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Improvement of somatic embryogenesis and plant regeneration of seven date palm (*Phoenix dactylifera* L.) cultivars: Effect of cytokinins and activated charcoal

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Abstract

This study presents a procedure for the rapid development of a large number of somatic embryos and shoots from seven date palm cultivars (Barthamuda, Sakkoty, Malkaby, Shamia, Khalas, Barhee and Medjool). Clusters of leaf meristem explants were cultured on Murashige and Skoog medium supplemented with 0.1 mg/L naphthalene acetic acid and cytokinins (benzyl adenine, kinetin or 2-isopentenyl adenine) at 0.05 or 0.1 mg/L, in the presence or absence of activated charcoal. Regeneration to newly formed embryos and shoot formation was significantly ($P<0.05$) promoted in all cultivars using a culture medium with 0.05 mg/L benzyl adenine in the absence of activated charcoal. The presence of charcoal was inhibitory to shoot formation in all cases, except on the medium with 0.05 mg/L benzyl adenine, where it stimulated 33% shoot formation. This medium plus activated charcoal is therefore the recommended one for shoot formation. The number of somatic embryos and shoots generated was greatest with cultivars Barthamuda, Barhee and Sakkoty, while Medjool had the lowest number. The healthy shoots were suitable for acclimatization to form plantlets in soil.

Key words: activated charcoal, cytokinins, date palm cultivars, shoot formation, somatic embryos

Abbreviations: AC: activated charcoal, ABA: abscisic acid, BA: benzyl adenine, IBA: indol butyric acid, 2iP: 2-isopentenyl adenine, Kin: kinetin, NAA: naphthalene acetic acid

Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious fruit tree of great economic importance for the arid regions of the Middle East Asia and Northern Africa. Hundreds of different local cultivars are grown in these regions to meet the local and export requirements. The propagation through seeds does not ensure the clonal quality of the cultivars. Vegetative propagation through shoot offshoot is therefore the traditional way of propagation. This is, however, not commercially practical, because it is limited by the number of offshoots produced during the palm´s lifetime, the low planting survival rate, and the possibility of spreading diseases and pests (Zaid and De Wet, 1999). The development of tissue culture propagation methods has the potential to produce date palm plantlets rapidly and on a large scale. Organogenesis and somatic embryogenesis are two major techniques currently used worldwide to mass-propagate date palm. Successful *in vitro* development of cells and tissues depends on many factors such as cultivar, type of explant, age and developmental stage of the explant as well as the composition of media and culture conditions. Several studies have shown effects of date palm genotype and cultivar for *in vitro* propagation (El- Hadrami, 1995; Al- Khayri and Al- Bahrany, 2004) and the improved induction of date palm somatic embryos by abscisic acid (Hassan, 2007). Cytokinins are known to promote growth of plant cell cultures, induction of somatic embryos and shoot regeneration including date palm (Tisserat, 1979).

Oxidation of phenolic compounds after wounding of the explants (known as browning) is often a major obstacle for successful explant survival (Mensulai *et al.*, 1993; Abohatem *et al.*, 2011). Activated charcoal adsorbs these toxic compounds. The situation for palms is controversial. Hilae and Te-Chato (2005) reported an inhibitory effect of activated charcoal in the medium on germination and maturation of oil palm embryos, whereas it had a beneficial effect on the germination of *P. dactylifera* somatic embryos originated from suspension culture (Zouine *et al.*, 2005). Micropropagation of selected clones of cherished cultivars is a goal for commercial growers, however the cultivars have very different media requirements for successful micropropagation (El- Hadrami *et al.*, 1995; Hassan, 2007; Abahmane, 2011), which need to be determined separately for each cultivar. The objective of this study was to evaluate the effect of phytohormones, especially cytokinins, and activated charcoal on regeneration and shoot formation from explants of seven commercial date palm cultivars with the aim to expand the application of micropropagation for the proliferation of many date palm cultivars.

Material and methods

Plant material: The study was carried out in the Central Laboratory of Date Palm Research and Development (Agricultural Research Center, Giza, Egypt) during 2008-2011. The date palm cultivars were obtained from the cultivar collection of the Agricultural Research Center, Giza, Egypt.

Cultivars: The selected date palm cultivars represent different fruit types of commercial value in different areas of the arid belt of Africa and Asia. Sakkoty, Barthamuda and Malkaby produce so-called dry fruits and these cultivars had been previously tested for micropropagation (Taha *et al.*, 2007). Sakkoty is the most important dry date palm in Upper Egypt, together with Shamia, which is a salt-tolerant cultivar. Barhee, Medjool and Khalas bear soft fruits and are especially cultivated in the Gulf region and the Arabian peninsula. Barhee has the highest yields, but suffers from pollination problems, Khalas is the most relished for its delicious taste, and Medjool is popular in the whole Arabian area.

Plant sterilization: The young off-shoots of seven date palm cultivars (Sakkoty, Barthamuda, Malkaby, Shamia, Barhee, Khalas and Medjool) used as source of explant materials were 2-4 years old, ranging in weight 5-7 kg and 50-70 cm in length. The selected off-shoots were transferred to the laboratory and prepared by removing the adventitious roots, fibrous sheaths and leaves. Leaves from off-shoots were removed until the white soft leaves were visible. The apical meristem with few leaf- primordia was used as explant material. Explants were washed by running tap water for 1 h. Thereafter, they were surface sterilized, under aseptic conditions. Firstly, by immersion in 60% v/v Chlorox (commercial bleach, 5.25% sodium hypochlorite) +2 drops of Tween 20 for 25 min, and then rinsing with sterilized distilled water twice. Secondly, by immersion in 0.1% mercuric chloride for 15 min and washing, and, finally, by immersion in 40% v/v Chlorox for 15 min, and subsequently rinsed three times with sterilized distilled water.

Embryo cultures: The sterilized shoot tips were longitudinally divided into several parts after removing the outer leaves which were affected by the sterilization treatment. Explants were cultured and transferred at one month intervals on solid MS medium (Murashige and Skoog, 1962) supplemented with 10 mg/L 2,4- D + 3 mg/L 2iP+ 40 g/L sucrose + 2 g/L activated charcoal (AC) + 4 mg/L ABA in addition to vitamins and glycine (Hassan, 2007)). After two months, the explants were subcultured at one month intervals, for six months, on similar fresh culture medium devoid of ABA to induce direct somatic embryogenesis. The formed embryos were subcultured on MS medium in absence of growth- regulators for 4 weeks until they were visible by naked eye. Clumps of somatic embryos, consisting of 3-4 embryos, were cultured on media with 0.1 mg/L NAA and 0.05 or 0.1 mg/L of BA, Kin or 2iP with or without activated charcoal for eight weeks (with transfer after 4 weeks). The explant cultures were then further subcultured three times, at 3 week-intervals, on fresh media and then the number of somatic embryos and shoots was recorded. Thus only, five transfers of cultures were performed to minimize carry-over effects.

Statistical analysis: The statistical analysis was performed with SigmaPlot 10 (Systat Software Inc., San Jose, CA, USA) statistical programs.

Results

Formation of somatic embryos: Usually, the successful transfer of micro-propagated plantlets from sterile media into soil is the bottle-neck for obtaining clonal plants, therefore a high number of embryos and plantlets thereof with shoots are a prerequisite for successful clonal propagation of desired cultivars. The following experiments were designed to elaborate optimal media compositions for that purpose. The plantlet stages are depicted in Fig. 1. After eight weeks the largest numbers of embryos and shoots were recorded with 0.1 mg/L NAA in conjunction with 0.05 or 0.1 mg/L of the cytokinins BA, Kin or 2iP with or without activated charcoal. Media with these phytohormone combinations were used for further three subcultures. Other concentrations of NAA (0.5 or 1.0 mg/L) or IBA (0.05, 0.1 or 1.0 mg/L) gave low numbers of embryos and shoots and were therefore not further subcultured.

Very different numbers of somatic embryos were obtained for the different cultivars and the different media. Still some general picture emerged: The culture without activated charcoal produced the highest embryo number per culture with M3 medium (mean: 36.6) and M4 medium (with mean: 30.1), both significantly higher than the other media, especially M2 and M6 media, where the lowest numbers were recorded (mean: 21.0 and 20.6, respectively) (Fig. 2a, Table 1). The highest individual value was noticed for Barthamuda on M3 medium (52 embryos/culture) followed by Sakkoty on M3 medium (49 embryos/culture), while the lowest one was for Medjool on M6 medium (12 embryos/culture). Comparison of the cultivars revealed that Barthamuda, Barhee and Sakkoty were best suited for somatic embryo regeneration, whereas Malkaby, Khalas and Medjool were the less productive (Table 1).

Activated charcoal in the media significantly decreased the number of formed embryos by about one third (Fig. 2b, Table 1) in all the six media. The impact of hormonal composition of media in presence of activated charcoal was similar to that without activated charcoal, *i.e.* M3 medium was significantly the best for all cultivars (except cv. Khalas where M3 medium was the worst) and M6 medium was the worst in all, except for Khalas and Medjool (Table 1). Also the performance of the different cultivars, in presence of activated charcoal, was similar as without charcoal, *i.e.*, Barthamuda and Barhee produced the highest number of embryos (Table 1).

Formation of shoots: Shoot formation is the ultimate goal for micropropagation, but it is known that media for best embryo formation is not necessarily best for shoot formation. Here, again M3 medium produced the highest number of shoots (mean: 21.0 shoots/culture) and M6 and M1 media had the lowest numbers (mean: 14.5 and 12.1 shoots/culture, respectively), but the differences between the media were less pronounced on the embryo formation (Fig. 3a, Table 2). In contrast, there were clear differences in the response of the cultivars. The significantly best cultivar was Sakkoty with mean of 25.3 shoots/culture followed by Malkaby with 20.3 shoots/culture, and the significantly least were Barhee with mean of 10.5 shoots/culture and Medjool with mean of 12.0 shoots/culture (Table 2). The highest individual value was obtained with Sakkoty on M3 medium (33 shoots/culture) and the lowest with Barhee on M1 and M6 media (9 shoots/culture).

Activated charcoal had a medium-specific effect on the number of formed shoots, because it significantly reduced the number per culture by one third (*i.e.* at the same ratio as it reduced embryo formation) in three of the media, except in M3 medium, where

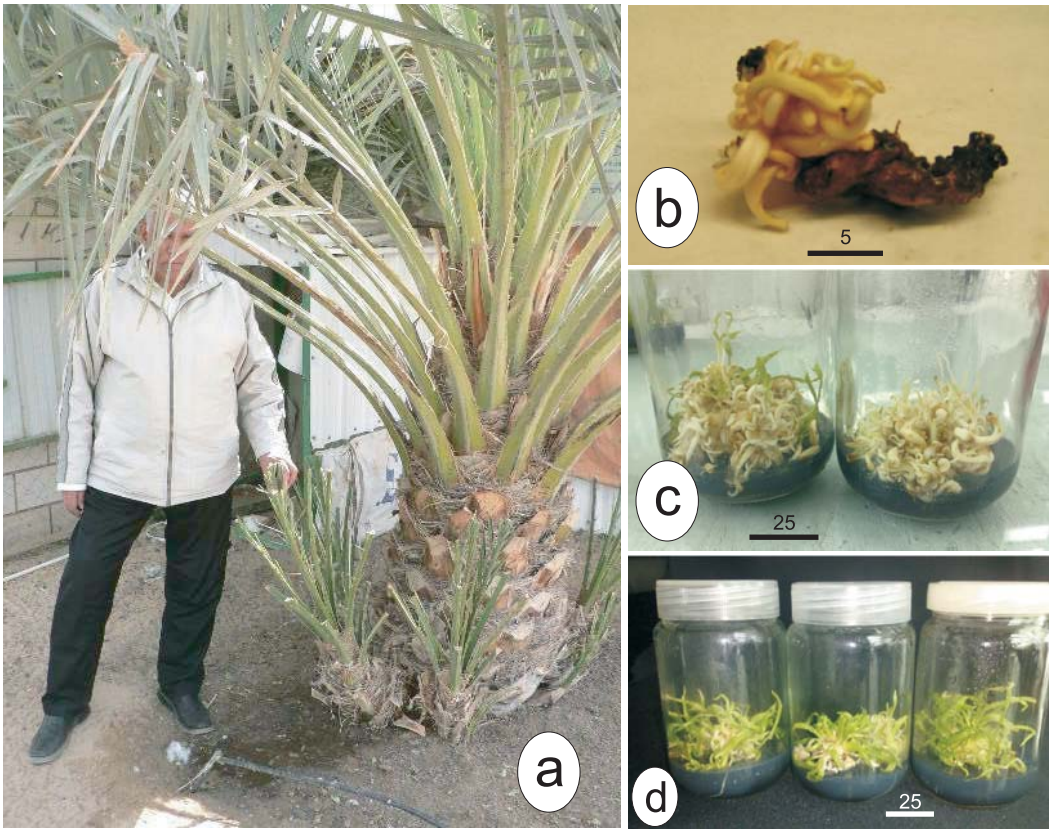

Fig. 1. Micropropagation sequences of date palm from mother plant to clonal offshoots. a: mother plant (cultivar Barthamuda), b: explants with embryonic clumps after 36 days in culture, c: small shoots formed after 12 weeks, d: shoots formed after further 9 weeks. Scale bars in mm.

Table 1. Significance of the treatment effect on embryo generation of different media and cultivars

Cultivar	Media						Cultivars	
	M1	M2	M3	M4	M5	M6	Mean	Significance
				Number of embryos (-AC)				
Sakkoty	ab	a	b	ab	ab	a	33.5±9.1	c
Malkaby	ab	ab	b	ab	ab	a	19.0±4.0	ab
Barthamuda	ab	a	b	ab	ab	ab	38.0±8.5	c
Shamia	a	a	c	b	a	a	24.0±5.4	b
Barhee	ab	a	b	b	ab	ab	34.2±7.8	c
Khalas	ab	ab	b	ab	ab	a	20.2±4.1	ab
Medjool	ab	ab	b	ab	ab	a	17.3±4.2	a
Comparison between media								
Mean±SD	26.4±8.0	21.0±4.5	36.6±10.6	30.1±12.1	24.9±7.5	20.6±6.8		
Significance	ab	a	c	bc	ab	a		
				Number of embryos (+AC)				
Sakkoty	a	a	c	a	a	b	20.2±6.2	bc
Malkaby	ab	ab	b	ab	ab	a	14.9±3.5	ab
Barthamuda	ab	ab	b	ab	ab	a	24.3±6.5	c
Shamia	b	b	d	c	c	a	17.5±5.3	b
Barhee	ab	ab	b	ab	ab	a	23.2±6.9	c
Khalas	ab	a	a	ab	b	a	13.5±1.7	ab
Medjool	ab	a	b	ab	ab	a	12.3±2.9	a
Comparison between media								
Mean±SD	18.8±6.9	15.9±4.9	25.0±8.0	18.3±4.3	18.4±4.0	11.6±1.6		
Significance	b	ab	c	b	b	a		
Comparison of –AC and +AC								
Significance (*P*)	0.002	0.002	<0.001	0.003	0.004	<0.001		
LSD (*P*=0.05)	Cultivar 0.52	Medium 0.48	Charcoal 0.20	Cultivar x Medium 1.27	Cultivar x Charcoal NS	Medium x Charcoal 0.68	Cultivar x Medium x Charcoal 1.80	

All media contained 0.1 mg/L NAA, in addition kinetin (M1 0.05 mg/L), (M2 0.1 mg/L), or BA (M3 0.05 mg/L), (M4 0.1 mg/L), or 2iP (M5 0.05 mg/L), (M6 0.1 mg/L). One Way Analysis of Variance, All Pairwise Multiple Comparison Procedures (Holm-Sidak method or Tukey Test) $P<0.05$. For pairwise comparison Mann-Whitney Rank Sum Test. Different letters indicate significant differences, NS = not significant. LSD = least significant difference.

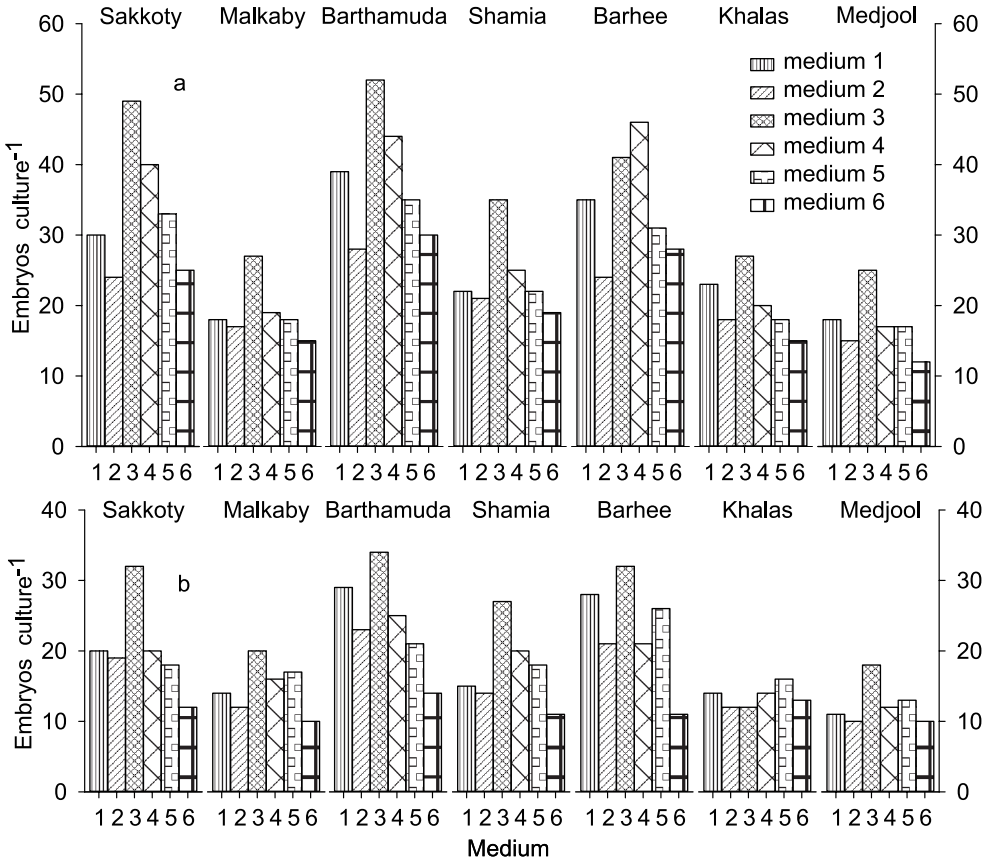

Fig. 2. Number of embryos per explant of date palm cultivars formed after 9 weeks in the media M1-M6 (designated as 1-6). a: media without activated charcoal, b: media with activated charcoal. All media contained 0.1 mg/L NAA, in addition kinetin (M1 0.05 mg/L, M2 0.1 mg/L), or BA (M3 0.05 mg/L, M4 0.1 mg/L), or 2iP (M5 0.05 mg/L, M6 0.1 mg/L).

it significantly increased the number by 33.33% (Fig. 3b, Table 2). Similar to the results without charcoal, Sakkoty produced the highest individual number of shoots (45 shoots/culture on M3 medium) followed by Malkaby on M3 medium with 39 shoots/culture, while Barhee recorded the least numbers (8 shoots/culture on M1, M2, M5 and M6 media). On all media, Sakkoty was the most productive for shoots followed by Malkaby, whereas Barhee was the least productive, however the differences between the cultivars were, except of Sakkoty, more without charcoal (Table 2).

Discussion

El- Hadrami *et al.* (1995) reported a protocol for the production of somatic embryos from shoot tips of date palm. Subsequently Fki *et al.* (2003) and Gadalla (2007) succeeded in regenerating date palm plantlets from suspension culture. However, suspension cultures carry the risk of producing high somaclonal variation, *i.e.* the desired properties of the mother plant may be changed in the micro-propagated shoots (reviewed in Al-Khalifah and Askari, 2011; El-Hadrami *et al.*, 2011). Therefore, meristem tip culture is a more reliable approach to conserve the clonal identity of the mother/elite plants. Hassan (2007) reported that direct somatic embryos of dry and semi-dry date palm cultivars, which resulted from ABA treatment and subculture on 0.1 mg/L NAA +0.05 mg /L IBA+ 1.0 g/L AC, exhibited good *in vitro* growth and were successfully transferred to the greenhouse with the "correct" phenotype. However, micropropagation systems often require optimization for different genotypes by modification of the culture medium, also as in case of date palms (Al-Khayri, 2010). Cytokinins are required for embryo production from cell cultures, but the cytokinin type and the appropriate concentrations vary from 0.1 to 5 mg/L for nearly each of the cytokinins (Abahmane, 2011). The promoting effect of BA compared with 2iP or Kin for date palm cultures was already noticed by Zouine and El Hadrami (2005). In our study M3 medium, containing 0.1 g/L NAA and 0.05 g/L BA, were the best medium for embryogenesis and shoot formation for all cultivars, despite large differences between the different cultivars in terms of embryo and shoot production, whereas the media with 0.1 g/L of 2iP or Kin (M2 and M6) were often the worst. Thus, a low concentration of BA is the best for date palm micropropagation and future experiments could prove whether even lower concentrations of BA would be better, especially since Taha *et al.* (2007) found that the best development of shoots occurred without hormones. Addition of activated charcoal reduced the formation of somatic embryos and shoots of all date palm cultivars and in all medium compositions, with exception of shoot formation in M3 medium. This medium-specific augmentation of shoot formation is puzzling. It may be argued that absorption of BA by activated charcoal may have further favored shoot formation, comparable to the shoot formation in the absence of hormones (Taha *et al.*, 2007). However, if really hormones would inhibit shoot formation, a positive effect of charcoal would be expected for all media, not only M3 medium, which however was not observed.

The cultivars Barthamuda, Sakkoty and Malkaby were consistently the most productive in terms of embryogenesis and

Table 2. Significance of the treatment effect on shoot generation of different media and cultivars

Cultivar	Media						Cultivar	
	M1	M2	M3	M4	M5	M6	Mean	Significance
				Number of shoots (-AC)				
Sakkoty	c	a	d	a	b	a	25.3±5.8	d
Malkaby	c	a	b	a	a	a	20.3±4.1	c
Barthamuda	ab	ab	b	ab	ab	a	15.2±3.3	b
Shamia	a	bc	c	bc	b	b	16.7±3.0	b
Barhee	a	ab	b	a	ab	a	10.5±1.7	a
Khalas	a	ab	b	ab	a	a	14.5±2.8	ab
Medjool	ab	ab	b	ab	ab	a	12.0±1.8	a
Mean±SD	12.1±2.0	17.4±4.6	21.0±6.5	16.4±4.9	16.6±6.4	14.5±5.6		
Significance	a	ab	b	ab	ab	a		
				Number of shoots (+AC)				
Sakkoty	b	a	d	b	c	a	20.7±11.9	b
Malkaby	a	bc	d	a	c	ab	17.8±10.2	ab
Barthamuda	a	a	b	a	a	a	12.8±6.1	a
Shamia	a	a	c	a	b	a	15.7±7.8	ab
Barhee	a	a	b	a	a	a	9.2±2.4	a
Khalas	a	a	c	ab	b	a	12.2±5.6	a
Medjool	a	a	b	a	a	a	10.8±4.3	a
Mean±SD	9.6±1.1	11.7±3.8	28.6±10.3	10.1±1.2	13.4±4.8	11.6±3.4		
Significance	a	a	b	a	a	a		
Comparison of –AC and +AC								
Significance (*P*)	<0.001	<0.001	0.015	<0.001	NS	NS		
LSD (*P*=0.05)	Cultivar 0.52	Medium 0.48	Charcoal 0.30	Cultivar x Medium 1.27	Cultivar x Charcoal NS	Medium x Charcoal 0.68	Cultivar x Medium x Charcoal 1.80	

All media contained 0.1 mg/L NAA, in addition kinetin (M1 0.05 mg/L), (M2 0.1 mg/L), or BA (M3 0.05 mg/L), (M4 0.1 mg/L), or 2iP (M5 0.05 mg/L), (M6 0.1 mg/L). Significance of the differences between the different media (M1-M6) and cultivars. All Pairwise Multiple Comparison Procedures (Holm-Sidak method or Tukey Test) $P<0.05$. For pairwise comparison Mann-Whitney Rank Sum Test. Different letters indicate significant differences, NS = not significant.

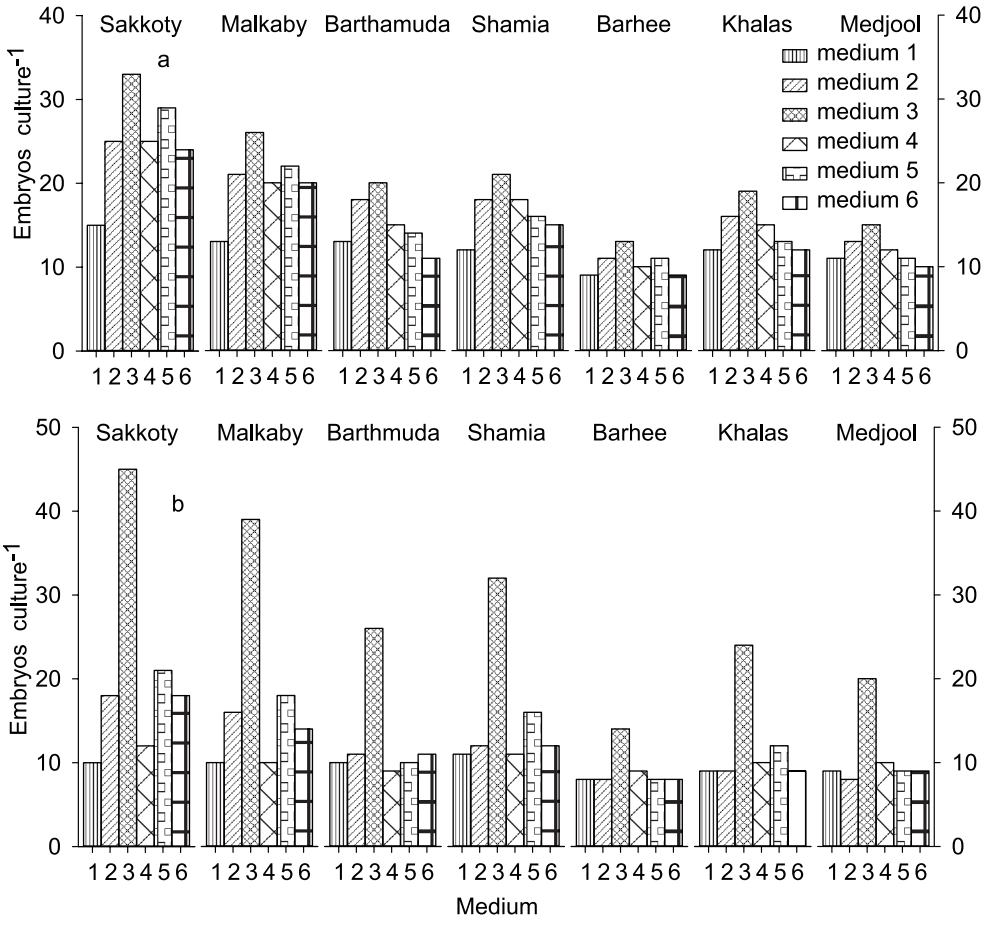

Fig. 3. Number of shoots per explant from embryonic cultures of date palm cultivars after 9 weeks in the media M1-M6 (designated as 1-6).

a: media without activated charcoal, b: media with activated charcoal. All media contained 0.1 mg/L NAA, in addition kinetin (M1 0.05 mg/L, M2 0.1 mg/L), or BA (M3 0.05 mg/L, M4 0.1 mg/L), or 2iP (M5 0.05 mg/L, M6 0.1 mg/L).

shoot formation. The present study compared seven cultivars including the cultivars Khalas, Medjool and Barhee, which produce soft dates and were successfully micro-propagated, although to only half of the success rate of Sakkoty and Barthamuda. The micropropagation and selection of Barhee clones (a high-yield cultivar) with less pollination failures than the usual trees as well as the micropropagation of Khalas, the most delicious and highly priced soft date is a promising goal for the future. The results of our study suggest that M3 medium in presence of activated charcoal is the recommended medium for shoot formation and plantlets which then may be acclimatized for the transfer to soil.

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