IN VITRO SELECTION AND IDENTIFICATION OF SOMACLONAL VARIANTS OF WHEAT RESISTANCE TOYELLOW RUST (*Puccinia striiformis*) Via RAPD Markers

Barakat,* M. N. and S.I. Milad

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ABSTRACT

Two in vitro selection methods have been used to examine the potential of using p-fluorophenylalanine (PFP) as in vitro mutagenic agent for developing mutant lines resistant to yellow rust (Puccinia striiformis). Two susceptible wheat cultivars; i.e., Gemmiza-1 and Sakha-69, were chosen for the in vitro selection experiment as they had the highest capacities to regenerate plants from mature embryo cultures. Sakha-61 was chosen because it is known for its resistance to yellow rust disease.

The results of direct in vitro selection method for tolerance to PFP indicated that the relative growth rate of callus and the percentage of embryogenic callus markedly decreased with increasing PFP concentration across wheat cultivars. The data, also, revealed that shoot formation either increased or decreased with increasing PFP levels in all cultivars. The results of stepwise in vitro selection method indicated that the callus weight markedly increased in response to increasing PFP levels. The percentage of shoot formation either decreased or increased with increasing PFP concentration in all wheat cultivars. Results in this study demonstrated that the stepwise method was more effective for shoot formation than the direct selection. The data of an artificial inoculation of the regenerated plants, derived from in vitro selection methods, showed that some somaclones derived from the PFP treatment were found to have mutated resistance to yellow rust disease of wheat. RAPD analyses were used for detecting polymorphism among the somaclonal variants derived from the in vitro selection procedures and the parental plants.

Key words : In vitro Selection, wheat, yellow rust resistance, RAPD markers

INTRODUCTION

The main target of the agricultural policy, in Egypt, is to increase the wheat production, in specific, as well as the other food crops, in general, to decrease the gap between wheat production 50-55%) and the annual requirements (about 12 million tons). In Egypt, wheat rust diseases are still the main factors for either eliminating or decreasing the longevity of the Egyptian wheat cultivars.

During the last three decades, yellow rust disease, caused by the fungal pathogen, *Puccinia striiformis*, was very destructive in different seasons and infected most of the Egyptian wheat cultivars; i.e., in 1967, 1995, 1997 and a slight epidemic that occurred in 1985 and 1998 (El-Daoudi *et al*, 1996).

One of the main objectives of wheat improvement program is to generate genetically diverse germplasm that has high yield potential, wide adaptation and durable resistance to important diseases, such as the rusts. Conventional breeding would probably be more efficient, if aided by modern tools, such as somaclonal variation and molecular markers.

The objectives of the present investigation were to compare two *in vitro* selection procedures for developing yellow rust mutant cell lines from two wheat cultivars and regenerate plants from the selected cells. Besides,the use of Molecular marker tools, such as RAPD method,were used for the detection of genetic polymorphisms among wheat somaclones and their parents.

MATERIALS AND METHODS

In vitro Selection Methods:

Two *in vitro* selection methods have been used to examine the potential of using p-fluorophenylalanine (PFP), as *in vitro* mutagenic agent, for developing mutant lines resistant to yellow rust disease. Two susceptible wheat cultivars; namely, Gemmiza-1 and Sakha-69, were chosen for the *in vitro* selection experiment as they had the highest capacities to regenerate plants from mature embryo cultures. Sakha-61 was chosen because it is known for its resistance to yellow rust disease. Only one medium protocol,which showed the greatest potential for shoot formation , was used for callus induction and ,also, has been used for the *in vitro* selection system. The callus induction medium was MS salts (Murashige and Skoog,1962), supplemented with 100 mg/L Inositol, 0.5 mg/L Nicotinic acid , 0.5 mg/L Pyridoxine – Hcl, 0.1 mg/L Thiamine –Hcl, 0.1 mg/L kinetin ,1.0 mg/L 2,4 –D, 3% sucrose and 0.7 % agar at pH 5.8. The regeneration medium was MS-medium, supplemented with 1.0 mg/L casein hydrolysate, 3% sucrose and 0.7 % agar

Direct *in vitro* selection method, using PFP:

The calli derived from mature embryos were transferred to a medium containing either 0, 100, 200, 300, 400 or 500 uM P-fluorophenylalanine (PFP). Each dish, containing five calli derived from embryo explant, was considered as one replication with ten replications for each genotype. The PFP was added to the medium as a filter sterilized solution of PFP.

Biotechnology Laboratory, Crop Science Department, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

The calli were maintained on each respective treatment for six weeks. The selected cell lines were transferred to the regeneration medium. Then, the *in vitro* traits were recorded.

Stepwise *in vitro* selection method, using PFP: The calli derived from mature embryos were transferred to the medium containing 100 μM/L PFP. They were monthly subcultured on a fresh medium with a gradual increasing concentration of PFP by 100 μM until it reached 500 μM. In the mean time, five replicates with five embryos were taken into regeneration media of each PFP concentration. Then, the calli were transferred to the regeneration medium free of PFP.

***In vitro* traits determination :**

Two *in vitro* traits were recorded before transferring the selected cells to regeneration medium. These *in vitro* traits were:

1- The growth weight of the calli.
2- The percentage of embryogenic callus (recorded for the direct selection method only).

Two *in vitro* traits were recorded after transferring the selected cell lines to the regeneration medium. These *in vitro* traits were:

1- The percentage of calli with green shoots, which exceeded one cm in length.
2- The percentage of calli with root formation.

Regenerated shoots were transferred to MS medium, supplemented with growth regulators for further development.

Statistical analysis :

Data for the selection methods to PFP were statistically analyzed as a two-factor experiment (cultivars and PFP treatments) in a split- plot design, with ten and five replicates, for direct and stepwise selection methods, respectively. All *in vitro* traits data, except for the callus weight, were subjected to arcsine transformation before statistical analysis. Comparisons among means were made, using the LSD test of significance. The data were analyzed by using SAS (1985) program.

Virulence survey of yellow rust in somaclonal variant lines :

Gemmiza-1, Sakha-69 and Sakha-61 wheat cultivars and their somaclonal variant lines were grown under greenhouse conditions at Sakha Agricultural Research Station, Agricultural Research Center (ARC), using the standard controlled environmental conditions of humidity and temperature, which allowed the development of yellow rust, in order to determine their resistance or susceptibility to yellow rust (*Puccinia striifomis*). Tests were carried, using a mixture of the common races of the pathogen under the Egyptian conditions. The plants were ready to be inoculated 7-10 days after sowing. Yellow rust estimation was recorded, using the (0-9) scale adopted by Mc Neal *et al.*(1971).

RAPD analysis for somaclonal variant lines and their parents:

Plant material:

PCR analyses were carried out by using genomic DNA from the wheat cultivars, Gemmiza-1, Sakha-69 and Sakha-61, and their somaclone lines obtained by the *in vitro* selection methods. The wheat cultivars, as well as their somaclone lines, were grown in pots.

DNA extraction:

Frozen young leaves (500 mg) were ground to a powder in a mortar with liquid nitrogen. The powder was poured into tubes containing 9.0 ml of warm (65°C) CTAB extraction buffer. The tubes were incubated at 65°C for 60-90 min. 4.5 ml chloroform/ octanol (24: 1) was added and tubes were rocked to mix for 10 min. and were centrifuged for 10 min. at 3200 rpm. The supernatants were pipetted off into new tubes and 6 ml isopropanol was added. After 60 min., the tubes were centrifuged for 10 min. and the pellets obtained were put in sterile Eppendorf tubes, containing 400 μl of TE buffer of pH 8.0 (10 mM Tris-HCl, pH 8.0 + 1.0 mM EDTA, pH 8.0). The DNA's from genotypes were, then, extracted and stored at -20°C until use.

PCR amplification :

Five primers (Table 6), from Pharmacia Biotech. (Amersham Pharmacia Biotech, UK Limited, England, HP79 NA), were tested in this Experiment to amplify the templated DNA.

Amplification reaction volumes were 25 μl, each containing 1 x PCR buffer with $MgCl_2$ (50 mM KCl, 10 mM Tris-HCl (pH = 9.0), 2 mM $MgCl_2$ and 1% trition x-100), 200 μM each of dATP, dCTP, dGTP and dTTP, 50 PM primer, 50 ng template DNA and 1.5 μ of tag polymerase. Reaction mixtures were overlaid with 15 μl mineral oil and exposed to the following conditions: 94°C for 3 min, followed by 45 cycles of 1 min. at 94°C, 1 min. at 36°C, 2 min. at 72°C and a final 7 min. extension at 72°C.

Amplification products were visualized with DNA marker on 1.6% agarose gel with 1x TBE buffer and detected by staining with an ethidium bromide solution for 30 min. Gels were, then, destained in deionized water for 10 min. and photographed on Polaroid films under UV light.

Data handling and cluster analysis:

Data were scored for computer analysis on the basis of the presence or absence of the amplified products for each primer. If a product was present in a genotype, it was designated "1 ", if absent it was designated "0 "after excluding the inreproducible

bands. Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products, were used to regenerate similarity coefficients, according to Jaccard (1908). The similarity coefficients were,then, used to construct dendograms, using the unweighted pair group method with arithmatic averages (UPGMA) employing the SAHN (Sequential, Agglomerative, Hierarchical and Nested clustering) from the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 1.80 (Applied Biostatistics) Program (Rohlf, 1993).

RESULTS AND DISCUSSION

Direct *in vitro* selection Method for Tolerance to PFP:

Callus weight : The analysis of variance for the effect of cultivars , PFP concentrations used and their interactions on the callus weight are presented in Table 1. Such analysis indicated that the callus weight was highly significantly influenced by differences in cultivars and PFP concentrations. However, the interaction between cultivars and PFP concentrations did not significantly affect callus weight.

Means of callus weight as affected by wheat cultivars and PFP concentrations, are summarized in Table 2. The effect of PFP on callus weight was consistent over the three wheat cultivars. However, the overall mean of Sakha-69 was less and significantly different from the other two cultivars. Furthermore, it was evident from the data (Table 2) that the callus weight significantly decreased in response to increasing PFP concentration (from 0.98 to 0.44 µM). Meanwhile, the interaction was insiginficant between the two studied factors (Table 2).

Embryogenic callus: Statistical analysis of embryogenic callus recorded significant differences among cultivars and PFP concentrations, whereas the interaction between the studied factors was insignificant (Table1).

Differences in response to ability of callus to form a mass of green nodules (embryogenic callus) were observed among different cultivars and PFP concentrations in the present investigation. The highest signigicant percentage of embryogenic callus, across the six PFP treatments,was obtained from the cultivar, Gemmiza-1 (42.73%), and it was significantly superior to the two other cultivars (Table 2). Furthermore, there were no significant differences between the cultivar, Saka-69(22.22%), and the cultivar, Saka-61(24.97), for embryogenic callus across the PFP concentrations (Table 2). The data, also, revealed that the percentage of embryogenic callus markedly decreased with increasing PFP concentration across wheat cultivars (Table 2). That is, from 69.03 to 9.16%.

Shoot formation : Statistical analysis of shoot formation revealed significant differences among cultivars, PFP levels and their interaction (Table 1).

Results in Table 2 showed that the cultivar, Gemmiza-1, produced the highest significant mean of shoot formation (14.41%) across the PFP levels and it was not significantly different from the cultivar Sakha-61(9.16%). The data, also, revealed that shoot formation either increased or decreased with increasing PFP levels in all cultivars. However, shoot formation of Sakha-69 cultivar markedly decreased with increasing PFP levels (Table 2). Moreover, data in Tables (1) and (2) indicated that the interaction was significant between wheat cultivars and PFP levels for shoot formation.The callus of Gemmiza-1 cultivar produced the highest percentage of shoot formation (19.63%) on the medium containing 300 µM PFP level (Table 2), whereas, that of Gemmiza-1 gave a low shoot formation (2.66%) on the medium containing 400 µM PFP level.

Stepwise *in vitro* selection method for tolerance to PFP:

Callus weight: The analysis of variance for the effects of cultivars and PFP concentrations used, and their interaction, on callus weight is presented in Table 3. The analysis indicated that the callus weight was highly significantly affected by cultivars and PFP concentrations and their interaction.

The effects of increasing PFP concentration on callus weight for the three cultivars are shown in Table 4. It was evident that the callus weight markedly increased in response to increasing PFP levels (from 0.44 to 5.62g). However, the increase was relatively higher in the cultivar, Gemmiza-1 (4.95g), than the two other cultivars (2.71 and 1.21g).

Shoot formation: Statistical analysis of shoot formation revealed highly significant differences among cultivars and significant ones among PFP levels (Table 3). However, the cultivar X PFP level interaction was not significant. The effects of increasing PFP concentration on the percentage of shoot formation for the three cultivars are shown in Table 4. It was evident that the percentage of shoot formation either decreased or increased with increasing PFP concentration in all wheat cultivars. However, the callus of Gemmiza-1 cultivar produced the highest percentage of shoot formation (36.70%) on the medium containing 100 µM PFP. Whereas, Sakha-69 gave no shoot formation (0.0%) in case of the medium 200 µM PFP level. Moreover, Sakha-61 cultivar, after being subcultured on the concentration ,300, it lost its capacity for shoot formation and no plant were generated after this subculture on the subsequent concentrations (400 and 500 µM PFP level).

Table 1: Analysis of variance for callus weight (g) embryogenic callus (%)[a] and shoot formation and their interaction, using direct *in vitro* selection method.

Source of Variance	D.F.	M.S.		
		Callus weight (g)	Embryogenic callus[a] (%)	Shoot formation (%)
Cultivar (A)	2	2.568**	7442.08**	820.93*
Error a	27	0.122	225.07	260.83
Concentration (B)	5	1.025**	14384.54**	935.75*
AXB	10	0.052	189.90	260.10*
Error b	135	0.114	330.09	201.82

a : Data were transformed to arcsine scale.
*,** : Significant at 0.05, 0.01 probability levels, respectively.

Table 2: Means of *in vitro* traits, as in fluenced by cultivars,PFP concentrations (µM) and their interaction, using direct *in vitro* selection method.

Cultivars	Concentrations of PFP (µM)						Cultivar mean
	C0	C100	C200	C300	C400	C500	
1- Callus weight (g)							
Gemmiza-1	1.24	0.81	0.96	0.86	0.64	0.68	**0.87**
Sakha-69	0.81	0.41	0.40	0.44	0.37	0.31	**0.46**
Sakha-61	0.89	0.71	0.68	0.47	0.46	0.44	**0.61**
Concentration mean	**0.98**	**0.64**	**0.68**	**0.59**	**0.49**	**0.44**	**-**
L.S.D.$_{(0.05)}$ for cultivar means = 0.13 and concentration means = 0.17							
2- Embryogenic callus (%)							
Gemmiza-1	84.69	46.15	36.35	18.36	18.36	24.82	**42.73**
Sakha-69	63.80	24.70	21.13	15.82	7.85	0.00	**22.22**
Sakha-61	58.61	37.74	26.89	15.94	7.97	2.66	**24.97**
Concentration mean	**69.03**	**36.20**	**31.35**	**22.70**	**11.39**	**9.16**	**--**
L.S.D.$_{(0.05)}$ for cultivar means = 5.98 and concentration means = 9.28							
3- Shoot formation (%)							
	19.63	10.63	15.97	19.63	2.66	16.97	**14.41**
Gemmiza-1	18.47	11.89	7.97	5.31	0.00	0.00	**7.28**
Sakha-69	10.39	14.31	19.63	5.31	0.00	5.31	**9.16**
Sakha-61							
Concentration mean	**16.16**	**12.28**	**14.84**	**10.08**	**0.89**	**7.43**	**--**
L.S.D.$_{(0.05)}$ for cultivar means = 6.05, concentration means = 7.25 and for their interaction = 12.45							

Root formation: Significant levels for the effect of cultivars and PEP levels used, and their interaction, on the percentage of root formation are presented in Table 3. The percentage of root formation was either significantly or highly significantly influenced by the differences in cultivars, PFP concentrations and their interaction.

The effects of increasing PFP concentration on the percentage of root formation for the three cultivars are shown in Table 4. It is evident from the table that the percentage of root formation for the different cultivars either decreased or increased with increasing PFP concentration. The cultivar, Gemmiza-1, had a high percentage of root formation with an average of 75.84%, followed by Sakha-69 (52.12%) and Sakha-61(25.85%) across the PFP levels (Table 4). Moreover, Gemmiza-1 cultivar recorded the highest value (90.0%) under 100,200 and 300 μ M PFP concentrations.

Results in this study demonstrated that the stepwise method was more effective for shoot formation than the direct selection.This might be explained by the gradual adaptation of the tissue to the step by step increase in level of PFP concentration. Similar results have been obtained by Barakat and Abdel-Latif (1996), using three *in vitro* selection methods for NaCl tolerance in wheat callus. They reported that the stepwise method of increasing NaCl in the medium was more effective for plant regeneration than other methods. Recently, Barakat *et al* (2004) reported that stepwise in vitro selection method was more effective than the *in vitro* direct selection method for developing heat tolerant cell lines from wheat cultivars and to regenerate plants from the selected cells.

On the other hand, the prolonged duration of selection might lead to a decrease in regeneration ability, as shown in Sakha-61,its capability of forming shoots was lost after transfering on the concentrations of 300, 400 and 500. This agreed with several investigators (Hartman *et al*, 1984; Barakat and Cocking, 1989). Barakat and Cocking (1989) reported that cultured cells lost their capacity for plant regeneration after several subcultures.Cultured cells frequently contained nuclei, showing various degrees of polyploidy, aneuploidy and chromosome abnormalities. This chromosomal variation often lead to loss of morphogenetic capacity. Therefore, basic information is needed to understand the types of gene action for shoot formation in wheat embryo culture. Barakat (1994) reported that dominance or dominant types of epistais might play a role in the immature embryo culture response to *in vitro* culture traits. In another study, information about the type and magnitude of genetic variation and the relative importance of additive and non-additive gene action types have been reported (Barakat, 1996).

Artificial inoculation of the regenerated plants derived from the *in vitro* selection method:

This section presents the disease scoring after artificial inoculation of the regenerated plants derived from the *in vitro* selection method. Disease estimation was recorded, using the (0-9) scale adopted by Mc Neal *et al* (1971). The results illustrated in Table (5) showed that the somaclones,derived from the control and PFP treatment, were found to have different resistance to yellow rust disease of wheat.

Table 3 : Analysis of variance for callus weight (g), shoot formation [a] (%) and root formation [a] (%), using stepwise *in vitro* selection method.

Source of variance	D.F.	M.S.		
		Callus weight (g)	Shoot formation[a] (%)	Root formation[a] (%)
Cultivar (A)	2	105.81**	2253.181**	18759.61**
Error a	12	1.387	201.350	327.03
Concentration (B)	5	66.677**	634.647*	1751.50*
AXB	10	13.846**	408.652	1751.28**
Error b	60	1.337	240.447	704.87

a : Data were transformed to arcsine scale.

*, ** : Significant at 0.05 and 0.01 probability level,respectively.

Table 4: Means of *in vitro* traits as influenced by cultivars, PFP concentrations (μM) and their interaction.

Cultivars	Concentrations of PFP (μM)						Cultivar
	S_0	S_{100}	S_{200}	S_{300}	S_{400}	S_{500}	mean
			1- Callus weight (g)				
Gemmiza-1	0.30	2.31	2.81	5.62	8.63	10.0	**4.95**
Sakha-69	0.49	1.07	1.57	3.23	5.38	4.54	**2.71**
Sakha-61	0.52	0.74	0.95	1.39	1.35	2.32	**1.21**
Concentration mean	**0.44**	**1.38**	**1.78**	**3.41**	**5.12**	**5.62**	--
L.S.D. $_{(0.05)}$ for cultivar means	= 0.66, PFP concentration means = 0.85 and their interaction = 1.95						
			2- Shoot formation (%)				
Gemmiza-1	23.54	36.70	31.39	18.47	26.32	13.16	**24.93**
Sakha-69	25.85	13.16	0.00	5.31	28.85	7.85	**13.50**
Sakha-61	18.47	10.63	18.47	0.00	0.00	0.00	**1.212**
Concentration mean	**22.62**	**20.63**	**16.62**	**7.93**	**18.40**	**7.00**	--
L.S.D. $_{(0.05)}$ for cultivar means	= 7.98 and for PFP concentration= 11.33						
			3- Root formation (%)				
Gemmiza-1	54.00	90.00	90.00	90.00	79.85	51.22	**75.84**
Sakha-69	33.70	54.00	23.79	56.78	79.85	64.63	**52.12**
Sakha-61	28.15	56.31	13.16	20.78	10.63	26.09	**25.85**
Concentration mean	**38.62**	**66.77**	**42.32**	**55.86**	**56.77**	**47.31**	--
L.S.D. $_{(0.05)}$ for cultivar means	= 10.17, concentration means = 19.39 and for their interaction = 44.66						

In most disease resistance mechanism, associated host molecules are products of secondary metabolism. Widholm (1987) proposed the selection of variants disturbed with respect to the appropriate biosynthetic pathway as a novel means of isolating disease –resistant variants. Many classes of resistance compounds were phenolic in nature, synthesized via the shikimic acid pathway. A pivotal step in phenolic biosynthesis was the conversion, catalysed by phenylalanine ammonia-layses, of phenylalanine into f- fluorophenylalanine acid. *In vitro* selection for resistance to the phenylalanine analogue P-fluorophenylalanine isolated cell lines, which over – accumulated phenolics (Palmer and Widholm, 1975; Berlin *et al*, 1982), due to possible overproduction of phenylalanine ammonia-layse. Selection for elicitor-induced phenylalanine analogue P-fluorophenylalanine – resistance may result in localized overproduction of phe nolics in response to pathogen challenge. Berlin *et al* (1982) reported that the resistant mechanisms included: (a) overproduction of phenylalanine and (b) increased activity of phenylalanine ammonia-layse, which could detoxify P- fluorophenylalanine by converting it to a secondary metabolite P-fluorocinnamic acid.

Increased phenylalanine ammonia –layse activity was associated with 2 to12-fold synthesis of phenolic, derived from phenylalanine. *In vitro* selection to fluroro-phenylalanine(PFP) resistant cell lines in higher plant tissue has been reported by several investigators (Palmer and Widholm, 1975; Niizeki and Fukui 1978 and 1983). Palmer *et al* (1975) investigated the effect of PFP in carrot and tobacco cell cultures. They obtained mutants resistant cells to PFP, increased level of phenylalanine and tyrosine were found in the mutant cell lines.

These amino acids were apparently converted into phenolic compounds, which were found to be six times.

Reddy *et al* (1994) estimated the total free phenols in the wheat constituted rust-resistant lines and susceptible recurrent parents. The biochemical estimation revealed that the total free phenols were relatively high in the respective constituted rust-resistant lines,compared with their recurrent susceptible parents.

Table (5): Disease estimation for an artificial inoculation of the somaclones, using the (0-9) scale adopted by Mc Neal *et al* (1971).

Somaclones		Score	Somaclones		Score	Somaclones		Score
cv.	Conc.		cv.	Conc.		cv.	Conc.	
Sk-69	100	0	Sk-69	400	0	Gem-1	100	1
Sk-69	100	0	Sk-61	100	0	Gem-1	100	8
Sk-69	100	5	Sk-61	100	0	Gem-1	200	0
Sk-69	100	3	Sk-61	100	0	Gem-1	200	0
Sk-69	100	8	Sk-61	100	0.	Gem-1	200	0
Sk-69	100	0	Sk-61	100	0	Gem-1	200	0
Sk-69	100	0	Sk-61	200	0	Gem-1	200	0
Sk-69	100	8	Sk-61	200	0	Gem-1	200	0
Sk-69	100	7	Sk-61	200	0	Gem-1	200	8
Sk-69	100	0	Gem-1	100	0	Gem-1	200	0
Sk-69	100	0	Gem-1	100	0	Gem-1	300	0
Sk-69	100	0	Gem-1	100	0	Gem-1	300	6
Sk-69	200	0	Gem-1	100	0	Gem-1	300	7
Sk-69	200	0	Gem-1	100	0	Gem-1	300	0
Sk-69	300	0	Gem-1	100	0	Gem-1	300	0
Sk-69	300	0	Gem-1	100	0	Gem-1	400	0
Sk-69	300	0	Gem-1	100	0	Gem-1	400	0
Sk-69	400	0	Gem-1	100	0	Gem-1	500	0
Sk-69	400	0	Gem-1	100	0	Gem-1	500	0
Sk-69	400	5	Gem-1	100	0	Gem-1	500	0
Sk-69	400	0	Gem-1	100	0			

Cultivar abbreviations:Sk-69 =Sakha-69,SK-61=Sakha-6 and Gem-1= Gemmiza-1.

In vitro selection of somatic wheat calli resistant to toxic fusarium metabolites, using double-layer and culture filterate techniques, were reported by several investigators (Yang *et al*, 1998; Abdalla *et al*, 2002). Inheritance of improved leaf rust resistance in somaclones of wheat have been reported (Oberthur *et al*, 1993). Somaclonal variations of some morphological traits and leaf rust reaction among regenerants, derived from callus of bread wheat cultures,were reported by Chauhan and Singh (1995). Wheat somaclones resistant to common rot and dark brown leaf spot blotch have been detected by using toxins of the common root rot pathogen (Wenzel and Foroughi-Wehr,1990). Similar recent studies were conducted on wheat to obtain *in vitro* variant lines resistant to Karnal bunt (Tando *et al*, 2000).

In conclusion, overall analysis of *in vitro* selection, as a tool to enhance disease resistance in plants, suggests that this technique has a significant potential to produce genetically improved plants (Jayasankar and Dennis, 2003). From the results of the present study, It could be suggested that the developed PFP mutagenesis procedure was found to be effective for isolation of variant lines resistant to yellow rust *In vitro* selection in wheat cell cultures, using PFP,might be valuable in attempts to increase the yellow rust resistance in wheat.

RAPD analysis for wheat cultivars and their somaclones:

Screening of polymorphic primers, among somaclones and their parents,where five primers were used to amplify the genome of the three wheat cultivars, Gemmiza-1, Sakha-69 and Sakha-61 and their somaclones. The five primers studied amplified a total of 58 DNA fragments. All of these amplified fragments were polymorphic. The number of bands, amplified per primer, ranged from eight (primers 1 and 5) to eighteen (primer 4) with a mean value of 11.6 bands per primer (Table 6). These values are rather high for RAPD amplification, compared to the average numbers of amplified bands recorded in other crops; namely, three fragments in *Triticum turgidum* L. (Joshi and Nguyen, 1993a), 4.3 fragments in *Solanum tuberosum* L. (Masuelli *et al*, 1995) and 6.7 in *Zea mays* L. (Heun and Helentjaris, 1993). Barakat *et al* (2000) reported that the same five primers were highly polymorphic across wheat genotypes as well as monogenic lines.

Figure (1) shows the amplification profiles, generated by primer-4 (AGCCAGCGAA) across the wheat cultivars and their somaclones. All of the eighteen scorable bands were polymorphic across the wheat genotypes and their somaclones.

A high level of polymorphism for DNA method was reported by Joshi and Nguyen (1993), but not by Devos and Gale (1992). Use of RAPD markers to determine the genetic diversity of diploid wheat genotypes have been reported by Vierling and Nguyen (1992). They reported that electrophoretic analysis of the amplification products revealed a higher incidence of polymorphism in *T. urartu* than in *T. monococcum*.

Table 6: Number of amplification and polymorphic products, using five primers, in wheat cultivars and their somaclones.

Primer number	Nucleotide sequence (5 `- 3`)	No. of amplification products(a)	No. of polymorphic products (b)	Polymorphism b/a (%)
1	GACCGCTTGT	8	8	100%
2	GGGTAACGCC	14	14	100%
3	TCGGCGATAG	10	9	90%
4	AGCCAGCGAA	18	18	100%
5	AGGTGACCGT	8	8	100%

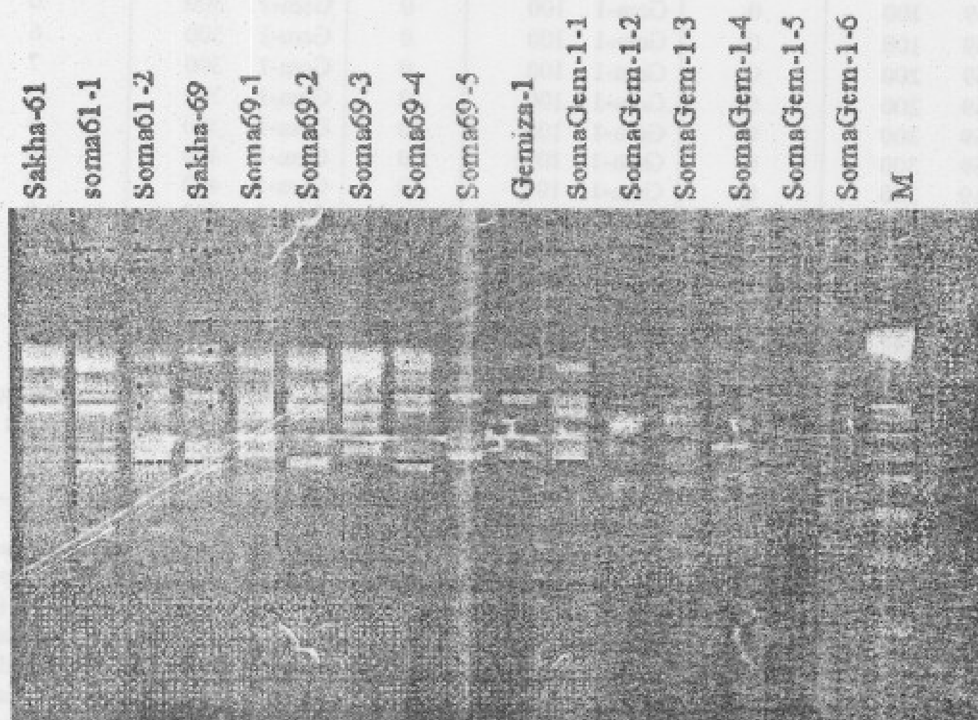

Fig.1 : RAPD polymorphic in wheat cultivars and their somaclones primer No .4 (5 AGCAGCGA 3).

Table 7: Simple matching coefficients of similarity matrix determined from analysis, using five primers that amplified 58 distinct products.

	Sakha-61	Som61-1	Som61-2	Sakha-69	Som69-1	Som69-2	Som69-3	Som69-4	Som69-5	Gemmiza-1	SomG1-1	SomG1-2	SomG1-3	SomG1-4	SomSG1-5	SomSG1-6
Sakha-61	**1.00**															
Som61-1	0.72	**1.00**														
Som61-2	0.49	0.54	**1.00**													
Sakha-69	0.32	0.34	0.47	**1.00**												
Som69-1	0.71	0.67	0.51	0.38	**1.00**											
Som69-2	0.41	0.46	0.40	0.38	0.46	**1.00**										
Som69-3	0.42	0.50	0.44	0.42	0.44	0.67	**1.00**									
Som69-4	0.41	0.49	0.46	0.37	0.46	0.53	0.73	**1.00**								
Som69-5	0.39	0.37	0.37	0.34	0.44	0.56	0.68	0.71	**1.00**							
Gemmiza-1	0.44	0.50	0.35	0.37	0.52	0.46	0.52	0.55	0.48	**1.00**						
SomG1-1	0.26	0.38	0.21	0.23	0.27	0.38	0.42	0.37	0.34	0.46	**1.00**					
SomG1-2	0.41	0.32	0.28	0.28	0.35	0.50	0.43	0.42	0.44	0.38	0.41	**1.00**				
SomG1-3	0.24	0.29	0.20	0.33	0.17	0.29	0.32	0.32	0.29	0.34	0.33	0.34	**1.00**			
SomG1-4	0.24	0.29	0.22	0.24	0.25	0.26	0.29	0.35	0.28	0.39	0.39	0.35	0.52	**1.00**		
SomG1-5	0.33	0.31	0.33	0.40	0.33	0.30	0.42	0.41	0.38	0.41	0.30	0.27	0.48	0.50	**1.00**	
SomG1-6	0.36	0.38	0.38	0.31	0.50	0.34	0.46	0.48	0.42	0.50	0.40	0.41	0.33	0.48	0.44	**1.00**

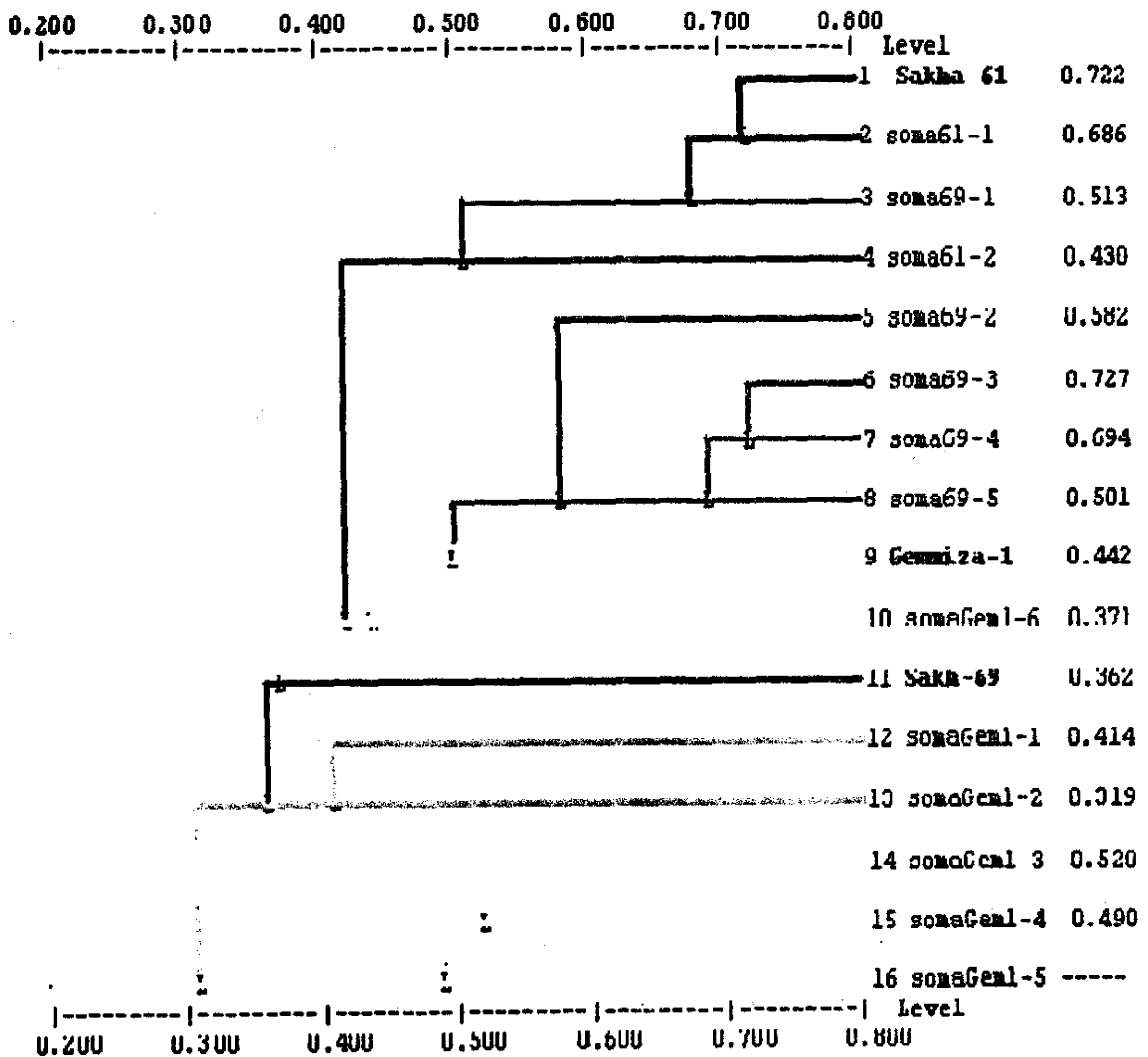

Fig: 2 Dendrogram of genetic distances, constructed by using RAPD data and the UPGMA method of clustering, shows DNA similarity between somaclonal variant lines and their parents.

Williams *et al* (1990) reported that polymorphism among individuals could arise through nucleotide change that prevented amplification by introducing either a mismatch at one priming site, detection of a priming site, insertions that rendered priming sites too distant to support amplification and insertions or deletions that changed the size of the amplified product.

The PCR technique has proved to be a powerful tool for the identification of polymorphism in cereals. Using wheat, barley, rye and wheat-barley addition lines, Weinging and Langridge (1991) detected polymorphism, using conserved semi-random and random primers. With different combinations of primers, they were able to detect both inter-and intraspecific diversities.

Two-hundred combinations of a pair of 10-mer primers were tested in wheat species to examine RAPD patterns; 25 combinations of primers produced informative bands (Nagaoka and Ogihara, 1997). They reported that the number of bands produced after the PCR reaction were approximately 220 bands in diploid wheats, 240 in tetraploid wheats and 260 in hexaploid wheats. Barakat *et al* (2000) used 26 primers of arbitrary nucleotide sequence to amplify DNA segments from the genomic DNA of six wheat genotypes, which had a high potentiality for shoot formation. One-hundred and 48 amplification products were obtained, out of which 128 showed polymorphism. They, also, reported that the RAPD markers, produced by primers, were used to construct a similarity matrix.

Cluster analysis :

One of the goals of the present study was to investigate the efficiency of RAPD markers in determining, accurately, the genetic relationship between wheat somaclones and their parents.

The RAPD markers, produced by five primers, were used to construct a similarity matrix (Table 7). Simple matching coefficients, ranging from 0.17 to 0.72, suggested a broad genetic base for wheat genotypes. Data in Table 7 presented the genetic similarity estimates of the 120 pairwise comparisons among the wheat cultivars and their somaclones, based on the 58 polymorphic bands. Figure (2) represents the clustering of wheat cultivars generated by UPGMA analysis of the parents; namely, Gemmiza-1, Sakha-69, Sakha-61 and their somaclones. Six clusters could be observed, the first cluster included Sakha-61, Soma 61-1, Soma 69-1 and Soma 61-2, while the second one included Soma 69-2, Soma 69-3, Soma 69-4 and Soma 69-5 . The third cluster included Gemmiza-1 and SomaGem 1-6 . The fourth cluster included only Sakha 69. The fifth cluster included SomaGem 1-1 and SomaGem 1-2 and the last one included SomaGem 1-3, SomaGem 1-4 and SomaGem 1-5.

These results indicated that RAPD technique could be successfully applied to species with very large genomes, like wheat, to obtain a proper characterization of genetic relationship. Nagaoka and Ogihara (1997) stated that RAPD markers were more easily handled and, thus, became more desirable to estimate genetic relationship among wheat genotypes. Barakat *et al* (2000) reported that wheat genotypes were classified into three clusters :- Giza cultivars and Sakha-69, Gemmiza-1 and Sohag-1.Their results revealed that the closest cultivars to Sakha-69 were Giza-167 and Giza-164. Sohag-1 and Gemmiza-1 were very different from other cultivars and quite distinct from each other.

Recently, RAPD markers have been used to characterize wheat somaclonal variants tolerant to heat stress and to compare them with their parents (Barakat and EL-Said, 2003). They reported that the genetic similarity among the fifteen genotypes ranged from 0.00 to 0.60. They , also, reported that wheat cultivars and their somaclones were classified into six clusters.

This study indicated that the use of RAPD technique to detect genetic variation at the level of DNA among wheat cultivars and their somaclones was sensitive and powerful. This would be of particular importance in the future, when dealing with *in vitro* selection to stress conditions.

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الملخص العربي

انتخاب معملي والتعرف على Somaclonal Variations المقاومة للصدأ الأصفر في القمح بواسطة RAPD Markers

أ.د. محمد نجيب بركات د. سناء إبراهيم محمد ميلاد

معمل التقنية الحيوية- قسم المحاصيل- كلية الزراعة-جامعة الإسكندرية

استخدمت طريقتان من طرق الانتخاب المعملي لتقدير مدى كفاءة استخدام مركـب(PFP) P-fluorophenylalanine كمـادة للانتخاب المعملي لإنتاج خلايا طافرة مقاومة لمرض الصدأ الأصفر.

تم اختيار صنفين من الأصناف القابلة للإصابة بالمرض و هما: جميزة ١ وسخا ٦٩ وتم اختيارهما حيث كانت قدرتهما عالية على إعطاء مجاميع خضرية عند الزراعة معملياً بواسطة الجنين الناضج. كما تم اختيار الصنف "سخا ٦١ "حيث أنه يعرف بمقاومته العالية لمرض الصدأ الأصفر.

وقد أظهرت نتائج الانتخاب المعملي المباشـر لتحمـل الـ PFP أن معـدل النمـو النـسبي للكـالس وكـذلك النـسبة المئويـة للـ embryogenic callus انخفضت مع زيادة تركيز الـ PFP مع كل الأصناف. وأظهرت النتائج أيضاً أن تكون المجاميع الخضرية كان يزيد أو يقل بزيادة مستوى الـ PFP في كل الأصناف.

أوضحت نتائج طريقة الانتخاب المعملي Stepwise أن وزن الكالس كان يزداد بزيادة مستوى الـPFP. وكانت نسبة تكون المجـاميع الخضرية إما تزداد أو تنخفض بزيادة تركيز الـPFP في كل أصناف القمح.

أوضحت نتائج هذه الدراسة أن طريقة الـStepwise كانت أكثر كفاءة من الطريقة المباشرة في تكوين المجاميع الخضرية. وقد أظهرت نتائج العدوى الصناعية للنباتات الناتجة من طرق الانتخاب المعملي أن بعض الـ Somaclons الناتجة من المعاملة بـالـ PFP كانـت عبارة عن طفرات مقاومة للصدأ الأصفر في القمح. استخدم تحليل RAPD لمعرفة الـ Polymorphism بين Somaclonal Variants الناتجة من الانتخاب المعملي والآباء المستخدمة.