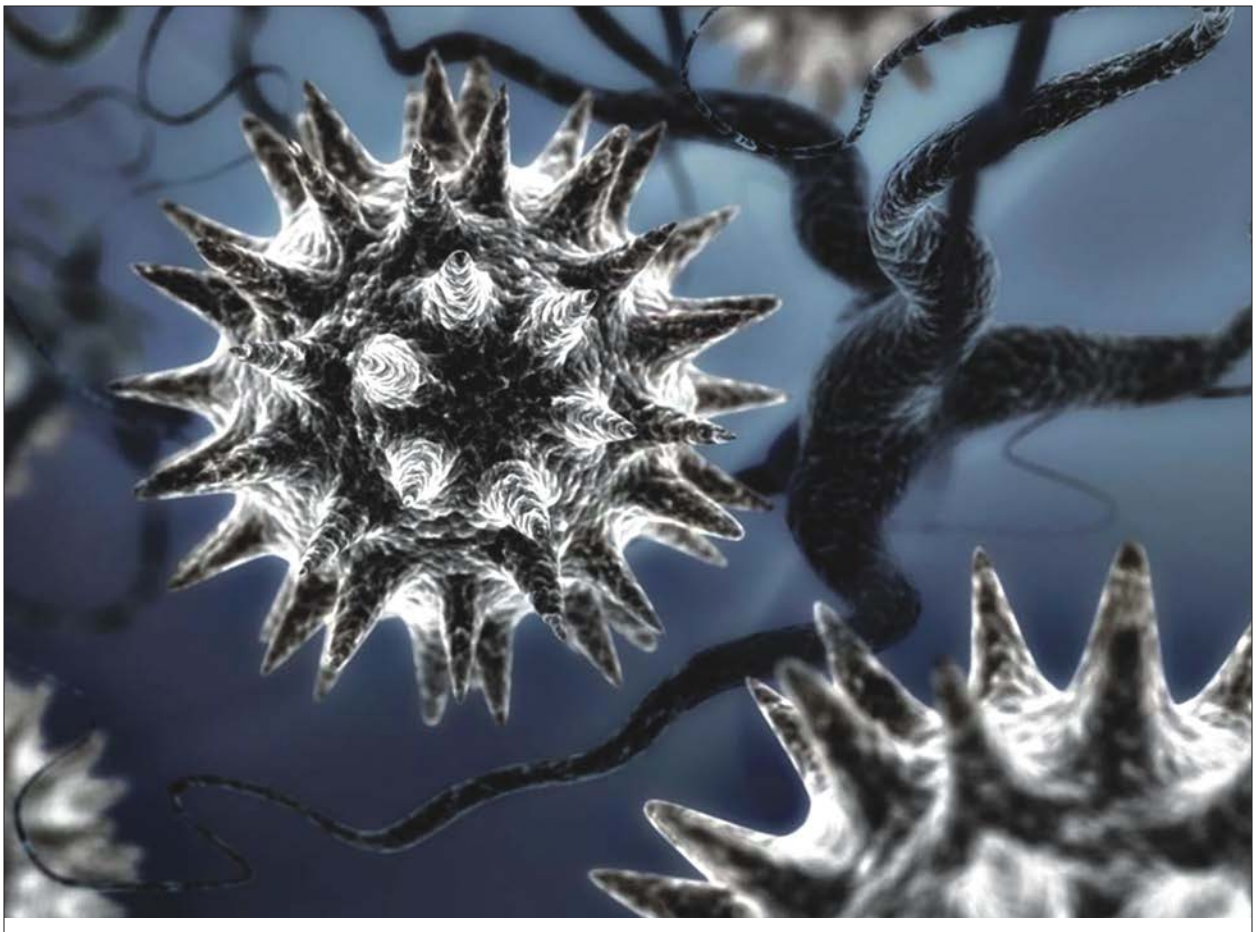

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Monitoring of Microtubers Virus Tested-Derived Potato Tissue Culture by DNA Fingerprint Analysis

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ABSTRACT

A simple ISSR-PCR as a routine method of microtuber PVY tested derived potato plantlets for somaclonal variations is aperquist for pricise monitoring of quality control during rapid mass micropropagation and effective management of microtubers genetic resoures. This study reports on the use of ISSR-PCR for detection of genetic variations in micripropagated potato plants. Microtubers PVY tested derived potato plantlets were screened using ISSR-DNA markers. Three ISSR primers were chosen as producing polymorphic DNA fragments differentiating the investigated plantlets and microtubers *in vitro*. Deoxyribonucleic acid fingerprint revealed genetic variations, 40% polymorphisms of therapeutic plantlets, approximately 50% of the analyzed potato plantlets with 4.5 polymorphic fragments per primer. While the DNA was isolated from microtubers produced using jasmonic acid and coumarin after ISSR amplification it was obvious that microtuber identical fragments profile. The frequency of somaclonal variations was found to be virus therapeutic and microtuberization inducers. The somaclonal variations were only detected in high jasmonic and coumarin concentrations. Although, minor morphological variations were recorded in the microtubers of some clones. The developed fragments profiles of different micropropagated clones were typical to that of the donor mother plants.

Key words: DNA fingerprint, ISSR-PCR, microtuber inducers, virus therapeutic

INTRODUCTION

The potato (*Solanum tuberosum* L.) is the fourth most important food crop worldwide after wheat, maize and rice (Salazar, 1996). Egypt also ranks among the world top potato exporters. Potato are subject to more than 40 viruses diseases (Jefferies *et al*., 2005). Viruses causes economic losses in potato yield depend on the virus, strain and cultivars, 15-25% by mild strain up to 65% by a severe strain.

Tuber-bearing *Solanum* sp., resistance to viruses failed to provide immune species (Singh *et al*., 1994). However, plants which are symptomless (tolerant) or resistant by mechanical inoculation were observed indicating that there was variation in reaction to virus.

Elimination of PVY by tissue culture technique is an important part of any general control program. A high percentage of potato virus free plantlets were obtained by meristem-tip culture (Cassels and Long, 1982).

Potato cultures used in production the Microtubers *in vitro* under controlled conditions. It is considered as an alternative method for the micropropagation of potato pathogen free, cultivars, tuberization *in vitro* is affected with several factors such as growth regulators in medium and environmental factors.

Presently, there are various methods available which can be used to detect and monitor tissue culture derived plants and cultivar identification, the most relable methods are the molecular marker techniques that identity the variance depending on the plant proteins, which are expressed from defined regions of DNA, or DNA polymorphisms. Inter-Simple sequence Repeats is a powerful technique for identification of genetic variation (Nagaoka and Ogihara, 1997). It has the distinct advantage of being technically simple and quick to perform requiring only small amounts of DNA compared to restriction fragments length polymorphism (RFLP) analyis (Welsh *et al*., 1991).

In the present study, the primary objectives were to use ISSR to examine the genetic integrity and uniformity of the important tissue culture and microtubers-derived PVY infected potato plants. In addition elimination of PVY via therapeutic methods (meristem-tip, chemotherapy and thermotherapy).

MATERIALS AND METHODS

Plant materials: PVY infected potato tubers cvs Diamond, Sponta and Lady Rossitti were obtained from Virology Lab. Microbiol. Dept. Fac. of Agric. Ain Shams Univ. The potato tubers were serologically tested by DAS-ELISA using specific polyclonal antibody.

***In vitro* propagation of potato plantlet:** Shoot tips were separated from tubers sprouts sterilized and cultured onto MS medium supplemented with (0.1 mg L^{-1}) thiamine-Hcl, (0.5 mg L^{-1}), Nicotinic acid (0.5 mg L^{-1}) Pyridoxin-HCL (0.5 mg L^{-1}) Glycine (1.0 mg L^{-1}) IAA and (2.5 mg L^{-1}) Kinetin, 30 gm L^{-1} Sucrose and 12 gm L^{-1} Bacto-Difco agar, the plantlets were multiplied *in vitro* as nodal cuttings in jars up to 5 subcultures intervals 21 days under 25°C and 2000 lux for 16 h light days.

Therapeutic

Meristem-tip culture: Twenty five potato sprouts (3-5 cm length) under the steromicroscope, the outer and primordia leaves were removed until the 2 youngest primordia leaves. The dome was excised with 0.2 to 0.3 mm length by scalpel. Excised dome meristems were cultivated on MS medium containing (0.1 NAA+0.5 Kinetin+2.25 mg L^{-1} Phytagel) then incubated for 21 days at 25°C and 2000 lux for 16 h light day. The plantlets were multiplied as nodal cutting in jars up to 5 subcultures.

Chemotherapy: The nodal cuttings of plantlets were transferred to MS medium containing 15, 30 and 45 mg L^{-1} concentrations of each virazole and 2 thiouracil as antivirus separated. The jars were incubated for 21 days under 25°C and 2000 lux for 16 h light days.

Thermotherapy: The nodal cuttings of plantlets were transferred to MS medium and incubated at 35°C and 2000 lux (light day) for 60 days (first group) and 75 days (second group).

Combination between chemotherapy and thermotherapy: The nodal cuttings of plantlets were transferred to MS medium containing 30 mg L^{-1} of each virazole and 2 thiouracil separated. The jars were incubated at 35°C and 2000 lux (light day) for 21 days.

The survival percentage was calculated on the plantlets for each therapeutic. The survival nodal cuttings were transferred to MS medium free antiviral and incubated at 25°C 2000 lux^{-1} for 16 h light days. After 4 weeks plantlets were tested against PVY by DAS-ELISA and calculated the percentage of plantlets PVY free of all therapeutic treatments.

DAS-ELISA tested: The mother potato tubers and all plantlets were resulted from tissue cultures of each treatments were tested using polyclonal antibodies specific for PVY by DAS-ELISA.

Tuberization of plantlets-PVY free: Fifty potato nodal cuttings of each cultivar derived from each treatment were transferred on MS liquid medium-hormone free. The cultures were incubated at 25°C 16 h^{-1} daylight. After 3 weeks, the residual of liquid medium was drawn and replaced by 50 mL jar^{-1} of the tuberogenic liquid medium supplement with 8% sucrose, (10 and 20 mg L^{-1}) coumarin and (0.5 and 1.0 mg L^{-1}) jasmonic acid. The cultures were incubated at 20°C 8 h^{-1} lightdays. After 10 weeks microtubers were harvested and calculated the number, size and weight per plantlet.

Statistical analysis: The differences among of means results were compared by Duncan's multiple range test (Duncan, 1955).

Genomic DNA isolation: Deoxyribonucleic acid of the potato plantlets-PVY tested derived therapeutic treatments and tuberization inducers was extracted following the method described by Kang and Yang (2004).

ISSR-PCR amplification: Three 11-meroligonucleotide primers (manufactured by Bioneer New technology certification from ATS Korea) were randomly chosen for this study. Polymerase Chain Reaction reactions were performed in a total volume of 10 μL of 10 X reaction buffer containing 2 mm $MgCl_2$, 2 μL dNTP at 0.2 mM, 0.1 uL (0.5 U) of taq DNA polymerase (Promega USA), 30 ng of genomic template DNA and 10 p moL primer (HB13, HB14 and HB15) in a UNOII thermocycler from Biometra.Polymerase Chain Reaction was initiated by a denaturation step at 94°C for 2 min and then the reaction was subjected to 45 cycles of 94°C for 30 sec; 44°C for 45 sec, 72°C for 1 min with final elongation step for 20 min at 72°C. In order to select the optimal conditions of the ISSR-PCR, different optimization were carried out.

Visualization and analysis of ISSR-PCR products: The amplified products were resolved by electrophoresis on a 1.5% agarose gel with ethidium bromide and visualized under UV. The presence and absence of fragments between samples were scored and data were transcribeted into binary fragment (1, 0, respectively). Based on the matrix of genetic similarity, cluster analysis was performed. The UPGMA method (unweighted pairgroup method with arithmetic averages) was used for clustering employing the NTSYS pc program (Bohlf, 2001).

RESULTS

Potato tubers (100 from each Diamont, Sponta and Lady Rossitti cultivars) were serologically tested by DAS-ELISA against PVY, PVX and PLRV. The results are revealed that 10, 8, and 11 out

Fig. 1: Shoot growth developing from 5 subculture of meristem tip on M.S medium supplemented with jasmonic acid and coumarin

Table 1: Percentage of plantlets survival and PVY free derived PVY infected potato tubers by tissue culture

*Therapeutic treatments		**Plantlets survival (%)		
Meristem Tip	0.3 mm	Healthy (78)	PVY infected (78)	***Plantlets-PVY free (%) (84)
Chemotherapy				
Virazole	15	100	85.7	61.5
	30	87.5	78.5	75.3
	45	75.0	51.3	87.7
**2-Thiouracil	15	92.5	65.3	62.5
	30	65.1	43.5	65.3
	45	35.5	26.5	83.5
Thermotherapy	35°C/4 weeks	95.1	85.0	57.5
	35°C/6 weeks	80.2	77.5	80.5
Combination	15 mg L^{-1} and 35°C/4 weeks	98.2	95.5	91.5

*Jar containing about 20 plantlets and 10 jars replicates for each treatment,** plantlets survival $total^{-1}$ treated plantlets, *** Plantlets PVY free $total^{-1}$ plantlets survival

of 100 tubers were infected with PVY only and free from PVX and PLRV for three cultivars, respectively. On the other hand, the other tubers were found mixed infection with three viruses (PVY, PVX and PLRV). PVY infected tubers used to micropropagation plantlets free from PVY and production microtubers using biotic inducers.

Meristem tips size 0.3 mm were cultivated on MS medium and incubated under convenient conditions, after 5 subcultures the meristems were developed to shoot growth Fig. 1. The survival and potato plantlets PVY free were 78 and 84%, respectively. The results were confirmed by DAS-ELISA (Table 1). Incorporation of Virazole and 2-thiouracil culture medium at conc. 15, 30, and 45 mg L^{-1} progressively increased the percentage of PVY-free plantlets with 61.5, 57.3, 87.7 62.5, 65.3 and 83.5, respectively. On the contrary, the percentage of platelets survival were decreased with increasement conc. of Virazole and 2-thiouracil (Table 1). The effect of Virazole proved to be some what phytotoxic in highest conc severe stunting, thin stem of plantlets, stunted leaflets and leaf narrows at 45 mg L^{-1}. While 2-thiouracil cau se deleterious effects on growth of plantlets shoots. It was observed that the phytotoxic effects was removed when subcultured on fresh medium without antiviral, these results were confirmed by DAS-ELISA.

Fig. 2: Microtubers showing growth abnormalities long, cylindrical narrow shapes, smooth skinned and increasing the eyes

Table 2: Potato microtubers production by tuberization inducers derived tissue culture

		Microtuber yield characteristics		
Tuberization inducers	Concentration (mg L^{-1})	Number	Size (mm)	Weight (gm)
Control	--	20.5^{a}	1.5^{a}	2.5^{b}
Jasmonic acid	0.5	25.4^{b}	1.8^{a}	1.7^{a}
	1.0	21.2^{a}	2.5^{ab}	2.3^{ab}
Coumarin	10.0	20.0^{a}	5.2^{c}	4.5^{c}
	20.0	45.3^{c}	4.5^{c}	2.8^{ab}

Each jar containing about 20 plantlets, yield characteristics calculated per jar. The results of yield were calculated average of 10 jar replicates

Potato plantlets incubated at 35°C on 2000 lux at 16 light days for 4 and 6 weeks were survived with 85 and 77.5% respectively. Whenever PVY elimination percentage were 57.5 and 75% respectively (Table 1). These plantlets were indexed for PVY isolate by DAS-ELISA.

The potato microtubers were produced from plantlets on MS medium incorporation with different jasmonic acid and coumarin concentration (Table 2). It was found that coumarin (20 mg L^{-1}) and (1.0 mg L^{-1}) jasmonic gave highly significant increasing in yield characteristics (number, size and weight) of microtubers. Where Coumarin (10 mg L^{-1}) and jasmonic acid (0.5 mg L^{-1}) gave non-significant increasing compared with MS medium without tuberization inducers (Table 2). On the other hand it was observed that, the high conc. of inducers due to increasing microtubers number on the contrary the low conc. of inducers due to decreasing microtubers size (Table 2). These tubers showing growth abnormality, long, cylindrical narrow shapes, smooth skinned and increasing the eyes (Fig. 2).

On based on the phenotypic polymorism which was clearly observed in microtubers *in vitro* (tisse culture derived plant lets cvs. Diamond, Sponta and Lady Rossitti a simple molecular marker for the identification of genetic stability or variation among tissue culture derived plants is required.

Preliminary ISSR analysis technique was performed to standaralize a reproducible protocol for potato genome analysis. It was found the PCR conditions, especially thermal profiles and source of tag polymerase resulted in disappearance or appearance of DNA fragments.

Table 3: DNA fragments amplified using ISSR primer by PCR of microtubers cv diamond derived tissue culture plantlets treated with comarin

	P1				P3				P11				
MW (bp)	C	M	T	Ch	C	M	T	Ch	C	M	T	Ch	Polymorphism
1272	-	-	+	+	-	-	-	+	-	+	+	+	Polymorphic
1150	+	+	-	+	+	+	-	-	+	-	+	+	Polymorphic
975	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
720	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
685	+	+	+	+	+	+	+	-	+	-	-	+	Polymorphic
422	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
352	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
186	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
Total (8)	7	7	7	8	7	7	6	6	7	6	7	8	

Table 4: DNA fragments amplified using ISSR primer by PCR of microtubers cv. sponta derived tissue culture plantlets treated with comarin

	P1				P3				P11				
MW (bp)	C	M	T	Ch	C	M	T	Ch	C	M	T	Ch	Polymorphism
1321	+	+	-	-	-	+	+	+	-	-	+	+	Polymorphic
1242	+	-	-	-	-	-	-	-	-	-	-	-	Unique
1178	-	-	-	-	-	-	-	-	-	-	-	+	Unique
985	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
735	-	+	-	-	-	-	-	-	-	-	-	-	Unique
621	+	+	-	+	+	-	-	-	+	+	+	+	Polymorphic
542	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
421	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
321	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
245	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
175	+	+	-	+	-	+	-	+	-	-	+	+	Polymorphic
Total (11)													

ISSR-PCR analysis were performed using three ISSR primers (primer-1, GAC (CAA)5, primer-3, CTG (AG) 8 and primer-11 (AG)8) of microtubers derived tissue culture plantlets treated with jasmonic acid and comarin for three potato cvs. diamond, sponta and lady rossitti. On the basis of the number (Table 6).

Intensity and reproducibility of ISSR fragments three primers were selected out of 10 intensity and reproducibility of ISSR fragments three primers were selected out of 10 ISSR primers which were previously tested (Data not shown). Fragments with the same mobility were treeted as identical fragments. Weak fragments with negligible intensity and smear fragments were both excluded from final analysis. Figure 2 and Table 3 and 4 demonstrate, the ISSR profiles obtained with three primers. The number of scored fragments were 14, 13 and 13 fragments per diamond, spounta and lady rossitti treated with jasmonic acid respectively with different molecular weight ranged from 157.5-125 bp, as well as 8, 11 and 10 fragments per diamond, spounta and lady rossitti potato cvs (Table 5, 9). Treated with comarin respectively with different molecular weight were ranged from 1321-125 bp. The DNA amplified fragments of microtubers resulted from potato

Table 5: DNA fragments amplified using ISSR primer by PCR of microtubers cv. diamond derived tissue culture plantlets treated with jasmonic acid

	P1				P3				P11				
MW (bp)	C	M	T	Ch	C	M	T	Ch	C	M	T	Ch	Polymorphism
1575	-	-	-	-	-	-	-	-	-	-	-	+	Unique
1350	+	+	+	+	-	+	-	-	-	+	+	+	Polymorphic
1225	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
1150	-	+	-	-	-	-	-	-	-	-	-	-	Unique
975	+	+	+	+	+	-	+	+	+	+	-	+	Polymorphic
825	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
700	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
625	-	-	+	-	-	-	-	-	-	-	-	-	Unique
550	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
425	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
325	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
245	-	-	-	-	-	-	+	-	-	-	-	-	Unique
200	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
125	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
Total ( )	10	11	11	9	9	9	10	9	9	10	9	11	

Table 6: DNA fragments amplified using ISSR primer by PCR of microtubers cv. sponta derived tissue culture plantlets treated with jasmonic acid

	P1				P3				P11				
MW (bp)	C	M	T	Ch	C	M	T	Ch	C	M	T	Ch	Polymorphism
1250	+	+	-	-	+	+	-	+	-	-	+	+	Polymorphic
1125	+	+	-	+	-	+	+	+	-	+	-	-	Polymorphic
1072	-	-	-	-	-	-	-	-	-	-	+	-	Unique
985	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
825	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
734	-	-	-	-	-	-	-	+	-	-	-	-	Unique
635	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
570	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
422	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
371	-	+	-	-	-	-	-	-	-	-	-	-	Unique
250	-	-	-	-	-	-	-	+	-	-	-	-	Unique
210	+	+	+	+	+	+	+	-	+	+	-	+	Polymorphic
175	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
Total	9	10	7	8	8	8	8	10	7	8	8	8	

plantlets PVY tested and sanitary with therapeutics merastem tip, thermotherapy and chemotherapy compared with healthy ones (controp treatment) were varied in number intensity and molecular weight.

The gentic stability among microtubers of three potato cultivars treated with jasmonic acid and comarin appeared identical DNA fragments (common amplified fragments MAF) with 23 (57.5%) and 17 (58.69%) respectively. The genetic variability of microtubers for three potato cultivars treated with jasmonic acid and comarin appeared the polymorphic amplified fragments (PAF) among potato cultivars were 8 with 20% and 9 with 31% as well as 9 and 3 unique fragments

Table 7: DNA fragments amplified using ISSR primer by PCR of microtubers cv. diamond derived tissue culture plantlets treated with jasmonic acid

	P1				P3				P11				
MW (bp)	C	M	T	Ch	C	M	T	Ch	C	M	T	Ch	Polymorphism
	C	M	T	Ch	C	M	T	Ch	C	M	T	Ch	
1225	-	-	+	+	-	-	-	+	-	-	+	+	Polymorphic
1150	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
975	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
845	-	-	+	-	-	-	-	-	-	-	-	-	Unique
730	+	+	-	+	-	-	+	+	-	-	+	-	Polymorphic
642	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
535	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
432	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
395	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
282	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
210	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
191	+	+	+	+	+	+	+	+	+	+	+	+	Monohicmorphic
105	+	+	+	+	-	+	-	-	+	+	-	-	Polymorp
Total ()	11	11	12	12	9	10	10	11	10	10	11	10	

Table 8: Polymorphism percentage of microtubers derived tissue culture plantlets treated with josmonic acid

	Polymorphism				Genetic markers				
Potato culture	TAF	MAF	PAF	Unique	MW	C	M	T	Ch
Diamond	14	8	2	4	1575	-	-	-	+
					1150	-	+	-	-
					625	-	-	+	-
					245	-	-	+	-
Sponto	13	6	3	4	1072	-	-	+	-
					734	-	-	-	+
Loady rossitti					371	-	-	+	-
					250	-	-	-	+
	13	9	3	10	845	+			
Total	40	23	8	9%					
% Polymorphism		56	20	22.5					

P1, P2, P3: ISSR primers. T: Thermotherapy C: Control, Ch: Chemotherapy MW: Molecular loeight, TAF: Total amplified fragments M: Tip merastim, PAF: Pohymorphic (specific) amplified fragments, MAF: Monomorphic (common) amplified fragments unique: frenetic markers

(genetic markers) with 22 and 10% respectively the genetic markers were 1150 bp formed in sanitary Diamond by merastimic tip, 625 and 245 bp by thermotherapy and 1575 bp by chemotherapy. In spounta cultivar were 1072 and 371 bp sanitary by thermotherapy, 734 and 250 sanitary by chemotherapy. As well as in lady rossitti cultivar were 845 by sanitary by thermotherapy. On the other hand spounta cultivar sanitary by merastimic tip 735 and by chemotherapy 1178 bp and healthy controp was 1242 bp. Only and not appeare genetic markers diamond and lady rossitti potato cultivare treated with comarin (Table 7).

Table 9: DNA fragments amplified using ISSR primer by PCR of microtubers cv. lady rossitti derived tissue culture plantlets treated with

	P1				P3				P11				
MW (bp)	C	M	T	Ch	C	M	T	Ch	C	M	T	Ch	Polymorphism
1225	+	+	-	-	-	-	+	+	-	+	+	+	Polymorphic
1075	-	-	+	+	-	-	+	+	+	-	-	+	Polymorphic
962	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
842	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
675	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
435	-	+	-	-	+	-	-	+	-	+	-	-	Polymorphic
335	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
270	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
175	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
125	+	+	+	+	+	+	+	+	+	+	+	+	Monomorphic
Total ()	8	9	9	8	8	8	9	10	8	9	8	9	

Table 10: Polymorphism percentage of microtubers derived tissue culture plantlets treated with comarin

	Polymorphism				Genetic markers				
Potato culture	TAF	MAF	PAF	Unique	MW	C	M	T	Ch
Diamond	8	3	5	-		-	-	-	-
Sponto	11	3	5	3	1242	+	-	-	-
					1178	-	-	-	+
					735	-	+	-	-
Loady rossitti	10	3	7	-	-	-			
Total	29	9	17	3					
% Polymorphism		30%	60%	10%					

P1, P2, P3: ISSR primers T: Thermotherapy, C: Control, Ch: Chemotherapy MW: Molecular weight, TAF: Total amplified fragments, M: Tip merastim, PAF: Polymorphic (specific) amplified fragments, MAF: Monomorphic (common) amplified fragments, Unique = Frenetic markers, M: DNA ladder C: Control M: Meristem tip T: Thermotherapy Ch: Chemotherapy

In vitro micropropagation of microtubers PVY tested from shoot tip, through the proliferation of meristem tip, treated with thermotherapy and or chemotherapy on MS medium treated with jasmonic acid and comarin incopartion individually on MS medium followed by shoot recovery give rise to two types of the microtubers (Fig. 3). The types can be clearly detected after prolonged periods of subculturing. Normal microtubers comparable to healthy control were the majority of the 3 month old culture and treated with jasmonic acid ( ) or comarin ( ) as a result of prolonged *in vitro* culture the plants were the same phenotypic (Table 8). On the contarary the culture treated with and ..... Mg L^{-1} jasmonic acid and comarin respectively showed variable phenotype. Phenotypic variations inducing microtubers.

In order to comarin the genetic stability or variation (at molecular level) of the microtubers resulted from potato polantlets treated with jasmonic acid and comarin on MS medium (*in vitro*, tissue culture derived) were screened with 3 ISSR primers (Table 10). The DNA was isolated from the level of 3 potato cultivars treated with jasmonic acid and comarin as well as non treated ones. After ISSR amplification, it was obvious that the sanitary potato plantlets treated 40 mg L^{-1} jasmonic acid and mg L^{-1} comarin showed non-identical ISSR profiles which observed polymorphism.

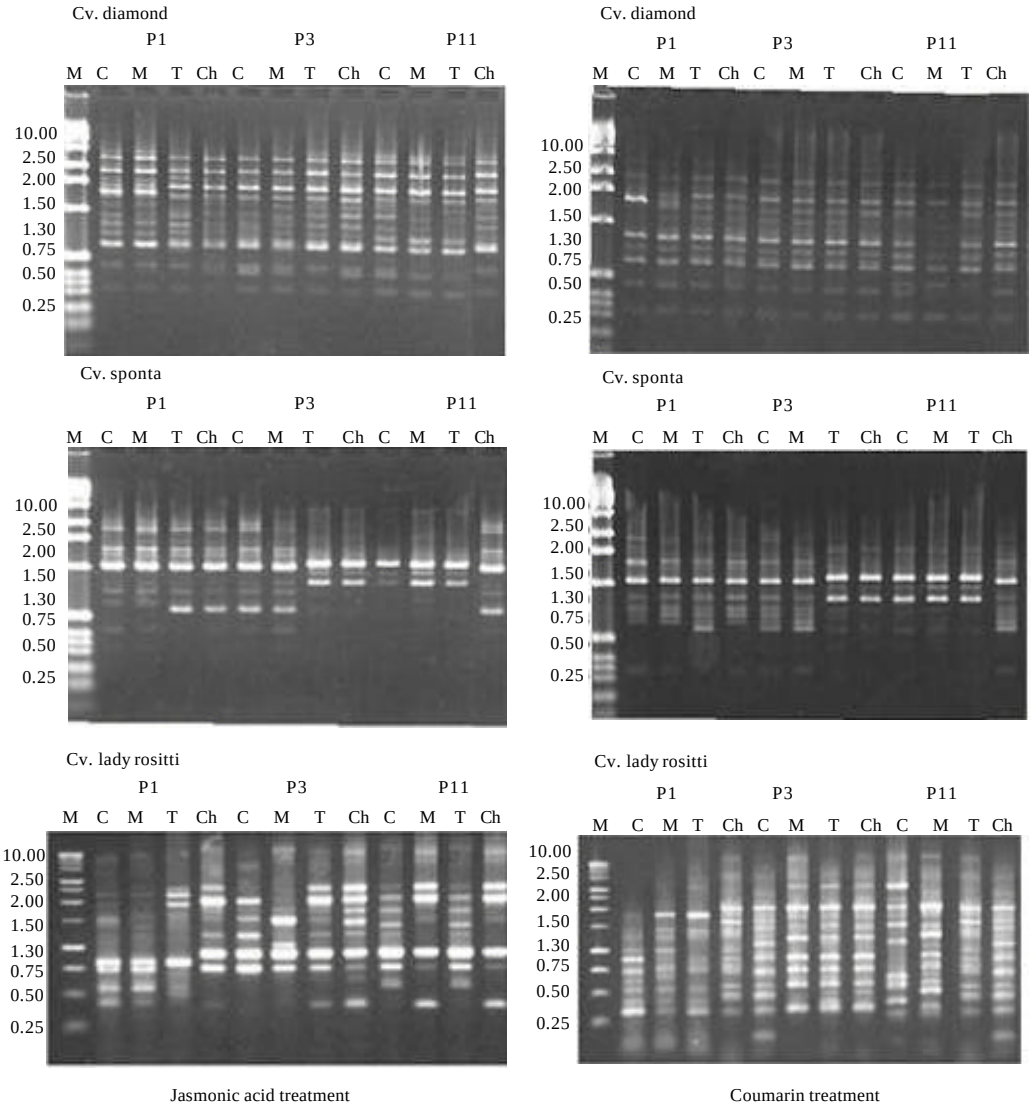

Fig. 3: Agarose gel (1%) electrophoresis analysis of ISSR-PCR amplified products from DNA isolated from microtubers of potato cultivars treated with jasmonic acid and Coumarin using 3 ISSR primers (p1, 3 and 11)

The microtubers produced from potato plantlets which treated with jasmonic acid and coumarin in MS medium, showed variations at morphological level compared with the control plant (mother plants) there were differences in morphological and yield (number, size and weight) microtubers. In order to confirm the genetic stability (at molecular level) oh the *in vitro* vegetatively micropropagated (tissue culture derived) microtubers, the quality of the microtubers tissue culture derived production was screened with the three random ISSR primers. The DNA was isolated form microtubers derived pantalets (produced using the tuberization inducers), after ISSR amplification, it was obvious that the all microtubers (produced using jasmonic acid and coumarin) identical fragment profile (i.e. No. of polymorphism was observed). As on example, Fig. 3 presented the identical fragments patterns of the fragment profiles 9 fragments are observed increase of

microtuber produced from plantlets treated with jasmonic acid and coumarin and their with out jasmonic acid and coumarin of the plantlets derived potato plants cv. diamond were amplified using 10 mer OP3 random primers.

DISCUSSION

Tissue culture techniques and potato breeing are well recognized tools for propagation of certified potato tubers and virus tested (Salazar *et al*., 1985). Although, there are many reference dealing with potto tissue culture e.g. Castro *et al*. (2000), Pruski *et al*. (2001), Arregui *et al*. (2003) and Sherin (2009). The analysis of tissue culture derived plants for somaclonal variation or mutation has yet to be established. ELTSA test was odne to etect PVY in shoot tip of mother plant and micropropagate shoots which are use to prouce microtubers certifie an PVY teste (Sherin, 2009) where the virus infect tissues were serologically reacte specifically with specific PVY antibodies. The specific virus antibodies was use to detect the presence of PVY in plant tissues and enable to detect many samples all at once.

Chemotherapy using thiouracil is an alternative *in vitro* technique traditionally used for virus elimination.

According to phytotoxicity of thiouracil we decided by low concentration 10 or 20 mg^{-1} thiouracil, while 30 or 40 mg^{-1} decreased survival rate and increased the percentage of PVY elimination. Antiviral compounds is too closely linked with normal metabolic processes in plants further some of substances that inhibit viruses appear to be phytotoxic or cause mutation in the treated plants.

Chemical analogous to purine and pyrimidine bases of nucleic acid that has been extensively tested against both DNA and RNA viruses. Many investigators demonstrated that their incorporation in culture media result in an increased percentage of virus free progeny. These chemicals prove to be effective in animal cell cultures by reucing the availability of ATP for DNA and RNA protein synthesis and GTP erivatives such as GP-glucose inactivation of intact virus particles presence inacell by breaking of their RNA or DNA (Kanovalov, 1990).

Screening of the thiouracil concentrations for virus elimination using tissue culture reveled that banding profiles obtained by three ISSR primers (primer-1, GAG (CAA)5, primer-3, CTG (AG)8 and primer-11, (AG)8) were enough to disting uishall the concentrations. The results indicated that ISSR technique is effective to develop genotype-specific banding patterns valuable for the mutant identification of the obtained resuts confirm. The usefulness and stability of ISSR markers for genetic variation etection. Our results are in agreement with Bornet *et al*. (2002). Who used ISSR markers to detect and assess the level of somaclonal variation, in tissues culture-erived potato plants, they have confirmed the value of ISSR markers for potato plants-derived tissue culture and selection of subbulture suitable for transplanting of genetic stability.

Since ISSR technique dose not require previous DNA sequence information and uses very small quantity of DNA, it is considered as one of the most widely used techniques for genetic diversity studies. However, there is a problem with ISSR regarding its reproducibility. The reproducibility of amplification profiles of ISSR is influence by any variation in the method used for DNA isolation (Bornet and Branchard, 2001), concentration or template DNA and primer. Tag DNA polymerases concentration, temperature of annealing number thermal cycles and mgclz concentration (Zietkiewicz *et al*., 1994). Several researchers have reported that the majority of ISSR ban are reproducible if one take care in developing a standardized protocol which is strictly followed in each

reaction (Meyer *et al.*, 1993). In order to ensure high ISSR reproducibility, it is essential to optimize then PCR reaction ISSR has been used for genetic stability and somaclonal variation in potato tissue culture (Sherin, 2009).

In our study, only three ISSR primers (20%) were able to generate polymorphisms among potato subculture on MS media. This results is in accord with what have found by Sherin (2009), where only three primers sufficient to identify all the studied material.

In this study, 11 primers were used in ISSR analysis to prove the clonal fidelity (i.g. genetic stability) of the tissue culture derived potato plants.

Identical banding patterns were observed with all primers tested. These results confirmed the genetic stability of the tissue culture derived potato plants. Molecular markers are believed to be reliable in monitoring variability at the DNA level in plants. ISSR technique was used by several research groups to examine genetic variability and it has been found to be very efficient an reliable (Nagaoka and Ogihara, 1997). As found in the present study, various investigators have observed the absence of variations in potato (Sherin, 2009) using ISSR technique, on the contrary, somaclonal variations were foundd in banana (El-Dougdoug *et al.*, 2007) and peach (Hashmi *et al.*, 1997).

In contrast to the ISSR resuls, minor morphological variations were observed in case of the leaves of tissue culture derived potato plants. The variability observed at the morphological level be caused by the clonal growth habit of the potato (Sherin, 2009).

These results mean that molecular tools are more reliable than the phenotypic observations for evaluating variations an monitoring genetic stability. It also highlights the need for alternative methods of definitive identification based on molecular techniques such as ISSR or amplified fragment length polymorphism (AFLP).

We demonstrated that ISSR analysis can detect sufficient polymorphism to differentiate among micropropagation of potato plants tissue culture derived potato plants and that it is suitable for studying their genetic relationships. This study is considered as a useful report on the assessment of genetic variability of cultivated potato genotypes by ISSR molecular markers. Our results showed a much higher level of genetic variability among subcultures of tissue culture-derived potato plants. While no variation was detected among the free subculture of tissue culture derived potato plants, which indicated higher genetic stability within each cultivar (Bornet *et al.*, 2002; Sherin, 2009).

There fore, the results of molecular characterization of potato cultivars and their genetic relationships provide important parameters for breading and can be used in the further development of new potato cultivars.

REFERENCES

Arregui, L.M., J. Veromendi and A.M. Mingo-Castel, 2003. Effect of gelling agents on *in vitro* tuberiztion of six potato cultivars. Am. J. Potato Res., 80: 141-144.

Bohlf, F.J., 2001. NTSYS-PC: Numerical Taxonomy and Multivariate Analysis System. Exeter Publishing Ltd., Setauket, New York.

Bornet, B. and M. Branchard, 2001. Nonanchored inter simple sequence repeat (ISSR) Markers: Reproducible and specific tools for genome fingerprinting. Plant Mol. Biol. Rep., 19: 209-215.

Bornet, B., F. Goraguer, G. Joly and M. Branchard, 2002. Genetic diversity in European and Argentinian cultivated potatoes (*Solanum tuberosum* subsp. *tuberosum*) detected by inter-simple sequence repeats (ISSRs). Genome, 45: 481-484.

Cassels, A.C. and R.D. Long, 1982. The elimination of potato virus X,S, Y and M in meristem and explant cultures of potato in the presence of virazole. Potato Res., 25: 165-173.

Castro, G., G. Abdala, C. Aguero and R. Tizio, 2000. Interaction between jasmonic and gibberellic acids on *in vitro* tuberization of potato plantlets. Potato Res., 43: 83-88.

Duncan, D.B., 1955. Multiple range and multiple F test. Biometrics, 11: 1-42.

El-Dougdoug, K.A., H.M.S. El-Harthi, H.M. Korkar and R.M. Taha, 2007. Detection of somaclonal variations in banana tissue culture using isozyme and DNA fingerprint analysis. J. Applied Sci. Res., 3: 622-627.

Hashmi, G., R. Huettel, R. Meyer, L. Krusberg and F. Hammerschlag, 1997. RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. Plant Cell Rep., 16: 624-627.

Jefferies, C., H. Barker and S.M.P. Khurana, 2005. Potato Viruses (and Viroid) and Their Management. In: Potato Production Improvement and Post-Harvest Management, Jefferies, C., H. Barker and S.M.P. Khurana (Eds.). Haworth's Food Products Press, New York.

Kang, T.J. and M.S. Yang, 2004. Rapid and reliable extraction of genomic DNA from various wild-type and transgenic plants. BMC Biotechnol., Vol. 4. 10.1186/1472-6750-4-20

Kanovalov, G.I., 1990. Application of virus desinhibitors for elimination of potato infection using the method of tissue culture, vastsi-Akademi. Navik. BSSr. Seryye. Biyalagich Nykh. Novilk, 6: 170-220.

Meyer, W., T.G. Michell, E.Z. Freedman and R. Vilgalys, 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. J. Clin. Biol., 31: 2274-2280.

Nagaoka, T. and Y. Ogihara, 1997. Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor. Applied Genet., 94: 597-602.

Pruski, K., P. Duplessis, T. Lewis, T. Astatkie, J. Nowak and P.C. Struik, 2001. Jasmonate effect on *in vitro* tuberization of potato (*Solanum tuberosum* L.) cultivars under light and dark conditions. Potato Res., 44: 315-325.

Salazar, L.F., 1996. Potato Viruses and their Control. International Potato Center, Peru, Pages: 214.

Salazar, L.F., L. Schilde-Rentschler and R. Lizarraga, 1985. Elimination of Potato Spindle Tuber Viroid from Potato by Cold Treatment and Meristem Culture. In: Subviral Pathogens of Plants and Animals: Viroids and Prions, Maramorosch, K. and J.J. McKelvey (Eds.). Academic Press, Orlando, FL., USA., pp: 137-150.

Sherin, A.M., 2009. Molecular studies on potato spindle tuber viroid. Ph.D. Thesis, Faculty of Agriculture, Sin Shams University, Egypt.

Singh, M., R.P. Singh and T.H. Somerville, 1994. Evaluation of tuber bearing solanum species for symptomatology, as diagnostic hosts, and source of immunity to potato virus Y necrotic strain (PVYN). Am. Potato J., 71: 567-579.

Welsh, J., C. Peterson and M. McClelland, 1991. Polymorphisms generated by arbitrarily primed PCR in the mouse application to strain identification and genetic mapping. Nucleic Acids Res., 19: 303-306.

Zietkiewicz, E., A. Rafalski and D. Labuda, 1994. Genome fingerprinting by simple sequence repeats (SSR) anchored polymerase chain reaction amplification. Genomics, 20: 176-183.