

# ***In vitro* propagation of selected aged date palm male clone via direct adventitious buds proliferation**

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## **ABSTRACT**

Very young invisible closed male spathes of selected aged date palm (*Phoenix dactylifera* L) clone was early detected and separated from the tree according to scheduled time previously calculated . Ethyl alcohol (70 %) spraying was used to the totally closed spathes for surface sterilization then, flamed under aseptic conditions followed by cold immersion (5-8 °C) in filter sterilized antioxidant solution containing citric and ascorbic acids 150 mg/L each for 2 hours, prior to dissecting.

Results indicated that immature spikelets (comb shape) which individually separated, shortened to single flowers (explants) and cultured on Modified MS (Murashige and Skoog 1962) medium (MMS) supplemented with IAA (0.1 mg/L) + NOA (0.1 mg/L) + Kin (0.5 mg/L) + 2iP (1.0 mg/L) was recorded higher explant survival (100 %), superior in adventitious buds formation (22.22 %) and scored the highest significant numbers of adventitious buds (2.5), buds fresh weight (2.67 g) and growth value (20.23) after 4 months of incubation under dark conditions followed by 2 months under light conditions.

Produced adventitious buds cultured on the same medium in addition to glutathione (20 mg/L) significantly increased fresh weight (9.74 g) and recorded higher no. of buds (13.33) and growth value (6.10) as compared with the control after 8 weeks of incubation.

The polyamine (PAs); Spermine (100 mg/L) obtained the highest significant no. of buds (32.89), higher fresh weight ( 10.50 g ) and growth value (9.56) after 8 weeks of incubation. Individual shoot clump cultured on basal MS medium supplemented with IBA (0.5 mg/L) achieved the highest significant values of root formation (100 %), number of roots (3.66) and root length (5.9 cm) after 8 weeks of incubation.

Growing mixture containing compost and perlite (1:1, v/v) recorded the highest significant number of leaves ( 3.77 ), leaf area (15.23 cm<sup>2</sup>) and plantlets survival (80 %) after three months in acclimatization. This work could encourage the availability to overcome somaclonal variation in date palm vitroplants since callus production was avoided.

**Key words:** *Phoenix dactylifera* L., tissue culture, , micropropagation, organogenesis, Inflorescence, male spathe, immature spikelet, PAs, glutathione, spermine

## INTRODUCTION

Research work has been ongoing for few decades to use inflorescence explants in micropropagation of date palm. The way to excise the immature inflorescence without damage to the mother tree, composition of the nutrient medium for direct organ initiation have remained hindrances to this technique over recent decades (**Abul-Soad, 2011**).

Date palm is one of the most ancient plants, grows in the regions of Middle East, North Africa, South Sahel, East and South Africa. Its sexually propagated hampers propagation of true-to-type genotypes due to heterozygosity (**FAO, 2010**). The vegetative propagation is carried out with the off shoots, produced from axillary buds situated at the base of the trunk during the juvenile life of palm tree. Offshoot production is slow; their numbers are limited, laborious and can't meet the rapidly growing demand of varieties (**Jain, 2012**). It is not surprising that little work has been done on date palm genetic improvement for developing new cultivars by traditional approaches. Therefore to speed up the date palm breeding programmes, particularly the areas where date palm is threatened by red weevil, devastating diseases like Bayoud and Brittle Leaf; as a source of bio-fuel, biotechnology would be of great help in overcoming these problems (**Jain et al., 2011**). In vitro multiplication of date palm has both certain advantages and disadvantages. Since in vitro propagation of date palm makes available a large number of cloned plants in a short time, there is risk to accelerate agro-diversity erosion. It could lead to reducing the number of cultivated varieties to a smaller group of cultivars of international reputation propagated by a few commercial tissue-culture laboratories. Contrary to this risk, date palm in vitro propagation allows the multiplication of very rare quality genotypes or of genotypes without offshoots ( **Ferry, 2011**). In this concern, **El. Korchi, (2007)** on Al Ain City date palm male reported that all attempts using traditional methods failed to propagate that male because it did not produce any offshoots, consequently, plant tissue culture using inflorescences as a source of initial explants was the only available way to propagate this tree. However, the operation is feasible with various types of explants: (1) Use of undifferentiated buds (**Ferry and Ruiperez, 1999**), apex or very young leaves. However, collecting this type of explants means sacrificing of the palm which is unique specimen of selected genotype; (2) Young leaves collected and cultivated, according to the technique used for the oil palm tissue culture (**Noiret et al. 1988**); (3) Young inflorescences. There is no need to sacrifice the palm when the flowering pattern is well established and the right extraction technique is used. One of the main technological factors limiting the use of this technique is the

production of abnormal plants, when plantlets are obtained by somatic embryogenesis. If this problem can be solved, this method of propagation could reduce the cost of production and, consequently, the selling prices of date palm tissue culture-derived shoots. At the moment, the supplied shoots with true-to typeness guarantee are produced by organogenesis. However, this technique does not offer the same propagation speed. It is a high labor-consuming process and consequently costly one. Furthermore, very few laboratories control organogenesis at an industrial scale. In date palm, flowering has long been considered a complex process regulated by intricate internal and external factors and its induction under *in vitro* culture has often been reported to present an extremely sophisticated venture. In fact, only a few studies have so far been carried out to investigate this phenomenon in date palm. (**Masmoudi-Allouche et al. 2009**). Date palm micropropagation was studied *via* female floral bud (**Drira, 1981, Drira and Benbadis, 1985 and Hegazy, 2008**). Immature inflorescence (**Fki et al. 2003**). Inflorescences of several species have been cultured *in vitro* (**Nitsh, 1963**). Date palm ovules, carpel tissue, parthenogenetic endosperm, and fruit stalk blackened within 24 hours after culturing on nutrient media, and subsequently died (**Reuveni and Kipnis, 1974**).

Also, cultures of date palm floral bud reproductive tissues and especially male anthers, usually turned brown and died after a few weeks in culture (**Tisserat et al., 1979**).

Moreover, **El. Korchi, (2006)** found that browning was more pronounced among date palm explants that had undergone disinfestation of the spikelets and among the nutrient media used for organogenesis. A high auxin level was speculated to be necessary to disrupt normal date palm development (**Eeuwens and Blake, 1977**). **Tisserat and De-Mason (1980)** confirmed that *in vitro* applications of auxins to media increase the frequency of visible expanded carpel's developing from supposedly date palm male flowers. **De-Mason and Tisserat (1980)** described the occurrence of apparent bisexual date palm flowers through a 2,4-dichlorophenoxyacetic acid (2,4-D) treatment of male flowers. They postulated however, that the staminodes in cultured pistil late flowers did not expand under used culture conditions. The apparent bisexual flowers harbored carpels without ovules. Vestigial female date carpels on surviving male flowers enlarged and became quite prominent (**Tisserat, 1979**). White friable callus usually initiated from the floral bud strand However, **Tisserat et al., (1979)** stated that in some cases, roots and embryos were initiated from date palm (**Tisserat, 1979**) found that roots have not been initiated on inflorescence rachis explants, which lack leaf or meristem tissue. Morphogenetic responses of date palm inflorescence culture were found dependent on the origin and physiological stage of the

explant (**Drira 1981**). Plantlets performance during acclimatization was determined to a large extent by the degree of autotrophy (**Kozai, 1993**). In addition, transfers of plantlets to greenhouse are depending primarily upon the quality and type of materials produced in the previous stages (**Hegazy et al. 2006 a; Hegazy, 2014**). Direct regeneration of vegetative buds minimizes the risk of somaclonal variation among regenerants. Moreover, the duration of culture period is limited by frequent renewal of the plant material. Actually, there are few laboratories that use this technique to produce date palm vitro-plants at the commercial level (**Ibrahim and Hegazy, 2001; Abahmane, 2011**). In fact, the few studies currently available in the literature indicate that *in vitro* propagation of male can be achieved from explant cultures of different date palm under particular *in vitro* culture conditions

The aim of this work was to study the availability of *in vitro* propagation to produce high quality aged date palm male clone which hasn't offshoots through direct adventitious buds proliferation on explant excised originally from very young immature spathes.

## **MATERIAL AND METHODS**

This work was carried out in the Plant Tissue Culture Department of the Genetic Engineering and Biotechnology Research Institute (GEBRI), Sadat City University during the period 2011- 2013.

### **Plant material preparation and sterilization:**

Very young invisible male spathes (5-8 cm) growing in leaf axil were separated from around 50 : 70 years- old trees of date palm male clone (Fig. 1-a), the farmers use to call any clone by the name Bora (group of trees initiated originally from one) and in our Bora, 17 aged male trees without any offshoots have high quality fertile, grown at Luxor governorate. During the winter season in November 15, invisible closed date palm spathes around 5-8 cm long, early grown in leaf axil, were separated carefully from the male trees through twisting the leaf outside to obtain the axil and dissect the spathe without any scratch to use as stock plant materials. After excision, it was immediately transferred to the laboratory. In a trial to avoid contamination, the totally closed young spathes were surface sterilized by sprayed with ethyl alcohol 70 % prior to transfer to aseptic cabinet. Once again under aseptic conditions, were sprayed with alcohol, then flamed. To minimize fast oxidative browning, cold immersion (5°C) in filter sterilized antioxidant solution containing citric and ascorbic acids (150 mg/L) each for 2 hours ( Fig. 1-b ) were used prior to dissecting. Spathes sheath removal was carried out under aseptic conditions using sterilized

scalpel to open and subsequently very young immature spikelets ( comb shape) were individually separated, shortened to single flowers (explants).

The MS basal medium (Murashige and Skooge, 1962) modified with [ casein hydrolyzed (1.0 g/L), glutamine (200 mg/L), biotin (0.5 mg/L), thiamine-HCl (10 mg/L), glycine (3.0 mg/L), Ca-pantothenate (10.0 mg/L), ascorbic acid (75 mg/L), citric acid (75 mg/L), Polyvinylpyrrolidone (PVP, 1.5 g/L), adenine sulfate (20 mg/L),  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (170 mg/L) and raised  $\text{KH}_2\text{PO}_4$  up to (220 mg/L), activated charcoal (1.5 g/L) and sucrose (40 g/L)] was used. The media pH was adjusted to 5.8 with 0.1 M KOH or 0.1 M HCl prior to gelling agent addition (agar 7.0 g/L). Media were dispensed in a glass tubes (2.5 x 20 cm & 2.5 x 25 cm Borosil ) capped with Bellco plastic caps containing 15 ml medium and autoclaved at 121°C and 1.2 Kg/cm<sup>2</sup> for 20 min.

### **Establishment of aseptic culture:**

Immature single flowers were cultured on modified MS medium (MMS) supplemented with the combination of auxin [ IAA (Indole-3- acetic acid); NOA (naphthoxy acetic acid)] and cytokinins [Kin (6-Furfurylaminopurine) & 2iP ( isopentenyladenine) were used (mg/L) ; Control (free hormone); IAA (0.1) + Kin ( 0.5 ) + 2iP (1.0); NOA (0.1) + Kin ( 0.5 ) + 2iP (1.0); IAA (0.1) + NOA (0.1) + Kin ( 0.5 ) + 2iP (1.0) and solidified with phyto- agar (7.0 g/L). Cultures were incubated for 4 months in total darkness in a growth room at 25±1°C., and recultured monthly on the same medium even without visual response. Nine test tubes (replicates) were used for each treatment

### **Adventitious buds proliferation:**

Cultures growing on the same medium and incubated in total darkness for 4 months followed by exposed to a 16-h photoperiod using ordinary fluorescent tubes with a light intensity of 1500 lux for 2 months. After the 6 months, data of survival %, adventitious buds formation %, no of adventitious buds, initial & final fresh weight (g) were recorded as well as growth value were calculated

### **Effect of some chemical compounds on adventitious buds proliferation:**

Adventitious buds produced from the best previous combination treatment were subjected to the same MMS medium with the superior growth regulators concentrations IAA (0.1 mg/L) + NOA (0.1 mg/L) + Kin ( 0.5 mg/L ) + 2iP (1.0 mg/L) in addition to :

**a- Glutathione** at the concentration of 0,10, 20, 30 (mg/L)

**b- Spermine** at the concentration of 0,50, 100, 150 (mg/L)

were used. Cultures were incubated under light with the same conditions previously mentioned. After 8 weeks, data of growth analysis i.e. no of adventitious buds, adventitious buds initial & final fresh weight (g) were recorded as well as growth value were calculated.

### **Root formation:**

Healthy shoots separated individually from the shoot clump and cultured on basal MS medium supplemented with different types of auxins at the same concentration were used; control (free hormone), IAA (0.5mg/L), IBA (0.5mg/L) and NAA (0.5mg/L). Cultures were incubated at  $25 \pm 1^\circ\text{C}$  with 16 h photoperiod (3000 lux). After 2 months, data of root formation %, number of roots, root length (cm) were recorded.

### **Acclimatization:**

Plantlets produced from rooting medium were removed from the tubes and rinsed under tap water and then the entire plantlet was completely immersed in distilled water for 2 h. Then, the plantlet roots only were immersed for 5 min in Benlate solution (0.5 %, w/v) containing 2 drops of Tween 20 as a fungicide treatment. Plantlets were individually planted with care in plastic pots (5 cm in diameter and 18 cm in length) filled with a growing mixture as follows: compost (call- val universal compost- England); compost and perlite (1:1, v/v); compost and vermiculite (1:1, v/v); compost and park chips (1:1, v/v). The plantlets were covered with transparent polyethylene sheets to raise the relative humidity around the plantlets. Potted plantlets were incubated for 30 days in acclimatization room at  $25 \pm 1^\circ\text{C}$ , relative humidity of (80-90 %) and 16 h photoperiod with a light intensity of 1500 lux. Acclimatization of plantlets was achieved through removing the plastic sheets progressively longer period each day till it totally removed after 30 days from transplanting. Plantlets were fertigated weekly with nutrient solution containing commercial fertilizer of NPK (Nitrolive, 0.5 g/L) at a ratio of 20: 20: 20. Plantlets were transferred to plastic greenhouse and were left to grow for another two months. After 3 months, all pots for each treatment were taken and the survival percentage, number of leaves/plantlet and leaf area ( $\text{cm}^2$ ) were recorded.

**Growth value:** Adventitious buds growth value were estimated according to the equation

of Ziv (1992). 
$$\text{GV} = \frac{Fw_F - Fw_i}{Fw_i} \quad \text{Where's}$$

GV = Growth value.  $Fw_F$  = Final fresh wt.  $Fw_i$  = Initial fresh wt.

**Statistical analysis:** Data were statistically analyzed by one factorial randomized complete design using the **SAS (1988)** package. The Least Significant Differences among levels of each treatment were compared using L.S.D. test at 5%, according to **Steel and Torrie (1980)**.

## Results and Discussion

### Adventitious buds proliferations:

Results in Table (1) and Fig. (1- c, d, e) showed that addition of auxin / cytokinin was found to have significantly enormous effect on buds proliferation . Results indicated that immature spikelets ( comb shape) which individually separated, shortened to single flower (explant) and cultured on Modified MS (Murashige and Skoog 1962) medium (MMS) supplemented with IAA (0.1 mg/L) + NOA (0.1 mg/L) + Kin (0.5 mg/L) + 2iP (1.0 mg/L) was recorded higher explant survival (100 %), superior in adventitious buds formation (22.22 %) and scored the highest significant numbers of adventitious buds (2.5), buds fresh weight (2.67 g) and growth value (20.23) after 4 months of incubation under dark conditions followed by 2 months under light conditions as compared with the control. In this regard, **Abul-Soad, (2011)** reported on date palm that research work has been ongoing for a few decades to use inflorescence explants in micropropagation of date palm. The way to excise the immature inflorescence without damage to the mother tree, composition of the nutrient medium for direct organ initiation have remained hindrances to this technique over recent decades inflorescence-based micropropagation holds great potential for the multiplication of recalcitrant male and female date palm individual trees and cultivars of commercial interests with limited populations. Spikelet explants are induced to produce

**Table (1): Effects of some growth regulators on adventitious buds formation of selected date palm male cultured *in vitro* under dark conditions for 4 months followed by light conditions for 2 months.**

Treatment (mg/L)	Growth parameters				
	Survival %	Adventitious buds formation		F. Wt (g)	Growth values
		%	No.		
Control	000 <sup>b</sup>	00.00 <sup>a</sup>	00.00 <sup>b</sup>	0.00 <sup>d</sup>	-1.00 <sup>d</sup>
IAA (0.1) + Kin (0.5) + 2iP (1)	100 <sup>a</sup>	00.00 <sup>a</sup>	00.00 <sup>b</sup>	1.16 <sup>c</sup>	8.67 <sup>c</sup>
NOA (0.1) + Kin (0.5) + 2iP (1)	100 <sup>a</sup>	00.00 <sup>a</sup>	00.00 <sup>b</sup>	2.12 <sup>b</sup>	18.27 <sup>b</sup>
NOA (.01) + IAA (0.1) + Kin (0.5) + 2iP (1)	100 <sup>a</sup>	22.22 <sup>a</sup>	2.50 <sup>a</sup>	2.76 <sup>a</sup>	20.23 <sup>a</sup>

Means within each column followed by the same letter are not significantly different at P= 0.05 according to the LSD test.

shining globular structures without a callus phase. Also, explants were exceptionally able to develop direct shoots. Concerning the effect of auxins / cytokins addition to the, modified MS medium on explant growth and morphogenesis, regardless of the type and the concentration. In this concern, **Ziv (1991)** stated that *in vitro* explant culturing necessitates a continuous supply of growth regulators to the culture medium. The most commonly used growth regulators to the culture medium are auxins and cytokinins supplied either singly or in combination at diverse ratios, depending on the species and the type of explant. The possibility of different hormonal receptors controlling growth and development is another new question, as well as whether different hormone types may compete for common receptor or at least operate in separate signaling pathways (**Timpte et al., 1995**).

Generally, obtained results simply showed that addition of cytokinins to the culture medium reflected positive response. In this regard, **Jiaqiang et al. (2003)** reported that cytokinin plays a critical role in plant growth and development by stimulating cell division and cell differentiation. Despite many years' research efforts, our current understanding of this hormone is still limited regarding both its biosynthesis and signaling. On the other hand, in carrot, **Tokuji and Kuriyama (2003)** reported that purine riboside, an anticytokinin, inhibited direct somatic embryogenesis, and this effect was nullified by the application of cytokinin. They added that cytokinin regulates the early stage of auxin-induced somatic embryogenesis in carrots.

### **Effect of some chemical compounds on adventitious buds proliferation:**

#### **a- Glutathione:**

Data presented in Table (2) and Fig. (1-g & h) showed that small cluster of adventitious buds ( 3 buds) cultured on MMS medium supplemented with the superior growth regulators concentrations IAA (0.1 mg/L) + NOA (0.1 mg/L) + Kin ( 0.5 mg/L ) + 2iP (1.0 mg/L) in addition to glutathione at the concentration of 20 mg/L significantly increased fresh weight (9.74 g ) and recorded higher no. of buds (13.33), and growth value (6.10) after 8 weeks of incubation as compared with the control. Similar results on somatic embryogenesis of date palm cv. Malakaby by **Hegazy et al (2009)** they found that, glutathione at the concentration of 20 mg/l recorded higher significant values of no. of embryos and embryos multiplication rate as well as higher fresh weight and growth value. Most of the inorganic nitrogen supplied in culture media is converted by plant tissues to amino acids, which are then assimilated into proteins; it should be possible to culture plants on media on in which amino acids are the only



**Table (2): Effects of glutathione concentrations on multiplication rate, fresh weight (g) and growth value of aged date palm male explant cultured *in vitro* for 8 weeks.**

Treatments	Growth character of proliferated adventitious buds		
	No.	fresh wt. (g)	Growth values
0.0	4.30 <sup>c</sup>	3.35 <sup>d</sup>	2.63 <sup>c</sup>
10	7.78 <sup>b</sup>	6.51 <sup>c</sup>	3.82 <sup>b</sup>
20	13.33 <sup>a</sup>	9.74 <sup>a</sup>	6.10 <sup>a</sup>
30	11.48 <sup>a</sup>	8.48 <sup>b</sup>	5.42 <sup>a</sup>

Means within each column followed by the same letter are not significantly different at P= 0.05 according to the LSD test.

nitrogen source. Amino acids provide plant cells with an immediately available source of nitrogen, and uptake can be much more rapid than that of inorganic nitrogen in the same medium (George, 1993). Exogenous applications of reduced glutathione (GSH) and oxidized glutathione (GSSG) promote growth of embryogenic tissue of white spruce during a 7-day subculture period. A statistically significant increase in fresh weight as well as RNA and DNA content was observed in the presence of GSH and GSSG during the last days in culture (Belmonte *et al.* 2005)

### **b- Spermine:**

Regarding the effects of polyamine (spermine ) concentrations on adventitious buds growth characters, data presented in Table (3) and Fig. (1- g & h) indicated that, cluster of adventitious buds ( 3 buds) cultured on MMS medium supplemented with the superior growth regulators concentrations IAA (0.1 mg/L) + NOA (0.1 mg/L) + Kin ( 0.5 mg/L ) + 2iP (1.0 mg/L) in addition to the polyamine (PAs); Spermine at the concentration of 100 mg/L recorded the highest significant no. of buds (32.89), higher fresh weight ( 10.50 g ) and growth value (9.56) after 8 weeks of incubation as compared with the control. However, elevated spermine concentration up to 150 mg/L has no significant effect on adventitious buds growth characters. On the other hand, control medium scored the least growth characters. Interestingly, it could be noticed that spermine at all concentrations markedly stimulated the numbers of proliferated adventitious buds and growth values this was accompanied by decreased in buds size (fresh weight) as compared with glutathione treatment. Similar results was obtained on polyamine by Hegazy (2008) who found on date palm floral buds "Selmy" that embryos cultured on modified MS medium in addition to putrescine (100 mg/L) obtained significant values of multiplication rate and growth value as

**Table (3): Effects of glutathione concentrations on multiplication rate, fresh weight (g) and growth value of aged date palm male explant cultured *in vitro* for 8 weeks.**

Treatments Sprmine (mg/L)	Growth character of proliferated adventitious buds		
	No.	fresh wt. (g)	Growth values
<b>0.0</b>	4.30 <sup>d</sup>	3.35 <sup>c</sup>	2.63 <sup>b</sup>
<b>50</b>	17.25 <sup>c</sup>	7.76 <sup>b</sup>	8.95 <sup>a</sup>
<b>100</b>	32.89 <sup>a</sup>	10.50 <sup>a</sup>	9.56 <sup>a</sup>
<b>150</b>	28.67 <sup>b</sup>	9.96 <sup>a</sup>	9.16 <sup>a</sup>

Means within each column followed by the same letter are not significantly different at p=0.05 according to the LSD test.

well as total soluble protein and PAL activity. **Hegazy and Abo shamaa, (2010)** on achieved direct date palm embryos cv. Medjool on modified MS medium contained putrescine (100 mg/L) . in addition, **Hegazy (2014)** found that propagules of date palm c.v Ekhlass cultured on MMS medium supplemented with putrescine (50 mg/L) recorded significantly the highest axillary buds multiplication rate, and growth value. In his concern, **Handa and Mattoo (2010)** reported that Biogenic amines putrescine, spermidine and spermine are ubiquitous in nature and have interested researchers because they are essential for cell division and viability, and due to a large body of their pharmacological effects on growth and development in most living cells. In addition, **Srivastava (2002)** published that, polyamines (PAs) are generally recognized as active regulators of plant growth. They are present in all cells, and their mMolar titer is responsive to physiological effects caused by many agents, such as hormones, light, and stress, but their precise mode of action in plant growth and development is still unclear.

### **Root formation:**

Regarding the effects of auxin types, data presented in Table (4) and Figure (1- i) indicated that, individual shoot clump cultured on basal MS medium supplemented with IBA (0.5 mg/L) were recorded the highest significant values of growth characters i.e. root formation (100 %), number of roots (3.66) and root length ( 5.9 cm) as compared with the control and the other studied auxin types treatments. Results are in accordance with those obtained by **Hegazy et al. (2008)** who found on individual shoot clump of date palm female inflorescence c.v Selmy cultured on basal MS medium (3/4 salts strength) supplemented with putrescine (100 mg/L) and IBA (0.5 mg/L) achieved the highest significant values of root formation %, number of roots and root length (cm) as well as PAL activity after 2 months of incubation. In addition, Srivastava (2002) who reported that, because auxin

**Table (4): Effects of different types of auxins on rooting stage of date palm male shoots culture *in vitro* for 8 weeks.**

Treatments Auxin (0.5 mg/L)	Roots Growth character		
	Formation %	No.	Length (cm)
<b>0.0</b>	22.22 <sup>b</sup>	0.67 <sup>c</sup>	0.7 <sup>c</sup>
<b>IAA</b>	33.33 <sup>b</sup>	0.89 <sup>c</sup>	0.9 <sup>c</sup>
<b>IBA</b>	100.00 <sup>a</sup>	3.66 <sup>a</sup>	5.9 <sup>a</sup>
<b>NAA</b>	44.44 <sup>b</sup>	2.10 <sup>b</sup>	2.3 <sup>b</sup>

Means within each column followed by the same letter are not significantly different at P= 0.05 according to the LSD test.

application caused a large increase in PAs content, it was suggested that auxins act through PAs to promote growth in this tissue. Similarly, IBA-induced root formation on mung bean hypocotyl cuttings was accompanied by 2 fold increase in putrescine content. Inhibition of this increase by PAs synthesis inhibitors decreased root formation. The decrease was reversed by the exogenous application of arginine or ornithine, (PAs synthesizer) suggesting that PAs are necessary for IBA- induced root formation. On the other hand, IAA (0.5 mg/L) was recorded the lowest growth characters among all auxin types tested.

### **Acclimatization:**

Data presented in Table (5) and Figure (1- J) showed that, growing mixture containing compost and perlite recorded higher plantlet survival (80 %), number of leaves/plantlet (3.77) and leaf area (15.23 cm<sup>2</sup>) as compared with those produced under the other growing mixture types. In this concern, some researchers explained the superiority of compost and perlite treatment on induction of higher plantlets survival % could be ascribed to their effects on sparring more suitable conditions for the growing roots. Perlite could hold three to four times its weight of water as well as it was most useful in increasing aeration in mixture (**Hartmann *et al.*, 1990; Hegazy *et al.*, 2006, Hegazy and Abo shamaa, 2010; Hegazy, 2014**). Compost might increase the organic matter content, which in turn improved the growing mixture physical condition in such way, increase the water holding capacity, prevented nutrients leaching and added mineral nutrients, might be a consequence of an increase in root surface area which in turn could increase water and mineral uptake from the soil. In this regard, **Picoli *et al.* (2001)** mentioned that failure of hyperhydric plants to grow when transferred to soil may often be due to malfunctioning of the leaf rather than the poor rootability. Reasons for this leaf malfunctioning are absence of epicuticular wax, stomatal abnormalities and reduced development of palisade tissue. Moreover, **Hegazy (2003)**

**Table (5): Effect of soil mixture type on growth characters of date palm male plantlets after 3 months in acclimatization stage .**

Treatments Growing mixture types	Growth character		
	Leaves no.	leaf area (cm <sup>2</sup> )	Survival % in acclimatization
Compost	2.82 <sup>b</sup>	14.17 <sup>c</sup>	40.00 <sup>b</sup>
Compost + perlite (1:1, v/v)	3.77 <sup>a</sup>	15.23 <sup>a</sup>	80.00 <sup>a</sup>
Compost + vermiculite (1:1, v/v)	3.21 <sup>ab</sup>	14.35 <sup>bc</sup>	46.67 <sup>b</sup>
Compost + park chips (1:1, v/v)	3.55 <sup>a</sup>	14.93 <sup>ab</sup>	50.00 <sup>b</sup>

Means within each column followed by the same letter are not significantly different at P= 0.05 according to the LSD test.

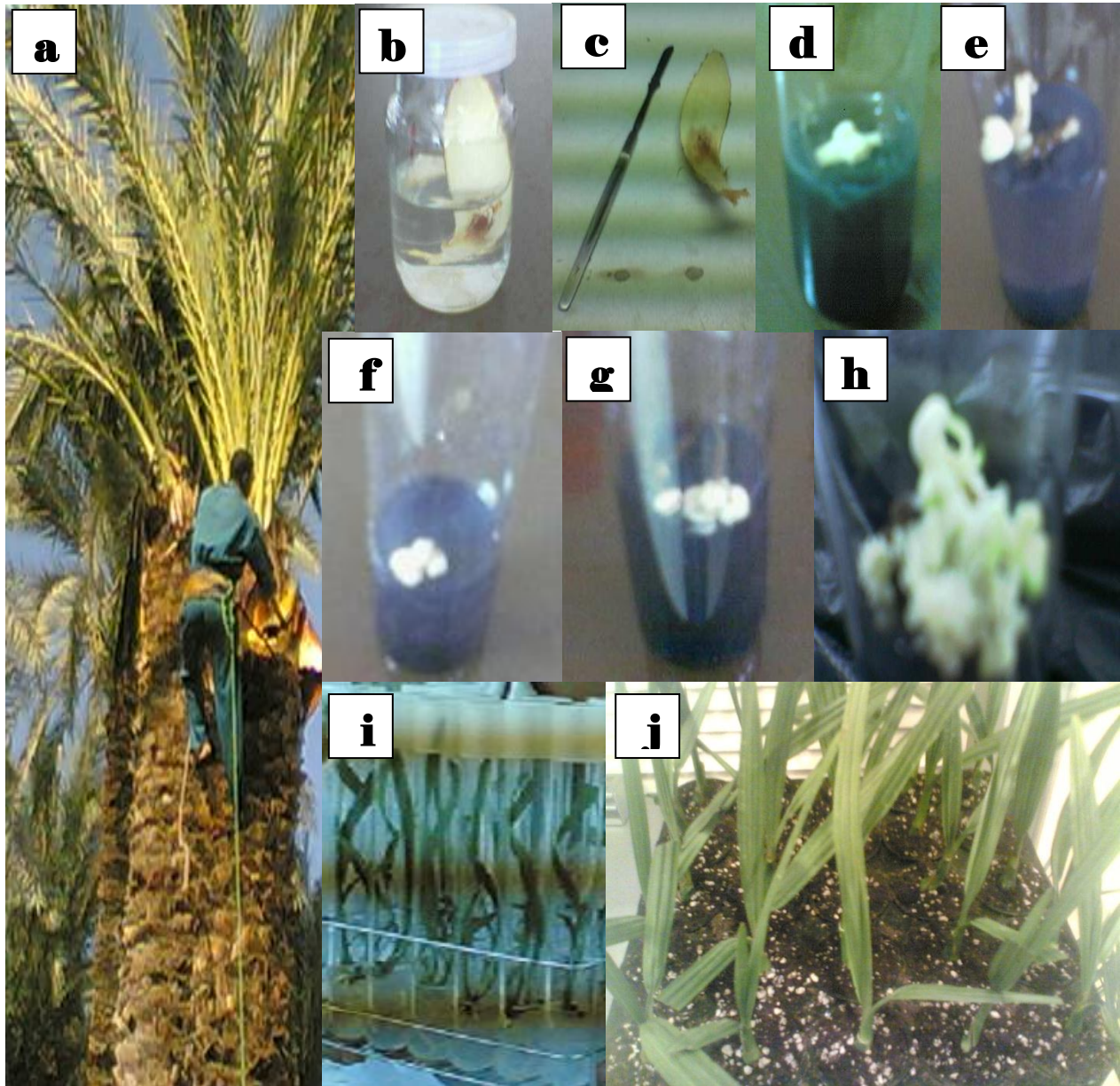
reported that soil culture represents the main source of water and nutrients for growing roots. Therefore, it must retain enough moisture, has sufficient porous so that excess water drains away, permitting adequate aeration to the roots and finally retains the nutrients in available form for plantlets to uptake. Thus, it appears that soil type consisted of compost and perlite could spare the aforementioned requirements, since perlite holds three to four times its weight of water, whereas compost may represent permanent source of available nutrients for the growing plantlets, which was reflected on the higher plantlets survival values obtained from this treatment.

### Conclusion

- Very young invisible closed male spathes of selected aged date palm (*Phoenix dactylifera* L) clone was early detected and separated from the tree according to scheduled time previously calculated . Ethyl alcohol (70 %) spraying was used to the totally closed spathe for surface sterilization then, flamed under aseptic conditions followed by cold immersion (5-8 °C) in filter sterilized antioxidant solution containing citric and ascorbic acids 150 mg/L each for 2 hours, prior to dissecting.
- Immature spikelet's ( comb shape) individually separated, shortened to single flower (explant) and cultured on Modified MS (Murashige and Skoog 1962) medium (MMS) supplemented with IAA (0.1 mg/L) + NOA (0.1 mg/L) + Kin (0.5 mg/L) + 2iP (1.0 mg/L) was recorded high in explant survival (100 %), superior in adventitious buds formation (22.22 %) and scored the highest significant numbers of adventitious buds (2.5), buds fresh weight (2.67 g) and growth value (20.23) after 4

months of incubation under dark conditions followed by 2 months under light conditions.

- Produced adventitious buds cultured on the same medium in addition to glutathione (20 mg/L) significantly increased fresh weight (9.74 g) and recorded higher no. of buds (13.33), and growth value (6.10) as compared with the control after 8 weeks of incubation.
- The polyamine (PAs); Spermine (100 mg/L) obtained the highest significant no. of buds (32.89), higher fresh weight ( 10.50 g ) and growth value (9.56) after 8 weeks of incubation.
- Individual shoot clump cultured on basal MS medium supplemented with IBA (0.5 mg/L) achieved the highest significant values of root formation (100 %), number of roots (3.66 ) and root length (5.9 cm) after 8 weeks of incubation.
- Growing mixture containing compost and perlite (1:1, v/v) recorded the highest significant number of leaves ( 3.77 ), leaf area (15.23 cm<sup>2</sup>) and plantlets survival (80 %) after three months in acclimatization.
- This work could encourage the availability to overcome somaclonal variation in date palm vitroplants since callus production was avoided.



**Fig.2.** *In vitro* propagation of selected aged date palm male clone via direct adventitious buds proliferation

**a-** Male tree during spathe isolation **b-** Male spathe after sprayed with ethyl alcohol ( 70 % ), flamed and soaking in sterilized antioxidant solution **c -** Male spathe ready for open and dissecting under aseptic conditions. **d-** Explant cultured after 3 months under dark conditions. **e, f-** Sequence of organogenesis development stage under dark conditions **g, h-** Direct adventitious bud proliferated under light conditions. **i-** Plantlets resulted from rooting stage. **j-** Healthy male plantlets after 3 months in acclimatization.

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### الملخص العربي

الإكثار المعملّي لمستعمرة ذكر نخيل تمر هرم منتخب من خلال تكون البراعم العرضية المباشرة

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هدفت الدراسة إلى إكثار مستعمرة ذكر نخيل تمر هرم منتخب من خلال تكوين البراعم العرضية المباشرة معمليا وذلك عن طريق زراعة الازهار المذكورة الغير ناضجة معمليا. تم حساب الوقت المناسب من العام مسبقا لفصل الاغريض المذكر في مرحلة ما قبل النضج وهو غير مرئي.

أدى رش الاغريض المذكر الغير ناضجة والمغلقة تماما بكحول الأيثيل 70% ثم رشها مرة أخرى في كابينة العزل المعقمة ثم اشعالها باللهب للتخلص تماما من التلوث ثم النقع المبرد في الثلجة لمدة ساعتين في محلول مفلتر من مضاد الاكسدة يحتوى على حمض الستريك وحمض الأسكوربيك (150 ملجم/لتر) الى التحكم الكامل في ظاهرة التلون البنى للنسيج خلال الزراعة والتحصين فيما بعد.

الاغريض المذكر الغير ناضجة بعد إزالة غطائها وفتحها تم تقطيعها الى ازهار مفردة لزراعتها على بيئة الزراعة أظهرت النتائج أن زراعة الازهار المفردة من الاغريض الغير ناضجة (تشبه شكل المشط) على بيئة موراشيجى وسكوج (1962) المعدلة المضاف إليها أندول حمض الخليك (0.1 ملجم/لتر) ونفثوكسى حمض الخليك (0.1 ملجم/لتر)، كايبتين (0.5 ملجم/لتر) وايزوبنتينيل ادنين (1.0 ملجم/لتر) قد حققت أعلى نسبة في البقاء حية (100%) ، وتفوقها في نسبة الاجزاء المنزرعة التي أعطت البراعم العرضية (22.22%) المباشرة وأعلى نسبة معنوية فى عدد البراعم العرضية (2.5) والوزن الطازج للبراعم (2.67 جم) وقوة النمو (20.23) بعد 4 شهور من الزراعة معمليا تحت ظروف الاظلام تبعها شهرين تحت ظروف الاضاءة.

زراعة البراعم العرضية في البيئة المعدلة مع إضافة جلوتاسيون (15 ملجم/لتر) سجلت زيادة معنوية فى الوزن الطازج (9.74 جم) وأعلى زيادة فى عدد البراعم (13.33 جم) وفي قوة النمو (6.10) بعد 8 اسابيع من الزراعة.

كما أنه بزراعة البراعم العرضية في البيئة المعدلة مع إضافة سيبرمين (100 ملجم/لتر) سجلت زيادة معنوية فى عدد البراعم العرضية المتكونة (23.89) وأعلى زيادة فى الوزن الطازج (10.5 جم) وفي قوة النمو (9.56) بعد 8 اسابيع من الزراعة.

أشارت النتائج أن استخدام بيئة الأساس لموراشيجى وسكوج (1962) مع إندول حمض البيوتريك (0.5 ملجم/لتر) فى بيئة تحتوى على آجار (7 جم/لتر) كان لها أكبر الأثر فى تحقيق أعلى نسبة للتجذير (100%) وفى عدد الجذور (3.66) وطول الجذور (5.9 سم) بعد 8 اسابيع من الزراعة.

كما أظهرت النتائج ان خلطة الزراعة المكونة من خليط من الكمبوست مع البيرليت (1:1 حجم/حجم) أعطت أعلى نسبة نجاح لنبيتات ذكور نخيل التمر في البقاء حية (80%) وكذلك أكبر عدد اوراق (3.77) ومساحة أوراق (15.23 سم<sup>2</sup>) بعد ثلاثة أشهر من الأقامة بالمقارنة بباقي التراكيب المختلفة لأنواع التربة تحت الدراسة

هذا العمل قد يشجع على إتاحة الفرصة للتغلب على التغيرات الجسمية التي تنشأ فى النبيتات الناتجة من الاكثار بالكلس.