obtained results are in agreement with those obtained by Esmail (2014) who reported that gamma irradiation at 15 Gy decreased the chlorophylls (a and b) content of *in vitro* Dracaena plant.

Table 5: Effect of different doses of gamma rays on shoot length and number of leaves during acclimatization stage of *Brunfelsia pauciflora*.

Radiation dose (Gy)	Shoot length (cm)	No. of leaves
0	8.3	12. 7
5	8. 7	13.3
10	11. 7	16.3
20	4.0	2.3
30	4.0	0.0
$LSD_{at\ 0.05}$	0.8	1.4

Table 6: Effect of different doses gamma rays on chlorophyll a,b and carotenoids contents (mg/g f.w) of *Brunfelsia pauciflora* plants leaves after three subcultures of multiplication stage.

Radiation dose (Gy)	Chlorophyll (a)	Chlorophyll (b)	Carotenoids
0	0.2	0.1	14.9
5	0.3	0.2	12.7
10	0.3	0.3	12.3
20	0.1	0.1	19.5
30	0.1	0.1	19.6
LSD at 0.05	0.1	0.1	3.1

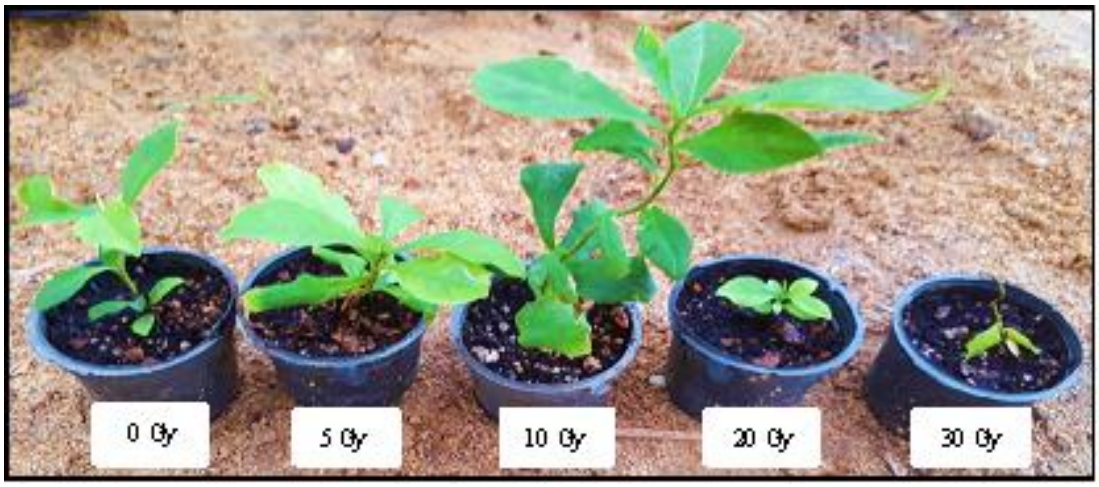

Fig. 3: Effect of different gamma rays doses (0, 5, 10, 20 and 30 Gy) on acclimatization stage of *Brunfelsia pauciflora*.

Effect of gamma irradiation on shootlets anatomical structure:

Anatomical studies of the stem:

Data presented in Table (7) and illustrated in Fig (4) dealing with microscopical measurements for sections through stem for revealed that thickness of stem was increased in all doses under study except dose of 30 Gy which showed decrease by 0.6%. Other doses cleared prominent increase and reached maximum at dose of 5 Gy by 46.5% over control, followed by dose of 10 Gy by 15.9% and finally the dose of 20 Gy by 13.3% over the control. Concerning the epidermal thickness it is clear that all doses except for 5 Gy dose increased the values by 50% over the control. The thickness of cortex exhibited only increase at the lowest dose (5 Gy), by 25% over control.

Table 7: Measurements in micron of certain histological features in transverse sections through the stem of *Brunfelsia pauciflora* plant treated with different doses of gamma rays.

Characterestics	Radiation dose				
	Zero Gy	5 Gy	10 Gy	20 Gy	30 Gy
Stem diameter	981.2	1437.5	1137.5	1112.5	975.0
Thick. of epi.	12.5	12.5	18.8	18.8	18.75
Thick. of cortex	250.0	312.5	187.5	212.5	162.5
Thick. of phloem	537.5	812.5	706.2	625	512.5
Thick. of xylem	37.5	125.0	137.5	62.5	37.5
Thick. of pith.	418.7	512.5	331.2	425.0	306.2

Anatomical studies of the leaves:

Data presented in Table (8) and illustrated in Fig (5) stated that thickness of both upper and lower epidermis were negatively affected with the applied gamma rays doses except for dose of 5Gy which didn't show any effect on this criteria .

Concerning mesophyll, data showed that its thickness exhibited an increase for both doses of 5 and 10 Gy and reached maximum value (225M) for the dose of 10 Gy, followed by 5 Gy which resulted in 175M i.e. 80 and 40% over the control, respectively. The other doses decreased mesophyll the values by -11.11 and - 25% for 20 and 30 Gy under the control, respectively. The midrib thickness showed an increase only at the doses of 5 and 10 Gy, which recorded increment by 63.6 and 45.4% over the control, respectively. The other doses decreased the values by 30.9 and 1.8% for 20 and 30 Gy under the control, respectively.

It's obvious from data mentioned before that both doses of 5 and 10 Gy verified a prominent results for most histological characters for the leaf and the stem which reflected into increments for both of shoot length and number of leaves as this might be logic, where the increase of mesophyll

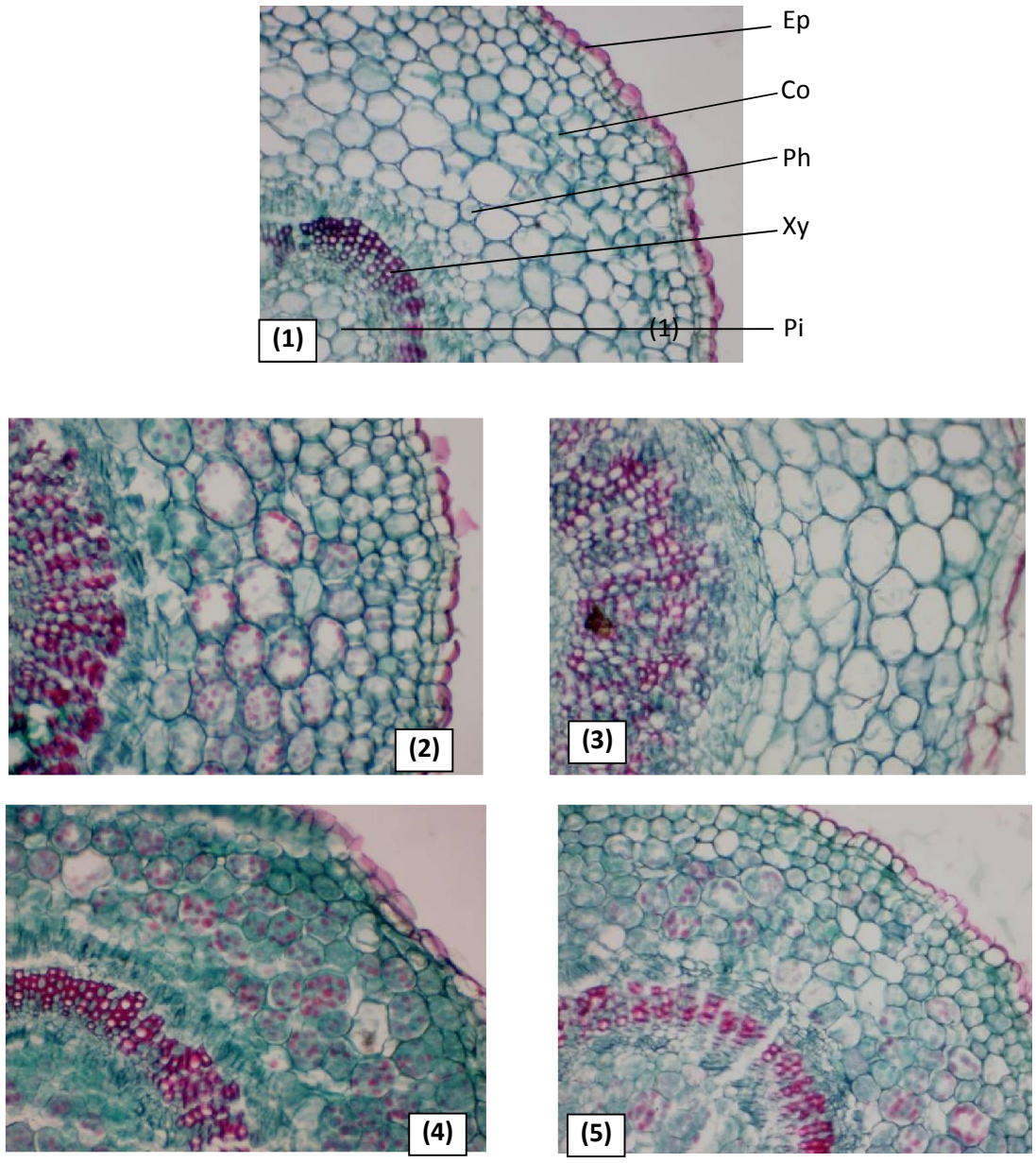

Fig. 4: Transverse sections through the middle part of stem of *Brunfelsia pauciflora* treated with different doses of gamma rays : (x =100)

1- Zero Gy 2- 5 Gy 3- 10 Gy 4- 20 Gy 5- 30 Gy
Ep: epidermis ; Co: cortex; Ph: phloem; Xy: xylem; Pi: pith.

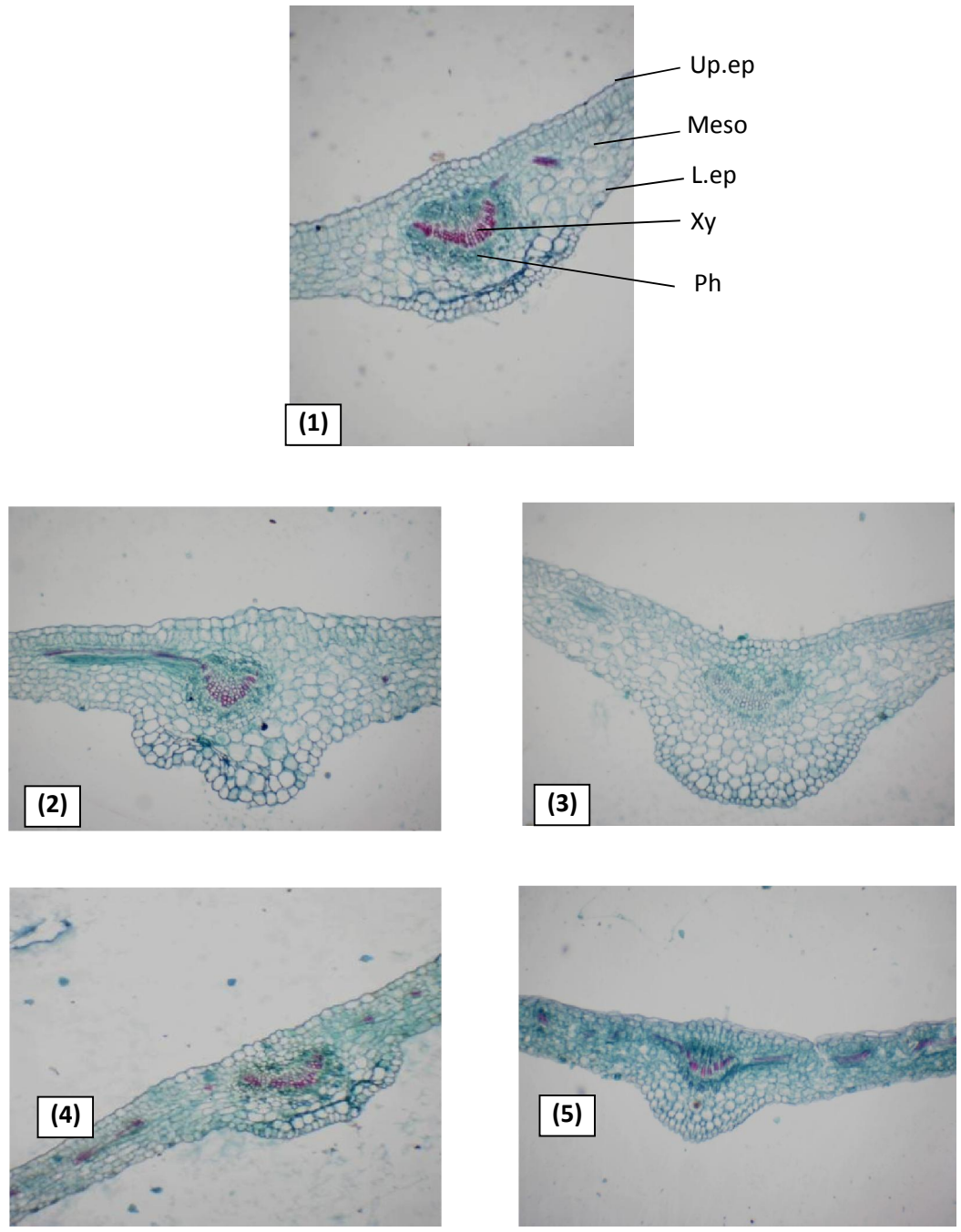

Fig. 5: Transverse sections of the leaf blade of *Brunfelsia pauciflora* plant treated with different doses of gamma rays: (x=100)

1- Zero Gy 2- 5 Gy 3- 10 Gy 4- 20 Gy 5- 30 Gy
Up: upper epidermis; Meso: mesophyl; L.ep: lower epidermis; Xy: xylem; Ph: phloem.

thickness can increase the exposed area to light to enhance photosynthesis especially palisade, also increasing in stem diameter shares to increase the morphological characters as a reflection of increasing the cortex, phloem, xylem and pith by increasing the thickness of these tissues xylem is the pathway for water and minerals, while phloem for feeding and this is responsible for this increase. These results agree with those of Sakr *et al*. (2013) who treated shoot tips of *Dracaena* with different doses of gamma irradiation (0, 5,10 and 15 Gy), and concluded that the application of 10 Gy induced highest value of the blade and mesophyll thickness and that might be attributed to the increase of palisade cells and number of chloroplasts per cell, acting as the primary site of photosynthesis in the leaves of those plantlets, this effects on increasing the efficiency of photosynthesis, as well the diameter of vascular bundle was related to other doses as, the application of 10 Gy exhibited increase for epidermis which shares for protection of stem.

Table 8: Measurements in micron of certain histological features in transverse sections through the leaf of *Brunfelsia pauciflora* plant treated with different doses of gamma rays.

Characterestics	Radiation dose				
	Zero Gy	5 Gy	10 Gy	20 Gy	30 Gy
Thick. of up.epi.	25.0	25.0	18.8	18.8	18.8
Thick. of lo. epi.	18.8	12.5	12.5	12.5	12.5
Thick. of mesophyll	125.0	175.0	225.0	112.5	100.0
Thick. of midribe	343.7	562.5	500.0	262.5	337.5
Thick. of phloem	43.7	37.5	25.0	18.7	37.5
Thick. of xylem	62.5	75.0	50.0	50.0	50.0

Effect of gamma irradiation on shootlets ISSR analysis of *in vitro Brunfelsia pauciflora* plants.

True-to-type clonal fidelity is one of the most important prerequisites in the micropropagation of any species. A major problem encountered with the *in vitro* culture is the presence of somaclonal variation amongst sub-clones of one parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissues or organs. In the present study, PCR based technique; ISSR was adopted for evaluating the variation between the mother plant and that obtained from explant treated with gamma irradiation at 0, 5, 10 or 20 Gy. Three ISSR primers were used in this study, the three ISSR primers produced good reproducible and scorable patterns and the amplification profiles were screened for the presence of polymorphisms among and within the mother plants and micropropagated gamma irradiated plants (Fig., 6).

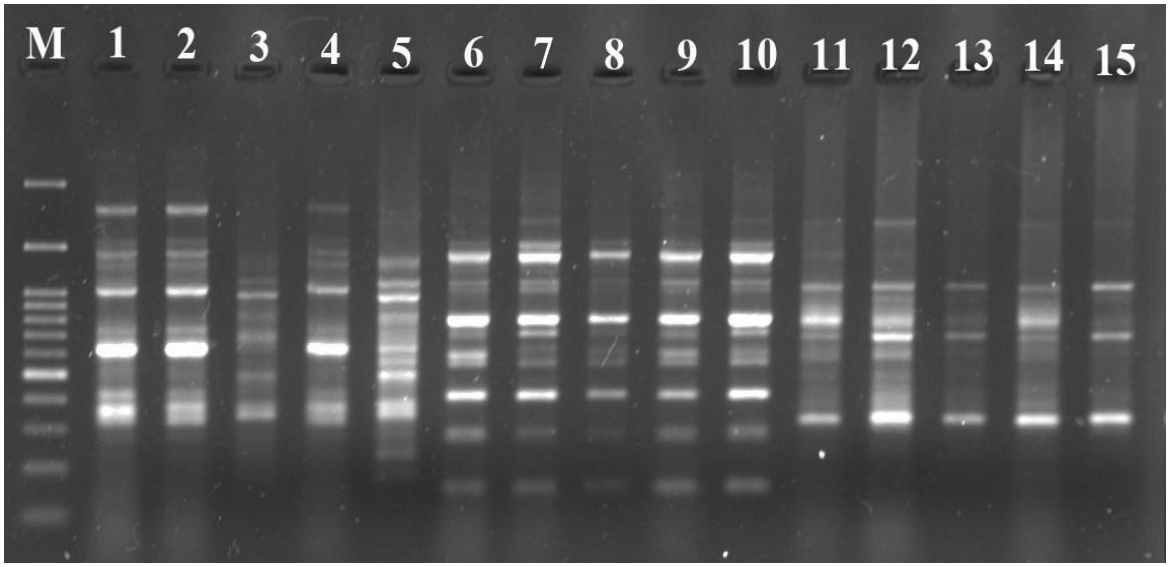

Fig 6: DNA amplification pattern obtained for ISSR primers:
HB-8: 1: mother plant, 2: zero Gy 3: 5 Gy 4: 10 Gy and 5: 20 Gy
HB-11: 6: mother plant, 7: zero Gy, 8: 5 Gy, 9: 10 Gy and 10: 20 Gy
HB-12: 11: mother plant, 12: zero Gy, 13: 5 Gy, 14: 10 Gy and 15: 20 Gy and M: 100 – 1500 pb DNA Ladder.

The three primers showed polymorphic bands obtained between the three primers. These results confirmed the true to tip between mother plant and the micropropagated plants only (sample number 1: control). However, between mother plant and irradiated plants at 5, 10 or 20 Gy enabled us to explore the DNA polymorphism in the collection of genotypes analyzed, and generate many polymorphic markers ensuring a good coverage of the genome.

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