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Cytogenetic changes and genomic DNA assay of Sudani and Masri Roselle varieties affected by different gamma irradiation doses

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Abstract: Roselle, *Hibiscus sabdariffa* L. is an important nutrimental, medicinal and pharmaceutical plant. Roselle is a tetraploid species and its flowers are cleistogamous. Therefore it is difficult to improve through conventional hybridization. Cytogenetic investigation and RAPD-PCR technique can identify the genotypes and be used as genetic markers. Seeds of Masri and Sudani Roselle varieties exposed to gamma rays doses zero, 40 and 80 Gray for the soaked seed category and zero, 160 and 320 Gray for the dry seed category were used in the present investigation. The treated seeds were sown for three summer seasons. Seeds of the last season were grown in Petri dishes for the determination of mitotic index and chromosomal abnormalities. All gamma doses reduced the mitotic activity. Different types of chromosomal abnormalities were occurred and increased with the gamma doses increasing. On the other hand, RAPD-PCR technique was used to detect the DNA profile changes affected by gamma doses. Eight out of fifteen random primers successfully amplified Roselle DNA fragments. The primers amplified different number of fragments, 30 monomorphic and 26 polymorphic amplicons. Sudani characterized by higher number of unique markers. The dose of 160 Gray was the more effective dose showing 3 positive and one negative marker. Primer OPD-18 exhibited both positive and negative markers and presented the highest number of unique markers. Finally, cytogenetic investigation and RAPD analysis proved that Sudani was more affected by gamma rays than Masri variety.

Key Words : Roselle, *Hibiscus sabdariffa,* gamma irradiation, mitotic index, chromosomal abnormalities, RAPD-PCR technique.

Introduction

Roselle or Karkadeh, *Hibiscus sabdariffa* L. (Family: Malvaceae) is an important plant grown in tropical regions of Africa, Asia and the Americas. It consumes for its seed, leaf, calyx and fiber production. Roselle is one of the most famous folk medicinal plants for various diseases treatment and microorganism growth limitation[1,2,3,4]. The red varieties of Roselle have antioxidant and cyclooxygenase inhibitory activity[5]. Also, Roselle inters in pharmaceutical and cosmetic industries[6]. Therefore, international trade of Roselle has increased steadily over the world market. Germany and the United States are large importers.

[7]Roselle is a tetraploid (2n=72 Chr.) species, thus its segregating populations need longer time for purification. Furthermore, [8] explained that Roselle flowers are cleistogamous. According to these facts, it is difficult to improve Roselle plant through conventional hybridization. On the other hand, [9,10,11] used irradiation

successfully for mutation in breeding of various plants. Whereas, [12,13]Mutagenesis has been improved many important traits such plant size, time of flowering and fruit color. [14] Irradiation is an adept mean for encouraging the expression of recessive genes and producing new genetic variations. However, cytogenetic studies are necessary to obtain information regarding the role of the genotypes response to a particular mutagen.

On the other hand, many complications of a phenotypic or biochemical based assay can be mitigated through direct identification of genotypes with DNA based assays[15]. Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) is a method amplifies random genomic DNA sequences using single, short arbitrary primers, and these can be used as genetic markers[10]. In addition, [16,17,18] reported that RAPD technique surveys numerous loci in the genome, so, it is particularly attractive for genetic distance analysis and similarity between closely related species.

The present work aims to evaluate the response of cytological changes and genomic DNA assay of Masri and Sudani Roselle varieties affected by different doses of gamma irradiation in dry and soaked seed categories.

Material and Methods

Materials

Seeds of each Masri and Sudani Roselle varieties were obtained from The Group of The Genetics and Breeding of Medicinal and aromatic Plants, Genetics and Cytology Department, National Research Centre (NRC), Egypt.

Seed irradiation with gamma rays and cultivation method

The seeds were divided into two categories; dry seed category where the air dry seeds were exposed to gamma radiation directly, and Soaked seed category where the seeds has been soaked in water for 10 hours before exposing to gamma rays. The seeds were exposed to gamma irradiation under Gamma Cobalt 60 Apparatus at the Nuclear Research Centre. The applied doses were 40 and 80 Gray for the soaked seed category, as well as 160 and 320 Gray for the dry seed category. Unexposed seeds were used as control. The doses rate of gamma rays was one Gray per 1.613 second. The irradiated and control seeds were sown in a new reclaimed sandy land (at Wadi Al-Natroun Village, Behira Governorate) using randomized complete block design for three successive summer seasons 2012-2014.

Investigation of mitotic index and chromosomal abnormality types

Some of the preserved seeds were prepared to grow in Petri dishes until seedling roots reached 1.5 – 3.0 cm in length, then the roots were cut and fixed in 3 absolute ethyl alcohol: 1 glycial acetic acid (V/V) for 24 hrs, Then saved in 70% ethyl alcohol and kept in refrigerator[19]. The mitotic index, percentage of total abnormalities and percentage of each type of chromosomal abnormalities were determined.

DNA extraction and RAPD analysis

The remained preserved seeds were grinded to a fine powder in liquid nitrogen. The genomic DNA was extracted using the Bio Basic Kit protocol according to the manufactory procedure (CANADA INC.). RAPD analysis was performed using fifteen 10-mer random primers to detect the polymorphism among the irradiated seeds and control (Table 1) produced from Operon Technologies (Metabion International AG). RAPD assay was performed as described by [20] with some modifications. PCR reaction was used in a final volume of 25 µl containing 12.5 of Master Mix (Bioteke), 2.5 µl of 5 µM for each primer, 50 ng of template DNA. PCR amplification was performed in PTC-100 PCR version 9.0 from M J Research-USA, Programmed for 95°C for 5 min (denaturation), 36 cycles of {94 °C for 1 min, 36 °C for 1 min (annealing), 72°C for 1min} and a final extension of 2 min at 72 °C. PCR products were analyzed using 1% agarose gel electrophoresis and visualized with ethidium bromide staining. Sizes of the fragments were estimated based on a DNA ladder of 100 bp (Fermentas, EU). Clear and distinct amplification products were scored for presence (1), absence (0) across the lines and the gel analysis applied by the programmer of (UVI gel version 12.4, 1999-2005,USA).

Table (1). List of the used primers and their nucleotide sequences in RAPD analysis.

No.	Primer	Sequence	No.	Primer	Sequence
1	OPA-16	5'-AGCCAGCGAA-3'	9	OPN-16	5'-AAGCGACCTG-3'
2	OPC-06	5'-GAACGGACTC-3'	10	OPN-19	5'-GTCCGTACTG-3'
3	OPC-16	5'-CACACTCCAG-3'	11	OPB-02	5'-TGATCCCTGG-3'
4	OPC-18	5'-TGAGTGGGTG-3'	12	OPD-02	5'-GGACCCAACC-3'
5	OPD-11	5'-AGCGCCATTG-3'	13	OPH-05	5'-AGTCGTCCCC-3'
6	OPD-18	5'-GAGAGCCAAC-3'	14	OPD-07	5'-TTGGCACGGG-3'
7	OPN-09	5'-TGCCGGCTTG-3'	15	OPG-10	5'-AGGGCCGTCT-3'
8	OPN-12	5'-CACAGACACC-3'			

Results and Discussion

Mitotic index

Mitotic index % (MI), abnormal cell mean and percentage of abnormality types for Masri and Sudani Roselle varieties affected by different gamma irradiation doses in soaked and dried seed categories were evaluated and shown in (Table 2). Cells number in mitosis and MI were significantly inhibited with increasing the irradiation doses in both Roselle varieties. Masri presented lower MI (71.22±2.22 and 50.74±0.57) than Sudani (79.79± 0.56 and 76.00± 0.23) affected by both gamma doses 40 and 80 Gray, respectively at the soaked seed category. Conversely with dry seed category, where Sudani had more MI inhibition (78.04±0.29 and 62.96±0.42) comparing with Masri (79.00 ± 0.62 and 74.97 ± 0.30) affected by 160 and 320 Gray, respectively.

However, abnormal cells mean was significantly increased with increasing the irradiation doses in both Roselle varieties. The highest abnormal cells mean was (3.90±0.33 and 10.95±0.16) stimulated by (80 Gray), the highest dose in the soaked seed category comparing for Masri and Sudani, respectively. Meanwhile, the highest abnormality mean in the dry seed category was (7.96±0.13 and 14.98±0.29) stimulated by (320 Gray), the highest dose in the dry seed category for the same respecting. Furthermore Sudani presented the higher abnormality mean than Masri variety in all gamma doses for both seed categories (Table 2).

The results of mitotic index (MI) proved that Masri showed more susceptibility to gamma rays at soaked seed category but lower susceptibility at dry seed category. Whereas, the result of abnormal cell mean proved that Sudani was more susceptible to gamma rays at both seed categories.

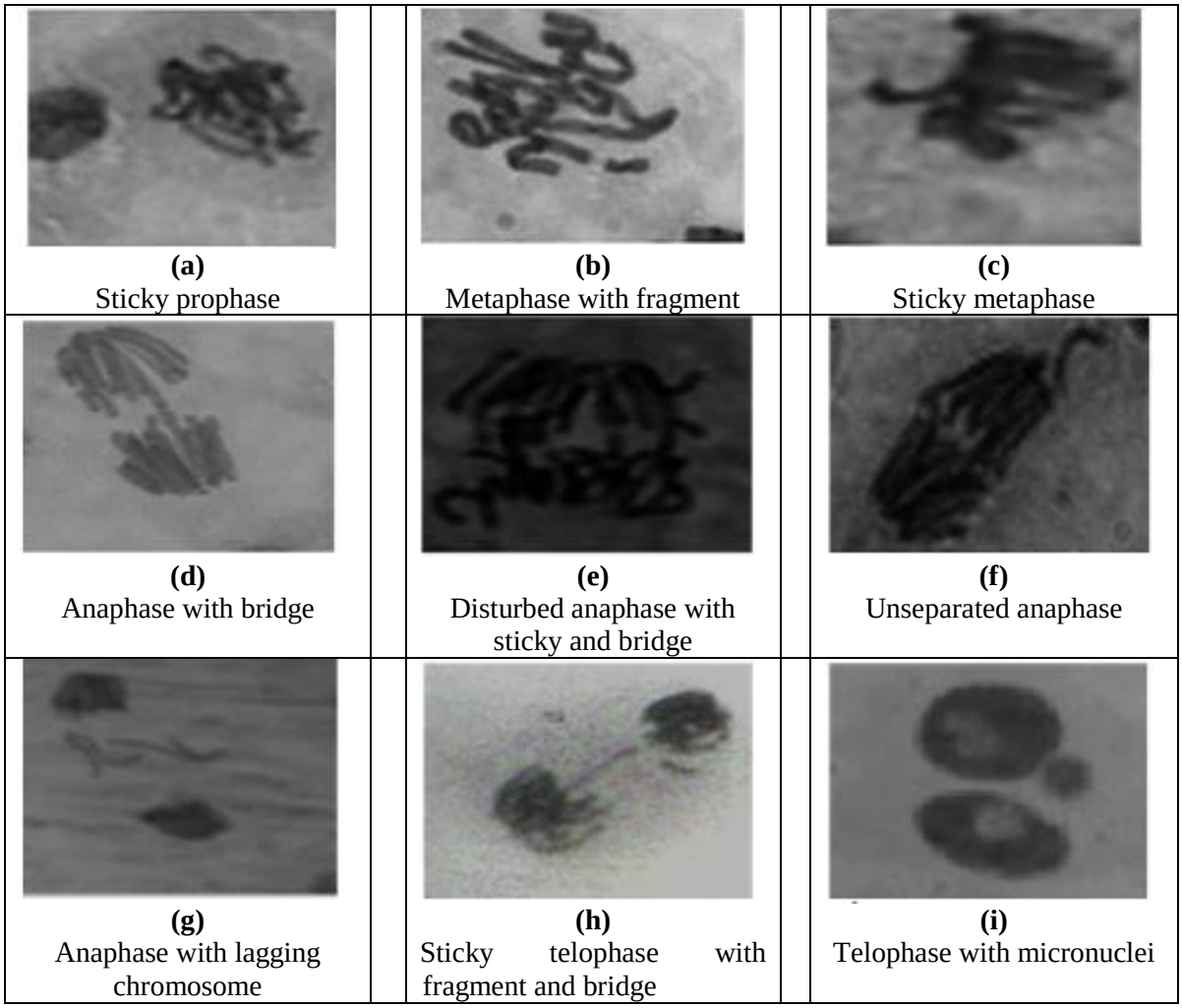
Many authors such as [21,22,23,24,25,26] reported that inhibition of mitotic activity is considered as a common effect induced by any mutagenesis stimulators such as radiations. [27] recorded that the decreasing ATP level and the pressure from the functioning of the energy production center causes inhibition in the DNA which reduces the mitotic activity. While, [28] stated that a blocking in the G2-phase of the cell cycle prevents the cell from entering mitosis.

Table (2). Percentage of mitotic index (MI), mean of abnormal cells and percentage of abnormality types for Sudani and Masri Roselle varieties exposed to different gamma irradiation doses in soaked and dried seed categories.

Category	Doses	Variety	No. of examined cells	No. of cells in mitosis	% of mitotic index MI	No. of abno-rmal cells	Mean of abno-rmal cells	% of each type of abnormality relative to the number of abnormal mitosis						
								Sticky meta-phase	Frag-ments	Bridg chrom-osome	Disturbed anaphase	Un separated anaphase	Lagging chrom-osome	Mico-nuclei
Soaked seed	Control	Masri	10853	9252	85.20** ± 2.29	114	1.23** + 0.38	25.43	25.43	11.40	14.91	2.57	14.91	5.26
		Sudani	10358	8553	82.57** ± 0.41	263	3.08** ± 0.14	19.77	28.52	10.27	17.11	3.04	17.11	3.80
	40 Gray	Masri	10183	7252	71.22** ± 2.22	177	2.44** + 0.13	15.82	33.33	09.60	13.55	5.08	14.69	7.93
		Sudani	10407	8106	79.79** ± 0.56	647	7.98** ± 0.34	21.62	30.00	8.33	18.33	1.00	16.67	5.00
	80 Gray	Masri	10309	5230	50.74** ± 0.57	204	3.90** ± 0.33	19.61	31.86	9.31	18.14	3.43	16.18	1.47
		Sudani	9988	7591	76.00** ± 0.23	831	10.95** ± 0.16	22.89	31.11	7.23	10.07	1.00	18.07	3.61
Dried seed	Control	Masri	10566	8954	84.74** ± 0.43	103	1.15** ± 0.04	9.71	31.07	12.62	15.53	7.77	18.45	4.85
		Sudani	10161	8443	83.09** ± 0.24	263	3.12** ± 0.13	20.91	36.12	11.41	13.31	5.70	9.51	3.04
	160 Gray	Masri	10236	8086	79.00** ± 0.62	407	5.04** ± 0.29	22.60	27.76	13.95	14.00	4.42	16.22	4.65
		Sudani	9890	7718	78.04** ± 0.29	686	8.89** ± 0.27	21.87	25.95	6.41	17.49	6.12	18.95	3.21
	320 Gray	Masri	10086	7561	74.97** ± 0.30	601	7.96** ± 0.13	21.63	29.45	10.85	14.98	3.33	15.81	3.33
		Sudani	10228	6440	62.96** ± 0.42	965	14.98** ± 0.29	23.63	25.90	8.29	19.48	4.46	14.30	3.94

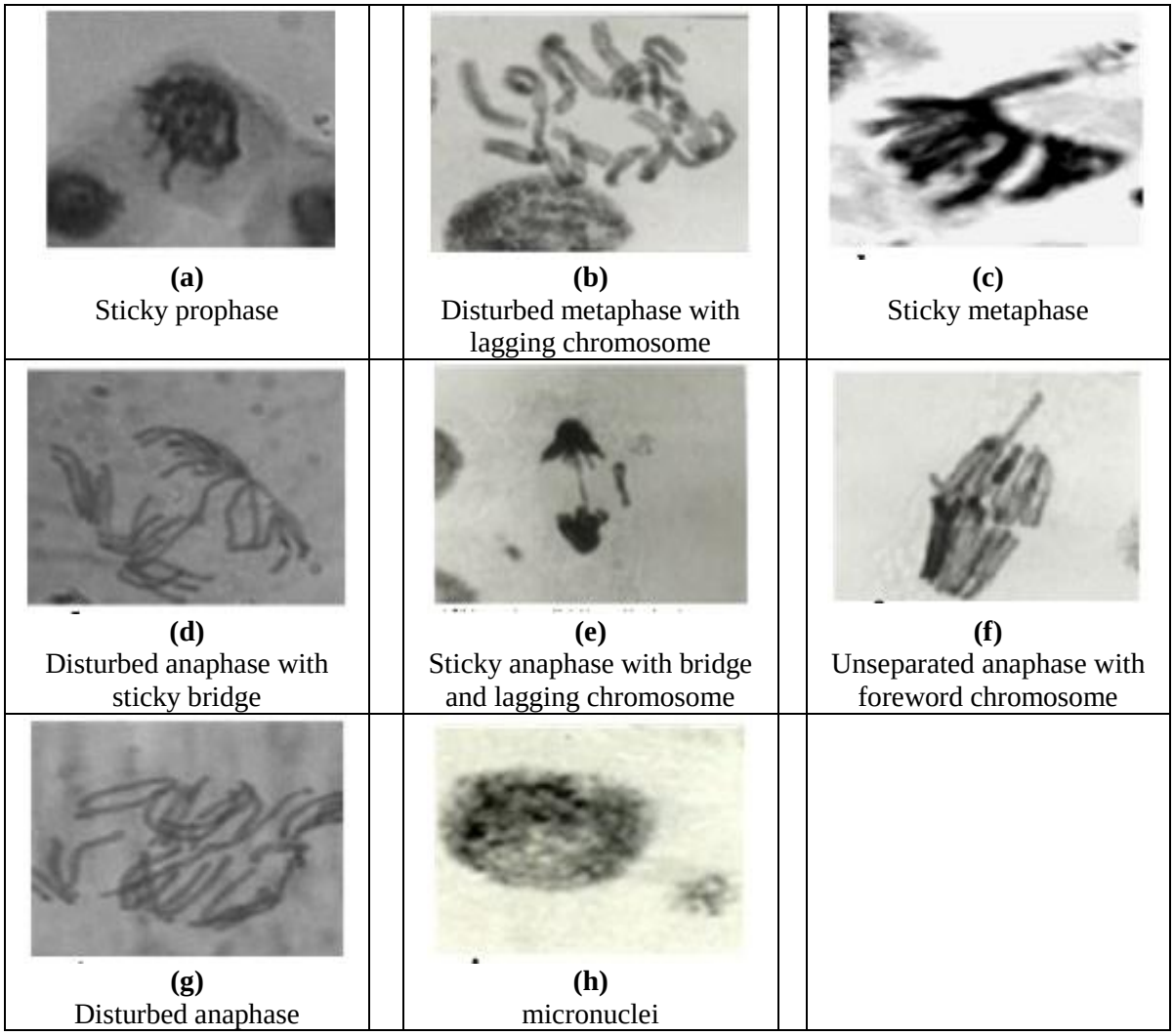
Chromosomal abnormality types

Chromosomal abnormalities or aberrations such as stickiness, fragments, bridges, disturbed chromosomes, unseparated anaphase, laggards and micronuclei were observed during the investigation as a result of the radiation impact (Figs. 1 & 2 and Table 2). Fragments followed by chromosomal stickiness were found to be the most common abnormalities, while unseparated anaphase down to micronuclei were the least aberrations in the different stages. The presence of stickiness in the chromosomes (Figs. $1_{(a,b,c,e\ \&\ h)}$ and $2_{(a,c,d\ \&\ e)}$) reflected highly harmful effect may be irreversible and lead to cell death[29]. Furthermore, [30] cleared that gamma rays affect the physiological properties of DNA and proteins, and form complexes with phosphate groups of nucleotides in the nucleic acids causing inhibition in protein synthesis.



(Fig. I). Abnormality types in mitotic cells of root-tip meristems in Sudani Roselle variety exposed to gamma irradiation.

Laggards chromosomes were also observed as a type of chromosomal abnormality (Figs. $1_{(g)}$ and $2_{(b\ and\ e)}$). [34]attributed Laggard chromosomes to irregular orientation of chromosomes. Meanwhile, [35]stated that Laggard chromosomes may be distributed randomly to either poles at anaphase I or II which result ultimately in euploidy. In fact, Laggards occurrence indicated the radiation effect (completely or partially) on the spindle apparatus. In addition to micronuclei type was appeared among the cytological aberrations (Figs. $1_{(i)}$ and $2_{(g)}$). Micronuclei may originate from chromosome lagging or fragment at anaphase[36]. [37] reported that micronuclei are true mutagenic aspects lead to loss the genetic material and regarded as a mutagenecity indicator.

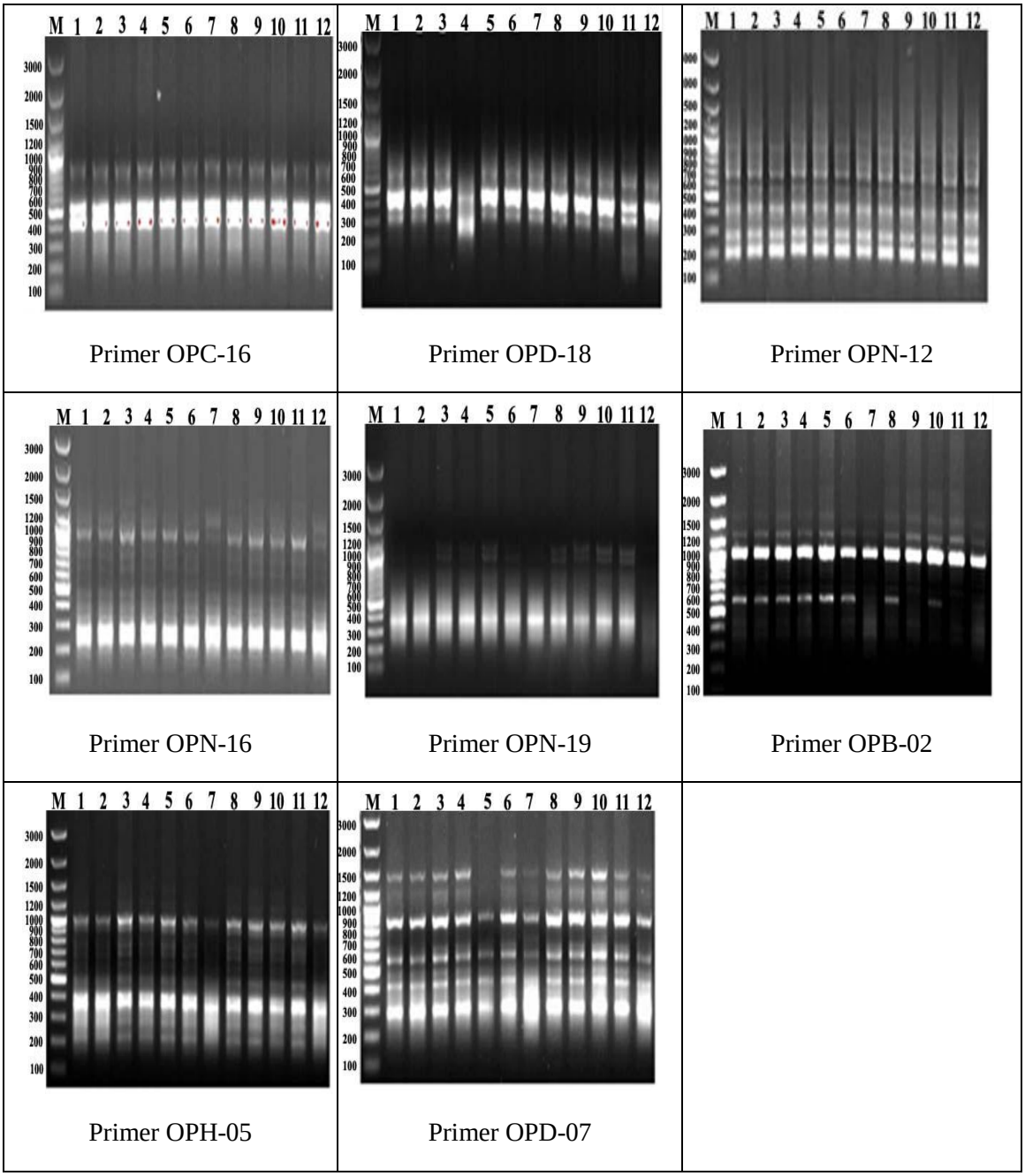


(Fig. 2). Abnormality types in mitotic cells of root-tip meristems in Masri Roselle variety exposed to gamma irradiation

RAPD banding profile and polymorphism

RAPD-PCR was used for detecting the DNA profile changes in both Roselle varieties affecting by the same doses of gamma rays (0, 40 and 80 Gray and 0, 160 and 320 Gray for the soaked and dry seed categories). Eight primers out of fifteen random 10-mer primers (OPC-16, OPD-18, OPN-12, OPN-16, OPN-19, OPB-02, OPH-05 and OPD-07) successfully amplified DNA fragments from Roselle DNA samples (Fig 3 and Table 3). The total number of amplified fragments differed among the used primers from four amplicons in the primer OPC-16 to ten amplicons in the primer OPD-07. The total number of DNA fragment (56 amplicons) amplified by all primers: 30 amplicons were monomorphic and 26 were polymorphic amplicons.

Primer OPN-12 amplified the highest number of monomorphic amplicons (6 amplicons), while primers OPD-18 and OPN-19 amplified the lowest number of monomorphic amplicons (2 amplicons). However, primers OPB-02 and OPD-07 amplified the highest number of polymorphic amplicons (6 amplicons), while primers OPC-16 and OPN-16 amplified only one polymorphic amplicon. Therefore, the primers expressed different levels of polymorphism ranged from 16.67 % in primer OPN-16 to 66.67% in primers OPD-18 and OPB-02 with an average level of polymorphism 44.69% per primer.



(Fig. 3). RAPD-PCR banding patterns of eight random 10-mer primers amplified DNA fragments for Masri and Sudani Roselle varieties affected by different doses of gamma irradiation in soaked and dry seed categories.

1, 2 and 3: Masri Roselle variety in soaked seed category, with Zero, 40 and 80 Gray gamma radiation doses, respectively. **4, 5 and 6**: Masri Roselle variety in dry seed category, with Zero, 160 and 320 Gray gamma radiation doses, respectively. **7, 8 and 9**: Sudani Roselle variety in soaked seed category, with Zero, 40 and 80 Gray gamma radiation doses, respectively. **10, 11 and 12**: Sudani Roselle variety in dry seed category, with Zero, 160 and 320 Gray gamma radiation doses, respectively.

Table (3). Number of amplicons, monomorphic, polymorphic and the percentage of polymorphism, as revealed by RAPD primers for Roselle plant.

NO	Primer	Total no. of Bands	Mono-morphic Bands	Poly-morphic Bands	Poly-morphism %	Band size
1	OPC-16	4	3	1	25.0	0.271-0.779
2	OPD-18	6	2	4	66.67	0.118-0.483
3	OPN-12	8	6	2	25.0	0.194-1.062
4	OPN-16	6	5	1	16.67	0.235-1.192
5	OPN-19	5	2	3	60.0	0.260-1.095
6	OPB-02	9	3	6	66.67	0.284-1.638
7	OPH-05	8	5	3	37.5	0.185-1.494
8	OPD-07	10	4	6	60.00	0.280-1.626
Total		56	30	26		
Average		7	3.75	3.25	44.69	0.239-1.100

RAPD analysis

Analysis of RAPD in Table (4) indicated negative and\or positive unique markers as response to different gamma doses treatment. Four positive and five negative unique markers were achieved for both Sudani and Masri varieties influenced by gamma irradiation. Sudani characterized by the highest number (6) of unique markers (3 positive and 3 negative), while Masri had (3) unique markers (1positive and 2 negative) proving that Sudani was more effected by gamma irradiation than Masri variety. On the other hand, 160 Gray was more effective dose showing 3 positive and one negative unique markers) ,while the dose of 320 Gray presented just one negative unique marker in the dry seed category of Sudani variety. Whereas, the doses of 40 and 80 Gray in soaked seed category did not exhibit any unique marker for both varieties.

However, the primer OPN-19 exhibited positive unique marker, while OPB-2 and OPH-5 primers exhibited negative unique markers, but primers OPD-7 and OPD 18 exhibited both positive and negative unique markers. The size of these unique markers ranged from 118 to 1626 bp. On the other hand the primer OPD-18 presented the highest number of unique markers (3) followed by OPB-02 and OPD-07, each presented 2 unique markers, while OPN-19 and OPH-05 produced just one unique marker.

Table (4). Positive and Negative unique RAPD markers generated from radiation treatments.

Variety	Category	Gamma doses	Positive unique marker		Negative unique marker		Grand total
			primer	Size/bp*	primer	Size/bp*	
Masri	Soaked Seed	Zero	---	---	---		zero
		40	---	---	---	---	
		80	---	---	---	---	
	Dry Seed	Zero	---	---	OPD18	483	3
		160	OPN-19	260	OPD-07	1626	
		320	---	---	---	---	
Sudani	Soaked seed	Zero	OPD-07	661	---	---	1
		40	---	---	---	---	
		80	---	---	---	---	
	Dry seed	Zero	---	---	OPB-02	607	5
			---	---	OPB-02	372	
		160	OPD-18	161	---	---	
			OPD-18	118	---	---	
		320	---	---	OPH-05	1201	
Total							9

bp*: base pear.

These changes in the RAPD–DNA profiles might be due to the effect of gamma rays on the structural rearrangements in DNA. [38] stated that appearance of new bands is usually resulting from different DNA stricture changes such as breaks, transposition and deletion. The results agreed with the finding of [10] who notes that the main changes in *Hibiscus Sabdariffa* as a response to gamma rays were changing in the RAPD–DNA profiles as appearance or disappearance of different bands with variation in their size base pare. Also [39] on potato, [40]

banana and Hamideldin and [41]on Mustard plant studied the effect of gamma irradiation using RAPD-DNA analysis and observed changes in the DNA bands.

Conclusion

The results of mitotic index, chromosomal abnormalities as well as RAPD analysis proved that Sudani was more affected by gamma irradiation and characterized by the highest number of unique markers. Induction of the chromosomal abnormalities pointed to the mutagenic potential of the applied doses of gamma radiation. The dose of 160 Gray was the highest effective dose presented three positive and one negative unique marker. The primer OPD-18 exhibited both positive and negative unique markers and presented the highest number of unique markers.

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