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BRIEF COMMUNCIATION

Growth, water content, and proline accumulation in drought-stressed callus of date palm

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Abstract

This study was conducted to examine the response of date palm (*Phoenix dactylifera* L., cvs. Barhee and Hillali) calli to water stress. Callus derived from shoot tip explants was inoculated in liquid Murashige and Skoog medium containing 10 mg dm^{-3} α-naphthaleneacetic acid, 1.5 mg dm^{-3} 2-isopentenyladenine, and 0 to 30 % (m/v) polyethylene glycol (PEG 8000) to examine the effect of water stress. After 2 weeks, callus growth, water content, and proline accumulation were assessed. Increasing water stress caused a progressive reduction in growth as expressed in callus fresh mass, relative growth rate, and index of tolerance. Both genotypes tested followed this general trend, however, cv. Barhee was more tolerant to drought stress than cv. Hillali. Increasing PEG concentration was also associated with a progressive reduction in water content and increased content of endogenous free proline.

Additional key words: index of tolerance, osmotic adjustment, relative growth rate, tissue culture, water stress.

Water availability is one of the principal limitations of crop production, particularly in the arid and semiarid regions where date palm is predominantly grown. The use of *in vitro* cultures to study stress responses is based on the fact that *in vitro* cultured cells behave similarly to cells of intact plants subjected to water deficit and salinity stress conditions (Attree *et al*. 1991). Moreover, species differing in drought tolerance at the whole plant level also usually exhibit differences in drought tolerance in cell cultures (Santos-Diaz and Ochoa-Alejo 1994a). Undifferentiated cells and callus cultures eliminate complications associated with genetic and morphological variability inherent to different tissues in whole plants. Moreover, cell culture systems eliminate all responses associated with water stress except those operative at the cellular level (Hasegawa *et al*. 1984, Newton *et al*. 1989). These studies involved the simulation of the effect of water stress by augmenting the culture media with polyethylene glycol (PEG) which induces osmoregulation through the accumulation of solutes such as proline.

Although, various *in vitro* studies focused on aspects of plant regeneration in date palm (Omar *et al*. 1992, El Hadrami and Baaziz 1995, Veramendi and Navarro 1996, Al-Khayri 2001, Al-Khayri and Al-Bahrany 2001), little is known about physiological parameters affecting date palm *in vitro* cultures. Recently, the behaviour of date palm callus subjected to salt stress was elucidated (Al-Khayri 2002). Reports about the response of date palm callus to water stress are lacking, hence, this research was conducted. Cell suspension cultures of two date palm cultivars were exposed to varying degrees of PEG-induced water stress to examine growth response, water content, and proline accumulation.

Shoot tips were separated from 3- to 4-year-old offshoots of date palm (*Phoenix dactylifera* L., cvs. Barhee and Hillali), surface sterilized in 70 % ethanol for 1 min followed by 15 min in 1.6 % m/v sodium hypochlorite containing a few drops of *Tween 20*, and rinsed

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Abbreviations: MS medium - Murashige and Skoog medium medium; PEG - polyethylene glycol; RGR - relative growth rate.
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with sterile distilled water four times. The tissue was then placed in chilled antioxidant solution consisting of 150 mg dm^{-3} each of ascorbic acid and citric acid to prevent browning. Whole leaf primordia and terminal tip longitudinal sections served as explants for culture initiation.

The basal culture medium consisted of Murashige and Skoog (1962) salts supplemented with [mg dm^{-3}] NaH_2PO_4 (170), myo-inositol (125), ascorbic acid (100), citric acid (100), glutamine (200), nicotinic acid (1), pyridoxine-HC1 (1), thiamine (1), biotin (1), glycine (2), calcium pentothenate (1), sucrose (30000), and purified agar (7000) (*Sigma*, St. Louis, USA). Media were adjusted to pH 5.7 with 1 M KOH, dispensed in *GA-7 Magenta* (*Sigma*) vessel (50 cm^3 per vessel) and autoclaved at 121 °C and 105 Pa for 15 min. The hormonal supplement and activated charcoal were added to the culture medium according to the culture stage.

Culture initiation medium was supplemented with 100 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 3 mg dm^{-3} 2-isopentenyladenine (2iP), and 1.5 g dm^{-3} activated charcoal (acid-washed, neutralized) (*Sigma*). These cultures were incubated in darkness at 24 ± 3 °C for 12 weeks during which they were transferred at 3-week intervals. The resultant callus was transferred to callus proliferation medium that contained MS basal salt augmented with 10 mg dm^{-3} α-naphthaleneacetic acid (NAA), 30 mg dm^{-3} 2iP, and 1.5 g dm^{-3} activated charcoal. After 3 weeks, the callus was transferred to MS basal medium containing 10 mg dm^{-3} NAA, 6 mg dm^{-3} 2iP, and 1.5 g dm^{-3} activated charcoal. Callus proliferation cultures were maintained at 24 ± 3 °C and a 16-h photoperiod of cool-white fluorescent light (irradiance of 50 μmol m^{-2} s^{-1}). After 9 weeks (3 sub-cultures), embryogenic callus was transferred to callus maintenance medium containing 10 mg dm^{-3} NAA and 1.5 mg dm^{-3} 2iP. To obtain sufficient amount of callus, these cultures were maintained in the dark at 24 ± 3 °C for 6 months after which they were used as a callus source for the current study.

To determine the callus response to drought stress, 2 g embryogenic callus was grown in 125-cm^3 flasks containing 50 cm^3 each of liquid maintenance medium supplemented with PEG 8000 in concentrations ranging from 0 to 30 % at 5 consecutive increments. 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 mass were determined and relative growth rates (RGR) based on fresh mass were calculated according to the following formula:

RGR = [In(final mass)-ln(initial mass)] / growth period

An index of tolerance (INTOL), used for comparing cultivar-related responses to stress conditions, based on RGR was calculated according to the following formula:

INTOL = RGR treatment / RGR control

Callus samples of known fresh mass were dried in an oven set at 65 °C for 48 h after which they were reweighed and the difference in mass was determined. The water content was expressed as a percentage of callus fresh mass.

Extraction and estimation of free proline were conducted according to the procedures described by Bates *et al*. (1973). Fresh callus, 500 mg per sample, was homogenized in 10 cm^3 of 3 % (m/v) aqueous sulphosalicylic acid and the homogenate was filtered through *Whatman No. 2* filter paper. In a test tube, 2 cm^3 of the filtrate was mixed with 2 cm^3 acid ninhydrin and 2 cm^3 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 cm^3 toluene, and the chromophore phase was aspirated from the aqueous phase. The absorbance was read at 520 nm using *LKB Novaspec Model 4049* spectrophotometer (*LKB Biochrom*, Cambridge, England).

The experiment was set up as a completely randomized 2 × 7 factorial design with six replications. Data were subjected to analysis of variance (*ANOVA*) and the means were separated, where appropriate, using the least significant difference (LSD) at 5 % significance. Standard deviations from the means were also calculated. The experiment was repeated twice to confirm results.

A common feature of *in vitro* cell and callus cultures in response to drought stress is the reduction in growth (Santos-Diaz and Ochoa-Alejo 1994b). Similarly, in date palm increasing water stress caused a progressive reduction in growth as expressed in callus fresh mass (Fig. l*A*). Differences in growth in response to water stress have been observed among genotypes of other plant species (Cress and Johnson 1987, Reddy *et al*. 1994, Santos-Diaz and Ochoa-Alejo 1994b, Tschaplinski *et al*. 1995). Concurrently, date palm callus fresh mass was significantly higher in cv. Barhee than cv. Hillali when callus was exposed to 10, 15, and 25 % PEG while other treatments resulted in a similar growth (Fig. l*A*). In both genotypes callus growth was significantly inhibited in response to as low as 5 % PEG. Both genotypes almost completely ceased growth when exposed to 20 % PEG beyond which callus fresh mass decreased. The critical concentration may vary depending upon the species. For example, it was 10 % and 25 % in *Capsicum annuum* and *Larrea tridentata*, respectively (Santos-Diaz and Ochoa-Alejo 1994a) while in *Populus trichocarpa* and *Populus deltoides* it was 20 % (Tschaplinski *et al*. 1995).

Steady reduction in RGR was also observed in response to increasing PEG-concentration in both cultivars (Fig. 1*B*). Although, controls of both cultivars exhibited similar RGRs, the RGRs were significantly altered in favour of cv. Barhee when PEG was added in

the culture medium. This difference diminished when callus was grown on 20 and 30 % PEG.

Callus growth was also expressed as INTOL to eliminate inherent differences associated with the relative growth rate of the two cultivars in response to stress. Based on INTOL, cv. Barhee exhibited higher tolerance to PEG-induced water stress (Fig. 1*C*). The INTOL values obtained followed a similar pattern as that of the RGR in both cultivars because the RGRs of the controls in both cultivars were almost identical.

The water potential gradient between the cells and surrounding nutrient medium caused by PEG results in cell dehydration. (Hasegawa *et al*. 1984). Increasing PEG concentration was associated with a progressive reduction in water content of date palm callus (Fig. 2*A*). This general trend was observed in both genotypes tested. Under non-stress condition, water content of cv. Hillali was higher than that of cv. Barhee. This relationship persisted when callus was treated with 5 % PEG, however, at higher PEG concentrations water content of cv. Barhee exceeded that of cv. Hillali. This suggests that the callus ability to retain water under water stress was better in cv. Barhee as compared to cv. Hillali. The differences between the two cultivars in relation to water content, however, were significant only at 5 and 15 % PEG.

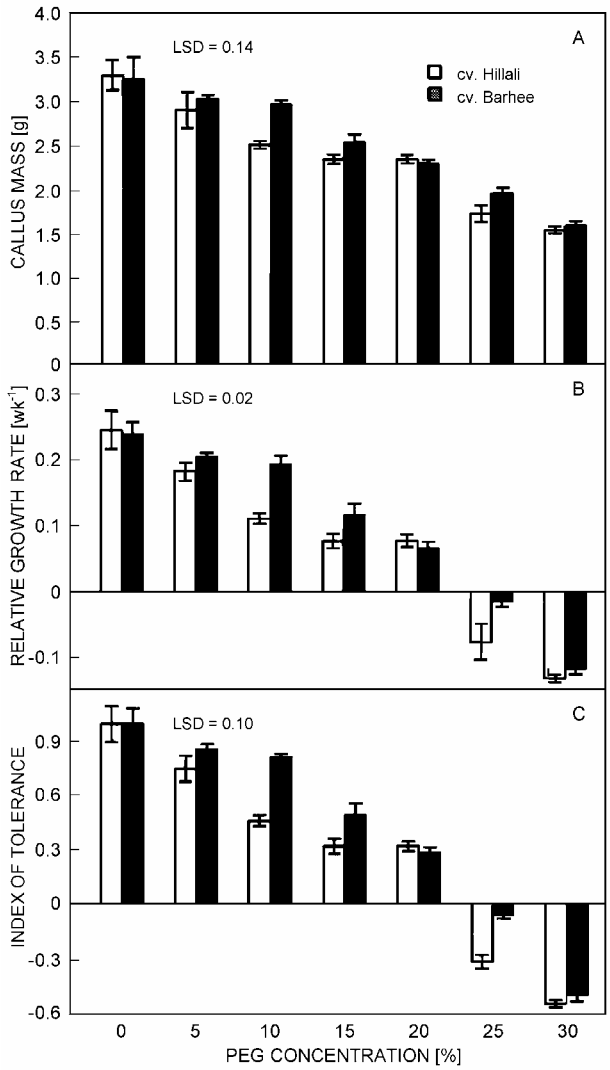

Fig. 1. Effect of polyethylene glycol concentration on callus mass (*A*), relative growth rate (*B*), and index of tolerance (*C*) of callus cultures in two date palm cultivars Barhee and Hillali. Means ± SD, $n = 6$.

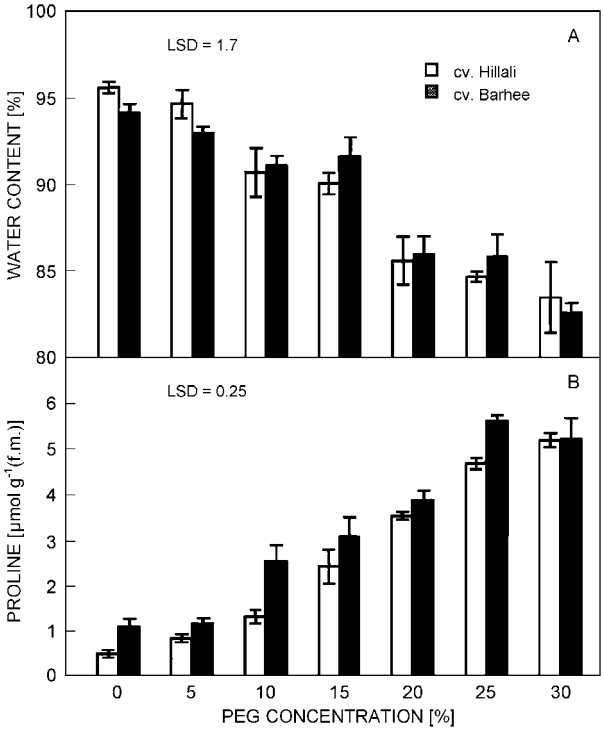

Fig. 2. Effect of polyethylene glycol concentration on water content (*A*) and proline accumulation (*B*) of callus cultures in two date palm cultivars Barhee and Hillali. Means ± SD, $n = 6$.

Osmoregulation through the accumulation of cellular solutes, such as proline, has been proposed as a possible means for overcoming water stress. Accumulation of endogenous free proline in plant tissues exposed to osmotic stress has been documented in various *in vitro* cultures (Hasegawa *et al*. 1984, Handa *et al*. 1986, Santos-Diaz and Ochoa-Alejo 1994b, Al-Khayri and Al-Bahrany 2002, Demir and Kocaçalişkan 2002, Fedina *et al*. 2002). In the current study, endogenous free proline content of date palm callus increased gradually in response to increasing PEG concentration (Fig. 2*B*). Both genotypes tested followed this general trend; however, they differed in their sensitivity to various PEG concentrations. At 5 % PEG, proline content was unaffected in relation to the PEG-free control in cv. Barhee. In contrast, cv. Hillali showed a significant increase in proline accumulation (Fig. 2*B*). The accumulation of proline in cv. Barhee callus significantly

exceeded that of cv. Hillali in response to all tested PEG concentration except at 30 %. Comparable to our finding, variability in the ability of proline accumulation in response to water deficit has been observed among cultivars of *Hordeum vulgare* (Naidu *et al*. 1992).

To summarize, callus growth and water content were shown to be negatively related to water stress induced by increasing PEG concentrations. Osmotic adjustment through the accumulation of proline was positively related to PEG concentration. Although growth was completely inhibited at 25 % PEG, proline accumulation continued to increase suggesting that the cells remained viable even at 30 % PEG. The study also demonstrated that drought tolerance differs between date palm cultivars. Under non-stress conditions, although both cultivars exhibited similar growth, cv. Barhee had lower water content and higher proline content as compared to cv. Hillali. These parameters translated to a higher tolerance in cv. Barhee when subjected to water stress conditions.

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