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In vitro production of date palm (*Phoenix dactylifera* L.) cv. 'Barhee' plantlets through direct organogenesis

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ABSTRACT: The effects of two inducing media (MS and ½ MS) and various levels of sucrose (30 and 60 g/l), 2ip (0, 1, 2, 3, 4, 6, 8 and 12 mg/l), 2ip (1.5, 3.0, and 4.5 mg/l) and NAA (0.2, 0.5, 1.0, and 2.0 mg/l) on in vitro shoot induction, multiplication, and root formation in *Phoenix dactylifera* L. cv. 'Barhee' were investigated respectively. The highest percentage of bud formation (43.7%) was observed in the MS medium. In addition, higher vegetative buds were obtained in the presence of 30 g/l sucrose. Interestingly, as the initiation medium was supplemented with 4 mg/l 2ip, bud formation was also considerably enhanced. More vegetative buds were achieved when 1.5 mg/l 2ip was applied to the multiplication medium and the differences between 3.0 and 4.5 mg/1 were insignificant (P<5%). However, higher levels resulted in higher abnormal shoots to regenerate into the complete plantlet. On the other extreme, the highest rooting was observed in the presence of 1.0 mg/l NAA and we had a rooting increase as NAA level was aggrandized. Accordingly, bud formation, multiplication, and rooting under in vitro conditions can be improved if appropriate culture medium and precise levels of sucrose, 2ip, and NAA were selected.

Key words: date palm, direct organogenesis, true to type, bud formation, micro propagation

INTRODUCTION

Belonging to the family of Arecaceae, Date palm (*Phoenix dactylifera* L.) is an out-crossed and perennial monocotyledonous tree which possesses critical economical and ecological importance in many countries of the Middle East and North Africa (Al-Khayri 2001; Asemota *et al.* 2007). Interestingly, in the arid areas, date palm is considered to be a vital inhabitant to the ecosystem as it protects the surrounding vegetation region against desert's perilous impacts and provides adequate microclimate to the under-store crops (Haddouch 1997).

Due to many barriers like seed dormancy, low rate of germination, and progeny variation (Chand and Singh 2004), date palm is not usually propagated by seed for commercial purposes. Conventionally, the multiplication of the species is mainly performed through off-shoots. Although, since this method of propagation is vegetative, it limits the expansion of the existing palm groves (Mustafa *et al.* 2013). Moreover, off-shoot multiplication is going to be hindered by another encumbrance such as slow propagation rate, transmission of - pathogens and insects, and low number of off-shoots production for a given period in the life time of a young palm tree (Gueye *et al.*, 2009). In order to tackle such problems, tissue culture technique is to be a great help (Junaid and Khan 2009). Nowadays, embryogenesis is what by which date palm propagation through tissue culture on large scale is carried out. To construct somatic embryos on a continuous basis from callus, high concentrations of plant growth regulators should be applied, though using high levels of plant growth regulators in the media may result in somaclonal variations (Jain *et al.*, 2011).

A number of reports witness that tissue culture, based on embryogenesis, causes many abnormal plantlets. McCubbin *et al.* (2000) maintained that tissue culture of date palms had made abnormal leaves with wide leaflets, slow growth rate and development, variegated leaves, non-flowering and low fruit setting. Abnormal multi-carpel flowers and fruits with 6-7 carpels were also reported to be produced as a side effect of tissue culture (Al-Wasel, 2000; Cohen *et al.*, 2004). Based on observation of 20 trees, originated by tissue culture propagation method, and 20 trees, made from off-shoot propagation method (control), a survey conducted by Damankeshan and Panahi (2013) which showed significant difference in many treats such as plant dwarfism, excessive vegetative growth, bleached white leaves, various color leaflet, leaf black burn, off-shoot misshapenness, twisted curly inflorescence, fruit set percentage, fruit shedding percentage, total number of primary flowers, number of seeded fruits, etc.

Producing buds from explants without passing through the callus phase is the other alternative method, named direct organogenesis which is less culprit of somaclonal variation among the other production method. The organogenesis technique comprises four steps: initiation of vegetative buds, bud multiplication, shoot elongation, and rooting. The first step plays that important role in fulfillment of the process so everything in that is literary hinged on it (Abahmane 2011).

So far, mass propagation of date palm through direct organogenesis was implemented by other researchers (Al-Khateeb 2006; Khierallah and Bader 2006; Hegazy and Aboshama, 2010; Khan and Bi 2012; Bekheet 2013). Recently, Bekheet (2013) studied on direct organogenesis of date palm from shoot tips of Zaghlool cultivar.

Murashige and Skoog (1962) medium are the most commonly used ones for performing tissue culture of date palm during the past three decades (Bhansali 2010). Too many reports proved the applicability of 2iP enhancing effect on in vitro organogenesis in other cultivars of date palm and several species (Bajji *et al.* 2013; Lattier *et al.* 2013). In this study, the effects of various levels of cytokinin in organogenesis (2ip) and sucrose in two media (MS and ½ MS) on in vitro direct organogenesis of *P. dactylifera* L. cv. 'Barhee' were well scrutinized.

MATERIALS AND METHODS

A. Explants Preparation and Sterilization

In this research, Date palm (*P. dactylifera* L.) cv. 'Barhee' was used as the object plant. Donor trees were grown in the Date Palm and Tropical Fruits Research Institute of Iran. Young off-shoots of Barhee cultivar with 2-3 years of age were selected and separated from the mother palm. To be dissected and gone under further empirical studies, older leaves of off-shoots were trimmed off and then they were transferred to the laboratory. Soon after, leaf fibers were cleaned from the base and the leaf-sheaths were removed one by one from the outer ring towards the center. During carrying the specimens care was taken to discard any kind of shock or damage probability to the core soft tissues. As concomitant of young and closely attached leaves (3.0-4.0 cm in width and 6.0-8.0 cm in length), excised apical meristems were pre-sterilized in ethanol (70%) for 1 min and then in sodium hypochlorite (5%) for another 10 min under a sterile laminar air flow hood. Besides, as an antioxidant, chilled aqueous solution of citric acid (150 mg l^{-1}) and ascorbic acid (100 mg l^{-1}) were added into a sterilized bottle in which afterwards the surface sterilized explants were then transferred and treated for 10 min. explants were rinsed with sterile distilled water after every solution. Final dissection was carried out acropetally at first and ultimately from several bases at a time. Reached shoot tips were divided into four parts and then cultured.

B. Initiation stage

In order to induce bud formation, divided explants transformed to Murashing and Skoog (1962) (MS) and 1/2 MS basal medium supplemented whit thiamine HCl (1 mg/), adenine sulfate (40 mg/l), pyridoxine (1 mg/l), calcium pantothenate (1 mg/1), L-glutamine (200mg/l), activated charcoal (2 gr/1), plant agar (7 gr/l), NAA (1 mg/l), NOA (1 mg/l) and BAP (1 mg/l). The pH was adjusted to 5.7-5.8 using HCl (1 N) and NaOH (1 N). The resulted media were autoclaved at 121°C for 15 min. To trigger the conditions, the explants were incubated in honey jars for one month in darkness. Hereafter, after passing this one-month stage, all explants were transferred to lighting conditions and were exposed to the light for 16 hours per day at 24 ± 1°C. Here by, data were collected from individual explants, which constitute this experimental research, and then these individual explants were averaged. Each treatment includes 16 replications which consist of 4 honey jars, 4 explants for per each. After six consecutive months, the number and percentage of in vitro bud formation were collected recorded and analyzed through a factorial-based completely randomized design with three factorial arrangements of 3 factors (the first factor was the concentration of MS basal medium, the second one stands for the concentration of sucrose (30 and 60 gr/l), and third factor is to be the concentrations of 2iP (0, 1, 2, 3, 4, 6, 8 and 12 mg/l).

C. Proliferation and elongation

For multiplication and growing the buds, bud clusters that showed the best characteristics in the previous stage (4mg/l 2ip) (Fig. 1A) were divided into small parts, so that at least with two buds, and then transferred onto the MS medium containing NAA (1.0 mg/l), BAP (1.0 mg/l), and different levels of 2ip (1.5, 3.0 and 4.5 mg/l). The cultures were incubated at 24 ± 1°C in 16 h of photoperiod and subculture was simultaneously carried out every four weeks, and data recorded after three month. At this the elongation medium was not used anymore because buds were grown and elongated enough (Fig.1C) to be considered as capable shoots. This was analyzed in completely randomized design (CRD) with 10 replications for each treatment and 2 buds per replication. Subsequently, Statistical Analysis was done by analysis of variance (ANOVA) (Gomez and Gomez, 1984). All statistical analysis were performed using SAS software package (SAS Institute, Inc 2001).

D. In vitro rooting

Aimed at inducing root (Fig. 1F), well-developed shoots (about 7 cm with at least 2 leaves) were transferred onto the MS medium supplemented with 3 various levels of NAA (0.2, 0.5, 1.0 and 2.0 mg/l).

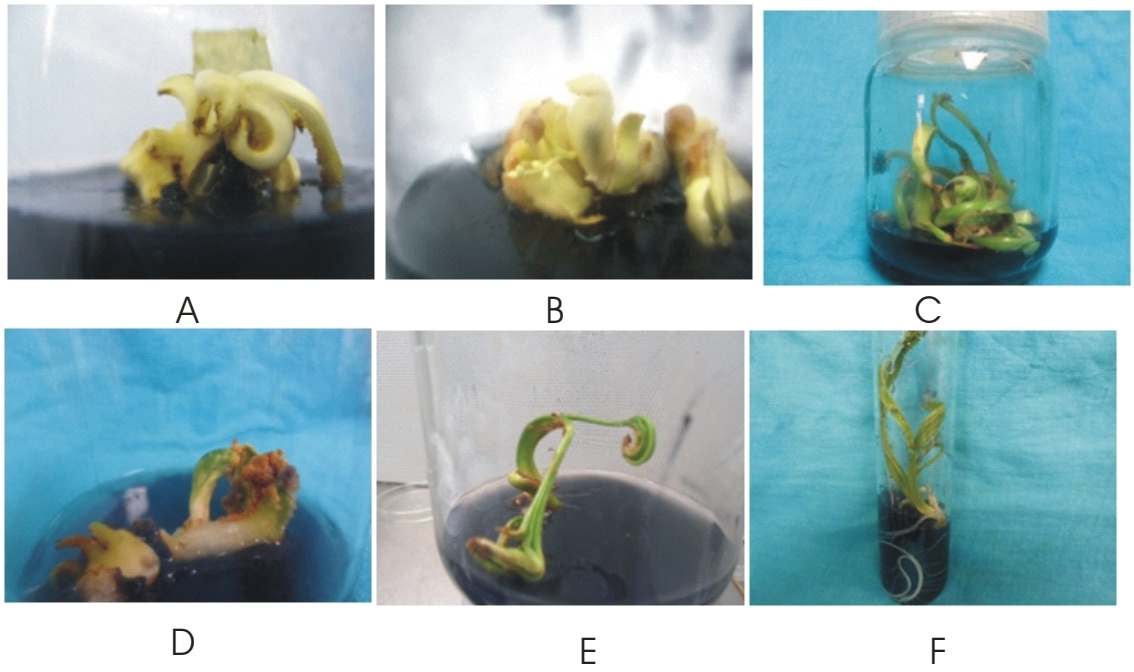

Fig. 1. The production process of date palm cv. Barhee plantlets by direct organogenesis, **A:** *in vitro* bud formation; **B:** *in vitro* flowering; **C:** bud multiplication; **D**, **E**: abnormal shoots; **F:** rooted plantlets.

Number of roots and roots length were recorded after eight week and were statistically analyzed by completely randomized design (CRD) with 11 replications and one explant per replication in test tube.

RESULTS

Bud formation was significantly affected by implementing both induction media and sucrose concentrations as well as different levels of 2ip treatment. Showing a significant different with1/2 MS medium (Table 1), MS medium is proved to manifest the highest percentage of bud formation (43.7%) and mean number of buds per explant. Also, highest number of buds (2.9 buds per explant) was formed when 30 g/l sucrose was applied to the induction medium (Table 2). Likewise, 2ip at 4 mg/l induced in vitro bud formation efficiently (Table 3).

Interestingly, the concentration of 4 mg/l of 2ip, resulting in 79.7% bud formation, is proved to be the optimal treatment for organogenesis. As for the average of the bud numbers, the concentration of 4 mg/l 2ip with an average of 6.0 buds per explant, showed the best results and had a significant difference with other concentrations (Table 3). At first with increasing the concentration of 2ip, increased organogenesis until 4 mg/l, then explants were bulkier and the organogenesis continued diminishing until the concentration of 12 mg/l 2ip, where the lowest rate (6.25%) was obtained, then gradually shifted to flowering and signs of flower formation were observed.

Table 1: Effect of culture medium on *in vitro* organogenesis (%) in *P. dactylifera* L. cv. 'Barhee'.

Medium culture	The mean number of buds per explant	Percentage of bud formation
MS	3.00 a	43.75 a
1/2MS	1.70 b	28.90 b

Results with the same letter are not statistically different.

Table 2: Effect of different concentration of sucrose on *in vitro* organogenesis (%) in *P. dactylifera* L. cv. 'Barhee'.

Concentration of Sucrose	The mean number of buds per explant	Percentage of bud formation
30 g/l	2.84 a	42.57 a
60 g/l	1.90 b	30.08 b

Results with the same letter are not statistically different.

Table 3: Effect of various levels of 2iP in combination with BAP (1mg/l) and NAA (1mg/l) on organogenesis percentage and bud number in *P. dactylifera* L. cv. 'Barhee'.

2iP Concentration mg/l	Organogenesis %	Mean squares number of buds/explant
0	15.62 d	1.06 e
1	31.25 c	2.75 c
2	45.31 b	2.56 c
3	70.31 a	3.81 b
4	79.68 a	6.00 a
6	31.25 c	1.87 d
8	10.93 d	0.69 ef
12	06.25 d	0.25 f

Results with the same letter are not statistically different.

As a result, produced inflorescence was shrunk to a flower axis which was covered with no spots. Undeveloped spots could be identified, at the bottom of the flower axis, as a small leaf. In vitro flowering was observed in the cultures treated with high levels of 2ip (12 mg/l) in high concentration of sucrose (60 g/l) (Fig. 1B).

Regenerated buds (Fig. 1A) were placed onto the MS multiplication medium. More buds were achieved (Fig. 1C) when the multiplication medium was supplemented with 1.5 mg/l 2iP and the differences between concentrations of 3.0 and 4.5 mg/1 were significant (Table 4). In addition, due to this fact that high level of 2ip treatment i.e. 4.5 mg/l results in forming abnormal leaves, so they could not be proliferated (Fig. 1D & E). In order to scrutinize all aspects of this experiment and for root formation, morphologically normal shoots were transferred onto the MS medium, containing various levels of NAA. After three months the shoots were developed enough (6.5 - 8.9 cm) to lead us to a sophisticated conclusion (Table 4).

Table 4: Effect of plant growth regulators on shoot proliferation and elongation of date palm cv. 'Barhee' after three month.

Growth regulators mg/l	Shoot number / explant	Shoot length
2iP (1.5), BAP (1), NAA (1)	6.7 a	8.90 a
2iP (3), BAP (1), NAA (1)	5.4 b	7.70 b
2iP (4.5), BAP (1), NAA (1)	4.6 b	6.50 c

Results with the same letter are not statistically different.

Table 5: Effect of different concentration of NAA on in vitro rooting in date palm cv. 'Barhee' after eight week.

NAA concentration mg/l	Rooting %	Average number of root/ shoot	Root length cm
0.2	72.7	3.2 d	11.0 a
0.5	90.9	4.1 c	8.4 b
1.0	81.8	5.8 a	7.4 b
2.0	81.8	5.0 b	5.4 c

Results with the same letter are not statistically different.

According to our results, the highest percentage of rooting (90.9 %) was achieved when 0.5 mg/l NAA mg/l was employed. The highest root number (5.8 root/shoot) was observed in the presence of 1.0 mg/l NAA and they decreased as NAA level was decreased (at 0.5 and 0.2 mg/l) or increased (2.0 mg/l). On the other hand, the higher root length was noted in the presence of 0.2 mg/l NAA; nonetheless, root length plunged as NAA level was lessened (Table 5).

DISCUSSION

For triggering organogenesis initiation, examined explants were transferred onto the MS and ½ MS media. Based upon our results, induction media significantly affected the percentage of bud formation. Since the highest percentage of bud formation (43.7%) was observed in MS medium, so our results revealed the superiority of MS medium over the other tested media. This result is consistent with Taha *et al.* (2001); they maintained that for bud propagation of date palm, the full-strength MS medium obtained more bud (8.80) than its half-strength. Working on somatic embryogenesis in date palm is also reported by other researchers (Abdolvand *et al.* 2014; Al-khateeb 2008 a). They found that in compared with full-strength MS medium, the half-strength MS medium produced higher somatic embryos. It is worthy to be noted that sucrose is used to meet the carbon needs of in vitro cultured explants. Our results also indicated that significantly higher buds were produced in the presence of 30 g/l sucrose in the induction medium. Similar results were also observed by Taha *et al.* (2001) and Al-khateeb (2008b).

Although it sounds that high levels of sucrose in culture medium could confer beneficial effects to somatic embryogenesis induction (Abdolvand *et al.* 2014; Al-khateeb 2008a), lower amounts of sucrose are required for shoot initiation and then multiplication through the direct organogenesis. On the other hand, boosting effect of high levels of sucrose (e.g. 65 g/l) on multiplication ration, elongation, and root formation from lateral bud of date palm cv. 'Sofedy' has been reported by Abdulwahed (2013) in Iraq. This discrepancy among the results may be due to the differences between genotypes of date palm.

In vitro date palm flowering is controlled by multifaceted factors such as light, photoperiod, pH of the medium, and nutrients. Several studies have also focused on the effect of plant growth regulators on in vitro flowering process in some species (Jain *et al* 2011). The significant effect of cytokinins on in vitro flowering was well-recognized and-comprehended in the literature (Wang et al 2001). The influence of BA (6-benzyladenine) or combined effect of BA with other phytohormones on early in vitro flowering has also been reported for different plant species (Hee *et al.* 2007; Jumin and Ahmad 1999; Sim *et al.* 2008; Wang *et al.* 2001; Galoch *et al.* 2002). In vitro flowering of date palm at initiation stage of organogenesis in our work, must be due to the use of high level of sucrose and cytokinins/auxins (2iP 12mg/l, BAP 1mg/l, NAA 1mg/l). Similarly, Masmoudi-Allouche *et al.* (2010) conducted an in vitro flower induction experiment on one year old date palm plantlets which was ran on basal MS medium, supplemented with sucrose (50 g/l) and phytohormones (NAA: 2.68 µM , BAP: 4.44 µM, Kin: 4.64 µM, IPA: 5.28 µM).

More vegetative buds (6.7) were achieved when 1.5 mg/l 2ip was applied into the multiplication medium. According to Bekheet and Saker (1998) cytokines, like 2iP, are essential components in culture medium, which are used for production of vegetative buds, could result in cell division and inhibition of apical dominance. The MS medium is widely used for multiplication of date palm production once it is supplemented with 3-4 mg/l 2ip (Al-Khalifa and Shanavaskhan 2012). There are many reports such as Bekheet (2013) that support using MS medium, supplemented with 5 mg/l 2iP and 2 mg/l BA, on cv. Zaghlool as well as Khierallah and Bader (2006) with the emphasis on 4 mg/l 2iP, 2 mg/l BA, 1 mg/l NAA and NOA on cv. Maktoom. Lower levels are also shown to be favorable for multiplication of date palm buds. Working on in vitro propagation of date palm cv. Sukry, Al-khateeb (2006) suggested that the highest propagation was taken place in the MS medium, supplemented with 0.05 mg/l Kinetin 0.025 mg/l 2ip, BAP, IAA, NOA, and NAA. The same results were also observed by Zaid *et al.* (2006) and Aaouine (2000).

Similarly, Khan and Bi (2012) came to this conclusion that after using 3 mg/l 2iP and BAP at initiation stage, the amount of cytokinins decreased to 0.5 mg/l Kinetin and BAP respectively. Also, they showed that employing a combination of two cytokinins (BAP and Kinetin) and one auxin (NAA) in multiplication stage proved more promising for making cultures with adequate number (7.95) of shoots with sufficient lengths (8.0 cm). In present study, shoots developed enough jus after three sub cultures without needing increasing the length, as we used combination of cytokinins (2iP and BAP) with auxin (NAA).

Afterwards, in order to carry out the root formation, well-developed and morphologically normal regenerated shoots were transferred onto the MS medium, containing various levels of NAA. Root formation is a fundamental stage in micropropagation of date palm, as it confers the subsequent success of production of free living date palm plants (Shaheen 1990). Our results revealed that, the highest number of root and rooting percentage were observed in the presence of 1.0 mg/l and 0.5 mg/l NAA respectively. The results (Table 5) showed that number of roots increased with the ascending concentrations of NAA until 1.0 mg/l, then decreased when 2.0 mg/l NAA was applied into the rooting medium. Comparing the effects of various concentrations of IAA, IBA, and NAA on in vitro rooting, Bekheet (2013) noted that NAA (1 mg/l) was the best for in vitro root formation in comparison with IAA or IBA at the same concentrations. The stimulating effects of NAA treatment on rooting has also been observed in other explants. Roots efficiently developed from leaf explants of date palm on the Eeuwen's induction medium, supplemented with 5-15 mg/L NAA (Asemota *et al.* 2007). On the other hand, Tissert (1984) reported that extraordinary rooting of date palm plantlets were gained on medium supplemented with 0.1 mg/l NAA. Table 4 shows that rising levels of NAA negatively affected the root length of date palm cv. Barhee plantlets.

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