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Full Length Article

Response of Different Date Palm Cultivars to Salinity and Osmotic Stresses using Tissue Culture Technique

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Abstract

Date palm (*Phœnix dactylifera* L.) is moderately tolerant to salinity and drought. However, information about the variation in salinity and drought tolerance among the date palm cultivars is limited. Thus, a method of *in vitro* propagation via somatic embryogenesis from shoot tips of date palm cvs. Barhi and Khalas under polyethylene glycol (PEG) induced osmotic stress or NaCl induced salinity stresses was investigated in this study. Highest induction frequencies of embryogenic calli occurred after 12 months on MS medium supplemented with 10 mg L^{-1} 2,4-D, 3 mg L^{-1} 2iP and 2 g L^{-1} activated charcoal. Optimum MS medium for higher frequency of matured somatic embryos and plant regeneration was recorded using 0.5 mg L^{-1} NAA, 3.0 mg L^{-1} 2iP of cv. Barhi and 1.0 mg L^{-1} NAA, 4.0 mg L^{-1} 2iP of cv. Khalas. The embryogenic callus of both cultivars was cultured on different levels of NaCl or PEG-induced stresses after 12 weeks for three subcultures. There were considerable differential responses between date palm cultivars about tolerance to salinity and osmotic stresses. However, the growth of cv. Barhi was completely ceased at levels above 300 mM NaCl or 25 g L^{-1} PEG while cv. Khalas continued to grow. This indicated that cv. Khalas is more tolerant to high salinity and osmotic stresses than the cv. Barhi. This study also showed that use of tissue callus is an effective way to monitor the response of date palm cultivars to salinity and osmotic stresses.

Keywords: Abiotic stresses; Somatic embryogenesis; *Phoenix dactylifera* L.; Plant growth regulators; Biochemical markers
Abbreviations: 2iP − 2-isopentenyladenine; AC-Activated charcoal; ABA-Abscisic acid; BAP− Benzyl Amino Purine; GA3-Gibberellic acid; IBA− 3-Indole Butyric Acid; MS – Murashige and Skoog medium; NAA − 2-Naphthalene Acetic Acid; Kn – Kinetin; PEG-Polyethylene glycol; PGR- plant growth regulators

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most widespread fruit crops in arid and semi-arid areas of the world, mainly Middle East, North and South Africa for at least 5000 years (Zohary *et al.,* 2012). It is widely grown in some Arab countries, including Saudi Arabia (Jain, 2012), and has recently been introduced to new area in California, South America, Mexico, Spain and India (Chao and Krueger, 2007). More than 300 types of date are produced within Saudi Arabia which considers the second largest producer of dates in the world (Assirey, 2015). The increase in date palm cultivation in this region is due to the availability of climatic needs for growth, as well as the ability of date palms to tolerate the climate stresses and to preserve ecosystems threatened by desertification. The high nutritional value of fruits made it a desirable crop among the inhabitants of Arab world. In addition, the cultivation of date palm generates significant employment opportunities in rural areas, and it is the main source of income for farmers. It also ensures the livelihoods and food security in rural areas (Jain *et al.,* 2011; Rajmohan, 2011).

Biotic and abiotic stresses remained a serious stress constraint facing several of date palm germplasm, which might lead to reduce in the genetic biodiversity (Djibril *et al*., 2005). Salt and drought in arid regions, such as Saudi Arabia, adversely affect productivity of most of the plants including date palm. Due to their negative impact, it is imperative to establish breeding programs to develop stress tolerant date genotypes. Screening and selection of date palms cultivars growing in farm of diverse salt and drought levels is an important step in breeding program to afford resources for enhancing the production of date palm under harsh environments (Al Kharusi *et al*., 2017), however, examining the field performance of date palm genotypes under these stresses are often inconclusive. Field trial is

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normally associated with non-uniform moisture availability and temperature fluctuations during the growing season. Also, it can be propagated sexually or asexually through seeds or offshoots, respectively. Propagation through seeds for the commercial production of elite genotypes is undesirable because it's weak genetic homogeneity (Tisserat, 1982). It is also due to significant differences in production capabilities, maturity of fruits quality and harvest time between vegetative propagated and seedlings plants (Zaid *et al.,* 2011). Propagation through offshoots is slow technique, limited number of offshoots, the survival of low life and the risk of disease transmission rate (Al-Khalifah and Askari, 2011). Nevertheless, propagation of date palm *via in vitro* culture using meristems explants provides effective substitutional to traditional methods (Quiroz-Figueroa *et al.,* 2006). Somatic embryogenesis and direct organogenesis are the common methods used for *in vitro* propagation of date palm (Abahmane, 2013). Somatic embryogenesis depends on the development of callus and increases its ability to multiply and quickly differentiate into shoots (McCubbin *et al*., 2000; Al-Khayri, 2013). Many of commercial laboratories use somatic embryogenesis as an alternative to organogenesis for rapid plant production and high multiplication efficiency (Al-Khateeb, 2006; Abahmane, 2011). However, organogenesis allows maintenance and production of true-to-type plants (Meziani *et al.,* 2015; Ma *et al.,* 2018) and uniform population of synchronously developing plant cells (Errabii *et al.,* 2006). The major issues in the *in vitro* propagation of date palm include tissue browning, hyperhydricity, shoot greening, precocious rooting, vitrification of tissue, deterioration of embryonic callus and callus formation on the bases of rooting plantlets (Al-Khateeb, 2008).

Accumulation of compatible solutes is an important adaptation strategy of plants under salinity and drought stresses (Hasegawa *et al*., 1984; Delauney and Verma, 1993). In date palm, accumulation of proline in callus tissue cultured under salt stress (Al-Khayri, 2002) or drought stress (Al-Khayri and Al-Bahrany, 2004) has been noted and linked with stress tolerance. Under stress, proline acts as an alternative resource for carbon and nitrogen, helps reducing oxidative damages, and stabilizing DNA and membrane protein (Szabados and Savoure, 2010). Tissue status of Na^+ and Cl^- concentration is another indicator of plant tolerance to salt stress (Munns and Tester, 2008). Specific ion toxicity may lead to reduction in cell expansion, disturbed cytosolic metabolism and oxidative damages (Wu *et al*., 2013).

In tolerant plants, Na^+ and Cl^- ions insinuate into plant cells and accumulate in the contractile vacuole, while for sensitive cultivars these accumulate in the cytoplasm (Kefu *et al*., 2003). Many plants have been adapted to salt stress by developing a capable prohibiting system, such as Na^+ exclusion from the root cells into the soil (Assaha *et al*., 2015; Fujimaki *et al*., 2015). Several studies confirmed that proline content, shoot Na^+ exclusion and tissue water status are effective markers for selection of date palm against salinity and drought stresses (Yaish, 2015; Jasim *et al*., 2016). However, to the best of our knowledge, no information is available reporting use of tissue culture techniques for evaluating the response of Saudi date palm cultivars to salinity and osmotic stresses. This study was, therefore, conducted to evaluate the response of *in vitro* propagated Saudi date palm cultivars to salinity and osmotic stresses using morphological, physiological and biochemical markers.

Materials and Methods

Offshoot preparation

To prepare offshoot, avoiding damage to meristematic region, a sharp knife was used to remove the external leaves and fibrous tissues until shoot tip was exposed. The shoot tip (3–4 cm wide, 6–8 cm long) was excised. The shoot tip was treated with antioxidant solution containing 100 mg ascorbic acid and 150 mg citric acid to prevent tissue browning caused by phenolic compounds.

Disinfection and Explant Removal

Under aseptic condition, the shoot tips were sterilized as described by Othmani *et al.* (2009). The shoot tips were washed with distilled water to eliminate any organic ruins. The tips were then soaked in benomyl and mancozeb fungicide solutions for 15 min and were rinsed three times with sterile distilled water. The shoot tips were then soaked in sodium hypochlorite (50%) containing 2 drops of tween-20, washed three times with sterile distilled water, and soaked in 0.2% $HgCl_2$ solution for 5 min. The shoot tips were then rinsed thoroughly with sterile distilled water. The young leaves surrounding the apical dome were gradually removed and shoot tip was cut into 2–4 pieces, then instantly moved to a prepared culture medium to avoid desiccation.

Culture Media and Incubation Conditions

The basal medium of Murashige and Skoog (1962) (MS) enhanced by NaH_2PO_4 (170 mg L^{-1}), calcium pantothenate (1 mg L^{-1}), sucrose (30 g L^{-1}), gelrite (2 g L^{-1}), activated charcoal (2 g L^{-1}), vitamins [*viz*., myo-inositol (100 mg L^{-1}), adenine (30 mg L^{-1}), pyridoxine-HCl (1 mg L^{-1}), glutamine (200 mg L^{-1}), nicotinic acid (1 mg L^{-1}), biotin (1 mg L^{-1})], plant growth regulators as per requirement for each *in vitro* stage, were used for micropropagation of date palm. The culture media were distributed in culture jars (170 mL/jar) and autoclaved. Explants were cultured on MS growth medium for 12–15 min, during this period explants were transferred every 4 weeks to fresh medium and sustained in dark at 25 ± 2°C to improve the initiation of the bud, moreover, to avoid the oxidation by phenolic compounds. The shoots were then maintained under lighting with a

photoperiod of 16 h with light intensity 3000 lux at 25 ± 2°C (Anjarne *et al.,* 2005).

In vitro Regeneration of Date Palm *Via* Somatic Embryogenesis

Callus formation stage: The explants were cultured on MS medium having various concentrations of 2,4-D (10–100 mg L^{-1}) mixed with N6-Δ²-isopentenyl adenine (3.0 and 6.0 mg L^{-1}). The cultures were incubated in the growth room at 25±2°C for 12 months at total darkness. Explants were re-cultured on the same concentrations mentioned above each for 8 weeks. Callus induction (%), adventitious shoot regeneration response and relative water content (%) [(fresh mass-dry mass)/(turgid mass-dry mass) × 100] were estimated after 12 months.

Somatic embryogenesis formation stage: Callus (500 mg fresh weight) was obtained from previous stage and cultured on MS basal medium containing 2-naphthalene acetic acid (NAA) at 0.5–2.0 mg L^{-1} in combination with 2.0–6.0 mg L^{-1} 2iP, 0.5 mg L^{-1} abscisic acid and 2 g L^{-1} activated charcoal. Each treatment had ten replicates and each replicate contained four explants. Explants were re-cultured on the same concentrations mentioned above each eight weeks. Cultures were re-assigned to light conditions with a photoperiod of 16 h. The culture was kept in growth room temperature at 25 ± 2°C with light intensity 3000 lux for 16 h photoperiod using cool white fluorescent lamps in the growth room for 8 weeks. Data were recorded as number and percentage of somatic embryogenesis formation.

Shoot elongation and rooting stage: Germinated embryos (2–3 cm) obtained from the previous stage, were cultured in MS basal medium with 0.2 mg L^{-1} gibberellic acid (GA_3) mixed with 1.0 mg L^{-1} indole butyric acid (IBA) to obtain shoot elongation and root formation. The cultured jars were incubated at 25 ± 2°C with light intensity 3000 lux for 16 h photoperiod using cool white fluorescent lamps in the growth room for 8 weeks. To encourage root formation, two-month-old healthy regenerated plantlets were cultured on MS medium supplemented with 1 mg L^{-1} IBA with 2 g L^{-1} activated charcoal and incubated under the same conditions as described above.

Acclimatization of regenerated plantlets: Two-month-old healthy redeveloped plantlets were individually removed from flasks, agar rinsed off, and then cultivating plants in plastic pots. These puts were filled with peat moss and sand (2:1 ratio). Then, pots were covered with polyethylene bags in a conservatory exposed to sunlight in 25 ± 2°C temperatures. After four weeks polyethylene sheet was removed, and plantlets were placed in a greenhouse (with 60–70% relative humidity and 25 ± 2°C temperature). Endurance efficiency, the percentage of plants that continue to exist upon transfer from *in vitro* to *ex vitro* habitat, was verified after four months.

Imposition of Salinity and Osmotic Stresses

Embryogenic callus of date palm cvs. Barhi and Khalas were cultured on MS basal nutrient medium in different conditions of PEG 6000 concentrations (5, 10, 15, 20 and 25 g L^{-1}) or NaCl (50, 100, 200, 300, 400 m*M*). Media were dispensed into the culture jars and topped with polypropylene closures. After that media were autoclaved for 20 min. at 121°C and 1.2 kg cm^{-2}. All jars for culture were incubated in the growth chamber for eight weeks under (25±2°C) and 16 h illumination of 3000 Lux (Mater, 1986). After 12 weeks data on callus growth (callus diameter), frequency of germination (germinated/embryo tested) and dried callus were recorded.

Proline Determination

Fresh leaves (100 mg) digested to determine leaf free proline by colorimetry method as described by Kefu *et al.* (2003).

Mineral Analysis

To estimate tissue Na^+, K^+ and Cl^-, fresh leaf tissues were freeze dried in liquid nitrogen. Dried ground leaf tissues (200 mg) were added to 5 mL HNO_3, and the solution was filtered through Whatman filter paper 42 to final volume of 50 mL. Leaf Na^+, K^+ and Cl^- were determined by flame photometer following Cresser and Parsons (1979).

Statistical Analysis

The experiment was carried out based on complete randomized design. Each of the experiments, excluding field performance study, was executed in five replicates with 20 samples per replication. For *in vitro* culture experiments, every single explant was treated as an experimental unit. Analysis of variance (ANOVA) was used to statistical analysis of experimental data using statistical software MSTATC. Differences between individual means were estimated according to Snedecor and Cochran (1982). All values are reported as means ± standard deviation.

Results

Embryogenic Callus Induction and Somatic Embryo Formation

A considerable variation in callus induction percentage and biomass was detected in parallel with an increase in auxins concentrations. After 12 months, the highest callus biomass production value was recorded with 10 mg L^{-1} 2, 4-D and 3 mg L^{-1} 2iP for both of cvs. Barhi and Khalas (Table 1 and Fig. 1). The embryogenic callus was successfully initiated through re-subculture primary callus at age 90 days on MS medium supplemented with 0.5 mg L^{-1} NAA and 3.0 mg L^{-1} 2iP (Table 2).

Table 1: Influence of growth regulators concentrations on callus induction, relative water content and adventitious shoot regeneration response of the date palm cvs. Barhi and Khalas via *in vitro* shoot tip section explants after 12 months

Growth regulators (mg L^{-1})		Callus induction percentage (%)		Relative water content (%)		Shoot regeneration	
2,4-D	2iP	Barhi	Khalas	Barhi	Khalas	Barhi	Khalas
10.0	2.0	56.5±0.5h	59.9±0.2h	42.3±0.5i	52.5±0.2h	+	-
10.0	3.0	100±1.2a	97.3±0.5b	86.5±1.3a	85.5±1.2a	+++	+++
10.0	4.0	92.7±0.8c	90.3±0.5c	80.3±0.7c	78.3±0.6d	+++	++
10.0	5.0	89.5±0.4d	87.8±0.4d	79.8±0.5d	83.8±0.2b	+++	+++
10.0	6.0	82.2±0.6e	81.3±0.9e	81.7±0.9c	80.7±1.0c	++	++
20.0	3.0	80.5±0.8e	77.4±0.2f	83.5±0.4b	81.4±0.6c	++	++
20.0	6.0	79.3±0.7e	76.4±0.5f	78.9±0.5d	70.8±0.2f	++	+
50.0	3.0	75.0±1.4f	78.7±1.4e	70.5±0.4f	73.2±0.8e	++	++
50.0	5.0	70.9±0.2f	73.5±0.8f	65.3±0.8g	55.9±0.7h	++	+
50.0	6.0	69.5±0.3g	65.4±0.9g	63.8±1.3g	60.8±0.9g	++	+
75.0	3.0	56.8±0.5h	58.5±1.3h	75.3±0.9e	78.3±0.8d	++	++
75.0	5.0	48.4±0.9i	52.9±1.2h	72.4±0.3f	70.4±0.5f	++	+
75.0	6.0	40.8±0.2i	45.8±0.5i	75.0±1.2e	70.0±0.6f	++	+
100.0	3.0	25.8±0.4k	30.9±0.8j	42.5±0.7i	72.5±0.7f	-	++
100.0	5.0	28.7±0.6k	32.8±1.0j	52.6±1.4h	58.6±1.4h	-	+
100.0	6.0	30.8±0.4j	34.0±0.6j	59.5±0.9h	62.5±0.9g	-	+

Values are presented by mean ± SE Same letters represent no significant differences between means at $P \leq 0.05$ level
Shoot regeneration are visually estimated as No regeneration= -, Poor= +, Good= ++, very good= +++

Table 2: Influence of growth regulators concentrations on number of somatic embryos, frequency of germination and frequency of conversion of germinated somatic embryos into plantlets of date palm cvs. Barhi and Khalas

Growth regulators (mg L^{-1})		Number of somatic embryosper embryogenic callus (0.5 g FW)		Frequency of germination percentage (germinated/embryos tested)		Frequency of conversion percentage (plantlets/germinated embryos tested)	
NAA	2iP	Barhi	Khalas	Barhi	Khalas	Barhi	Khalas
0.5	2.0	304.45±0.35e	365.24±1.05e	85.15±0.18g	86.25±1.05g	90.25±0.49i	91.36±0.88h
1.0	2.0	380.48±0.87e	395.25±0.83e	80.25±0.28h	83.47±0.62g	89.25±0.56j	90.25±1.09i
1.5	2.0	268.25±0.65f	270.38±0.59f	84.58±0.35g	89.92±0.55f	90.68±0.93i	91.35±0.58h
2.0	2.0	252.45±0.38f	268.48±0.77f	80.35±0.72h	84.59±0.71g	91.35±0.66h	92.48±0.33g
0.5	3.0	585.25±0.58a	586.49±0.92a	95.45±0.85a	93.50±0.59b	98.52±0.49a	97.82±0.69b
1.0	3.0	520.28±0.32c	535.18±1.07c	91.25±0.19d	93.45±0.26b	95.24±0.88d	97.05±0.17b
1.5	3.0	550.25±0.49c	565.79±0.65b	90.85±0.52e	92.45±0.93c	94.30±0.71e	93.08±0.29f
2.0	3.0	477.33±0.69d	490.48±0.44d	89.73±0.11f	90.48±0.86e	92.26±0.33g	93.69±0.48f
0.5	4.0	578.15±0.89b	581.35±0.50b	88.52±0.36f	90.95±0.75e	95.25±0.37d	97.89±0.69b
1.0	4.0	582.25±1.09b	587.40±0.20a	93.45±0.38b	94.68±0.59a	97.58±1.04b	99.58±0.72a
1.5	4.0	450.42±0.98d	459.45±0.19d	91.25±1.08d	92.59±0.16c	96.52±0.55c	98.06±0.55a
2.0	4.0	463.58±0.20d	472.49±0.45d	89.85±0.39f	90.58±0.52e	94.85±0.89e	95.45±1.20d
0.5	6.0	560.25±1.04b	568.54±0.39b	92.52±0.82c	93.84±0.99b	94.29±1.12e	96.25±0.88c
1.0	6.0	498.52±0.59d	502.04±0.22c	92.54±0.33c	93.90±0.47b	95.28±0.78d	96.85±0.52c
1.5	6.0	550.75±0.68c	558.69±0.35c	92.89±0.72c	93.59±0.36b	94.57±0.29e	95.45±0.99d
2.0	6.0	535.27±0.82c	546.78±0.61c	93.05±0.53b	93.70±0.80b	95.65±1.32d	96.89±0.105c

Values are presented by mean ± SE Same letters represent no significant differences between means at $P \leq 0.05$ level

Somatic Embryo and Plantlet Formation of Date Palm

The number of somatic embryos per 0.5 g fresh weight of embryogenic callus were 585.25 and 587.40 with a frequency of germination percentage 95.45%; 94.68% and percentage of plantlets germinated embryos 98.52% and 99.58% for cv. Barhi and cv. Khalas, respectively. These observations were recorded on MS medium supplied by 0.5 mg L^{-1} NAA, 3.0 mg L^{-1} 2iP, 5 mg L^{-1} ABA and 2 g L^{-1} activated carbon; 1.0 mg L^{-1} NAA, 4.0 mg L^{-1} 2iP, 5 mg L^{-1} ABA and 2 g L^{-1} AC, respectively (Table 2 and Fig. 2).

Effect of NaCl salt and PEG Treatments

Callus proliferation: Initial mass (500 mg) from callus was cultured on MS medium (Murashige and Skoog, 1962) supplied by concentration of NaCl varies from 0 to 400 m*M* or 5, 10, 15, 20 and 25 g L^{-1} osmotic stress. Under low concentrations of NaCl (50–100 m*M*) and (5–15 g L^{-1} PEG 6000), callus growth was improved in both cvs., while higher concentrations of NaCl and PEG decreases the callus weight, in contrast to the control. Moreover, growth was completely stopped at 300 m*M* NaCl and 20 g L^{-1} osmotic stress; 400 m*M* NaCl and 25 g L^{-1} osmotic stress of cv. Barhi and cv. Khalas, respectively (Fig. 3).

Proliferation Stage

Embryogenic callus obtained after 15 months were cultured on MS medium containing 10 mg L^{-1} 2, 4-D and 3 mg L^{-1} 2iP of callus induction and MS medium containing 0.5 mg L^{-1} NAA and 3 mg L^{-1} 2iP of somatic embryos formation with different concentrations of osmotic stress (5, 10, 15, 20 and 25 g L^{-1}) or NaCl (50, 100, 200, 300, 400 m*M*). Data showed in Table (3) indicated the passive effect of NaCl

Table 3: Influence of osmotic and salt stresses on callus diameter, percentage of dried callus and frequency of somatic embryo germination percentage in date palm cvs. *Barhi* and *Khalas* cultivar after 12 weeks

Treatments	Callus diameter (cm)		Percentage of dried (dead) callus		Frequency of germination percentage (germinated/embryos tested)	
	Barhi	Khalas	Barhi	Khalas	Barhi	Khalas
Control	1.98 ±0.72b	2.05.±0.40a	0.00±0.00k	02.09±0.11j	96.30±0.68b	95.25±0.51b
PEG 5 g L^{-1}	1.97±0.37b	1.99±0.34b	0.00±0.00k	00.00±0.00k	98.05±0.35a	97.38±0.79a
PEG 10 g L^{-1}	1.82±0.28d	1.92±0.31c	10.31±0.24i	8.25±0.73i	92.48±0.48c	91.98±0.56c
PEG 15 g L^{-1}	1.65±0.17e	1.87±0.49d	17.45±0.31h	12.32±0.39i	75.45±0.18e	72.78±0.81e
PEG 20 g L^{-1}	1.40±0.15f	1.49±0.62f	43.55±0.38e	38.65±0.56f	62.54±0.79f	58.25±0.92g
PEG 25 g L^{-1}	0.92±0.09c	1.25±0.53g	80.68±0.72b	67.19±0.24d	13.68±0.25i	25.18±0.28h
NaCl 50 m*M*	1.93±0.58c	2.00±0.89a	00.00±0.00k	00.00±0.00k	95.23±0.96b	95.00±0.39b
NaCl 100 m*M*	1.47±0.42f	1.90±0.73c	23.25±0.33g	19.62±0.29g	90.36±0.85c	88.63±0.25d
NaCl 200 m*M*	1.25±0.29g	1.49±0.38f	45.28±0.42e	32.25±0.16f	42.25±0.27g	48.60±0.60g
NaCl 300 m*M*	0.24±0.08i	0.45±0.16h	78.35±0.15c	71.98±0.27c	21.40±0.19h	25.28±0.72h
NaCl 400 m*M*	0.00±0.00j	0.13±0.05i	100.0±0.00a	83.75±0.54b	00.00±0.00j	12.45±0.73i

Values are presented by mean ± SE same letters represent no significant differences between means at $P \leq 0.05$ level

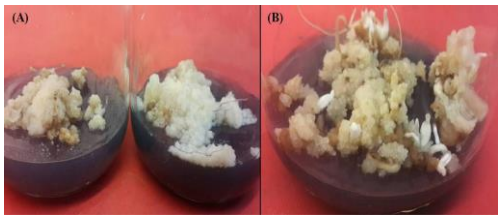

Fig. 1: *In vitro* callus induction and Initiation of somatic embryogenesis from pieces of shoot tips of date palm cv. Barhi and Khalas. (A): Induction of callus after 12 months on MS medium supplemented with 10.0 mg L^{-1} 2, 4-D and 3.0 mg L^{-1} 2iP. (B): Induction of embryogenic callus after five months on the surface of primary callus that was induced from callus cultured during three months on MS medium supplemented with 0.5 mg L^{-1} NAA and 3.0 mg L^{-1} 2iP

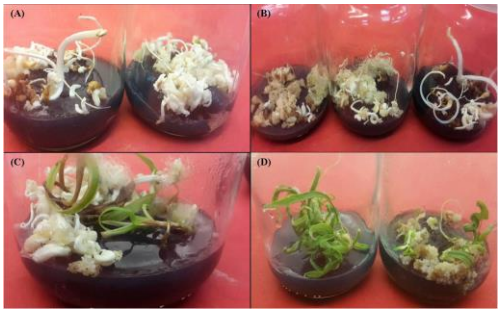

Fig. 2: Improved somatic embryo production and plant regeneration of date palm (*Phoenix dactylifera* L.) cvs. Barhi and Khalas. (A): Matured somatic embryos derived from callus cultured for two months on MS containing with 0.5 mg L^{-1} NAA, 3.0 mg L^{-1} 2iP, 5 mg L^{-1} ABA and 2 g L^{-1} activated charcoal (AC) of Barhi cultivar. (B): Matured somatic embryos derived from callus cultured for two months on MS medium supplemented with 1.0 mg L^{-1} NAA, 4.0 mg L^{-1} 2iP, 5 mg L^{-1} ABA and 2 g L^{-1} activated charcoal (AC) of Khalas cultivar**.** (C and D): plantlet obtained from a converted somatic embryo

treatments on callus growth, percentage of dried callus and frequency of somatic embryo germination percentage after 12 weeks. The Highest mean value of callus growth (1.93, 2.00) was observed at 50 m*M* NaCl, compared to control treatment (1.98, 2.05), following by 100 m*M* (1.47, 1.90), 200 m*M* (1.25, 1.49), 300 m*M* (0.24, 0.45) for cv. Barhi and cv. Khalas, respectively although callus of cv. Khalas recorded the minimum value of callus growth (0.13) under 400 m*M* NaCl, nevertheless cv. Barhi under was not capable of survive under this concentration. The highest callus growth (callus diameter) was obtained for cv. Barhi (1.97) and cv. Khalas (1.99) under 5 g L^{-1} osmotic stress in contrast to the control, while the highest frequency of germination (%) (germinated/embryos tested) were obtained under 10 g L^{-1} osmotic stress of both cultivars.

Sodium, Potassium and Chloride Ions Content

In the current study, the effect of different concentrations of salinity on mineral contents in cvs. Barhi and Khalas were investigated. The results indicated a positive relationship was observed between salinity levels and Na^+, Cl^- ion contents, while on the other side there was an inverse relation between salinity level and K^+ ion content. Na^+ accumulation in leaves of cv. Khalas was considerably lower than Na^+ accumulation in the cv. Barhi. Cultivar Khalas was more tolerant to salt stress than Barhi cultivars. This can be clarified by the following: cv. Khalas was less in Na^+ absorption and more in K^+ contents in leaves in contrast to the Barhi cultivar (Fig. 4). In addition, significant reduction in K^+/Na^+ ratio in shoots were decreased as NaCl levels increased in the growth medium.

Proline Contents

The measurement of proline content as a biochemical parameter was applied in this study to determine the nature of the two tested cultivars in terms of ability to resist salt and drought stresses. Our results showed a significant increase in proline content with an increase in the PEG

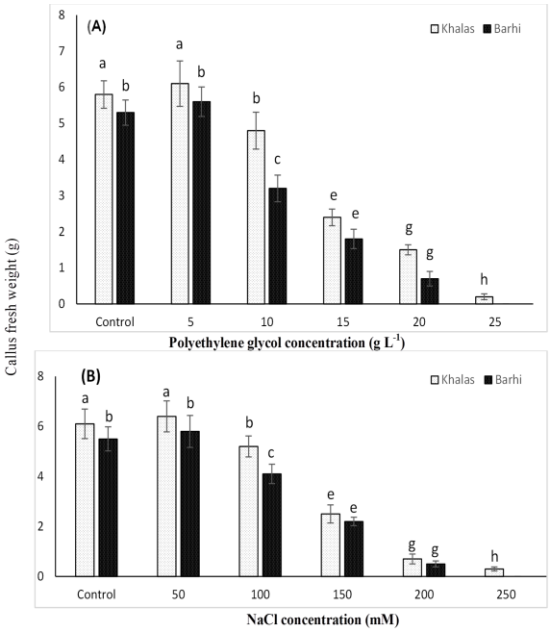

Fig. 3: Influence of (A) osmotic and (B) salt stresses oncallus fresh weight of two date palm cvs. Barhi and Khalas callus

levels in the growth medium. Depending on the level of PEG added to the growth medium, the proline content in cv. Khalas recorded values ranging from 32.85 to 83.26 μg g^{-1} of leaves fresh weight at 15 to 25 g L^{-1} of osmotic stress, respectively, while in cv. Barhi proline increased quickly and significantly from 29.48 to 62.68 μg g^{-1} of leaves fresh weight at 15 to 20 g L^{-1} of osmotic stress, respectively (Fig. 5). Similar results were also observed when NaCl was applied, where the lower and upper value of proline content was the higher in cv. Khalas (38.5 to 73.52 of μg g^{-1} leaves fresh weight) compared to the cv. Barhi (29.58 to 61.58 μg g^{-1} of leaves fresh weight).

Discussion

The challenges facing the Earth, such as climate change, high temperature, desertification, land salinity and the prevalence of diseases, have become steadily increasing, which has a detrimental effect on the productivity of crops and therefore inadequate food, which has contributed to the increasing in the hunger worldwide, especially African countries. Therefore, agricultural research institutions have taken care of breeding programs in the field of improving agricultural cultivars to overcome these environment stresses. With the advent of Third Technology Revolution, and for failure conventional breeding techniques to maintain velocity for food requirements and to present adequately rapid and professional systems for crop development, plant breeders have introduced and applied several new technologies in plant breeding programmes to generate

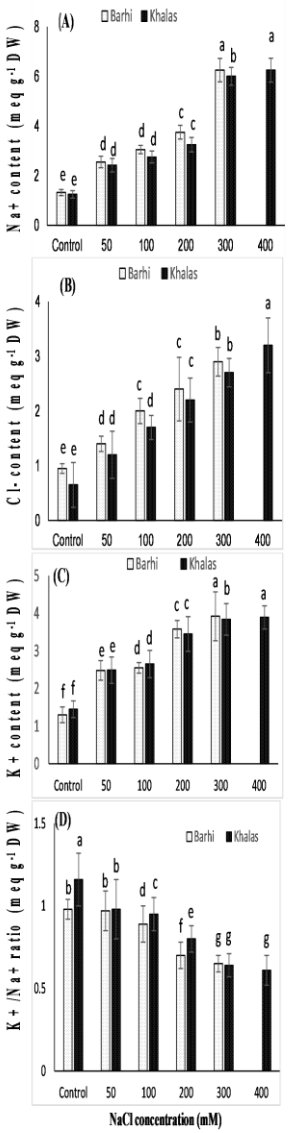

Fig. 4: Influence of salt stresses on (A) leaf Na, (B) leaf Cl, (C) leaf K and (D) leaf K^+ /Na^+ of two date palm cvs. Barhi and Khalas

plants with desirable characters of greater yield, nutrition and resistance to diseases, one of the most important new breeding technique that used in biotechnology including tissue culture technology (Debergh and Read, 1991). Genetic resources that have beneficial qualities are the effective tool for plant breeders for improvement and high productivity of crops (Buck and Hamilton, 2011). Therefore, our study was interested in the selection of two cultivars of date palms under *in vitro* culture condition to recommend any of these two cultivars have benefit types of traits that qualify it to be a distinct genetic source that can be recommended to use by plant breeder.

Based on the studies that confirmed the effectiveness

of somatic embryogenesis technique to increase palm date productivity (Fki *et al*., 2011), in this study, we worked to obtain *in vitro* culture optimization protocol of date palm *via* indirect somatic embryogenesis and to obtain the best proportion of plantlets able to survival under greenhouse or natural condition. Jain (2012), Mazri (2014, 2015) pointed out that success in the application of this technology is affected by various factors, including the cultivars, such as genetic structure and explant type, and others including the component of medium in terms of growth hormones and conditions of incubation.

After 12 months, the highest callus biomass production value and relative water content was recorded on MS medium supplemented with 10.0 mg L^{-1} 2, 4-D and 3.0 mg L^{-1} 2iP for both of cvs. Barhi and Khalas, while MS medium supplemented with 10.0 mg L^{-1} 2, 4-D and 3.0 mg L^{-1} 2iP or 10.0 mg L^{-1} 2, 4-D and 5.0 mg L^{-1} 2iP was the best in getting the highest value of shoot regeneration (Table 1). The selection of shoot tip explant and MS medium, with modification in the type and quantity of some compounds such as mineral elements, vitamins and anti-corneal compounds to obtain the growth of friable callus was relied on the previous studies results. Whereas Badawy *et al.* (2005) recorded that whenever MS medium strength was decreased from full strength to half or quarter strength, this led to a significant reduction in friable callus formation. They also detected that shoot tip explant was the salient in promoting callus formation compared to axillary buds and leaf primordia. The production of friable callus was desirable, compared to compact callus, for higher efficiency to produce shoot and root in next stages. Also, Saeed *et al.* (2017) found that imbibition of meristem tissue in solution with 8 mg L^{-1} 2,4-D was found to be most favorable combination for callus induction, and embryogenesis in wheat.

The results suggested that 2,4-D plays a key role in the somatic embryos induction and friable callus formation. This effect of 2,4-D has been explained based on it acts as a signal cascade trigged to start a hyperpolarization of membrane polypeptides (Barbier-Brygoo *et al.,* 1989; Zuo *et al.,*2002). Despite, our results confirm the 10.0 mg L^{-1} 2,4-D and 3.0 mg L^{-1} 2iP was the optimum concentration to callus mass production. The asymmetry in terms of 2,4-D concentration of the results between different studies to 2,4-D requirement for callus induction depends on type of explant and genotype used. On the other hand, cvs. Barhi and Khalas maintained relatively high RWC (>60%) in most of the treatment with 2,4-D and 2iP (Table 1). Highest value of RWC was recorded under treatment with 10.0 mg L^{-1} 2, 4-D and 3.0 mg L^{-1} 2iP for cv. Barhi (86.5%) and cv. Khalas (85.5%). The results stated that RWC is a contributing factor in nature of callus formation (friable or compact) data not presented. Etienne *et al.* (1991) stated that water status in the callus is apparently important for initiating somatic embryogenesis, and RWC appear to be good physiological marker of it embryogenic state.

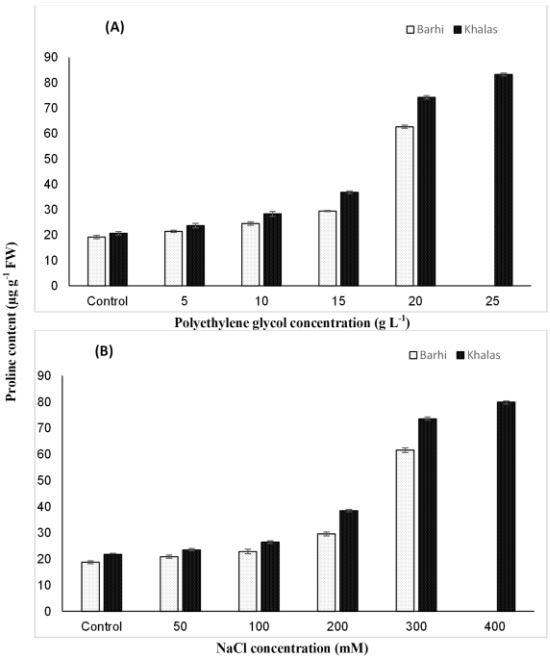

Fig. 5: Influence of (A) osmotic and (B) salt stresses on tissue proline contents in date palm cvs Barhi and Khalas

For further improvement and maturation of somatic embryogenesis, frequency of germination and conversion into plantlets, MS basal solid medium supported by NAA at (0.5–2.0 mg L^{-1}) in combination with 2iP (2.0–6.0 mg L^{-1}) were tested in present of 0.5 mg L^{-1} ABA and 2 g L^{-1} AC. It is possible to induce somatic embryogenesis, germination and conversion from shoot tip explant of date palm in the presence of NAA as auxin and 2iP as cytokinin, it stimulated leaf and root growth. NAA at (0.5 and 1 mg L^{-1}) and 2ip at (3 and 4 mg L^{-1}) proved to be the more effective comparing to other concentration for induce somatic embryogenesis, germination and conversion in both cultivars cv. Barhi and cv. Kahalas, respectively (Table 2). Effect of NAA on the conversion of matured somatic embryos was also reported by Sane´ *et al.* (2006), Othmani *et al.* (2009) on culture of date palm cv. Amsekchi and cv. Boufeggous, respectively. Average number of somatic embryo (50.66), frequency of germination (83.50%) and frequency of conversion (94.50%) were recorded when 1 mg L^{-1} NAA was applied (Othmani *et al.,* 2009). Similar requirements of cytokinin such as 2ip (Badawy *et al.,* 2005), BAP (Fki *et al.,* 2003; Zouine *et al.,* 2005) and KIN (Meziani *et al*., 2015) to promote the growth, development, enhance shoot morphogenesis and maturation into date palm have been observed. Transfer of germinated embryos of date palm cultivars to MS medium supported by 1 mg L^{-1} NAA and 1 mg L^{-1} BAP; 22.5×10^{-6} 2,4-D and 1.4×10^{-5} BAP, respectively resulted in the construction of vital plantlets showing impartial shoot and root growth (Fki *et al.,* 2003; Zouine *et al.,* 2005). The presence of auxin with cytokinin is important and essential for the

initiation and propagation of cells to somatic embryo (Michalczuk *et al.,* 1992; De Jong *et al.,* 1993).

Addition to the effect of PGR on the quality of somatic embryogenesis, also, ABA and AC reported to have the beneficial effects of promote somatic embryogenesis in date palm (Maruyama *et al.,* 2007; Rai *et al.,* 2011). The stable concentration of ABA and AC at 0.5 mg L^{-1} and 2 g L^{-1}, respectively was used during the various stages of the experiment to induce maturation of somatic embryos**.** ABA plays a role in controlling several physiological and biochemical processes that increase protein and lipid content during maturation of somatic embryos (Pliego-Alfaro *et al.,* 1996; Nakagawa *et al.,* 2001; Sghaier *et al.,* 2009). On the other side, adding AC at 0.1 g L^{-1} to MS medium was essential to enhance the maturation of somatic embryos (Othmani *et al.,* 2009). Despite the importance of AC to prevent the development of abnormal plantlets and reduce toxic phenolic exudates during plant embryogenesis stages (De Touchet *et al.,* 1991; Pullman and Gupta, 1991; Teng, 1997).

The elongation phase is necessary in the conversion of shoot bud to plantlets (Mazri, 2012). Thus, he tested the effect of cultivars used, type of medium (solid or liquid/full or half strength) and PGR on shoot elongation in date palm. Liquid medium was better than the solid medium, full or half strength medium did not have any effect, but PGR did. For this reason, different concentration of GA3 and IBA were tested to select the best doses of GA3 and IBA for shoot elongation in date palm. Based on the results of this experiment, the high value of shoot elongation was observed on the nutrient medium supplemented with 0.2 mg L^{-1} GA3 in combination with 1.0 mg L^{-1} IBA, data not presented.

The selection of cultivars of date palms, either *in vitro* or *in vivo*, under stresses conditions of salinity and drought is often done in many experiments using NaCl and PEG, respectively (Djibril *et al.,* 2005; El-Rabey *et al.,* 2015; Jasim *et al.,* 2016; Al Kharusi *et al.,* 2017). Na^+ and Cl^- ions are the most destructive elements are exposed to salinity stress (Flowers *et al.,* 2014), while PEG simulate the mechanical caused by the withdrawal of cellular water due to low water availability and is not expected to penetrate plant tissue rapidly (El-Hadrami *et al.,* 2011c). The presence of sodium chloride in the plant growth medium leads to often induces secondary stresses (Al Kharusi *et al.,* 2017). The pattern of selection *in vitro* for drought tolerant cultivars based on PEG is less time consuming, allow quick identification of tolerant cultivars (Badiane *et al.,* 2004). In the current study, screening of both cv. Barhi and cv. Khalas under different concentration of NaCl (50, 100, 200, 300, 400 m*M*) and PEG6000 (5, 10, 15, 20 and 25 g L^{-1}) was tested by measuring morphological markers such as callus growth (callus dimeter), frequency of germination % (germinated/embryo tested) and % of dried callus; biochemical marker such as proline, Na^+, K^+ and Cl^-.

In this study, NaCl and PEG successes as an osmotic stress agent to identify differences in salinity and drought tolerance for both cultivars Barhi and Khalas. There was a significant inhibited of both NaCl and PEG on values of callus growth, frequency of germination and K^+/Na^+ while the values of callus dead, proline, Na^+, K^+ and Cl^- were high. Cv. Khalas recorded the highest values for the callus growth, frequency of germination and K^+/Na^+ traits and the lowest values for the callus dead, proline, Na^+, K^+ and Cl^- traits compared to the cv. Barhi (Table 3). Increasing in NaCl treatment was inhibited the growth of both cvs. but cv. Barhi was stopped completely growing and die under 400 m*M* NaCl, while they managed to grow under the highest concentration of osmotic stress (25 mg L^{-1}). This effect may be attributed to NaCl inhibits the absorption of water and mineral elements by the roots, as a result, the plant suffers from a lack of growth requirements and the emergence of the symptoms of lack of growth based on the resistance of each plant (Munns, 2002).

The physiological basis of salt tolerance in date palm was found to be based on Na and Cl concentration in leaves and keeping up the K content (Alrasbi *et al.,* 2010). In our study, highest values of these ions were recorded in parallel with increasing in NaCl treatments. The susceptible cv. Barhi had much higher values for Na^+ and Cl^- than tolerant cv. Khalas (Fig. 4). The physiological explanation of the ability of some cultivars to grow and to be more tolerant under stresses conditions compared to others may be due attributed to its ability to restrict Cl^- movement in tissues, thus Cl^- become less harmful of the photosynthetically active (Jasim *et al.,* 2016). In the current study increasing K^+ values have a direct correlation with increased concentration of NaCl and cv. Khalas has a highest value of K^+ comparing with cv. Barhi. K^+ is essential ions to control the electrolyte leakage and consequently maintains membrane integrity (Anschütz *et al.,* 2014). Na^+, K^+ and K^+/Na^+ ratio in several investigations reported to be a physiological marker to determinate of salt stress tolerance (Craig and Moller, 2010).

In the present study, NaCl and PEG increased accumulation of proline and it was slightly higher in cv. Khalas than that in cv. Barhi (Fig. 5). Djibril *et al.* (2005) pointed out that the level of proline is directly correlated with the NaCl concentration in the date palm callus. Yaish (2015) reported that the leaf proline levels increased by 9.3- fold under salt treatment and 2.5-fold under drought treatment than the control treatment. The increase in proline content under abiotic stresses compared to control was interpreted as one of the mechanisms that help the plant to adapt of different abiotic stresses (Hayat *et al.,* 2012). Proline is considered a compatible osmotic, it is working inside the tissues to adjust osmotic pressure (Al-Mansoori *et al.,* 2006). Although the present study and several previous studies (Al-Enezi and Al-Kayri, 2012) have supported the importance of the proline combination as a biochemical marker to improve plant tolerance for salinity and drought in date palm, nevertheless Yaish (2015) showed that proline content is an unsuitable molecular marker for salinity and drought in conventional plant breeding programs.

Conclusion

The date palm (*P. dactylfera* L.) cv. Khalas can better tolerate salinity and drought stresses. The plant acclimatization was successful after four months in *ex-vitro*. Growth medium containing GA3 in combination with 1.0 mg L^{-1} IBA may be used to have better stem elongation and root formation *in-vitro.* Use of tissue callus is an effective way to monitor the response of date palm cultivars to salinity and osmotic stresses.

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