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In Vitro Selection for Water Stress Tolerant Callus Line of *Helianthus annus* L. Cv. Myak

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ABSTRACT

Cell cultures of sunflower (*Helianthus annus* L. cv. Myak) were established from callus tissues inoculated in MS liquid medium supplemented with 1.0 mg/L NAA (naphthalene acetic acid), 0.1 mg/L KN (kinetin). PEG 6000 was added to the medium to induce water deficit. Water stress tolerant callus line was isolated by plating the cell suspension on agar-solidified medium containing the same solute potential of PEG (polyethylene glycol). This selected line grew better than the non-selected one on various levels of water deficit induced by PEG. Moreover, the selected line accumulated more K^+, Na^+ and N but less Ca^{++} and P than the non-selected line. The proline level showed a positive correlation with the degree of tolerance to water stress, which suggests that proline accumulation accompanies survival and growth in drought environment. With the exception of polysaccharides, the sugar contents of both callus lines significantly increased with increasing PEG concentration. Protein profile of both selected and non-selected callus lines shows the presence of four major and three minor polypeptide bands. No qualitative differences have been obtained between both callus lines in presence or absence of water stress. No *de novo* proteins have been produced under PEG-induced water stress conditions. DNA banding pattern indicated the occurrence of four *de novo* DNA fragments in the non-selected callus line exposed to -0.8 and -1.0 MPa osmotic stress. Exposing selected callus line to water stress was accompanied by the induction of a unique DNA fragment of an approximate size of 571 bp. The better performance of selected line under water stress may be attributed to its greater osmotic adjustment in relation to non-selected line.

Key Words: *In vitro* selection; Water stress tolerance; PEG; *Helianthus annus* L.

INTRODUCTION

The maximum potential of horticulture crops is seldom attained because of limitations on morphological and physiological processes imposed by stress (Krizek, 1981). Water is becoming increasingly limited in many areas of agricultural production and water deficit is among the most important environmental factors that limit crop productivity. Drought is an environmental stress which is a major barrier to productivity of agricultural crops throughout the world. Crops exposed to this stressful environment are observed initially to have reduced growth rates. If water stress is more severe the response is manifested visually in a number of specific and recognizable symptoms (Rains, 1989).

Sunflower (*Helianthus annus* L.) is one of the most edible oil producing crops in the world. However, in Egypt, still there is shortage of sunflower cultivation because of various factors. Breeding for water stress tolerance by the traditional methods is time consuming procedure (Dorffling *et al*., 1993). Cell culture techniques have proved useful in many areas of plant research. One of the areas in which the *in vitro* selection approach has been used effectively is plant breeding. Selection process can be applied at either the cell population level or on regenerated plants from cell cultures, and followed by selection in conventional field plots (Barakat & Abdel-Latif, 1996; Barakat & Al-Haris, 1998). In fact, plant cell and tissue culture techniques allow screening of a very large population of cells and regenerated plants in a small space and in a much more controlled environment than in conventional field trials. This is possible only when a trait is amenable to *in vitro* selection, and is expressed and transmitted in the regenerated plants and their progenies. *In vitro* selection for cells exhibiting increased tolerance to water stress has been reported (Bressan *et al*., 1981; Harms & Oertli, 1985; Sabbah & Tal, 1990; Borkird *et al*., 1991; Barakat & Abdel-Latif, 1995; El-Haris & Barakat, 1998; Jain, 2001; Barakat *et al*., 2002).

Polyethylene glycol (PEG) of high molecular weights, have long been used to stimulate water stress in plants (Ruf *et al*., 1967; Kaufman & Eckard, 1971; Corchete & Guerra, 1986). PEG of high molecular weight is a non-penetrating inert osmoticum lowering the water potential of nutrient solutions without being taken up or being phytotoxic (Lawlor, 1970).

In this study, isolation and characterization of PEG-tolerant (water stress tolerant) cell lines using cell suspensions of the drought-sensitive sunflower (*Helianthus annus* L. Cv. Myak) has been described as the first step for the possible drought-tolerance improvement of this plant species.

MATERIALS AND METHODS

Callus cultures were established from hypocotyl explants of *Helianthus annus* Cv. Myak obtained from The Agricultural Research center, Giza, Egypt. The culture medium was that of Murashige and Skoog (MS, Murashige & Skoog, 1962) containing 1.0 mg/L NAA (naphthalene acetic acid), 1.0 mg/L KN (kinetin) and 30 g/L sucrose. The medium was adjusted to pH 5.8, solidified with 0.8% agar and autoclaved at 121°C for 20 minutes. Cultures were maintained in 16 hours photoperiod at 28°C ±1. After establishment, calli were subcultured at four weeks intervals on fresh medium. Suspension cultures were established by inoculating callus (5 g fresh weight) into a honey jar containing liquid medium. These cultures were kept on a rotatory shaker at the same conditions described earlier for callus cultures.

Selection procedure. The selected line was obtained by subculturing, every four weeks, on fresh liquid medium supplemented with 200, 270, 295 or 310 g/L of PEG 6000 to induce water potential of -0.4, -0.6, -0.8 or -1.0 MPa, respectively (Mansour & Al-Mutawa, 2000). The suspension cultures were then allowed to settle for about 30 minutes and most of the supernatant was decanted. These cells (or small aggregates of cells) were then inoculated on agar-solidified medium supplemented with 270 g/L PEG 6000 (-0.6 MPa). The selected line was then grown and subcultured every four weeks on fresh medium with increase in PEG (-0.8 & -1.0 MPa). The selected line was then grown and subcultured on fresh medium containing 295 g/L PEG 6000 (-0.8 MPa) since -1.0 MPa was lethal for almost all cells. After ten passages (about eight months) on PEG selection media, necrotic tissues were removed from callus and the surviving cells from the PEG-containing medium were considered water stress-tolerant. These cells were subcultured on fresh medium with the same level of PEG (-0.8 MPa) for almost three months and was designated as selected callus line. The control callus was subcultured on PEG-free medium and was designated as non-selected callus line.

Characteristics of the selected callus line. For the measurement of water stress-tolerance, callus pieces from the two callus lines (selected and non-selected) were transferred to fresh medium with final concentrations of 0, -0.4, -0.6, -0.8 or -1.0 MPa. Four callus pieces per dish and 5 replicate dishes per treatment were used. At the end of four weeks, callus fresh weight was measured.

Elements, carbohydrates and proline measurements. Sodium, potassium and calcium were determined using emission spectrophotometry (PYE UNICAM SP 191). Nitrogen was determined by Kjeldahl digestion method (Haynes, 1980). Phosphorus was determined photometrically as phosphomolybdate (John, 1970).

Carbohydrates were measured using Naguib method (1964) and direct reducing sugars were estimated according to Nelson (1944). Proline content was measured colorimetrically according to Bates (1973).

Protein analysis. Protein content was determined according to Bradford (1976).

Electrophoresis. Samples (30 µg protein) were loaded on 12.2% SDS-polyacrylamide gel and allowed to run at constant electric current (25 mA) for five hours. Molecular weight markers of 218, 131, 86, 43, 33, 19.3 and 7.2 KD were run alongside the sample proteins. The gel was then stained for 6 hours with 0.125% coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid and de-stained in methanol: acetic acid: water (5 : 1 : 4, V : V : V) for several hours.

DNA extraction. The method of Doyle and Doyle (1987) was followed to extract and determine DNA.

PCR amplification. Amplification reaction volumes were 25 µl, each containing 1x PCR buffer with Mg Cl_2 (50 mM KCl; 10 mM Tris-HCl (pH = 9.0); 2 mM Mg Cl_2 and 1% trition x100), 200 µM each of dATP, dCTP, dGTP and dTTP, 50 PM primer, 50 ng template DNA and 1.5 unit 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.

Statistics. Data were statistically tested using Student's t-test for comparison between means of treatments.

RESULTS AND DISCUSSION

Fresh weight. From Table I, it is obvious that the fresh weight of both callus lines decreased with increasing PEG concentration. The selected line exhibited the highest cell mass production on -0.4 MPa. On the other hand, growth of the non-selected callus line was sharply decreased when they were exposed to -0.4 MPa or higher. Similar results were reported in tomato (Bressen *et al*., 1981; Handa *et al*., 1983a), carrot (Fallon & Philips, 1989), wheat (Barakat & Abdel-Latif, 1995b), groundnut (Purushotham *et al.*, 1998) and chili pepper (Santos-Diaz & Ochoa-Alejo, 1994).

Table I. Effect of water stress induced by PEG 6000 on fresh weight of selected and non-selected sunflower cell lines. Data represent means of five replicates ± SE. All treatments of the selected line are significant at 1% level compared with the control

Cell lines	Fresh weight (g)	
Water stress (MPa)	Non selected	Selected
0.0	10.107 ± 0.102	7.713 ± 0.099
- 0.4	7.750 ± 0.107	8.457 ± 0.151
- 0.6	3.057 ± 0.098	5.717 ± 0.141
- 0.8	1.727 ± 0.075	4.320 ± 0.113
- 1.0	0.650 ± 0.031	0.887 ± 0.046

Table II. Effect of water stress induced by PEG on K^+, Na^+, Ca^{++}, N and P (%) of selected (S) and non-selected (NS) callus lines. Data represent means of five replicates ±SE. All treatments of selected line are significant at 1% level compared with the control

Treatment	Potassium		Sodium		Calcium		Nitrogen		Phosphorus	
MPA	NS	S	NS	S	NS	S	NS	S	NS	S
0.0	1.52±0.05	3.92±0.11	0.19±0.01	0.36±0.01	0.74±0.04	0.63±0.06	0.64±0.02	1.31±0.04	0.74±0.01	0.38±0.02
- 0.4	1.87±0.06	2.94±0.05	0.22±0.01	0.27±0.01	0.71±0.01	0.70±0.01	0.65±0.09	1.37±0.06	0.70±0.09	0.21±0.02
- 0.6	1.98±0.06	3.18±0.27	0.28±0.01	0.34±0.01	0.79±0.01	0.71±0.01	0.83±0.15	3.56±0.29	0.33±0.02	0.21±0.01
- 0.8	2.04±0.05	1.56±0.14	0.32±0.01	0.39±0.01	0.76±0.03	0.86±0.01	1.93±0.11	3.14±0.21	0.73±0.03	0.18±0.01
- 1.0	1.13±0.00	2.24±0.14	0.18±0.01	0.27±0.01	0.71±0.02	0.47±0.01	1.13±0.03	2.64±0.08	0.19±0.01	0.34±0.02

Elements. Table II, shows the concentrations of ions at different water stress levels tested. Selected line generally accumulated more Na^+, K^+ and N as compared with non-selected callus line. However, non-selected line accumulated more Ca^{++} and P than the selected one (except at -0.8 and -1.0 MPa for Ca^{++} and P, respectively). Similar results have been reported by Santos-Diaz and Ochoa-Alejo (1994). In their study, PEG-tolerant cell clones of chili pepper accumulated more Na and K than the non-selected cells. In agreement with our finding, the previous authors also indicated that non-selected cells had more Ca that of the selected one. Potassium was found to be much contributed to osmotic potential in relation to other ions (Santos-Diaz & Ochoa-Alejo, 1994), which is also the case in our study. Greater concentrations of accumulated ions obtained in selected line may have adaptive significance under water stress in order to decrease the cell water potential to keep water absorption going on and thus to prevent water loss (Morgan, 1984; Munns, 1988). Higher accumulation of ions suggested more osmotic adjustment in selected line and this was reported to be associated with tolerance to water stress (Morgan, 1984).

Table III. Effect of water stress induced by PEG 6000 on proline content in selected and non-selected sunflower cell lines. Data represent means of five replicates ± SE. All treatments of the selected line are significant at 1% level compared with the control

Cell lines	Proline content (mg/g)	
Water stress (MPa)	Non selected	Selected
0.0	0.543 ± 0.028	0.650 ± 0.012
- 0.4	0.810 ± 0.012	1.567 ± 0.041
- 0.6	0.931 ± 0.012	2.307 ± 0.055
- 0.8	1.218 ± 0.006	3.160 ± 0.026
- 1.0	2.155 ± 0.030	3.757 ± 0.119

Proline. Proline content was increased with PEG in selected and non-selected callus lines. However, the selected callus line accumulated more proline than the non-selected one at all PEG concentrations used (Table III). Proline has been shown to be accumulated in a number of plant tissues in response to different types of osmotic stresses (Yancey *et al.*, 1982; Watad *et al*., 1983; Rudulier *et al*., 1984; Chandler & Thorpe, 1987; Paek *et al*., 1988; Kumar & Sharma, 1989; Jain *et al*., 1991b; Thomas *et al*., 1992; Verbruggen *et al*., 1993; Santos-Diaz & Ochoa-Alejo, 1994; Barakat & Abdel-Latif, 1995; Purushotham *et al*., 1998).

Proline accumulation under water deficit has been mainly recognized as an osmotic agent restricted to the cytoplasm (Handa *et al*., 1983, 1986; Duncan & Widholm, 1987; Mansour, 2000). There is a controversy in the literature as to whether proline accumulation is related to resistance to drought or it is not so related and may even be the result of damage (Hanson *et al*., 1979; Mansour, 2000). This is because in some cases drought sensitive cultivars accumulate more proline than tolerant ones (Hanson & Hitz, 1982; Galiba *et al*., 1989; Mansour, 2000). However, in the present investigation, the proline levels showed a positive correlation with the degree of tolerance to PEG which suggests that proline accumulation accompanies survival and growth in water deficit.

Carbohydrates. With the exception of polysaccharides, the sugar content of both selected and non-selected callus lines significantly increased with increasing PEG concentration (Table IV). The increase was more in the selected line as compared with the non-selected one (except reducing sugars

Table IV. Effect of water stress induced by PEG on reducing sugars, di- and polysaccharides (mg/g) of selected (S) and non-selected (NS) callus lines. Data represent means of five replicates ±SE. All treatments of selected line are significant at 1% level compared with the control

Treatment	Reducing sugars		Disaccharides		Polysaccharides	
MPa	NS	S	NS	S	NS	S
0.0	21.29 ± 0.6	7.64 ± 0.01	70.81± 0.11	154.9± 0.35	159.10± 0.18	205.64± 0.08
- 0.4	118.45± 0.03	16.44± 0.04	122.95± 0.29	174.82± 0.12	137.94± 0.18	148.55± 0.23
- 0.6	143.91± 0.04	107.61± 0.28	122.98± 0.39	223.77± 0.23	114.02± 0.06	126.03± 0.31
- 0.8	149.94± 0.09	181.55± 0.02	144.66± 0.28	236.67± 0.17	110.76± 0.06	91.86± 0.08
- 1.0	172.43± 0.34	202.50± 0.19	201.25± 0.17	249.63± 0.05	89.77± 0.34	59.33± 0.17

at low solute potential). On the other hand, PEG treatment causes a decrease in the polysaccharides content in both selected and non-selected callus lines. The decrease in polysaccharide may be explained by its degradation into disaccharide and monosaccharide, which were increased under water stress. Similar observation has been reported by Kikuta and Richter (1988), and Kameli and Losel (1996) who found reduced level of polysaccharide and increased di- and monosaccharide concentrations under water deficit. It is possible that sugars may be involved in osmotic adjustment under water deficit.

Protein analysis. Fig. 1 shows the protein pattern of the non-selected callus line (lanes 2 to 6) as well as the selected callus line (lanes 7 to 11) exposed to 0.0, -0.4, -0.6, -0.8 or -1.0 MPa, respectively. Analysis of this pattern using Gel Pro-analyzer Software indicated that protein profile of both selected and non-selected callus tissues showed the presence of four major polypeptides. The first one was between 209 and 230 KD, the second one at 40 KD, the third one at 34 KD and the fourth one was at 7 KD. The profile also showed the presence of three minor bands at 170, 186 and 20 KD. The intensity of the bands of non-selected callus line at -0.6 MPa and selected callus lines at -0.4 MPa were less than other treatments. Variations in bands intensity, high accumulation of some polypeptides and less accumulation of others, in *in vitro* cultured materials could be attributed to chromosomal instability of calli survived under stress conditions (Chretin *et al.*, 1992) or to quantitative differences in polypeptides of different treatments.

No qualitative differences have been obtained in the polypeptides of selected and non-selected callus lines or their controls. This result suggests that exposing the sunflower callus tissues to PEG-induced water stress does not stimulate the production of *de novo* protein. Contradictory results have been reported by Ramagopal (1987a) who observed the accumulation of a 26 KD protein in drought-stressed barley seedlings.

DNA analysis. Fifteen random primers were used in the present study to identify the mutagenic alterations among the selected and non-selected sunflower callus lines. Six primers (Table V) were succeeded to generate DNA products. Primer Z_6 indicated the occurrence of four *de novo* DNA fragments in the non-selected callus lines exposed to –0.8 & -1.0 MPa osmotic stress (Fig. 2). These fragments were not detected in other treatments. Two of these fragments have approximate size of 1814 and 1693 bp. The other two have approximate size of 2030 and 2027 bp. Exposing selected callus line to water stress induced by PEG was accompanied by the induction of a unique DNA

Table V. Primer sequences used to detect polymorphism between the selected and non-selected callus lines exposed to water stress

Primer	Nucleotide Sequance 5' TO 3'
A_1	CAGGCCCTTC
A_2	TGCCGAGCTG
B_5	TCGCCCCTTC
B_6	TGCTCTGCCC
Z_5	CAGCACCGCA
Z_6	GTGCCGTTCA

Fig. 1. SDS- polyacrylamide gel electrophoresis of non-selected (lanes 2 to 6) and selected (7 to 11) callus lines exposed to 0.0, -0.4, -0.6, -0.8 or -1.0 MPa, respectively. Lane (1) represents the molecular weight markers

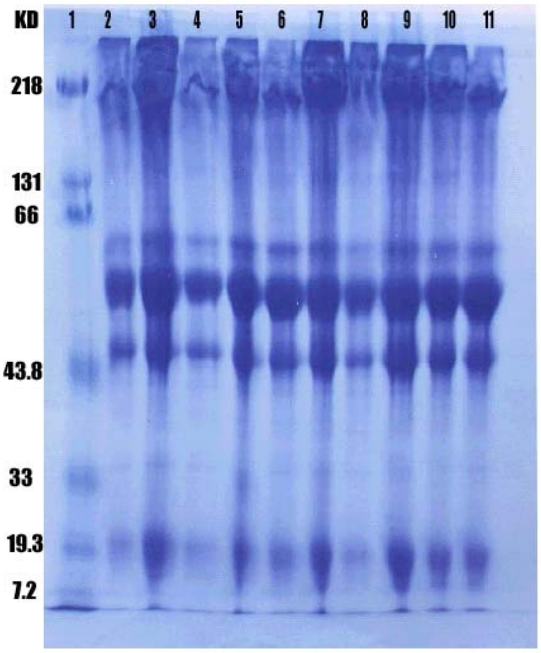
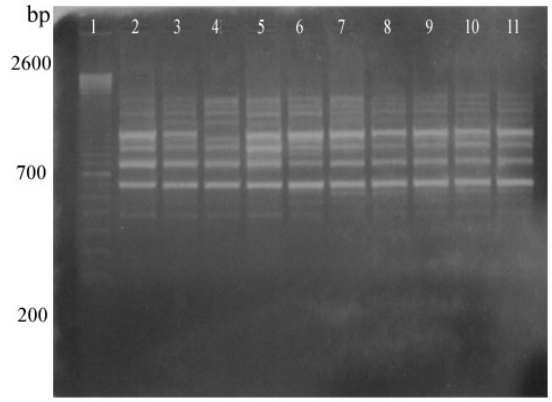


Fig. 2. RAPD profile generated by the primer Z_6 detecting DNA changes among non-selected (lanes 2 to 6) and selected (lanes 7 to11) callus lines exposed to 0.0, -0.4, -0.6, -0.8 or -1.0 MPa, respectively. Lane 1, represents the DNA molecular size markers



fragment of an approximate size of 571 bp. This fragment has not been detected in the non-selected callus line. Changes in genomic DNA of selected and non-selected callus lines were also observed with the other five primers. As one might expect, these changes should have been reflected on RNA and protein patterns which has not been recorded in the present study. This could be attributed to the sensitivity of the RAPD-DNA technique which monitors any minor changes in the genome.

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