SOMACLONAL VARIATION FROM MATURE EMBRYO EXPLANTS OF SOME EGYPTIAN BARLEY GENOTYPES

SHAIMAA E. RASHAD[1], F. M ABDEL-TAWAB[2], EMAN M. FAHMY[2] AND M. M SAKR[3]

1.Microbial Genetics Dept., National Research Center, Egypt
2.Genetics Dept., Faculty of Agriculture, Ain Shams University, Egypt
3.Plant Biotechnology Dept., National Research Center, Egypt

Plant tissue culture manipulation has been a good source of genetic variability for the improvement of crops. Somaclonal variation is the term used to describe the variation seen in plants that have been produced by plant tissue culture. Chromosomal rearrangements are an important source of this variation.Somaclonal variations (SCV) present among regenerated plants may not be stable because of physiological disturbances and epigenetic causes. Plants regenerated from *in vitro* cultures possess an array of genetic and epigenetic changes(Temel *et al.,* (2014).

Wang and Wang (2012) reported the occurrence of elevated frequency of SC with calli age. However, in advanced somaclonal generations, these genetic causes may be eliminated and whatever variation is found, it is eventually fixed and can be potentially used in crop improvement programs (Kole *et. al*., 2006).

Application of proteins, isozymes, randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSRs) have been used to detect the genetic variations in nuclear, mitochondrial and chloroplast genomes. Molecular studies on somaclonal variation in wheat and barley have demonstrated that the electrophoretic pattern of storage proteins could be useful for assessing somaclonal variation. Meanwhile, protein patterns provided useful information about similarities and variations among and within taxa. Many investigators employed protein polymorphism to assay genetic fingerprint and diversity in a wide range of plants (Breiman *et. al*., 1987).

Isozymes variation in some regenerates was heritable through the seed generations. Somaclones with morphological character variations were distinguished from their original varieties by zymogram changes included deletion of specific enzyme bands, appearance of new bands, and band intensity(Nan *et. al.*, 1991).

Egypt. J. Genet. Cytol., 49:103-121, January, 2020

Web Site (*www.esg.net.eg*)

RAPD markers were used to assess genetic diversity among barley varieties. They were useful for evaluation of genetic diversity between different winter barley cultivars(Ciulca *et. al*., 2010).

The present investigation Investigation the influence of different media composition on the embryogenic response and regeneration of the different barley genotypes. Confirmation the genetic stability of regenerated calli through testing the possible somaclonal variation using molecular genetic markers such as protein SDS-PAGE, isozymes and RAPD-PCR.

MATERIALS AND METHODS

Plant materials

Eight barley genotypes (*Hordeum vulgare* L.) with different genetic backgrounds were used in this investigation, their code numbers, names and pedigrees are shown in Table (1). Barley grains were provided by the Barley Research Department, Field Crops Research Institute, ARC, Giza, Egypt.

Methods

Callus maintenance and selection

Culture phase I

After two sub-cultures on medium M1 (MS media were supplemented with 30 g/L maltose and 3.7 g/L gel rite, and pH was adjusted to 5.7, in addition to 2 mg/L 2,4-D, 1 g/L casein, 690 g/L proline 5 mg/L thiamine and 500 mg/L inositol). Two months later, the embryos were scored for the formation of callus with embryogenic morphology. Embryogenic callus tissue was selected and maintained for 2-3 weeks, then transferred to new medium according to Abumhadi *et. al*., (2005).

Culture phase II

The developed calli were maintained on MS medium containing 1 mg/L 2,4-D and 0.5 mg/L BAP. The formed calli were transferred to regeneration medium. They were continuously subcultured on the same fresh medium every 3-4 weeks during a period of 4-6 months.

Regeneration protocol

Direct shoot and root organogenesis

After one subculture on maintenance media, calli of the eight barley genotypes were placed on solidified MS basal salt medium (Murashige and Skoog, 1962). Bright light (8:16 h. dark: light photoperiod) at 22-25°C, were applied. These cultures usually regenerate shoots and roots, in two weeks.

Indirect shoot and root organogenesis

The three genotypes; Giza130, El-Kasr and Giza126 were cultured on callus induction medium M1. Calli were transferred to the media Ma, Mb and Mc as shown in Table (2) for 4-6 months for shooting. The less developed green plant-

lets and shoot-like structures were sub-cultured once more on regeneration medium. They were continuously sub-cultured on the same fresh medium every 2-3 weeks during a period of 4-6 months.

Somaclonal variation

Somaclonal variation was assessed intensively in the previous three barley cultivars using biochemical and molecular genetics analyses according to Ruíz *et. al.,*(2002).

Biochemical andMolecular genetic studies

SDS-PAGE of water-soluble protein fraction

SDS-PAGEof water-soluble protein fraction was performed according to Cooper *et. al.,* (1986).

Isozymes electrophoretic conditions

Polyacrylamide gel electrophoresis (Native-PAGE) was used to study isozymes variations of peroxidases and esterases from young leaves of the three barley cultivars according to Stegman *et. al*., (1985).

Isozymes visualization

Peroxidases

After electrophoresis, gels were immersed in 100 ml of developing solution, which consisted of0.025 g benzidine dihydrochloride, 0.5 ml glacial acetic acid and H_2O (dd) up to 100 ml. Twenty drops of 1% hydrogen peroxide (H_2O_2) were added to the reaction mixture just before staining. The gels were incubated at room temperature in the dark until the bands appeared. The reaction was stopped with tap water and gels were photographed (Graham *et al*., 1964).

α-esterases

After electrophoresis, the gels were immersed in 100 ml of 100 mM Na-phosphate buffer (pH 6.0) containing 0.50 mg α-naphthyl acetate and 0.50 mg fast blue RR salt.

Na-phosphate buffer of 100 ml was prepared by 61 ml of 0.2M of disodium phosphate (Na_2HPO_4) and 39 ml of 0.2M of monosodium phosphate ($NaHPO_4$). The gels were incubated at 37°C in the dark until brown bands appeared. The gels were rinsed, photographed and fixed with fixation buffer according to Scandalios (1964).

RAPD-PCR

Genomic DNA extraction using CTAB

DNA extraction from plant tissue can vary depending on the material used. Essentially, any mechanical means of breaking down the cell wall and membranes without their degradation were required (Križman *et. al*., 2006).

Polymerase chain reaction (PCR) conditions

RAPD-PCR reactions were performed according to Williams *et. al.,* (1990). PCR reaction mixture components and program conditionswere illustrated in Tables (3 and 4), respectively. Five RAPD-primers (Operon Technologies, Inc), shown in Table (5) were used with the studied genotypes.

Agarose electrophoresis

The amplification products were analyzed by electrophoresis in 2 % agarose gel in TAE buffer stained with 0.2 µg/ml ethidium bromide and photographed under UV light.

RESULTS AND DISCUSSION

Biochemical genetic analysis (protein and isozymes) and molecular genetic analysis (RAPD-PCR) were applied to assess the impact of somaclonal variations in the three barley genotypes (El-Kasr, G130 and G126).

Biochemical and molecular genetic analyses

SDS-PAGE electrophoresis

The SDS-polyacrylamide gel electrophoresis of proteins for five samples of one control and four regenerates of genotype Giza130 is shown in Fig. (1) and Table (6). The profiles comprised a total of five bands with apparent molecular weights ranged between68 and 37KDa. Two polymorphic bands appeared in regenerated plants, while was absent in the control. The results indicated polymorphism which due to somaclonal variation.

The SDS-PAGE of proteins for five samples of one control and four regenerates of genotype El-Kasr appeared in Fig. (2) and Table (7). The profiles comprised of a total of seven bands with apparent molecular weights ranged between111 and 23 KD. Four polymorphic bands appeared in regenerates plants when compared with the control. The results confirmed the presence of somaclonal variation.

The SDS-PAGE of proteins for five samples of one control and four regenerates of genotype G126 is shown in Fig. (3) and Table (8). The profiles comprised of a total of nine bands with apparent molecular weights ranged between110 and 22 KD. Three polymorphic bands appeared in regenerates and were absent in the control, while the band at MW 110 was present in the control and absent in regenerate six. TheSDS-PAGE patterns indicated the occurrence of somaclonal variation of the regenerates compared with their respective control.

Isozymes electrophoresis

α-esterase (Est)

Generally, α-esterase acetate exerted highly polymorphic patterns among the studied genotypes. Est is a gene family which hydrolyzes ester bond in lipid to produce plant energy for biochemical reactions. The esterase banding patterns are presented in Fig. (4) and Table (9). The

results for the three barley cultivars, El-kasr, G126 and G130 revealed a total of four bands, they were monomorphic in one cultivar; G 130 which showed stability, while the other two genotypes; El-Kasr and G126 exhibited somaclonal variation compared with their respective controls.

Peroxidase (Prx)

Peroxidase electrophoretic patterns of the fifteen samples of the three barley genotypesare present in Fig. (5) and Table (9). The results for the three barley cultivars, El-kasr, G126 and G130 revealed a total of three bands, G130 showed stability, while the other two genotypes; El-Kasr and G126 gave somaclonal variation when each control was compared with its four regenerates of the two barley genotypes.

RAPD-PCR

In the present study, the genetic variability among barley populations based on RAPD-PCR analysis was studied. Five primers were used and produced informative products resolvable by agarose gel electrophoresis and succeeded to give polymorphism among samples as shown in Figs (6-10) and Tables (10-14).

The results of primer Op X11 are shown in Fig. (6) and Table (10). They gave amplification products with the fifteen barley samples with a total of eight polymorphic bands. Molecular sizes ranged between 350-1000 bp. El-Kasr showed somaclonal variation for three bands at MS of 800, 500 and 380 bp, which appeared in the control only. At the same time, G126 showed presence of three fragments at MS of 1000, 800 and 700bp in the four regenerates when compared with its control and the other regenerates which indicated somaclonal variation. On the contrary, G130 showed genetic stability with no somaclonal variation.

The results of primer Op T08 are shown in Fig. (7) and Table (11). They gave amplification products for the fifteen barley samples; with a total of 14 polymorphic bands. MS ranged between 1100 bp to 300 bp. El-Kasr showed somaclonal variation for three bands at 1100, 630 and 340 bp. At the same time, G126 showed presence of two fragments at MS of 580 and 480 bp in the fourth regenerates only when compared with the control and the other regenerates which indicated somaclonal variation. On the contrary, G130 showed genetic stability.

The results of primer Op C19 are shown in Fig. (8) and Table (12). They gave amplification products for the fifteen barley samples with a total of 12 polymorphic bands. Their molecular sizes ranged between 1200 bp to 360 bp. El-Kasr showed somaclonal variation for six bands at 1200, 600, 500, 490, 420 and 360 bp. At the same time, G126 showed presence of six polymorphic bands at MS of 1200, 870, 800, 530, 525 and 380 bp. The results indicated the occurrence of somaclonal variation for the regenerates of each of El-kasr and G126, while it re-

vealed no somaclonal variation for those of G130.

The results of primer Op D13 appear in Fig. (9) and Table (13). They gave nine amplification products with the fifteen barley samples. A total of 15 polymorphic bands appeared; with different molecular sizes ranged between 800 bp to 190 bp. El-Kasr showed somaclonal variation for two bands at 650 and 500 bp. At the same time, G126 showed presence of five fragments at molecular sizes of 420, 370, 260, 230 and 190 bp indicated somaclonal variation. On the contrary, G130 showed genetic stability.

The results of primer Op X17 are shown in Fig. (10) and Table (14). They gave amplification products with the fifteen barley samples with a total of 14 polymorphic bands; with different molecular sizes ranged between 1400 bp to 330 bp.El-Kasr showed somaclonal variation for eight bands at 1300, 1200, 830, 810, 790, 700, 520 and 330 bp. At the same time, G126 showed somaclonal variation of five fragments at MS of 1400, 1200, 560, 540 and 520 bp. The somaclones of each of El-kasr and G126 revealed somaclonal variation compared with their respective controls, while those of G130 indicated stability with no somaclonal variation.

This was in agreement with Aydin *et al.* (2016) who stated that,in rye, genetic variations, and especially somaclonal variations, are undesirable and detected this variation using RAPD analysis. In addition, Atanassov *et. al.,* (1998) detected the occurrence of somaclonal variation in four Bulgarian varieties of barley by the application of SDS-PAGE and RAPD-PCR analysis.

SUMMARY

Somaclonal variation is used to describe the occurrence of genetic variants derived from *in vitro* procedures; it is also called culture-induced variation. Many plant biotechnology applications use plant tissue culture as a tool, and the unpredictable nature of these variations represents a serious problem, especially for the commercial applications of such technology. Such variations in micro propagated material, such as genetic and epigenetic modifications, need to be characterized. Biochemical genetic analysis (protein and isozymes) and molecular genetic analysis (RAPD-PCR) were applied to study somaclonal variations in three barley genotypes (El-Kasr, G130 and G126).

In conclusion, this study demonstrated the establishment of an influence of different media composition on the embryogenic response and regeneration of the different barley genotypes. Biochemical and molecular genetic analysis of protein, isozymes and RAPD-PCR proved to be efficient tools for detecting the occurrence of somaclonal variation in the three barley genotypes El-kasr, G126 and G130.

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Table (1): Code number, names, abbreviations and pedigrees of the eight barley genotypes.

Code number	Genotype name	Abbreviations	Pedigrees
1	Giza123	G123	Giza 117/FAO 86
2	Giza124	G124	Giza 117/ Bahteem 52// Giza 118/ FAO 86
3	Giza125	G125	Giza 117/ Bahteem 52// Giza 118/ FAB 86
4	Giza126	G126	Baled Bahteem/SD 729-POR12762-BC
5	Giza130	G130	BF 891 M – 584 Sel., 4AP
6	Giza2000	G2000	Giza 117/ Bahteem 52// Giza 118/ FAO Giza 121 /3/ 86
7	El-Kasr	El-Kasr	17Km west MarssaMatrouh
8	Awlad Ali	Awlad Ali	143Km east MarssaMatrouh

Table (2): Components of maintenance indirect regeneration media.

Media code	Maintenance	Indirect regeneration
Ma	Control	MS media, 1mg/L 2,4-D, supplemented with0.5 mg/L BAP
Mb	4mg/L 2,4-D,50µg/L BA	MS medium freehormones
Mc	6 mg/lL 2,4-D,100 µg/LBA	half-MS mediumsupplemented with 0.5 mg/L 2,4-D and 1.0mg/L zeatin

Table (3): PCR reaction mixtures.

Compound and concentration	Amount per reaction
PCR buffer10X	2.5 µL
Taq polymerase (Alliance) 5 units/µl	0.25 µL
Nucleotides mixture (dNTPs) 5 mM	2.0 µL
Primer10 pmole	3.0 µL
Genomic DNA20-50 ng	3.0 µL
Double distilled water Total volume	14.25 µL 25 µL

Table (4): PCR program.

PCR step	Temperature (°C)	Time (min)	No. of cycles
Initial activation	°C	3	1
Denaturation	°C	2	36
Annealing	°C	1	
Extension	°C	2	
Final extension	°C	10	1
Cooling down	°C	until use	

Table (5): RAPD primers used in PCR reaction.

Primer code	Sequence (5'→3')
Op X11	GGAGCCTCAG
Op T08	AACGGCGACA
Op C19	GTTGCCAGCC
Op X17	GACACGGACC
Op D13	GGGGTGAGGACGA

Table (6): The presence (+) and absence (-) of protein bands of the barleygenotypeG130 as revealed by SDS-PAGE.

MW(KDA)	CONTROL	REGENERATED PLANTS			
LANE1	LANE2	LANE3	LANE4	LANE5	LANE6
68	-	+	+	+	+
62	+	+	+	+	+
51	-	+	+	+	+
48	+	+	+	+	+
37	+	+	+	+	+

Table (7): The presence (+) and absence (-) of protein bands of the barleygenotypeEl-Kasr as revealedby SDS-PAGE.

MW KDA	CONTROL	REGENERATED PLANTS			
LANE11	LANE2	LANE3	LANE4	LANE5	LANE6
111	+	+	+	+	+
94	-	+	+	+	-
68	-	+	+	+	+
54	-	+	+	+	+
46	+	+	+	+	+
30	+	+	+	+	+
23	-	+	+	+	+

Table (8): The presence (+) and absence (-) of protein bands of the barleygenotypeG126 as revealed by SDS-PAGE.

MW KDA	CONTROL	REGENERATED PLANTS			
LANE1	LANE2	LANE3	LANE4	LANE5	LANE6
110	+	+	+	+	-
97	+	+	+	+	+
77	-	-	+	+	+
59	+	+	+	+	+
52	+	+	+	+	+
46	+	+	+	+	+
32	-	+	+	+	+
28	+	+	+	+	+
22	-	+	+	+	+

Table (9): The presence (+) and absence (-) of bands in the two isozymes systems (Est and Prx) among the fifteen barley genotypes.

Genotype															
	El-Kasr					Giza 126					Giza 130				
Isozymes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
α- Est	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	-	+	+	+	-	-	+	-	+	-	-	-	-	-	-
	+	-	-	-	-	+	-	+	-	+	+	+	+	+	+
Prx	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	+	-	-	+	-	-	-	-	-	-	+	+	+	+	+
	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-

Table (10): The presence (1) and absence (0) of amplified DNA fragments that produced by Op X11 primer with the fifteen barley genotypes.

MS	El-Kasr					G126					G130				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1000	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
800	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
700	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
500	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
450	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
400	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0
380	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
350	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1

Table (11): The presence (1) and absence (0) of amplified DNA fragments that produced by Op T08 primer with the fifteen barley samples.

MS	El-Kasr					G126					G130				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1100	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0
1000	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0
850	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
800	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
700	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0
630	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
600	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
580	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
500	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
480	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
430	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0
390	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
340	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0

Table (12): The presence (1) and absence (0) of amplified DNA fragments that produced by Op C19 primer with the fifteen barley genotypes.

MS	El-Kasr					G126					G130				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1200	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0
870	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0
800	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1
600	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
530	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0
525	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
510	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
500	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
490	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
420	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
380	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
360	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0

Table (13): The presence (1) and absence (0) of amplified DNA fragments that produced by Op D13 primer with the fifteen barley samples.

MS	El-Kasr					G126					G130				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
800	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
650	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
510	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
500	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
420	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
370	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
260	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
230	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
190	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0

Table (14): The presence (1) and absence (0) of amplified DNA fragments that produced by Op X17 primer with the fifteen barley samples.

MS	El-Kasr					G126					G130				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1400	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
1300	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
1200	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
830	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
810	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
790	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
700	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
600	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
560	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
540	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
520	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
330	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0

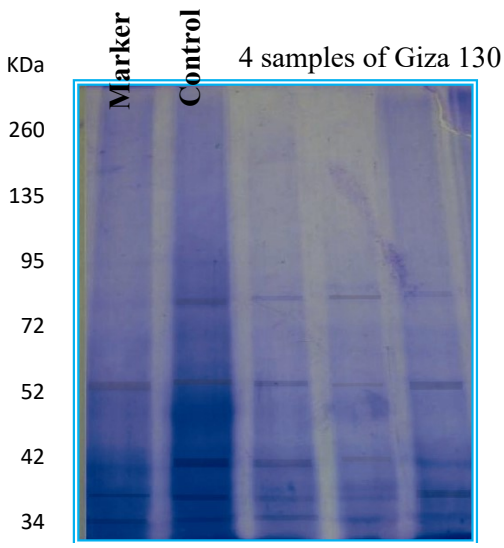

Fig. (1): SDS-PAGE protein profiles of Giza 130; lane 1 molecular weight protein marker (KDa), lane 2 control and lanes 3-6 four regenerates.

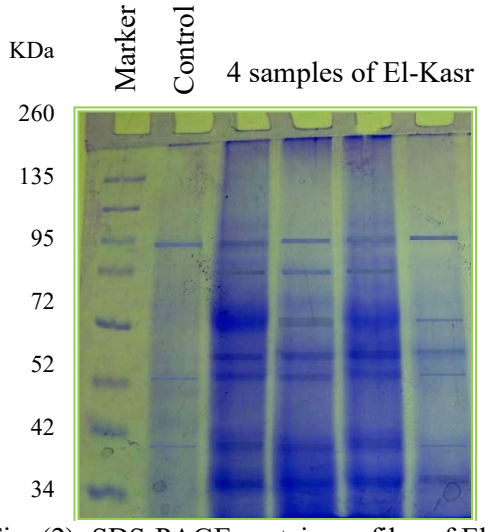

Fig. (2): SDS-PAGE protein profiles of El-Kasr; lane 1 Molecular weight protein marker (KDa), lane 2 control and lanes 3-6 four regenerates.

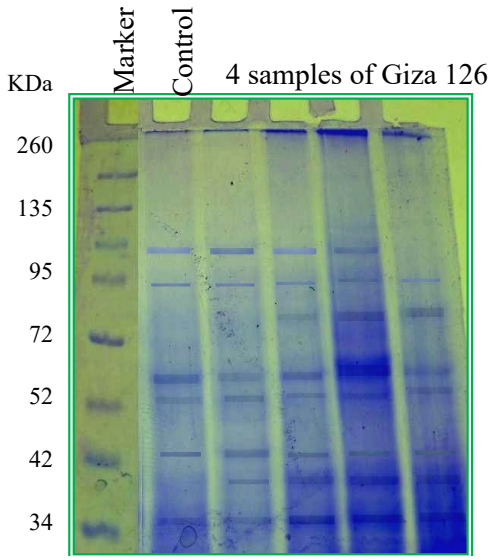

Fig. (3): SDS-PAGE protein profiles of G126; lane 1 Molecular weight protein marker (KDa), lane 2 control and lanes 3-6 four regenerates.

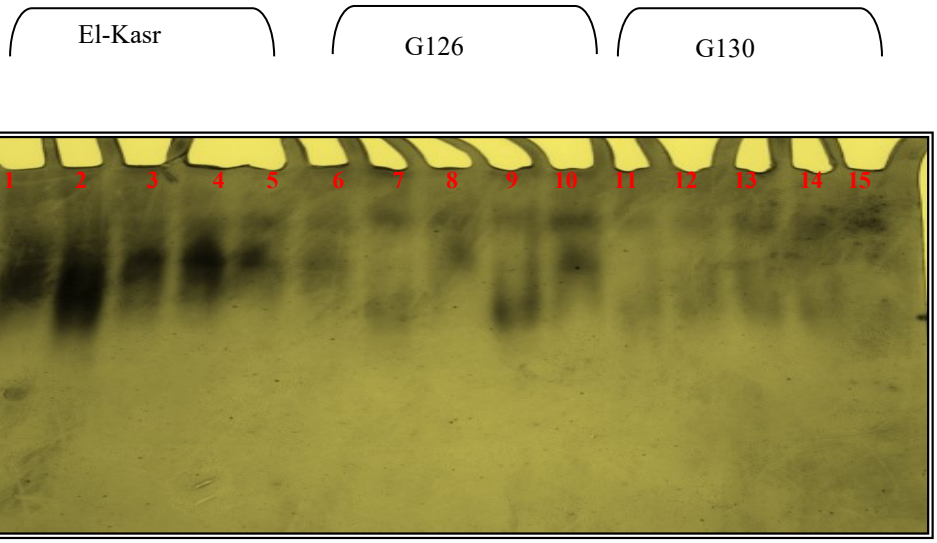

Fig. (4): Zymogram of α-Esterase (Est.) in the three barley genotypes (El-Kasr, G126 and G130)**.** Each one is represented by five samples control (1, 6 and 11) and the others are four regenerates for each genotype.

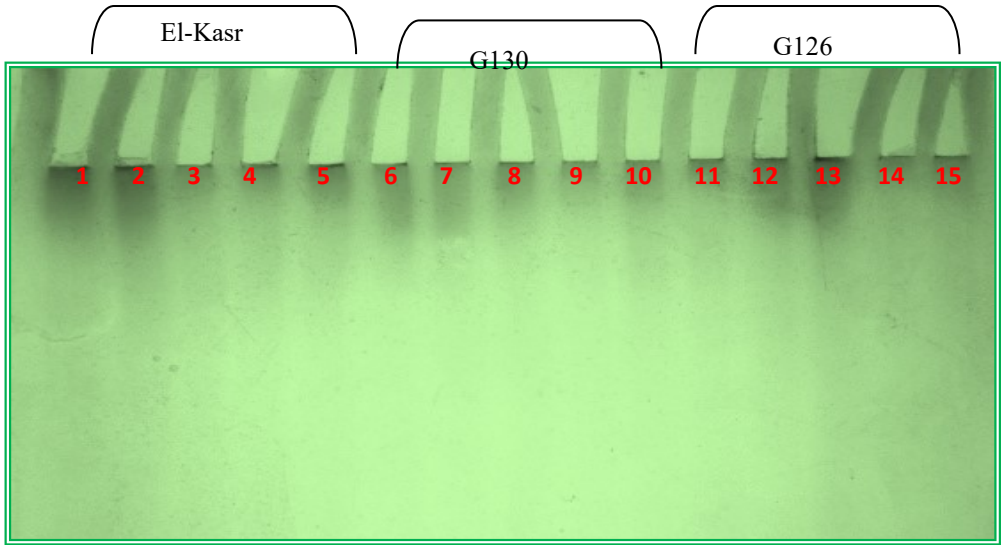

Fig. (5):Zymogram of peroxidase (prx.) in the three barley genotypes (El-kasr, G126 and G130). Each one is represented by five samples; control (1, 6 and 11) and four regenerates.

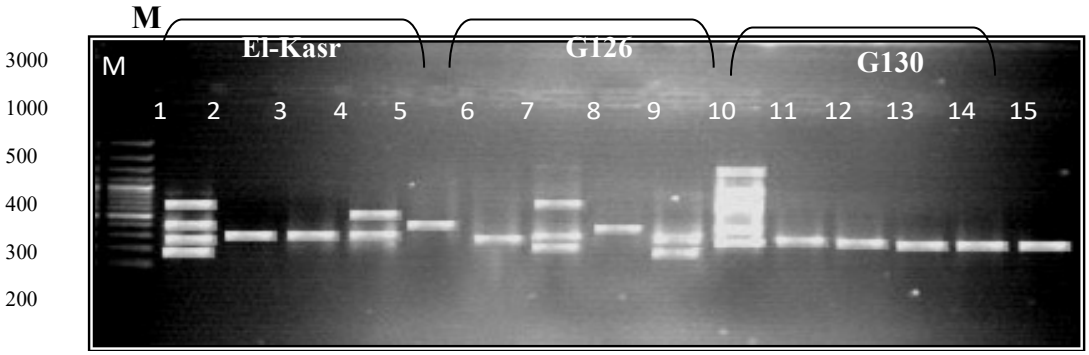

Fig. (6): DNA polymorphisms in three barley genotypes (El kasr,G126 and G130) with primer Op X 11. Each one is represented by five samples;control (1, 6 and 11) and four regenerates.

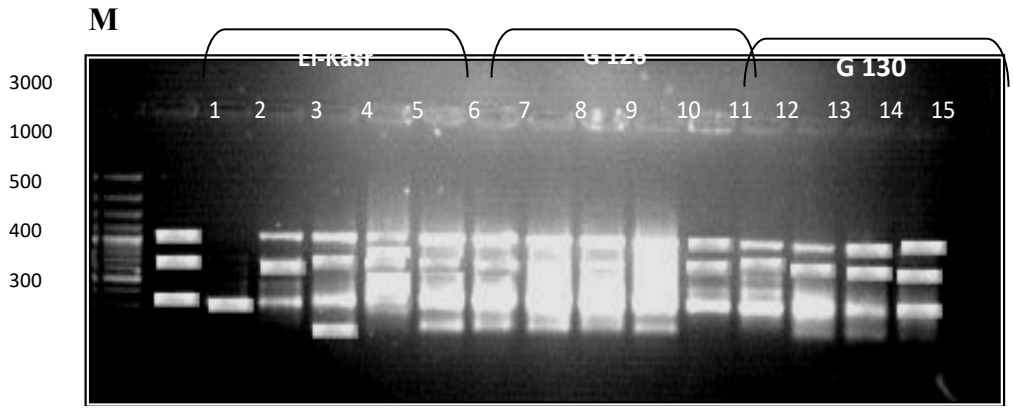

Fig. (7): DNA polymorphisms in three barley genotypes (El kasr, G126 and G130) with primer Op T08. Each one is represented by five samples; control (1, 6 and 11) and four regenerates.

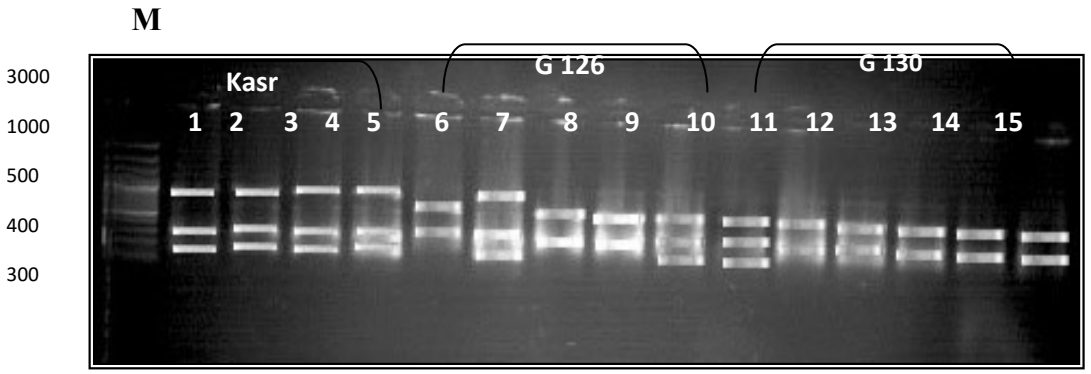

Fig. (8): DNA polymorphisms in three barley genotypes (El-Kasr, G126 and G130) with primer Op C19. Each one is represented by fives amples;control (1, 6 and 11) andfour regenerates.

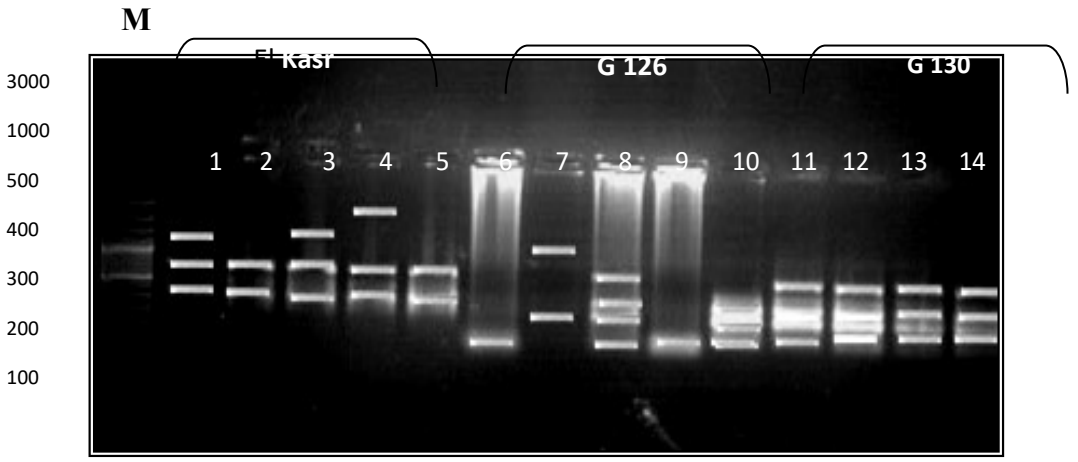

Fig. (9): DNA polymorphisms in three barley genotypes (El-Kasr, G126 and G130) with primer Op D13. Each one is represented by five samples; control (1, 6 and 11) and four regenerates.

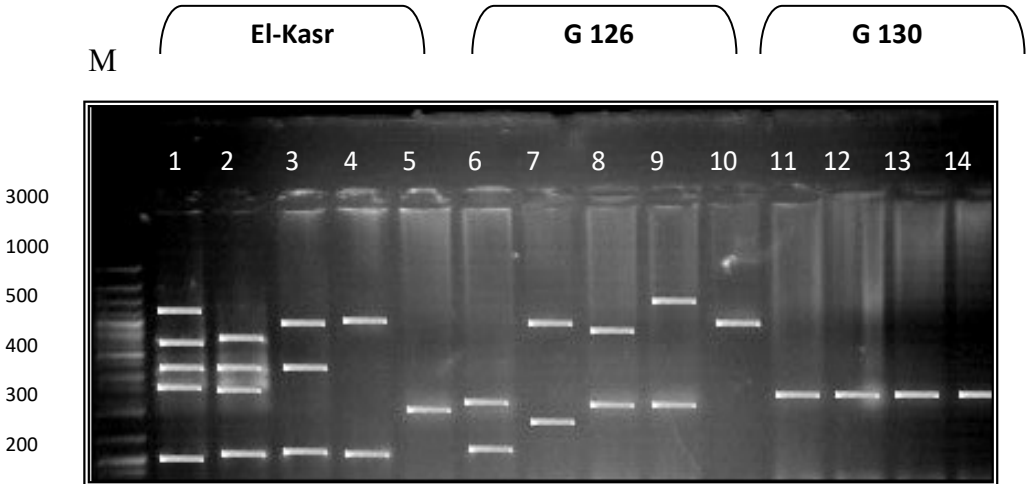

Fig.(10):DNA polymorphisms in three barley genotypes (El-Kasr, G126 and G130) with primer Op X17. Each one is represented by five samples; control (1, 6 and 11) and four regenerates.