In vitro genetic improvement of *Jatropha curcas* L using gamma ray to induce salinity tolerance.

Elsayh S. A. A. [1]; Sarhan, A. Z.[2]; Amira Sh. Soliman[3]; Rayan A. O. and El-Shishtawy H.[4]

[1]Horticulture Research Institute, Agriculture Research Center, Giza, Egypt.
[2]Ornamental Horticulture Department, Faculty of Agriculture, Cairo University, Giza, Egypt.
[3]Natural Resources Department, Institute of African Research and Studies, Cairo University, Giza, Egypt.
[4]Secretariat of the National biosafety Committee Agricultural Genetic Engineering Research Institute (AGERI), Agriculture Research Center (ARC), Giza, Egypt.
Corresponding author: sayedelsayh@yahoo.com

Abstract: This work aimed to induce mutation in *Jatropha curcas* L.in order to increase the salinity tolerance of it. The reaction was performed by exposing healthy callus to gamma ray at 0.0, 5.0, 10.0, 15.0, 20.0 and 25.0 Kr, and then the regenerated shoots were treated with sea salt concentrations at 0.0, 25.0, 50.0, 90.0 and 100.0% during two successive seasons of 2013 and 2014. Results showed that treated shoots resulted from both 20 and 25 Kr doses of gamma ray had a high survival percentages (93.3 % and 80.0% respectively) when treated with 90% of sea salt. In addition, shoots treated with gamma ray 20 and 25 kr gave a high survival percentage when treated with sea salt 100% (86.7 % and 66.7%, respectively). Significant differences were found between control and plants treated with sea salt in shoot number. Plants treated with 20 and 25 Kr doses and 90% concentration of sea salt gave 2.8 and 2.4 shoot/explants respectively. Treated plants with gamma ray 20 Kr dose and 90% sea salt showed shoot length of (3.6) compared to control treatment (3.4). Leave number gave the highest value in treatment 20 Kr and 90% sea salt (5.6). RAPD analysis had successfully generated reproducible polymorphic products. Five oligodecamers of operon kits (OPA-11, OPA-12, OPB-06, OPB-07, and OPD-15. A total of 66 amplicons from five primers were produced in PCR amplification reaction and the number of amplified DNA fragment by each primer ranged from 10 to 17 fragments. OPD-14 amplified the highest number of fragments (17 bands) while, OPB-07 produced the lowest number of bands (10).Finally, irradiation with 20 and 25 Kr dose and treatment with 90% and 100% sea salt resulted in high percentage of survival.
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Key words: *Jatropha curcas* L., Sea salt concentration, Induced mutagenesis, RAPD-PCR, mutation breeding, bio-diesel oil.

1.Introduction

Jatropha curcas L. belongs to family Euphorbiaceae (Heller, 1996), that is one of the important oil seed crops and a potential source of vegetable oil as a replacement for petroleum and in particular, the production of biodiesel (King *et al*., 2009). It is native to tropical America (Openshaw, 2000 and Tatikonda *et al*., 2009) and has been introduced into many parts of the tropics and subtropics (Basha and Sujatha, 2007). This plant is grown well in marginal soils in semiarid tropics (Sunil *et al*., 2008). Physical and chemical mutagen studies on experimental mutagenesis in this plant are very limited besides, mutation breeding studies in *J. curcas* carried out in Thailand using fast neutrons and isolated dwarf or early flowering mutants from the M3 generation, but the potential productivity of these variants under intensive cultivation conditions was not proved (Sakaguchi and Somabhi, 1987). India has led to induction of cotyledonary variables in *J. curcas* (Pandey and Datta, 1995). The studies of induced mutation in *Jatropha curcas* were performed by exposing the healthy and dry seeds to gamma ray viz., 5, 10, 15, 20 and 25 Kr doses. Songsri *et al*.)2011) studied the effects of different levels of gamma radiation on germination percentage, survival percentage and growth of five physic nut genotypes. The seeds were treated with six levels of gamma radiation (0, 200, 400, 600, 800 and 1000 Gray. Pandey and Datta, (1995) studied sensitivity to γ radiation with a view to mutation breeding in this potential oil seed crop, the effects of various γ ray doses on cotyledons were studied. DNA is the starting point of all molecular evidences related to any improvement technique in plants and contains several responsive genes in their genome. A large number of genes with potential roles in salt stress responses have been identified using genetic screening and genome wide expression studies (Yeh *et al.*, 2012).

Plants tolerate such stresses by modulating multiple genes and by coordinating the expression of genes in different pathways (Vinocur and Altman, 2005). Nowadays, molecular marker, have become an important tool to evaluate genetic diversity in plants

more efficiently. Among various molecular markers, the random amplified polymorphism DNA (RAPD) is most widely used. It does not require prior genomic information and are simpler and more economical than other DNA marker techniques.

Therefore, this study aims to investigate the effect of different gamma ray and sea salt concentrations on increasing the salinity resistance of *Jatropha curcas L.* plants under salt stress.

2-Material and Methods:

2.1. Materials:

2.1.1. Sample:

Leaves of *Jatropha curcas* L was used as an explant

2.1.2.Rooting media

Developed shoots were transferred for root induction on ¾ MS medium supplemented with IBA at 0.0, 1.0, 2.0, 3.0 and 5.0 mg/l. For *ex vitro* establishment, well rooted plantlets were rinsed thoroughly with sterile water to remove residual nutrient from the plant body.

2.1.3. Primers

RAPD-PCR reactions were conducted twenty-eight random 8-mer primers. Their codes and sequences are shown in (Table 1).

Table (1): Sequence of the five arbitrary primers assayed in RAPD-PCR

No.	Primer	Sequence (5´-3´)
1	OPA-11	TCTGTGCTGG
2	OPA-12	AGCCAGCGAA
3	OPB-06	CATCCCCCTG
4	OPB-07	TGCTCTGCCC
5	OPD-14	TTCCCCCGCT

2.2.Methods:

2.2.1- Sterilization of the explants

Leaves of *Jatropha curcas* L which was used as an explant were excised from 4 months old plant and were surface sterilized by cleaning thoroughly under running tap water for 20 minutes and washed with commercial detergent followed by running tap water and rinse with distilled water. The explants were then exposed to three concentrations of surface sterilization by commercial (Clorox, 5.25% w/v sodium hypochlorite, NaOCl). The first one by 10% Clorox for 20 min followed by thoroughly washing with sterilized water for one time. The second one by 15% Clorox for 20 min. and then washing the explants with sterilized distilled water and the three one by 20% Clorox for 20 min and then washing the explants with sterilized distilled water for three times all surface sterilized solutions contained one drop of tween 20 per 100 ml solution (Tunawidjaja *et al.,* 1998).

2.2.2. Culture condition:

Margins of expanded leaves (10-12 mm long) were removed and the remaining part was cut transversely to the midrib into two portions. Then explants (leaves) were dissected into small pieces (0.3 cm). The explants were cultured on MS (Murashige and Skoog, 1962) basal medium containing 3.0% sucrose (W/V), 0.6% agar, 2,4-D at (1.0, 2.0 and 4.0 mg/l) and PH was adjusted to 5.8 by NaOH and HCL, the media were autoclaved at 121˚C for 20 minutes before using.

2.2.3. Callus induction mesearment:

For callus induction cultures were incubated in a culture room at 25±1˚C centigrade under 16/8 hr photoperiod by cool white fluorescent tubes.

The survival percentage and callus formation percentage were measured after 4 weeks of culturing.

2.2.4. Gamma irradiation treatment:

Callus induction was used in this experiment. The callus were exposed to different concentrations of gamma ray 0.0, 5.0, 10.0, 15.0, 20.0 and 25.0 Kr and then cultured on MS medium supplemented with BA at 3.0 mg/l + IAA at 3.0 mg/l for differentiation. Each treatment contained of 3 jars, the experiment re-culture three times every 4 weeks, the data recorded as survival percentage, Embryo percentage, average of embryo number and average of embryo length.

2.2.4. Salinity treatment:

Shoots from previous experiment which was applied by gamma ray were treated with different concentrations of sea salts and then shoots from each treatment were cultured on the best medium of multiplication which consist of MS medium supplemented with BA at 3.0 + IBA at 0.8 mg/l. In this experiment we used different levels of sea salt at 0.0, 25.0, 50.0, 90.0 and 100.0% with the planted which treated by 0.0, 10.0, 15.0, 20.0 and 25.0 Kr of gamma ray.

The following data was recorded as survival percentage; shoot number, shoot length and leaves number after 4 weeks.

2.2.5. Acclimatization

The regenerated plantlets were then transferred to plastic cups containing sterile soil, sand, compost (1:1:1) , covered with polythene and maintained in tissue culture conditions. The well-developed plantlets were transferred to bigger earthen pot, kept in greenhouse and finally transferred to the field.

2.2.6. Randomly amplified polymorphic DNA (RAPD-PCR)

- Genomic DNA extraction

Total genomic DNA was extracted from young and fresh leaves of *Jatropha curcas* L. tested genotypes then bulked DNA extraction was performed using DNA easy Plant Mini Kit (QIAGEN). DNA concentration was quantified spectrophotometrically (Gene Quant, Amersham Pharmacia Biotech) and

DNA quality was examined by electrophoresis in 0.8% agarose.

- DNA amplification

RAPD-PCR reactions were conducted twenty-eight random 8-mer primers. Their codes and sequences are shown in (Table 1). Amplification reactions were performed in 30 µl total volumes according to Williams *et al*. (1990). The amplification procedures were carried out in a DNA thermocycler (MWG-BIO TECH Primuse) programmed as follows: initial pre-denaturation step at 94°C for 5 min, followed by 45 cycles of 1 min at 94°C, 90 sec 36°C and 2 min at 72°C followed by 7 min incubation period at 72°C. The amplification products were stored at 4°C before analysis.

- DNA electrophoresis

The amplified products were separated in 1.2% agarose gel electrophoresis using 1x Tris–Boric acid–EDTA buffer, (5 µl Ethidium bromide was added to the melted gel after the temperature became 55^{o}C).

2.2.7. Statistical analysis:

Experiments were designed in randomized complete design. Each treatment was performed in six jars containing five explants and each experiment was replicated three times. Data were subjected to analysis of variance by MSTAT (1990) Computer statistical analysis program. Duncan's multiple range test at 5% level of significance (P=0.05) was used for means comparisons according to (Snedecor and Cochran, 1980).

RAPD gels were processed using quantity one software (Bio-Rad) which identifies DNA fragments using an optimized set of parameters (as reported in quantity one user guide for version 4.2 Windows Bio-Rad Laboratories) which was manually adjusted by visual inspection.

3. Results and Discussion:

3.1.Data recovered due to Salinity and Mutation gamma ray treatments:

Table (2) shows the effect of gamma ray on the growth of callus *Jatropha curcas* L. Differences in values of irradiated callus and sea salt treatments at different levels (0.00, 25%, 50%, 90% and 100%) was highly significant at 0.05% level. In case of survival percentage, data in table (2) and fig (1) showed significant differences among control results.

Data showed that irradiation with 10 Kr dose and treatment with 25%, 50%, 90% and 100% sea salt concentrations resulted in survival percentage of 70.3%, 66.6%, 33.3% and 22.2%, respectively compared to control 88.8%. Also, gamma ray dose 15 Kr and treatment with 25%, 50%, 90% and 100% sea salt resulted in survival percentage was 66.6%, 44.4%, 22.2% and 11.1%, respectively compared to control 98.0%.

Also gamma radiation with 20 Kr dose and treatment with 90% and 100% sea salt resulted in survival percentage of 93.3 % and 86.7%, respectively compared to control 100%. On the other hand, irradiation with 25 Kr dose and treatment with 90% and 100% sea salt resulted in survival percentage of 80.0 % and 66.7%, respectively compared to control 90%. It can be concluded that the radiation induced mutagenesis in combination with *in vitro* culture has proved effective in the induction of novel genetic variation, selection and multiplication of the mutant clones aimed at crop improvement (Biswas *et al*., 2002; Zhu *et al.,* 2004; Patade and Suprasanna, 2008). In addition, higher doses of radiation cause chromosomal damage in plant meristematic cells, deceleration of the cell cycle, and delay of mitosis, which significantly affect overall plant regeneration and development. While an increase in radiation doses boosts mutation frequency, it also increases damage to the plant (Sharabash, 2001; Alikamanoğlu, 2002; Hewawasam *et al.,* 2004; Gulsen *et al.,* 2007; Toker *et al.,* 2007).

Data also showed significant differences among control and plant treated with sea salt in number of shoots in 20 and 25 Kr dose and 90% sea salt were 2.8 % and 2.4%, respectively decreased number of shoots compared to control 3.0% and 2.7%, respectively. While, all treatments were 2.9%, 2.9%, 2.6% and 2.3%, 2.2% and 1.9%, respectively in 25%, 50%, and 100%, sea salt respectively. This behavior may be attributed where in salt stress cause decline in callus growth and survival in response to salt stress and nutritional imbalance resulting from interference by Na+, which increasing amounts of Na+ destabilize osmotic potential, creating a highly toxic environment to plant cells, even with the aid of defense mechanism of antioxidant enzymes, and leaving callus slimy or dead **[**Saif *et al.,* 2001 and Atak *et al.,* 2004]. Moreover, increasing salt concentration results lower percent tissue water content and membrane damage to cells. This effect presumably arises from dehydration of cells through low water potential or nutritional imbalance because of interference of salt ions with essential nutrients [Babaoğlu *et al.,* 2004]. It is now well documented that, reactive oxygen species-ROS production is increased under saline conditions (Hasegawa *et al.,* 2000) and their higher concentrations in the absence of any protective mechanism seriously disrupt normal metabolism of plants through oxidation of membrane lipids, proteins and nucleic acids (Noctor and Foyer, 1998).

Treated plants 20 Kr dose and 90% sea salt showed decreased shoot length 3.6% compared to control 3.4%, while other treatments; 3.2%, 3.9%, and 3.4%, respectively in 25%, 50% and 100% sea salt respectively. Regarding the number of leaves, results

in table (2) and fig (2) indicated that the 20 and 25 Krad gamma rays and treated 90% and 100% induced significant enhancement on number of leaves 5.6% and 3.9%, respectively. Compared to control 3.8% and 4.00% and the other treatments resulted in the lowest number of leaves as compared to control 3.8%, 4.8% and 3.3%, 4.00% in 25%, 50% sea salt, respectively. These results were in harmony with Patade and Suprasanna (2009), Yaycili and Alikamanoğlu (2012) and Nikama *et al.* (2015).

Recommendation:

Gamma radiation with 20 Kr dose and treatment with 90% and 100% sea salt resulted in survival percentage of 93.3 % and 86.7%, respectively, compared to control 100%. On the other hand, irradiation with 25 Kr dose and treatment with 90% and 100% sea salt resulted in survival percentage of 80.0 % and 66.7%, respectively compared to control 90%.

Table (2). Effect of different gamma ray and sea salt on the Survival percentage, number shoots, shoots length and number leaf of *Jatropha curcas* L. during 2013 and 2014

Gamma ray	Sea salt concentrations	Survival percentage	Number Shoots	Shoots length	number Leaf
Kr 10	Control	88.8 [a]	3.2 [b]	3.1 [a]	3.3 [a]
	25%	70.3 [b]	6.3 [a]	2.4 [a]	3.6 [a]
	50%	66.6 [b]	2.0 [bc]	2.8 [a]	3.3 [a]
	90%	33.3 [c]	1.0 [c]	2.4 [a]	3.0 [a]
	100%	22.2 [c]	0.7 [c]	2.6 [a]	3.0 [a]
LSD (0.05)		**9.10**	**1.42**	**1.68**	**1.62**
Kr 15	Control	98.0 [a]	5.3 [a]	3.63 [a]	3.57 [a]
	25%	66.6 [b]	2.0 [b]	2.50 [a]	3.30 [a]
	50%	44.4 [c]	1.3 [b]	3.20 [a]	3.00 [a]
	90%	22.2 [d]	1.3 [b]	3.10 [a]	3.60 [a]
	100%	11.1 [d]	0.3 [b]	2.40 [a]	3.00 [a]
LSD (0.05)		**8.18**	**1.93**	**1.53**	**1.52**
Kr 20	Control	100 [a]	3.0 [a]	3.4 [a]	3.8 [a]
	25%	96.6 [ab]	2.9 [a]	3.2 [a]	3.8 [a]
	50%	77.7 [c]	2.9 [a]	3.9 [a]	4.8 [a]
	90%	**93.3 [ab]**	2.8 [a]	3.6 [a]	5.6 [a]
	100%	86.7 [bc]	2.6 [a]	3.4 [a]	5.0 [a]
LSD (0.05)		**7.09**	**1.48**	**1.48**	**1.48**
Kr 25	Control	90 [a]	2.7 [a]	3.0 [a]	4.0 [a]
	25%	77.7 [bc]	2.3 [a]	2.4 [a]	3.3 [a]
	50%	55.5 [d]	2.2 [a]	3.2 [a]	4.0 [a]
	90%	**80.0 [ab]**	2.4 [a]	2.6 [a]	3.8 [a]
	100%	66.7 [cd]	1.9 [a]	2.8 [a]	3.9 [a]
LSD (0.05)	**8.18**	**1.48**		**1.48**	**1.48**

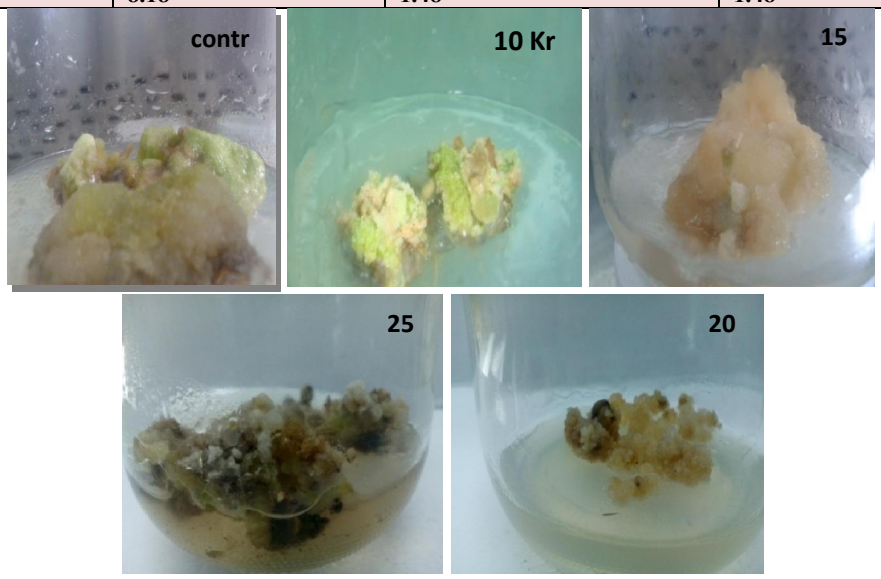

Fig. (1): Effect of different doses of gamma ray on callus percentage of *Jatropha* curcas

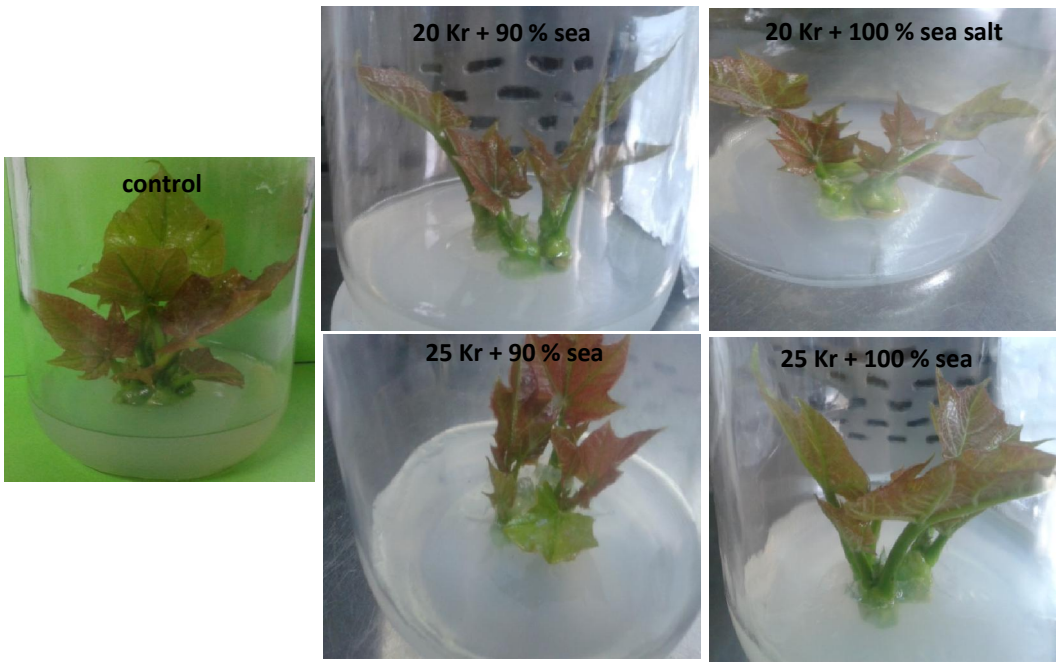

Fig. (2): Effect of gamma ray on plant treatment by (0.00, 25%, 50%, 90% and 100%) sea salt

Fig. (3): The best rooting percentage at 3.0 mg/l IBA and acclimatization stages of jatropha.

3.2.RAPD-PCR molecular analysis.

Data of the amplified fragments using twenty-eight random 8-mer primers for the five *Jatropha curcas* L. revealed success in amplifying DNA fragments. Polymorphism levels differed from one primer to the other. The number of total amplified fragments (TAF) and polymorphic bands (PB) for each primer, amplified fragments (AF) and specific markers (SM) for each jatropha using the twenty-eight random 8-mer primers are shown in (Table 3). Only five primers (OPA-11, OPA-12, OPB-06, OPB-07, and OPD-15) were used to generate RAPD profiles from *Jatropha curcas* L. Genomic DNA fragment were successfully amplified from *Jatropha curcas* L by using all of the five primers used in this experiment (Fig. 4). The characteristics of fragment primers summarized in (Table 3) and the following are the amplification results of the *Jatropha curcas* obtained by the examined primers.

A total of 66 amplicons from five primers were produced in PCR amplification reaction, and the number of amplified DNA fragment by each primer ranged from 10 to 17 fragments. OPD-14 amplified the highest number of fragments (17 bands) while, OPB-07 produced the lowest number of bands (10). The size of these fragments ranged from 150.684 to 3342.091 bp. All the used primers produced polymorphic bands (Table 3). The number of polymorphic was 27 Primer OPB-06 revealed the highest number of polymorphic bands (10) while, the lowest number polymorphic band (2) was detected by OPB-07. The number of Monomorphic bands was (17) Primer OPB-07 highest number of Monomorphic bands (7), while, the lowest number (1) was detected by OPA-12 and OPB-06. Therefore, the percent of polymorphism revealed by the different primers ranged from 20.00%% to 66.66% detecting genetic variation, similarity coefficients varied from 100 to 58

across all the samples (Table 4). In the dendrogram, the samples diverged into three groups A, B, C. Group A contained 3, 5, while group B included 3, 4, 5 and 1, 2 were in Group C (Fig. 4). These results are in harmony with Yaycili and Alikamanoğlu 2012, Bhumi *et al.*, 2013 and Dhillon *et al.*, 2014).

Table (3): Polymorphism and its percentage as detected by RAPD marker

Primer name	Total number of band	Monomorphic bands	Polymorphic (without Unique)	Polymorphism (%)
OPA-11	11	5	5	45.45%
OPA-12	12	1	8	66.66%
OPB-06	16	1	10	62.25%
OPB-07	10	7	2	20.00%
OPD-14	17	3	7	41.17%
Total	66	17	27	
Average	13.3	3.4	5.4	

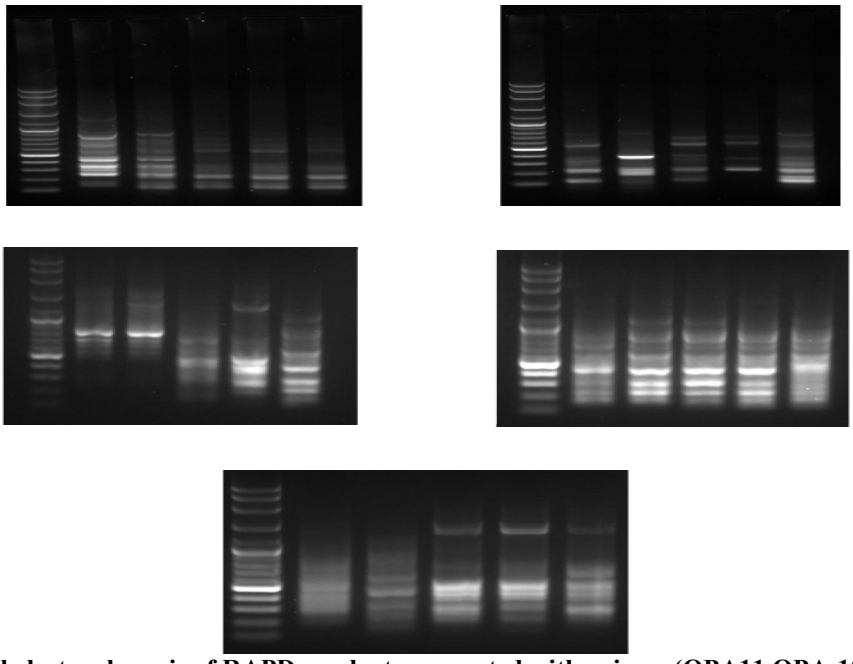

Fig. 4. Agarose-gel electrophoresis of RAPD products generated with primer (OPA11,OPA-12, OPB-06, OPB-07 and OPD-15 in four selected *Jatropa curcas* and control.

Genetic similarity based on RAPD markers:

Table (4): Genetic similarity as detected RAPD

Gamma ray 0.00	Control 100	S 1	S 2	S 3	S 4
10 Kr	72	100			
15Kr	67	64	100		
20Kr	67	58	79	100	
25Kr	66	58	80	78	100

Results of similarity index among the five jatropha based on RAPD-PCR with the five primers using UPGMA computer analysis is shown in **(Table 4)**. The highest similarity value recorded was (80 %), which was observed between 2 and 4, while the lowest similarity recorded value was (58) between 1 and each of 3, 4. A dendrogram for the genetic relationships among the five gatropha across the five primers results was carried out and is shown in **(**Fig. 5).

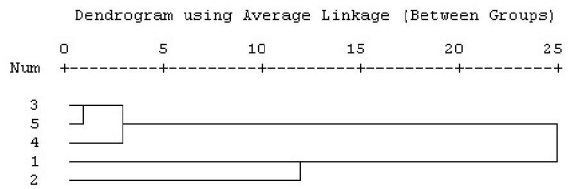

Fig. (5): Dendrogram of correlation similarity using average linkage (Between Groups) among the different *Jatropha curcas* based on RAPD results.

Conclusion:

Irradiation *Jatropha curcas* with 20 and 25 Kr dose when it grow in 90% and 100% sea salt is recommended for increasing the tolerance and resistances salinity of *J. curcas,* as well as, enhancing height growth and height quality crop, and that led to increase horizontal expansion in the production of bio-fuel plants to reduce the use of food crops in the new area.

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