Molecular Diversity Analysis of Two Irradiated Potato Varieties *in vitro* Revealed by Random Amplified Polymorphic DNA

Ayman El-Fiki[1]*, Zakia Adam[2], Thoria Mohamed[2], Shaimaa Sobieh[2] and Amal Salah[1]

[1]National Centre for Radiation Research and Technology, Nasr City, Cairo, Egypt.
aymana.elfiki@eaea.org.eg, adly4000@yahoo.com

[2]Ain Shams University, Faculty of Women for Arts, Science and Education, Cairo, Egypt.
zakiaadam123@gmail.com, thoria.rashad@hotmail.com, ssobieh@yahoo.com

*Corresponding author: aymana.elfiki@eaea.org.eg

Abstract

Potato buds cvs. Spunta and Valor were cultured *in vitro* on MS solid medium supplemented with 0.2 mg $^{-1}$ BAP. The resulting plantlets were irradiated with gamma radiation doses 10, 20, 30 and 40Gy. Irradiated single node pieces were transferred onto fresh MS with BAP. Plantlets survival percentage calculated after eight weeks, gamma radiation caused decrease in the survival percentage of micropropagated buds in both Spunta and Valor cultivars ranging from 91.4 % to 28.5% in cv. Spunta and 93.3% to 30.47% in cv. Valor, comparing with the values of the two non-irradiated cultivares 95.2% and 96.2% respectively. Microtubers produced from irradiated plantlets were decreased with increasing gamma radiation doses with changes in size and numbers. The estimated proline content in irradiated plantlets was increased with increasing gamma radiation dose. The genomic DNA of non-irradiated (control) in two cultivars and eight radiation treatments was amplified with 10 RAPD primers that generated 54 polymorphic bands. The highest number of genetic similarity was 0.9672 showed between irradiated plantlets with dose 20 and 30Gy in cv. Valor. However, the highest genetic distance was 0.3995 observed between irradiated plantlets with dose 20Gy in cv. Valor and 30Gy in cv. Spunta. The dendrogram generated by cluster analysis distinguished the irradiated plantlets genetically.

Keywords: *in vitro*, polymorphism DNA, Potato, RAPD, Radiation.

Introduction

Potato is a crop of worldwide importance and is an integral part of the diet of a large proportion of the world's populations. The development of efficient *in vitro* culture methods has facilitated the usage of mutation techniques for improvement of both seed and vegetative propagated crops. Mutation induction in combination with *in vitro* culture techniques may be the effective methods for plant improvement **(Novak (1991).** Plant breeders suffer from the lack of availability or existence of required genotypes. Therefore, induced genetic diversity is the basic requirement of plant breeding in developing plant varieties. Gamma rays belong to ionizing radiation and interact to atoms or molecules to produce free radicals in cells. These radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy,

biochemistry and physiology of plants depending on the irradiation level. These effects include changes in the plant cellular structure and metabolism, e. g. dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system and accumulation of phenolic compounds (**Kovacs and Keresztes, 2002; Kim *et al*., 2004; Wi *et al*., 2005**). Molecular genetic markers have become useful tools in providing a relatively unbiased estimation of genetic diversity and phylogeny in plants **(Clegg, 1990)**. Several different PCR techniques for DNA fingerprinting have been developed during the last decades, each one with specific advantages and disadvantages. Random amplified polymorphic DNA (RAPD) is the simplest and fastest DNA-based techniques in genetic similarity studies **Gwanama *et al*., (2002).** A number of scientists have used RAPD markers to study polymorphism in various plants (**Ortiz *et al*., 1997; Ranade *et al*., 2002; Rout and Das, 2002; Samal *et al*., 2003**). In potato, RAPD technique has been used mainly for identification and genetic characterization of cultivars **(Bered *et al*., 2005**). This work is an attempt to increase genetic variability in potato (Valor and Spunta) cultivars using gamma radiation as physical mutagen and forming molecular diversity analysis through RAPD marker.

Materials and Methods

Plant materials

Buds from two commercial tuber seeds (cvs. Spunta and Valor) were excised and surface sterilized by dipping in Clorox (30%) for ten minutes followed by three rinses in sterile distilled water. The buds were cultured on solid MS medium (**Murashige and Skoog, 1962)** without hormone. Micropropagation began after 6 - 8 weeks when the plantlets were about 10 - 12 cm high. The culture was maintained by cutting into single nodes and transferring them onto MS medium supplemented with 0.2 mg^{-1} BAP. The pH of the culture medium was adjusted to 5.7 before autoclaving and incubated in growth chamber at 25°C ± 2 under photoperiod 16h (Figure1).

Gamma irradiation

Irradiation was carried out with ^{60}Co source at the dose rate 10 Gy/ 23 min. 34 sec. at National Centre for Radiation Research and Technology, Cairo, Egypt. Mass cultures of *in vitro* grown plantlets derived from single nodes were treated with different doses of gamma rays (10, 20, 30, 40 Gy).

Fresh weight, dry weight and water content

The fresh weight of the samples was determined directly after taking the samples. Dry weights were determined after drying the samples for 48 h at 80 °C. The water content was calculated from the difference between fresh and dry weight.

Acclimatization

Irradiated plantlets cultivated in sterile jars containing peat moss and sand with ratio 1:1. Plastic caps were removed and covered with puncture plastic sheets. After one week the plastic cover sheets were removed and the plantlets were left in growth chamber one week before transfer to the green house.

Tuberization

The irradiated plantlets were transferred to liquid medium MS mixture, 8% sucrose, 2.0 mg^{-1} BAP. The pH of the culture medium was adjusted as above before autoclaving. The cultures were incubated in a growth chamber at 20°C under a photoperiod of 8 h at 400 lux

for 3-4 months. The resulting microtubers were cultured in green house for macrotubers production.

Proline content estimation

The proline content was estimated according to **Beatles *et al*., (1973)** in irradiated and non- irradiated plantlets.

Genomic DNA isolation

Total genomic DNA was isolated according to the protocol described by **Anderson *et al.,* (1992)** in irradiated and non-irradiated potato plantlets cvs. Spunta and Valor

RAPD amplification

Fifteen different primers were chosen arbitrarily. The 10-mer primers used were synthesized by Metabion International AG (Inc. Germany). Primers sequences (’5-’3) were as shown in Table 1. Amplification reactions were performed in a 50 µl volume, containing: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl2, 200 µM each of dNTPs, 1 µM primer, 30 ng of genomic DNA, 1.5 U of *Taq* DNA polymerase. The reaction mixture was overlaid with two drops of mineral oil, incubated for 3 min at 94 °C for initial denaturation, and then amplified for 35 cycles consisting of 1 min at 94 °C, 30 s at 32.3 °C and 1.30 min. at 72 °C, followed by 7 min incubation at 72 °C. Amplification products were separated by gel electrophoresis on 1.0% agarose and visualized under UV illumination after staining with ethidium bromide and photographed.

Data analysis

The size of RAPD fragments were estimated by comparison with the DNA marker. RAPD fingerprints were recorded in the binary form (1 = presence of a band and 0 = absence of a band). All data were scored twice by two independent scorings. A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram by using POPGENE package Version 3.5 **(Yeh *et al*., 1999).**

Results and Discussion

Potato (Spunta and valor) cultivars plantlets were exposed to different doses of gamma radiation 10, 20, 30 and 40 Gy. Gamma irradiation caused decrease in the survival percentage of micropropagated buds in both cultivars Spunta and Valor to 91.4, 81.9, 68.5 and 28.5 % and 93.3, 87.6, 79, and 30.47 % comparing with the two value of the two non-irradiated treatments (95.2% and 96.2), respectively as shown in Figure 2. The shoot lengths of both cultivars in irradiated plantlets were decreased with increasing gamma radiation doses (Figure 3). Also, the fresh and dry weights were decreased with increase gamma radiation dose in both cultivars (Figure 4). The resulting microtubers were also decreased in number and size with increasing gamma radiation doses. The radiation sensitivity result based on survival percentage, shoot length and tuberization of irradiated and non-irradiated plantlets showed that a significant reduction was observed with increasing gamma dosage. These results were in accordance with radiation sensitivity test done by **Hasegawa *et al*., 1995; El-Fiki *et al*., 2015 and 2016** for tobacco, **El-Fiki (1997)** for potato, **El-Fiki *et al*., (2005a and b)** for alfalfa, **Norfadzrin *et al*., (2007)** for tomato and okra and **Kiong *et al.,* (2008)** for *Orthosiphon stamineus*.

Gamma rays are often used on plants in developing varieties that are agriculturally an economically important and have high productivity potential (**Jain *et al.,* 1998)**. They are useful for mutations in breeding programs and *in vitro* mutagenesis in order to develop required features of plants and increase the genetic variability.

Proline content estimation

Proline content in irradiated potato plantlets with doses of 10, 20, 30 and 40 Gy and non-irradiated plants was estimated. Gamma radiation doses had a possitive impact on proline content in both cultivars. The results showed that the proline content was increased with increasing gamma radiation dose (Figure 5). The most crucial function of plant cell is to respond to gamma stress by developing defense mechanisms. This defense may be affected by alteration in the pattern of gene expression **Corthals *et al*., (2000),** which may led to modulation of certain metabolic and defensive pathways **Zolla *et al*., (2003)**.

Total genomic DNA from non-irradiated (control) and eight radiation treatments of potato cvs Spunta and Valor were used as templates for RAPD finger printing (Figure 6). The total number of bands (150) were obtained by using 10 primers were selected based on the quality and repeatability of the amplified bands. The band size ranged from 13061 bp to 159 bp (Table 2). The maximum number of bands were 12 and 13 in Spunta and Valor cultivars respectively which produced by primer OP- B07. However, the minimum numbers of bands were 5 produced by primers OP- B11, OP- L12 in cv. Spunta and OP- L12, OP- L16 and OP- Z03 in cv. Valor. All the ten primers were polymorphic and generated 30, 24 polymorphic bands in both cultivars respectively (Table 3).

Treatments Specific Markers

All the ten primers were gave specific markers. A total of 30 and 24 specific markers were generated in Spunta and Valor cultivars respectively. The highest numbers of specific marker (8) have been obtained from Primer OP- B07. While the lowest number of specific marker (1) have been obtained from 4 primers in both cultivars (Table 4).

Genetic Relationships in Treatments

The genetic similarity and genetic distance between the eight gamma radiated treatments and non-irradiated treatments of both potato cultivars Spunta and Valor are presented in (Table 5). The Nei's genetic identity was the highest (0.9672) in treatments pairs 30 and 20Gy in cv. Valor. However, the lowest genetic identity was (0.6707) in treatments pairs 30Gy in cv. Spunta and 20Gy in cv. Valor. On the other hand, the highest Nei's genetic distance was (0.3995) between the two treatments 20Gy in cv. Valor and 30Gy in cv. Spunta. Whereas, the lowest Nei's genetic distance was (0.0333) within irradiated Valor with dose 20Gy.

RAPD Based Genetic Relationships

A dendrogram based on **Nei's, (1972)** genetic distance using unmeasured pair group method of arithmetic mean (UPGMA) was established with 10 gamma irradiation treatments in potato cvs. Valor and Spunta (Figure 7). These treatments segregated into two main clusters. The first cluster contains non-irradiated Valor and Spunta and gamma irradiation doses 10, 20, 30 and 40Gy to cv. Valor. The second cluster includes other gamma radiation doses 10, 20, 30 and 40Gy of cv. Spunta. Molecular markers have become an effective tool and a means by which both intra- and inter-species genetic diversity is evaluated and characterized. Marker systems are distinguished by the extent (i.e., magnitude) of their informativeness, which in turn depends on the degree of polymorphism. The concept of

polymorphism is used to determine the genetic variability in the population, which in recent decades has become the subject of intense study by various disciplines (genetics, ecology, botany, zoology and some others). Examples of this are numerous and obvious (**Chesnokov and Artemyeva, 2015**). The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes (**Williams *et al*., 1990, Welsh and McClelland, 1994**). The simplicity and applicability of the RAPD technique have captivated many scientists' interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterization of the genome. Although the RAPD method is relatively fast, cheap and easy to perform in comparison with other methods that have been used as DNA markers, the issue of reproducibility has been of much concern since the publication of the technique. In fact, ordinary PCR is also sensitive to changes in reaction conditions, but the RAPD reaction is far more sensitive than conventional PCR because of the length of a single and arbitrary primer used to amplify anonymous regions of a given genome. This reproducibility problem is usually the case for bands with lower intensity. The reason for bands with high or lower intensity is still not known. Perhaps some primers do not perfectly match the priming sequence, amplification in some cycles might not occur, and therefore bands remain fainter. The chance of these kinds of bands being sensitive to reaction conditions of course would be higher than those with higher intensity amplified with primers perfectly matching the priming sites. The most important factor for reproducibility of the RAPD profile has been found to be the result of inadequately prepared template DNA (**Welsh and McClelland, 1994**). Differences between the template DNA concentration of 2 individuals DNA samples result in the loss or gain of some bands (**Bardakci, 1996**). The cultivars identification using RAPD markers is well-documented in studies of molecular characterization (**Bianchi *et al*., 2003, Crochemore *et al*., 2004**). Fingerprinting based on this marker type was used for identification and characterization of potato cultivars in North America (**Sosinski and Douches 1996**), Australia (**Ford and Taylor 1997**) and India (**Chakrabarti *et al.,* 1998**).

Conclusion

The usage of gamma radiation as a tool for genetic variations in plants is good and efficient. Gamma irradiation had a negative impact on growth rate, number and microtubers size of potato plantlets. Also, the use of RAPD as a genetic marker is still the fastest, easiest, cheapest and effective way to differentiate between varieties and treatments.

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Table 1: The list of 15 primer's names and their sequences

No	Name	Sequence	No	Name	Sequence
1	OP-A02	TGC CGA GCT G	9	OP-C08	TGG ACC GGT G
2	OP-A12	TCG GCG ATA G	10	OP-F06	AGG TGC GTC C
3	OP-B-01	CTG TCG TCG T	11	OP-L12	GGG CGG TAC T
4	OP-B07	AGG TGA CCG T	12	OP-L13	ACC GCC TGC T
5	OP-B10	CTG CTG GGAC	13	OP-L16	AGG TTG CAG G
6	OP-B11	CAG CAC TGC T	14	OP-L20	TGG TGG ACC A
7	OP-B12	CCT TGA CGC A	15	OP-Z03	CAG CAC CCC A
8	OP-C02	GTG AGG CGTC			

Table (2): Sequence of 10 primers selected and tested, the numbers of DNA fragments amplified and the name and size a bases (bp) in potato cvs. Spunta and Valor.

primer's **name**	**Sequence** '5-'3	Spunta		Valor	
		Total No. of Bands	**Band size range/bp**	**Total No. of Bands**	**Band size range/bp**
OP-B01	**CTG TCG TCG T**	**9**	**1399 – 218**	9	1399 – 218
OP-B07	**AGG TGA CCG T**	**12**	**1568 – 189**	13	1568 - 189
OP-B11	**CAG CAC TGC T**	**5**	**863 – 220**	7	863 – 220
OP-B12	**CCT TGA CGC A**	**9**	**1874 - 177**	10	2655 – 177
OP-F06	**AGG TGC GTC C**	**8**	**1491 – 187**	9	13061 - 187
OP-L12	**GGG CGG TAC T**	**7**	**1211 - 193**	8	10703- 193
OP-L13	**ACC GCC TGC T**	**7**	**1699 –**	5	841 – 294

			255		
OP-L16	**AGG TTG CAG G**	**5**	**430 – 144**	5	430 – 144
OP-L20	**TGG TGG ACC A**	**6**	**835 – 221**	6	835 – 221
OP-Z03	**CAG CAC CCC A**	**5**	**591 – 159**	5	591 – 159
Total		**73**		77	

primer's Spunta Valor

Table (3): Number and percentage of polymorphic loci obtained in 10 potato cvs. Spunta and Valor gamma radiation treatments

	Gel polymorphism				Gel polymorphism			
	Total band No.	Monomorphic bands	polymorphic bands	Polymorphism (%)	Total band No.	Monomorphic bands	polymorphic bands	Polymorphism (%)
OP-B01	9	6	3	33.33%	9	6	3	33.33%
OP-B07	12	4	8	66.66%	13	11	2	15.38%
OP-B11	5	4	1	20.00%	7	4	3	42.85%
OP-B12	9	7	2	22.22%	10	6	4	40.00%
OP-F06	8	3	5	62.50%	9	4	5	55.55%
OP-L12	7	6	1	14.28%	8	7	1	12.50%
OP-L13	7	3	4	57.14%	5	3	2	40.00%
OP-L16	5	3	2	40.00%	5	3	2	40.00%
OP-L20	6	4	2	33.33%	6	5	1	16.66%
OP-Z03	5	3	2	40.00%	5	4	1	20.00%
Total	73	43	30	41.09%	77	53	24	31.16%

Table (4): Used primers and the specific RAPD markers generated for gamma radiation treatments.

Table 5: Genetic similarity (above diagonal) and genetic distance (below diagonal) values among of 10 gamma radiation treatments in potato cvs. Spunta and Valor.

Primer No.	Spunta	Valor
	Specific band marker (bp)	Specific band marker (bp)
OP-B01	1399, 852, 684	1399, 852, 684
OP-B07	1568, 1272,1050, 870, 626, 455, 372,189	1568 , 220
OP-B11	410	410 , 346 , 289
OP-B12	1874 , 177	2655 , 1874 , 1109 , 177
OP-F06	1491 , 959 , 637 , 427 , 352	13061 , 1491 , 959, 637 , 352
OP-L12	1211	10703
OP-L13	1699 , 841 , 294 , 255	841 , 294
OP-L16	430 , 328	430 , 328
OP-L20	835 , 610	835
OP-Z03	591 , 391	591

ID	1	2	3	4	5	6	7	8	9	10
1	****	0.8800	0.8559	0.8559	0.8757	0.9167	0.7740	0.7986	0.7235	0.8389
2	0.1278	****	0.7163	0.7650	0.7540	0.8984	0.8311	0.7461	0.8408	0.7542
3	0.1556	0.3336	****	**0.9672**	0.9006	0.8244	0.7067	0.7671	**0.6707**	0.8721
4	0.1556	0.2678	**0.0333**	****	0.8684	0.8389	0.7219	0.7671	0.7206	0.8209
5	0.1327	0.2824	0.1047	0.1411	****	0.8610	0.7912	0.7842	0.6985	0.8729
6	0.0869	0.1071	0.1931	0.1757	0.1496	****	0.8463	0.8542	0.7720	0.8346
7	0.2562	0.1850	0.3472	0.3259	0.2341	0.1669	****	0.8820	0.8877	0.8780
8	0.2249	0.2929	0.2652	0.2652	0.2431	0.1576	0.1256	****	0.8034	0.8936
9	0.3237	0.1734	**0.3995**	0.3277	0.3587	0.2588	0.1191	0.2189	****	0.7795
10	0.1757	0.2821	0.1369	0.1974	0.1360	0.1809	0.1301	0.1125	0.2491	****

1= Cont. Valor
2= 10Gy Valor
3= 20Gy Valor
4= 30Gy Valor
5= 40Gy Valor
6= Cont. Spunta
7= 10Gy Spunta
8= 20Gy Spunta
9= 30Gy Spunta
10= 40Gy Spunta

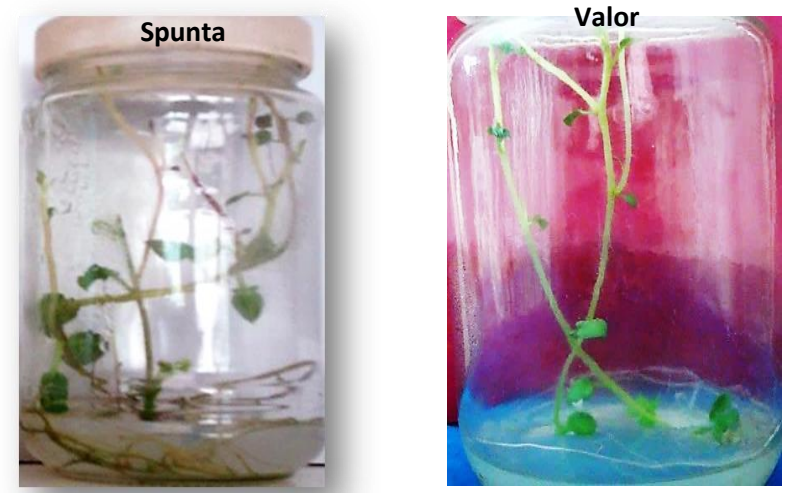

Figure 1: Micropropagation of Spunta and Valor cultivars

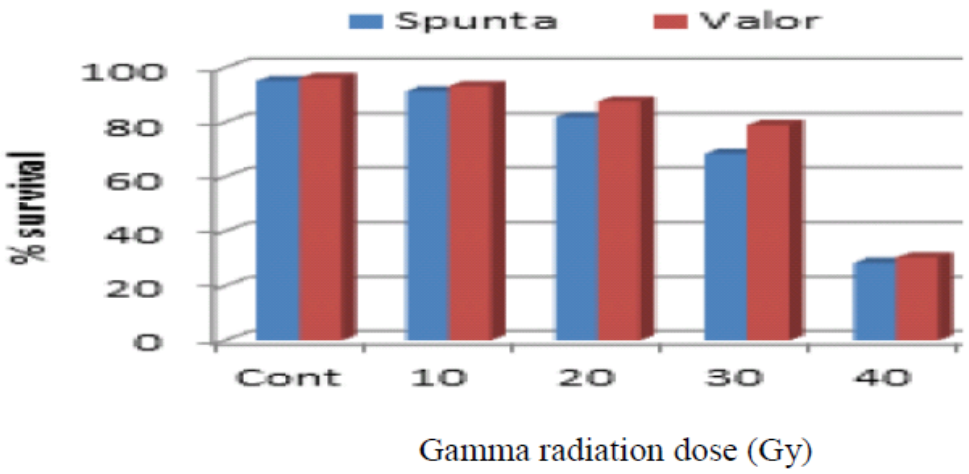

Figure 2: Gamma radiation impact on potato survival percentage cvs. Spunta and Valor

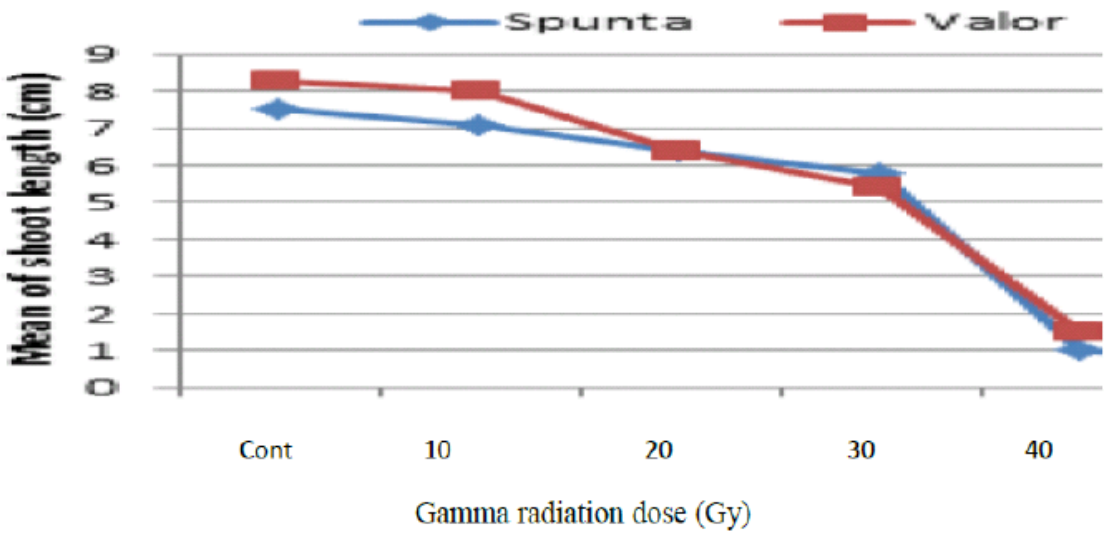

Figure 3: Gamma radiation impact on potato shoot length cvs. Spunta and Valor

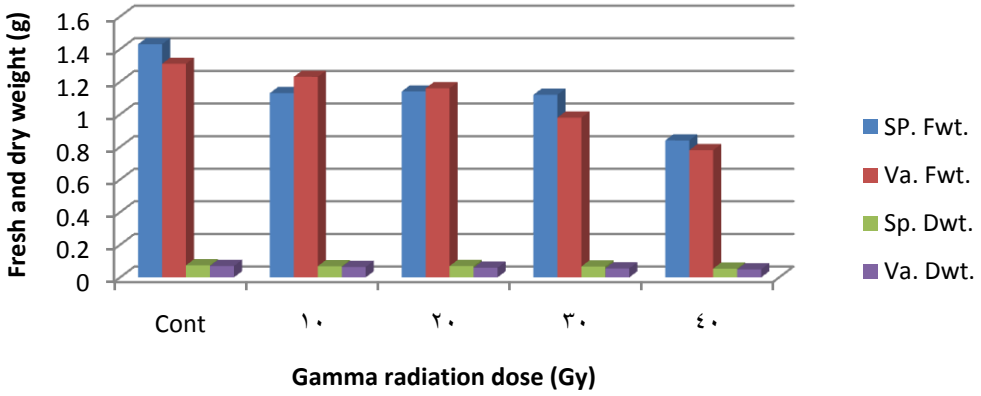

Figure 4: Gamma radiation impact on potato plantlets fresh and dry weight cvs. Spunta and Valor

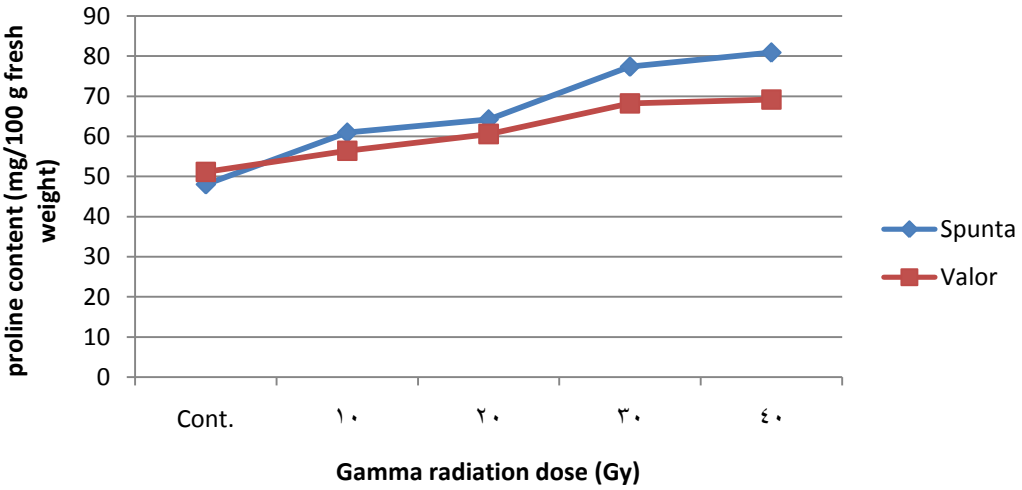

Figure 5: Gamma radiation impact on proline content in potato plantlets cvs. Spunta and Valor

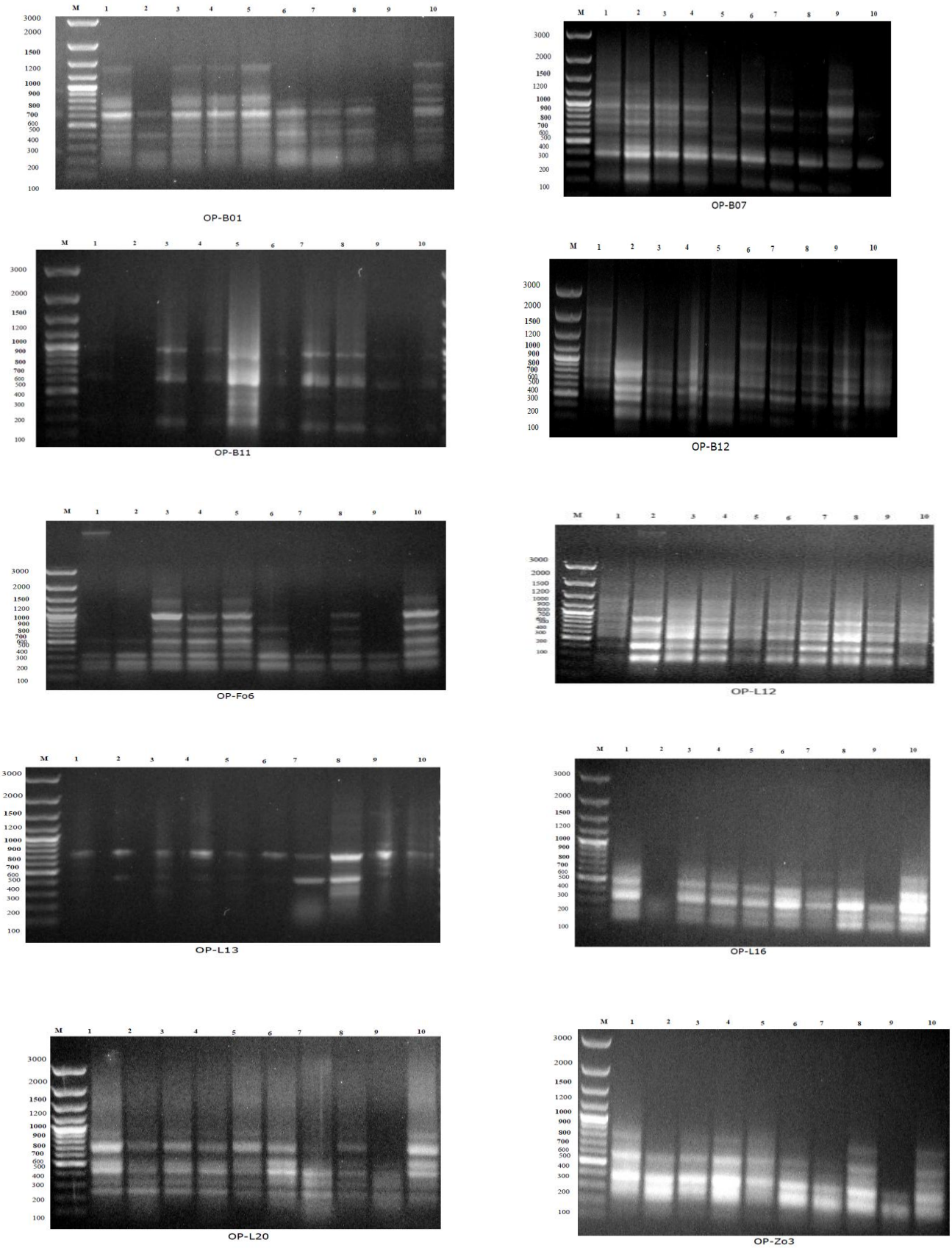

Figure 6: DNA RAPD profiles of non-irradiated and irradiated Spunta and Valor cultivars by using10 primers.

M Marker ,1- Valor Control, 2-V 10 Gy, 3-V20Gy, 4- V 30Gy, 5- V40Gy, 6- Spunta control, 7- S 10Gy, 8- S 20Gy, 9- S30Gy, 10- S40Gy

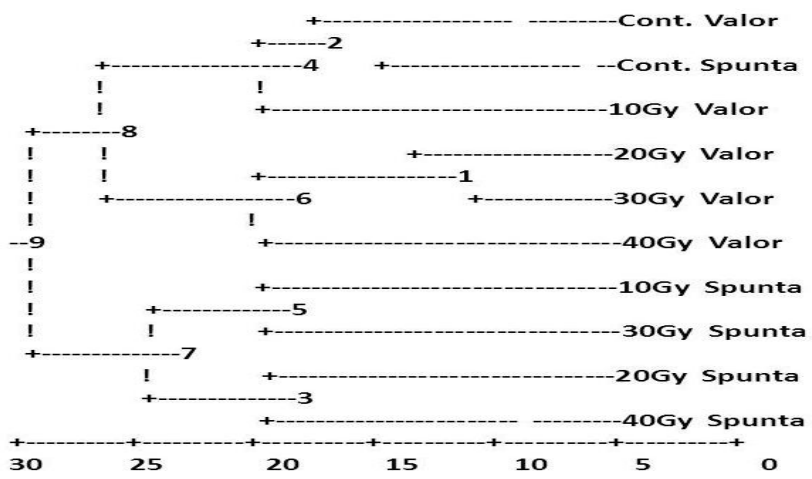

Figure 7: Dendrogram of two non-irradiated treatments and eight gamma radiation doses treatments of potato cvs. Spunta and Valor.

الملخص العربى

تحليل الإختلافات الجزيئيه فى صنفين من البطاطس المشععه معمليا بواسطة التضخم العشوائى متعدد الأشكال للحمض النووى

أيمن الفقى[1] وزكيه أدم[2]وثريا رشاد[2] وشيماء سلمى[2] وأمل صلاح[1]

1 قسم بحوث المنتجات الطبيعية - المركز القومى لبحوث وتكنولوجيا الاشعاع –هيئة الطاقة الذرية

مدينة نصر- القاهرة- مصر

[2] كلية البنات للأداب والعلوم والتربية - جامعة عين شمس - القاهرة- مصر

تمت زراعة براعم البطاطس صنفى إسبونتا و فالور على بيئة موراشيج وسكوج مضافا إليها 0.2 ملجم / لتر بنزايل أدينين. وتم تعريض النباتات الناتجة إلى جرعات إشعاعية من أشعة جاما 10، 20، 30، 40 جراى. ثم نقلت القطع المشععة على بيئة موراشيج وسكوج مضاف اليها 0.2 ملجم / لتر بنزايل وتم حساب نسبة الحيوية للنباتات بعد ثمانية أسابيع. ومن خلال تسجيل النتائج وجد أن أشعة جاما قد تسببت في فقد 91.4 إلى 28.5٪ في صنف إسبونتا و 93.3 إلى 30.47٪ في صنف فالور و ذلك بالمقارنة مع قيمة النباتات غير المشععة (الكنترول) 95.2 و 96.2% فى الصنفين على الترتيب. وانخفضت الدرنات المنتجة من النباتات المشععة مع زيادة جرعة الإشعاع جاما مع التغيرات في الحجم والعدد. كما تم تسجيل زيادة فى محتوى البرولين في النباتات المشععة مع زيادة الجرعة الإشعاعية. كذلك تم إجراء تفاعل البلمرة المتسلسل PCRبتقنية التكرارت العشوائية RAPD وذلك بإستخدام 10 بادئات ، وقد سجلت إجمالى 54 حزمة متباينة الأشكال فى كلاالصنفين ومع ثمانية من المعاملات الإشعاعية. وأظهرت النتائج أعلى نسبة قرابة وراثية 0.9672 بين النباتات المشععة مع جرعة 20 و 30جراى في صنف إسبونتا. ، و كانت أقل نسبة قرابة وراثية 0.3995 لوحظت مع جرعة 20جراى في صنف فالور و 30 فى الصنف سبونتا. وتم تمييز النباتات المشععة وراثيا عن طريق تحليل الحزم بواسطة الشجره الوراثيه .