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Effect of shock and gradual drought by PEG on callus growth and proline accumulation in sour orange (*Citrus x aurantium*)

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Abstract. The responses of sour orange (*Citrus x aurantium*) callus to different concentration of polyethylene glycol (PEG), induced water stress including callus growth, water content and proline accumulation was studied. To study callus growth in response to two different treatment of PEG-8000 (shock and gradual), 100 mg callus was grown in test tube containing 12.5 ml of solid media supplemented with PEG (MW 8000) at 0, 2, 4, 6, and 8 g/L. Results revealed that increasing water stress initiated by increasing PEG concentration caused a progressive reduction in callus fresh weigh. A significant reduction in callus weight and water content were noticed in response to 4 g/L PEG but the inhibitory concentration was identified to be 8 g/L PEG, whereas, a significant increase in callus weight and water content were noticed in 0, and 2 g/L PEG. Increasing PEG concentration in cultured media caused increase in proline accumulation. In conclusion, this study showed that gradual treatment was better than shock treatment in all studied parameters.
Key Words: Drought stress, PEG, callus, *in vitro*, sour orange.

Introduction. Drought involves the absence of rainfall for a period of time, long enough to cause moisture depletion in plant tissue (Mitra 2001).

Drought is an environmental stress which causes important agricultural losses particularly in arid and semi arid areas (Rai et al 2011). However, tolerances of the crop to drought has not been very well defined and still not clear what aspect of the plant morphology or physiology is the most important factor for drought tolerance. Many efforts should be achieved and still needed in order to define clear target for improving drought tolerance (Zhang 1999).

Plant cell and tissue culture have been an useful tool to study stress tolerance mechanism under *in vitro* condition (Bajji et al 2000), and also used to obtain drought–tolerance plant assuming that there is correlation between cellular and *in vivo* plant response (Mohamed et al 2000).

The drought stress could be induced in the plant cell culture by adding different plant cell cultures by adding different compounds to the nutrient medium such as polyethylene glycol (PEG) which stimulates water stress by acting as osmotic agent which reduces the potential of the medium in where the cells are growing (Gulati & Jaiwal 1994).

PEG of high molecular weight is a non–penetrating inert osmoticum lowering the water potential of the nutrient solution without being taken up or being phytotoxic (Lawlor 1970).

Ion and osmotic homeostasis are necessary for plant to be salt and drought tolerant. Osmotic homeostasis is accomplished by accumulation of compatible osmolytes in the cytosol for intracellular osmotic homeostasis (Hasegawa et al 2000). These include the accumulation of endogenous free proline which contributes in preventing dehydration and cellular damage via balancing the osmotic potential of the cytoplasm with surrounding environment (Handa et al 1982; Santos-Diaz & Ochoa-Alejo 1994).

Citrus, one of the most important agricultural commodities in the world has crossing problem, however, citrus varieties and its close relatives are known to possess important agricultural characters (i.e., disease tolerance of environmental stresses) (Koc et al 2008).

The objective of this study is to examine the response of *Citrus x aurantium* (sour orange) callus to varying degree of PEG–induced water stress by two types of selective methods has been suggested: (a) shock treatment, in which cultures are directly subjected to high concentration; (b) step wise long term treatment, in which cultures were exposed to stress with gradual increase in concentrations of selecting agent. The period between concentrations was 4 weeks. Callus fresh weight, water content and proline accumulation in both treatments were determined.

Material and Method

This study was conducted at Plant Tissue Culture Laboratory, College of Agriculture, Basra University during the period 2012-2013. Healthy seeds were collected from sour orange fruits (Figure 1A), as following.

Preparation of sterilized explant. The fruits were cut by sharp sterilized knife and the nucellus tissues (Figure 1B) were separated and were sterilized with 1.05 % solution of commercial clorax (NaOCl) which contained 2-3 drops of Tween -20 for 15 minutes with autoclaved distilled water inside the laminar airflow chamber.

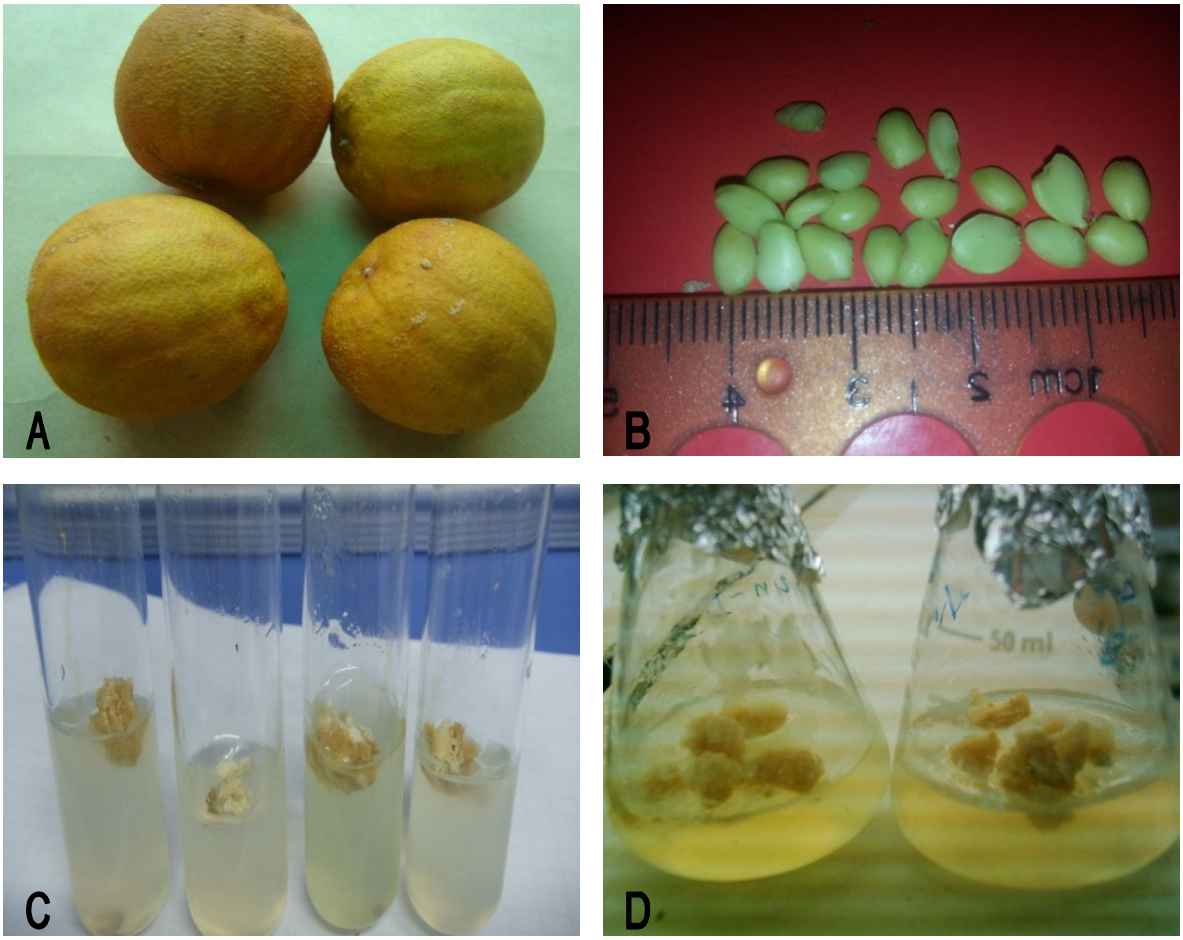

Figure 1. (A) Fruits of sour orange, (B) Nucellus tissues, (C,D) Callus growth in 8 mg/L 2,4-D and 1mg/L BA media.

Preparation of media and callus induction. The sterilized explants were cultured on MS medium (Murashige & Skoog 1962) containing 30 g/L sucrose, 8 mg/L 2,4-D, 1

mg/LBA, 6 g/L agar, 1 g/L PVP (polyvinylpyrrolidone), 1 mg/L of some vitamins and 1mg/L of some amino acids. The medium pH was adjusted to 5.7 ± 0.1 prior to autoclaving at 121 °C and at 1.1 Kg/cm^2 pressure for 20 minutes. The cultures were incubated and growth under dark for 4 weeks.

Stress treatments. 30 days old calli were used in this experiment (Figure 1 C & D). Calli (100 mg) were placed on culture media containing five different concentrations of PEG (8000) (0, 2, 4, 6, and 8 g/L), for two types treatment (shock and graduate). These cultures were maintained under 24 h light (1000 Lux) conditions at 25 ± 1 °C for 4 weeks. Each treatment was included 6 replicates of test tubes.

Statistical anaylsis. The experiment was designed as a factorial experiment (two type of treatments x PEG concentration) with a Completely Randomized Design (CRD) was used in all laboratory studies with at least six replications. All data were subjected to the analysis of variance procedures and treatment means were compared using the Reviesd Least Significant Difference (RLSD) at level 0.05 (Sendecor & Cochran 1982).

Results and Discussion

Fresh weight and water content of callus. Results of this study (Table 1-2) revealed that increasing water stress induced by PEG-8000 caused progressive reduction in callus fresh weight and callus water content of sour orange in either the effect of sudden (shock) and gradual treatments.

Significant callus growth and in callus water content inhibition was observed in response to as low as 2 g/L PEG. Increasing PEG from 4 to 6 and 8 g/L PEG caused reduction in callus fresh weight and in callus water content respectively. These results mean that callus fresh weight and callus water content on MS media containing 0.0 g/L PEG (control) gave the highest callus weight and callus water content (0.2176 g, 90.18 %, 0.2106 g, 90.18 %) respectively, while the lowest callus fresh weight and callus water content were observed in 8 g/L PEG (0.1236 g, 80.32 %) and (0.1356 g, 82.55 %) in shock and gradual treatments respectively. This was because PEG-8000 in solid media lowers water potential of the medium that adversely affect cell division leading to reduced callus growth (fresh weight) (Ehsanpour & Razavizadeh 2005).

Further, the decrease in water content caused a decrease in cell turgor pressure and consequently reduction in callus growth as expressed in callus fresh weight (Al-Bahrany 2002). Additionally, environment stress such as drought increases superoxide dismutase and peroxidase activities, which are implicated in cell membrane damage in sensitive species, then caused a decrease in plant growth (Tahir et al 2003).

In general, the media which containing higher PEG induced osmotic stress might be due to reduced cell division, shrinking imbalance due to reduced loss of cell turgor, nutritional imbalance due to reduced up take of water, increase in electrolyte leakage and decrease in cell water contents with increasing stress (Lokhande et al 2010).

Table 1

Effect of PEG concentration on callus fresh weight (g)

Treatment	*Concentration of PEG g/L*					*Mean of treatment*
	0	*2*	*4*	*6*	*8*	
Shock	0.2176	0.2090	0.1523	0.1353	0.1236	0.1675
Gradual	0.2106	0.2090	0.1706	0.1660	0.1356	0.1784
Mean of concentration	0.2140	0.2090	0.1615	0.1506	0.1297	-
R.L.S.D 0.05	type of treatment = 0.00408		Concentrations = 0.00645		Interaction between treatments = 0.00913	

The effect of water stress on the slowdown of cell division and elongation by loss of turgor has been widely reported (Al-Bahrany 2002; Abdul–Qadir & Al-Ka'aby 2011). Similar results were obtained by Hassan et al (2004), El-Houssine & Mohammed (2012),

Wani et al (2010), Mahmood et al (2012) in sunflower, rice, and wheat respectively. They reported that, the inclusion of PEG into medium of cultured plant cells diminished a gradient favoring water movements into cell and if the PEG concentration was high enough, the gradient would be reversed and causes water to leave the cell and suggested that the growth of callus decreased rapidly as the concentration of the PEG increases.

Table 2

Effect of PEG concentration on callus water content (%)

Treatment	*Concentration of PEG g/L*					*Mean of treatment*
	0	*2*	*4*	*6*	*8*	
Shock	90.18	90.41	85.34	82.13	80.32	85.68
Gradual	90.18	87.08	86.50	83.93	82.55	86.05
Mean of concentration	90.18	88.75	85.92	83.03	81.44	-
RLSD 0.05	Type of treatment = 1.46		Concentrations = 2.32		Interaction between treatments = 3.28	

Proline accumulation. The stressed calli (100 mg) were used to estimate proline accumulation. Water stressed callus cultures exhibited higher levels of free proline content as compared to the control. Results shows (Table 3) that proline content of sour orange callus was increased gradually in response to increasing PEG simulated water stress in both treatments (shock and graduate), it means that concentration of PEG (8 g/L) gave the highest accumulation of free proline content (22.50 and 20.03 µg/g dry weight) respectively in both treatments, while the lowest proline content was obtained in the 0.0 g/L PEG (13.97 and 13.97 µg/g dry weight) in both treatments.

Generally, ions and osmotic homeostasis are necessary for plants to be salt and drought tolerant. Osmotic homeostasis is accomplished by accumulation of compatible osmolytes in the cytosol for intercellular osmotic homeostasis (Hasegawa et al 2000). These include the accumulation of endogenous free proline which contributes to prevent dehydration and cellular damage via balancing the osmotic potential of cytoplasm with surrounding environment (Handa et al 1982; Santos-Diaz & Ochoa–Alejo 1994)).

Additional proline may act as an enzyme protector, stabilizes membranes and cellular structures during hostile conditions, detoxifies free radicals by forming long–lived adducts with them and affects solubility of various proteins by interacting with hydrophobic residue (Hong et al 2000), proline may also serve as an organic nitrogen reservoir ready to be used after stress relief to sustain both amino acid and protein synthesis (Sairam & Tygai 2004).

Accumulation of proline in plant tissues exposed to osmotic stress has been well established in cell and callus cultures (Al-Khayri & Al-Bahrany 2002; Abdul-Qadir & Al-Ka'aby 2011; Mahmood et al 2012; Farshadfara et al 2012) in rice and wheat respectively.

Table 3

Effect of PEG concentration on callus proline content (µg/g)

Treatment	*Concentration of PEG g/L*					*Mean of treatment*
	0	*2*	*4*	*6*	*8*	
Shock	13.97	14.06	15.66	16.90	22.50	16.62
Gradual	13.97	14.06	14.02	16.03	20.03	15.62
Mean of concentrations	13.97	14.06	14.84	16.46	21.26	
RLSD 0.05	type of treatment = 0.311		Concentrations = 0.492		Interaction between treatments = 0.696	

Shock and gradual treatments. Results shown in table 1 – 2 revealed that gradual treatment caused significant callus growth (fresh weight) and callus water content, and gave the highest value (0.1784 g, 86.05 %), respectively in callus cell than the shock treatment which gave the lowest value in fresh weight and callus water content (0.1675 g, 85.68 %), while as gradual treatment improved free proline accumulation in callus cell when increased concentration of PEG in medium (Table 3).

Because of the gradual treatment by PGE gave the enough time to the callus to form number of genes that form osmo–proteins (shock proteins) which considers as anti-drought system, but this proteins were not formed when calli were exposed to shock treatments of PEG (Ziegler 1990; Antoniw et al 1980). These results are similar to those reported by Alouda (2006) in barley and Abdul-Baker (2012) in tomato who observed that gradual treatments were better than shock treatments.

The effect of PEG stress on callus browning and necrosis in sour orange (Figure 2) showed that callus cultures became compact, necrotic and brown when exposed to media containing PEG at concentration 8 g/L after 16 weeks of incubation in light. But no such necrotic and browning were found in the calli culture in media containing 2 g/L PEG, where, the calli in this concentration became incompact with white-greenish color and formed primary vegetative adventitious shoots.

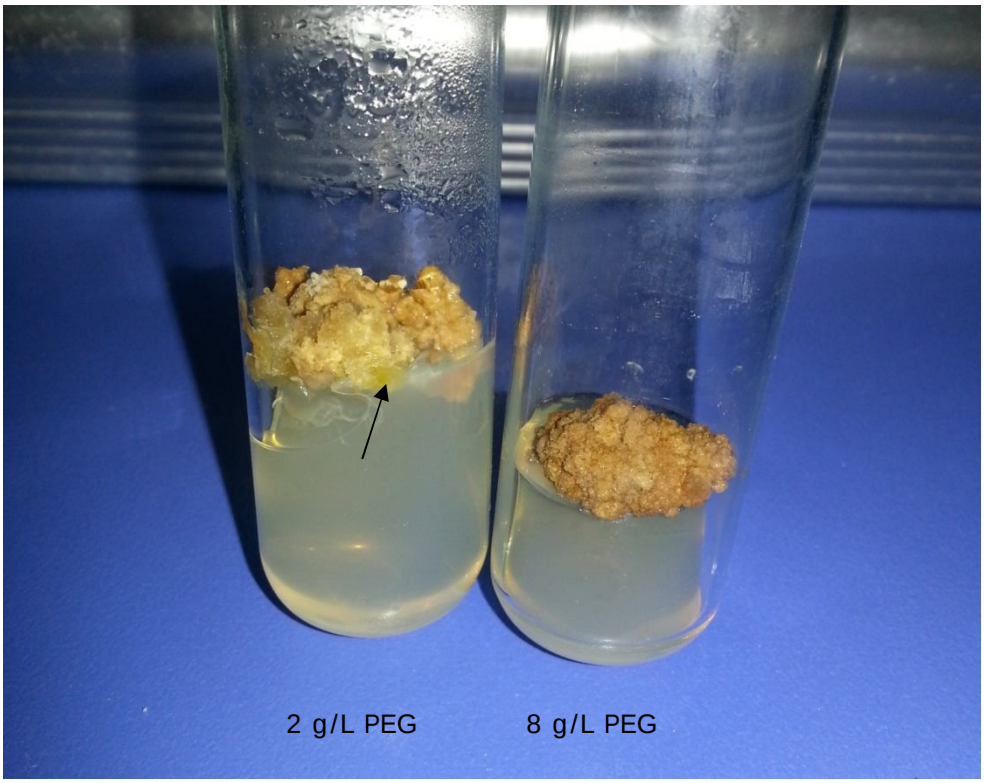

Figure 2. The effect of PEG stress on calli, white and primary shoot in media containing 2 g/L PEG and brown and necrotic in 8 g/L PEG.

Conclusions. This study has characterized the response of sour orange callus to drought stress. Results showed that increasing the PEG-8000 concentration in media, inhibited callus growth and its water content, whereas, free proline content was increased and accumulated in callus cells of sour orange. However, gradual treatment showed best results in all studied traits as compared with shock treatment.

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