Direct Organogenesis of Date Palm (*Phoenix dactylifera* L.) for Propagation of True-to-Type Plants

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### Key words
- Date palm
- Direct organogenesis
- RAPD analysis

### ABSTRACT

This study aimed to develop an efficient method for micropropagation of true-to-type date palm plants through direct organogenesis. Nodular cultures were obtained from shoot tips on MS medium plus 2 mg/l 2ip and 1 mg/l NAA. Among combinations of 2ip and 2,4-D added to culture medium for direct shoot buds proliferation, 5 mg/l 2ip alone gave the highest organogenesis frequency. For in vitro multiplication, culture medium amended with 5 mg/l 2ip + 2 mg/l Kin gave the maximum shoot bud proliferation and shoot bud length. The response of three (1, 5, and 10 mg/l) concentrations of silver nitrate in the presence of 2ip regarding shoot bud multiplication was examined. Supplementation of culture medium with 5 mg/l silver nitrate was superior to the other concentrations used. For in vitro rooting, NAA (1 mg/l) was the best for in vitro root formation in comparison with IAA or IBA at same concentration. Acclimatization was achieved by transferring the plantlets into pots contained equal volumes of peat moss and vermiculite under high humidity. Tissue cultured plantlets were subjected to assessment of genetic stability using RAPD analyses. The patterns of DNA amplification showed a very high level of genetic similarity between regenerated plants and their mother plant.

### Introduction

Date palm (*Phoenix dactylifera* L.) tree is one of the important fruit crops cultivated in arid and semi-arid regions. The tremendous advantages of the tree are its resilience, its requirement for limited inputs, its long-term productivity and its multiple purposes attributes (Bircher 1990). Due to the high degree of genetic heterozygosity and dioecious nature of date palm, sexual propagation method cannot be used for propagation the cultivars of interest in a true-to-type manner. It has to be propagated vegetatively by the offshoots which arise from the base of mother plants. This method of propagation is limited since mother plant produces 15 to 20 offshoots during palm's life, depending on the cultivar and this reduces considerably the expansion of the existing palm growers (Zaid, and de Wet 1999). One more disadvantage of this method is the spread of dangerous diseases and pests such as Bayoud disease or Red Palm Weevil which can be transported by contaminated offshoots. To satisfy the increasing demand of healthy date palm plants for national and international markets, it necessary to develop alternative methods of vegetative propagation to produce large numbers of disease-free plants from selected genotypes. The use of tissue culture is the most suitable approach for large-scale plant propagation of such recalcitrant crop. High quality and uniform planting material can be multiplied on a year-round basis under disease-free conditions anywhere irrespective of the season and weather.

The date palm micropropagation process, like other large-scale plant propagation processes, carries a number of risks. Off-types, that is, non true-to type and genetically not identical to the mother plant, may be among the resulting plants (Moghaib et al. 2011). Date palm is micropropagated by two main methods, the first is known as propagation by the somatic embryogenesis and the other is by forming auxiliary buds (organogenesis). Somatic embryogenesis method is characterized by giving in vitro plants in a comparatively, shorter time as well as high propagation ranges. But the most disadvantage of this method is the possibility of mutation and abnormalities occurrence during growing in vitro which appear in the field later on. In this respect, several attempts of date palm regeneration have been done based on somatic embryogenesis (Sharma et al. 1986; Madhuri and Shankar 1998; Bekheet et al. 2001). Conversely, the most advantage of micropropagation via organogenesis is getting in vitro plantlets highly identical in their genetic and vegetative characteristics with the mother plant (Tisserat, 1984; Abo El-Nil 1986; Aaounie 2000). The organogenesis technique consists of four steps: initiation of vegetative buds; bud multiplication; shoot elongation and rooting. The success of this technique is highly dependent on the success of the first step (Abahmane 2011). Hence, direct organogenesis presents an advantage of use of low concentrations of plant growth regulators and consequently callus-phase is avoided.
In vitro propagation of date palm from several genotypes through organogenesis has been studied using various meristematic explants including shoot tips and lateral buds (Zaid, and Tisserat 1983; Al Khateeb 2006). In Egypt, Bekheet and Saker (1998) studied the impact of growth regulators on direct and indirect shoot bud proliferation from shoot tips of Zaghloul cultivar. Likewise, an effective rapid method of in vitro multiplication of shoot buds of date palm was developed by Taha et al. (2001). Recently promising protocol for mass propagation of seven cultivars i.e., Zaghloul, Samany, Hayany, Amhat, Siwy, Selmy and Malakaby of date palm through direct organogenesis was established (Hegazy, 2008; Hegazy and Aboshama, 2010). On the other hand, the occurrence of cryptic genetic defects arising via somaclonal variation in the regenerates can seriously limit the broader utility of the micropropagation system (Salvi et al. 2001). Factors such as explants source, time of culture, number of subcultures, plant growth regulator, genotype and media composition are capable of inducing in vitro variability (Yu et al. 2008). It is, therefore, imperative to establish genetic uniformity of micropropagated plants to confirm the quality of the plantlets for its commercial utility. In this context, molecular markers have been used in the detection of variation or confirmation of genetic fidelity during micropropagation (Tyagi et al. 2007). Genetic stability in tissue-cultured date palm was examined by Random Amplified Polymorphic DNA (RAPD) analysis (Saker et al. 2000; Sanchez et al. 2003; Othmani et al. 2010). The objectives of this study are to develop an efficient method for in vitro propagation of date palm through direct organogenesis technique and assess the genetic stability of in vitro raised plantlets using RAPD analysis.

Materials and Methods

Plant material and sterilization

Young offshoots (2-3 years old) of date palm cv. Zaghloul were detached from mother palm. Outer leaves and fibrous tissues at their bases were removed gradually until exposure of the shoot tip zone. Sheathing leaf base enclosing the very young leaves of the heart of the offshoot was left in place to protect it from disinfection solutions. Shoot apices (3 cm) with small part of sub-meristematic tissues were excised under air laminar flow. The explants were taken and kept in antioxidant solution (100 mg/l ascorbic acid + 150 mg/l citric acid). Sterilization of explants was done using 70 % ethanol for 1 min and 2.5 % sodium hypochlorite for 20 min. Explants were then rinsed three times with sterile distilled water.

Initiation stage and organogenesis

External leaves were removed under complete aseptic conditions. Then shoot tips were excised and cultured on Murashige and Skoog (1962) (MS) medium supplemented with 2 mg/l 2ip + 1 mg/l NAA in addition of 100 mg/l myo-inositol, 40 mg/l adenine sulfate, 170 mg/l NaH_{2}PO_{4}, 200 mg/l H_{3}PO_{4} and, 1 mg/l thiamine HCl and 3 g/l activated charcoal (AC) (Figure 1A). Cultures were incubated in the dark condition to reduce phenolic secretions from the explants. The nodular cultures were obtained after three sub-culturing (five weeks interval) on same composition fresh medium. For direct proliferation of adventitious shoot buds, 2ip in different concentrations were added alone and in combination with 3 mg/l of 2,4-D and cultures were incubated at 16/8 light/dark day. Frequency of organogenesis and number of shoot buds per culture were registered after five weeks of culturing.

In vitro multiplication

To maximize numbers and growth of the in vitro proliferated shoot buds of date palm, bud clusters were divided into small clumps (1.5 cm height) and re-cultured on medium contained 5 mg/l of 2ip alone or combined with of BA or kin (2 mg/l for each). In another experiment, silver nitrate was examined for its potential in in vitro shoot bud multiplication of date palm cv. Zaghloul. For this propose, various concentrations (1, 5 and 10 mg/l) of silver nitrate was added to MS medium contained 5 mg/l of 2ip. In this stage, the medium was amended with 3 g/l AC and the cultures were incubated at normal light condition (16 hr photoperiod). The number of proliferated shoot buds and means of shoot buds height (cm) were recorded after five weeks of culturing in both two experiments.

In vitro rooting

The multiplied microshoots were elongated and prepared for root formation. For in vitro root formation, 1 mg/l of each IAA, IBA and NAA were added to free-AC culture medium. The elongated shootlets (5-6 cm) were individually cultured in 25 x 150 mm culture tubes containing the rooting media. The tubes were incubated at normal light condition. After eight weeks, percentages of root formation and root numbers of each treatment were recorded.

Culture conditions and statistical analysis

Tissue culture media were solidified with 0.7 % agar (initiation and multiplication stages) and 1.75 g/l gerlite (rooting stage) and they were adjusted to pH 5.8 before autoclaving at 121°C and 1.5 lb/Mp for 25 min. Cultures were normally incubated at 25 ± 2°C and 16 hr photoperiod provided by white fluorescent tubes (3000 lux light intensity). Experiments were run in completely randomized design and data were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1967).
Acclimatization

For adaptation of in vitro rooted plantlets to the free-living conditions, the healthy rooted plantlets were taken out from the culture tubes and adhering gerlile was removed in running tap water and then disinfected by soaking in benlate solution (1 g/l) for 20 min. Then plantlets were transplanted into plastic pots (10 cm diameter) containing peat moss and vermiculite (1:1). The pots were covered with clear polyethylene bags which were sprayed with water to maintain a high relative humidity. Plants were watered with distilled water. Water supply was monitored very carefully during the first month of acclimatization. Covers were gradually removed within eight weeks.

RAPD analysis

Extraction of genomic DNA

The genomic DNA of date palm was isolated using the protocol of Doyle and Doyle (1990) from both in vitro and mother plants. Small pieces (0.5 g) of leaf tissue of date palm samples were frozen in liquid nitrogen in Eppendorf tubes and homogenized in 500 L of extraction buffer (2% CTAB, 1.4 m NaCl, 20 mm EDTA pH 8.0, 100 mm Tris-HCl, pH 8.0, 0.1 m β-Mercatethanol). The extract was incubated at 60°C for 20 min. To this, 500 L phenol: chloroform: isoamyl alcohol (24:24:1) were added and mixed by vortexing for 30 sec followed by centrifugation at 10,000 xg for 5 min at room temperature and aqueous phase was transferred to another tube. This was once again extracted with 500 L chloroform: isoamyl alcohol (24:1) in Eppendorf tube. To the aqueous phase, 0.6 volume of isopropanol was added, genomic DNA was precipitated and the fibrous genomic DNA was spooled. Genomic DNA was then washed three times with 70% ethanol, vacuum dried, dissolved in TE containing 10 mg mL-1 RNase and incubated at 37°C for 30 min, followed by extraction with phenol: chloroform: isoamyl alcohol (24:24:1) and the aqueous phase was transferred to a fresh tube. Thereafter, the genomic DNA was precipitated by adding 0.3 m sodium acetate, pH 5.2 (final concentration) and 2.5 vol of ethanol and collected by centrifugation at 10,000 xg for 20 min at 4°C. The pellet was washed with 70% ethanol, vacuum dried and dissolved in TE.

PCR amplification

RAPD analysis was carried out using four oligonucleotide primer (9-10 mer) i.e., A11 (5/- TGCGACCTG -3/), A12 (5/- GAGGGTGCCG -3/), A6 (5/ CCCTACCAGC -3/), and A13 (5/ CACCTTTCCT -3/) to detect the polymorphism among the in vitro and mother plants. The amplification was carried out in 25 1 reaction volume containing DNA master mix 12.5 μl (PCR buffer, MgCl2, dNTPs, Taq DNA polymerase), primer 2 μl, template DNA 2 μl and sterilized distilled water 8.5 μl. PCR amplification was performed for 40 cycles, using UNO thermalcycler of Biometra (Germany) as follows: one cycle at 92 °C for 2 min then 40 cycles at 94°C for 30 s, 36°C for 1 min and 72°C for 30 sec (for denaturation, annealing and extension, respectively). Reaction mixture was finally incubated at 72°C for 10 min. The amplification products were analyzed by electrophoresis in 1% agarose in TBE (Tris-Borate-EDTA) buffer (pH 8.0) in presence of DNA ladder (Promega) which used as a marker with a molecular size of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. Then the gel was stained with ethidium bromide (0.2 mg /ml) and photographed under UV light. The banding patterns generated by RAPD-PCR marker analysis were compared to determine the variability of vitro and mother plants of date palm.

Results and Discussion

Direct shoot buds proliferation

In this work, organogenesis of date palm cv. Zaghlool based on the direct regeneration of vegetative buds from the cultured shoot tips was achieved. The development of shoot tips took place on 2 mg/l 2ip + 1 mg/l NAA containing medium and protrusions of white tissue swellings were noticed emerging from different places of the tissues after three subcultures (five weeks interval). For direct regeneration, the initiated nodular cultures were sub-cultured on medium contained different levels of 2ip alone or in combination with 2,4-D. Results reveal that shoot bud differentiation was observed with medium incorporated by 2ip. Organogenesis frequency was increased as increasing of 2ip in culture medium (alone or combined with 2,4-D). The highest percentages (85 %) of direct organogenesis was achieved when MS +5 mg/l 2ip was used. On this medium, shoot tips resulted average 4.50 shoot bud per culture (Figure 1B and Table 1). On the other hand, low differentiation responses was noticed and few shoot buds were produced from nodular cultures during single culture passage (five weeks) on the low concentrations of 2ip either when it was added alone or in combination with 2,4-D. It is important here to mention that the proliferated buds were able to multiplicity several times before elongation.

In vitro plant regeneration is a complex phenomenon involving different biochemical mechanisms for its progression. It is the process of the activation and regulation of certain enzymes at specific times for organogenesis (Abbasi et al. 2007). Plant growth regulators have a significant influence on shoot regeneration during the initial induction phase. The growth, differentiation and organogenesis of tissues become feasible only on the addition of one or more plant regulators to a medium. In this study, it was found that more than half number of shoot tips responded to direct shoot regeneration on medium containing 5 mg/l 2ip alone or combined with 3 mg/l 2,4-D. It is obviously noticed that the cytokinin 2ip is the main factor affecting the direct shoot bud proliferation of date palm cv. Zaghlool. Direct organogenesis presented here as proliferation of shoot buds, is usually used to produce clonal plants that are true-to-type, and therefore unorganized callus phase was avoided. There are some reports which show the parallel results with our study. Khierallah and Bader (2007)
stated that in vitro direct bud formation of date palm var. Maktoom was achieved by culturing of shoot tips for 16 weeks on MS medium supplemented with 2 mg/l 2ip, 1 mg/l BA, 1 mg/l NAA and 1 mg/l NOA. Direct organogenesis has also been successfully established from the shoot tip explants of date palm cv. Medjool by Hegazy and Aboshama (2010). They reported that axillary bud proliferation occurred under dark condition after three subcultures on MS medium supplemented with 2ip (1 mg/l), kin (1 mg/l), BA (1 mg/l) and NOA (0.5 mg/l). When these shoot buds were transferred under light condition onto the same medium containing putrescine (150 mg/l), about 55 % of them showed direct somatic embryo formation. Also, direct shoot regeneration system for date palm cv. Dhakki was reported by (Khan and Bibi 2012). Shoot tips were cultured on media supplemented with 1 mg/l NAA, 3 mg/l 2IP and 3 mg/l BA for initiation stage.

Table 1. Organogenesis frequency and number shoot buds of date palm cv. Zaghlool proliferated on MS medium contained different combinations of 2ip and 2,4-D.

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Organogenesis (%)</th>
<th>No. of shoot buds/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS + 1 mg/l 2ip.</td>
<td>10</td>
<td>1.50 ± 0.10</td>
</tr>
<tr>
<td>MS + 2 mg/l 2ip.</td>
<td>20</td>
<td>1.95 ± 0.15</td>
</tr>
<tr>
<td>MS + 3 mg/l 2ip.</td>
<td>50</td>
<td>2.30 ± 0.09</td>
</tr>
<tr>
<td>MS + 4 mg/l 2ip.</td>
<td>70</td>
<td>3.10 ± 0.20</td>
</tr>
<tr>
<td>MS + 5 mg/l 2ip.</td>
<td>85</td>
<td>4.50 ± 0.17</td>
</tr>
<tr>
<td>MS + 1 mg/l 2ip +3 mg/l 2,4-D.</td>
<td>10</td>
<td>1.20 ± 0.11</td>
</tr>
<tr>
<td>MS + 2 mg/l 2ip + 3 mg/l 2,4-D.</td>
<td>15</td>
<td>1.55 ± 0.10</td>
</tr>
<tr>
<td>MS + 3 mg/l 2ip + 3 mg/l 2,4-D.</td>
<td>30</td>
<td>2.50 ± 0.30</td>
</tr>
<tr>
<td>MS + 4 mg/l 2ip + 3 mg/l 2,4-D.</td>
<td>40</td>
<td>3.30 ± 0.19</td>
</tr>
<tr>
<td>MS + 5 mg/l 2ip + 3 mg/l 2,4-D.</td>
<td>60</td>
<td>5.50 ± 0.25</td>
</tr>
</tbody>
</table>

Mean ± SE, n= 20

Effect of growth regulators on shoot bud multiplication

In this experiment, the formed shoot buds were divided into small clumps and cultured MS medium supplemented with 5 mg/l of 2ip in combination of BA or kin (2 mg/l of each) in order to increase shoot buds proliferation. Results presented in Table 2 reveal that, both BA and kin generally enhanced in vitro shoot buds multiplication when they were combined with 5 mg/l 2ip. The maximum number of shoot buds per culture (8.10) and shoot length (3.9 cm) were recorded on 5 mg/l 2ip + 2 mg/l Kin containing medium. When shoot buds were cultured on this medium, their proliferation was very intensive (Figure 1C). With respect of the effect of BA, data showed that the medium supplemented with 5 mg/l 2ip + mg/l BA proved effective for achieving high number of shoots (6.60) with appropriate length (2.5 cm). In contrast, the culture of shoot buds on free-hormone MS medium decreases enormously its proliferation ability (Table 2).

Table 2. Multiplication of shoot buds of date palm cv. Zaghlool on MS medium containing 5 mg/l 2ip in combinations with 2 mg/l of BA or kin.

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>No. of shoot buds /culture</th>
<th>Height of shoot buds (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS hormone-free</td>
<td>1.50 ± 0.15</td>
<td>1.8 ± 0.05</td>
</tr>
<tr>
<td>MS + 5 mg/l 2ip</td>
<td>4.40 ± 0.10</td>
<td>2.0 ± 0.09</td>
</tr>
<tr>
<td>MS + 5 mg/l 2ip + 2 mg/l BA</td>
<td>6.60 ± 0.22</td>
<td>2.5 ± 0.10</td>
</tr>
<tr>
<td>MS + 5 mg/l 2ip +2 mg/l kin</td>
<td>8.10 ± 0.11</td>
<td>3.9 ± 0.12</td>
</tr>
</tbody>
</table>

Mean ± SE, n= 20

In vitro shoot buds multiplication is mainly phytohormone dependent. Cytokinins are generally known to reduce the apical meristem dominance and induce both axillary and adventitious shoots formation from meristemical explants (Madhulatha et al. 2004). Our finding in this experiment indicated that, 2ip in combinations with BA and Kin obviously enhanced shoot bud multiplication of date palm cv. Zaghlool. It is evident from the results that medium supplemented with 5 mg/l 2ip and 2 mg/l Kin revealed as the most effective combination for maximum shoot bud proliferation. This result is consistent with other results where 2ip and BA have been used for in vitro multiplication of date palm (Al-Marri and Al-Ghamdi 1997; Al-Khateeb et al. 2002). Likewise, Taha et al. (2001) achieved an effective rapid method of in vitro multiplication of shoot buds of date palm cv. Zaghlool was using medium contained high levels of 2ip. However, in a study of micropropagation of date palm cv. Sukry, Al-Khateeb (2006) reported that low hormone concentrations promoted formation of new buds while high concentrations resulted in abnormal growth without any sign of budding or shoot formation. The best combination that gave a good multiplication rate was (mg/l): Kin (0.2), 2-iP (0.1), BA (0.1), IAA (0.1), NOA (0.1) and NAA (0.1). In their work on Khals cultivar of date palm, Aslam and Khan (2009) reported that the best shoot multiplication rate was obtained with 7.84 mM of BA. The highest frequencies of shoot regeneration and number of shoots per an explant were obtained on solid MS medium as compared to liquid medium.

Effect of silver nitrate on shoot bud multiplication

Silver nitrate has proved to be a very potent inhibitor of ethylene action and is widely used in plant tissue culture. This part of study was conducted to examine the effect of silver nitrate on shoot bud multiplication of date palm cv Zaghlool grown in vitro. The response of different (1, 5 and 10 mg/l) concentrations of silver nitrate combined with 2ip regarding shoot bud multiplication is presented in Table 3. The results showed that addition of silver nitrate (1-10 mg/l) in
Likewise, formation is a critical stage in date palm micropropagation, as it governs the subsequent success of production of NAA or IBA for in vitro root formation of date palm cv. Zaghlool at 5 mg/l. Usually, to culture medium -silver nitrate- was added to establish a root system of in vitro. The establishment of and effectual root system of in vitro is vital for subsequent success throughout acclimatization to autotrophic condition. Usually in vitro grown microshoots are rooted in an auxin-enriched medium to give rise to plantlets. In this work, the individual effect of IAA, IBA and NAA on in vitro rooting of date palm cv. Zaghlool was examined. Our finding reveals that rooting of elongated microshoots was achieved upon supplementation of full-strength MS medium with 1 mg/l of each IAA, IBA or NAA. No root formation was observed on MS hormone-free medium. Using NAA was more efficient than IAA and IBA at the same concentration which gave rise 85 % rooting (Table 4). With this treatment, white and strong roots began to appear within three weeks. After five weeks of culturing, vigorous and healthy root system were developed in the absence of silver nitrate. It was notable that when concentration of silver nitrate was 1 mg/l, the adventitious bud induction rate reached the 5.50 shoot bud per culture. However, the most promotive effect of silver nitrate on shoot bud multiplication was observed at 5 mg/l which gave 6.80 shoot buds per culture and 3.30 cm shoot bud length. When the concentration of silver nitrate was increased to 10 mg/l, there was fall in number of shoot buds per explant and average shoot length.

Table 3. Multiplication of shoot buds of date palm cv. Zaghlool on MS medium containing various concentrations (5, 10 and 10 mg/l) of silver nitrate combined with 5 mg/l 2ip.

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>No. of shoot buds /culture</th>
<th>Height of shoot buds (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS + 5 mg/l 2ip</td>
<td>4.20 ± 0.10</td>
<td>2.00 ± 0.07</td>
</tr>
<tr>
<td>MS + 5 mg/l 2ip + 1 mg/l silver nitrate</td>
<td>5.50 ± 0.12</td>
<td>3.11 ± 0.11</td>
</tr>
<tr>
<td>MS + 5 mg/l 2ip + 5 mg/l silver nitrate</td>
<td>6.80 ± 0.20</td>
<td>3.20 ± 0.15</td>
</tr>
<tr>
<td>MS + 5 mg/l 2ip + 10 mg/l silver nitrate</td>
<td>6.20 ± 0.33</td>
<td>3.30 ± 0.25</td>
</tr>
</tbody>
</table>

Mean ± SE, n = 20

Silver nitrate is a potent inhibitor of ethylene action, and ethylene is considered to suppress shoot organogenesis in vitro. Silver ions in the form of nitrate, such as AgNO₃, play a major role in influencing somatic embryogenesis, shoot formation and efficient root formation (Bais et al. 2000). In the present study, we found that supplementation of culture medium with silver nitrate combined with 2ip obviously enhanced shoot bud multiplication of date palm cv. Zaghlool presented as shoot bud number and shoot bud length. The concentration of 5 mg/l of silver nitrate was the most effective in the induction of multiple shoot buds compared to other concentrations. Similar results have also been reported by Al-Khayri and Al-Bahrainy (2001). They stated that silver nitrate promoted callus proliferation and enhanced the formation and elongation of somatic embryos of date palm. They added, the action of silver nitrate was clearly modified by the addition of 2IP. The number of resultant embryos was the highest on 25 M AgNO₃ in the presence of 0.5 M 2IP. This treatment also caused maximum embryo elongation. Another study was conducted to examine the effect of silver thiosulphate (STS) and glutamine at various concentrations on direct organogenesis and shoot multiplication of date palm Barhi and Maktom cvs. (Bader and Khierallah 2009). Results obtained indicated that the addition of STS at 90 M and glutamine at 0.7 mM gave the highest number of adventitious bud formation (8.1 and 9.4 buds per explant for Barhi and Maktom respectively) after 16 weeks of culturing. In their study on in vitro culture of Zinnia angustifolia cv. Starbright, Anantasaran and Kanchanapoom (2008) elucidated that the addition of 2 mg/l silver nitrate to culture medium resulted in improvement of the regeneration frequency. Likewise, in micropropagation of pomegranate (Punica granatum L.), Patil et al. (2011) stated that nodal explants grown on MS medium containing (mg/l) 1.8 BA, 0.9 NAA, 1 silver nitrate and 30 adenine sulphate had the highest proliferation rate in multiplication stage.

In vitro rooting

The establishment of and effectual root system of in vitro is vital for subsequent success throughout acclimatization to autotrophic condition. Usually in vitro grown microshoots are rooted in an auxin-enriched medium to give rise to plantlets. In this work, the individual effect of IAA, IBA and NAA on in vitro rooting of date palm cv. Zaghlool was examined. Our finding reveals that rooting of elongated microshoots was achieved upon supplementation of full-strength MS medium with 1 mg/l of each IAA, IBA or NAA. No root formation was observed on MS hormone-free medium. Using NAA was more efficient than IAA and IBA at the same concentration which gave rise 85 % rooting (Table 4). With this treatment, white and strong roots began to appear within three weeks. After five weeks of culturing, vigorous and healthy root system were induced (Figure 1D). In addition of early response, the number of roots as well as root length was higher on 1 mg/l NAA containing medium.

Table 4. In vitro root formation on microshoots of date palm cv. Zaghlool after five weeks of culturing on MS medium containing IAA, IBA and NAA (1 mg/l of each).

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Root formation (%)</th>
<th>Number of root /shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS hormone-free medium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MS + 1 mg/l IAA</td>
<td>20</td>
<td>3.00 ± 0.15</td>
</tr>
<tr>
<td>MS + 1 mg/l IBA</td>
<td>40</td>
<td>3.50 ± 0.10</td>
</tr>
<tr>
<td>MS + 1 mg/l NAA</td>
<td>85</td>
<td>5.90 ± 0.30</td>
</tr>
</tbody>
</table>

Mean ± SE, n = 20

Root formation is a critical stage in date palm micropropagation, as it governs the subsequent success of production of free living date palm plants (Shaheen, 1990). Moreover, the continuous formation of lateral roots is a vital part of establishing a root system and enables plants to react with developmental plasticity to changing soil conditions. In the present study, NAA was found to be superior over the IBA or NAA for in vitro root formation of date palm cv. Zaghlool. Addition 1 mg/l gave the maximum percentage of root formation, numbers and length. In this context, the role of NAA as an effective auxin in root formation has been established in date palm micropropagation by several researchers. Al -Kaabi
et al. (2001) found that addition of NAA at 1 and 2 mg/l induced root on the in vitro grown date palm. However, Abo El-Nil (1986) obtained rooted plantlets from date palm shoots grown in vitro using full strength MS-medium containing 0.2 mg/l NAA. Otherwise, Belal and El-Deeb (1997) induced roots on in vitro proliferated shoots of date palm cvs. Zaghlool and Samany using MS-medium supplemented with 3 mg/l NAA + 0.5 mg/l Kin. In this connection, Sidky et al. (2007) reported that plantlets transferred onto MS medium at half strength supplemented with 0.1 mg/l of NAA, 1 g/l of AC, 40 or 50 g/l of sucrose and 4 mg/l of paclobutrazol, increased thickness of plantlets, accelerated root formation and promoted secondary root formation. On the other hand, Ibrahim (1999) reported that the concentration of inorganic salts plays an important role in root induction on in vitro grown shoots of date palm. However, Hassan et al. (2008) mentioned that sucrose at the highest concentration significantly increased root formation of date palm tissues.

**Acclimatization**

The acclimatization phase is the most important stage in the protocol of date palm micropropagation because if not optimized, the whole process will be inefficient. Factors affecting the successful production of free-living date palm, including length of plantlets, strength of root system, humidity conditions, and number of leaves and composition of the soil have been reviewed. In the present study, successful adaptation of vitro plantlets of date palm cv. Zaghlool was obtained by transplanting well rooted plantlets into pots contained equal volumes of peat moss and vermiculite under high humidity conditions (Figure 1F). The high survival and may be due to the healthy and well developed root system and the composition of transplanting medium. Similar procedure has been reported by Tisserat (1984) who elucidated that high survival rate was obtained when date palm plantlets with 2-3 foliar leaves and of shoot length greater than 10 cm (with a well-developed adventitious root system) were transplanted in pots containing a mixture of peat moss and vermiculite. However, Othmani et al. (2009) reported that rooted plantlets of date palm cv. Deglet Nour were hardened through growing in liquid medium containing half the strength of MS, coupled with incubation under high intensity illumination prior to transfer to the soil mixture.

**RAPD analysis**

In the present investigation the clonal fidelity of in vitro raised regenerants of date palm cv. Zaghlool was tested using RAPD marker. For this purpose, DNA isolated from in vitro raised plantlets was compared with those isolated from mother plant from which the explants were taken. For RAPD analysis, four primers i.e., A11, A12, A6 and A13 were screened with the DNA of the date palm samples. The patterns of DNA amplification using the four different primers are shown in Table 5 and Figure 2. The all primers generated reproducible and scorabe RAPD profiles. These produced multiple band profiles with a number of amplified DNA fragments ranging from 6 to 9. The total number of fragments produced by four primers was 31 with an average of 7.75 fragments/primer. The maximum number (9) of fragments was amplified with primer A11, while the minimum number (6) was amplified with primer A6. No polymorphism was detected with three (A11, A6 and A13) primers. However, two polymorphic fragments were observed only with primers A12. This corresponds to average level of polymorphism of 6.25%. The sizes of the polymorphic fragments were 400 and 700 bp.

<table>
<thead>
<tr>
<th>Primer code</th>
<th>Total amplicons</th>
<th>Monomorphic amplicons</th>
<th>Polymorphic amplicons</th>
<th>% polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>A11</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>A12</td>
<td>8</td>
<td>6</td>
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</tr>
<tr>
<td>A6</td>
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<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>A13</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>29</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Average</td>
<td>7.75</td>
<td>7.25</td>
<td>0.5</td>
<td>6.25</td>
</tr>
</tbody>
</table>

Table 5. Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism as revealed by RAPD markers among the vitro and mother plants of date palm cv. Zaghlool.

In clonal mass propagation, it is immensely important to maintain genetic uniformity of in vitro raised progenies. Among molecular markers, RAPD has been used as a reliable method to identify clones and cultivars and to assess somaclonal variation. In the present study, the detected 31 amplicons from the four primers showed that there were a high similarity within the micropropagated and the mother plants of date palm cv. Zaghlool. The low level of polymorphism reflects the genetic fidelity of the micropropagated plantlets and the mother plant and indicates that direct regeneration minimizes the risk of somaclonal variation among regenerants. From this study it emerges that the direct regeneration offers tremendous potential propagation true to type plants of date palm. Similar results have been reported by Othmani and de Wet (2005). In their study on micropropagation of date palm, they stated that the genetic risk is to be relatively low (1 to 3 % per regeneration cycle) for adventitious shoots and much greater (up to 10 % per regeneration cycle) for adventitious somatic embryos. Conversely, Gurevich et al. (2005) reported that a significant level of genetic variation was detected among in vitro regenerated plant of date palm cv. Madjool using Amplified fragment length polymorphism (AFLP) technique. In this context, two tissue culture methods i.e., organogenesis and somatic embryogenesis were compared for somaclonal variation in the resultant plants using AFLP analysis (Al Kaabi et al. 2005). The frequency of variation at the DNA level was low in plants regenerated through organogenesis. Furthermore, a survey of embryogenesis-derived trees in the field identified a relatively high level of morphological abnormalities. Recently, Moghaieb et al. (2011) examined the genetic similarity between the mother plants of the unknown and Ferhi cultivars and several plants regenerated in vitro.
from both cultivars by RAPD analysis using 10 random primers. The data indicate that the regenerated plants from the unknown and Ferhi cultivars showed 36.2 and 37.8% polymorphism and sharing 63.8 and 62.2% of similarities, respectively with their mother plants.

Figure 1. In vitro propagation of date palm through direct organogenesis: A) shoot apices cultured on MS medium + 2 mg/l 2ip + 1 mg/l NAA + 3 g/l activated charcoal, B) direct shoot bud proliferation using MS medium + 5 mg/l 2ip, C) shoot bud multiplication on MS medium containing 5 mg/l 2ip + 2 mg/l Kin + 3 g/l activated charcoal, D) in vitro root formation on MS medium + 1 mg/l NAA and E) adaptation of plantlets to free-living conditions.

Figure 2. RAPD profiles for vitro (left) and mother plants (right) as detected with primers A11 (1), A12 (2), A6 (3) and A13 (4). M: DNA marker.

Abbreviations
AFLP- amplified fFragment length polymorphism; BA- benzyladenin; 2, 4-D- 2,4-dichlorophenoxyacetic acid; IAA- indoleacetic acid; IBA- indolebutyric acid; 2ip- N6-(2-Isopentyl)adenine; kin- kinetin; NAA- naphthaleneacetic acid; NOA-naphthoxyacetic acid; RAPD- random amplified polymorphic DNA.

References


