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Callus Growth and Proline Accumulation in Response to Polyethylene Glycol-induced Osmotic Stress in Rice, *Oryza sativa* L.

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Abstract: The responses of Hassawi rice (*Oryza sativa* L.) callus was studied to varying degrees of polyethylene glycol (PEG)-induced water stress including callus growth, water content and proline accumulation. To characterize callus growth in response to PEG, 2.5 g embryogenic callus was grown in 125 ml flasks containing 50 ml each of liquid MS medium supplemented with PEG (MW 8000) at 0, 50, 100, 150, 200, 250 and 300 g l^{-1}. Results revealed that increasing water stress induced by increasing concentration of PEG caused a progressive reduction in callus fresh weight. Significant reduction in callus weight was observed in response to 50 g l^{-1} PEG, but the inhibitory concentration was identified to be 200 g l^{-1}. Increasing PEG concentration was also associated with a progressive reduction in callus water content, which caused increase in proline accumulation reaching significant increase over the control at 100 g l^{-1} PEG. This study serves as a precursor for genetic improvement efforts to enhance the tolerance of Hassawi rice to water stress.

Key words: Drought stress, PEG, relative growth rate, tissue culture, *in vitro*

Introduction

Biotechnological approaches are indispensable tools to achieve crop improvement, but application of such approaches requires the availability of a regeneration system for the crop of interest. Although *in vitro* regeneration of rice (*Oryza sativa* L.) has been well documented (Heyser *et al*., 1983; Oard and Rutger, 1988; Rueb *et al*., 1994; Al-Khayri *et al*., 1996), only recently a tissue culture system for Hassawi rice was developed (Al-Khayri and Al-Bahrany, 2000). This highly nutritious brown rice is a land race variety indigenous to Alhassa area, eastern Saudi Arabia which is predominately a hot, arid region characterized stress conditions for plant growth. Improvement of agronomic characteristics such as tolerance to water stress conditions would be of a paramount importance for growing this indigenous important crop in such an arid area.

Cell and callus cultures provide controlled, uniform environment for studying physiological and biochemical processes in plants, particularly mechanisms operative at the cellular level such as water stress responses (Hasegawa *et al*., 1984). To simulate the effect of water stress *in vitro*, researchers have incorporated polyethylene glycol (PEG) in the culture medium (Handa *et al*., 1982; Bhaskaran *et al*., 1985; Newton *et al*., 1986; Newton *et al*., 1989). This compound is a high molecular weight, non-ionic, non-plasmolysing that simulates drought stress in cultured cells in a similar manner to that observed in the cells of intact plants subjected to drought conditions (Hohl and Schopfer, 1991; Attree *et al*., 1991). Drought stress induces osmotic adjustment through the accumulation of solutes. These include the accumulation of endogenous free proline which contributes to preventing dehydration and cellular damage by balancing the osmotic potential of the cytoplasm with surrounding environment (Handa *et al*., 1982; Santos-Diaz and Ochoa-Alejo, 1994a, 1994b). Moreover, overproduction of endogenous free proline in response to water stress can be used as a biochemical marker beneficial for selecting drought tolerant biotypes for crop improvement (Aspinall and Paleg, 1981). In rice, various *in vitro* studies have focused on physiological and biochemical aspects related to salt and drought stress (Lutts *et al*., 1996; Reddy and Vajranabhaiah, 1996; Chauhan and Prathapasenan, 1998; Al-Khayri and Al-Bahrany, 2002). Genotype differences among rice cultivars in relation to callus responses to stress have been observed (Reddy *et al*., 1994).

This study was conducted as a precursor for future work aimed at the improvement of Hassawi rice through somaclonal selection of biotypes with increased tolerance to water stress. Because the behaviour of this rice in response to PEG-induced stress is unexploited, this study was conducted to determine the inhibitory concentration to serve as the basis for somaclonal selection. The objective was to examine the responses of Hassawi rice callus to varying degrees of PEG-induced water stress including callus growth, water content and proline accumulation.

Materials and Methods

Culture establishment and callus induction was according to previously described procedures (Al-Khayri and Al-Bahrany, 2000). Mature rice seeds, cv. Hassawi, were dehusked manually and surface sterilized for 1 min in 70 % ethanol, followed by 30 min shaking in 2.6% w/v sodium hypochlorite containing 3 drops Tween 20 per 100 ml solution, then rinsed several times in sterile distilled water. The seeds were cultured on MS salts (Murashige and Skoog, 1962) supplemented with 1 mg l^{-1} thiamine-HCl, 1 mg l^{-1} pyridoxine-HCl, 1 mg l^{-1} nicotinic acid, 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose, 1.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg l^{-1} 6-furfurylaminopurine (kinetin). The medium was solidified with 8 g l^{-1} agar [Agar-agar/Gum agar] (Sigma Chem Co, St. Louis, MO) and adjusted to pH 5.8 with 1N KOH. Culture medium was dispensed in 150 x 25-mm culture tubes (15 ml medium per tube) and autoclaved at 121°C and 1 x 10^5 Pa (1.1 kg/cm^2) for 15 min. The cultures were incubated at 24± 2°C under a 16-h photoperiod of cool-white fluorescent light (40 μmol m^{-2} s^{-1}). After 4 weeks, the resultant calli were separated from the explants and transferred to callus maintenance medium containing 1.25 mg l^{-1} 2,4-D to encourage further callus proliferation. Calli were maintained for an additional 12 weeks by transferring to a fresh maintenance medium dispensed in 125 ml flasks (50 ml per flask) after which the calli were used to study the response to PEG.

To characterize callus growth in response to PEG, 2.5 g embryogenic callus was grown in 125 ml flasks containing 50 ml each of liquid maintenance medium supplemented with PEG (MW 8000) at 0, 50, 100, 150, 200, 250 and 300 g l^{-1}. The cultures were placed on a gyratory shaker set at 150 rpm for 2 weeks. To determine the effect of PEG concentration on callus growth, callus fresh weight was determined and relative growth rate (RGR) based on fresh weight was calculated according to the following formula: RGR = [ln(final weight)-ln(initial weight)]/weeks.

To study the effect of PEG treatments on water content, callus samples of known fresh weight were dried in an oven set at 65° C for 48 h after which they were reweighed and the differences in weight were determined. The water content was expressed as a percentage of callus fresh weight.

To examine the effect of PEG treatments on proline accumulation in response to PEG, 500 mg fresh callus samples were used for extraction and estimation of free proline according to Bates *et al.*

Table 1: Analysis of variance for Hassawi rice callus weight, relative growth rate (RGR), water content and free proline accumulation

		Callus weight		Relative growth rate		Water content		Free proline content	
Source	df	MS	*p*-value	MS	*p*-value	MS	*p*-value	MS	*p*-value
PEG conc.	6	4.3193	< 0.0001	0.0688	< 0.000121	0.5713	< 0.0001	18.7317	< 0.0001
Error	49	0.0982		0.0011		1.1032		0.0216	

P-values less than 0.05 are significant.

(1973). The samples were homogenized in 10 ml of 3% (w/v) aqueous sulphosalicylic acid and the homogenate was filtered through Whatman No. 2 filter paper. In a test tube 2 ml of the filtrate was mixed with 2 ml acid ninhydrin and 2 ml glacial acetic acid and incubated in 100 °C water bath for 1 h. The reaction mixture was terminated by placing in ice bath, extracted with 4 ml toluene and the chromophore phase was aspirated from the aqueous phase. The absorbance was read at 520 nm using LKB Navaspec Model 4049 spectrophotometer.

The experiment was set up as a completely randomized single factor design with eight replications. The main factor was PEG concentration at seven levels. Data were subjected to analysis of variance (ANOVA) and the means were separated, where appropriate, using the least significant difference (LSD) at 5% significance. To confirm results, the experiment was repeated twice.

Results and Discussion

Callus growth: Decreased cell growth must be the most sensitive response of the plant to water stress, since cell growth is quantitatively related to cell turgor which decreases with increased dehydration. Dehydration induced by PEG reduces the availability of water and thus turgidity and growth (Heyser and Nabors, 1981). In a number of plant species, researchers have observed inhibitory effect of PEG on growth and proliferation of callus expressed in fresh weight (Santos-Diaz and Ochoa-Alejo, 1994b; Heyser and Nabors, 1981; Bornman and Huber, 1979). In rice also PEG-induced water stress was shown to inhibit callus growth (Reddy and Vajranabhaiah, 1996). Previous studies have shown that the response of *in vitro* cultures to water stress is related to genotype (Santos-Diaz and Ochoa-Alejo, 1994b; Cress and Johnson, 1987; Tschaplinski *et al.*, 1995). In rice, genotype differences in response to water stress have been observed (Reddy *et al.*, 1994). Therefore, it is pertinent to determine the response of this particular rice genotype, Hassawi rice, which is adapted to the adverse environmental conditions typical of xeric climate which predominate the Saudi Arabia. The present study revealed that increasing water stress induced by PEG caused a progressive reduction in callus fresh weight of Hassawi rice (Fig. 1A). Significant callus growth inhibition was observed in response to as low as 50 g l^{-1} PEG. As PEG concentration was increased to 100 g l^{-1} further significant reduction in callus fresh weight occurred. Increasing PEG to 150 g l^{-1} caused no significant change; however, at 200 g l^{-1} callus fresh weight was significantly further reduced. Beyond this concentration, no significant differences in callus fresh weight were noted. It is worth noting that even at these concentrations callus growth occurred at 20% increase over the initial weight of inoculums. This growth, however, is considered minor as compared to the nearly 100% weight increase exhibited by the callus grown on the PEG-free control. Based on these observations, 200 g l^{-1} PEG is considered the critical inhibitory level which can be used in selection for drought tolerant cell lines of Hassawi rice. The critical inhibitory level may vary depending upon the species. For example, the inhibitory PEG level in *Capsicum annuum* L. and *Larrea tridentate* were 10% and 25%, respectively (Santos-Diaz and Ochoa-Alejo, 1994a), while the inhibitory level for *Populus trichocarpa* Torr. & Gray and *Populus deltoids* Bartr. was 20% (Tschaplinski *et al.*, 1995). Based on the resultant fresh weight, callus growth

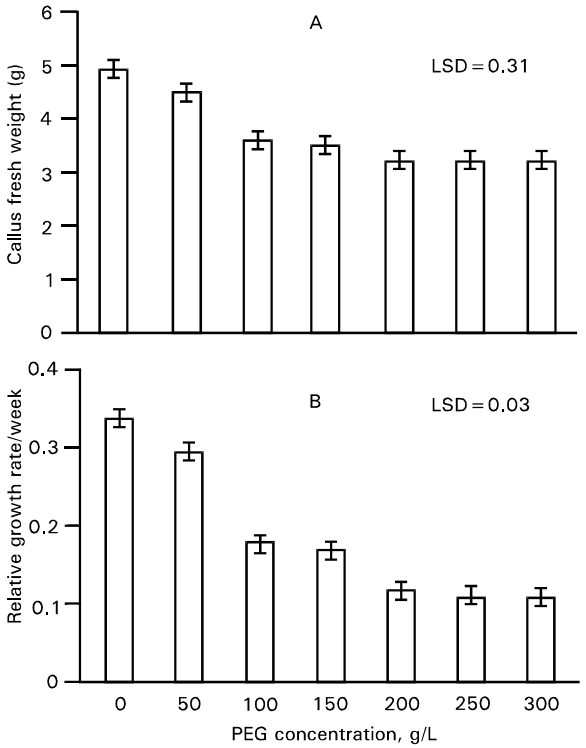

Fig. 1: Response of Hassawi rice (*Oryza sativa* L.) to polyethylene glycol concentration in relation to (*a*) callus weight and (*b*) relative growth rate

expressed in RGR followed the same pattern as callus fresh weight (Fig. 1B). Reduction in RGR was observed in response to increasing PEG concentration.

Water content: It has been established that the water potential gradient between the cells and the nutrient medium caused by PEG results in dehydration of the cells (Hasegawa *et al.*, 1984; Heyser and Nabors, 1981). Previous studies have shown that increases in osmotic stress by PEG was accompanied by steep decline in moisture content of tissues (Heyser and Nabors, 1981; Bornman and Huber, 1979). In Hassawi rice also increasing PEG concentration was associated with a progressive reduction in callus water content (Fig. 2A).

Proline accumulation: Improving crop resistance to osmotic stresses is a major goal of agricultural biotechnology and can be facilitated by the use of biochemical markers, such as proline analysis. This is based on the fact that certain plants have evolved high capacity to synthesize and accumulate non-toxic solutes, predominantly in the cytoplasm as part of an overproduction mechanism to raise osmotic pressure and thereby maintain both turgor and the driving gradient for water uptake under osmotic stresses (Hare *et al.*, 1999). Accumulation of proline in plant tissues exposed to osmotic stress has been well established in cell and callus cultures (Hasegawa *et al.*, 1984; Santos-Diaz and Ochoa-Alejo, 1994b; Al-Khayri and Al-Bahrany, 2002; Handa *et al.*, 1986).

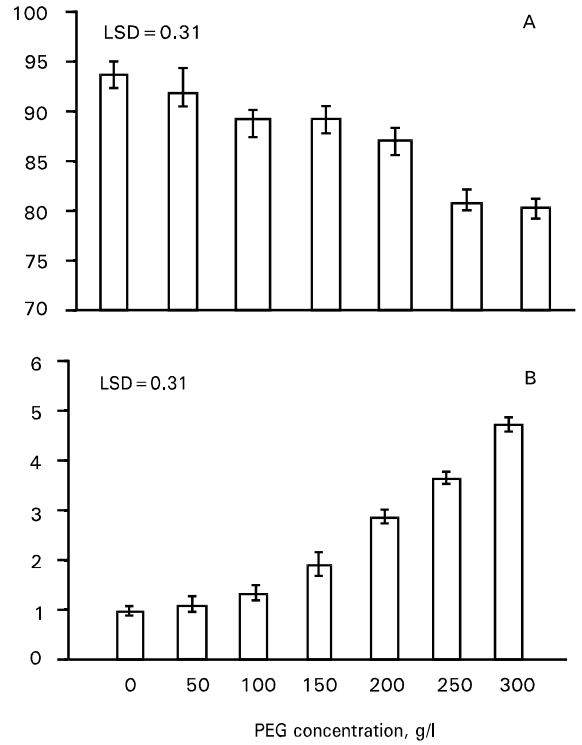

Fig. 2: Response of Hassawi rice (*Oryza sativa* L.) to polyethylene glycol concentration in relation to (*a*) water content and (*b*) free proline accumulation.

In the current study, proline content of Hassawi rice callus was shown to increase gradually in response to increasing PEG-simulated water stress (Fig. 2B). Low level of PEG was observed to cause slight increase in proline content; however, significant increase in proline content was seen on 100 g l^{-1}. The level of proline accumulation continued to rise even when increases in growth diminished.

This study has characterized the response of Hassawi rice cell cultures to water stress. Results have shown that increasing PEG concentration caused increased water stress as indicated by the decrease in water content which in turn stimulated the proline synthesis and increased its accumulation. This is evident by the highly significant negative correlation (Pearson correlation coefficients = -0.929) between proline and water content of callus exposed to drought stress. The decrease in water content caused a decrease in cell turgor pressure and consequently reduced callus growth as expressed in fresh weight; hence, significant positive correlation between callus weight and water content (Pearson correlation coefficients = 0.779). This understanding of Hassawi rice callus and the information obtained related to physiology and growth will facilitate the recovery of tolerant Hassawi rice biotypes.

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