ORIGINAL ARTICLE JGEB Vol. 5, No. 1, 2007
ISSN:1687-157X

Development of Suspension Culture System for *In Vitro* Propagation of Date Palm

Mahmoud M. Saker[1], Mai A. Allam[1], Amina H. Goma[2] and Abd El-Zaher M. H.[2]

[1]Plant Biotechnology Department, National Research Center, Dokki, [2]Pomology Department, Faculty of Agriculture, Cairo University, Egypt

ABSTRACT

A reproducible and applicable protocol for *in vitro* propagation of date palm cv. Sewi (Egyptian semi-dry date palm cultivar), using suspension culture system was established, as a first step towards industrial production of date palm offshoots using bioreactors. The investigated factors included the efficacy of liquid and solid media, identification of the best liquid medium for the maturation and development of somatic embryos and the effect of activated charcoal. The obtained results revealed insignificant effect of NAA (Naphthalene acetic acid) on the number of somatic embryos after two months of culturing in liquid system (shake flask system). The highest number of somatic embryos (129 embryos/ flask) was recorded in liquid basal MS medium, followed by (120.2 embryos/flask) on basal MS supplemented with 0.2 mg/l NAA. The obtained data also indicated that the number of somatic embryos formed in liquid system was about 2 to 10 times greater than that on the same corresponding solid media. Addition of activated charcoal has a positive effect on the number of somatic embryos in both solid and liquid systems. It could be concluded that, an improved and efficient protocol for date palm regeneration through somatic embryogenesis using suspension culture system was developed and further studies are needed to confirm the validity of the developed system on bioreactor level (scaling up).

Key Words: *Phoenix dactylifera* L., Somatic embryos, Suspension culture

Corresponding Author: Mahmoud Saker
E-mail : sakrmahmoud@yahoo.com

Journal of Genetic Engineering and Biotechnology, 2007, 5(1-2): 51-56

INTRODUCTION

Date palm *(Phoenix dactylifera* L.*)*, is a member of family *Palmaceae (Arecaceae)*, inhabit tropical and sub-tropical habitats and the majority of palms are found in the old world *(Moore, 1973)*. The nutritional quality of date palm fruits is well-known and documented *(Toutain, 1967)*. In addition to its nutritional value, palm tree tolerates adverse environmental conditions (abiotic stresses) and is important in reducing desertification and sanding-up problems. This situation makes palm tree is the major plantation crop in Arabic world *(Shaheen, 1986)*. Increasing date palm productivity can be achieved through increasing the productivity of the existing trees or expanding of palm cultivated area dry areas, which are unsuitable for growing of traditional crops. Both ways constitute a big dilemma, because extensive breeding programs for the selection of superior date palm clones through traditional methods are tedious efforts due to the long life cycle and strongly heterozygous nature of the palm tree. Moreover, insufficient and expensive cost of offshoots (planting materials) is another serious limiting factor, which hinders expansion of palm cultivated area, renewing of existing palm orchards and improving its productivity. Therefore, serious efforts to benefit from endless applications of biotechnology are a must, to overcome date palm dilemma *(Saker and Moursy, 2003)*.

Although, several works have been published describing some culture media for organogenesis or somatic embryogenesis of date palm *(EL-Harmadi, 1995 and Fki et al., 2003)*, few trails of suspension culture technique have been achieved in date palm. Successful culture of oil palm *(Elaeis guineensis Jacq.)* suspension cells in a bioreactor was reported by *Gorret et al. (2004)*. This method might be efficient for mass propagation of *Phoenix dactylifera (Fki et al., 2003)*. In this context, a protocol was described by *Fki et al. (2003)* for large-scale micropropagation of date palm cv. Deglet Nour. Clonal plants were regenerated from somatic embryos derived from highly proliferating suspension cultures. Friable embryogenic calli were initiated from both leaves and inflorescence explants. Suspension culture consisting of pro-embryonic masses was established from calli showing high competency for somatic embryogenesis.

This study was proposed to optimize various parameters affecting successful establishment of cell suspension

culture system and mass production of date palm somatic embryos in suspension culture system, as a key step towards industrial production of date palm somatic embryos in bioreactors.

MATERIALS AND METHODS

Establishment of suspension cultures:

In this study, friable embryogenic callus (nodular-shaped embryos) developed from shoot tip explants of date palm (cv Sewi) was used *(Saker et al., 2006)*. To establish suspension cultures, friable and loose embryogenic callus pieces (500 mg) were transferred aseptically to 50 ml liquid media in 250 ml Erlenmeyer flasks. The content of the flask was filtered using a sieve (500 µm diameters) and the filtrate (homogenous embryogenic suspension cultures) was incubated on rotary shaker at 125 rpm at 23 ± 2°C under a 16-8 h. photoperiod. The cultures were subcultured each 15 days by total renewal of medium after decantation. The culture medium contained MS basal salts, 170 mg/l KH_2PO_4, 10 mg/l thiamine-HCl, 200 mg/l glutamine and 30 g/l sucrose.

Effect of solid and liquid media and activated charcoal on somatic embryo formation:

To identify the best type of media on the formation of somatic embryos, four different types of MS media, namely E1, E2, E3 and E4 were tested (Table 1). To study the efficacy of the liquid culture system, the effect of the same four media in liquid form (E1L, E2L, E3L and E4L) and in the solid form (E1S, E2S, E3S and E4S) were also tested. To study the effect of activated charcoal (AC), the same aforementioned four media in solid (2 g/l phytagel) and liquid forms supplemented with or without 1 g/l activated charcoal (AC) on the number of somatic embryo per flask were also tested. Data, expressed as the increment in the number of somatic embryos, were recorded during 8 weeks (4 subcultures).

RESULTS

Effect of liquid media with activated charcoal:

The effect of the four different liquid media supplemented with AC on the number of somatic embryos through four subcultures was presented in Table (2). Data of this table indicated that there is a significant increase between the control media (E1L) and other tested media after two months of culturing in liquid media. The highest number of somatic embryos (129) was recorded in (E1L) medium, followed by (120.2) in (E2L) medium. However, media (E3L and E4L) had a highly significant difference comparing with control, with a recorded number (13.2) and (38.4), respectively. No significant differences were obtained between E1L and E2L in the first subculture (10.2 and 9.8), respectively. The same observation was obtained in subcultures 3 and 4. In the case of E1L medium, no significant differences were recorded between the number of somatic embryos in weeks of the first subculture and in the weeks of the fourth subculture. However, significant differences were obtained between the weeks of the second and the third subcultures. In the case of medium E2L, no significant between the first three weeks and the last two weeks was recorded. The other weeks (4, 5, 6 and 7) had significant differences between them. It could observed that the first two weeks and the last two weeks had no significant changes between them in the tested media E3L and E4L, but significant differences were noticed in the other weeks. (Figure 1) shows the effect of the four different liquid media on somatic embryos formation.

Effect of solid media with activated charcoal:

Data presented in Table (3) showed the effect of the same four tested media, in a solid case, i.e. E1S, E2S, E3S and E4S on the number of somatic embryos. A glance on data summarized in this table indicate clearly that the overall all yield of somatic embryos in liquid system (Table 2) is 10 folds greater than the yield on the same corresponding solid medium (Table 3). For instance, the maximum yield from somatic embryos in E1L medium at the end of the fourth subculture was 129 and on the same solid medium (E1S) was 17.2. Similar to the same trend recorded in the case of liquid media, a gradual increase in the number of somatic embryos was recorded as the number of subcultures increased. Also, the medium E1 gave the best number of somatic embryos. In conclusion, comparison between the liquid and solid media (Tables 2 and 3) clearly showed that the number of somatic embryos formed onto liquid media was 2 to 10 times greater than the number of somatic embryos produced on the same corresponding solid media. Figure 2 shows the effect of solid media with and without AC on somatic embryos formation.

Effect of liquid and solid media without activated charcoal:

Tables (4 and 5) showed the effect of liquid and solid types of the four tested media without activated charcoal on the number of somatic embryos. A comparison between the two tables indicated that the number of somatic embryos on liquid media is usually higher than the number on the same corresponding solid media. In the liquid and solid forms of the medium E1; E1L and E1S; The total yield of somatic embryos at the end of the fourth subculture was 17.2 and 11.6, respectively. Similarly in the other three media, the yield of somatic embryos on liquid system is about 1.5 to 2 times greater than the yield on the corresponding solid media. The highest significance increase in the number of somatic embryos on liquid media was recorded in medium E2L (16.2) and E2S (8.4). In the case of liquid medium (E1L), no significant increase in number of somatic embryos was observed during the last week of the

second, the third and the fourth subcultures. Also, no significant increment in number of somatic embryos throughout the four subcultures was recorded (Table 4). In case of solid media, data of Table (5) revealed non-significant increment in the number of somatic embryos from first to fourth weeks in media E1S and E2S. However, there was a high significant increase from the fourth to eighth week. That was not the same with E3S medium, which recorded non-significant increase between the first and second weeks, while the weeks from the third to the eighth recorded a significant increment in the number of somatic embryos. Figure (3) shows the effect of the liquid media with and without AC on somatic embryos formation.

Table 1: Composition of the four different media used in this study.

E1	E2	E3	E4
30 mg/lsucrose 4.4 mg/l MS 70 mg/l thiamine-HCl 70 mg/l KH2PO4	E1 Medium, 0.1 mg/l NAA	E1 Medium, 125 mg/l myo inositol, 200 mg/l glutamine, 100 mg/l ascorbic acid, 100 mg/l citric acid, 1 mg/l nicotinic acid, 1 mg/l pyrodoxine-HCl, 2 mg/l glycine, 1 mg/l panthothianic	E1 Medium, 10 mg/l 2,4-D, 3 mg/l 2iP, 200 mg/l glutamine

Table 2: Effect of liquid media supplemented with AC on number of somatic embryos after 8 weeks (4 subcultures – 2 weeks for each subculture).

Medium \ Week	1	2	3	4	5	6	7	8
E1L (Control)	10.20 ^{aG}	26.8 ^{aF}	42.0 ^{aE}	62.8 ^{aD}	86.4 ^{aC}	115.2 ^{aB}	126.6 ^{aA}	129.0 ^{aA}
E2L	09.80 ^{aH}	22.2 ^{bG}	40.8 ^{aF}	60.6 ^{aE}	82.0 ^{bD}	107.4 ^{bC}	116.6 ^{bB}	120.2 ^{aA}
E3L	00.80 ^{cF}	01.6 ^{dF}	03.4 ^{cE}	05.2 ^{cD}	08.2 ^{dC}	011.8 ^{dB}	012.2 ^{dAB}	013.2 ^{cA}
E4L	04.00 ^{bG}	06.0 ^{cF}	11.4 ^{bE}	21.6 ^{bD}	29.8 ^{cC}	036.0 ^{cB}	037.0 ^{cAB}	038.4 ^{bA}

Each value is the mean of 5 replicates. Small letters compared between subcultures of the same medium, Capital letters compared between the media in the same subculture. Mean separation by LSD (0.05).

Table 3: Effect of solid media supplemented with AC on number of somatic embryos, after 8 weeks (4 subcultures – 2 weeks for each subculture).

Medium \ Week	1	2	3	4	5	6	7	8
E1S (Control)	0.80 ^{aE}	1.60 ^{aDE}	2.40 ^{aCD}	3.20 ^{aC}	8.80 ^{aB}	9.60 ^{aAB}	10.00 ^{aA}	10.40 ^{aA}
E2S	0.60 ^{abE}	1.40 ^{aE}	1.80 ^{aE}	3.60 ^{aD}	6.60 ^{bC}	8.60 ^{aB}	09.80 ^{aAB}	10.00 ^{aA}
E3S	0.00 ^{bC}	0.00 ^{bC}	0.60 ^{bBC}	1.20 ^{bAB}	1.40 ^{dAB}	1.40 ^{cAB}	01.60 ^{cA}	01.80 ^{cA}
E4S	0.40 ^{abC}	0.60 ^{bBC}	1.20 ^{abBC}	1.60 ^{bB}	4.00 ^{cA}	4.20 ^{bA}	04.60 ^{bA}	04.80 ^{bA}

Each value is the mean of 5 replicates." Small letters compared between subcultures of the same medium, Capital letters compared between the media in the same subculture. Mean separation by LSD (0.05).

Table 4: Effect of liquid media without AC on number of somatic embryos, after 8 week (4 subcultures – 2 weeks for each subculture).

Medium \ Week	1	2	3	4	5	6	7	8
E1L (control)	1.60 ^{aF}	2.20 ^{bF}	4.20 ^{bE}	8.00 ^{aD}	10.60 ^{aC}	14.80 ^{aB}	16.80 ^{aA}	17.20 ^{aA}
E 2L	1.40 ^{aF}	2.80 ^{abF}	5.40 ^{aE}	8.80 ^{aD}	10.80 ^{aC}	14.00 ^{aB}	15.20 ^{bAB}	16.20 ^{aA}
E3L	0.20 ^{bE}	0.60 ^{cE}	1.20 ^{cDE}	1.80b ^{cCD}	2.80 ^{cBC}	3.80 ^{cAB}	4.20 ^{dA}	4.80 ^{cA}
E4L	1.40 ^{aD}	3.60 ^{aC}	4.00 ^{bC}	4.60 ^{bBC}	5.40 ^{bB}	6.80 ^{bA}	7.40 ^{cA}	7.60 ^{bA}

Each value is the mean of 5 replicates." Small letters compared between subcultures of the same medium, Capital letters compared between the media in the same subculture. Mean separation by LSD (0.05).

Table 5: Effect of solid media without AC on number of somatic embryos, after 8 week (4 subcultures – 2 weeks for each subculture).

Medium \ Week	1	2	3	4	5	6	7	8
E1S (control)	0.40 ^{aF}	1.00 ^{bF}	2.20 ^{aE}	3.80 ^{aD}	5.60 ^{aC}	8.20 ^{aB}	11.00 ^{aA}	11.60 ^{aA}
E2S	0.60 ^{aE}	0.80 ^{bE}	2.80 ^{aD}	4.20 ^{aC}	5.60 ^{aB}	7.40 ^{aA}	08.00 ^{bA}	08.40 ^{bA}
E3S	0.20 ^{aC}	0.40 ^{bC}	0.60 ^{bBC}	0.80 ^{cBC}	1.40 ^{cB}	2.40 ^{cA}	02.60 ^{dA}	03.20 ^{dA}
E4S	0.80 ^{aC}	1.80 ^{aBC}	2.00 ^{aBC}	2.40 ^{bB}	4.20 ^{bA}	5.00 ^{bA}	05.20 ^{cA}	05.40 ^{cA}

Each value is the mean of 5 replicates. Small letters compared between subcultures of the same medium, Capital letters compared between the media in the same subculture. Mean separation by LSD (0.05).

Figure 1: Date palm embryogenic cultures developed in liquid culture system using four different media, namely E1L, E2L, E3L and E4L. All media contained activated charcoal (AC). Photos were taken after two months of cultivation.

Figure 2: Development of somatic embryos on solid E1S and E2S with activated charcoal (+AC) and without activated charcoal (-AC)

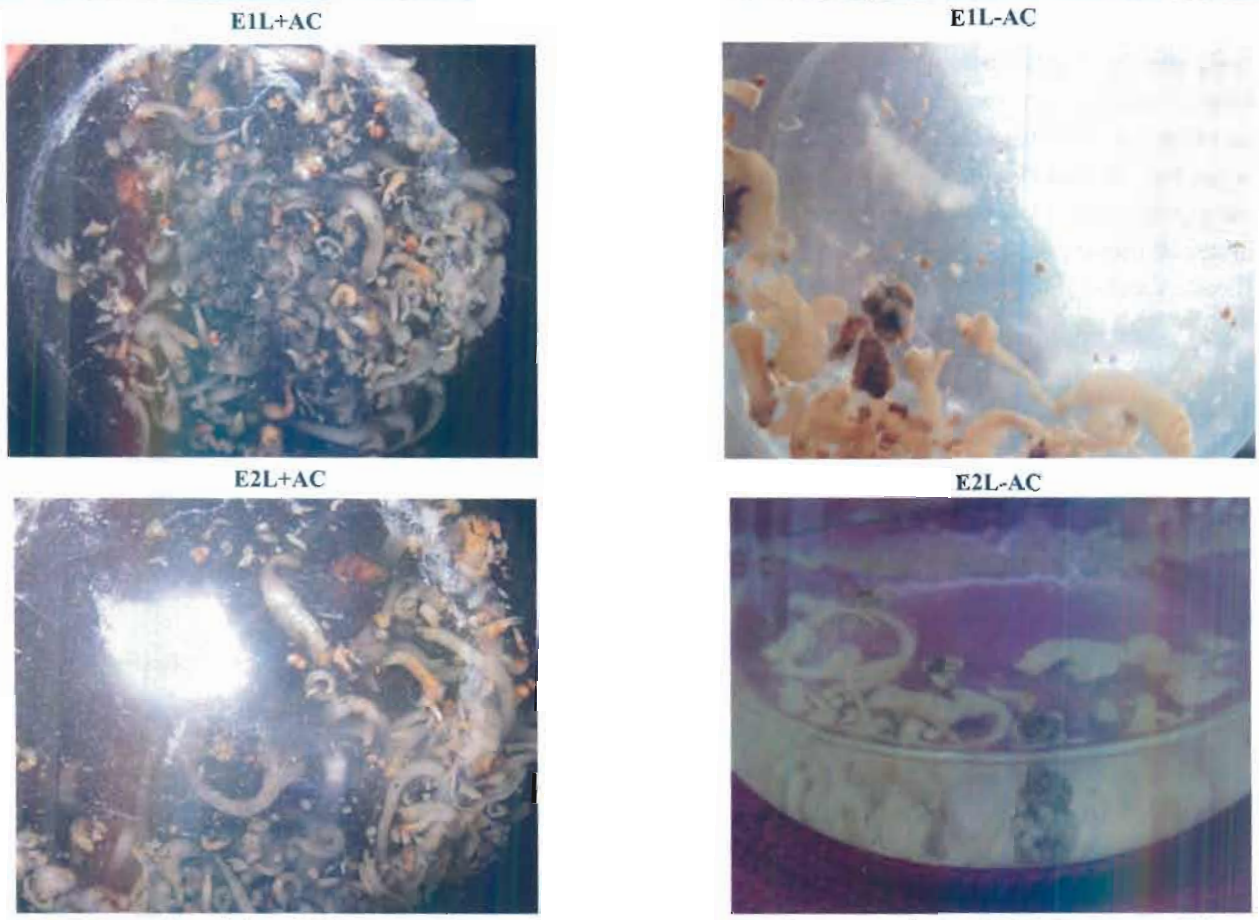

Figure 3: Effect of activated charcoal (AC) on the number of somatic embryos after 2 months of culturing on medium E1L and El2.

DISCUSSION

The obtained data can be concluded as follows:

Liquid culture system is a promising system for rapid mass propagation of date palm and the yield of somatic embryos in liquid media is about ten times greater than that on solid media, the yield of somatic embryos in liquid media supplemented with activated charcoal is about 10 times greater than that on the corresponding medium free of activated charcoal and hormone-free medium (E1) is the best medium for production of somatic embryos. The successful production of huge numbers of somatic embryos in liquid culture system described herein on hormone-free medium can be seen as a successful step towards large scale production (industrial production) of date palm offshoots and artificial seeds using bioreactors. Also successful formation of somatic embryos on hormone-free medium will definitely reduce and minimize the level of somaclonal variations in tissue culture-derived date palm plantlets. And will facilitate future genetic transformation studies in date palm.

The superiority of liquid system was reported by many authors, among them, *Fki et al. (2003)*. They reported that liquid medium (MS salts, MS vitamins, Fe-EDTA, Sucrose, Myo-inositol, Glycine, Glutamine, KH_2PO_4, Adenine, 2,4-D and Activated charcoal) was found to be drastically increase the production of somatic embryos, whereas only 10±2 embryos were recovered from 100 mg callus (FW) on solid medium, the same amount of callus produced up to 200±10 embryos in liquid medium after a one month, the yield on liquid medium is about 20 times greater than that on solid medium. Similarly, *Houng et al. (1999)* and *Zouine et al. (2005)* found that the development of somatic embryos in solid media was very slow, taking 4-6 months in vitro. In addition, the number of somatic embryos produced in liquid media was significantly ($p<0.05$) higher than in solid media. They added that the inoculation of plant suspension cultures into fresh medium results in activation of cell metabolism. A relatively short lag phase of growth is followed by a rise in the cell number (high mitotic activity in the exponential phase). These results were also conferred by *Cvikrova et al. (1999)*.

In this context, *Gorret et al. (2004)* initiated suspension culture by bioreactor in oil palm. They demonstrated that embryogenic oil palm cells can grow successfully in a stirred-tank bioreactor with an increase in biomass of 3.5 fold per month compared with values reported for shake flask cultures. Another advantage of production of date palm somatic embryos in the liquid system is the bipolarity of the developed embryos, typical to zygotic embryos, i.e. bipolar formation of the apical and root meristems *(Fki et al., 2003 and Zouine et al., 2005)*. The beneficial effects

of activated charcoal during the formation and germination of somatic embryo reported herein confirms the great role of activated charcoal on somatic embryogenesis. This positive effect was also reported by *Zouine et al. (2005)*. In this context, *Komamine (1997)* added that activated charcoal prevented the development of abnormal plantlet and enhanced somatic embryogenesis, shoot formation, plant recovery and rooting.

The results of the present study indicated that basal medium (hormone-free medium) is the best one for somatic embryos production. This observation disagrees with that of *Zouine et al. (2005)*. They observed that the highest production of embryos was obtained with BSTN cultivar (71 embryos/flask) in liquid media contained 2,4-D and glutamine. They also mentioned that it is possible that the increasing osmotic pressure resulting from the combination of 2,4-D (4.5 × 10-7 M 2,4-D), glutamine (6.7×10-4M) and ABA in the liquid medium might be responsible for embryo production and maturation of two date palm cultivars (JHL and BSTN). Our results are in agreement with *Bhaskara and Smith (1992)*. They reported that somatic embryogenesis in date palm occurred only in the absence of 2,4-D. *Kreuger et al. (1995)* obtained the same results with cultures of Cyclamen persicum. The great increment in the number of somatic embryos in liquid media may be due to the easy and available nutrients to the cells or perhaps due to the big surface area of the cells directly exposed to the nutrient *Duval et al. (1995)*.

Acknowledgement:
This work was funded by ICGEB research grant no. CRP/ EGY03-03

REFERENCES

Bhaskaran, S. and Smith, R. H. 1992. Somatic embryogenesis from shoot tip and immature inflorescence of *Phoenix dactylifera* CV. Barhee. Plant Cell Reports **12**(1):22-25.

Cvikrová, M., Binarová, P., Cenklová, V., et al. 1999. Reinitiation of cell division and polyamine and aromatic monoamine levels in alfalfa explants during the induction of somatic embryogenesis. Physiologia Plantarum **105**(2):330-337.

Duval, Y., Aberlenc, F. and de Touchet, B. 1995. Use of embryogenic suspensions for oil palm micro-propagation. In ISOPB International Symposium on Recent Development in Oil Palm Tissue Culture and Biotechnology. Kuala Lumpur: International Society for Oil Palm Breeders. pp. 38-47.

El Hadrami, I., Cheikh, R. and Baaziz, M. 1995. Somatic embryogenesis and plant regeneration from shoot-tip explants in *Phoenix dactylifera* L. Biologia Plantarum **37**(2):205-211.

Fki, L., Masmoudi, R., Drira, N. and Rival, A. 2003. An optimised protocol for plant regeneration from embryogenic suspension cultures of date palm, *Phoenix dactylifera* L., cv. Deglet Nour. Plant Cell Reports **21**(6):517-524.

Gorret, N., Bin Rosli, S. K., Oppenheim, S. F., et al. 2004. Bioreactor culture of oil palm *(Elaeis guineensis)* and effects of nitrogen source, inoculum size and conditioned medium on biomass production. Journal of Biotechnology **108**(3):253-263.

Huong, L. T. L., Baiocco, M., Huy, B. P., et al. 1999. Somatic embryogenesis in Canary Island date palm. Plant Cell, Tissue and Organ Culture **56**(1):1-7.

Komamine, A. (1997). Activated charcoal affects morphogenesis and enhances sporophyte regeneration during leaf cell suspension culture of *Platycerium bifurcatum*. Plant Cell Rep., **17**: 77-83.

Kreuger, M., Postma, E., Brouwer, Y. and Van Holst, G. J. 1995. Somatic embryogenesis of Cyclamen persicum in liquid medium. Physiologia Plantarum **94**(4):605-612.

Moore, H. E. Jr. 1973. The major groups of palms and their distribution. Gentes Herbarium **11**:27-141.

Saker, M. M., Adawy, S. S., Mohamed, A. A. and El-Itriby, H. A. 2006. Monitoring of cultivar identity in tissue culture-derived date palms using RAPD and AFLP analysis. Biologia Plantarum **50**(2):198-204.

Saker,M. M. and Moursy,H. A. 2003. Sep 16-19; Transgenic date palm: A new era in date palm biotechnology. Proceeding of the International Conference on Date Palm. King Saud University, Qaseem, Saudi Arabia.

Shahin,M. A. and Said,A. A. 1986. Effect of activated charcoal on tissue culture in date palm. Proceeding of the Second Symposium on the Date Palm in Saudia Arabia. pp. 107-113. Al-Hassa, Saudi Arabia, King Faisal University.

Toutain, G. 1967. Le palmier dattier: Culture et production. Al Awamia 25:83-151. Cited by FAO (2004). FAO production year book. Food and Agriculture Organization of the United Nation, Rome.

Zouine, J., El Bellaj, M., Meddich, A., et al. 2005. Proliferation and germination of somatic embryos from embryogenic suspension cultures in *Phoenix dactylifera*. Plant Cell, Tissue and Organ Culture **82**(1):83-92.