Micropropagation of Date Palm (*Phoenix dactylifera* L.) var. Maktoom through Direct Organogenesis

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Abstract

This study aimed to determine the best combinations of plant growth regulators and other conditions in order to achieve organogenesis and multiplication directly from shoot tips of date palm (*Phoenix dactylifera* L.) var. Maktoom without callus formation so as to avoid any possibility of undesirable genetic variability. Results revealed that MS modified medium supplemented with 2.0 mg/L 2ip, 1.0 mg/L BA, 1.0 mg/L NAA and 1.0 mg/L NOA was the best for bud formation from shoot tip after 16 weeks (6.2 bud per explant). Subculturing the formed buds on liquid agitated multiplication medium supplemented with 4.0 mg/L 2ip, 2 mg/L BA, 1.0 mg/L NAA and 1.0 mg/L NOA gave the optimum average of buds number (12.6 buds). In elongation stage MS medium with 0.5 mg/L GA_3 and 0.1 mg/L NAA enhanced plantlet length to 5.3 cm. Optimum rooting percentage 90% was achieved when shoots were transferred to a medium with 1.0 mg/L NAA. The average root number after 8 weeks was 5.4 with 9.0 cm length. Rooted shoots (plantlets) were transplanted in small pots containing a mixture of peatmoss and perlite (2:1) and placed in plastic tunnels or in a greenhouse. The survival percentage was 85% after 3 months when the plants were transferred to bigger pots. These results define a successful protocol for the in vitro propagation of Maktoom cv. date palm.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is a dioecious tree and its cultivation has extended to Iraq and most Arab countries. It also occupies special significance for its distinguished economic, nutritional, esthetic, historic and social values. It is propagated traditionally by seeds or offshoots, but because of heterozygosity the plantlets produced from seeds are not identical, are lesser in quality than the mother plant, and are approximately 50 % male. Therefore, propagation by offshoots is better, but the numbers produced from the tree are limited, especially from superior and rare cultivars, so it cannot satisfy the need to establishing new groves. The use of plant tissue culture to supplement propagation by offshoots is necessary. Since the first attempts at date palm propagation by tissue culture (Shroeder, 1970; Reuveni, 1972), two methods of propagation have appeared: the first was direct organogenesis and the second was somatic embryogenesis through embryogenic callus produced from explants. Important successes were reported of direct oganogenesis by some researchers in axillary branching of shoot tips (Tisserat, 1984; Hameed, 2001). Al-Maari and Al-Ghamid (1997); Al-Khateeb et al. (2002), were successful in enhancing adventitious bud formation on shoot tips. The second method, the production of somatic embryos from embryogenic callus, has been reported by many researchers (Al–Khalifah, 2000; Al-Musawi, 2001; Al–Khayri, 2003). Despite the fact that the second method is the most commonly used in commercial plant tissue culture labs, it involves the possibility of undesirable genetic variability in the derived plants which is not apparent until the fruiting stage. The first method enables the production of plants that are genetically identical and true-to-type with the mother plant, therefore this method represents an effective means of large scale vegetative propagation of date palm. The aim of this study was to determine the best combinations of plant growth regulators and culture growth conditions to stimulate the initiation and multiplication of adventitious buds directly from shoot tips (without callus formation), to elongate these buds to shoots, to root and acclimatize the plantlets in order to transfer them successfully to soil.

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MATERIALS AND METHODS

Explants Preparation and Sterilization

Young offshoots of Maktoom cultivar (2 – 3 years old) were chosen and detached from the mother palm. Offshoots were dissected acropetaly until the shoot tips appeared. Shoot tips of 3 cm (apical meristem with soft inner leaves) were excised with immature fiber 2 cm in diameter and then applied in autioxidant solution consisting of 150 mg/L cirtic plus 100 mg/L ascorbic acid (Tisserat, 1991). Explants were sterilized in commercial bleach (sodium hypochlorite) 20 % containing eight drops of tweed – 20 as emulsifier for 20 minutes with vacuum, and rinsed three times with sterile distilled water. Then they were transferred to petri dishes and all leaf primordia were removed except two pairs surrounding the apical meristem.

Initiation Stage

The medium used in the initiation stage (Table 1) was composed of MS (Murashise and Skoog, 1962) plus the following (in mg/L): thiamine – HCl, 1.0; pyridoxine – HCl, 1.0; adenine sulfate $2H_2O$, 50; myo-inositol, 100; $NaH_2\ PO_4$. 2 H_2O, 170; glutamine, 200; sucrose, 30000 activated charcoal, 2000 and agar-agar 7000. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl, before the addition of agar. Media were dispensed into culture jars with 25 ml in each, then covered with polypropylene caps. All vials with media were autoclaved at 121°C and 1.04 kg/cm² for 15 minutes. Apical meristems were cultured into the jars aseptically in a laminar air flow cabinet and cultures were incubated in the dark to reduce phenolic secretions from the explant for one month (Fig. 1). The apical meristems were then removed and divided longitudinally into four equal segments and cultured on media of the same composition supplemented with benzyl adenine (BA), kinetin, isopentenyladenine (2ip), naphthaleneacetic acid (NAA) and naphthoxyacetic acid (NOA) (Table 1). The activated charcoal was changed by 2 g/L of polyvenypyroledone (PVP).

All cultures were incubated in a culture room under low light intensity of 1000 lux for 16 hours daily at 27 $\pm$ 1°C for four weeks. They were subculture four times at four week intervals until the buds had initiated, at which time data was recorded. There were ten replicates of each treatment.

Multiplication Stage

The formed buds were divided into small clumps, each one containing not less than three buds, and cultured on medium of the same composition except for the hormones. Depending on the initiation stage, kinetin was removed and 2ip was added in various concentrations (1.0, 2.0, 4.0, 6.0, mg/L) plus 1.0 mg/L of NAA and NOA. There were ten replicates for each treatment. Cultures were incubated under the same conditions as above. Subculture was carried out every four weeks, and data recorded after eight weeks. The physical status of the medium was evaluated when buds were transferred to liquid medium containing the best combination of plant growth regulators achieved from the multiplication stage. Stationary medium and rotating medium on an orbital shaker (40 rpm) were examined. Results were recorded after eight weeks and compared with those on solid medium.

Elongation Stage

In order to increase the shoot length, shoots were transferred to elongation medium with the same composition except the addition of GA_3 in various concentrations (0.1, 0.3, 0.5, and 1.0 mg/L) in the presence of 1 mg/L NAA with ten replicates for each treatment. Data were recorded after eight weeks, while the subculture was done every four weeks.

Rooting Stage

Resultant shoots were transferred to test tubes (one shoot / test tube) containing 25

cm³ of rooting medium consisting of MS salts and the following (in mg/L) : Thiamine HCl 0.4, myo–inositol 100, sucrose 60000 and agar 7000. The auxin NAA was added separately in different concentrations (0.1, 0.3, 0.5, and 1.0 mg/L). There were ten replicates for each treatment, and cultures were incubated in a culture room at 27 ± 1 ° C and 1000 lux light intensity for 16 hours daily. Rooting percentage, average number of roots and root length were recorded after two months of culture.

Acclimatization Stage

There were two stages of acclimatization. Firstly, the rooted shoots were taken out of the test tube and the root system was washed with tap water to remove the medium. Every washed plantlet was transferred to a new test tube containing 20 cm³ of MS salts and was incubated for two weeks at 27 ± 1°C with 3000 lux light intensity for 16 hour days.. Subsequently,all plastic covers were removed for one week in the culture room. Secondly, the plantlets were washed with distilled water and treated with fungicide (Benlet 2g / L) for 10 minutes, then transplanted into peat moss and perlite alone or into a mixture (1 : 1, 1 : 2 and 2 : 1). Plants were placed in pots with a 10 cm diameter, that were filled with a peat/perlite mixture and placed in a greenhouse or under plastic tunnels. Pots were irrigated with 1/2 strength MS salts and plastic covers were removed gradually for eight weeks. The survival of acclimatized plants was then recorded. The experimental design used in this study was a randomized complete block design (RCBD), and wherever there was a significant effect, less significant difference (LSD) was used to compare means at 5 % level of probability.

RESULTS AND DISCUSSION

Initiation Stage

It was clear that the type and concentration of cytokinin affected the response percentage as well as the formation of buds (Table 2). No response was noticed among explants cultured on media free from cytokinin (medium 2, Table 1) or those supplemented with 0.1 mg/L kinetin (medium 3, Table 1). The medium containing 2 mg/L 2ip plus 1 mg/L BA gave a better result in terms of growth response percentage (80 %) and average bud formation (6.2 bud), (Fig. 2). These results indicated the superiority of BA over other cytokinins (kinetin and 2ip) for the initiation and development of buds (Fig. 3).

Multiplication Stage

The multiplication of buds was slightly more enhanced by the addition of 2ip than BA. The average number of buds formed was 4.5 buds when 6.0 mg/L 2ip was added, which was significantly better with other concentrations, e.g. 4.2 buds with 4.0 mg/L of BA (Table 3). The highest number of the buds was 8.6 when 4.0 mg/L 2ip was combined with 2 mg/L BA and 1 mg/L NAA. This number was significantly higher than all other treatments (Fig. 4). This result shows the optimum combination of plant growth regulators for high multiplication rates. This result is consistent with other results where BA and 2ip have been used in the medium for initiation and multiplication of date palm in vitro (Al–Marri and Al–Ghamdi, 1997; Bekheet and Saker, 1998; Al- Khateet et al., 2002). For the effect of physical status of the medium, results indicated that the agitated liquid medium significantly increased the number of buds which was 12.6 in comparison with 8.6 buds in solid medium and 5.8 bud with stationary liquid medium (Table 4). This effect could be attributed to the increase of nutrient availability and uptake in the liquid medium compared to the solid medium, as well as the movement of explants which lead to gas exchange, and removal of some mineral deficiency symptoms that occur in solid medium (Pierik, 1987).

Elongation Stage

GA_3 had a positive effect on the elongation of shoots produced in the

multiplication stage (Table 5). Shoot length increased with the increasing GA_3 concentration in the medium, but some malformations were noticed at 1.0 mg/L in spite of its superiority over other treatments. The average length of shoots was 7.4 cm, but they were slender and difficult to root and transplant. Therefore, the concentration of 0.5 mg/L was considered a better treatment with an average shoot length of 5.3 cm which was significantly different from other treatments. This result directs our attention to the well-know role of gibberellins in the elongation of the plant cells (1PG SA, 1998).

Rooting Stage

Results of rooting are shown in Table 6. They indicated that the addition of NAA leads to an increase in rooting, with the concentration 0.5 mg/L resulting in the best rooting percentage (90%) and average root length (5.4cm), which were significantly different from other treatments. No reduction in root length was observed with the increasing of auxin concentration. It is known that auxins play an active role in root formation by the induction of root initials (IPGSA, 1998). Our results are not consistent with those by El-Hammady (1999) using NAA. He noticed that the average root length decreased with the increasing auxin concentration. Many researchers have mentioned the importance of NAA in the rooting of date palm shoots in vitro (Al-Maari and Al-Gamdi, 1997; Tisserat, 1984; Mater, 1990), (Fig. 5).

Acclimatization Stage

Acclimatization of plantlets derived from tissue culture confirmed the efficiency of the method used, where the transformation of rooted shoots to the MS salts solution and increasing light intensity enhanced the plantlets for photosynthesis and then changing from heterotrophic to autotrophic status. The gradual lifting of plastic covers both in the culture room and the greenhouse assisted in formation of the cuticle layer (Fig. 6) and regulation of stomatal action. In spite of the significant differences in the percentage of acclimatization successes, the mixture containing 2 peatmoss: 1 perlite was the best and gave 80% survival of acclimitaized plants (Table 7, Fig. 7). According to these results, we can say that all steps in this study were practically successful and could be used as a protocol for micropropagation of date palm, Maktoom cv.

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Tables

Table 1. Plant growth regulators used in initiation stage.

Conc. mg/L					Medium No.
NOA	NAA	2ip	Kinetin	BA	
-	-	-	-	-	1
1.0	1.0	-	-	-	2
1.0	1.0	-	1.0	-	3
1.0	1.0	-	2.0	-	4
1.0	1.0	-	3.0	-	5
1.0	1.0	1.0	-	-	6
1.0	1.0	2.0	-	-	7
1.0	1.0	3.0	-	-	8
1.0	1.0	-	-	1.0	9
1.0	1.0	-	-	2.0	10
1.0	1.0	-	-	3.0	11
1.0	1.0	-	2.0	1.0	12
1.0	1.0	2.0	1.0	-	13
1.0	1.0	2.0	-	1.0	14
1.0	1.0	1.0	1.0	1.0	15

Table 2. Effect of various concentrations of cytokinin and its interaction in the percentage of response and the number of buds formation from the apical meristem of date palm, Maktoom cv. after 16 weeks of culture.

Medium No.	Number of Buds	%Response
2	0.0	0
3	0.0	0
4	0.3	10
5	0.4	10
6	1.8	20
7	2.7	40
8	3.2	50
9	3.3	30
10	4.2	60
11	3.8	50
12	3.5	40
13	3.7	50
14	6.2	80
15	3.4	20
L .S .D	2.62	38

Table 3. Effect of BA and 2ip and its interaction on adventitious bud multiplication for Maktoom cv. after 8 weeks of culture in the presence of 1 mg/L of NAA and NOA.

2ip Mg/L BA	0.0	1.0	2.0	4.0	6.0	AVE BA
0.0	3.0	3.3	3.7	4.2	4.5	3.74
1.0	3.2	4.0	4.6	5.5	4.9	4.44
2.0	3.8	4.3	5.2	8.6	5.4	5.46
4.0	4.2	4.6	5.4	6.3	5.0	5.10
6.0	3.5	3.7	4.3	5.2	4.1	4.16
AVE 2ip	3.54	3.98	4.64	5.96	4.78	
			LSD **:** Interaction, BA = 0.2, 2ip = 0.2 =0.5			

Table 4. Effect of physical status of the medium on adventitious bud formation for Maktoom cv. after 8 weeks.

Physical status	Buds number
semi solid	8.6
Stationary liquid	5.8
Agitated liquid	12.6
LSD	0.78

Table 5. Effect of various concentrations of GA_3 on the elongation of shoots produced from Maktoom cv. after 6 weeks in presence 1 mg/L NAA.

GA_3 Conc	Shoots Length (cm)
0.0	2.23
0.1	3.12
0.3	4.58
0.5	5.30
1.0	7.42
LSD	0.34

Table 6. Effect of different concentrations of NAA in the percentage of rooting, roots number and root length after 8 weeks.

NAA Conc. mg/l	Rooting %	Average of root / shoot	)Roots length cm
0.0	20	0.3	6.2
0.1	50	2.3	8.0
0.3	80	4.4	6.8
0.5	90	5.4	4.7
1.0	70	3.0	4.1
LSD	42	0.49	0.68

Table 7. Effect of culture mixture on the percentage of acclimatization for date palm plant, Mektoom cv.

Culture mixtures	Survival **%**
peatmoss	60
perlite	50
peatmoss : perlite(1:1)	70
peatmoss: perlite(2:1)	70
peatmoss : perlite(1:2)	80
LSD	0.49

Figures

Fig. 1. Quarter of apical meristem (explant) after four weeks in the dark on hormone free medium.

Fig. 2. The development of explant to adventitious buds of Maktoom cv. on MS medium plus (mg/L) BA 1, 2ip 2, NAA, and NOA 1 after 16 weeks .

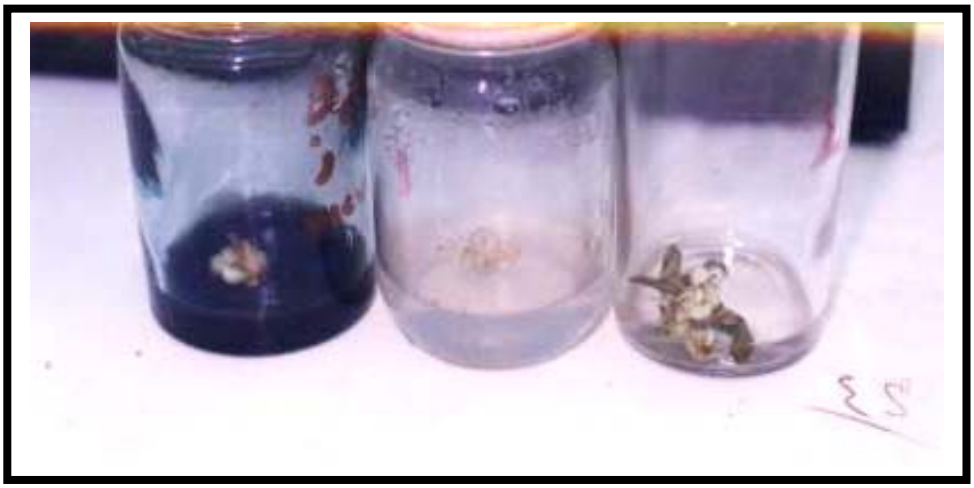

Fig. 3. Apical meristem development stages of Maktoom cv. to adventitious bud after 8, 16 and 24 weeks of culture .

Fig. 4. Bud multiplication of Maktoom cv. on agitated liquid medium supplemented with (mg/L) BA, 2, 2ip, 4, NAA and NOA 1 after 8 weeks.

Fig. 5. Stages of microprpagation of Maktoom date palm culture, from the left (initiation, multiplication, elongation and rooting) .

Fig. 6. Acclimazation of Maktoom date palm under the plastic tunnel in the greenhouse.

Fig. 7. Date palm plants in green house after 8 months after acclimatization.