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Optimisation of *in vitro* micropropagation of several date palm cultivars

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Abstract

Rapid and efficient propagation methods that avoid the use of mutagenic growth regulators are required for date palm breeding and cultivation. The effects of different growth regulators on micropropagation of six female cultivars of date palm including Barhee, Medjool, Khalas, Khadrawi, Nemeishi and River Gem and the male cultivar Jarvis were invstigated. The experiments were conducted *in vitro* using floral explants of female and male mature inflorescences. Embryogenic callus induction and proliferation were significantly higher (93%) in the woody plant nutrient culture medium (WPM) supplemented with 5mg l^{-1} Thidiazuron (TDZ). The addition of zeatin to the medium was most effective for shoot regeneration from callus and this enhanced regeneration frequency and the average number of shoots obtained per explant. The highest number of shoots/explant (16) was obtained using 2 mg l^{-1} zeatin in WPM and WPM with 0.5 mg l^{-1} GA_3 and 2 mg l^{-1} zeatin enhanced shoot length significantly.
Bud initiation and adventitious shoot formation 16 weeks after flower culture was enhanced by WPM supplemented with 1.5 mg l^{-1} 2,4-D and 5 mg l^{-1} 2ip. Sub-culturing of formed buds on a multiplication medium supplemented with 3.0 mg l^{-1} zeatin and 0.3 mg l^{-1} NAA produced the highest average number of buds. Adventitious shoot elongation (up to 7.64 cm) was maximized using a WPM medium with 0.5 mg l^{-1} GA_3 and 3 mg l^{-1} zeatin.
Optimum rooting (81%) was achieved when shoots were transferred to a medium with 0.2 mg l^{-1} NAA. The survival rate was 80% and the plants were subsequently transferred to bigger pots and acclimatized for field planting. No phenotypic differences were observed among the regenerants. This observation was validated using a set of five highly polymorphic iPBS and SSR markers.

Keywords: Inflorescence, Organogenesis, Somatic embryogenesis, Woody Plant Medium.
Abbreviations: 2,4,5-T_Trichlorophenoxyacetic acid, 2,4-D_2,4-dichlorophenoxyacetic acid, 2iP_2-isopentenyladenine, B5_Gamborg medium, BA_Benzyladenine, GA_3_Gibberellic acid, MS medium_Murashige and Skoog medium, NAA_α-naphthaleneacetic acid, PGRs_Plant Growth regulators, SE_Somatic embryo, TDZ_Thidiazuron, WPM_Woody plant medium, Zeatin_Zeatin riboside.

Introduction

Date palm, *Phoenix dactylifera* L., is an economically important fruit tree in the desert areas of the Middle East and North Africa (Al-Khayri, 2007). The number of date palm cultivars worldwide is estimated to be as high as 5,000 (Jaradat and Zaid, 2004). Date palm cultivars, such as Barhee, Medjool, Khalas, and Khadrawi, are the most popular worldwide because of their desirable flavor. However, their fruit is more expensive than other cultivars. Date palms are traditionally planted from offshoots. However, establishing new date palm plantations using this method is slow because a limited number of offshoots are produced during the lifespan of a tree. Seed-propagated palms do not bear true to type fruit due to high levels of heterozygosity and require up to 7 years before fruiting (Othmani et al., 2009).
Tissue culture is used to vegetatively propagate many plant species (Murashige, 1974). However, culturing woody species in general (King, 1974) and monocotyledonous plants in particular has seldom been successful (Staritsky, 1970; Bhojwani et al., 1977). Thus there has been interest in techniques that produce large numbers of plants from tissue. *In vitro* micropropagation has become an essential and effective means of renewing and extending palm plantations (Al-Khayri and Naik, 2017; Smith and Aynsley, 1995). Researchers have focused on large-scale micropropagation of date palm since 1970 using either somatic embryogenesis or organogenesis (Singh and Shekhawat, 2009).
Efficient methods for obtaining true to type plants using explant culture were developed (Muter, 1991). Smith and Thomas (1973) successfully stimulated plantlet formation from adventitious buds by callus induction of date palm roots *in vitro*. Today, most commercial laboratories worldwide use somatic embryogenesis from shoot-tip

explants (Mazri and Meziani, 2015). However, deriving explants from offshoots requires a high-auxin media and the method has significant short-comings, these include endogenous bacterial contamination, browning, somaclonal variation and long production duration as micropropagation from offshoot tips takes approximately 3 years. Viable alternative explant techniques that overcome these limitations are not available.

The culture potential of explant inflorescences was tested to develop direct (Abul-Soad et al., 2004) and indirect somatic date palm embryos (Drira and Al-sha'ary, 1993; Abulsoad et al., 2005; Sidky et al., 2007). This technique is widely employed to produce normal plantlets from many other plants (Chevreau et al., 1989; Yepes and Aldwinckle, 1994; Chen and Chang, 2002). The origin and ontogeny of indirect somatic date palm embryos have been extensively reviewed (Sané et al., 2006; El Dawayati et al., 2012).

Plant growth regulators influence tissue culture success because they regulate cell division and tissue and organ differentiation (Jennifer et al., 2010). Most tissue culture studies have focused on the effects of different auxins and concentrations on explant cultures of date palm (Othmani et al., 2009; Eke et al., 2005) and macaw palm (Moura et al., 2009). The auxin 2,4-dichlorophenoxyacetic acid (2,4-D), most effectively triggers this pathway (Gaj, 2004). However, many phytohormones induce somaclonal variation during prolonged explant subculture on a high-auxin medium (Abul-Soad et al., 2002). On the other hand, thidiazuron (TDZ) was successfully used *in vitro* to induce adventitious shoot formation and promote axillary shoot proliferation (Lu Chin-Yi, 1993). TDZ can stimulate cell division in soybean callus, induce adventitious shoot formation in tobacco leaf discs and stimulate radish cotyledon expansion (Thomas and Katterman, 1986).

A suitable growth regulator that avoids the use of high concentrations of strong auxins such as 2,4-D is required for commercial date palm cultivation. This study aimed to develop a rapid and efficient propagation method with suitable medium, growth regulator(s) and explant types. The genetic fidelity of the micropropagated plants was assessed using iPBS and SSR markers to detect somaclonal variants.

Results and discussion

Somatic embryogenesis

Although shoot proliferation methods using adventitious shoots and auxillary buds have been widely used for *in vitro* propagation, somatic embryogenesis is more common in woody plants (Germanà and Lambardi, 2016). In date palm, somatic embryogenesis is favoured mainly because of its greater potential for mass propagation (Al-Khateeb, 2008; Al-Khayri, 2001; El Hadrami et al., 1999; Fki et al, 2003; Othmani et al., 2009). Furthermore, plant regeneration via somatic embryogenesis could have several advantages over organogenesis, including single cell origin, automated large-scale production of embryos in bioreactors and the production of synthetic seeds (Giri et al., 2004).

Three different types of media (MS, WPM and B5) were evaluated on the cultivar Barhee, chosen because of its importance worldwide. Tissue-culture-derived clones of this cultivar are much sought after on the global market because of the high quality of its dates that can be consumed semi-ripe (Khalal). TDZ was the most effective growth regulator in embryogenic callus induction and it was therefore used at the effective concentration of 5 mg l^{-1} in all three culture media and in combination with various concentrations of 2,4-D and NAA to ascertain whether TDZ alone is sufficient for embryogenic callus induction/direct somatic embryo-genesis.

WPM was the most efficient of the three salt media in embryogenic callus induction (Supplementary Table 1). Furthermore, it was observed that TDZ alone at a concentration of 5 mg l^{-1} or in combination with 7 mg l^{-1} 2,4-D or 10 mg l^{-1} NAA were the most effective for callus induction. Thus, WPM supplemented with these three levels of growth regulators were used in all subsequent experiments on all cultivars.

Effect of the selected growth regulators on days to embryogenic callus initiation and callus induction efficiency

Significant differences between growth regulator treatments for the time to callus initiation from the inflorescences as explants were observed (Figure 1). Two cultivars (River Gem and Nemeishi) initiated embryogenic calli effectively from inflorescences within 20 days on medium supplemented with 5 mg l^{-1} TDZ. The treatment with TDZ (5 mg l^{-1}) alone led to significantly faster responses than combinations with either 2,4-D or NAA in all cultivars. The average time to callus initiation across all cultivars was 26.4, 46.6 and 62.1 days in the presence of 5 mg l^{-1} TDZ (H1), 5 mg l^{-1} TDZ + 7 mg l^{-1} 2,4-D (H2) and 5 mg l^{-1} TDZ + 10 mg l^{-1} NAA (H3), respectively. Significant differences were observed among cultivars and the response patterns were similar regardless of the combination of growth regulators. The male cultivar (Jarvis) took longer to initiate callus (36 days for H1 treatment to 71 days for H3 treatment) compared to all the female cultivars.

The effect of growth regulators on the percentage of callus induction is shown in Supplementary Table 2. TDZ at 5 mg l^{-1} was very callogenic and significantly different from the effect of TDZ combined with either 2,4-D or NAA in all cultivars except Khalas. In this cultivar, the combination of TDZ with either 2,4-D or NAA exhibited lower callus induction rates. Furthermore, the primary callogenesis from floral explants appeared highly dependent on genotype. Khadrawi and River Gem appeared highly callogenic (93%), whereas Medjool had the least responsive explants (73.3%) when TDZ was used alone. Although inconsistent, the male cultivar (Jarvis) showed less callogenesis compared to female cultivars indicating that its flowers were less responsive to exogenous application of growth regulators and suggests the need for a stressful pre-treatment before inoculation.

These observations support those of Ipeki and Gozukirmizi (2003) and Sidky and Zaid (2011), who successfully induced embryogenesis and increased the number of somatic embryos from explants initially cultured on media supplemented with 10 mg l^{-1} TDZ alone. However, they contrast with other studies that identified 2,4-D as crucial to the development of embryogenic callus (Abul-Soad, 2012; Kurup et al., 2014). The current study limited somaclonal variation by minimising or avoiding the use of strong auxins like 2,4-D in date palm micropropagation. However, 100

mg/L 2,4-D was reported in many date palm studies (Eshraghi et al., 2005; Al-Khayri, 2010; Al-khayri, 2011) which is a high dose that may have induced somaclonal variation (Fki et al., 2011). Furthermore, the use of TDZ with various types of salt media helped optimize and minimize the concentration of TDZ.

Auxins or substances with auxin like activity such as TDZ are generally necessary for the induction and proliferation of cells that later differentiate into somatic embryos (De Jong et al. 1992; Michalczuk et al. 1992). Although auxins are the best studied inducers of embryogenic cells, they are not unique in the ability to mediate the transition from somatic cells to embryogenic cells (Hutchinson et al., 1996).

Thidiazuron did not only substitute for the auxin–cytokinin requirement of the induction process in the current study, but also augmented the number of somatic embryos and increased the embryogenic response of the floral explants with normal growth and development. A similar response was observed in Geranium by Gill *et al.* (1993) and Visser *et al.* (1992) and has also been observed in many other species, including peanut and tobacco (Victor et al., 1999; Laloue and Pethe, 1982).

The TDZ induced somatic embryogenesis within a relatively short exposure time in the current study highlights its effective substitution for the combined auxin and cytokinin requirements of somatic embryogenesis. Its high stability in culture media and persistence in plant tissues was described by Mok and Mok (1985) on lima bean. Sexena et al. (1992) suggested that TDZ helps establish the optimal internal balance between cytokinin and auxin required for the induction and expression of somatic embryogenesis in *Solanum melongena*. Furthermore, TDZ can increase nucleoside levels as reported in tobacco (Laloue and Pethe, 1982) and promote the synthesis and accumulation of purines (Capelle et al., 1983). Huetteman and John (1993) demonstrated that low TDZ concentrations more efficiently induced organogenesis or somatic embryogenesis than other cytokinins, particularly in recalcitrant woody species.

Effect of growth regulators on callus fresh weight of seven cultivars after 8 weeks of culture

Different growth regulators influenced the fresh weight of callus (Figure 2). The average callus fresh weight after 2 months of culture was significantly higher under TDZ (5 mg l^{-1}) alone compared to the NAA treatment, however it did not differ from the 2,4-D treatment. Average weights of 259 and 260 mg were observed for the H3 and H2 treatments, respectively, after 60 days of culture. The increased callus weight in the TDZ-containing medium without 2,4-D was probably a result of weaker cytokinin and auxin activity which promoted cell division (El-Hammdy et al., 1999; Chukwuemeka, 2005). The effect of TDZ might also be linked to the oxidation of nutrients and the formation of growth-related enzymes (Rabechault et al., 1976) which can be exploited by tissues for cell division and growth.

Effects of growth regulators on callus morphology

The inflorescence-derived callus in the TDZ medium was characterized by a white color and granular appearance and subsequent development of globular opaque bodies (Figures 3 & 4). This type of callus is considered to be primary embryogenic callus (Omar 1988; Nazeri et al. 1993; Mater 1986 and Jasim 1999). Brownish coloured and compact calli were observed in the media containing 2,4-D and NAA. Histological (Figure 3O) and scanning electron microscopy (Figures 3P-3R) analyses showed the development of the globular embryos. Histological examination of swollen floral explants showed the formation of new parenchymatous cells which caused the explants to swell. The newly formed cells are small and densely packed which is characteristic of meristematic cells. Small individualized embryogenic masses and globular embryos can be observed embedded in the degenerating parenchyma (Figure 3O).

Plantlet regeneration

Zeatin alone was more effective in plantlet regeneration than 2ip or NAA combined with either 2ip or zeatin in all cultivars after the fourth subculture (Supplementary Table 3). Zeatin alone at 2.0 mg l^{-1} generated the highest average number of plantlets (14.14 plantlets). The combination of NAA with 2ip had no significant effect on the conversion rate of somatic embryos to plantlets in all cultivars with the exception of Barhee and River Gem. The addition of NAA increased the conversion rate from 60 to 71.4% and from 53.6 to 73.5% in Barhee and River Gem, respectively. These observations are supported by Sidky and Zaid (2011) who reported that TDZ at low concentration (1.0 mg l^{-1}) was optimal for the maturation and growth of embryos.

Direct organogenesis and proliferation of adventitious bud clusters

Organogenesis is the process by which explants undergo changes that lead to the formation of a unipolar structure (shoot or root primordium) with vascular connections to parent tissues (Darwesh et al., 2011). In date palm this involves the direct formation of adventitious buds on the floral explant without the production of an intervening callus (Supplementary figures 1 and 5). Regeneration through adventitious organogenesis is slower but avoids the risk of somaclonal variation (Bekheet et al., 2001; Taha et al., 2001; Al Khateeb, 2008).

Date palm direct organogenesis from floral explants comprises the following steps: adventitious bud initiation, shoot bud multiplication, shoot elongation, rooting and plantlet acclimatization (Abahmane, 2011; Al Maari, Al Ghamdi, 1998). The success of this regeneration pathway depends on several factors, which are presented in the following sections.

Effects of culture media, culture conditions and growth regulators on adventitious bud initiation

Three different types of media, viz., MS, WPM and B5 were evaluated in preliminary experiments on the cultivar Barhee. Each medium was tested with different concentrations of 2ip, 2,4-D, NAA and 2,4,5-T to determine the optimal salt medium and growth regulator(s). 2ip was found to be essential for adventitious bud initiation and was subsequently used at the effective concentration of 5 mg l^{-1} with all three culture media in combination with 2,4-D, NAA and 2,4,5-T to investigate the effect of auxin addition on bud

Table 1. Effects of various auxin concentrations combined with 2ip at 5 mg/l on number of adventitious buds in explants of seven date palm cultivars after 16 weeks of culture.

Variety	1.5 mg l^{-1} 2,4-D	1.0 mg l^{-1} NAA	1.0 mg l^{-1} 2,4,5-T
Barhee	9	6	7
Medjool	8	5	5
Khalas	12	10	11
Jarvis	10	6	8
Nemeishi	11	10	9
Khadrawi	7	5	6
River Gem	8	7	7

L.S.D. (growth regulators) = 0.4361, L.S.D. (variety)=0.6661, L.S.D. (growth regulators x variety) =1.1537.

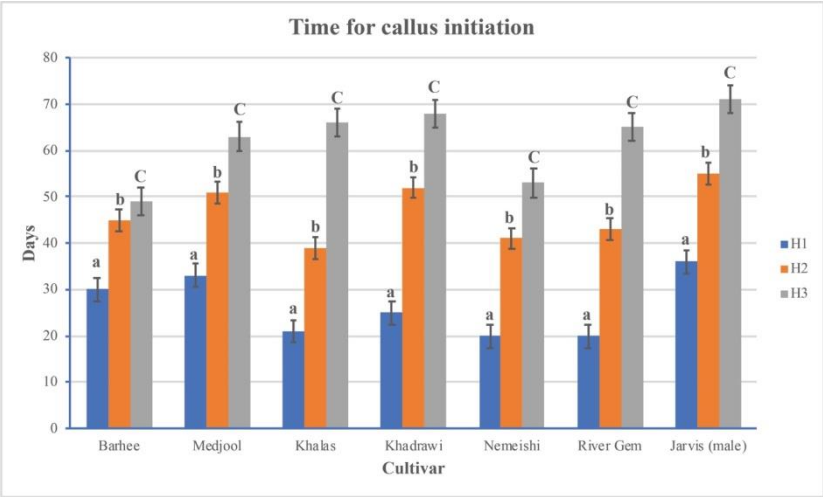

Fig 1. Time to embryogenic callus initiation from inflorescence explants in WPM supplemented with different combinations of growth regulators for seven date palm cultivars. H1= 5 mg l^{-1} TDZ; H2= 5 mg l^{-1} TDZ + 7 mg l^{-1} 2,4-D; H3= 5 mg l^{-1} TDZ + 10 mg l^{-1} NAA. Bars on columns represent standard errors and different letters on top of columns means significant difference.

Table 2. Nutrient medium composition for micropropagation from inflorescence explants.

Medium composition	Growth regulator(s)	Response
WPM + 30 g sugar	5 mg l^{-1} TDZ	Callus initiation
WPM + 30 g sugar	2 mg l^{-1} Zeatin	Callus multiplication & shoots regeneration
WPM + 30 g sugar + charcoal 3 g/l	1.5 mg l^{-1} 2,4-D + 5 mg l^{-1} 2ip	Buds initiation
WPM + 30 g sugar	3 mg l^{-1} Zeatin + 0.3 mg l^{-1} NAA	Inflorescence proliferation
WPM + 30 g sugar + charcoal 0.5g/l	0.2 mg l^{-1}NAA	Rooting

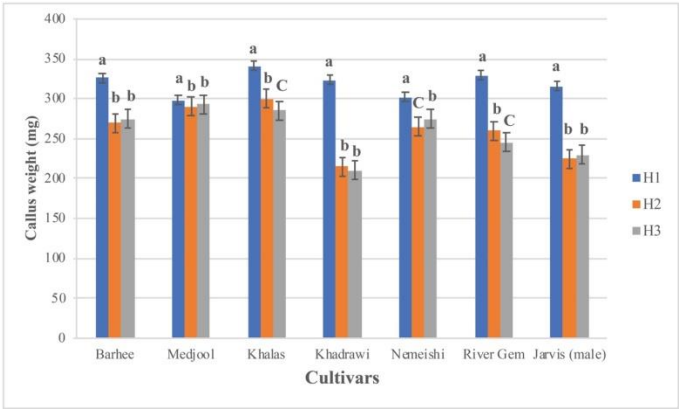

Fig 2. Effects of growth regulators on fresh callus production in different date palm cultivars. H1= 5 mg l^{-1} TDZ; H2= 5 mg l^{-1} TDZ + 7 mg l^{-1} 2,4-D; H3= 5 mg l^{-1} TDZ + 10 mg l^{-1} NAA. Bars on columns represent standard errors and different letters means on top of columns significant difference.

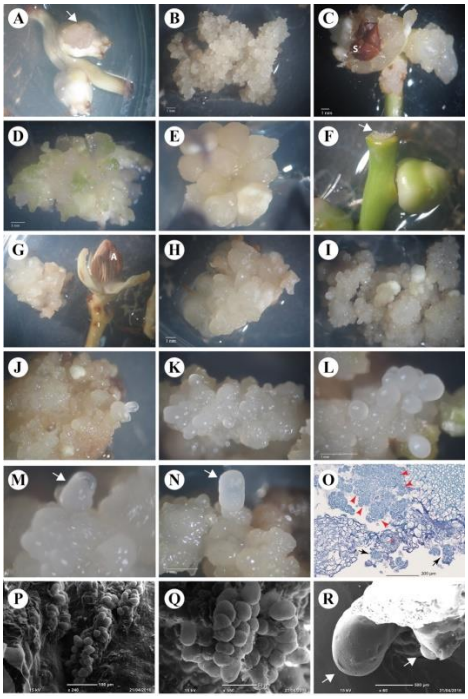

Fig 3. Indirect embryogenesis and callus development in female inflorescences in (A-F, I-R) and male inflorescence in G-H. **(A)** Inflorescence explant on semi-solid medium with a mass of callus initiated on the perianth surface. **(B)** Friable embryogenic callus with granular appearance. **(C-D)** Compact callus masses arising from perianth and the three carpels and showing browning and degenerating carpels in C. **(E-H)** Compact nodular primary callus initiated on the basal part of the flowers and the abscission zone as shown in F (white arrow). **(I-N)** Embryogenic calli with different stages of differentiating somatic embryos (late heart stage in M and torpedo stage in N) but mainly at the globular stage dispersed all over the surface of the callus. (O) Histological section of the embryogenic callus showing the dense meristematic zones surrounded by degenerating parenchymatous cortex (black arrows) and proembryonic masses (arrowheads) and spherical globules made of meristematic cells (star). The presence of meristematic zones in primary callus indicated the acquisition of competence. **(P-R)** SEM micrographs showing differentiated somatic embryos on the surface of the embryogenic callus. Abbreviations: A, Anther; C, carpel; S, stigma.

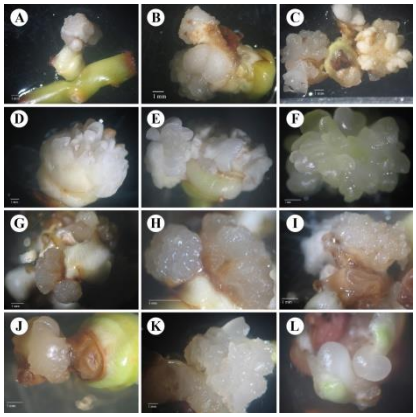

Fig 4. Direct initiation and differentiation of somatic embryos on floral date palm explants inoculated on WPM medium supplemented with plant growth regulators. **(A-F)** Initiation and differentiation of somatic embryos on flower carpels and perianth of the cultivar Barhee showing somatic embryos mostly at late heart stage in D and cotyledonary stage in F. **(G-I)** Initiation and differentiation of somatic embryos on flower carpels of the cultivar Khalas showing globular embryos. **(J-L)** Initiation and differentiation of somatic embryos on flower carpels and perianth of the cultivar Khadrawi.

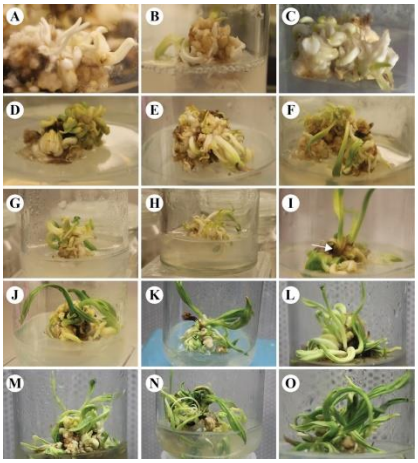

Fig 5. Direct initiation and differentiation of adventitious shoots on floral date palm explants cultured on WPM supplemented with plant growth regulators. **(A-E)** Proliferation of *Phoenix dactylifera* "Barhee" adventitious bud cluster (caulogenic capacity). **(F-L)** Shoots elongation after treatment with GA_3. **(M-O)** High inflorescence proliferation.

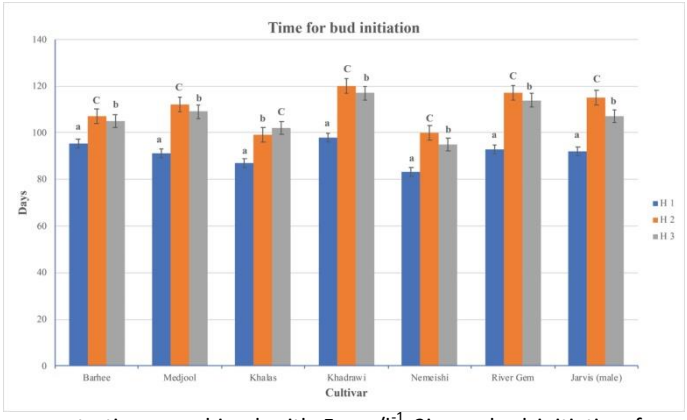

Fig 6. Effect of various auxin concentrations combined with 5 mg/l^{-1} 2ip on bud initiation from the inflorescence explants of different date palm cultivars. H1=1.5 mg l^{-1} 2,4-D + 5 mg l^{-1}2ip; H2=1.0 mg l^{-1} NAA + 5 mg l^{-1} 2ip; H3=1.0 mg l^{-1} 2,4,5-T + 5 mg l^{-1} 2ip. Bars on columns represent standard errors and different letters means on top of columns significant difference

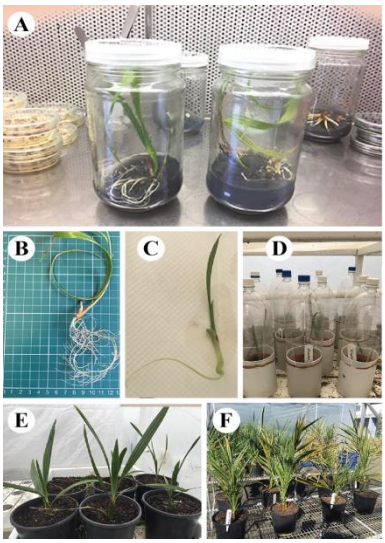

Fig 7. Roots formation and *Ex vitro* hardening of Barhee date palm in a microclimate room under controlled conditions. **(A-C)** Adventitious bud-derived plantlets (organogenesis) with roots formation induced by the addition of NAA. **(D)** Somatic embryo-derived plantlets with well-developed shoot and root structures ready to be transferred to pots for acclimatization. **(E-F)** Hardened Barhee date palm plants growing in 20 cm diameter pots during acclimatization in a microclimate room in **E** and under a net in **F**. Cutting mat divisions = 1 cm in B-C and scale bar = 1 cm in D.

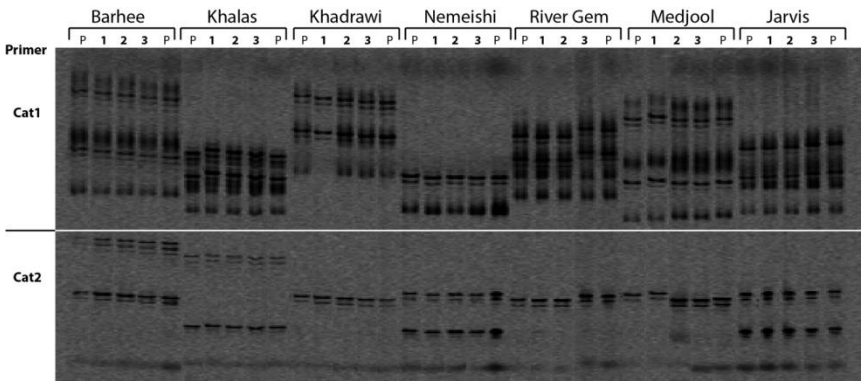

Fig 8. SSR profile of 3 *in vitro* regenerated plants and the parent plant (P) of each of 7 date palm cultivars using primers Cat1 and Cat2. The developed SSR profiles were typical to that of mother plant of the tested cultivars.

initiation and inflorescence proliferation (Supplementary Table 4).

WPM was confirmed as the most efficient of the three salt media for adventitious bud initiation. The 2ip at a concentration of 5 mg l^{-1} in combination with 1.5 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA or 1.0 mg l^{-1} 2,4,5-T were the most effective combinations at promoting bud initiation and inflorescence proliferation. Thus, these three combinations of growth regulators in WPM were used in all subsequent experiments on all cultivars. No bud formation was observed in explants without plant growth regulators and cultivating explants in the dark for one month showed some positive influence on bud induction since floral explants cultivated in the light immediately after inoculation often became necrotic (turned blackish brown) because of the emission of large quantities of phenolic compounds. The excessive release of phenolic compounds; a characteristic of the 'Barhee' cultivar after exposure of explants to light, could be partially overcome by culturing explants for 15 days on culture media supplemented with activated charcoal (1.5g/L).

Effects of growth regulators on time to bud initiation and inflorescence proliferation

Different combinations of auxin with 5 mg l^{-1} 2ip stimulated the emergence of lateral buds from inflorescence explants. The concentration of 1.5 mg l^{-1} 2,4-D with 5 mg l^{-1} 2ip (H1) produced optimal budding initiation (Figure 6). That is, the lateral bud was initiated within an average of 91.2 days, which was significantly earlier than those of the other treatments (110 days for 2ip combined with 1 mg l^{-1} NAA (H2) and 107 days for 2ip combined with 1 mg l^{-1} 2,4,5-T (H3)).

Auxin influences plant elongation and cell division by promoting nuclear division (Prisca Campanoni and Peter Nick, 2005; Catherine Perrot-Rechenmann, 2010). In the current experiment, the low 2,4-D concentration was effective in initiating budding. Zaid (1993), Bekheet and Saker (1998) and Jasim (2002) also reported that the cytokinin-to-auxin ratio determines the growth direction of cells. At high cytokinin-to-auxin ratios vegetative growth was preferred; an observation consistent with earlier reports (Sudhersan and Abu-El-Nil 2004).

Effect of auxin on the number of buds initiated from the inflorescence of explants in seven date palm cultivars:

Significant differences in the number of buds produced per explant from flower tissue under different auxin concentrations were observed (Table 1). 2,4-D (1.5 mg l^{-1}) combined with 2ip at 5 mg l^{-1} initiated the formation of a significantly higher number of buds (9.28 buds averaged over all cultivars) than those of the other treatments (7.0 buds for 1 mg l^{-1} NAA and 7.57 buds for 1 mg l^{-1} 2,4,5-T). Hence, 2,4-D combined with 2ip was considered an appropriate medium for the promotion of bud formation and later multiplication. The bud sources were the cells exposed to the medium. These cells lost their differentiation status and were re-differentiated by the medium components. The cells then developed and grew into buds through the same morphogenesis as the buds of leaf primordia (Thorpe, 1978). The emergence of buds from *in vitro* tissue culture has been recorded in many species. However, only a few sources reported similar induction in date palm (Abhman et al., 2001).

Effects of zeatin, BA, and 2ip on adventitious bud multiplication after 8 weeks of culture in the presence of 0.3 mg l^{-1} NAA.

Zeatin treatment significantly affected the multiplication of the adventitious buds resulting from the cultivation of three adventitious buds (Supplementary Table 5). The bud multiplication was enhanced more by zeatin than by 2ip and BA. An average of 11.6 buds were formed over all cultivars at 3 mg l^{-1} zeatin; this number significantly exceeded those formed using other substances (9.3 buds for 2 mg l^{-1} BA + 0.3 mg l^{-1} NAA and 9.1 buds for 4 mg l^{-1} 2ip + 0.3 mg l^{-1} NAA). The combination of different concentrations of auxins and cytokinin may have induced a hormonal balance that stimulated the vegetative multiplication of the occasional buds. These results are consistent with earlier observations that zeatin was two to three times more effective than 2ip in promoting shoot induction and proliferation (Zaid 1993; Hamid 2001; Debnath and McRae 2001). Zeatin was also more effective than other cytokinins in multiplying shoots (Cüce et al., 2013).

The current study revealed an optimal combination of plant growth regulators for high multiplication rates.

Other studies also reported that BA and 2ip can multiply date palm tissue *in vitro* (Al-Marri and Al-Ghamdi, 1997; Bekheet and Saker, 1998; Al-Khateeb et al., 2002). Clearly, low auxin concentrations in the presence of cytokinin stimulated the multiplication of adventitious buds. The adventitious buds derived from different date palm cultivars then exhibited different multiplication ratios possibly because of genetic factors. These findings confirmed the significance of cytokinins and their influence on the initiation of shoot differentiation and the frequency of shoot proliferation.

Effects of various GA_3 concentrations on shoot elongation

GA_3 positively affected the elongation of shoots produced in the multiplication stage (Supplementary Table 6). Shoot-length increased with increasing GA_3 concentration in the medium; however, some malformations were noted at 1.0 mg l^{-1} including thin growth and distortions of the slender leaves which then became difficult to root and transplant. Apart from good elongation and strong growth, the average cultivar shoot length was 7.64 cm under 0.5 mg l^{-1} GA_3, which was significantly better than other treatments. However, different cultivars showed different responses to GA_3 with the male cultivar Jarvis showing the highest elongation (9.49 cm). These results confirm the well-known role of GA_3 in plant cell elongation (1PG SA, 1998). The gibberellins cause elongation by encouraging sub-apical meristem cell proliferation and elongation and help dissolve polysaccharides into simple sugars that benefit the plant tissue (Brian, 1959). These findings were consistent with the earlier reports of Hamid (2001) and Zaid and DeWet (2005).

Rooting Stage

Effects of different NAA concentrations on the rooting percentage, number of roots, and root length after 12 weeks

Rooting of the plantlets derived from the occasional buds help complement vegetative propagation by tissue culture. Excised shootlets (5-10 cm height) were transferred onto rooting medium and 0.2 mg l^{-1} NAA produced a higher rooting rate than other concentrations (Supplementary Table 7). Similarly, the number of roots formed in each plant under 0.2 mg l^{-1} NAA differed significantly from those produced in other concentrations. The average root length of 5.63 cm was significantly longer than other treatments.
Auxins are known to play an active role in root formation (IPGSA, 1998) and the first cell splitting for root initiation depends largely on auxins. The highest rooting rate and root number were attained at 0.1–0.5 mg l^{-1} NAA which was consistent with earlier findings (AL-Maarri and AL-Ghamidi 1997; EL-Hammady et al. 1999). El-Hammady et al. (1999) noted that high auxin levels inhibit root formation and reduce root number while root length did not decrease with increasing auxin concentration. The optimised media with proper supplements including the most efficient growth regulators to advance the micropropagation process from callus induction or adventitious budding to rooting is summarized in Table 2.

Hardening and acclimatization

The plantlets were hardened in a sterilized mixture of peat moss and perlite (1:1) inside a microclimate room (Figure 7). Increasing light intensity enhanced the plantlet photosynthesis and promoted the change from heterotrophic to autotrophic status. The gradual lifting of the plastic covers in the microclimate culture room helped to harden the plantlets and a survival rate of about 80% was achieved. Thus, the procedures evaluated were practically feasible and effective for micropropagating date palm.

Molecular analysis of genetic fidelity of regenerated plantlets

No phenotypic differences were observed among the regenerants. However, molecular markers can efficiently assess the genetic fidelity of various species after *in vitro* culture (Bagali et al., 2010). The genetic fidelity and variability of micropropagated date palm plants were therefore assessed using SSRs (Figure 8) and iPBS (Supplementary Figure 2) markers.
The fidelity study showed no genetic variation between plants derived from the same cultivar maintained in the field and those maintained *in vitro*. Thus, the high genetic fidelity of the micropropagated plants was confirmed. However, these markers do not cover the whole date palm genome and there is always the possibility that mutations were not detected by the markers used.

Materials and Methods

Explant source

The inflorescences of 10-15 years old 6 female date palm cultivars (*P. dactylifera* L. cv. Barhee, Medjool, Khalas, Khadrawi, Nemeishi, and River Gem) were selected for propagation. One male cultivar, Jarvis (15 years old tree) was chosen for *in vitro* culture. Mature inflorescences of these cultivars were collected from South Australia and the Northern Territory. Spathes were excised from mother date trees just before they split open and subsequently transferred to a refrigerator set at 4 °C until used.

Surface sterilization

The outer surface of the protective spathes was cleaned with cotton and sprayed with 70% (v/v) ethanol. The external hairs of each entire spathe were then removed using a flame applied for a few seconds under aseptic conditions. The spathe was cut to expose the inflorescence. All floral strands were cut into 1–2 cm-long pieces containing 2-3 flowers prior to culturing on artificial medium (Supplementary Figure 3). These uncultured explants can be stored in sterile Petri dishes in a refrigerator at 4 °C for 1–6 weeks.

Medium preparation, culture conditions and preliminary experiments

Floral explants were cultured on woody plant basal medium with vitamins (Lloyd and McCown, 1981) supplemented with 30 g l^{-1} sucrose, 5 g l^{-1} Agargellan (PhtoTechnology

Laboratories, Product No.: A133), 1.5 g l^{-1} activated charcoal and various concentrations of plant growth regulators. The media were adjusted to pH 5.7 and autoclaved for 15 min at 121 °C and 1×105 Pa (1.1 kg cm^{-1}). MS medium was used in addition to WPM and B5 media in a preliminary experiment to test the effect of salt medium and growth regulators on micropropagation of the cultivar Barhee. The explants were cultured in callus induction medium or inflorescence proliferation medium and maintained in darkness at 24 ± 3 °C for 8 weeks, during which they were subcultured at 3-week intervals.

For callus induction, TDZ (0.0–6 mg l^{-1}), 2,4-D (0.5–12 mg l^{-1}) and NAA (1.0-12 mg l^{-1}) were filter-sterilized before being added to the autoclaved culture media. Five replicates (55 mm diameter Petri dishes) per treatment and seven explants were placed in each Petri dish on the surface of 5 ml semi solid medium. For inflorescence proliferation, 2,4-D (0.5–12 mg l^{-1} with 0.5 mg l^{-1} increment), 2,4,5-T (0-2 mg l^{-1} with 0.5 mg l^{-1} increment), NAA (0-2 mg l^{-1} with 0.5 mg l^{-1} increment) and 2iP (0-6 mg l^{-1} with 1.0 mg l^{-1} increment) were used to culture floral explants. Seven explants were placed in each Petri dish (5 ml medium) and the regenerated plantlets were visible after 12–24 weeks.

Explants that responded well were transferred into the maturation medium and sub-cultured once or twice. The explants were then transferred gradually to illuminated conditions with a photoperiod of 16 h/day using cool-white fluorescent light. Light intensity varied between 3,000–6,000 lux illumination depending on the multiplication stage. The growth room temperature was maintained at 27 ± 1 °C during illumination. The optimal salt medium and growth regulator concentration was determined and used in all subsequent experiments.

Embryogenic callus production in seven cultivars of date palm

The effects of different growth regulators (TDZ, 2,4-D, and NAA) on days to callus induction (number of days from explant inoculation to callus initiation), callus induction percentage (calculated as the percentage of explants with callus) and callus fresh weight were determined. The medium with WPM salts was replicated 15 times for each treatment. Combinations of TDZ at 5.0 mg l^{-1} with 2,4-D (0.5–12 mg l^{-1}) and NAA (1.0-12 mg l^{-1}) were used as treatments after which the explants were incubated at 27 ± 1 °C in the dark. The initial appearance of the callus was recorded and the percentage of explants forming callus determined after 3 months of culturing. The floral explants were sub-cultured three times at 4-week intervals and data were recorded following published protocols (Jasim, 2000; El-Hammady et al., 1999).

Direct inflorescence proliferation (direct organogenesis) in seven cultivars of date palm

The growth regulators 2,4-D (0.0-2.0 mg l^{-1}), 2,4,5-T (0.0-2.0 mg l^{-1}) and NAA (0.0-2.0 mg l^{-1}) in combination with one 2iP concentration (5 mg l^{-1}) were selected for culturing floral explants in WPM. Seven floral explants were placed in each Petri dish (5 ml medium) with five replicates for each treatment. The cultures were transferred to fresh media four times at 4-week intervals. All cultures were incubated in a culture room under darkness or a low light intensity of 1000 lux for 16 h daily at 27 ± 1 °C for 4 weeks, after which data were recorded. The regenerated plantlets were visible after 12–24 weeks.

Plantlet regeneration

Different culture media were tested for callus growth and shoot development from floral tissues. The concentration of plant growth regulators and rates of multiplication and germination of somatic embryos are important considerations for effective micropropagation. Explants with initiated globular embryos were transferred to woody salt Lloyd and McCown media (1981) supplemented with zeatin (2 mg l^{-1}), 2ip (3 mg l^{-1}), NAA (3 mg l^{-1}) with zeatin (2 mg l^{-1}), or 2ip (3 mg l^{-1}) with NAA (3 mg l^{-1}). The explants were incubated at 27 ± 1 °C at a light intensity of 1000 lux for 16 h/day. The numbers of shoots formed, plants produced and the percentage of plantlets derived from calli were determined after 3 months of culturing. A sub-culture was then conducted every 6 weeks. Shoots were successfully regenerated in all the media tested and measurements were made at the end of 6 weeks of culture and five replicates were prepared for each treatment.

An inflorescence proliferation experiment was then performed using buds that formed on multiplication media. These buds were divided into three-bud clusters to multiply the occasional buds obtained from previous experiments to examine the effects of treatments on the multiplication of occasional buds. Inflorescence-derived shoots regenerated from the male and some selected female genotypes were evaluated using culture media containing woody salt Lloyd and McCown (1981) supplemented with zeatin (3 mg/l), 2ip (4 mg/l), or BA (2 mg/l). The formed buds were divided into small clumps containing not less than three buds each and cultured in the presence of the different hormone treatments. Depending on the initiation stage, five replicates were adopted for each treatment. The cultures were incubated under the same conditions mentioned above and sub-cultured every 4 weeks and data were recorded after 8 weeks.

Elongation Stage

Shoots from callus regeneration were transferred to elongation media with the same composition, except for the addition of GA_3 in various concentrations (0.1, 0.2, 0.5, and 1.0 mg l^{-1}) in the presence of 2 mg l^{-1} zeatin to increase the shoot length. Ten replicates were prepared for each treatment. Materials were sub-cultured every 4 weeks and data captured after 8 weeks.

Rooting stage

Branches regenerated from callus and those grown from occasional buds were rooted to obtain a plant that could be transferred to soil. The rooting medium consisted of WPM salts and 60 g l^{-1} sucrose, 0.5 g l^{-1} activated charcoal, and 7 g l^{-1} agar. The auxin NAA was added separately in different concentrations (0.0, 0.2, 0.5, and 1.0 mg l^{-1}). Treatments were replicated ten times and the cultures were incubated in a culture room at 27 ± 1 °C and 1000 lux light intensity for 16 h daily. The rooting results including number of roots and

root length were recorded daily after 3 months and subcultures were taken every 4 weeks.

Acclimatization

Date palm plantlets were acclimatized using the method described by Abul-Soad (2011). The plantlets were removed from jars and the rooted plantlets were washed with distilled water to remove residual gel or medium. The plantlets were then immersed in 0.5% (w/v) fungicide solution (Benlate 2g l^{-1}) for 5 min before planting into 250 mm plastic pots containing a 1:1 (v/v) soil sterilized mixture of peat moss:perlite. The plants were maintained under natural day light and high relative humidity (85%–90%) by using a transparent plastic bottle. The cover was removed after eight weeks to allow the plants to develop under microclimate greenhouse conditions at 28 ± 2 °C. The plants were watered once a week and sprayed with fungicide as needed. The acclimatized plants were transferred to a shade house for further hardening at 6 – 8 months before transfer to an open field.

Microscopy methods

Cultures were examined under a binocular microscope (ZEISS Stemi 2000-c stereomicroscope) and photographed with a digital camera. For histological observations, floral explants and embryogenic calli at various stages of development were fixed in formalin acetic alcohol (FAA; 5 parts formalin: 5 parts glacial acetic acid: 90 parts 50% ethanol (v/v/v)) and stored in 70% ethanol. They were dehydrated through an ethanol series (50, 70, 95, and 100%) and then embedded in paraffin with a melting point 58–60 °C for microtoming. Serial sections were cut with a rotary microtome (Leica RM2255, Germany) at 8 µm thickness and subsequently stained with safranin-O and Fast Green FCF (Ahmad et al., 2009, Sass, 1958). They were then dehydrated through an alcohol series to absolute ethanol and mounted in DPX (BDH, Poole, UK). Samples were digitised using a slide-scanning microscope (ZEISS AxioScan Z.1, Germany).
Floral explants and embryogenic calli at various developmental stages were fixed in formalin acetic alcohol (FAA) (5:5:90 formalin, glacial acetic acid and 50% ethanol (v/v/v)) for 24 hours and stored in 70% ethanol for electron microscopy. Fixed tissues were dehydrated in a graded ethanol series (50, 70, 95, and 100%). Samples were then critical point dried in CO_2 using a BAL-TEC030 critical point dryer (Bal-Tec, Balzers, Leichtenstein) and mounted on sticky tape affixed to aluminum stubs and coated with 20 nm gold–palladium in a sputter coater (Edwards E306 A:Edwards Vacuum Systems, Crawley, UK). The mounted specimens were examined with a JEOL Neoscope Benchtop scanning electron microscope (Nikon Instruments Inc., NY, U.S.A) operating at an accelerating voltage of 15 kV and images were captured digitally with an installed digital camera.

The genetic fidelity of in vitro cultured plants

The total genomic DNA of the donor mother plant and the *in vitro* raised clones of each cultivar were extracted from young leaf tissue (200 mg). DNA was isolated following the methods described in Ahmed et al. (2016, 2017). iPBS and SSR analysis was conducted using 3 iPBS primers (2074, 2381 and 2374) and 2 SSR primers (Cat1 and Cat2). These primers showed the highest polymorphism among date palm cultivars previously (Ahmed et al., 2016; 2017). The primers were used to detect the polymorphism among the plantlets derived from indirect somatic embryogenesis compared to the mother plants. Three regenerated plants from each cultivar were chosen randomly and fingerprinted along with the elite donor mother plant.

Recording data and statistical analysis

Data were analyzed by ANOVA and means were separated using Fisher's protected least significant difference at the 5% probability level. All analyses were performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA).

Conclusion

This study provides an efficient and simple *in vitro* protocol for the mass propagation of both female and male cultivars from fully emerged mature inflorescences following simple sterilization without chemical disinfectants and antioxidants. The inflorescence removal protocol did not adversely impact the mother tree and plantlets were quickly re-generated with a minimum number of subcultures, thus reducing the risk of somaclonal variation. WPM with 5 mg l^{-1} TDZ provided the best callus initiation and WPM with 2.0 or 3.0 mg l^{-1} zeatin optimized shoot initiation. GA_3 was necessary for shoot elongation when used at a concentration of 0.5 mg l^{-1} in WPM. NAA (0.2 mg l^{-1}) applied to WPM gave the highest number of roots and longest roots compared to all other concentrations. An *in vitro* hardening step was a prerequisite for the successful transfer of vitroplants in soil. DNA banding profiles obtained from two types of PCR based markers (iPBS and SSR) showed almost complete uniformity between mother trees and micropropagated progeny.

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