

Micropropagation of Five Date Palm Cultivars Through *in vitro* Axillary Buds Proliferation

K.W. Al-Maarri^(*) and A. S. Al-Ghamdi^(*)

ABSTRACT

Tissue culture process of date palm propagation via bud proliferation is fully described. Adventitious plantlets were obtained from shoot tips, axillary buds and the bottom of young leaves of five date palm cultivars (*Sefri, Zahdi, Rizeez, Khalas and Hillaly*). After four to six months, sometimes more, the cultured explants gave some signs that budding was beginning. The time required to bud initiation and multiplication varied according to different cultivars. The addition of activated charcoal to the rooting medium had no beneficial effect on root quality of date palm *in vitro* produced plants. Rooting percentage and the quality of roots were improved on medium containing 1/2 Murashige and Skoog salts with high concentration of sucrose, 100 mg. L⁻¹ inositol, 0.4 mg. L⁻¹ thiamine and 0.2 mg. L⁻¹ naphthalene acetic acid. Using this new method of acclimatization increased the rate of plantlets survival. The procedures presented in this paper may offer a plausible method to produce large number of genetically uniform palms of the best date genotypes.

Key Words: Micropropagation, Date Palm, Organogenesis, Tissue culture.

Abbreviations: cv, Cultivar ; (MS) Murashige and Skoog salt ; BAP, 6-benzylaminopurine; IAA, Indole Acetic acid; IBA, indole-3-butyric acid; NAA, naphthalene acetic acid; K, kinetin; NOA, naphthoxy acetic acid; 9ip, 9(γ-γ dimethylallylamino purine; P.V.P., polyvinyl-pyrrolidone.

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Micropropagation *in vitro* de 5 Cultivars de Palmier-Dattier par Prolifération des Bourgeons Axillaires

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RESUME

La technique de multiplication, par organogénèse, de cinq cultivars du palmier dattier (*Phoenix dactylifera* L.) est décrite. Des vitro plants des cultivars: (*Sefri*, *Zahdi*, *Rizzez*, *Khalas* et *Hillaly*) ont été obtenus, à partir d'apex, de bourgeons axillaires et d'ébauches foliaires. Quatre à six mois après la mise en culture, des explants ont commencé à initier des bourgeons. Le temps nécessaire pour la formation et la multiplication des bourgeons axillaires varie selon les cultivars.

L'addition du charbon actif sur le milieu d'enracinement n'a donné aucun effet positif sur l'enracinement et sur la qualité des racines formées. Le meilleur pourcentage de rhizogénèse avec des racines de bonne qualité ont été obtenus sur milieu contenant 1/2 solution minérale de Murashige et Skoog, additionné de 100 mg. L⁻¹ inositol, 0,4 mg. L⁻¹ thiamine, 0,2 mg. L⁻¹ acid naphthalène acétique, et 20 g.L⁻¹ sucrose. Un pourcentage de reprise très élevé a été obtenu par l'utilisation d'une nouvelle technique de transfert, sous conditions naturelles.

Mots-Clés: Organogénèse, Micropropagation, Palmier-dattier, culture *in vitro*.

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INTRODUCTION

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The date palm (*Phoenix dactylifera* L) is a dioecious tree which is grown widely, particularly in the Middle East. Date palm can be propagated either by seeds or from offshoots. When plants are grown from seeds, about half of the palms turn out to be males and the other half would be female, which can be identified only at the time of flowering. Moreover, the plants obtained through seeds are genetically heterogeneous. Consequently, for uniformity of the orchards date palms cultivars should be propagated through offshoots only.

Mass production of date palm from the offshoots is not possible because of the limited number of offshoots produced during the lifetime of the palm tree (Reuveni *et al.*, ١٩٧٢; Sharma *et al.*, ١٩٨٠). However, tissue culture techniques may offer a plausible method to produce a large number of genetically uniform palms (Tisserat, ١٩٧٩).

Two main ways are useful for *in vitro* date palm plant propagation: the first one is bud proliferation with no callus formation. The second is the somatic embryogenesis from a proliferating callus.

Several reports dealing with tissue culture in palms have appeared in the literature, they are: Ammar and Benbadis, ١٩٧٧; Poulain *et al.*, ١٩٧٩; Rhiss *et al.*, ١٩٧٩; Tisserat, ١٩٧٩; Beauchesne *et al.*, ١٩٨٩; Sharma *et al.*, ١٩٨٤; Drira and Benbadis, ١٩٨٥; Al-Maarri and Al-Ghamdi, ١٩٩٥.

Somatic embryogenesis is a very important method for plant propagation, but it requires further genetic investigations to be employed with safety for true to type progeny in some monocotyledons as oil palm *Elaeis guineensis*. These methods may involve some risks of abnormality or disorder. Abnormal blooming was reported by Corley *et al.*, (١٩٨٦) on *in vitro* produced plants obtained through somatic embryogenesis with a callus phase in the oil palm. The technique of axillary bud proliferation, produces *in vitro* plants which are typical of their cultivars (Ferry *et al.*, ١٩٨٨). By using this method it is possible to obtain a great homogeneity and no more such abnormalities or disorders. One of the major obstacles to the practical application of plant tissue culture to mass production of date palm has been the difficulty of successful transfer of plantlets from *in vitro* conditions to a soil medium.

The aim of this research was to provide a detailed procedure for the propagation of plantlets *in vitro* via bud proliferation with no callus formation from several Saudi date palm cultivars.

MATERIALS and METHODS

Offshoots (۲-۳ Years old) of date palm cultivars: *Sefri*, *Khalas*, *Rezziz*, *Zahdi* and *Hillaly* were taken off from the field of the "Date Palm Reseach Center in King Faisal Uninversity" and used as a source of palnt material.

Offshoots were separated form field-grown trees. Leaves were trimmed and tied in one bundle. The basal part of the offshoot was cleaned by removing the leaf fibers. The final size of the offshoot heart is about ۲ to ۳ cm in width, and ۴ to ۶ cm in length. At this step, plant material was submerged in a chilled acqueous anti-oxidant solution containing ۱۵۰ mg ascorbic acid and ۱۰۰ mg citric acid per liter of distilled water.

Plant material was sterilized by dipping in ۲% clorox (sodium hypochlorite ۵,۲۵%) solution and ۵ drops of tween ۸۰ per liter for ۱۵ minutes, then washed three times with sterilized distilled water containing ۱۵۰ mg. L⁻¹ ascorbic acid and ۱۰۰ mg. L⁻¹ citric acid (Al-Maarri and Al-Ghamdi, ۱۹۹۵).

The offshoot heart was dissected under sterile laminar air flow hood. Terminal and lateral buds and the bottom of young leaves, mostly the axillary part between the leaves and the underlying tissue were put in cultrue. The top end of these leaves with the future folioles were cut out and discarded.

In the primary experiments, more than ۳۰ media were tested. MS(Murashige and Skoog, ۱۹۶۲) and Beaucesne (۱۹۸۹) were used. They were supplemented with growth regulators like auxins (NAA, IAA, IBA, NOA, and ۲,۴-D), cytokinins (۳ip, K and BAP). These hormones were applied either alone or in combinations. Both liquid and agar media were

tested.. Only those media in which explants responded favorably have been described in (Table ١).

The basal medium used was composed of Murashig and Skoog's (١٩٦٢) mineral solution to which were added ١٧٠ mg.L⁻¹ NaH₂PO₄·٢H₂O, ٣٠ g.L⁻¹ sucrose and ٧ g.L⁻¹ agar. The vitamins and phytohormones were added to basal medium either alone or in combination at different concentrations. The detailed composition of the media is given in Table (١) according to each step of culture process as miligrams per liter. The pH of all media was adjusted to ٥,٧ with NaOH ١N and HCl ١N, before adding the agar and autoclaving for ١٥ minutes at ١٢١ °C.

The culture process of date palm propagation via bud proliferation with no callus formation, included the five following steps: Initial culture, bud initiation, bud multiplication and elongation of shoots, rooting and transplanting of plantlets from tubes to natural conditions.

This experiment included ٥ different cultivars. Four offshoots were cultured from each cultivar. Each offshoot produced between ١٢ to ١٥ explants, eventually about ٤٨ to ٦٠ explants were produced in eachcultivar. The experiment was conducted twice for each cultivar. The explants were inoculated into test tubes (١٥٠ x ٢٤ mm) with ١٧ ml of medium (A) in the dark. They were subcultured twice on the same medium at monthly intervals. They were then transfered to medium (B), under a photoperiod of ١٦ h at ٢٥ μ mol. m⁻² s⁻¹.

Table (١) :Composition of media used in date palm multiplication.
Composition des milieux utilisés pour la multiplication du palmier-dattier.

Media Composition	Initial culture Media (A)	Bud initiation Media (B)	Bud multiplication Media (C)	Rooting Media (D)
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MS salt	MS	MS	MS	MS/γ
Sucrose (g. L ⁻¹)	३.	३.	३.	१.
Agar (g. L ⁻¹)	γ	γ	γ	γ
Thiamin HCl (mg. L ⁻¹)	१	१	१	.,ξ
Inositol (mg. L ⁻¹)	१०.	१०.	१०.	१०.
Pyridoxine (mg. L ⁻¹)	१	१	१	-
Biotin (mg. L ⁻¹)	१	१	१	-
Nicotinic acid (mg. L ⁻¹)	१	१	१	-
Glutamine HCl (mg. L ⁻¹)	१०.	१०.	१०.	-
Ca Pantothenate (mg. L ⁻¹)	१	१	१	-
Adenine sulfate (mg. L ⁻¹)	ξ०	ξ०	ξ०	-
Kinetin (mg. L ⁻¹)	-	γ	.,γ	-
BAP (mg. L ⁻¹)	-	१	.,१	-
γ ip (mg. L ⁻¹)	.,०-०.,	१	.,१	-
NAA (mg. L ⁻¹)	१.,०-०.,	१	.,१	.,γ
NOA (mg. L ⁻¹)	१.,०-०.,	१	.,१	-
IAA (mg. L ⁻¹)	१.,०-०.,	१	.,१	-
P.V.P (g. L ⁻¹)	γ.,	-	-	-

Subcultures were made at monthly intervals on the same medium (B) until the apparition of bud initiation.

The budding tissues formed on medium (B) were cut into two to three segments and subcultured on medium (C) (Table 1). Subcultures were made at monthly intervals on the same medium to obtain a continuous source of shoots. Three experiments were repeated in this stage to calculate the rate of shoot multiplication and elongation (number of shoots able to be transferred to rooting medium, γ, ० to ० cm in length formed per explant each subculture).

Shoots formed on the multiplication medium were separated and transferred to the rooting medium (D). Basal rooting medium supplemented with different concentrations of NAA (., .,γ, ., ० and १ mg. L⁻¹) were tested. The effects of different concentrations of sucrose (३., ०. and १.g. L⁻¹) and

activated charcoal were investigated. For rooting experiments, 24 experiment of the cv, "*Hillaly*" and 24 of "*Khalas*" were used per treatment. Each experiment was conducted twice. All cultures were maintained at a constant temperature 20 ± 1 °C in a growth room. The light conditions were different according to the step of culture. At the beginning, the cultures were put in darkness, they were then transferred to 16h photoperiod by fluorescent tubes at $20 \mu \text{mol. m}^{-2} \text{s}^{-1}$.

Two methods were used to acclimatize date palm *in vitro* produced plants under greenhouse conditions. In the first one; the plantlets were removed from the culture vessels, washed and transferred into small pots containing different kinds of media (perlite, peat moss, potting soil and sand). Plantlets were protected from dessication under a shaded, high humidity plastic cover. After 10 days, the plantlets were gradually exposed to a lower degree of humidity. Twelve plantlets of the cv. *Khalas* and 12 *Hillaly* were transferred to each kind of soil media.

In the second method, the plantlets were removed from the vessels, washed and transferred to beakers (200 ml) containing 100ml of distilled water, covered with big beakers, and kept in the laboratory (24 °C) at low humidity (50%). After 10 days, covers were removed, then two days later, plantlets were transplanted into small pots without covering. Different kinds of soil media (Perlite, peat moss, potting soil and mix peat moss with perlite 1:1) were used. Twelve *in vitro* produced plants of the cv. *Khalas* and 12 of *Hillaly* were transferred on each treatment. All plantlets were kept in a greenhouse at 24 °C with natural illumination.

RESULTS and DISCUSSION

In the primary experiments Beauchesne's media (1989) were used for multiplication of two cultivars *Rizez* and *Sefri*, but no good results were obtained. Consequently, some modifications in kind and concentrations of hormones were applied in more than 30 primary experiments. These

modifications permitted to determine the suitable media for all steps of culture which have been described in Table (١).

Initial Culture

The first observation after culturing the tissue was the low frequency of explant contamination. The method of surface sterilization employed was very effective in obtaining contaminant free-cultures. Within the first week of culture the contamination percentage was low, between ١٠ - ٢٠% in various cultivars. In the following weeks of culture, the browning rate of explants was ٢٥ - ٣٥%. The presence of Polyvinyl-pyrrolidone (PVP) in the media enhanced the percentage of explant survival which was between ٤٠ - ٦٠% in various cultivars (Table ٢).

Table (٢). Growth response of various date palm cultivars cultured *in vitro*.

Croissance des différents cultivars du palmier-dattier cultivés *in vitro*.

Media Cultivars	Survival explants (%)	Explant developed (%)	Time for bud initiation (Months)	Time for shoot formation (Months)	Time for plantlet production (Months)
<i>Khalas</i>	٦٠	٣٠	٤	٦	٨
<i>Rizzez</i>	٥٠	٢٠	٤	٦	٨
<i>Hillaly</i>	٥٢	٢٥	٦	٨	١٠
<i>Sefri</i>	٤٥	٢٠	٦	٨	١٠
<i>Zahdi</i>	٤٠	٢٠	٨	١٠	١٢

٤٨ explants per culture, were subcultured every four weeks, time was calculated from the date of initial culture.

Each number represents the mean of two experiments.

Browning of the tissue and medium usually results in premature death of the explant. Addition of charcoal to the nutrient media improved the survival of explants (Tisserat ١٩٧٩; Zaid and Tisserat ١٩٨٣). In our case Polyvinyl-pyrrolidone was successfully employed to combat explant browning in date palm culture; this absorbent had been used also by Rhiss *et al.*, ١٩٧٩. Moreover, using anti-oxidant solution (citric acid and ascorbic acid) with

sterile distilled water for rinsing explants after sterilization was effective to retard browning in date palm explants. Furthermore, the culture date plays a major role in the process of browning (Al-Maarri and Al-Ghamdi ١٩٩٥).

The type of tissue clearly influenced the morphogenic potential of explants cultured *in vitro*. In the case of shoot tips, within a few weeks of culture, tip derived explants initiated leaves and grew considerably.

Most of the cultured lateral buds failed to grow in culture and usually turned brown and died. A limited number of lateral buds (about ١٠ %) developed and initiated new leaves. These explants could be transferred to the rooting medium (Table ١) to regenerate plantlets. Furthermore, new adventitious buds were formed on the proximal region of the leaves issued from the initial axillary buds.

After approximately four to six weeks, the bottoms of some young leaves cultured on the initial medium gave some signs of developments. Most of them differentiated, enlarged and turned green. Some of these subcultured leaves, eventually (about ٢٠%) formed buds after ٤ to ٦ months of culture. These budding tissues were transferred to multiplication medium.

Bud Initiation

The development of explants was very slow. The explants were subcultured on the same medium (B) at monthly intervals until the apparition of bud initiation (Fig. ١). There was little difference in the initial growth response of explants in various cultivars. The positive response percentage was from ٢٠ to ٣٠% (Table ٢).

After four to six months, sometimes more, the cultured explants gave some signs that budding was starting and a clear difference was observed among cultivars in the time necessary to bud initiation. In *Khalas* cultivar, the bud initiation was observed after four months, but in *Zahdi* cultivar, the budding began after 8 months (Table 1). Our finding confirmed the observation of Beauchesne *et al.*, (1989) in other date palm cultivars.

Bud initiation was obtained from shoot tips, lateral buds and at the base of young date leaf after several months of cultures. Meanwhile, the morphogenic potential of explants was 50%, 10% and 20% respectively.

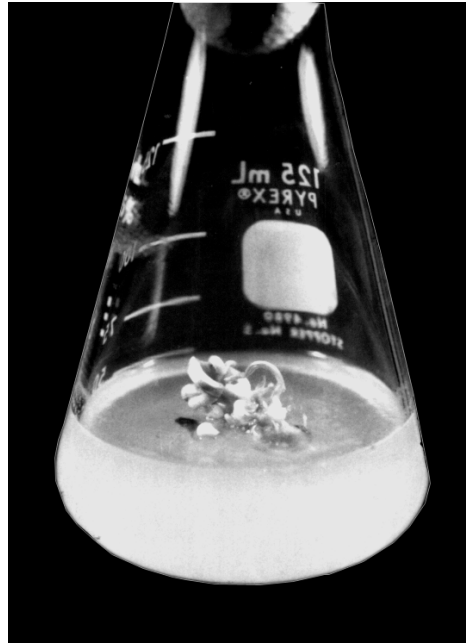


Fig. 1. *In vitro* date palm propagation: cluster of buds on medium B obtained from leaf primordia.

The shoot tip showed the best response in comparison to lateral buds and young leaves. These observations are in agreement with the finding of Tisserat (1999).

Shoots multiplication and elongation

The budding tissues formed in the second step, were transferred to the multiplication medium (C) (Table 1). After 30 to 40 days, the buds were found to form shoots, with good leaves (Fig. 2). It was necessary to separate each shoot from the others and transfer them to the rooting medium. The other parts of the tissue were put again on a multiplication medium to obtain a continuous source of shoots.

There was a clear difference in the multiplication rate of shoots in various cultivars cultured on medium (C) however, no significant differences were found between *Sefri*, *Zahdi* and *Rizzez*. Meanwhile, no clear difference in the mean of shoot elongation in various cultivars was observed. *Khalas* cultivar produced the highest shoot average and was significantly better than the other cultivars (Table ٣). The multiplication rate calculated in this study includes only the number of shoots ٢,٥ to ٥ cm in length per explant that were able to be transferred to rooting medium. Moreover, all other shoots formed less than ٢cm in length were not included in the calculation.



Fig.٢. *In vitro* date palm propagation:bud multiplication and elongation on medium C.

In these experiments it was observed that the multiplication rate was very slow because it seems that some correlative inhibitions exist between enlarged shoots and the other parts of the budding tissue. Meanwhile, the shoot elongation was obtained on the same medium. Beauchesne process includes shoot multiplication and elongation in two steps separately (Beauchesne *et al.*, ١٩٨٩).

Table (٣). Average number of shoot multiplication rate in various date palm cultivars.

Taux moyen de multiplication des différents cultivars du palmier-dattier cultivés *in vitro*.

Cultivars	Multiplication rate	Elongation Mean (cm)
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<i>Sefri</i>	1,31 a	3,30 a
<i>Hillaly</i>	1,70 b	3,20 a
<i>Khalas</i>	2,10 c	3,20 a
<i>Zahdi</i>	1,20 a	3,06 a
<i>Rizzex</i>	1,20 ab	3,16 a

12 explants were cultured on medium (C) from each cultivar in each experiment. Treatments with the same letter are not significantly different at the 5 % level (LSD test). Each number represent the mean of three experiments.

ROOTING

Shoots formed on the multiplication medium were separated and transferred to the rooting medium (D). After approximately 20 days of culture, no notable differences in the percentage of rooted shoots were observed among cultivars. Indeed, the rooting percentage was very high, (more than 80% in different cultivars) and sometimes all explants transferred were rooted.

Different NAA concentrations were investigated for their effect on adventitious root formation. The best quality of root formation was obtained with 0,2 mg.L⁻¹ of NAA, while 0,2 and 1 mg.L⁻¹ of NAA increased the callus formation and the root thickness; these factors affected root quality and decreased the percentage of *in vitro* produced plants survival which were transferred *ex vitro*.

The effect of different sucrose concentrations on rooting medium (30, 50 and 70 g.L⁻¹) was investigated on two cultivars *Khalas* and *Hillaly*. The results showed that the rooting percentage was between 80 to 90 percent in the different sucrose concentrations. Meanwhile, the appearance of rooted shoots on medium containing 70 g.L⁻¹ was healthier than shoots rooted on the other media.

The addition of activated charcoal to the rooting medium had no beneficial effect on root quality of *in vitro* produced plants. In this case, roots formed were thin and very long with no secondary root formation. Rooting percentage and root quality of explants were better on rooting medium devoid of charcoal (Fig. ٣).

The best quality of roots was obtained on medium containing 0.5 mg.L^{-1} NAA, 50 mg.L^{-1} of sucrose and without charcoal. Beauchesne process did not include rooting step, but it was very important in our process (Beauchesne *et al.*, ١٩٨٩).

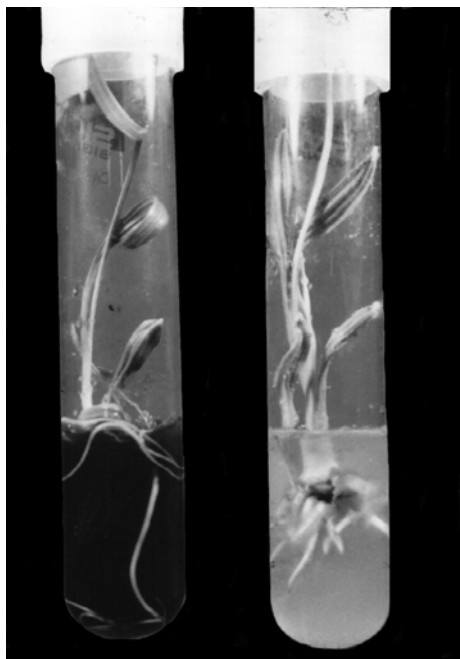


Fig.٣. *In vitro* date palm propagation: Comparison of the rooting response on medium D with or without charcoal.

Transplantation of rooted shoots

The plantlets used in this study were ٧ to ١٢ cm long with two to three leaves and good rooting system.

After one month of acclimatization, using the first method, the success percentage was very low (between ١٠ - ٢٠% in various soil used). Moreover, the experiment was repeated to improve the level of survival, but no good results were obtained.

Applying the second method of acclimatization increased the rate of survival of plantlets in natural conditions. The percentage of plantlets survival was more than 90% in various media used for transplanting (Table 4). In the meantime, the best plantlet survival was obtained from plantlets transferred to pots holding mixture of perlite and peatmoss (1:1).

This new method of acclimatization was conducted several times from *in vitro* produced plants of all multiplied cultivars and was found to be very effective. The rate of survival was more than 80% in all experiments.

Applying this new method of acclimatization, permits to obtain a high percentage of plantlets survival and solves the major obstacle to mass propagation of date palm through tissue culture, in our conditions.

Table (4). Effect of type of soil media on date palm *in vitro* produced plants acclimatization.

Effect de type du sol sur acclimatation des vitroplants de Palmier-dattier.

Soil Media	% of plantlets survival	
	<i>Khalas</i>	<i>Hillaly</i>
Perlite	92,7.	84,4.
Peatmoss	84,4.	70,0.
Potting soil	84,4.	70,0.
Perlite + peatmoss (1:1)	92,7.	92,7.

92 *in vitro* produced plants of *Khalas* and 92 of *Hillaly* were transferred of each treatment. Treatments are not significantly different at 0% level L.S.D. Test.

The process of *in vitro* culture for the date palm described in this article, was intended to avoid callus formation and somatic embryogenesis. The technique of axillary budding proliferation produce plantlets which are true to type for their variety (Beauchesne *et al.*, ١٩٨٩; Ferry *et al.*, ١٩٨٨).

A practical method of palm tissue culture was achieved by the production of plantlets via organogenesis. This study is focussed on elite date cultivars of the Al-Hassa area in Saudi Arabia. Research is still required to improve commercial application techniques and to develop more suitable media for shoot multiplication.



Fig. ٤: Date palm vitropalnt after ٣ months in the greenhouse.

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