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Full Length Article

In vitro Propagation of Date Palm Cultivars Magdoul and Safwai through Somatic Embryogenesis

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Abstract

The success of genetic conservation, cloning and possible genetic transformation requires an effective micropropagation protocol *in vitro*. For date palm (*Phoenix dactylifera* L.), there seems to be a lot of reported genotypic variation influencing optimum *in vitro* protocols. Two different cultivars of date palm Safawi and Magdoul were investigated to establish efficient protocol for callus induction, somatic embryogenesis, shoot proliffaration *in vitro* and plant acclimization *ex vitro*. The highest value of callus induction and relative water content (RWC) was reached at 25.0 mg L^{-1} 2, 4-D and 5.0 mg L^{-1} 2iP; 10 mg L^{-1} 2, 4-D and 8.0 mg L^{-1} 2iP for cvs. Safawi and Magdoul, respectively. The highest percentage of callus producing somatic embryos were 92.25% and 96.18% obtained on MS medium fortified with 6.0 mg L^{-1} 2iP with 1.0 or 3.0 mg L^{-1} NAA for cvs. Safawi and Magdoul, respectively. High root formation occured on 2 and/or 2.5 mg L^{-1} NAA alone or with of 0.5 mg L^{-1} IBA. Regenerated plantlets were successfully acclimatized *ex vitro* with a 55–70% survival rate. The present article gives an update of the current approaches of date palm micropropagation with emphasis on the plant regeneration through somatic embryogenesis. It highlights key factors that influence *in vitro* differentiation and evaluated somatic embryos from embryogenic lines established from two cultivars with respect to their ability to germinate and be converted into plantlets. © 2020 Friends Science Publishers

Keywords: Date palm; *In vitro* culture; Micropropagatio; Plant growth regulators; Somatic embryogenesis

Introduction

Date Palm (*Phoenix dactylifera* L.) is one of the most important horticultural agricultural crops in the countries of the Middle East, where the weather and soils are compatible with the agricultural needs of the palm plant (Jain 2012). The Kingdom of Saudi Arabia is considered the second largest producer of high-quality dates in the world (Assirey 2015). Although there are about 150 million palm trees planted around the world (Al-Khayri *et al.* 2018), this number has recently been subject to annual decreases due to environmental factors such as desertification and salinization accompanied by insect problems and physiological disease. Furthermore, ineffectiveness of sexual reproduction by seed and limitations in asexual reproduction by offshoot cuttings (macro-propagation) are limiting the replanting or expansion of date palm plantations (Al-Mayahi 2015).

To improve the rapid propagation of palms and avoid the negative aspects of using propagation with seeds or stem cuttings, plant breeders are resorting to using modern techniques of *in vitro* culture. Tissue culture techniques using the planting of various explants such as leaf, shoot tip, or somatic embryos are an effective alternative method compared to conventional methods of propagation (Brar and Khush 1994; Aldhebiani *et al.* 2018). Without success in obtaining an effective plant propagation protocol *in vitro*, the development in the use of other biotechnology techniques such as regeneration of transgenic plants and *in vitro* conservation of plant such as cryopreservation will be limited (Idowu *et al.* 2009). Accordingly, scientific research in this field is aimed at developing effective methods with a high success rate in the effectiveness of propagation and homogeneity (Rai *et al.* 2012; Reetika *et al.* 2019) and genetic stability of micropropagated plantlets (Eshraghi *et al.* 2005).

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Somatic embryogenesis has been advocated for rapid propagation *in vitro* and has proven to be effective for large-scale propagation of date palm and *in vitro* raised plants have been demonstrated to be genetically similar to mother plants (Faqir *et al.* 2019). This technique however, does carry some risk as it may be subject to failure due to the physiological disturbances experienced by the explants during culture (Mazri and Meziani 2015). The most well-known problems indicated by previous research are tissue browning which ofter leads to explant death (Mazri and Meziani 2015), hyperhydricity due to acumulation of water in the cultured explants (McCubbin and Zaid 2006) and precocious rooting which leads to a decrease in the multiplication efficiency of shoot buds (Khateeb 2008). In order to prevent browning, activated charcoal, PVP, ascorbic acid and citric acid have applied and added to the medium (Boufis *et al.* 2014). Mazri and Meziani (2013) and have indicated that increasing PGRs, amonium concentration and using liquid medium may lead to increasing hyperhydricity.

Date palm somatic embryo (SE) culture goes through the standard SE protocol of embryogenic callus induction, somatic embryo formation, somatic embryo growth, maturation and conversion (germination). Each of the steps in this process are often affected by many factors such as genotypes (Parrott *et al.* 1989; Kumar *et al.* 2020), plant growth regulator (PGRs) especially the combination ratio of auxin and cytokinin (Fatima and Anis 2012; Boufis *et al.* 2014; Abdolvand *et al.* 2014; Zayed *et al.* 2020), texture of the culture media (Al-Khayri 2011; Al-Khayri and Naik 2017; Salama 2019), time periods of mother plant material collection and size of explant (Moon *et al.* 2005) and intensity and quality of light (Meziani *et al.* 2015). The objective of the current study was to investigate the role of different concentrations and combinations of 2, 4-D, 2iP, NAA, IBA and kinetin to induce callus and somatic embryogenesis formation from shoot tip explants. We also evaluated somatic embryos from embryogenic lines established from two different date palm cultivars Safawi and Magdoul with respect to their ability to germinate and be converted into plantlets. Establishment of an optimized protocol for micropropagation of date palm will contribute to the success of both genetic transformation and cryopreservation experiments which will the subject of our future research.

Material and Methods

Plant material preparation and disinfection

Two high quality popularly grown Saudi Arabian date palm (*Phoenix dactylifera* L.) cultivars Safawi and Magdoul, according to (Lieb *et al.* 2019; Steingass *et al.* 2020), were chosen and applied in the current study. The study was conducted from March. 2018 until the end of Sept. 2019 year at Tissue Culture Units, Biological Sciences Dep., Fac. of Science, University of Jeddah, SA with cooperation with T issue Culture and Biotechnology Labs., Maryout Research Station, Desert Research Center, Ministry of Agriculture, Cairo, Egypt.

Offshoots (cuttings) of mother trees were washed with distilled water (DW), and stored until processed further. An offshoot was taken and shoot tip explants (8–10 cm in length) exposed and excised using a sharp knife. Shoot tips was surface sterilized using following procedure: 1) immersion in 50% Clorox (NaOCl at 5.25%) containing 2 drops of Tween-20 for 30 min followed by washing three times with sterilized distilled water (SDW), 2) immersion in 0.2% $HgCl_2$ solution for 5 min and rinsed with SDW three times, 3) shoot tip explants were then divided into 4 sections. All steps of the disinfection procedure were performed in a Laminar Air Flow "Hood" and aseptic conditions were applied accordingly (Aldhebiani *et al.* 2018).

Induction of calli

Shoot tip explants were cultured on induction medium (M1) including: 1) 4.4 mg L^{-1} MS (Murashige and Skoog 1962), 2) 170 mg L^{-1} Na H2 PO4, 3) 125 mgL^{-1} *myo*-inositol, 4) 200 mg L^{-1} glutamine, 5) 100 mg L^{-1} ascorbic acid, 6) 100 mg L^{-1} citric acid, 7) 5.0 mg L^{-1} thiamine-HCl, 8) 1.0 mg L^{-1} nicotinic acid, 9) 1.0 mg L^{-1} pyridoxine-HCl, 10) 30 mg L^{-1} sucrose, 11) 7.0 g L^{-1} agar, 12) 2.5 g L^{-1} activated charcoal and 13) 2.0 g L^{-1} gelrite, supplemented with different concentrations (10, 25, 50, 75 and 100 mg L^{-1}) of 2, 4-D alone or in combination with (2.0, 3.0, 5.0, 6.0 and 8.0 mg L^{-1}) of 2iP in order to optimize the best callus induction media. Four replicates of five of petri dishes (20 total) were set-up for each treatment and each petri dish cultured with 5 explants. To stimulate induction and growth of callus, the cultures were incubated in total darkness in the growth room at 25 ± 2°C for 12 months (period between August 2018 to July 2019), then callus induction percentage and RWC (%) was assessed accordingly (Elmeer and Hennerty 2008) and data was analzed as shown in Table (1).

Somatic embryo formation

Following on from callus induction 5–10 mg fresh weight of healthy callus was cultured on MS basal medium containing NAA at various rates (0.5, 1.0, 2.0, 3.0 and 4.0 mg L^{-1}) either alone or in combination with various concentrations of 2iP (1.0, 2.0, 4.0, 6.0 and 8.0 mg L^{-1}), each treatment comprised of five jars each with four callus pieces and was replicated 10 times. The cultured jars were grown in a growth room for 10 weeks at 25 ± 2°C under cool white fluorescent lamps with an intensity 70 μmol m^{-2} s^{-1} for 16 h photoperiod. After ten further weeks, data were recorded as number and percentage of somatic embryogenesis formation and analyzed (Table 2).

In vitro germination of somatic embryogenesis

The induction of somatic embryogenesis was carried out on with MS medium containing different concentrations of 2iP

at (2.0, 4.0 and 6.0 mg L^{-1}) alone or in combination with kinetin (2.0 and 3.0 mg L^{-1}) and IBA (0.5 and 1.0 mg L^{-1}). The experiment was designed and cultured in the same conditions as described in the previous stage. After 2 months the number of shoots formed and shoots length were recorded and analyzed (Table 3).

Rooting and acclimatization of regenerated plantlets

To obtain shoot elongation and root formation from *in vitro* germination of somatic embryos, MS culture was used supplemented with different concentration of NAA (1.0, 1.5, 2.0 and 2.5 mg L^{-1}) alone or in combination with IBA (0.5, 1.0 and 1.5 mg L^{-1}) in the presence of 2.5 g L^{-1} activated charcoal. Each was treatment replicated five times and each replicate was represented by three jars with each jar containing two plantlets. The cultures were kept in the same conditions as described for the somatic embryogenesis formation stage. Four weeks later, the following measurements were recorded: percentage of plantlets with root formation (%), root length (cm) and number of roots per plantlet (no.).

Acclimation of *in vitro* date palm plantlets followed the following procedure:

1) covers of cultured jars were opened, healthy date palm plantlets (5–8 cm long) gently removed and washed with tap water to remove any residual of traces the medium attached to the roots, 2) plantlets directly rinsed for three min in 1.0 mg L^{-1} Ridomil solution as a fungicide, 3) plantlets transferred to plastic pots filled with peat moss and sand (1:1 v/v), 4) Pots were covered with two layers of polyethylene, the lower layer was transparent while the upper layer was opaque, and placed in a greenhouse at 27°C under 16 h with cool-white fluorescence tubular lamp 50 µmol m^{-2} $s^{-1}$and watered with freshly modifed Hoagland nutrient solution (Cramer *et al.* 1986), 5) after two weeks, the opaque covers were removed, and the transparent cover was punctured to reduce the humidity, 6) after a further four weeks the transparent cover was removed and ten weeks later percentage of survived transplants (%) was recorded.

Statistical analysis

All experiments were arranged in a Randomized Complete Block Design (RCBD). Analysis of variance (ANOVA) and the calculation of LSD or Duncan's Mulitple Range test (0.005) was undertaken using CoStat software programme, version no. 6.4. All values were reported as means ± standard error according to Snedecor and Cochran (1989).

Results

Effect of PGRs

With the exception of the medium with 10 mg L^{-1} 2, 4-D 0.0 mg L^{-1} 2iP treatment all media hormone combinations induced callus in both cultivars (Safawi and Magdoul) of date palm (Table 1). However not all hormone combinations were equally effective and the highest induction rates (89.30%; 91.50%) were achieved with 25 mg L^{-1} 2, 4-D and 5 mg L^{-1} 2iP for cv. Safawi and 10 mg L^{-1} 2, 4-D and 8 mg L^{-1} 2iP for cv. Magdoul, respectively, after incubation for 12 months under dark regimes compared with other treatments (Fig. 1). These hormone combinations were also amongst the highest for Relative Water Content and Shoot Induction. Also, the optimum rate (+++) of shoot regeneration was recorded of cv. Safawi at 25 mg L^{-1} 2, 4-D with 5 or 3 mg L^{-1} 2iP and 10 mg L^{-1} 2, 4-D with 8 mg L^{-1} 2iP, while in cv. Magdoul 10 mg L^{-1} 2, 4-D with 6 or 8 mg L^{-1} 2iP and 100 mg L^{-1} 2, 4-D with 0.0 mg L^{-1} 2iP were more efficiency (+++) comparing with other treatments (Table 1). In contrast, MS medium supplemented with 75 mg L^{-1} 2, 4-D and 2.0 mg L^{-1} 2iP were less effective of callus induction percentage (17.05%; 14.35%) in cvs. Safawi and Magdoul, repectively. On the other hand, less relative water content percentage (36.28%; 30.05%) was recorded under MS medium supplemented with 25 mg L^{-1} 2, 4-D and 50 mg L^{-1} in the absent of 2iP of cvs. Safawi and Magdoul, repectively. It is noted that no shoot regeneration growths were recorded either when 2-4-D applied alone at 10, 25, 50 and 75 mg L^{-1} without adding hormone 2iP in both cultivars, or when it applied at 100 mg L^{-1} with different concentration of 2iP in cv. Safawi.

Somatic embryogenesis

The efficiency of somatic embryogenesis from callus samples explants was evaluated on MS basal medium containing different concentrations of 2iP alone or incombination with different concentration of NAA as described above in material and methods section. The results indiacted the somatic embryo (SE) formation was achieved in all media hormone combinations but ranged from 28% to 96% (Table 2). There was a strong correlation between the proportion of callus forming SE's and the number of SE's induced. The best hormone combinations differed between the 2 varieties with 6 mg L^{-1} 2iP + 1 mg L^{-1} NAA being the best for cv. Safawi and 6 mg L^{-1} 2iP + 3 mg L^{-1} NAA being best for cv. Magdoul (Fig. 2).

Somatic embryos germination and development of plantlets

All media hormone combinations showed the ability to promote SE germination (%) and the production of shoots (no.) and length of shoot (cm), the value ranged from 32% to 92%, 1.50 to 12.6 and 0.9 cm to 3.89 cm, repectively (Table 3). There was a strong correlation between germination % and the mean number of shoots produced and the shoot length such that the best media also gave the highest number of shoots and the tallest plantlets. For the cv. Safawi the best medium contained 6 mg L^{-1} 2iP + 3 mg L^{-1} Kin + 0.5 mg L^{-1} IBA, whilst for cv.

Table 1: The effect of different combination of 2,4-D and 2iP as a growth regulators on callus induction (%), relative water content (%) and adventitious shoot regeneration response of the *Phoenix dactylifera* L. cvs. Safawi and Magdoul using shoot tip section explants after 12 months

Growth regulators (mg L^{-1})		Callus induction (%)		Relative water content (%)		Shoot regeneration	
2,4-D	2iP	Safawi	Magdoul	Safawi	Magdoul	Safawi	Magdoul
10.0	0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-	-
25.0	0.0	20.40 ± 0.27j	16.53 ± 0.36i	25.38 ± 0.92h	36.28 ± 0.43f	-	-
50.0	0.0	25.30 ± 0.23j	12.45 ± 0.22i	28.42 ± 0.57g	30.05 ± 0.22f	-	-
75.0	0.0	45.30 ± 0.11h	25.15 ± 0.57g	38.09 ± 0.72f	42.25 ± 0.19e	-	+
100.0	0.0	65.43 ± 0.32f	79.04 ± 0.62b	41.21 ± 0.77e	74.18 ± 0.67b	+	+++
10.0	2.0	55.22 ± 0.74g	40.18 ± 0.56e	46.05 ± 0.59e	50.40 ± 0.49d	++	++
10.0	3.0	57.02 ± 0.44g	45.20 ± 0.59e	48.30 ± 0.29e	52.45 ± 0.81d	+	++
10.0	5.0	62.09 ± 0.84f	65.50 ± 0.74c	52.21 ± 0.50d	54.55 ± 0.19d	+	++
10.0	6.0	79.10 ± 0.48b	83.22 ± 0.38b	54.10 ± 0.47d	77.05 ± 0.83b	++	+++
10.0	8.0	82.15 ± 0.12b	91.50 ± 0.39a	56.45 ± 0.18d	79.90 ± 0.37a	+++	+++
25.0	2.0	78.25 ± 0.53c	72.34 ± 0.85b	65.38 ± 0.38c	68.18 ± 0.52c	++	+
25.0	3.0	80.34 ± 0.35b	74.45 ± 0.58b	69.29 ± 0.66b	69.25 ± 0.69c	+++	++
25.0	5.0	89.30 ± 0.53a	78.06 ± 0.36b	71.45 ± 0.39a	55.40 ± 0.72d	+++	++
25.0	6.0	77.19 ± 0.66c	77.18 ± 0.49b	70.05 ± 0.57b	58.34 ± 0.39d	++	+
25.0	8.0	74.22 ± 0.78d	75.02 ± 0.33b	68.54 ± 0.91c	69.62 ± 0.15c	++	+
50.0	2.0	72.06 ± 0.47e	76.54 ± 0.55b	59.55 ± 0.68d	62.55 ± 0.77c	+	+
50.0	3.0	66.76 ± 0.23f	70.04 ± 0.96b	58.30 ± 0.48d	60.20 ± 0.59c	+	+
50.0	5.0	52.22 ± 0.43g	63.20 ± 0.78c	61.23 ± 0.55c	64.19 ± 0.71c	+	+
50.0	6.0	50.19 ± 0.65g	61.30 ± 0.28c	63.05 ± 0.48c	66.05 ± 0.82c	+	+
50.0	8.0	44.03 ± 0.67h	48.65 ± 0.33e	65.20 ± 0.58c	67.08 ± 0.60c	+	+
75.0	2.0	17.05 ± 0.34k	14.35 ± 0.43i	28.28 ± 0.82g	32.20 ± 0.71f	+	+
75.0	3.0	28.33 ± 0.21j	19.52 ± 0.35h	39.50 ± 0.62f	40.25 ± 0.53e	+	+
75.0	5.0	32.25 ± 0.72i	29.18 ± 0.84g	45.03 ± 0.57e	44.32 ± 0.61e	+	+
75.0	6.0	42.16 ± 0.45h	38.54 ± 0.76f	47.15 ± 0.45e	46.05 ± 0.67e	+	+
75.0	8.0	49.15 ± 0.55h	43.25 ± 0.87e	48.08 ± 0.22e	49.30 ± 0.80e	+	+
100.0	2.0	72.55 ± 0.76d	66.15 ± 0.68c	28.20 ± 0.75g	50.52 ± 0.25d	-	++
100.0	3.0	59.20 ± 0.35g	68.05 ± 0.26c	52.04 ± 0.39d	60.08 ± 0.61c	-	++
100.0	5.0	53.40 ± 0.88g	62.30 ± 0.63c	50.28 ± 0.31d	58.05 ± 0.29d	-	++
100.0	6.0	44.52 ± 0.67h	52.22 ± 0.46d	58.02 ± 0.94d	62.42 ± 0.33c	-	++
100.0	8.0	43.30 ± 0.38h	49.16 ± 0.33e	59.55 ± 0.88d	69.50 ± 0.49c	-	++

Values are means ± standard error of three replicates from two experiments. For each cultivar, bars with the same letters are not significantly different at $P \leq 0.05$ level. Shoot regeneration are visually estimated as No regeneration= -, Poor= +, Good= ++, very good= +++

Table 2: Influence of plant growth regulators concentrations on percentage of somatic embryos formation /explant and average number of somatic embryos / 1.0 g FW of callus of the date palm cvs. Safawi and Magdoul after 10 weeks

Growth regulators (mg L^{-1})		Percentage of somatic embryos formation/explant		Average number of somatic embryos/1.0 g FW of callus	
2iP	NAA	Safawi	Magdoul	Safawi	Magdoul
1.0	0.5	28.65 ± 0.52j	32.25 ± 0.85h	210.75 ± 0.55j	130.40 ± 0.67j
2.0	0.5	42.49 ± 0.65h	39.35 ± 0.37h	338.25 ± 0.29h	180.95 ± 0.39i
4.0	0.5	48.35 ± 0.59h	42.25 ± 0.59g	340.20 ± 0.62g	190.00 ± 0.53i
6.0	0.5	52.70 ± 0.25g	52.15 ± 0.27f	350.00 ± 0.18g	195.25 ± 0.77i
8.0	0.5	59.25 ± 0.22g	55.25 ± 0.55f	352.88 ± 0.51g	220.35 ± 0.49h
1.0	1.0	65.90 ± 0.35f	45.28 ± 0.68g	378.15 ± 0.88f	310.00 ± 0.92g
2.0	1.0	82.50 ± 0.92d	62.14 ± 0.33e	420.15 ± 0.90d	330.25 ± 0.33g
4.0	1.0	90.45 ± 0.58c	66.25 ± 0.69e	432.70 ± 0.95c	345.85 ± 0.79g
6.0	1.0	96.18 ± 0.15a	72.25 ± 0.49d	465.25 ± 0.38a	482.30 ± 0.17d
8.0	1.0	92.65 ± 0.59b	70.25 ± 0.22d	438.40 ± 0.62b	489.35 ± 0.31d
1.0	2.0	35.22 ± 0.77i	38.18 ± 0.19h	310.18 ± 0.44i	280.87 ± 0.55h
2.0	2.0	38.40 ± 0.63i	40.00 ± 0.25g	328.00 ± 0.29h	258.14 ± 0.39h
4.0	2.0	41.25 ± 0.81h	51.35 ± 0.50f	325.14 ± 0.35h	320.48 ± 0.73g
6.0	2.0	48.55 ± 0.39h	52.00 ± 0.58f	339.85 ± 0.77g	389.15 ± 0.65f
8.0	2.0	50.25 ± 0.25g	55.40 ± 0.90f	348.45 ± 0.12g	395.25 ± 0.45f
1.0	3.0	39.28 ± 0.44i	68.45 ± 0.88e	350.00 ± 0.52g	402.27 ± 0.28f
2.0	3.0	43.58 ± 0.96h	74.35 ± 0.42d	338.25 ± 0.78g	480.50 ± 0.69d
4.0	3.0	62.35 ± 0.19f	88.25 ± 0.17c	371.25 ± 0.38f	558.75 ± 0.75b
6.0	3.0	73.10 ± 0.33e	92.25 ± 0.56a	410.22 ± 0.29e	568.25 ± 0.50a
8.0	3.0	75.40 ± 0.71e	90.35 ± 0.25b	412.75 ± 0.75e	542.28 ± 0.88c
1.0	4.0	55.30 ± 0.88g	67.28 ± 0.91e	349.55 ± 0.39g	335.12 ± 0.29g
2.0	4.0	68.25 ± 0.39f	69.35 ± 0.15e	380.65 ± 0.45f	363.70 ± 0.91g
4.0	4.0	48.30 ± 0.47h	55.65 ± 0.72f	338.25 ± 0.92h	280.25 ± 0.78h
6.0	4.0	60.15 ± 0.66f	68.25 ± 0.49e	370.25 ± 0.20f	465.25 ± 0.68e
8.0	4.0	69.25 ± 0.32f	69.75 ± 0.33e	382.39 ± 0.89f	471.15 ± 0.60e

Values are means ± standard error of three replicates from two experiments. For each cultivar, bars with the same letters are not significantly different at $P \leq 0.05$ level

Table 3: Influence of plant growth regulators concentrations on somatic embryos germination percentage, average number of shoots formation and average shoots length of the date palm Safawi and Magdoul after 2 months

Growth regulators (mg L^{-1})			Somatic embryos germination (%) (germinated/embryo s tested)		Mean number of shoots formation/(1.0 g callus)		Mean length of shoots/explant (cm)	
2iP	Kin	IBA	Safawi	Magdoul	Safawi	Magdoul	Safawi	Magdoul
2.0	2.0	0.0	32.35 ± 0.26i	36.25 ± 0.18g	1.50 ± 0.29h	2.75 ± 0.98h	0.95 ± 0.19f	1.04 ± 0.28g
4.0	2.0	0.0	38.25 ± 0.59h	36.85 ± 0.22g	1.65 ± 0.78h	2.82 ± 0.73h	1.05 ± 0.26e	1.38 ± 0.66g
6.0	2.0	0.0	40.15 ± 0.44g	55.29 ± 0.75e	2.25 ± 0.66g	3.70 ± 0.48g	1.69 ± 0.34d	2.84 ± 0.43c
2.0	3.0	0.0	38.28 ± 0.78h	36.45 ± 0.55g	1.58 ± 0.57h	2.78 ± 0.22h	0.98 ± 0.26f	1.69 ± 0.36f
4.0	3.0	0.0	38.75 ± 0.33h	38.65 ± 0.92g	2.59 ± 0.48g	3.65 ± 0.67g	1.45 ± 0.15e	1.72 ± 0.76e
6.0	3.0	0.0	59.85 ± 0.29e	69.55 ± 0.63d	2.85 ± 0.19g	3.90 ± 0.50g	1.72 ± 0.66d	2.95 ± 0.92b
2.0	2.0	0.5	37.38 ± 0.93h	40.25 ± 0.66f	2.95 ± 0.33g	3.45 ± 0.18g	1.08 ± 0.35e	1.49 ± 0.47f
4.0	2.0	0.5	40.48 ± 0.49g	45.39 ± 0.49f	3.65 ± 0.92f	5.25 ± 0.55f	1.63 ± 0.41d	1.68 ± 0.22f
6.0	2.0	0.5	63.25 ± 0.77d	81.40 ± 0.84b	4.49 ± 0.85e	6.87 ± 0.72e	2.75 ± 0.84b	2.86 ± 0.46c
2.0	3.0	0.5	46.29 ± 0.58f	40.69 ± 0.99f	4.15 ± 0.77e	5.89 ± 0.33f	1.15 ± 0.55e	1.75 ± 0.74e
4.0	3.0	0.5	78.55 ± 0.99c	69.75 ± 0.29d	6.25 ± 0.86c	8.58 ± 0.91d	1.55 ± 0.29d	1.89 ± 0.36e
6.0	3.0	0.5	90.45 ± 0.52a	76.55 ± 0.26c	8.45 ± 0.39a	9.75 ± 0.27c	2.95 ± 0.39a	3.05 ± 0.73b
2.0	2.0	1.0	55.25 ± 0.26e	57.45 ± 0.82e	3.67 ± 0.38f	5.39 ± 0.49f	1.38 ± 0.62e	2.15 ± 0.55d
4.0	2.0	1.0	56.85 ± 0.88e	59.44 ± 0.78e	5.90 ± 0.27d	8.25 ± 0.44d	2.15 ± 0.91c	2.93 ± 0.62b
6.0	2.0	1.0	75.45 ± 0.26c	92.55 ± 0.65a	6.35 ± 0.45c	12.65 ± 0.62a	2.92 ± 0.49a	3.89 ± 0.39a
2.0	3.0	1.0	57.39 ± 0.49e	69.15 ± 0.16d	4.28 ± 0.31e	5.91 ± 0.15f	1.25 ± 0.77e	1.78 ± 0.96e
4.0	3.0	1.0	73.68 ± 0.79c	79.38 ± 0.39c	6.69 ± 0.95c	9.72 ± 0.88c	2.05 ± 0.82c	1.92 ± 0.64e
6.0	3.0	1.0	85.15 ± 0.60b	89.40 ± 0.56b	7.35 ± 0.44b	10.40 ± 0.48b	2.78 ± 0.96b	3.82 ± 0.38a

Values are means ± standard error of three replicates from two experiments. For each cultivar, bars with the same letters are not significantly different at $P \leq 0.05$ level.

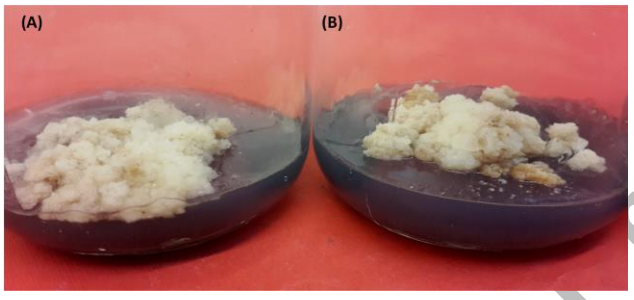

Fig. 1: *In vitro* callus induction from pieces of shoot tips of date palm cvs. Safawi and Magdoul. **A**) Induction of callus on MS medium supplemented with 25 mg L^{-1} 2,4-D and 5.0 mg L^{-1} 2iP in the presence of 2.5 g L^{-1} activated charcoal of cv, Safawi after 12 months. **B**) Induction of callus on MS medium supplemented with10 mg L^{-1} 2,4-D and 8.0 mg L^{-1} 2iP of cv. Magdoul after 12 months

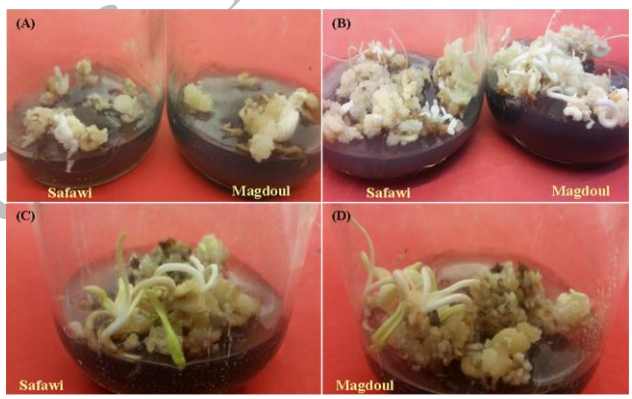

Fig. 2: Somatic embryogenesis formed from callus cultured on MS containing with 6.0 mg L^{-1} 2iP and 1.0 mg L-1 NAA of cvs. Safawi and Magdoul (**A**) and 6.0 mg L^{-1} 2iP and 3.0 mg L^{-1} NAA of cvs. Safawi and Magdoul cultivar in the presence of 2.5 g L^{-1} activated charcoal after 10 weeks (**B**). Somatic embryos germinated on MS medium supplemented with 6.0 mg L^{-1} 2iP, 3.0 mg L^{-1} Kin and 0.5 mg L^{-1} IBA of cv. Safawi (**C**) and 6.0 mg L^{-1} 2iP and 2.0 mg L^{-1} Kin and 1.0 mg L^{-1} IBA of cv. Magdoul in the presence of 2.5 g L^{-1} activated charcoal after 2 months (**D**)

Magdoul the best medium contained 6 mg L^{-1} 2iP + 2 mg L^{-1} Kin + 1 mg L^{-1} IBA (Fig. 3A and B). The highest value of SE germination (%) and the production of shoots (no.) and length of shoot (cm) was recorded of cv. Magdoul comparing with cv. Safawi (Table 3).

Rooting formation and acclimatization of regenerated plantlets

In this investigation isolated single shoots of cvs. Safawi and Magdoul were maintained in the dark for seven days following with incubation under 16 day/ 8 night photoperiod for three weeks to induce rooting as clarified in Fig. (3C). All media hormone combinations were capable of the induction of roots in shoots obtained from SE but they were not all equally effective with the proportion of shoots forming roots ranging from 38% to 98% **(**Fig. 4). For the cv. Safawi the best medium was 6 mg L^{-1} NAA + 0.5 mg L^{-1} IBA and for cv. Magdoul the best was 2.5 mg L^{-1} NAA + 0 mg L^{-1} IBA (Fig. 4). The data showed that the highest rooting percentage (96.72%) were obtained on MS medium supplemented with 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} IBA in the present of 2.5 g L^{-1} activated charcoal. Also, under this treatment the highest significant differences were observed of mean number of roots/shoot (6.85) and mean length of roots/shoot (5.94 cm) of cv. Safawi compared with other treatments. While, the highest shoot forming roots percentage (98.25%), the highest mean number of

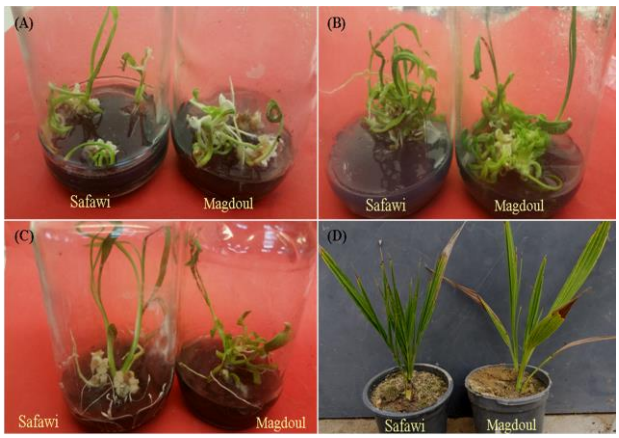

Fig. 3: Plantlet obtained from a converted somatic embryo of date palm cvs. Safawi and Magdoul on 6.0 mg L^{-1} 2iP and 2.0 mg L^{-1} Kin and 1.0 mg L^{-1} IBA in the presence of 2.5 g L^{-1} activated charcoal under light after six weeks (**A**) and after eight weeks (**B**). Development of roots obtained on MS medium supplemented with 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} IBA of cv. Safawi and 2.5 mg L^{-1} NAA of cv. Magdoul in the presence of 2.5 g L^{-1} activated charcoal (**C**). Plants after acclimatization to free living conditions (**D**)

roots/shoot (7.45) and mean length of roots/shoot (6.25 cm) of cv. Magdoul was obtained on MS medium supplemented with 2.5 mg L^{-1} NAA in the present 2.5 g L^{-1} activated charcoal compared with other treatments (Fig. 3C).

In vitro plantlets with well-formed roots were transplanted to a sand: soil media, then placed back in the same growth chamber, for primary hardened, for 8 weeks and then transferred to the greenhouse. After 6–8 months new leaves had developed **(**Fig. 3D). During the acclimatization period, the results indicated that 55–70% of plantlets successfully survived and all had survived two months later. Plants with healthy growth were then transferred to the open field and both cvs. Safawi and Magdoul Plants gave showed a good normal growth in soil and appeared phenotypically normal.

Discussion

The results clearly demonstrated that 25 mg L^{-1} 2, 4-D with 3.0 mg L^{-1} 2iP and 10 mg L^{-1} 2, 4-D with 8 mg L^{-1} 2iP gave high rates of callus induction with high relative water content for varieties Safawi and Magdoul, respectively **(**Table 1). It is not surprising that the two varieties had different optimal media hormone combinations as it has been shown by Arimarsetiowati and Prastowo (2020) that different responses of induction of callus and formation of embryogenic calli can be induced by different 2, 4-D and 2iP concentration ratios. Such differences are attributed to genetic factors among cultivars. Ozias–Akins and Vasil (1982) showed that low levels of 2, 4-D stimulate the division of cells but increasing the level beyond 2 mg L^{-1} can inhibit cell division in wheat. Furthermore, treating

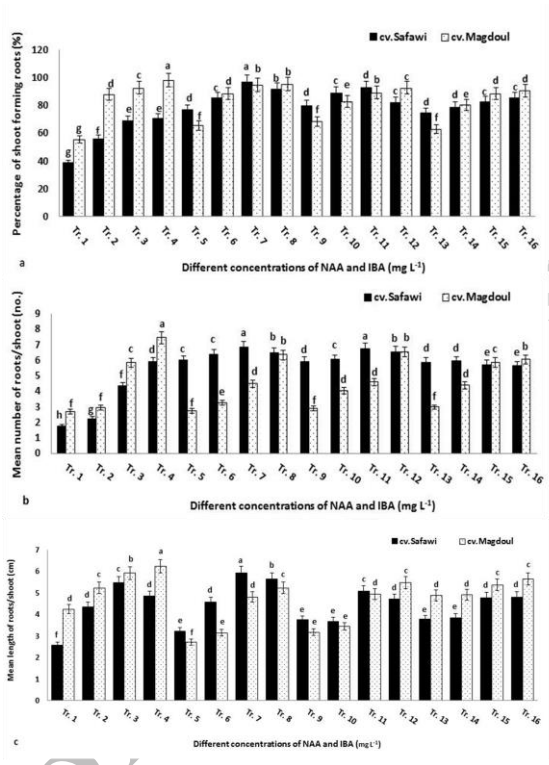

Fig. 4: Effect of MS medium supplemented with different concentrations of auxins; NAA and IBA on the a) Percentage of shoot forming roots, b) mean number of roots/shoot (no.), c) mean length of roots/shoot (cm) of date palm cvs. Safawi and Magdoul after 4 weeks. Tr.1, Tr.2, Tr.3, and Tr.4 = 1, 1.5, 2.0 and 2.5 mg L^{-1} NAA, respectively; Tr.5 = 1 mg L^{-1} IAA and 0.5 mg L^{-1} IBA, Tr.6 = 1.5 mg L^{-1} IAA and 0.5 mg L^{-1} IBA, Tr.7 = 2 mg L^{-1} IAA and 0.5 mg L^{-1} IBA, Tr.8 = 2.5 mg L^{-1} IAA and 0.5 mg L^{-1} IBA, Tr.8 = 1 mg L^{-1} IAA and 1 mg L^{-1} IBA, Tr.10 = 1.5 mg L^{-1} IAA and 1.0 mg L^{-1} IBA, Tr.11 = 2 mg L^{-1} IAA and 1.0 mg L^{-1} IBA, Tr.12 = 2.5 mg L^{-1} IAA and 1 mg L^{-1} IBA, Tr.13 = 1 mg L^{-1} IAA and 1.5 mg L^{-1} IBA, Tr.14 = 1.5 mg L^{-1} IAA and 1.5 mg L^{-1} IBA, Tr.15 = 2 mg L^{-1} IAA and 1.5 mg L^{-1} IBA, Tr.16 = 2.5 mg L^{-1} IAA and 1.5 mg L^{-1} IBA

callus cells with high concentrations of 2, 4-D may lead to increased cytoplasm concentration with a consequent significant reduction in relative water content acting as an induced osmotic stress (Pan *et al.* 2010). Under high 2, 4-D the protein DRT102 which plays an important role in DNA replication and cell division my be suppressed (Pasternak *et al*. 2002) and lead to the decreased fresh weight and total cell number (Ozias–Akins and Vasil 1982). Also, it is proven that adding auxin (*e.g*., 2, 4-D) to artficial growth medium stimulates the cell to increase cells division and discourages the process of differentiation. Thus, this contributes to the orientation of the cell to induce diverse developmental processes such as somatic embrygenesis and established root induction (Aderkas and Bonga 2000). The difference between cultivars in optimum induction medium was in agreement with Sané *et al.* (2012) who also reported a difference in the rate of callus formation for different

cultivars of palm. Furthermore, Gonzalez *et al.* (2001) and Rathore *et al.* (2020) also reported a strong dependence of callus induction on genotype and induction media used.

Percentage of somatic embryos formation/explant and average number of somatic embryos per 1.0 g FW of callus showed a varied response in the palm cultivars studied. The presence of 2iP at higher concentration (6.0 mg L^{-1}) in combination with 1.0 or 3.0 mg L^{-1} NAA gave the best results. While under low concentration of 2iP (1.0, 2.0 and 4.0 mg L^{-1}) induction was significantly lower (Table 2). This indicated that somatic embryoegensis required a high concentration of cytokinins and a low or medium concentration of auxin. Similar results have been recently reported in *Digitalis lanata* Ehrh (Bhusare *et al.* 2020). Removal of 2, 4-D from the somatic embryogensis medium results in a lowering of amounts of endogenous antioxidants (glutathione, ascorbic acid, vitamin E), which stimulates somatic embryo development an hypothesis was purported by Aderkas and Bonga (2000).

The different concentrations of 2iP alone or in combination with Kin or IBA were highly effective for somatic embryo germination and plantlet development in both varieties studied (Table 3). In most plant species, the success of plant tissue culture *in vitro* depends on the combinations between auxin and cytokinin. While both are essential to promote and control cell division, only the cytokinins stimulate the formation of the shoot system whilst auxins stimulate the formation of the root system (Meziani *et al.* 2019). The differential ability of cytokinins in induction of shoots is frequently attributed to factors such as stability, mobility, and the rate of conjugation and oxidation of the hormones (D'Onofrio and Morini 2005). In this study significant differences were found between the cultivars under the same hormone treatments, for example a concentration of 6.0 mg L^{-1} 2iP plus 3.0 mg L^{-1} and 0.5 mg L^{-1} Kin recorded the highest value of somatic embryos germination % (90.45 ± 0.52) in cv. Safawi, but for cv. Magdoul germination % was only 76.55 ± 0.26. This can be explained on the basis of the different genotypes giving rise to differences in their endogenous hormone contents. Thus the accumulation and sensitivity to hormone concentrations in media could explain the genotypic variations of embryogenic potential (Hadrami *et al*. 1995; Hadi *et al*. 2015).

The stage of root formation is the last and critically important stage in the tissue culture procedure and is a key step in micropropagation because without it plants cannot be weaned and develop *in vivo* status (Klerk *et al.* 1997). To obtain a high percentage of rooted shoots, many studies indicate the importance of exogenous auxins such asIAA, IBA and NAA (Abdelaziz *et al.* 2019; Kumar *et al.* 2020). In our study combinations of NAA and IBA had a positive impact on inducing root, mean number of roots/shoot and mean length of root/shoot (Fig. 4). The study found thatNAA at 2.5 or 2.0 mg L^{-1} in combination with low concentrations of IBA at 0.0 or 0.5 mg L^{-1} was the best concentration to induce roots (96.72 ± 0.91; 98.25 ± 0.84), number of roots/shoot (6.85 ± 0.25; 7.45 ± 0.52) and mean length of root/shoot (5.94 ± 0.75; 6.25 ± 0.62) of cvs. Safawi and Magdoul, respectively. Higher auxin concentrations has been linked to the production of ethylene and degradative metabolites in tissues which impede root formation. Sanchez *et al.* (2020) also noted that rooting is promoted at low auxin concentrations and inhibited at high (supraoptimal) concentrations with Iraca palm (*Carludovica palmata* Ruíz & Pavón). It is worth noting that IBA is more commonly used to induce root compared to IAA and NAA, but in the current study, NAA has been used because: 1) it is considered best in the case of plants that have a high auxin-oxidase activity, 2) it is stable and persistent in the tissue in it is free form, 3) it is taken up faster and 4) low concentrations have been shown to be efficient to produce roots in other species (Smulders *et al.* 1990). Plantlet acclimatization was successfully achieved with a good survival rate of 55–70% which is similar to other studies on date palm (Mazri and Meziani 2015). Indeed the survival rate in previous studies of date palm recorded 50% (Mazri 2015), 60 % (Kurup *et al.* 2014), 60–80% (Othmani *et al.* 2009), 72–84% (Al-Khayri 2010) and 92% (Meziani *et al.* 2015). This variation in the survival rate (minimum of 50% and maximum rate of 92%) has not been discussed before, but the explanation is probably due to variations in genotypes studied, the method of acclimatization followed and the micropropagation technique used.

Conclusion

The present study provides a new optimised protocol of *in vitro* propagation of two high quality popularly grown Saudi Arabian date palm (*Phoenix dactylifera* L.) This protocol will be useful for continous production of somatic embryos/plantlets for *ex vitro* transplantation and genetic conservation via *in vitro* germplasm bank for those cultivars. The approach used here with the systematic variation in hormone combinations is also a sensible approach for optimization with other elite date palm cultivars.

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Author Contributions

EMRM, NMSK and HIAS planned the experiments, carried all the experiment lab work and collected the data, OAA wrote the literature review and interpreted the results, EMRM, HIAS and MPF made statistically analyzed of the data and made illustrations and MPF made the language editing.

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