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USE OF INTER SIMPLE SEQUENCE REPEAT-POLYMERASE CHAIN REACTION (ISSR-PCR) TECHNIQUE FOR DETERMINATION OF THE EFFECT OF FAST NEUTRON IRRADIATION ON *VICIA FABA* SEEDS

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ABSTRACT

The effect of low doses of fast neutrons on the DNA genome of broad bean (*Vicia faba*) mature seeds was determined**.** In this study, inter simple sequence repeat (ISSR)-polymerase chain reaction (PCR) tool was used to analyze the genetic alteration of treated seeds of *V. faba* using six ISSR primers. Results showed that each primer gave rich and clear patterns with bands ranged from 179 to 1256 bp. Among the 61 ISSR fragments, 21 were representing the effect of the treatment of *V. faba* seed samples**.** ISSR markers clearly and easily distinguish all the tested samples and also discriminate between fast neutron-treated and untreated seeds. The dissimilarity values ranged 42 to 47% between the fast neutron-treated seeds and the control seed (untreated) and from 27 to 38% between the fast neutron-treated seeds. The dendrogram resulting from a UPGMA cluster analysis comprised two main distinct clusters, the first one including the control *V. faba* seed (untreated) and the second, includes all samples from the same kind of treatment divided into three groups: the S4 (group A), S2 and S3 (group B) and S1 (group C), these groups being separated at a similarity level.

INTRODUCTION

The effects of fast neutron irradiation on DNA were studied (Spotheim-Maurizot *et al*., 1990; Yamaguchi and Waker, 2007). The influence of dose rate on the effectiveness of a neutron irradiation was investigated using growth inhibition in *Vicia faba* bean roots as biological system. d (50) + Be neutron beams produced at the cyclotron CYCLONE of the University of Louvain-la-Neuve were used, at high and low dose rate, by modifying the deuteron beam current (Van dam *et al*., 1983; and Beauduin *et al*., 1989).

The availability of a variety of DNA markers, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and intersimple sequence repeat (ISSR) has enabled researchers to investigate genetic diversity among various plant species across natural populations (Wolfe *et al*., 1998; Gabrielsen and Brochmann 1998; Knox and Palmer, 1999; Deshpande *et al*., 2001, Archak *et al*., 2003). Among these, PCR-based techniques of random multilocus analysis (RAPD, AFLP, and ISSR) have been successfully used in genotyping, genome mapping and phylogenetic studies in horticultural crops such as strawberry (Korbin *et al*., 2002);

oybean (Ferreira *et al*., 2000) and potato (Prevost and Wilkinson, 1999).

The ISSR technique is similar than RAPD, except that ISSR primers consist of a di- or trinucleotide simple sequence repeat with a 5' or 3' anchoring sequence of 1–3 nucleotides. Compared with RAPD primers, the ISSR primers sequence is usually larger, allowing for a higher primer annealing temperature, which results in greater band reproducibiliy than RAPD markers (Culley and Wolfe, 2000). These have been successfully used to assess genetic variation in plants such as citrus (Fang and Roose, 1997), *Viola pubescens* (Culley and Wolfe, 2000), potato (Prevost and Wilkinson, 1999), and *Oryza granulate* (Qian *et al*., 2001).

The present study aimed to determine the effect of fast neutron irradiation on the DNA genome of *V. faba* seeds by using ISSR-PCR.

MATERIALS AND METHODS

Plant materials and irradiation process

Mature seeds of *V. faba* L. cv. G 461 were obtained from the Field Crops Research Institute, Agricultural Research Center (ARC), Giza, Egypt**.** The seeds were screened for uniformity of size, and then divided into five groups (S1, S2, S3, S4 and S5) each of 25 seeds. Seeds of S1, S2, S3 and S4 were moistured (24 h) and irradiated with fission neutrons from Cf^{252} point source ($\bar{E} \approx 2Mev$) to fluencies $2.5x10^5$, $3x10^6$, $1.5x10^8$ and $1.5x10^9$ n/cm^2, respectively, based on the method of Magda *et al*., (2003). The source of fission neutrons was manufactured by Radiochemical Center, Amersham, England. S5 group was kept unirradiated (control sample). These irradiated seed groups as well as the control seeds were considered as F0 germination. All seeds (irradiated and control) were planted in pots 25 cm diameter, under greenhouse conditions in the Faculty of Agriculture, Zagazig Univ., to yield the next germination (F1). Five F1 seeds representing each group (irradiated: S1, S2, S3 and S4 as well as control (S5) were used for determination of the effect of fast neutron irradiation on DNA *via* ISSR analysis.

Seeds preparation: All fast neutron-irradiated broad bean mature seeds as well as control were surface sterilized by soaking them in 70% ethanol for 30 seconds, then rinsed in sterile water before transferring to 20% commercial household bleach for 10-15 min with 1-2 drops of Tween 20 followed by washing 5-6 times with sterile distilled water ($d.H_2O$)**.** On sterilization, the seeds were germinated in a pre-autoclaved wet cotton pads, placed in 10 cm glass jars and covered with aluminum foil followed by incubation at 28°C±2 and under 16 hr photoperiod provided from cool white fluorescent lamp (3000 lux).

In vitro grown seedlings (10-13 days old) were used for DNA extraction and ISSR-PCR analysis.

Extraction of genomic DNA: About 100 mg of seed materials were used for DNA extraction according to CTAB method of Lassner *et al*. (1989). DNA concentration was estimated with a Biophotometer and by gel analysis. PCR reactions were conducted using six ISSR primers.

Primer used: Codes, name and sequences of the ISSR-PCR primers listed in Table -1 were used.

Table-1: List of primers used, their sequence, melting temperature and annealing temperature.

Codes	Primer names	Sequences	Tm	Ta
P12	ISSR 1	CAC(TCC)7	55.9	59-50
P22	807	(AG)8T	46.6	51-42
P23	811	(GA)8C	46.4	51-42
P25	852	(TC)8AA	47.6	51-42
P27	3	(CA)8AT	50.0	55-46
P35	ISSR 35	TCGA(CA)7	57.6	62-53

ISSR-PCR: The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR-buffer, 1.2mM $MgCl_2$, 0.2 mM dNTPs, 50pmol primer, 1 µl *Taq* DNA polymerase (ABgene) and 50ng template DNA. Temperature cycling was performed on MJ Research PTC-200. The amplification profile consisted of initial denaturation of the template DNA at 94ºC for 4 min, followed by 10 cycles of 94ºC for 45s, touchdown one-degree decrement for annealing temperature started with 5ºC above Tm for each primer for 30s and 72ºC for 2 min followed by 25 cycles of 94ºC for 45s, last annealing temperature for 30s (Table-1) and 72ºC for 2 min and final extension of 72 for 5 min.

DNA electrophoresis: The amplification products were visualized in an ultraviolet transilluminator, after horizontal electro-phoresis in 2.2 % agarose gel, using the TBE 1X buffer, the being stained with ethidium bromide (Sambrook *et al*., 1989).

ISSR-PCR analysis: Unequivocally scorable and consistently reproducible amplified DNA fragments were trans-formed into binary character matrix (1= presence, 0 = absence). The similarity matrix was determined by Dice Co-efficient method. In addition, clustering of all characters was determined by the unweighted paired group method with average algorithm (UPGMA) (Sneath and Sokal, 1973). Analyses were done using the Phoretix 1D software from Nonlinear Dynamics.

RESULTS AND DISCUSSION

The effect of fast neutrons on the biological properties of barley seeds has studied by Kovács *et al*., (1977) and Kovács *et al*., (1979). They determined its effect on germinability of barley seeds and on the chlorophyll content of the seedlings according to the dose used (Kovács *et al*., 1977) and on variation of total nucleic acid content and ultrastructure in barley leaves vs. dose (Kovács *et al*., 1979). Beauduin *et al*., (1989) carried out a radiobiological intercomparison of clinical neutron beams for inhibition the growth system of *V. faba*. In Egypt, Elshafey *et al.,* (1991) studied the effect of fast neutron on the growth and yield of wheat.

The first studies employing ISSR markers were published in 1994 (Zietkiewicz *et al.*, 1994; Gupta *et al*., 1994). The initial studies focused on cultivated species, and demonstrated the hyper variable nature of ISSR markers (Wolfe and Liston, 1998). Their results clearly demonstrated the utility of ISSR markers for addressing questions of hybridization and diploid hybrid speciation.

In this study, six ISSR primers were used successfully to identify the effect of the fast-neutron irradiation on *V. faba* seeds

compared with untreated seeds (control) on genetic makeup level. The primers gave a total of 61 amplified fragments with 77.04% totally dissimi-larity, ranged from five bands (IS-1) to 16 bands (IS-5) and dissimilarity occurred by the irradiation ranged from 20% (IS-1) to 100% (IS-6) (Tables 2 and 3).

Dissimilarity percentage were shown in Table-4, the most dissimilar was between control and S2 (47%) and the least dissimilar was between control and S4 (42%) whereas the mean of dissimilarity between control (untreated) and treated seeds was 44%. The dissimilarities between the irradiated seeds ranged from 27 to 38 %. The lowest dissimilarity was found between S1&S2 and S2&S3 (27%) followed by 28% between S2 and S4, 31% between S1 & S4, 33% between S3 & S4 and 38% between S1 & S3.

The dissimilarity between the untreated seeds (control) and the treated one induced by the exposure to different doses of the fast-neutron irradiation have represented by appearing a new bands and/or disappearing ones. This alteration of genetic makeup of the *V. faba* was observed by using the ISSR-PCR markers as shown in Figure-1.

The genetic dendrogram of treated and untreated seeds of *V. faba* was resulting from a UPGMA cluster analysis based on estimates of Dice's coefficient of similarity obtained from ISSR marker. The dendrogram (Figure-2) comprised two main distinct clusters, the first one including the control *V. faba* seed (untreated) and the second, includes all samples from the same kind of treatment divided into three groups: the S4 (group A), S2 and S3 (group B) and S1 (group C), these groups being separated at a similarity level.

In conclusion we found that exposure to different doses of fast-neutron irradiation on *V. faba* cv. G 461 seeds did not cause death to the seeds but lead to appearing new fragments and/or disappearing fragments and make alteration in genetic level. When these new fragments isolated, identified and sequenced we can recognize the role of these fragments and associated loci impact in different metabolic pathways.

Table-2. Analysis of DNA polymorphisms of ISSR-PCR of broad bean seeds treated with different doses of fast neutron irradiation (S1, S2, S3 and S4) and control (untreated broad bean seed, S5) using six ISSR primers primer.

Fragments #	Fragment sizes (pb)	S1	S2	S3	S4	S5 (Cont.)
P12-1	**606**	0	0	0	0	**1**
P12-2	352	1	1	1	1	1
P12-3	242	1	1	1	1	1
P12-4	200	1	1	1	1	1
P12-5	179	1	1	1	1	1
P22-1	1256	1	1	1	1	1
P22-2	564	1	0	0	1	0
P22-3	509	1	1	1	1	1
P22-4	401	0	1	1	0	1
P22-5	320	1	1	1	1	1
P22-6	260	1	1	0	1	0
P22-7	208	0	0	**1**	0	0
P22-8	200	0	0	0	0	**1**
P23-1	996	0	0	0	1	1
P23-2	974	0	1	1	0	0
P23-3	813	1	0	1	1	1
P23-4	709	1	0	1	0	0
P23-5	644	1	1	1	1	1
P23-6	525	1	1	1	1	1
P23-7	476	1	1	1	1	1
P23-8	434	0	0	**1**	0	0
P23-9	425	1	1	0	1	0
P23-10	324	0	1	1	0	1
P23-11	302	1	0	1	0	0
P23-12	297	0	1	0	1	0
P23-13	238	0	0	0	1	1
P23-14	218	0	1	1	0	0
P25-1	1180	0	0	**1**	0	0
P25-2	763	0	0	0	**1**	0
P25-3	754	**1**	0	0	0	1
P25-4	737	0	1	1	0	0
P25-5	678	1	1	1	1	1
P25-6	574	1	1	1	1	**0**
P25-7	481	0	0	1	0	1
P25-8	412	1	1	1	1	0
P25-9	354	0	0	**1**	0	0

0: Absent. 1: Present.

Table (2): Continue.

Fragments #	Fragment sizes (pb)	S1	S2	S3	S4	S5 (Cont.)
P27-1	937	1	0	0	0	1
P27-2	856	0	0	0	**1**	0
P27-3	800	1	1	1	1	0
P27-4	715	0	0	0	0	**1**
P27-5	667	1	1	1	1	1
P27-6	548	1	1	1	1	1
P27-7	516	1	1	1	1	**0**
P27-8	467	**1**	0	0	0	0
P27-9	451	0	1	1	1	0
P27-10	389	0	**1**	0	0	0
P27-11	357	1	1	0	0	0
P27-12	347	0	0	1	1	0
P27-13	337	0	0	0	0	**1**
P27-14	300	0	0	**1**	0	0
P27-15	274	0	0	0	**1**	0
P27-16	238	0	1	1	1	0
P35-1	781	0	0	0	0	**1**
P35-2	656	0	0	0	0	**1**
P35-3	541	0	0	1	1	1
P35-4	518	1	1	1	0	0
P35-5	449	1	1	0	1	1
P35-6	391	0	0	1	1	0
P35-7	385	1	1	0	0	0
P35-8	349	0	0	0	0	**1**
P35-9	313	**0**	1	1	1	1

Table-3: Total amplified fragments (TAFs); polymorphic fragments (PFs) and dissimilarity percentage of six ISSR primers used to determine the effect of fast neutron irradiation on the DNA genome of *V. faba* seeds by using ISSR-PCR.

Primers	TAFs	PFs	Dissimilarity (%)	Band range (bp)	
				From	To
IS-1	5	1	20.00	179	606
IS-2	8	5	62.50	200	1256
IS-3	14	11	78.50	218	996
IS-4	9	7	77.75	354	1180
IS-5	16	14	87.50	238	937
IS-6	9	9	100.0	313	781
Total	61	47	77.04		

Table-4: Dissimilarity percentage between different treated and untreated seeds of *V. faba* (variety name) based on ISSR-PCR marker.

Samples	S1	S2	S3	S4	Control
S1	0				
S2	27	0			
S3	38	27	0		
S4	31	28	33	0	
Control	44	47	45	42	0

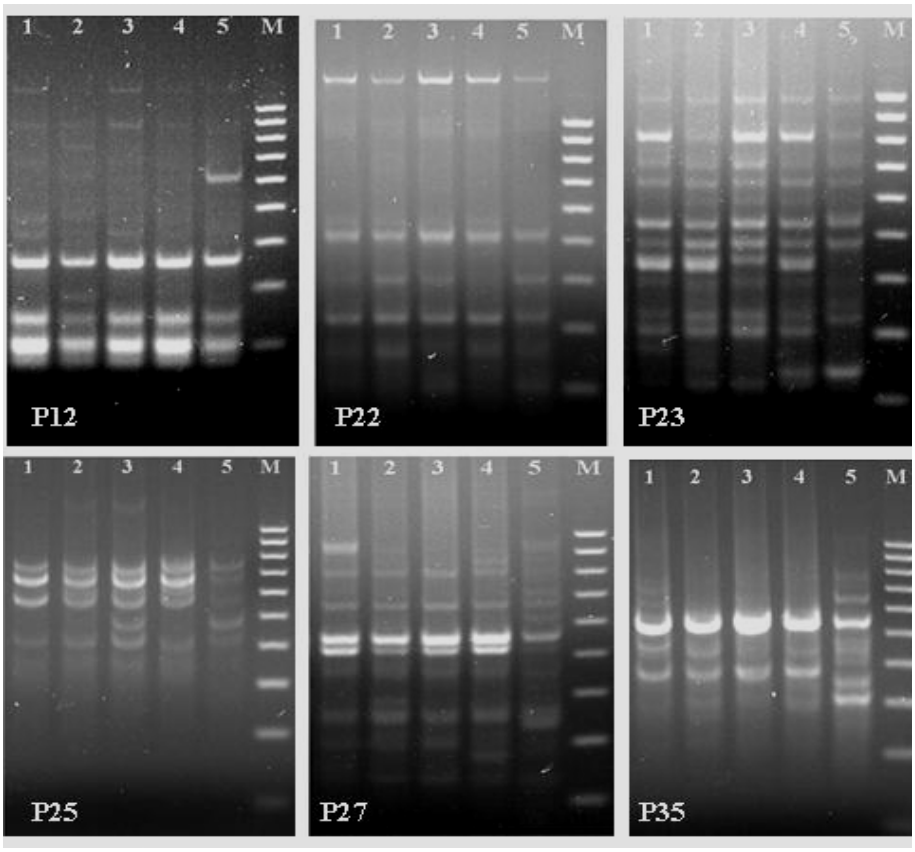

Figure-1: 2.2% agarose gel electrophoresis shows DNA polymorphisms of ISSR-PCR of broad bean seeds treated with different doses of fast neutron irradiation (S1, S2, S3 and S4) and control (untreated broad bean seed, S5) using six ISSR primers.

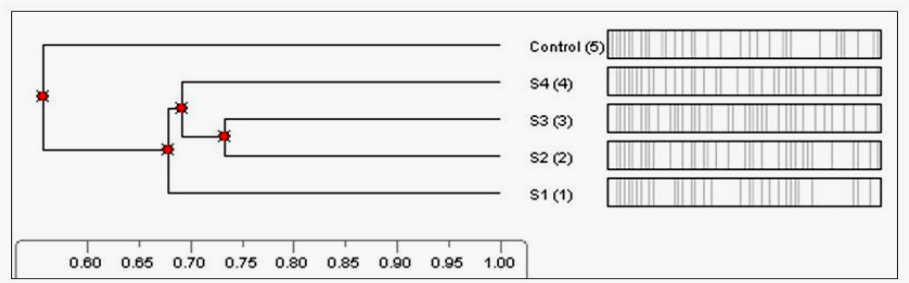

Figure-2: A dendrogram shows the similarities between four broad bean seeds treated with different doses of fast neutron irradiation (S1, S2, S3 and S4) and control (untreated broad bean seed, S5) based on ISSR-PCR using 6 ISSR primers.

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