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Using Low Levels of Seawater to Enhance Growth and Development of Date Palm Embryogenic Cultures

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Abstract: Salinity is generally investigated as a stress factor for growth, but not utilized for improving or increasing growth and development of *in vitro* plants. Salinity tolerated plants may generally resist or need low level of saline for their growth and development. These plants such as date palm can be growth promoted by incubating in low salinity level medium. This research investigated the level of salinity that could improve growth and development of embryogenic cultures of date palm cv. Malkaby. The effect of seawater levels (0.0, 500, 1000, 1500 and 2000 ppm.) were examined on callus fresh weight, number of secondary embryos, germinated embryos and leaves. In addition, percentage of rooting, root number, root length and root thickness were determined. Seawater at the level of 500 and 1000 ppm. enhanced callus fresh weight. However, 1500 ppm. achieved the highest number of secondary somatic embryos, germinated embryos, root percentage and root number. Leaf chlorophyll a, b and carotinoide, total indols, phenols, sugars and proline content of resulted plantlets could explain the results obtained.

Keywords: Callus, *Phoenix dactylifera*, proline, salinity, somatic embryo

INTRODUCTION

High salinity in the growing medium usually retards plant growth, yield and quality of horticultural crops. Selection of relatively resistant horticultural crops is a necessity for the utilization of lands prone to Salinization, like arid and semi-arid areas, for horticultural production (Wahome, 2003). Salinity tolerant plants like date palm trees are preferred to utilize at these areas. Lower salinity does not inhibit growth and sometimes, can improve growth of tolerated plants. Mills *et al*. (2001) studied the effect of different kinds of salts on the shoot development of salt-tolerant jojoba clones. Salinity enhanced growth and significantly promoted leaf expansion. Al-Khayri (2002) studied the effect of NaCl concentrations on date palm callus and found that, the highest value of callus growth was occurred under the lowest concentration. In addition, Sotiropoulos and Dimassi (2004) studied the *in vitro* response of kiwifruit to various concentrations of NaCl and boron (B) in the culture medium. Low concentrations of NaCl (10 and 20 mM) could stimulate shoot proliferation.

Micropropagation especially organogenesis and somatic embryogenesis are increasingly becoming an important method for large–scale propagation of date palm (El-Bellaj, 2000). Several studies were conducted to enhance somatic embryogenesis of date palm; some through changing of culture medium components (Al-Khayri, 2010; Hassan *et al*., 2007; Hassan and Taha, 2012), technical methods (Othmani *et al*., 2009; Ibrahim *et al*., 2012) and pretreatments (Mustafa *et al*., 2013). Exposing to stress could be another method to improve growth and development of *in vitro* plants (Taha, 2009, in jojoba).

This investigation aimed to study the effect of salinity in growth and development of embryogenic cultures of date palm cv. Malkaby using low levels of seawater.

MATERIALS AND METHODS

The present study was carried out through 2011-2013 at Biotechnology of Fruit Trees Laboratory, Pomology Department, National Research Centre, Cairo, Egypt.

Plant materials and *in vitro* callus establishment: Off shoots, 5-7 kg in weight, were separated from adult date palm trees of cv. Malkaby and brought to the lab. Leaves were carefully removed and the terminal portion of the shoots composed of the apical meristem, numerous leaves primordial and subapical tissue, was immediately placed in an antioxidant solution of Tochopherol at 400 mg\L and incubated in the refrigerator for 24 h at 5°C (from our previous data which published recently; Mostafa *et al*., 2013). Afterward, apical shoots were disinfected by immersion in 70% Clorox of 5.25% sodium hypochlorite solution for 20 min with 2-3 drops of Tween 20 then washed three times with sterilized distilled water. Under aseptic conditions, shoot tips were cut longitudinally into

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several parts. Explants were initially cultured on a callus induction medium consisted of MS inorganic salts (Murashige and Skoog, 1962), 100 mg/L glutamine, 10 mg/L 2,4-Dichloro-phenoxy acetic acid (2,4-D), 3 mg/L iso-pentenyl adenine (2 iP), 1.5 g/L activated charcoal, 30 g/L sucrose and solidified with 2 g/L Gelrite. The pH was adjusted to 5.7-5.8 prior to addition of Gelrite. Medium was dispensed into 25×150 mm culture tubes at the rate of 25 mL per tube. Tubes were capped with polypropylene closures then autoclaved at 121°C and 15 ibs\ins^2 for 15 min. Cultures were maintained in the dark at 25±2 for 36 weeks and subcultured with 6 weeks intervals.

Callus cultures: Induced callus (1.0 g) were transferred to fresh medium, with the previous components, supplemented with various low levels of seawater (41000 ppm) at 0.0, 500, 1000, 1500 or 2000 ppm. The media were adjusted to pH 5.8 and dispensed at 20 mL\glass jar of 150 mL. Jars were capped with polypropylene closures then autoclaved at 121°C and 15 ibs\ins^2 for 15 min. Fresh weight of callus after four subcultures with six weeks intervals were determined.

Embryo cultures: Clumps of formed embryos (2-3 embryos) derived from callus cultures were transferred to fresh medium (half strength MS) supplemented with 0.1 mg/L NAA+0.05 mg/L BA+1.0 g/L AC (Ibrahim *et al*., 2009) and various low levels of seawater (0.0, 500, 1000, 1500 or 2000) then dispensed into 20 mL\glass jar of 250 mL. Number of secondary embryos, germinated embryos per embryo and leaves per plantlet were determined after four subcultures with three weeks intervals.

Rooting stage: Plantlets of Malkaby cv.; 7-9 cm in length were cultured on solid then liquid half strength MS medium supplemented with 0.1 mg/L NAA (Eke *et al*., 2005) in addition to the previously mentioned seawater levels for 12 weeks (6 weeks interval) to study their effects on root formation. The media were dispensed into 25×250 mm long tubes. Percentage of rooted plantlets, average number of roots (per rooted plantlet) and root length were determined. Root thickness is determined by degrees according to Pottino (1981) as followed: thin (1), low thick (2), average thick (3) and thick (4).

Acclimatization: Plantlets were transferred to the greenhouse and transplanted into 18×8 cm pots containing a mixture of peat: perlite (2:1 by volume) and placed for further growth and hardening under polyethylene sheets in a green house with 27±2°C under sunlight and 80-90 % relative humidity.

Physiological analysis:

Determination of pigments, total indoles and phenols: Chlorophyll a, b and carotenoid, total indols and phenols were determined in plantlets leaves according to the method of Arnon (1949).

Total Soluble Sugars (TSS): TSS were determined in plantlets leaves according to the method of Fales (1951).

Proline content: Leaf proline content was determined by a spectrophotometric assay as described by Bates *et al*. (1973).

Data analysis: Data were analyzed as one-way completely randomized design and mean values were separated by Steel and Torrie (1980) at a 5% level of significance.

RESULTS

Callus cultures: Data in Table 1 show that, increasing levels of seawater from 0.0 to 1500 ppm. in subculture two, increased callus fresh weight per gram compared with the control. Whereas, the highest callus fresh weight was produced from the levels of 500 or 1000 ppm. while, the lowest callus fresh weight was produced from the level of 2000 ppm (Fig. 1, the first row). The same results were obtained from subculture four, as the levels of 500 and 1000 ppm. gave the highest callus fresh weight per gram. Callus from the control treatment of subculture four approached the surpassed results which means that, it took longer time to reach the same weight of surpassed results. Increasing levels of seawater to 2000 ppm. decreased callus fresh weight per gram in subculture two as well as subculture four.

Embryo cultures: Data in Table 2 revealed that, all levels of seawater concentration achieved higher number of secondary embryos and germinated embryos

Table 1: Effect of low levels of seawater on callus fresh weight (g/g) after two and four subcultures

Treatment	Callus fresh weight after Sub 2	Callus fresh weight after Sub 4
Control	1.69c	3.31b
500 ppm	2.26a	3.57a
1000 ppm	2.18a	3.53a
1500 ppm	1. 82b	1.88c
2000 ppm	1. 38d	1.02d

Means with different letters within each column were significantly different at 5% level. Sub: subculture

Table 2: Effect of low levels of seawater on embryo development after four subcultures

Treatment	Second embryo n	Germinated embryo n	Leaf number
Control	3.14d	8.33d	4.4a
500 ppm.	9.33b	8.56c	4.18b
1000 ppm.	9.33b	8.57c	3.76c
1500 ppm.	9.78a	14.0a	3.35d
2000 ppm.	5.29c	8.86b	3.23e

Means with different letters within each column were significantly different at 5% level

Table 3: Effect of low levels of seawater on rooting stage after 12 weeks

Treatment	Rooting %	Root number	Root length	Root thickness
Control	60.00	1.33c	4.40a	1.20e
500 ppm	60.00	3.00b	4.44a	1.63d
1000 ppm	66.67	3.00b	3.97b	1.78c
1500ppm	68.18	3.20a	3.66c	2.00b
2000 ppm	62.50	3.00b	2.70d	2.20a

Means with different letters within each column were significantly different at 5% level

Table 4: Effect of low levels of seawater on physiological analysis of planted leaves

Treatment	TSS	Indol	Phenol	Chlorophyll A	Chlorophyll B	Carotenoid	Proline
Control	20.1e	1.25c	0.65c	0.90c	0.20c	0.104a	1.098d
500 ppm	21.9d	1.20c	1.27a	2.60a	1.90a	0.271a	1.187cd
1000 ppm	24.5c	3.56b	0.89b	1.30b	0.40b	0.191a	1.299bc
1500 ppm	26.4a	4.68a	0.82bc	0.50d	0.20c	0.173a	1.4360b
2000 ppm	25.7b	4.68a	0.80bc	0.50d	0.10c	0.103a	1.7600a

Means with different letters within each column were significantly different at 5% level. TSS: total soluble sugars

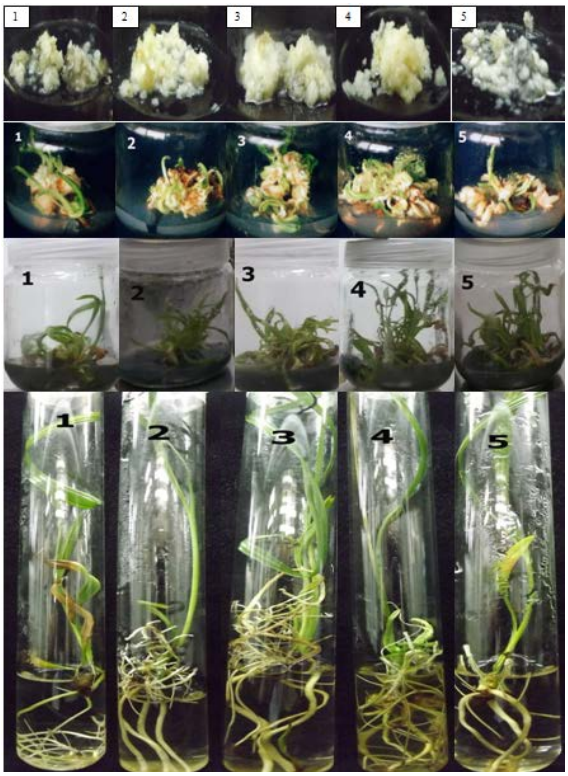

Fig. 1: Effect of low levels of seawater on growth and development of date palm explants cv. Malkaby. First row: callus cultures, second: embryo cultures, third: germinated embryos and forth: rooting stage. Treatments 1, 2, 3, 4 and 5 were seawater levels: 0, 500, 1000, 1500 and 2000 ppm., respectively

compared with the control, as for 1500 ppm. surpassed other levels and gave the highest average number of secondary as well as germinated embryos. Meanwhile, increasing levels of seawater to 2000 ppm. Decreased number of secondary embryos compared with the lower levels (Fig. 1, second and third row).

Concerning leaf number, increasing the level of seawater decreased the average number of leaves significantly compared with the control as the lowest significant number of leaves was appeared with the highest level of seawater.

Rooting stage: Data in Table 3 showed that, seawater levels (1000, 1500 and 2000 ppm.) increased rooting percentage as the level of 1500 ppm. gave the highest percentage. In addition, the level of 1500 ppm. gave the highest average number of roots followed by other levels while, the lowest average number of roots was related to the control (Fig. 1, fourth row). Increasing levels of seawater increased the root thickness significantly as roots produced from the level of 2000 ppm. showed the highest thickness. Meanwhile, increasing levels of seawater from 1000 to 2000 ppm. decreased the root length significantly compared with the control and the level of 500 ppm.

Physiological analysis: Data in Table 4 revealed that, increasing seawater levels increased total soluble sugars compared with the control. The highest TSS content was appeared at the level of 1500 ppm. followed by 2000 ppm. while, the lowest TSS content was appeared at the control. In addition, the levels of 1500 and 2000 ppm. surpassed other levels and the control in total indoles content. The level of 500 ppm. did not have any significant differences from the control. We can also observe that, the level of 500 ppm. achieved the highest content of total phenols, chlorophil A, B significantly and carotinoide insignificantly followed by the level of 1000 ppm. Increasing the level of seawater from 1000 to 2000 ppm. decreased these values. Concerning proline content, increasing seawater levels increased proline content in the leaves as, the highest proline content was achieved at the level of 2000 ppm.

DISCUSSION

Callus cultures showed an increase in callus fresh weight per gram with increasing levels of seawater (500 and 1000 ppm.) compared with the control. It is obvious that presenting callus to these levels enhanced their response at earlier subcultures in contrast with no treated callus (the control). Al-Khayri (2002) studied the effect of various concentrations of sodium chloride on callus induction and proliferation of date palm. It was found that NaCl at a concentration of 25 μM had a stimulatory effect on the proliferation of callus and a concentration of 125 μM was inhibitory and harmful.

Chretien *et al*. (1992) cultured jojoba callus for 2 months in medium either without salt (control) or with 150 mM NaCl. Those that survived and adapted to a salt medium grew more quickly than controls. In addition, fresh and dry weight of jojoba explants were increased until the medium salinity level then decreased at high salinity levels (Roussos and Pontikis, 2003).

It is obvious that, presenting date palm embryos to seawater levels enhanced multiplication of secondary embryos and development (germinated embryos) compared with the control. Growth enhancement due to the presence of salinity has been reported for various *in vitro* cultures such as *Suaeda aegyptiaca* (Eshel, 1985), *Prunus cerasifera* peach rootstock (Dimassi-Theriou, 1998) and date palm (Al-Khayri, 2002). In addition, lower level of seawater induced shoot multiplication of two jojoba clones (Fayek *et al*., 2010). The positive effect of NaCl on plant growth may be due to the increased osmolarity (Flowers and Lauchli, 1983).

Increasing seawater levels increased total soluble sugars and total indols content compared with the control. Total soluble sugars in leaves of two young Iranian commercial olive cultivars (‘Zard’ and ‘Roghani’) increased with an increase in salinity up to 80 mM but decreased with additional increase in salinity (Mousavi *et al*., 2008). In addition, soluble carbohydrate concentration of two sugar beet cultivars significantly increased with increasing salt stress. Starch concentration of leaves also increased with increasing salinity (Dadkhah, 2010). It was reported that the elevated levels of the total soluble and insoluble carbohydrates to the shoot and root are considered to be playing an important role in the osmotic adjustment (Dhanapackiam and Ilyas, 2010).

The level of 500 ppm. achieved the highest content of total phenols, chlorophyll A and B. Those were reduced with increasing the level of salinity. Similarly, the total chlorophyll content of the leaves of *Sesbania grandiflora* seedlings increased when grown at 10 and 20 mM NaCl and decreased at 40 and 50 mM treatments (Dhanapackiam and Ilyas, 2010). In addition, an increase in chlorophyll contents was observed in six genotypes of rice under salinity (Alamgir and Ali, 1999). The fast rise in variable chlorophyll fluorescence yield increased in leaves of sugar beet plants treated with salt suggesting stimulation of photosystem II activity relative to photosystem I (Smillie and Nott, 1982).

In our investigation, proline content was obviously increased with increasing salinity but at the lowest level of salinity no difference was observed in proline content. These findings are the same with that of Al-Khayri (2002) who indicated that, in date palm cultures, proline content increased gradually as the concentration of NaCl increased. At 25 mM NaCl, proline content was unaffected compared with the control. However, when the level of NaCl was increased to 50 mM and over, significant accumulation of proline occurred. Also, Woodward and Bennett (2005) stated that, the shoot proline levels significantly increased in two salt tolerant clones of *Eucalyptus camaldulensis* when exposed to 100 mM NaCl in the shoot multiplication medium compared with 50 mM and the control. Early work by Joshi *et al*. (1962) underlines the hypothesis that amino acid metabolism are integrally involved in plant responses to salinity. Later studies (Flowers *et al*., 1977; Sacher and Staples, 1985) have shown that true vascular halophytes often produce copious amounts of nitrogenous compounds (e.g., proline and betain), while salt-sensitive vascular glycophytes generally produce carbohydrates (e.g. polyols and sucrose), to counteract the osmotic stress of salt. A number of roles have been proposed for proline in salinity tolerance. The first is that it acts as a store of energy that can be rapidly broken down and used when the plant is relieved of stress. The second is that it acts as an osmolyte and reduces the osmotic potential of the cell, thus reducing toxic ion uptake (Hare *et al*., 1998). In this case, the latter is more likely, with the salt tolerant plants not only producing more proline when stressed, but also having, in most cases, no significant drop in the chlorophyll content. This indicates that the increase in proline is reducing the physiologically detrimental effects of the salt (Delauney and Verma, 1993; Hare *et al*., 1998).

CONCLUSION

Low levels of salinity enhanced growth and multiplication of *in vitro* tolerant plants, particularly, date palm plants. In addition, increasing of proline, total soluble sugars and total indols content in leaves are useful for growth and development of date palm *in vitro* cultures.

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