Journal of Applied Sciences Research, 3(11): 1589-1599, 2007

Development of Drought Tolerant Double Haploid Wheat Using Biochemical Genetic Markers on *In vitro* Culture

[1]Abdelsamad, A., [2]O.E. El-Sayed and [2]Hayam, F. Ibrahim

[1]Department of Biochemistry, Faculty of Agriculture, Cairo Univ.
[2]Genetics and Cytology Dept., National Research Centre, Dokki, Cairo, Egypt.

Abstract: Embryogenic calli induced from mature embryos of four selected wheat (*Triticum aestivum* L.) genotypes, consisted of two double haploid (DH) and two varieties; Giza 168 and Sakha 93 were used in the study. They were selected from ten DH genotypes and five varieties, which were grown under drought stress at the Experimental Station of the National Res. Center and then germinated in Petri dishes under 10, 20 and 30% PEG. Significant differences of genetic responses were observed for the four wheat genotypes at 10 and 20% PEG for callus induction, callus fresh weight, growth index, relative water content, relative tolerance percentage and regeneration efficiency. DH-1 followed by DH-2 and then Sakha 93 were superiors in sequence for *in vitro* PEG-tolerance. Biochemical genetic markers, such as SDS-PAGE of water soluble and non-soluble proteins and five enzymes; EST, SOD, PRX, GOT and MDH were used to determine the genetic variations among the four wheat genotypes at 10 and 20% PEG. SDS-PAGE analyses displayed eight and four newly induced water soluble and non-soluble protein bands, respectively which were expressed and synthesized in response to an altered environment (10 and 20% PEG). Moreover, five and eight other water soluble and non-soluble proteins were inhibited, respectively at the two drought stress. Electrophoretic analysis of the five enzymes revealed specific appearance of some new isozyme bands and lack of several isozymes in DH-1 and DH-2 drought-tolerant genotypes as compared with the other two wheat genotypes. As a result, proteins and isozyme polymorphisms were furnished reliable markers for discriminating the improved drought tolerant wheat genotypes.

Keywords: Bread wheat genotypes, double haploid, embryogenic calli, drought tolerant, SDS-proteins and isozymes markers.

INTRODUCTION

Abiotic stress is a major limiting factor in agricultural crop production in many countries and the main abiotic stresses of economic importance include drought and salinity[17]. Because of periodic drought and limited area, the rain fed uplands did not support sufficient output of cereal crops to meet domestic demand; approximately 32% of the wheat growing regions in developing countries experience some type of drought stress during the growing season[25] .

Polyethylene glycol (PEG) has long been used *in vitro* culture to reduce water potential of nutrient solutions and to stimulate water stress without the risk of being taken up by the plants[9]. Thus, cell cultures survive under water stress can be selected and raised as drought resistant cell lines. Dragiiska *et al.*[11] developed a system for *in vitro* selection during somatic embryogenesis in alfalfa using PEG as a selective agent for osmotolerance. Abdel Hady *et al.*[1] subjected embryogenic calli of five wheat cultivars to *in vitro* selection for drought tolerance using 5, 10 and 20% PEG. Zhu *et al.*[37] studied the effects of different conc. of PEG-simulated drought stress on *Pinus sylvestris* seeds germination.

One way of increasing productivity in stressful environments is to breed crops more tolerant to stress. However, success in breeding for tolerance has been limited because tolerance to stress is controlled by many genes and their simultaneous selection is difficult[12], complexity of the several tolerance mechanisms involved, tremendous effort is required to eliminate undesirable genes that are also incorporated during breeding[29] and there is a lack of efficient selection procedures particularly under field conditions[27].

The improvement of high yielding wheat varieties suitable for growing under different stress conditions by plant breeding requires genetically pure lines; either to be used as parents for mating in breeding processes or

Corresponding Author: Abdelsamad, A., Department of Biochemistry, Faculty of Agriculture, Cairo Univ.

to be distributed as new cultivars produced from breeding programs. The production of these pure lines by conventional breeding practices is time consuming process needs several generations and could lead to a delay in new varieties production. One of the solutions for this problem is the production of haploid plants via anther culture. This procedure usually needs only one generation to be conducted and could accelerate the production of new varieties with improved traits[3]. This study aims to develop some promising double haploid wheat genotypes, which have favorable response and tolerant to drought stress through *in vitro* culture using some biochemical genetic marker techniques.

MATERIALS AND METHODS

Plant Materials: Fifteen hexaploid bread wheat (*Triticum aestivum* L.) genotypes comprising ten doubled-haploids (DH) and five varieties provided from Division of national Wheat Res. program, Agricultural Research Center, Giza were used for selection of drought tolerance. Wheat grains of the ten double haploid (DH) genotypes and five varieties; Giza 157, Giza 168, Sakha 93, Sakha 8 and Sids-1 were grown under drought stress conditions at the Experimental Station of the National Res. Center in Shalakan, El-kalyobia. In order to determine the appropriate concentrations of PEG, which will be used in callus induction and to verify the most drought tolerant genotypes, the 15 wheat genotypes were germinated in Petri dishes under different PEG conc. (0, 10, 20 and 30%). Five of the ten double haploid genotypes and two wheat varieties, which characterized by their tolerant to drought were selected for callus induction.

Callus Induction of the Selected Wheat Genotypes: The five selected DH genotypes and two wheat varieties were used as sources of mature embryos. Mature grains were harvested from main spikes, surface-sterilized in 70% (v/v) ethanol for 1 min and in commercial bleach (5% sodium hypochlorite) for 30 min, and then washed several times in sterile distilled water. mature embryos were aseptically excised from caryopsis and placed with the scutellum upwards on solid agar medium in sterile Petri dishes for 14 days at 26±1°C in continuous darkness. The agar medium contained the mineral salts of Murashige and Skoog (MS)[20] and 20 mg sucrose, 2 mg 2,4-D, and 7 mg agar. The media were adjusted to pH 5.8 and autoclaved for 20 min at 121°C and 1.1 kg/cm^2 pressure. After callus induction, calli were subcultured at four weeks intervals on fresh MS medium.

***In vitro* Selection of Drought Tolerance Cell Lines and Plant Regeneration:** One month growing calli were immediately transferred to MS medium with polyethylene glycol (PEG) 10,000 as drought stress with concentrations 0, 10 and 20% for 30 days stress period according to Bajji *et al.*[4]. The calli were maintained on their respective treatments for two subcultures (4 weeks each). The callus was incubated in darkness at 24° C. Two of the five selected double haploid genotypes (DH-1 and DH-2) and two wheat varieties (Giza 168 and Sakha 93) could be successfully induced enough callus weights under drought stress and were used for protein and isozyme analyses. calli were transferred to regeneration medium which contained MS basal salts without 2,4-D[8] and maintained for 5 weeks at 26±1°C in a 16 h light (2000 lux), 8 h dark photoperiod. The remainder of medium is the same as previously described. Individual cultures were then scored for the regeneration.

Statistical Analysis: Five replicates were made for the control and for 10 and 20% PEG treatments and each jar contained of five calli for each wheat genotype. Five different characters were estimated. Callus induction percentage, callus fresh weight (mg) before drought treatments, growth index (GI) or increasing value of callus fresh weight was calculated as $(W_1 - W_0)/ W_0$, where W_0 is the weight before treatments and W_1 the final weight after eight weeks of treatments, relative water content (RWC) was calculated by the following formula: (FW-DW)/DW, whereas FW and DW were the callus fresh and dry weights, respectively. Relative tolerance (Rt) percentage was calculated for each wheat genotype at different drought levels by using the following formula: Rt % = (value under stress x 100)/ value at 0.0% stress level. Reduction percentage was calculated for each character as the difference between the two drought stress.

SDS-PAGE Analyses of Proteins and Isozymes: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to[19]. Calli of mature embryo cultures of the four selected wheat genotypes under drought stress were ground in liquid nitrogen and 1 ml of water soluble extraction buffer was added. After centrifugation for 10 min at 12,000 rpm under 4° C the supernatant was collected. Water non-soluble buffer was added to each pellet and left in refrigerator overnight, then centrifuged at 12,000 rpm under 4° C for 10 min.[4]. Electrophoresis was carried out at 4° C and the gel was stained with silver nitrate according to the method of Goldberg and Warner[14] until the bands were clearly observed. Gel bands were scanned and analyzed using

Gel Doc Bio-Rad system. isozymes were separated by horizontal starch gel electrophoretic method according to Wendel and Stuber[36]. Calli were placed in eppendorf tubes and immediately homogenized in 35µl of extraction buffer. The methods of slicing and staining of the gel were applied according to Shaw and Prasad[31]. Five enzymes; EST, SOD, PRX, GOT and MDH were extracted from the calli of the four genotypes under different PEG concentrations.

RESULTS AND DISCUSSIONS

Callus Induction of the Four Wheat Genotypes: The callus induction from mature embryos of the four wheat genotypes became visible within one week of culturing. the formed embryogenic calli were nodular and milky white to cream (yellow) in color regardless of genotype and compactness in surface morphology (Fig. 1-a). The callus induction percentages showed difference between genotypes; whereas DH-1 displayed 99% followed by Sakha 93 with 84.2% and DH-2 with 79.3%, while Giza 168 showed the lowest percentage with 46.1% (Table 1). Callus fresh weight estimated after four weeks of culturing showed difference among the four wheat genotypes, whereas Sakha 93 revealed the highest weight value (64.1) with more than two folds than the other three genotypes and Giza 168 displayed the lowest weight value (26.2), while the genotype mean was 37.3 as shown in Table (1).

This result suggested a positive relationship between callus induction and callus weight and implies that an increase in callus induction in wheat genotypes will accompany an increase in culture efficiency. In this study, mature embryo culture was used to initiate callus induction. However, immature embryos are the most frequently used as explants for the tissue culture, although it has many disadvantages. For example, the growth stage of immature embryo appropriate for isolation is strictly limited, suitable embryo size for tissue culture varies with varieties and environmental conditions and growth of donor plant and immature embryo isolation are all time-consuming, expensive and laborious. Alternatively, the use of mature embryo is easy to handle and available at any time[34]. The obtained data in Table (1) obviously revealed that culture responses were greatly influenced by the wheat genotypes and also emphasized a profound effect of genotypes on callus induction capacity, which is in agreement with extensively reports on callus induction, for example in durum wheat[7] and in bread wheat[15]. Birsin and Özgen[6] reported the genotype effects on callusing ability from triticale mature embryo cultures. Gandonou *et al.*[13] observed significant differences between nine sugarcane genotypes for callus induction capacity, embryogenic response and plant regeneration ability indicating that these criteria are genotype dependent.

Table 1: Callus induction percentage and callus fresh weight/mg after four weeks of the four wheat genotypes.

Wheat genotypes	Callus induction (%)	Callus freshweight (mg)
DH-1	99.0	31.4
Sakha 93	84.2	64.1
DH-2	79.3	27.3
Giza 168	46.1	26.2
Genotype mean	77.2	37.3

In general, callus induction used as an efficient character for assessment of culture responses from mature embryo in wheat genotypes. The callus fresh weight is provided a more concise quantitative character for the development rate of callus. In plant tissue cultures, a desirable genotype is expected to possess high callus induction. However, numerous studies have shown the absence of such a relationship between callus induction and plant regeneration capacity and thus, the independence of these characters from each other. On the contrary, Birsin *et al.*[5] suggested that genotypes with high callus induction also caused an increase in the number of plants transferred to soil.

Effects of Drought Stress on Some Characters and Regeneration Efficiency: Callus induction from mature seeds of the four genotypes under 0, 10 and 20% PEG was shown in Fig. (1-a). Callus growth index (CGI) showed remarkable differences in the means of increasing value of the selected calli among the four genotypes; whereas DH-1 was relatively the better with the highest callus increasing values at 10 and 20% PEG (1614 and 1128), respectively as presented in Table (2). DH-2 recorded the second better genotype in callus increasing value with 1099 and 1057 at 10 and 20% PEG, respectively. However, Sakha 93 was the third better genotype with 493 and 436 at 10 and 20% PEG, respectively. Giza 168 displayed the lowest mean of increasing value at the two successive drought levels with 142 and 137, respectively.

The relative water content of callus tissues of the four wheat genotypes increased with increasing in drought concentration in culture medium. The callus tissues of Giza 168 exhibited more water content with an average mean of 8.2 followed by DH-1 with an average mean of 6.2 comparing with the other two genotypes, which estimated 4.0 and 4.1 for DH-2 and Sakha 93, respectively. The total average at 10 and 20% PEG was significantly higher (5.6) than the average means of DH-2 and Sakha 93 as shown in Table (2). The relative tolerance percentages (RT%) of the four wheat genotypes were considered according to

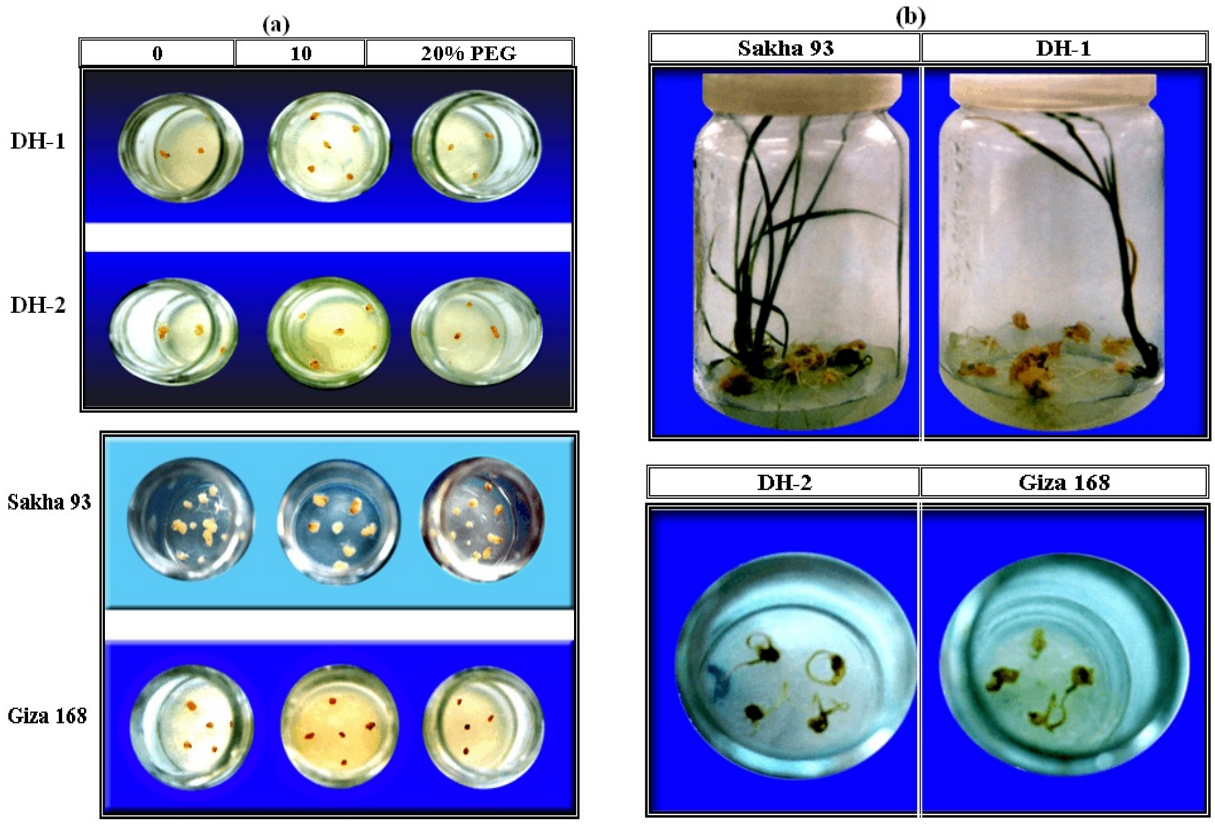

Fig. 1: (a) Callus induction of four wheat genotypes under different PEG concentrations (0, 10 and 20%). (b) Plant regeneration on 2,4-D free MS medium from two month-old callus of four wheat genotypes at 20% PEG.

the difference occurred under the two PEG conc. as shown in Table (2), whereas the reduction percentage from 10 to 20% PEG was the lowest in Sakha 93 with -6% followed by DH-2 with 2%. The other two genotypes were higher in reduction percentages than the two previous genotypes with 7% in Giza 168 and 35% in DH-1, while the average reduction percentage (8%) was lower only than DH-1. The four wheat genotypes were able to produce callus and in this regard, calli derived from the mature embryos of Sakha 93, DH-1 and DH-2 were more able to adapt to higher drought levels than Giza 168, which indicating the superiority of such genotypes for drought tolerance *in vitro*. Plant regeneration on 2,4-D free MS medium from two month-old callus of the four wheat genotypes under different PEG conc. was presented in Fig. (1-b). Sakha 93 revealed the highest percentage of regeneration mean at 10% PEG with 38.2%, followed by DH-1 with 30.1%, while DH-2 and Giza 168 displayed lower percentages than the other two genotypes with 32.4 and 12.5%, respectively. At 20% PEG, regeneration mean percentages were significantly decreased in the four genotypes. Among the four genotypes, Sakha 93 also showed higher percentage of plant regeneration (24.3%) compared with the other three genotypes in the same order like 10% PEG.

Therefore, in other *in vitro* culture studies included different species, it was demonstrated that genotype differences in callus growth index was found among different genotypes[33]. Moreover, regeneration capacity of plant tissue is genetically controlled and specific for each genotype[26].

Effects of Drought Stress on the Protein Patterns of Wheat Calli: SDS-PAGE profile of water soluble proteins extracted from the calli of four wheat genotypes; Giza 168, Sakha 93, DH-1 and DH-2 treated with 0, 10 and 20% of PEG concentrations is presented in Fig. (2-a). SDS-PAGE analysis revealed 31 protein bands with different molecular weights ranged from 289 to 29 kDs as shown in Table (3). Among such protein bands, ten were varied in some wheat genotypes at different PEG concentrations, while the other 21 protein bands were commonly detected.

Table 2: Callus growth index (CGI), relative water content (RWC), relative tolerance percentage (RT) and regeneration efficiency of the four wheat genotypes measured after eight weeks at different PEG conc.

Characters	PEG conc. (%)	Wheat callus genotypes				PEG stress average
		Sakha 93	DH-1	DH-2	Giza 168	
CGI	Control	782	1797	1116	467	1041
	10	493	1614	1099	142	837
	20	436	1128	1057	137	690
	Genotype mean	570	1513	1091	249	856
RWC	Control	2.7	5.7	3.4	6.8	4.7
	10	4.3	6.4	4.1	8.1	5.7
	20	5.3	6.6	4.5	9.7	6.5
	Genotype mean	4.1	6.2	4.0	8.2	5.6
RT%	10	96.7	93.2	90.2	121	100
	20	102.5	65.7	88.7	113	92.4
	Reduction %	-6	35	2	7	8%
Regeneration efficiency %	Control	51.7	49.0	30.2	18.0	37.2
	10	38.2	30.1	23.4	12.5	26.1
	20	24.3	16.8	11.6	6.3	14.8
	Genotype mean	38.1	32	21.7	12.3	26.0

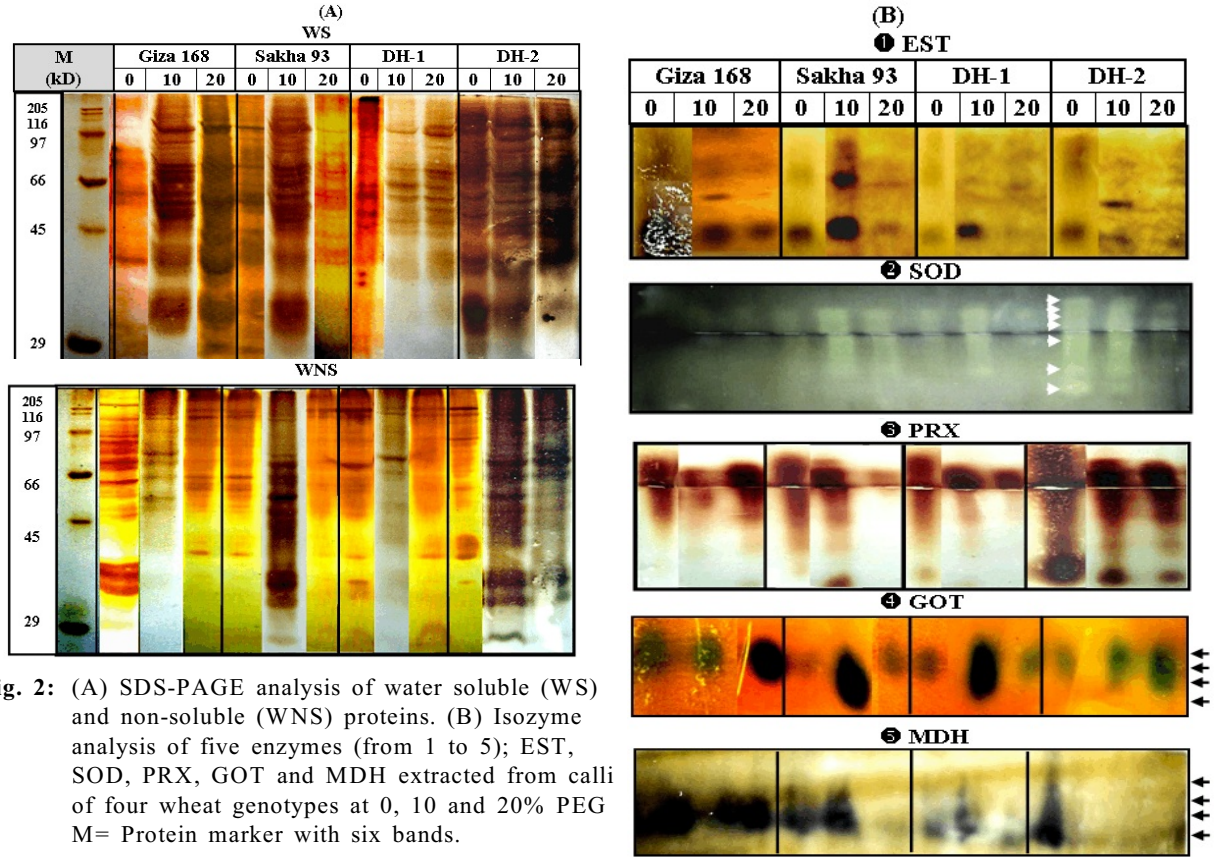

Fig. 2: (A) SDS-PAGE analysis of water soluble (WS) and non-soluble (WNS) proteins. (B) Isozyme analysis of five enzymes (from 1 to 5); EST, SOD, PRX, GOT and MDH extracted from calli of four wheat genotypes at 0, 10 and 20% PEG M= Protein marker with six bands.

Table 3: Induced and inhibited water soluble (WS) and water non-soluble (WNS) protein bands of four wheat genotypes at 0, 10 and 20% PEG.

Protein bands		Band (kD)	Giza 168			Sakha 93			DH-1			DH-2		
			0	10	20	0	10	20	0	10	20	0	10	20
I. induced	WS	289			+		+	+						
		106.5					+	+						
		87					+	+						
		54		+	+			+						
		42.5					+	+		+	+			
		39.5											+	+
		35.5		+	+								+	+
		30.5											+	+
	Total	8		2	3		4	5		1	1		3	3
	WNS	184					+			+				
		185			+									
		53			+					+	+			
		33											+	
		44			+								+	
	Total	5		0	3		1	0		2	1		2	0
Total bands		14 bands		2	6		5	5		3	2		5	3
II. inhibited	WS	164							+					
		39.5	+	+		+	+							
		35.5				+								
		30.5										+	+	
		294	+											
	Total	5	2	1		2	1		1			1	1	
	WNS	300							+			+		
		289	+						+					
		172				+	+		+	+				
		80							+					
		53				+						+	+	
		45	+	+		+	+							
		37				+	+		+			+		
		4				+	+							
	Total	8	2	1		5	4		5	1		3	1	
Total bands		13 bands	4	2		7	5		6	1		4	2	

Nine of the ten variable protein bands were obviously affected by PEG concentrations either by disappearance or induction according to their appearance on the gel. The water soluble protein bands of untreated calli of the four wheat genotypes were varied in numbers, whereas Sakha 93 revealed the lowest numbers (26 bands), Giza 168 and DH-2 (28) and DH-1 (29). Moreover, DH-2 displayed all the 31 proteins under 10% PEG and the highest numbers of proteins with 30 bands at 20% PEG. The other three genotypes revealed fewer bands than DH-2 (Table 3). The genetic analysis of the four wheat genotypes showed some unique and distinctive protein bands in the controls (untreated), which disappeared at the two PEG conc. For example, three protein bands with molecular weights 164, 235 and 29 kDa appeared in DH-1, Sakha 93 and Giza 168, respectively. While, two other protein bands with 39.5 and 30.5 kDa appeared in the control and under 10% PEG and disappeared under 20% PEG in Giza 168 and Sakha 93 (39.5 kD) and DH-2 (30.5 kD). On the other hand, some protein bands were induced under the two PEG conc. and disappeared in the controls. Eight induced protein bands appeared under 10 and 20% PEG among the four genotypes, while two induced proteins with 164 and 54 kDa appeared only at 20% PEG in Giza 168 and Sakha 93, respectively. Sakha 93 showed the highest induced proteins with 5 bands, while DH-1 revealed one induced band with 42.5 kD (Table 3).

The profile patterns of water non-soluble proteins extracted from the calli of four wheat genotypes under different PEG conc. using SDS-PAGE are presented in Fig. (2-a). The analysis revealed a total of 29 protein bands ranged from 300 to 4 kD, not all of them were detected in any sample, whereas, 18 bands were commonly detected in the four genotypes under 0, 10 and 20% PEG with different molecular weights (Table 3). The other eleven variable bands were used for genetic characterization of the four wheat genotypes under different PEG conc. Based upon the presence of total number of protein bands, the four genotypes were decreased at 20% PEG comparing with numbers in the control, while at 10% PEG they gave the same numbers as the controls in Sakha 93 and DH-2 with total numbers 28 and 27, respectively or they increased one band in Giza 168 or decreased two bands in DH-1. On the other hand, five induced protein bands were stimulated to display among the four wheat genotypes at 20% PEG, while one band with 53 kD was displayed at 10 % PEG in DH-1. Thereby, Giza 168 showed three induced bands with 185, 53 and 4 kDa followed by DH-1 and DH-2 with two different bands (184 and 53 kDa) in DH-1 and (33 and 18 kDa) in DH-2, while Sakha 93 exposed one induced band with 184 kD (Table 3). Meanwhile, drought stress affected and inhibited some distinctive water non-soluble protein bands at 10% PEG, which they were appeared at the control. For instance, band with 300 kD in (DH-1 and DH-2), band with 289 kD in (Giza 168 and DH-1), band with 80 kD in DH-1, band with 53 kD in Sakha 93 and band with 37 kD in (DH-1 and DH-2). Consequently, the genetic characterization of the four wheat genotypes under 10% PEG showed that DH-1 was strongly affected in four bands vs. two bands in DH-2, while both of Giza 168 and Sakha 93 were less affected in one band only by drought stress at 10 % PEG. Moreover, drought stress inhibited some other water non-soluble proteins at 20% PEG, which they were appeared at 10% PEG. For example, four bands with 172, 45, 37 and 4 kDa were inhibited at 20% PEG in Sakha 93 and one protein band with different molecular weights; 45, 172 and 53 kDa in Giza 168, DH-1 and DH-2, respectively. Thus, Sakha 93 was mostly affected by drought stress at 20% PEG than the other three genotypes.

Table (3) summarized the induced and inhibited water soluble (WS) and water non-soluble (WNS) protein bands of four wheat genotypes at 0, 10 and 20% PEG. Whereas, SDS-PAGE analysis displayed eight and four newly induced water soluble and non-soluble protein bands, respectively were expressed and synthesized in response to an altered environment under two drought stress concentrations of PEG 10 and 20%. In contrary, five and eight other water soluble and non-soluble proteins were inhibited, respectively at the two drought stress concentrations of PEG 10 and 20%. Dragiiska *et al.*[11] developed a system for *in vitro* selection during somatic embryogenesis in alfalfa using PEG as a selective agent for osmotolerance. PEG is described also as a non-ionic water-soluble polymer, which is not expected to penetrate intact plant tissues rapidly[22].

In the present study, SDS-PAGE analysis displayed some newly induced proteins expressed in response to drought stress in the four wheat genotypes. These results were agreed with many reports, for instant Visser[35] reported that the drought tolerance has been shown to be a highly complex trait, influenced by many different genes and should not be regarded as a unique heritable trait, but as a complex of often fully unrelated plant properties and altered gene expression including new patterns of protein. Plomion *et al.*[23] suggested that drought stress caused profound alterations in cellular metabolism, such as protein functions, variation of protein amounts between non-stressed and stressed conditions, simultaneous study of protein expression, and protein pattern differences between genotypes. They reported also that, numerous

genomic regions were involved in a complex regulation of those alterations at the molecular level. Moreover, Quarrie[24] reported that using quantitative trait locus (QTL) analysis is became important to study the genetic control of yield under a range of environments more droughty, it then becomes possible to identify whether yield is always controlled by the same set of genes or new genes become important for yield as the stress increases. Seventy-eight proteins with significant quantitative variation (increase or decrease) were obtained among two lines of maize (Zea mays L.) and their hybrid, submitted to progressive water stress for 10 days[28]. Moreover, found some newly protein markers for drought tolerance were induced in wheat cultivars under different PEG concentrations[1], they reported that the newly induced protein bands could be used as markers for indirect selection to the drought tolerance and this could support the development of drought tolerant wheat cultivars.

Genetic Effects of Drought Stress on Wheat Isozyme Patterns: Eelectrophoretic analyses of five enzymes were performed in four wheat genotypes under different drought stress. These enzymes were organized into four groups according to their general enzyme functions; hydrolase (EST, SOD), oxidoreductase (PRX), transferase (GOT) and dehydrogenase (MDH).

On the basis of the presence of esterase (EST) isozyme bands in each callus sample, the four wheat genotype at different PEG conc. displayed five bands (fig. 2-b). Four banding patterns were grouped according to the appearance of the five bands at different PEG conc. (Table 4). The banding pattern (E1) that comprised all the obtained five bands was existed in the four untreated wheat genotypes and also at the low drought stress level (10%) in DH-1. Sakha 93 was affected by disappearance of two bands numbers 1 and 2 at 10% PEG, while Giza 168 and DH-2 were affected by disappearance of one band number 1. With increasing PEG from 10 to 20%, Sakha 93 was much affected by absence of an addition band number 3, followed by Giza 168 with absent in one band number 2. The two double haploid genotypes showed similar genetic response at 20% PEG with banding pattern (E2).

Superoxide dismutase (SOD) isozymes of four wheat genotypes at different drought stress revealed a total of seven (Fig. 2-b), whereas three bands occurred in the anodal and four in cathodal direction (Table 4). The untreated DH-2 was only the genotype that displayed all the seven bands with banding pattern (S1), followed by untreated Sakha 93 that disappeared in two bands with banding pattern (S3), while both of Giza 168 and DH-1 were disappeared in three bands with similar pattern (S4). No difference due to drought stress was observed for Sakha 93 at 10 and 20% PEG Giza 168 and DH-2 revealed the same behavior as Sakha 93 at 10% and 20% PEG, respectively. However, at 10% PEG, DH-1 was uniquely characterized by two addition bands comparing with the control. At 20% PEG, Giza 168 and DH-1 revealed similar behavior with similar pattern (S6), while DH-2 showed a uniquely banding pattern (S5) that distinguished by the absent of three bands (Table 4).

Peroxidase (PRX) isozyme analysis revealed differences in the banding profiles under PEG conc. with a total of seven bands, whereas three bands occurred in the anodal and four in cathodal direction (Fig. 2-b and Table 4). In the cathodal direction, the three bands were presented in the four untreated wheat genotypes and in Sakha 93 at 10% PEG. band number 3 was commonly disappeared at 10% PEG in the other three genotypes and also in the four wheat genotypes at 20% PEG. Among the four anodal direction bands, three bands were consistently appeared in the control and at 10% PEG in all genotypes. At 20% PEG, Sakha 93 was strongly affected by the absent of all the four anodal bands, followed by DH-1 that affected by the absent of three bands, while DH-2 was not affected in the anodal bands by drought stress at any PEG conc. (Table 4).

A total of four bands grouped into three categories were observed for GOT isozyme profile (Fig. 2-b and Table 4). Sakha 93 and DH-1 characterized by the presence of all the four bands at 10% PEG. Two bands 1 and 2 were commonly detected in all wheat calli. Giza 168 and DH-2 showed three bands at all PEG conc. with (G2) and the same banding pattern was observed in the control of Sakha 93 and at 20% PEG, as well as in the control of DH-1. With an exclusion of band number 4; Giza 168, Sakha 93 and DH-2 were not affected dramatically by drought stress at the two PEG levels, whereas they revealed similar banding pattern (G2) as the control, while DH-1 was effectively affected at 20% PEG. A maximum of four bands were detected for MDH isozymes at drought stress according to their mobilities among zymogram (Fig. 2-b and Table 4). The four untreated wheat showed four bands, as well as Giza 168, Sakha 93 and DH-1 at 10% PEG, while DH-2 severely affected with disappearance of three MDH bands at 10 and 20% PEG. Sakha 93 displayed a similar behavior with the same banding pattern at 20% PEG as DH-2.

The modifications of gene expression due to environmental stress are a common response in the metabolism of plant cells. Gene activation due to environmental stimuli plays an extremely important role in the adaptation of plants to unfavorable conditions

Table 4: Isozyme fingerprint classification of four wheat genotypes at different PEG conc. using different banding patterns of four enzyme systems.

Wheat genotype	PEG %	Hydrolase															oxidoreductase									Transferase					Dehydrogenase				
		EST bands					P	SOD bands							P		PRX bands							P		GOT				P		MDH			P
		*1	2	3	4	5		-1	-2	-3	-4	5	6	7			-1	-2	-3	4	5	6	7			*1	2	3	4			*1	2	3	4	
Giza 168	Cont.	+	+	+	+	+	E1		+	+	+	+			S4		+	+	+	+	+	+		P3		+	+	+		G2		+	+	+	+	M1
	10		+	+	+	+	E2		+	+	+	+			S4		+	+		+	+	+		P4		+	+	+		G2		+	+	+	+	M1
	20%			+	+	+	E3		+	+		+			S6		+	+		+	+	+		P4		+	+	+		G2		+	+	+		M2
Sakha 93	Cont.	+	+	+	+	+	E1		+	+	+	+	+		S3		+	+	+	+	+	+	+	P1		+	+	+		G2		+	+	+	+	M1
	10			+	+	+	E3		+	+	+	+	+		S3		+	+	+	+	+	+	+	P1		+	+	+	+	G1		+	+	+	+	M1
	20%				+	+	E4		+	+	+	+	+		S3		+	+						P6		+	+	+		G2		+				M4
DH-1	Cont.	+	+	+	+	+	E1		+	+	+	+			S4		+	+	+	+	+	+	+	P1		+	+	+		G2		+	+	+	+	M1
	10	+	+	+	+	+	E1	+	+	+	+	+	+		S2		+	+		+	+	+	+	P2		+	+	+	+	G1		+	+	+	+	M1
	20%		+	+	+	+	E2		+	+		+			S6		+	+			+			P5		+	+			G3		+	+			M3
DH-2	Cont.	+	+	+	+	+	E1	+	+	+	+	+	+	+	S1		+	+	+	+	+	+	+	P1		+	+	+		G2		+	+	+	+	M1
	10		+	+	+	+	E2	+	+	+	+	+	+	+	S1		+	+		+	+	+	+	P2		+	+	+		G2		+				M4
	20%		+	+	+	+	E2		+		+	+	+		S5		+	+		+	+	+	+	P2		+	+	+		G2		+				M4

and promotes the appearance of specific proteins[21]. In addition, proteins and isozyme polymorphisms are good indicators of response to biotic and abiotic stresses[10].

The results of Italianskaya[18] were supported our finding in the present study, where the author obtained some drought-resistant bread wheat variants through cell selection for osmotic stress resistance with PEG as selective agent and electrophoretic analysis of esterase and peroxidase revealed the specific appearance of new isozyme bands and lack of several isozymes in drought-resistant somaclones as compared with the parental cultivars. On the other hand, the increase substantially in superoxide dismutase (SOD) activity has been shown to confer resistance to drought stress only in the roots of *Chrysanthemum morifolium*[16]. Safarnejad[30] showed that the activity of catalase (CAT) isozyme increased in the tolerant genotypes of Alfalfa (*Medicago sativa* L.), but remained unchanged in the parent when they were subjected to PEG stress. In general, employment of previously described isozyme electrophoretic analysis revealed that the four enzyme systems were useful for displaying the effects of drought stress among the four wheat genotypes, because the four enzymes possess numerous gene loci that code different molecular forms. combined class pattern analysis was performed to obtain better focusing of the four genotypes under drought stress. By so doing, it was possible to identify each wheat genotype by a unique class pattern combination. Therefore, the grouping of the five isozyme patterns was genetically characterized the four genotypes with a unique banding pattern which could be used as a discriminated genetic fingerprint for each genotype alone under drought stress as shown in Table (4). This was agreed with Abdel-Tawab *et al.*[2] who used a combination of four systems of electrophoresis fingerprinting to identify each of 31 *Vicia faba* L. accessions by a unique class patterns. As reported by Soltis and Soltis[32], enzyme electrophoretic analyses using some enzyme systems, such as esterase and peroxidase isozymes are often good indicators of response to biotic and abiotic stresses.

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