Journal of Sciences, Islamic Republic of Iran 33(2): 125 - 132 (2022) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

A 2,4-D-Free Combined Direct Organogenesis-Indirect Somatic Embryogenesis Protocol for Mass Propagation of Date Palm (*Phoenix dactylifera L.*) *cv.*Barhi

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Received: 2 November 2021 / Revised: 25 January 2022 / Accepted: 2 March 2022

Abstract

A 2,4-D-free protocol for indirect asexual embryogenesis from root explants of date palm cv. Barhi has been established. Different mixtures and concentrations of plant hormones were applied for direct root induction from juvenile leaf explants of the apical meristem in the offshoot shoot terminal. The highest percentage of induced roots was obtained on the Murashige and Skoog (MS) medium complemented with NAA (1 mg L^{-1}), NOA (3 mg L^{-1}), IBA (1 mg L^{-1}), and 2ip (0.1 mg L^{-1}). The highest percentage of induced callus, both embryogenic and non-embryogenic callus, was achieved from *in vitro*-induced roots cultured on the Murashige and Skoog medium complemented with BAP (2 mg L^{-1}) and NAA (0.1 mg L^{-1}). The highest number of somatic embryos was acquired on the Murashige and Skoog medium complemented with BAP (2 mg L^{-1}) and NAA (0.1 mg L^{-1}). Successful rooting of date palm plantlets achieved on the PGR-free ½ MS medium following three weeks. The endurance value of rooted plantlets obtained from indirect somatic embryogenesis pathway, was 70.32%. The developed protocol would be applicable for rapid and efficient mass propagation of elite cultivars of date palm with less risk of generating genetic instability.

Keywords: *Phoenix dactylifera* L., Direct organogenesis, Micropropagation, Somatic embryos, Plant growth regulator.

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most overriding crops grown mainly in desert regions of the south of Asia, North Africa and the United States. As well as nutritional value (vital minerals, vitamins, and amino acids), date fruits have many health-beneficial consequences namely antioxidant, antibacterial, antifungal, and anti-inflammatory activities, along with inhibitory effects on proliferation of human tumor cells [1].

Classical breeding methods have been used to improve date palm. However, because of the date palm's lengthy living, these approaches are often time-consuming. Conventional proliferation of date palm via offshoots has limitation, as only 10–15 offshoots can be

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produced during the life cycle of a date palm tree. In addition, genetic heterogeneity is responsible for variability in field performance of date palm seedlings [2]. Gender identification is another problem in date palm breeding, as it takes 5-7 years for a palm to reach sexual maturity [3].

The developmental process of the date palm sector mainly depends upon an efficient propagation system [4]. Biotechnological methods are able to complete the traditional breeding methods of date palm in a more rapid manner.

Cell and tissue culture is a technology which is largely utilized for quick micropropagation of various plants, including date palm. Due to its intrinsic restrictions linked to the traditional propagation of date palms utilizing offshoots or seeds, cell and tissue culture now has become a viable substitute method for large scale micropropagation of commercialized varieties [5, 6].

Decades after attempting to micropropagate date palm, scientists have found that a variety of factors, including explant origin, explant lifetime, explant dimentions, light strength and status, temperature, culture medium pH, plant hormones and the type of culture medium can affect the efficiency of date palm circumstances under the *in vitro* conditions [5-8].

Exogenously applied plant growth regulators (PGRs) have great impact on enhancing efficiency of somatic embryogenesis process. Inclusion of auxins, mainly in the form of 2,4-dichlorophenoxyacetic acid (2,4-D), to the culture is generally considered to be essential to initiate asexual embryogenesis in many species [11, 12]. This auxin however can cause epigenetic and genetic alterations in the plant cells [13, 14].

The plant growth regulator 2,4-D at 100 mg/l is generally required to produce callus in the induction step of date palm, which leads to the genetic instability. Abnormal embryo production has been repeatedly announced in various species [11]. The rate of these abnormalities are assumed to be affected by the physiological status or somaclonal variation, which can consequently affect the morphology of the resulting plants [11]. Depending on the genotype, some culture conditions in the culture medium can improve embryo quality by reducing abnormalities [11]. It is well known that 2,4-D disrupts auxin homeostasis in embryos when included in the culture medium [11]. Therefore, lower concentrations of 2,4-Dichlorophenoxyacetic acid, e.g., 0.1 mg L^{-1} is recommended to initiate asexual embryogenesis and calli production [4]. Fki *et al*. (2017) reported efficient indirect asexual embryogenesis in date palm using immature leaves explants on the MS medium accommodating little concentration of 2,4-Dichlorophenoxyacetic acid (0.1 mg L-1) [2].

Besides reducing the amount of 2,4-Dichlorophenoxyacetic acid included in the calli emergence stage, the second method is the complete elimination of 2,4-D, in which a concomitant occurrence of direct organogenesis and somatic embryogenesis methods can be observed. McCubbin and Zaid (2007) tried to introduce the conjunction of organogenesis and embryogenesis procedures for date palm propagation [15]. Mazri *et al* (2017), for instance, reported that they first produced adventitious buds through direct organogenesis on stem apical meristem explants, and then they induced callus and somatic embryogenesis on the adventitious bud explants without using 2,4-D [16]. A well planned mass micropropagation in date palm *cv*. Medjool through combined pathways of direct organogenesis and asexual embryogenesis has been announced by Mazri *et al.* (2018) [17].

One of the objectives of the present study is the complete elimination of 2,4-D by using a combination of direct root organogenesis and somatic embryogenesis methods in order to completely eliminate this genetic instability factor. For this purpose, rhizogenesis was initially induced on the juvenile leaves of the apical meristem, and then callus and somatic embryos were generated on the derived root explants. Another objective of the current study is to lay out a simple protocol for propagation of Barhi cultivar that does not require experienced technicians and is easily applicable.

Regeneration on root explants in tissue culture methods has always been an attractive and innovative method. This approach entails a number of advantages. It prevents overuse of offshoots, the production of which is very low and limited. In addition, prevents damage to the donor tree when the offshoots are separated. Furthermore, prevents the reduction of fruiting by preventing excessive consumption of inflorescences. Moreover, this method uses the explants which are disease-free and available regardless of the season. Gueye *et al.* (2009) investigated cultivation of proximal part of immature leaves explants of date palm on a medium complemented with 1 µM 1-naphtaleneacetic acid intending to initiate direct rhizogenesis [18]. They performed histological studies throughout the culture period and noted precocious cell reactivation in the pluripotent cells of the perivascular sheaths and three phases of organogenic competence acquisition, determination and morphological differentiation (morphogenesis) [18].

Materials and Methods

Plant substance and explant establishment

Juvenile leaves of the offshoots of date palm *cv*. Barhi, adjoining the apical shoot meristem, were used as initial explants for direct root regeneration. For this purpose, suckers, measuring 5–7 kg, were isolated from the mature palms and their outer leaves were gradually removed. Several cuts were made at the base until the white leaves appeared. Shoot tip region (~2-3 cm width and 4-6 cm length) was cleansed under the streaming drinking water for 30 minutes and afterwards put down in antioxidant solution consisting of ascorbic acid (140 mg L^{-1}) and citric acid (140 mg L^{-1}) for 15 min to stop browning [19]. Shoot tip tissue was then submerged in Benomyl fungicide for 15 min. Then, surface disinfection was conducted by submerging shoot tip tissue in 70% ethanol for 1 minute, accompanied by three pulses cleansing with sterile distilled water and submerging in 1.6% (w/v) sodium hypochlorite (30% v/v) for 15 minutes and finally rinsing four pulses with sterile distilled water. Under a laminar airflow workbench (JAL TAJHIZ®, JTLHC1 Model, Iran), outer leaves of sterilized shoot tip termini were removed in succession to reach immature leaves bordering the apical shoot meristem. Then, juvenile leaf explants (measuring less than1cm in length) were isolated from apical dome and cultured for direct root regeneration.

Direct root regeneration

The culture medium for direct root induction was consisted of the Murashige and Skoog medium [20] complemented with four dissimilar mixtures of PGRs depicted in Table 1. The pH of the medium was regulated to 5.8 ± 0.2 prior to prepending 6 $gL^{-1}$of plant agar (Duchefa, Netherlands). Then, culture media were autoclaved at 121°C for 15 minutes. Hormonal combinations were based on previously carried out experiments (not published) and the investigation was directed in the form of completely randomized design with three Petri dishes as replicas and one sample for each treatment. Juvenile leaf explants were cultured horizontally on the abaxial side in transparent container accommodating 50 mL of the Murashige and Skoog medium complemented with aforementioned PGRs combinations plus 30 gL^{-1} sucrose and 1.5 gL^{-1} activated charcoal. Culture vessels were then incubated in a phytotron at 24 °C with 60–65% corresponding dampness under 16/8 (light/dark) illumination. After seven weeks, the percentage of induced roots on the proximal parts of juvenile leaves was recorded. Samples of the treatment with highest regeneration rate transferred to the next step.

Induction of embryogenic and non-embryogenic calli

*Directly induced roots explan*ts were sectioned into individual pieces (measuring 2-3cm in length) and cultivated horizontally on the medium complemented with dissimilar assemblages of Benzylaminopurine (BAP) (0, 0.5, 1, 2, and 3 mg L^{-1}), Naphthalene acetic acid (NAA) (0.1 mg L^{-1}), sucrose (30 gL^{-1}) and 1.5 gL^{-1} activated charcoal. Hormonal combinations were based on previously carried out experiments (not published) and the investigation was set out in a completely randomized design with six replicas (as Petri dishes) and four samples. Culture vessels containing segmented root explants were then incubated in a phytotron at 27 ± 1 °C with 60–65% corresponding dampness under 16/8 (light/dark) illumination. Well-ordered subcultures were carried out each four weeks and percentages of induced embryogenic and non-embryogenic calli were documented at the end of the 12th week of the inquiry. Samples of the embryogenic calli of the treatment with highest regeneration rate transferred to the next step.

Indirect asexual embryogenesis

The induced embryogenic calli were individually transferred to the MS culture medium complemented with different assemblages of BAP (1, 2, and 3 mg L^{-1}),

Table 1. Effect of applied combinations and concentrations of plant growth regulators on direct root induction of date palm cv. Barhi. Juvenile leaf explants were cultured for 7 wk on the MS medium supplemented with different plant growth regulators, activated charcoal (1.5 g L^{-1}), and sucrose (30 g L^{-1}).

Plant growth regulator (mg L^{-1})							Root induction (%)	Root length (cm)
IAA	NAA	NOA	IBA	2ip	BAP	TDZ		
0	1	3	1	0.1	0	0	75.43±3.37[a]	5.00±0.37[a]
2.5	2.5	0	0	0.1	0	0	7.02±0.12[b]	0.5±0.21[b]
1	1	1	0	0	1	0.5	2.02±0.11[c]	0.2±0.11[c]
0	0	0	0.6	0	0.6	0	0.00±0.00[d]	0.00±0.00[d]

Values represent the mean± standard error from three replicate trials each with one sample. For each variable and genotype, values followed by the same letters are not significantly different at the P<0.05.

BAP 6-benzylaminopurine, *IAA* Indole-3-acetic acid, *IBA* Indole-3-butyric acid, *MS* Murashige and Skoog, *NAA* Naphthalene acetic acid, *NOA* Naphthoxyacetic acid, *TDZ* Thidiazuron, *2ip* 6-(dimethylallylamino) purine

NAA (0.1 mg L^{-1}), sucrose (30 gL^{-1}) and 1.5 gL^{-1} activated charcoal. The cultivations were preserved at 22±3 °C and 16/8 (light/dark) illumination for twelve weeks and number of induced embryos from each callus was counted at the end of culture period. Hormonal combinations were based on previously carried out experiments (not published) and experiment was a Completely randomized design with six replica (as Petri dishes) and four samples. Samples of the treatment with highest regeneration rate transferred to the next step.

Rooting and acclimatization

The induced somatic embryos were cultured on the hormone free Murashige and Skoog medium for 14 days to reach full maturation. For root induction, mature somatic embryos were individually conveyed to experimental tubes containing the half strength PGR-free Murashige and Skoog medium for three weeks. Plantlets with fully developed roots (~3-4 cm) were conveyed to glass jars carrying 50 mL of the half strength PGR-free Murashige and Skoog medium for fully establishment for 4 weeks. To remove all traces of agar, the rooted plantlets were cleansed with distilled water and then planted in disinfected peat moss : perlite (1:1) in nylon container, covered with translucent nylon cups, and kept at a greenhouse condition (24 ± 1°C, 90% relative humidity). After one week, transparent covers were removed and plantlets were conveyed to larger nylon containers including perlite: peat moss: farm soil (1: 1: 1) in a greenhouse with lower relative humidity (55%). The survival rate of plantlets was calculated after one week.

Statistical evaluation

Statistical evaluations inclusive of analysis of variance (ANOVA) and means comparison analysis, were conducted employing SAS® (SAS Institute Inc., Cary, NC) software. Assumption about the distribution and homogeneity of the variance of the errors was conducted by normality test using the software of SAS. Using Duncan's multiple range test means were compared at 5% ($P \leq 0.05$) probability level.

Results

Direct Root Regeneration on Juvenile Leaves Explants

Direct root induction of individually cultured juvenile leaves (measuring less than 1cm in length) monitored seven weeks after establishment of explants on the culture medium (Fig. 1a). Mean comparisons of

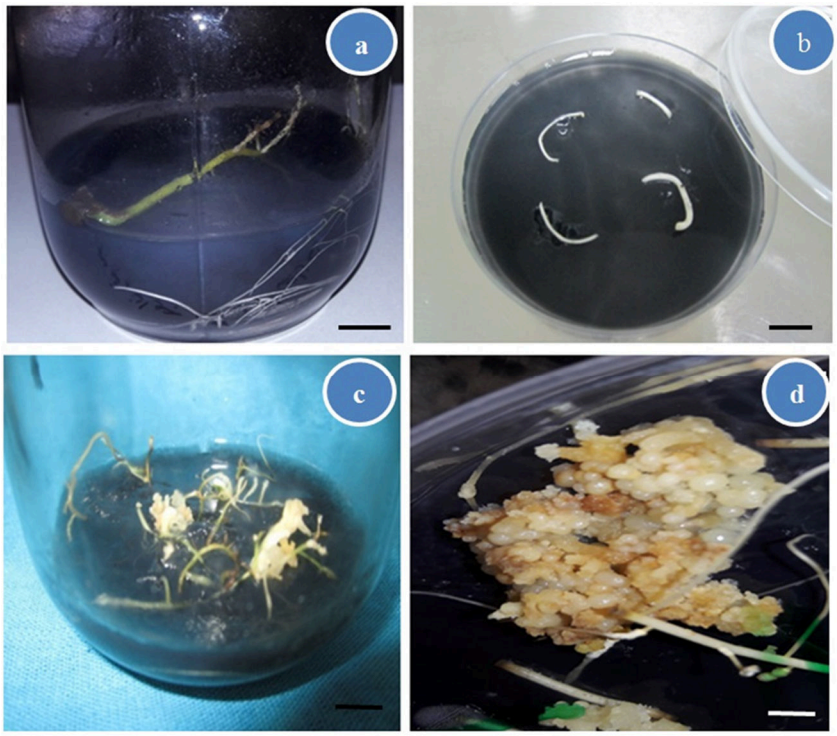

Figure 1. Indirect somatic embryogenesis of date palm cv. Barhi. **(a)** Direct root induction of juvenile leaf explant on the MS medium supplemented with different combinations and concentrations of PGRs (bar=2cm). **(b)** Establishment of *in vitro*-induced root explants on the callus induction medium (bar=2cm). **(c)** Embryogenic and non-embryogenic calli of date palm cv. Barhi (bar=2cm). **(d)** Somatic embryos of date palm cv. Barhi (bar=1cm).

induced roots revealed that there was significant difference between applied concentrations of PGRs, as the highest ratio of initiated roots observed on the Murashige and Skoog medium complimented with NAA (1 mg L^{-1}), Naphthoxyacetic acid (NOA) (3 mg L^{-1}), Indole butyric acid (IBA) (1 mg L^{-1}), and 6-(dimethylallylamino) purine (2ip) (0.1 mg L^{-1}). The Murashige and Skoog medium supplemented with 0.6 mg L^{-1} IBA and 0.6 mg L^{-1} BAP was not successful in root induction from immature leaves explants of *Phoenix dactylifera* cv. Barhi. The most exorbitant mean of length of *in vitro*-induced roots was also attributed to the Murashige and Skoog medium complimented with NAA (1 mg L^{-1}), NOA (3 mg L^{-1}), IBA (1 mg L^{-1}), and 2ip (0.1 mg L^{-1}) (Table 1).

Callogenesis on In Vitro-Induced Roots

Segments of the *in vitro*-induced root explants (measuring ~2-3 cm in length) were used for callus induction and indirect asexual embryogenesis of *Phoenix dactylifera* cv. Barhi (Fig. 1b). Callus induction was initiated 3 months after establishment of *in vitro*-induced root explants on the culture medium (Fig. 1c). Results of mean comparisons analysis revealed significant differences between applied concentrations of BAP in terms of both embryogenic and non-embryogenic calli at 5% probability level. The highest percent of embryogenic calli were perceived on the MS medium complimented with 2 mg L^{-1} BAP and 0.1 mg L^{-1} NAA; however, there was no embryogenic callus induction on the MS medium complimented with 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA. Ratio of non-embryogenic callus was more than embryogenic callus in all investigated concentrations of BAP (Table 2).

Somatic Embryogenesis

Embryogenic callus was conveyed to the Murashige and Skoog medium complimented with dissimilar concentrations of BAP (1, 2, and 3 mg L^{-1}) along with a continual concentration of NAA (0.1 mg L^{-1}). Following 12 weeks, asexual embryos were detected at the exterior of induced calli (Fig. 1d). Results of mean comparisons analysis showed that there was significant differences between applied concentrations of BAP in the matter of number and size of induced asexual embryos. The most exorbitant number of asexual embryo was perceived on the MS medium complimented with 2 mg L^{-1} BAP and 0.1 mg L^{-1} NAA, as long as lowest number of asexual embryos was obtained on the MS medium complimented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA. The most exorbitant and lowest means of dimensions of induced asexual embryos were also obtained through the application of 2 and 1 mg L^{-1} of BAP discretely (Table 3).

Rooting and Acclimatization

Maturation of induced somatic embryos occurred on the hormone free Murashige and Skoog medium after

Table 2. Effect of applied concentrations of BAP plant growth regulator on callus induction of *in vitro*-induced roots of date palm cv. Barhi. *In vitro*-induced roots were cultured for 12 wk on the MS medium supplemented with concentrations of BAP, activated charcoal (1.5 g L^{-1}), and sucrose (30 g L^{-1}).

Plant growth regulator (mg L^{-1})		Embryogenic callus (%)	Non-embryogenic callus (%)
BAP	NAA		
0	0.1	0.00±0.00^{d}	0.00±0.00^{e}
0.5	0.1	0.00±0.00^{d}	25.00±2.64^{d}
1	0.1	25.00±2.31^{b}	54.16±2.43^{b}
2	0.1	45.83±4.21^{a}	66.66±3.45^{a}
3	0.1	16.66±1.65^{c}	29.16±1.39^{c}

Values represent the mean± standard error from six replicate trials each with four samples. For each variable and genotype, values followed by the same letters are not significantly different at the P<0.05.
BAP 6-benzylaminopurine, *MS* Murashige and Skoog, *NAA* Naphthalene acetic acid

Table 3. Effect of applied concentrations of BAP plant growth regulator on somatic embryogenesis of date palm cv. Barhi. Embryogeniccalli were cultured for 12 wk on the MS medium supplemented with concentrations of BAP, activated charcoal (1.5 g L^{-1}), and sucrose (30 g L^{-1}).

Plant growth regulator (mg L^{-1})		Number of somatic embryos	Somatic embryo size (mm)
BAP	NAA		
1	0.1	3.85±0.37^{c}	0.98±0.11^{c}
2	0.1	7.85±0.75^{a}	1.93±0.13^{a}
3	0.1	4.86±0.46^{b}	1.40±0.21^{b}

Values represent the mean± standard error from six replicate trials each with four samples. For each variable and genotype, values followed by the same letters are not significantly different at the P<0.05.
BAP 6-benzylaminopurine, *MS* Murashige and Skoog, *NAA* Naphthalene acetic acid

Figure 2. Rooting and acclimatization of date palm plantlets regenerated from indirect somatic embryogenesis pathway. **(a)** Root induction of individually cultured plantlets of date palm cv. Barhi on the half strength PGR-free MS medium (bar=2cm). **(b)** A fully matured plantlet of date palm cv. Barhi on the half strength PGR-free MS medium (bar=3cm). **(c)** Hardening of *in vitro* regenerated plantlets (bar=3cm). **(d)** Acclimatized plantlet of date palm (bar=3cm).

two weeks. In the course of rooting stage, shoot length, rooting percentage, root length, and plantlet quality are crucial factors. All mature embryos isolated from callus, which were individually conveyed to experimental tubes including PGR free half strength Murashige and Skoog medium, exhibited well-developed roots (~3-4 cm) following 3 weeks (Fig. 2a). plantlets derived from somatic embryos with well-developed root system were transformed into the high quality plantlets with average root length of 81.1 mm and shoot length of 40 mm after 4 weeks in 50 ml jars containing hormone-free ½ MS medium (Fig 2b). The acclimatization rate of survived plants after cultivation in 200-ml pots (Fig. 2c) and then 8×10 cm pots (Fig. 2d) was 70.32%.

Discussion

In the experiments of tissue culture of *Phoenix dactylifera*, the implementation of auxin hormones such as 2 4-dichlorophenoxyacetic acid, which is used relatively more than other auxins, is crucial for callus initiation and somatic embryogenesis induction [21]. One of the major challenges dealing with asexual embryogenesis is the use of 2,4-dichlorophenoxyacetic acid in the early stages and one of the solutions is the 2,4-D-free method presented in this research. Studies have shown that Barhi cultivar is highly susceptible to genetic changes during micropropagation and the present protocol by removing 2,4-D, which is one of the important factors in causing genetic changes during micropropagation, has provided a suitable method for propagating this cultivar.

Indirect asexual embryogenesis is a appliance with higher capacity for widespread micropropagation of *Phoenix dactylifera*. Genetic and epigenetic modifications are the reasons of formation of abnormal somatic embryos when high accumulation of or extended display to 2,4-dichlorophenoxyacetic acid

happens in *in vitro* culture procedure [11]. In addition to optimal type and concentrations of PGRs, optimal number of sub-cultures is very important to reduce genetic instability of *in vitro* regenerated *Phoenix dactylifera* plants [22]. The aim of the present investigation was to set up a well-organized indirect somatic embryogenesis protocol in cv. Barhi of date palm without applying 2,4-D.

Micropropagation of date palm can be carried out using different tissues including root explants [23]. One of the most useful methods that has been devised for the first time in Iran without using 2,4-D is to use the roots of tissue cultured date palm plantlets as an explant, which succeeded in producing embryos and finally producing plantlets of date palms cv. Medjool by Roshanfekrrad *et al.* (2017) [23]. In their study, the roots were excised from *in vitro* plantlets produced through the indirect somatic embryogenesis using high concentrations of 2,4-D [23]. Thus it is possible to make genetic changes due to the use of 2,4-D, while the root explants of the present study where obtained without the use of 2,4-D. In the present study, date palm tissue cultured plantlets were regenerated for the first time in Iran from the roots produced *via* direct organogenesis.

Mazri *et al*.(2017) announced asexual embryogenesis from segments of the adventitious buds of *Phoenix dactylifera* cv. Najda [16]. In comparison with Mazri *et al*.(2017) which has presented a combination of direct shoot organogenesis and indirect asexual embryogenesis, the present study has presented a mixture of direct root organogenesis and indirect somatic embryogenesis. Direct shoot organogenesis is a time consuming process that requires experienced technicians, and in contrast, direct root organogenesis is a relatively short and easy process. Therefore, the present protocol can provide a quick, easy and economical way to commercialize date palm propagation. Shortening of culture time in culture medium can be an important factor in increasing the genetic stability of this sensitive cultivar. The established protocol is applicable for safe mass micropropagation of elite varieties of *Phoenix dactylifera*.

Our finding in this investigation is that in the first step of the present study, we implemented the direct organogenesis method to produce the root organ and then we turned the roots into the complete plants *via* the somatic embryogenesis method.

In the present study, BAP along with NAA led to the successful embryogenic calli induction and asexual embryogenesis in date palm cv. Barhi. The comparative proportion of auxin to cytokinin is a vital element in asexual embryo induction, proliferation and differentiation [24]. NAA is seldom capable of initiating asexual embryos in *in vitro* studies [25]; however, a low concentration of NAA along with a cytokinin can promote both shoot induction and later rooting. Zayed (2017) reported germination of well-matured somatic embryos of date palm on the Murashige and Skoog medium holding 0.1 mg L^{-1} NAA [26].

Mass transfer of *in vitro* micropropagated plantlets out of culture, with a high survival rate, is one of the key factors to establish a successful micropropagation system. An efficient acclimatization recipe with a stepwise reduction of relative humidity was introduced in the present study.

Conclusions

Because of extended life cycle and long durations of breeding programs, *in vitro* regeneration plays a principal task in large-scale micropropagation of *Phoenix dactylifera*. Asexual embryogenesis is one of the most well organized pathways for propagation of *Phoenix dactylifera*. However, auxins applied for somatic embryogenesis induction, especially 2,4-D, are the most important factors causing genetic instability. In addition, initial explant preparation from the shoot tips of young offshoots leads to the destruction of the mother plants. Combined direct organogenesis and indirect asexual embryogenesis has been registered for large scale micropropagation of *Phoenix dactylifera* cv. Barhi in the present study. In the first step, juvenile leaves (measuring less than 1cm in length) of the shoot apical meristem were used as explants for direct root induction. Then, *in vitro* induced roots were utilized as preliminary explants for indirect asexual embryogenesis.The MS medium supplemented with 1 mg L^{-1} NAA, 3 mg L^{-1} NOA, 1 mg L^{-1} IBA, 0.1 mg L^{-1} 2ip led to the largest quantity of roots. BAP (2 mg L^{-1}), with a low concentration of NAA (0.1 mg L^{-1}) in the MS medium, was the best PGR combination for both embryogenic calli initiation and somatic embryo initiation. The regenerated roots of the present study can be used to provide sufficient explants for further somatic embryogenesis studies and establish more efficient protocols through investigating other influential factors. The herein described protocol would be applicable for efficient and non-destructive propagation of valuable cultivars of date palm. Our protocol will be useful to conquer impediments encountered with the utilization of shoot apical dome explants or inflorescences and to reconstruct groves demolished by pests and diseases.

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