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Original Article

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

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Abstract

Potato buds cvs. 'Valor' and 'Spunta' were cultured *in vitro* on MS solid medium with 0.2 mg^{-1} BAP. The resulting plantlets were irradiated with gamma radiation doses 10, 20, 30, 40 and 50 Gy. Irradiated segments were transferred onto fresh MS with BAP and plantlets survival percentage was calculated after eight weeks. Gamma radiation caused the death of 3.8% to 81% in cv. 'Spunta' and 2.9% to 83.9% in cv. 'Valor'. Microtubers produced from irradiated plantlets were decreased with increasing gamma radiation doses, with notable changes in shape, size and numbers. The proline contents in irradiated plantlets were steady increase with gamma radiation doses. The genomic DNA of the two cultivars and ten radiation treatments was amplified with 10 RAPD primers that generated 53 polymorphic bands. The highest number of genetic identity was 0.9672 showed between irradiated plantlets with 20 and 30 Gy in cv. 'Valor'. However, the highest genetic distance was 0.3995 observed between irradiated plantlets with dose 20 Gy in cv. 'Valor' and 30 Gy 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 basic part of the diet of a large proportion of the world's populations (FAO, 2008). The development of efficient *in vitro* culture methods has facilitated the use of mutation techniques for improvement of both seed and vegetatively 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 ray is an ionizing radiation where they react with atoms or molecules to produce free radical in cells. Radicals may have harmful effect or act on rearranging the cell components and this effect may appear on the morphology, physiology, biochemistry and anatomy depending on radiation doses. 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). Over the past decades, PCR techniques have been developed for DNA fingerprinting, each one with specific advantages and disadvantages. Random amplified polymorphic DNA (RAPD) is the simplest and fastest of 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 has been used mainly for the identification and genetic characterization of cultivars (Bered *et al*., 2005).

The hereby work was an attempt to increase genetic variability in potato ('Valor' and 'Spunta') cultivars using gamma radiation as physical mutagen and molecular diversity analysis through RAPD marker.

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Materials and Methods

Plant material

Buds from tubers of 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 the buds were thereafter incubated in growth chamber at 25 °C ± 2 under photoperiod 16 h.

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, 50 Gy). A further 100 *in vitro* grown plantlets were irradiated at each dose.

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 and 72 h at 80 °C. The water content was calculated from the difference between fresh and dry weight.

Acclimatization

Irradiated plantlets were cultivated in sterile jars containing peat moss and sand with a ratio of 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 containing half strength MS salt mixture, 8% sucrose, 2.0 mg $^{-1}$ BA. 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. As another way for tuberization, were through acclimatized plantlets cultured on pots containing peat moss and sand 1:1.

Proline content estimation

The proline content was estimated according to Batels *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. 'Valor' and 'Spunta'.

RAPD amplification

Fifteen different primers were chosen arbitrarily. The primers used in the current experiment were 10-mer synthesized by Metabion International AG (Inc. Germany). Primers sequences (5`-3`) are 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 sec 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 ethidiam bromide and photographed.

Data analysis

The size of RAPD fragments were estimated by comparison with the 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, 40 and 50 Gy. The impact of gamma irradiation caused a decrease in the percent survival of micropropagated buds in both cultivars to 91.4, 81.9, 68.5, 28.5 and 14.2% and 93.3, 87.6, 79 and 30.47%, respectively, as shown in Fig. 1. The shoot lengths of both cultivars in irradiated plantlets were decreasing with increasing gamma radiation doses (Fig. 2). Also, the fresh and dry weights decreased with increased gamma radiation dose in both cultivars (Fig. 3). The resulting microtubers were also decreasing in number and size with increasing gamma radiation doses. The results showed by the radiation sensitivity test based on survival percentage, shoot length and tuberization of irradiated and non-irradiated plantlets, that significant reduction was observed with increasing gamma dosage. Generally, gamma irradiation can be used to obtain varieties that are economically important in agriculture, with high productivity and quality (El-Fiki, 1997; Jain, 2010). 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. Many mutant varieties, which are resistant to biotic and abiotic stress and with high quality, have been developed (Jain *et al*., 2013). Several attempts of mutagenic treatment on cultured anthers have been reported in higher plants (Sangwan and Sangwan, 1986; MacDonald *et al*., 1988; Ling *et al*., 1991; El-Fiki *et al*., 2015). These results were in accordance with radiation

sensitivity test done by Hasegawa *et al*. (1995), El-Fiki *et al*. (2015, 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*.

Proline content estimation

Proline content in irradiated potato plantlets with doses of 10, 20, 30 and 40 Gy and non-irradiated plants was estimated. Irradiated potato plantlets with gamma radiation doses had a positive impact on proline content in both cultivars. The results showed that the proline content increased with increasing gamma radiation dose (Fig. 4).

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).

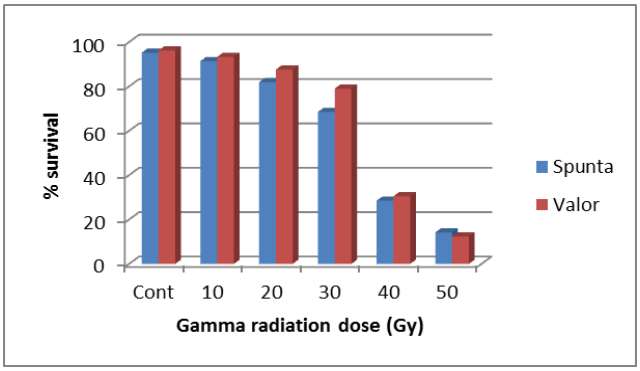

Fig. 1. Gamma radiation impact on potato bud survival percentage cvs. 'Spunta' and 'Valor'

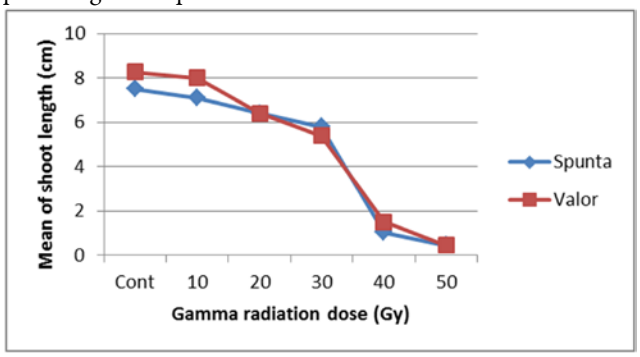

Fig. 2. Gamma radiation impact on potato shoot length cvs. 'Spunta' and 'Valor'

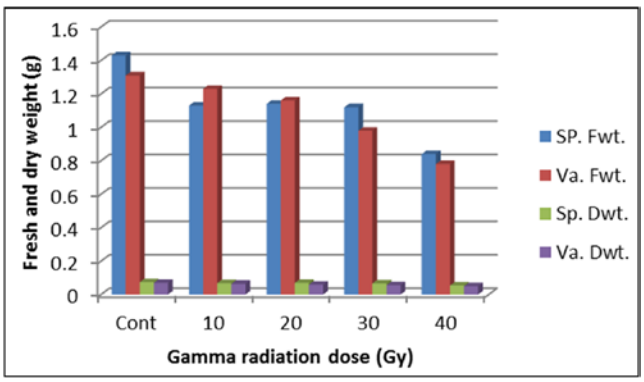

Fig. 3. Gamma radiation impact on potato plantlets fresh and dry weight cvs. 'Spunta' and 'Valor'

Total genomic DNA from ten radiation treatments of potato cvs. 'Spunta' and 'Valor' were used as templates from RAPD finger printing. Of the 15 RAPD primers tested, 10 primers were selected based on the quality and repeatability of the amplified bands. Altogether, 146 bands were obtained, with an average of 14.6 bands per primer and the band size ranging from 13.061 Kbp to 0.159 Kbp (Table 2). The maximum number of bands was of13, produced by primer OP- B07. However, the minimum numbers of bands was 5, produced by primers OP- B11, OP- L13, OP- L16 and OP- Z03. The polymorphic bands ranged from one in primers OP- B11, OP- L12, OP- L20 and OP-Z03, to eight bands in primer OP- B07. The primer OP-B07 produced the maximum number of bands (12) with non-irradiated cv. 'Spunta ' and (13) with non-irradiated and irradiated cv.' Valor ' by dose 10 Gy. However, the minimum number of bands observed in cvs. 'Spunta' and 'Valor ' was 5 bands with primers OP- B11, OP- L16, OP- Z03 and OP- L13. These bands have emerged as a result of gamma irradiation with primers OP- B11 (10 Gy), OP- L16 (non-irradiated, 10 and 40 Gy) and OP- Z03 (20 and 40 Gy) in cv. 'Spunta ', while in cv. 'Valor ' these bands were observed with primers OP- L16 and OP- Z03 (non-irradiated, 20, 30 and 40 Gy) and OP- L13 (20 and 30 Gy). All the ten primers were polymorphic. A total of 146 bands were amplified of which 53 (36.3%) polymorphic across radiation treatments (Table 3).

Treatments specific markers

All ten primers gave specific markers. A total of 37 specific markers were generated. The highest numbers of specific marker was 9 and was from primer OP- B07, while the lowest number of specific marker (1) was obtained from OP- L12 in both cultivars under study. There was a great similarity in the obtained specific markers except primer OP-L12 in both cultivars (Table 4).

Genetic relationships in treatments

The genetic identity and genetic distance to ten gamma radiation treatments of both potato cultivars 'Valor' and 'Spunta ' 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 30 Gy in cv. 'Spunta ' and 20 Gy

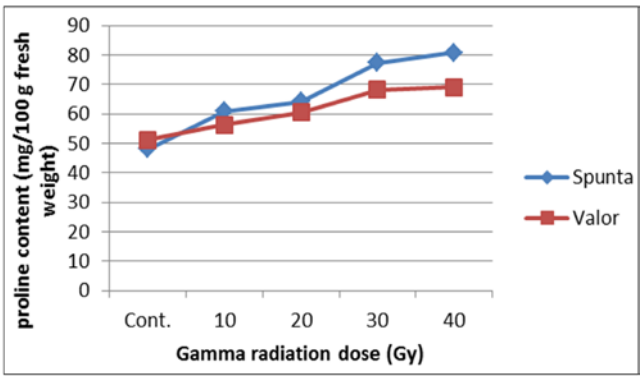

Fig. 4. Gamma radiation impact on proline content in potato plantlets cvs. 'Spunta' and 'Valor'

Table 1. The list of 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 primers tested, the numbers of DNA fragments amplified and the size a kilobases (Kbp) in potato cvs. 'Valor' and 'Spunta'

Primer No.	Sequence	'Valor'		'Spunta'	
		No. of bands	Band size range/kbp	No. of bands	Band size range/kbp
OP-B01	CTG TCG TCG T	9	1.399 - 0.218	8	1.399 - 0.218
OP-B07	AGG TGA CCG T	13	1.568 - 0.189	12	1.568 - 0.189
OP-B11	CAG CAC TGC T	7	0.863 - 0.220	5	0.863 -0.220
OP-B12	CCT TGA CGC A	9	2.655 - 0.177	8	1.874 - 0.177
OP-F06	AGG TGC GTC C	9	13.061 -0.187	7	1.491 - 0.187
OP-L12	GGG CGG TAC T	8	0.193 , 0.274	7	0.193 , 0.274
OP-L13	ACC GCC TGC T	5	0.841 – 0.294	7	1.699 , 0.255
OP-L16	AGG TTG CAG G	5	0.328 , 0.144	5	0.328 , 0.144
OP-L20	TGG TGG ACC A	6	0.835 , 0.221	6	0.835 , 0.221
OP-Z03	CAG CAC CCC A	5	0.391 , 0.159	5	0.391, 0.159
Total		76		70	

Table 3. Number and percentage of polymorphic loci obtained in 10 potato gamma radiation treatments on cvs. 'Valor' and 'Spunta'

Primer No.	'Valor'				'Spunta'			
	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%	8	5	1	37.5%
OP-B07	13	11	2	15.83%	12	4	3	66.66%
OP-B11	7	4	0	42.85%	5	4	0	20.00%
OP-B12	9	6	2	33.33%	8	6	1	25.00%
OP-F06	9	4	4	55.55%	7	2	3	71.42%
OP-L12	8	7	0	12.50%	7	6	1	14.28%
OP-L13	5	3	2	40.00%	7	3	2	57.14%
OP-L16	5	3	2	40.00%	5	3	2	40.00%
OP-L20	6	5	1	16.66%	6	4	1	33.33%
OP-Z03	5	4	1	20.00%	5	3	2	40.00%
Total	76	53	17	30.26%	70	40	16	42.85%

in cv. 'Valor '. On the other hand, the highest Nei's genetic distance was (0.3995) between the two treatments 20 Gy in cv. 'Valor' and 30 Gy in cv. 'Spunta '. Whereas, the lowest Nei's genetic distance was (0.0333) within irradiated 'Valor' with dose 20 Gy.

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 ' (Fig. 5). These treatments segregated into two main clusters. The first cluster contained non-irradiated 'Valor ' and 'Spunta ' with 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 '. The highest genetic distance level was (6.14499) observed between group 6 and dose 40 Gy in cv. 'Valor'. However, the lowest genetic distance level was (1.52699) showed between group 4 and 2.

Molecular markers have become an effective tool by which both intra- and inter-species genetic diversity can be 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 a population, which in recent decades has become the subject of intense study by various disciplines (genetics, ecology, botany, zoology and others). Examples of this are numerous and obvious (Chesnokov and Artemyeva, 2015). The development of PCR techniques and random primers led to amplify a set of randomly distributed loci in any genome facilitating 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. The main reason for the success of the RAPD analysis is that one can obtain 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. The PCR is very sensitive to any changes that may occur in the 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 kind 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-

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

	'Valor'	'Spunta'
Primer No.	Specific band marker (bp)	Specific band marker (bp)
OP-B01	1.399, 0.852, 0.684	1.399, 0.852, 0.684
OP-B07	1.568, 0.220	1.568, 1.272, 1.050, 0.870, 0.626, 0.455, 0.372, 0.189
OP-B11	0.410, 0.346, 0.289	0.410
OP-B12	2.655, 1.874, 1.109, 0.177	1.874, 0.177
OP-F06	13.061, 1.491 , 0.959, 0.637, 0.352	1.491, 0.959, 0.637, 0.427, 0.352
OP-L12	10.703	1.211
OP-L13	0.841, 0.294	1.699, 0.841, 0.294, 0.255
OP-L16	0.430, 0.328	0.430, 0.328
OP-L20	0.835	0.835, 0.610
OP-Z03	0.591	0.591, 0.391

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

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

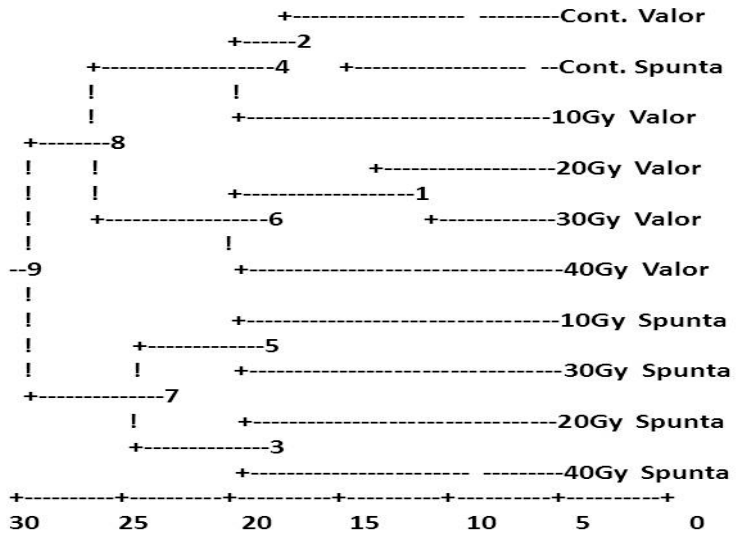

Fig. 5. Dendrogram of ten gamma radiation doses treatment of potato cvs. 'Valor' and 'Spunta'

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). 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 of 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).

Conclusions

The use of gamma radiation as a tool for inducing genetic variations in plants represents an efficient method. Gamma irradiation had a negative impact on growth rate, number and microtubers size of potato plantlets. In addition, the use of RAPD as a genetic marker remains the fastest, easiest, cheapest and effective way to differentiate between varieties and treatments.

Acknowledgements

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