*Bull. Fac. Agric., Cairo Univ., 57 (2006): 789-802.*

# IMPROVED METHOD FOR THE MICROPROPAGATION OF DATE PALM (*Phoenix dactylifera* L.) THROUGH ELONGATION AND ROOTING STAGES

(Received:21.6. 2006)

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## ABSTRACT

Differentiated somatic embryos of date palm *cv.* Zaghloul cultured onto nutrient medium containing different growth promoters (NAA and $GA_3$) and activated charcoal to enhance vegetative shoots elongation. Quantitative and qualitative improvements in shoots elongation and root development have been obtained throughout three subcultures of somatic embryos. Inclusion 0.1 mg $l^{-1}$ NAA and 3.0 g $l^{-1}$ AC in elongation medium promoted shoot length and leaf width as well as reduced vitrification. Using $GA_3$ at concentration more than 0.5 mg $l^{-1}$ produced delicate shoots. The nutrient medium contained 0.1 NAA mg $l^{-1}$ only was the best medium for root formation in comparison with other treatments. The ideal shoots for transplanting should have 3-4 broad leaves, thick trunk and 4-5 adventitious roots with secondary roots.

*Key words: activated charcoal, date palm, elongation gibberellic acid,NAA, rooting, tissue culture.*

## 1. INTRODUCTION

Utilizing multiple somatic embryos of date palm (*Phoenix dactylifera* L.) in multiplication stage for many subcultures formed

weak and delicate shoots. However, the proliferated propagules failed to find their ability for elongation to normal plants fitting for rooting and transplanting (Abul-Soad *et al.*, 2002b).

Root hairs are formed from the epidermal layer of the root and are responsible to increase the surface of absorption, making the uptake of water and minerals much more rapid. As a result of this the *in vitro* plantlets will develop as quickly as possible and the time of obtaining *in vitro* plantlets suitable for transplanting will increase. Thus root hairs formation was principle in rooting process of date palm plantlets. By this the date palm plantlets can be successfully transplanted (Fosket, 1994).

Zaid and Tisserat (1983) reported that the best rooting results was noticed on medium without charcoal with 0.1 mg/liter NAA. Tisserat (1984) mentioned that adventitious rooting was obtained readily after re-culturing separated shoots to an agar nutrient medium containing 0.1 mg/liter NAA without charcoal, following 8-16 weeks in culture. Date palm plants may be obtained by transferring individual young plants to MS medium supplemented with 0.1 mg/l NAA to enhance rooting (Omar, 1988).

In monocots, the cells in the center of the vascular cylinder do not differentiate into tracheary elements, but remain as undifferentiated parenchyma and are collectively called pith (Fosket, 1994).

More one study examined the effect of different plant growth regulators on shoot proliferation of date palm during multiplication stage (Abul-Soad *et al.*, 2002a). On the other side, a little information was available about elongation stage, although the importance of this stage. It represents the neck of bottle to produce viable date palm plantlets fitting for adaptation. It is necessary to enhance shoot elongation to shorten the time required from somatic embryo to plantlet. It was observed that producing new shoots or doing so many subcultures in multiplication stage produced weakly and delicate shoots. However, outbreak of bacterial contamination in subcultured shoots. One thing could be noticed was leaves fall during transfer cultures to fresh medium. This kind of shoots were not able to produce roots or continued in growth and development. Some of propagators go to get plantlets from somatic embryos directly without proliferation to sustain quick and vigorous growth. By this way they loss the advantages of proliferation.

The objective of this work aimed to improve the elongation and rooting of small shoots to viable plantlets in a short time, as well as study the role of incorporation some stimulators into the nutrient media of elongation stage in parallel with the form of shoots. Because of the suitable medium will differ by the shape of shoots. Thus using homogenous cultures was very essential to obtain genuine results.

## 2. MATERIALS AND METHODS

### 2.1. Plant material

Repeated embryos (RE) or multiple embryos ( Zaid *et al.*, 2004 a) derived from tissue culture were picked up (Abul-Soad *et al.*, 2004). Fifteen propagules were selected for each treatment. There was a desire to select similar propagules of somatic embryos. Each propagule was cultured in an individual small jar (150 $cm^3$) in the first culture. Then, propagules were transferred in large jars (350 $cm^3$). After that, the individual plantlets were transferred onto free-hormone medium in long-test tubes (25 cm in length and 2.5 cm in diameter).

### 2.2. Media preparation

The basal nutrient medium employed through this study contained Murashige & Skoog (MS) inorganic salts (1962) supplemented with (in 0.5 mg $l^{-1}$):

0.5 nicotinic acid; 0.5 pyridoxine-HCl; 0.4 thiamine-HCl; 100.0 myo-inositol; mg $l^{-1}$2.0 glycine; 6 000.0 agar (win-lap.) and 30 000.0 sucrose. Seven treatments were used in this experiment as follows:

1. MS (with vitamins).
2. MS+ 3 $gl^{-1}$ activated charcoal (AC).
3. MS+ 0.1 $mgl^{-1}$ naphthalene acetic acid (NAA).
4. MS+3 $gl^{-1}$ AC+0.1 $mgl^{-1}$ NAA.
5. MS+0.1 $mgl^{-1}$ gibberellic acid ($GA_3$).
6. MS+0.5 $mgl^{-1}$ $GA_3$.
7. MS+1.0 $mgl^{-1}$ $GA_3$.

### 2.3. Culture conditions and data recorded

*In vitro* cultures were incubated under light intensity of 3000 lux with a photoperiod of 16 hrs in a temperature-controlled room at 27 ± 2 °C. Data collection and re-culturing were performed at 6 weeks intervals. Those that were viable and showed indications of response

were selected for further subculture. During this stage, many observations and the following data were recorded:

1. Average shoots number/culture.
2. Average shoots length/culture (cm).
3. Average roots number/plantlet.
4. Average roots length/culture (cm).
5. Leaf width (mm).
6. Vetrification [it is expressed as scores and presented as follows, similar to the shoot growth which was estimated (as scores) according to the method described by Pottino (1981) and Abul-Soad (1999)].

a. Negative growth results = 1
b. Below average growth = 2
c. Average growth = 3
d. Above average growth = 4
e. Excellent growth = 5

**2.4.Statistics**

In each treatment, 15 Jars each containing 1 propagule (Cluster of shoots somatic embryos). Factorial Randomized Complete Block Design was used and data were subjected to analysis of variance. Separation of means among treatments was determined using L.S.D test at 5%, according to Steel and Torrie (1980).

## 3. RESULTS AND DISCUSSION

Initial clusters of somatic embryos of date palm cv. Zaghloul (Fig. 1a) elongated into individual healthy plantlets. The growth parameters were recorded during three subcultures.

**3.1. Shoot number**

Data represented in Table (1) show that the shoot number significantly increased in the presence of $GA_3$ in the elongation medium. The average shoot number was 20.0 shoot/culture when the elongation medium was incorporated 1.0 mg $l^{-1}$. As well as the average shoot number increased by increasing the $GA_3$ concentration in the elongation medium.

On the other side, the shoot number significantly increased by increasing the subculture number. The average shoot number was 12.9 and 15.1 shoot/culture in subculture 1 and 2 ($S_1$ and $S_2$), respectively. While the interaction effect between chemicals and subcultures

**Table (1): Effect of MS modified medium supplemented with NAA, $GA_3$ and AC & subculture number on the growth and development of date palm (Zaghloul *cv.*) somatic embryos.**

| Characters (B) | Avg. shoots no./culture | | | | Avg. shoots length (cm) | | | | Avg. roots no./plantlet | | | | Avg. roots length (cm) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chemicals(A) ($mgl^{-1}$) | $S_1$ | $S_2$ | $S_3$ | Mean (A) | $S_1$ | $S_2$ | $S_3$ | Mean (A) | $S_1$ | $S_2$ | $S_3$ | Mean (A) | $S_1$ | $S_2$ | $S_3$ | Mean (A) |
| MS | 10 | 10 | 10 | 10.0d | 4 | 10 | 14 | 9.3cd | 1 | 1 | 1 | 1.0cd | 0.5 | 2 | 4 | 2.2e |
| MS+ 3000 AC* | 8 | 8 | 8 | 8.0e | 6 | 8 | 10 | 8.0d | 1 | 2 | 2 | 1.7bc | 2 | 5 | 7 | 4.7c |
| MS+ 0.1 NAA** | 15 | 20 | 20 | 18.3b | 9 | 14 | 16 | 13.0b | 2 | 4 | 5 | 3.3a | 4 | 8 | 9 | 7.0a |
| MS+3000 AC+0.1 NAA | 12 | 14 | 14 | 13.3c | 12 | 16 | 20 | 16.0a | 2 | 2 | 2 | 2.0b | 6 | 6 | 8 | 6.0b |
| MS+0.1 $GA_3$*** | 9 | 14 | 14 | 12.3c | 5 | 10 | 12 | 9.0d | 1 | 1 | 1 | 1.0cd | 5 | 7 | 7 | 6.3ab |
| MS+0.5 $GA_3$ | 16 | 20 | 20 | 18.7ab | 7 | 10 | 15 | 10.7c | 0 | 1 | 1 | 0.7d | 3 | 3 | 4 | 3.7d |
| MS+1.0 $GA_3$ | 20 | 20 | 20 | 20.0a | 6 | 9 | 9 | 8.0d | 1 | 1 | 1 | 1.0cd | 6 | 6 | 8 | 6.7ab |
| **Mean (B)** | **12.9b** | **15.1a** | **15.1a** | | **7.0c** | **11.0b** | **13.7a** | | **1.1b** | **1.7a** | **1.7a** | | **3.8c** | **5.1b** | **6.7a** | |

*Activated charcoal (AC); **œ-Naphthalene acetic acid (NAA); ***Gibberellic acid ($GA_3$).

Each treatment contains 15 propagules. Means followed by the same letters aren't significantly different.

L.S.D at 0.05 AB 2.8 2.4 1.3 1.5

showed significant differences. The presence of NAA and $GA_3$ greatly enhanced the shoot formation. This result is in agreement with Zaid and Tisserat (1983) who stated that Subcultures shoot tip and leafy lateral bud callus on nutrient media devoid of charcoal and supplemented with 0.1 mg/l NAA produced adventitious plantlets

**3.2. Shoot length**

The main aim of this study was to encourage the shoot elongation as quickly as possible. At the same time, obtaining healthy shoots able to initiate good-root system after shoot elongation. The obtained data in Table (1) reveal that, addition of 3 g $l^{-1}$ activated charcoal (AC) + 0.1 mg $l^{-1}$ NAA to the nutrient medium significantly raised the shoot length. Hence, the average shoot length was 16.0 cm. Sub-culturing elongated shoots of date palm significantly increased the shoot length from $S_1$ to $S_3$. The average shoot length was 7.0, 11.0 and 13.7 cm for $S_1$, $S_2$ and $S_3$, respectively. The interaction effect showed that, the small embryos of date palm after differentiation could elongate considerably to reach 20 cm after 18 weeks in culture onto the elongation medium which composed of MS basal medium supplemented with 0.1 mg $l^{-1}$ NAA and 3.0 g $l^{-1}$ AC. These results are in agreement with Ibrahim *et al.*, in (1999) who reported that the addition of AC to differentiation media usually improved embryos elongation rates compared to without AC. Also, data in Table (1) reveal that not only the activated charcoal stimulated the shoot elongation but also the addition of NAA in the elongation medium. Gadalla *et al.*, (2003). It was observed that the average shoots length in MS+ 3000 mg $l^{-1}$ AC treatment was 8.0 cm, whereas it was 16.0 cm when the NAA added to the elongation medium. Actually, the combined effect of AC and NAA significantly increased the shoots length in comparison with other treatments.

**3.3. Root number**

Addition of NAA to the elongation medium significantly raised the average root number, as shown in Table (1). The average roots number was 3.3 per plantlet. In other wise the root formation ranged from 3 – 4 roots per plantlet. While, the other treatments significantly decreased the root formation. In fact, the purpose of this study not form roots for elongated shoots but enhancement for shoots elongation. After that, induce adventitious roots on the base of long shoots. Naturally, root formation was occurred during the shoots

elongation. But most of the formed roots were primary roots. The plantlet depends on the primary roots to ensure the food in the beginning. But for the successful transplanting they need to form adventitious roots. Thus, trimming of primary roots in the beginning of the elongation stage or using negligible concentrations of NAA will enhance shoot elongation. This result is in harmony with Abul-Soad et *al.*, (2003) who reported that adventitious embryos formation continues alongside embryo maturation and germination (Proliferation of secondary embryos) giving multiplying asynchronous cultures. Although, well-formed somatic embryos have a primary root meristem, not all germinated embryos develop a primary root.

On the other hand, the average root number was significantly increased by subculture. Where it was 1.1 and 1.7 roots per plantlet in $S_1$ and $S_{2,3}$ , respectively.

**3.4. Root length**

Root length was enhanced mainly by $GA_3$ and NAA. Presence of $GA_3$ in the elongation medium not stimulated root formation but enhanced root length. $GA_3$ aid the roots to proceed their growth and developments. Data in Table (1) show that, the average roots length was 6.7 cm in the presence of 1.0 mg $l^{-1}$ $GA_3$ compared with the control treatment (without promoters), where it was 2.2 cm.

On the other side, there was a significant difference among different subcultures. Hence the average roots length was increased by increasing the subculture number.

**3.5. Leaf width (leaf diameter)**

It was observed that the leaf width or the diameter of elongated shoots tend to expand in width during the elongation stage. But when the elongated shoots exposed to exceptional conditions like over heat or growth deficiency, leaves tend to twist on its self and became thin and delicate (Fig. 1b). Whenever the leaf wide for the elongated shoots was big, the more growth and development will be happened. Data in Table (2) show that the leaf width significantly increased when the nutrient medium contained AC and 0.1 mg $l^{-1}$ NAA. The average leaf width was 10.0 mm, whereas it was 4.7 mm in the control medium. From other side, inclusion AC in the nutrient medium significantly increased the leaf width. Where, it was 7.3 mm. It is noticed that the leaf width improved by increasing subculture number. The average leaf width was 4.0 in $S_1$ and went up to 5.9, and

**Table (2): Effect of MS modified medium supplemented with NAA, $GA_3$ and AC & subculture number on Leaf width and Vetrification of date palm (Zaghloul cv.) somatic embryos during elongation stage.**

| Characters (B) / Chemicals(A) ($mgl^{-1}$) | Leaf width (mm) | | | | Vetrification | | | |
|---|---|---|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | Mean (A) | $S_1$ | $S_2$ | $S_3$ | Mean (A) |
| MS | 3 | 5 | 6 | 4.7c | 20 | 5 | 5 | 10.0c |
| MS+ 3000 AC* | 5 | 8 | 9 | 7.3b | 5 | 5 | 5 | 5.0d |
| MS+ 0.1 NAA** | 4 | 5 | 7 | 5.3c | 40 | 60 | 5 | 35.0a |
| MS+3000 AC+0.1 NAA | 8 | 10 | 12 | 10.0a | 5 | 5 | 5 | 5.0d |
| MS+0.1 $GA_3$*** | 3 | 5 | 6 | 4.7c | 20 | 40 | 5 | 21.7b |
| MS+0.5 $GA_3$ | 4 | 6 | 7 | 5.3c | 5 | 5 | 5 | 5.0d |
| MS+1.0 $GA_3$ | 2 | 2 | 3 | 2.3d | 20 | 5 | 5 | 10.0c |
| **Mean (B)** | **4.0c** | **5.9b** | **7.1a** | | **16.4b** | **17.9a** | **5.0c** | |

*Activated charcoal (AC); **œ-Naphthalene acetic acid (NAA); ***Gibberellic acid ($GA_3$). Vetrification is expressed as scores.
Each treatment contains 15 propagules. Means followed by the same letters aren't significantly different.
L.S.D at 0.05 AB 1.5 2.8


Fig. (1). (a) Somatic embryos used in the experiment, (b) delicate and thin elongated shoots (left Jar), and vigorous elongated shoots with wide leaves (right jar).

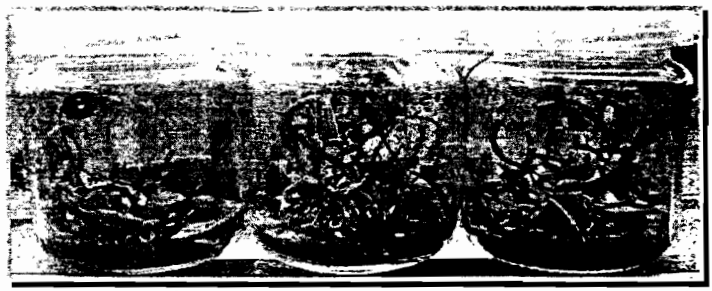

Fig. ( 2). Somatic embryos after 6 weeks in culture onto MS modified medium supplemented with 0.1 mg $l^{-1}$ NAA + 3 g $l^{-1}$ activated charcoal.

Fig. (3). 3 months-date palm plantlets cultured into long tubes to produce adventitious roots after elongation onto MS basal medium supplemented with 0.1 mg $l^{-1}$ NAA.

7.1 in $S_2$ and $S_3$, respectively. There were significant differences among interactions between growth promoters and subculture number. The basal salts of MS supplemented with 3 g $l^{-1}$ AC + 0.1 mg $l^{-1}$ NAA after 4.5 months ($S_3$) resulted in the highest leaf width (1.2 cm in diameter).

**3.6. Vitrification**

Hyperhydricity or water retention in organs of cultures during differentiation or proliferation is considered as a big problem. This phenomenon made vegetative shoots soft and glassy. Glassy or vitrificated shoots were not able to elongate to normal shoots. This phenomenon observed by Gaspar *et al.*, (1995) who reported that Hyperhydric malformations affecting shoots under micropropagation, a phenomenon called vitrification or hyperhydricity, appear as resulting from an inability of the organs to adapt completely their whole defense battery composed of enzymes against activated oxygen species and of soluble reductants, in front of several simultaneous stresses due to *in vitro* culture conditions.

In respect, this the effect of promoters in the elongation medium, culturing somatic embryos in first subculture onto 0.1 mg $l^{-1}$ NAA produced new shoots. Some of these new shoots were vitrificated. This may be attributed to the effect of NAA as auxin which stimulated shoot proliferation .Zaid *et al.*, (2004 b) But the other growth promoters mainly encourage the growth and development of vegetative shoots.

Regarding the effect of subculture number on vitrification, it was observed that the vitrification significantly increased in $S_2$ in some treatments. Selection viable shoots or plantlets to proceed their growth and development in third subculture decline sharply vitrification (Fig. 2).

It is not required to induce roots during elongation stage of date palm. But it is plausible to concentrate on the shoot elongation firstly, then induce root on the trunk of the viable shoots. Although inducing roots during elongation stage might help small shoots for growth and development. The bad thing is some of the elongated shoots were still weak and delicate. They became short, thin and pale-color shoots with a many adventitious shoots. This made them not able to grow in a quick and normal way. It is important to get balance between shoot and root systems development during the elongation and rooting stages to ensure success and save transplanting.

One of the most important benefits for quick elongation of somatic embryos was the low percent of bacterial contamination. It was observed that, cultures of date palm which continue in growing within test tubes of culture vessels more than one year produced contaminated plantlets. Thus, no bacterial contamination was noticed through the three subcultures of experiment.

It is recommended that culturing small propagules of date palm on the nutrient medium containing 0.1 mg $l^{-1}$ NAA and 3.0 g $l^{-1}$ AC in elongation medium to enhance shoot elongation throughout first two subcultures. After that, transfer the healthy shoots into rooting medium (0.1 mg $l^{-1}$ NAA) for 1 – 2 re-cultures (Fig. 3). Finally, using $GA_3$ at 0.1 – 0.5 mg $l^{-1}$ to encourage shoot and root elongation.

It can be concluded that some treatments exhibited advantages in shoot elongation only and the other for growth vigor. It is a good idea to culture vegetative shoots during elongation stage on the suitable medium for shoot elongation. Secondly, transfer vigorous shoots onto rooting medium. Induction roots for vegetative shoots during elongation stage encouraged shoot elongation. The reverse was found in proliferation stage. The ideal shoots for transplanting should have 3-4 wide leaves, thick trunk and 4-5 adventitious roots with secondary roots.

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## طريقة محسنة للإكـــثار الدقيق لنخيـــل البلح خلال مرحلتي الإستطالة و التجذير

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### ملـــخص

تمت زراعة الأجنة الجسمية المتكشفة لنخيل البلح علي وسط غذائي يحتوي علي منشطات نمو مختلفة (نفثالين حامض الخليك NAA ،وحـــامض الجبريلـــك $GA_3$ و الفحم المنشط AC) لتنشيط استطالة الأفرع الخضرية. تم الحصول علي تحسينات كمية و نوعية لاستطالة الأفرع و تطور الجذور خلال ثـــلاث نقـــلات للأجنة الجسمية. شجع إضافة ٠,١ مليجرام/لتر NAA و ٣ جرام/لتر فحم منشط علي استطالة الأفرع ، عرض الورقة (قطر الورقة) ، و كذلك قلل من الظـــاهرة الزجاجية. اعطي استخدام $GA_3$ بتركيز أعلي من ٠,٥ مليجرام/لتر افرع ضعيفة. كان الوسط الغذائي المحتوي علي ٠,١ مليجرام/لتر NAA فقط هو أفضل وسط لتكوين الجذور بالمقارنة بالمعاملات الأخري. يجب ان تحتوي الأفـــرع المثاليـــة للنقل للصوبة علي عدد ٣- ٤ أوراق عريضة ، ذات غلاظة لقاعدة النبـــات ، و ٤- ٥ جذور عرضية عليها جذور ثانوية.

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (٥٧) العدد الرابع (أكتوبر ٢٠٠٦) : ٧٨٩-٨٠٢.