

## COMPARISON AMONG DIFFERENT AUXINS AND CYTOKININS TO INDUCE DATE PALM (*PHOENIX DACTYLIFERA* L.) SOMATIC EMBRYOGENESIS FROM FLORAL BUDS

NAJAMUDDIN SOLANGI<sup>1</sup>, ADEL AHMED ABUL-SOAD<sup>2</sup>, GHULAM SARWAR MARKHAND<sup>1</sup>, MUSHTAQUE AHMED JATOI<sup>1</sup>, TAHIRA JATT<sup>1</sup> AND ABDUL AZIZ MIRANI<sup>1</sup>

<sup>1</sup>Date Palm Research Institute, Shah Abdul Latif University, Khairpur 66020, Sindh, Pakistan

<sup>2</sup>Horticulture Research Institute, Agricultural Research Center, Giza, Egypt

\*Corresponding author's email: najamsolangi@gmail.com

### Abstract

The influence of different auxins and cytokinins combinations in inducing callus and somatic embryogenesis (SE) in two top commercial date palm cultivars of Pakistan Aseel and Dhakki were studied. In addition, the role of different concentrations of NaOCl (10%, 20%, 40% and 50%) in successful surface sterilization and as well as the influence of the spathe's dimension (17, 28 and 32 cm in length) to induce callus were also investigated. The findings obtained showed varietal variation to induce somatic embryogenesis. The results obtained showed using 50% NaOCl solution produced significantly the lowest mortality and contamination percentages. The spikes' explant obtained from immature inflorescence of 17 cm long and 6.2 cm in width gave significantly the highest callus formation in both cultivars. The MS basal medium supplemented with 2.0 mg L<sup>-1</sup> 2,4-D and 0.5 mg L<sup>-1</sup> 2iP formed white compact callus in the 87.3% of cv. Aseel and 84.3% of cv. Dhakki explants. On the other hand, the initiation medium provided with 2.0 mg L<sup>-1</sup> NAA and 0.5 mg L<sup>-1</sup> 2iP significantly induced callus in cvs. Aseel (90.3%) and Dhakki (78%). Differentiation of the embryogenic callus occurred using 0.05 mg L<sup>-1</sup> 2,4-D and 2.0 mg L<sup>-1</sup> 2iP and the highest induction of non-repeated embryos (NRE) were achieved in most of cultures in cvs. Aseel (88.3%) and Dhakki (86%). But the direct shifting of embryogenic callus on PGRs free MS medium under full darkness after 9 months of initiation produced repeated embryos. These findings will expectedly help to define the appropriate size of spathe and plant growth regulators formula for somatic embryogenesis for commercial production of date palm.

**Key words:** Antifungal, *Phoenix dactylifera* L., Callus, Somatic embryos, Auxins, Cytokinins.

### Introduction

The date palm is the type specimen of the genus *Phoenix* of *Arecaceae* family, diploid species with dioecious nature and one of the major horticultural crop dispersed under the warm climatic conditions worldwide (Hazzouri *et al.*, 2015; Abul-soad *et al.*, 2017; Al-Qurainy *et al.*, 2020). Pakistan is always ranked among the top dates producing and exporting countries with an ancient cultivation trend dating back to Indus Civilization (Jatoi *et al.*, 2009; Markhand *et al.*, 2010). Date palm can be propagated by seeds and vegetatively using offshoots. However, micropropagation is the preferred mean to produce vast number of true-to-type plants and free from pests (Abul-Soad, 2011; Al-Khalifah & Askari, 2011; Jatoi *et al.*, 2015).

Somatic embryogenesis is an efficient pathway as compared to other methods (Quiroz-Figueroa *et al.*, 2006; Abul-Soad, 2011) which provides large-scale production (Zaid & de Wet, 2002; Fki *et al.*, 2011; Abul-soad *et al.*, 2017). The need for date palm propagation materials is increasing with an estimate of 1-2 million plants per year in the international market (Jain, 2007). This made the commercial labs outside the date palm origin to work on developing viable somatic embryogenesis protocols (Abul-Soad & Mahdi, 2010).

The attempts of micropropagation of date palm started since 1970s using the shoot tip explants of young offshoots (Abul-Soad *et al.*, 2002). But recently inflorescence explants have been proved valuable and promising to micropropagate palms especially those at risk of danger because of many advantages compared to the shoot tip (Abul-Soad, 2011). Moreover, the applied technique of floral buds through direct somatic embryogenesis produced very low percentage of

somaclonal variations after open field fruiting, and in some varieties with no variations at all (Mirani *et al.*, 2019). It is worth to mention that such protocol should be optimized for each cultivar as genotype, explant age and auxin type-dependent somatic embryogenesis process (Abul-Soad & Al-Khayri, 2018; Abul-Soad *et al.*, 2017).

Protocols established mostly for tissue culture of date palm done with offshoot derived explants (terminal shoot tip, primordial leaves and axillary buds) while very less work has been done using the potential of floral buds or inflorescence explants (Abul-Soad, 2011; Al-Mayahi, 2013; Jatoi *et al.*, 2015; Jatoi *et al.*, 2019). In addition, there is scarcity of literature available regarding evaluating the efficient role of different auxins and cytokinins on date palm somatic embryogenesis using floral buds (Abul-Soad, 2012). Therefore, this research has been conducted to explore the potential of inflorescence explants, size of spathe and the influence of different PGRs for *In vitro* production of date palm plants.

### Material and Methods

The experiments were conducted in the Biotech Lab of Date Palm Research Institute (DPRI), Shah Abdul Latif University (SALU), Khairpur, Sindh, Pakistan.

**Plant material:** The healthy plants of the main cultivars of Pakistan Aseel and Dhakki grown in the Research Orchard of DPRI were selected to get immature inflorescences. The immature inflorescences of different sizes (17, 28 and 32 cm long) were then excised from them prior to emergence. Excised inflorescences were preserved in clean plastic bags and transferred to the lab for surface sterilization.

**Explant preparation:** The intact inflorescences (Fig. 1A) were immersed into fungicide solution (2 g L<sup>-1</sup> Bavistin-DF) for one minute without shaking and washed with running tap water for 60 seconds. Under the laminar air-flow cabinet, different concentrations (10%, 20%, 40% and 50%) of sodium hypochlorite (NaOCl) solutions were used for 5 min with addition of few drops of Tween-20. The spathes were washed with distilled sterilized water 3 to 4 times.

The outer hard cover/sheath of each inflorescence was removed carefully without damaging the spikelets of inflorescence bunch under aseptic conditions (Fig. 1B). The spikelets were cut into 3 cm long (each explant possessed 8-10 immature florets) and cultured on different concentrations of the induction media containing auxin and cytokinin (Fig. 1C).

**Media preparations and culture conditions:** The Murashige & Skoog (1962) medium supplemented with different combinations of auxins (2,4-D and NAA) and the cytokinin 2iP specified to callus and embryo formation stage of date palm cultures was used (Table 1). The cultures were kept under complete dark conditions at 27±2°C in growth room. Subculturing of explants on fresh medium was done after every 3 weeks of interval.

#### Statistical analysis

Every treatment has accommodated the two studied cultivars, 3 replicates in each cultivar and an individual spikelet explant was cultured in a culture tube. The data is presented as means and the significance of differences among means were obtained using LSD test at  $p < 0.05$  using Statistix 8.1 software. A set of measurements: explant mortality %, contamination % and survival %, average callus and embryo formation % were taken after each subculture.

#### Results and Discussion

The findings obtained in the current study induced successfully callus and embryo formation using different concentrations of auxins and cytokinins from the date palm inflorescence explants in relation to strong impact of the inflorescence size. This in addition, a viable surface sterilization protocol using NaOCl was developed.

#### Influence of different NaOCl concentrations on surface sterilization of cvs. Aseel and Dhakki inflorescences:

The treatment of NaOCl solution of 50% (5.25% NaOCl) proved the best in terms of getting high survival % and the lowest contamination % and mortality % in both studied cultivars (Table 2). According to the protocol developed by Abul-Soad (2011), there was no need to sterilize spikelets explants exist within the intact immature spathe. Cracks in the outer spathe cover occurred during its excision from the parent tree or even during the transfer from field to the laboratory contaminated whole initial *In vitro* cultures within few days. The spikelets inside the cover sheath were contaminants-free and gave excellent results in tissue culture protocols of date palm (Abul-Soad *et al.*, 2007; Abul-soad *et al.*, 2008; Abul-Soad & Mahdi, 2010). This is what was noticed in all explants during initiation stage, however few explants contaminated with fungus during successive subcultures most probably due to the improper handling during culturing. Up to 90% of all *In vitro* cultured explants were free from all type of contaminants and developed into healthy compact callus (Table 2). While, cracks in the spathes can increase contamination rate up to 100%.

Data presented in Table 2 showed that survival% of spikelets explants in both cultivars increased by increasing the NaOCl from 10% to 50%. The NaOCl concentration (50%) recorded with significantly highest survival (90%) and lowest contamination (4%) and mortality (6%) in cv. Aseel while in cv. Dhakki maximum survival (88%) and lowest contamination (7%) and mortality (5%) was observed on same treatment of NaOCl. On the contrary decreased survival, increased contamination and mortality of the explants were observed when the spathe was surface sterilized with 10% NaOCl (Table 2).

It is worth to mention that browning in the explants was controlled by quick shifting (3 weeks interval) of explants onto fresh medium during initiation stage. Since, shifting of explants to fresh medium was found crucial to avoid further oxidation of phenolic compounds exuded by explants on induction medium. Cultures growth was better without adding antioxidants in the medium because the inflorescence cultures exude least phenolic compounds as compare to offshoots' explants.

**Table 1. Media composition for somatic embryogenesis of date palm (cvs. Aseel and Dhakki) from immature inflorescence.**

Growth stage	Medium composition (mg L <sup>-1</sup> )			
	Salts	Additives	Auxins	Cytokinins
Initiation	Micro and Macro salts of MS*	30000 Sucrose + 6000 Agar + Vitamins of MS + 170 KH <sub>2</sub> PO <sub>4</sub> + 200 Glutamine + 40 Adenine Sulphate	M1**. 2,4-D (0.1, 0.5, 1.0, 2.0) M2. NAA (0.1, 0.5, 1.0, 2.0)	M1. 2iP (0.1, 0.5) M2. 2iP (0.1, 0.5)
Differentiation	Micro and Macro salts of MS	30000 Sucrose + 6000 Agar + Vitamins of MS + 170 KH <sub>2</sub> PO <sub>4</sub> + 200 Glutamine + 40 Adenine Sulphate	M1. 2,4-D (0.01, 0.02, 0.04, 0.05) M2. NAA (0.01, 0.02, 0.04, 0.05)	M1. 2iP (1.0, 2.0) M2. 2iP (1.0, 2.0)

\*Murashige and Skoog (1962) medium; \*\*Medium

KH<sub>2</sub>PO<sub>4</sub> = Potassium dihydrogen phosphate, 2,4-D = 2,4-Dichlorophenoxyacetic acid, NAA =  $\alpha$ -Naphthalene acetic acid, 2iP = 2-isopentenyl adenine

**Table 2. Effect of different concentrations of NaOCl solutions used for surface sterilization of immature inflorescences of date palm (cvs. Aseel and Dhakki).**

NaOCl (%)	cv. Aseel			cv. Dhakki		
	Survival %	Contamination %	Mortality %	Survival %	Contamination %	Mortality %
10	17 c	70 a	13 a	21 c	72 a	7 ab
20	22 c	67 a	11 a	20 c	70 b	10 a
40	65 b	25 b	10 a	70.6 b	6.6 c	6.3 b
50	90 a	4 c	6 b	88 a	7 c	5 b
LSD at $p < 0.05$	0.0001***	0.001**	0.045*	0.0001***	0.0001***	0.05*

Means on the same column with different letters are significantly different at  $p < 0.05$

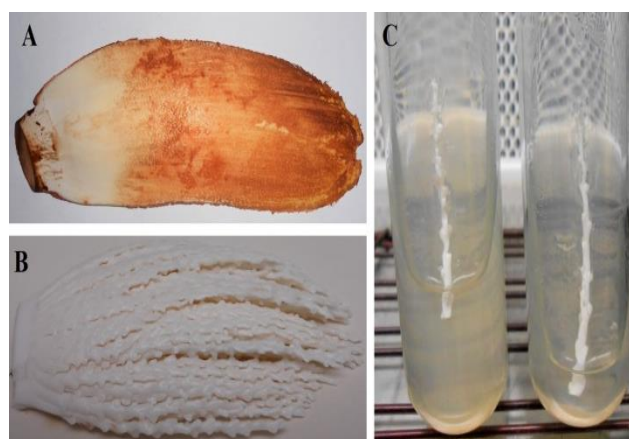


Fig. 1. Explant preparation, (a) Date palm inflorescence without any crack, (b) The bunch of spikes after removal of outer protective sheath of inflorescence, (c) the culturing of spikelets on initiation medium.



Fig. 2. Callus induction of floral buds obtained from 17 cm long immature inflorescence.



Fig. 3. Spike explants excised from 32 cm long inflorescence produced least callus.

#### Impact of the size of inflorescences on inducing callus and somatic embryogenesis:

The results obtained proven the impact of the size, i.e. age of date palm inflorescences in playing a vital role in callogenesis (callus formation). In date palm micropropagation, several factors like genotype, explant type, culturing duration and kind and concentrations of plant growth hormones had a strong influence for producing embryogenic callus (Mazri & Meziani, 2015; Abul-Soad *et al.*, 2017; Abul-Soad & Al-Khayri, 2018). The explants obtained from the inflorescences of 17 cm in length resulted in better initiation response with significantly highest callogenesis from floral buds of cv. Aseel (85%) and cv. Dhakki (70%), as shown in Table 3 and Fig. 2. This is compared to the explants obtained from inflorescences of 32 cm long that produced little amount of callus or failed to induce callus at all (Fig. 3). The medium size inflorescence of 28 cm long which was excised from a mother tree at the end of 1<sup>st</sup> week of February showed average callus percentage (30.3% in cv. Aseel and 32.6% in cv. Dhakki) whereas the inflorescence of 32 cm long induced significantly the lowest callogenesis percentage (12 % in cv. Aseel and 16% in cv. Dhakki). Not to mention that these timings could differ according to the ambient climatic conditions which control the time of explant excision (Abul-Soad & Al-Khayri, 2018). In case of the appropriate immature inflorescence size of 17 cm long according to the current study, the callogenesis was rapid and took only a single month to convert into initial callus while explants from inflorescences of 28 and 32 cm long consumed 2-3 months to induce callus. Not only longer time but also callus could not grow further from them. Since, the size (length and width) of date palm inflorescences is genotype-dependent hence the findings obtained in current study can be used as a reference for other date palm cultivars that might show similar inflorescence size. Recently and after the success of novel trail to use immature inflorescences for micropropagation of date palm (Abul-Soad, 2011), other reports followed the usage of immature inflorescences (Abahmane, 2013). Also, it is recommended to use the immature female inflorescences (cv. Siwi) of 6-7 cm in size and the excision time from 15<sup>th</sup> to 30<sup>th</sup> January (Zayed & Abd Elbar, 2015). In other cultivars like Kashoo wari, Gajar, and Dedhi from Khairpur, Pakistan the immature inflorescences were excised in early spring and successfully induced somatic embryogenesis (Jatoi *et al.*, 2015).

#### Influence of auxins and cytokinins on callogenesis from floral buds of date palm:

There were significant differences obtained among the different combinations of auxins and cytokinins to induce callogenesis in both date palm cultivars. In particular, the highest callogenesis occurred significantly in cv. Aseel (90.3%) using 2 mg L<sup>-1</sup> NAA + 0.5 mg L<sup>-1</sup> 2ip while in cv. Dhakki the significantly highest values (78%) were recorded using 2 mg L<sup>-1</sup> 2,4-D + 0.5 mg L<sup>-1</sup> 2ip. It seemed that floral explants of cv. Aseel responded much more to the NAA than the 2,4-D. On the other side, the significant lowest values were recorded in explants of both cultivars cultured onto media formulations of 0.1 mg L<sup>-1</sup> 2,4-D or NAA along with 0.1 mg L<sup>-1</sup> 2ip (Table 4). This is indicating the high requirement of date palm cells from the auxins and cytokinins. Therefore, the balance should be in the favor of auxin on the account of the 2iP as the same concentrations from auxins and 2iP poorly produced callus,

keeping in view the role of NAA to induce relatively more callogenesis. It is stated that auxins like 2,4-D are extensively used to induce embryogenic cultures in date palm and other plant species (Evans *et al.*, 1981).

Different other reports (El Hadrami & Baaziz, 1995; Fki *et al.*, 2003; Eshraghi *et al.*, 2005) observed the positive influence of several applications of 2,4-D beside other hormones to encourage the embryogenic capability of callus in different cultivars of date palm.

The efficiency of spikelet explants in callus induction increased when the medium provided with 2 mg L<sup>-1</sup> 2,4-D (Fig. 4) and gradually decreased to low percentage of callus induction on 0.1 mg L<sup>-1</sup> 2,4-D along with the all tested treatments of 2iP. The three treatments of 2,4-D (0.5, 1.0, 2.0 mg L<sup>-1</sup>) along with 2iP at 0.1, 0.5 mg L<sup>-1</sup> formed whitish compact callus. The callus formed in a way that initial explant swelled, particularly, the florets existing on the spikelet which then completely converted into compact callus (Fig. 2). Florets took 4 months to be converted completely into callus. Further growth of callus was achieved on same media until the formation of pro-embryos. Abul-Soad *et al.*, (2007) found that the addition of auxins mainly 2,4-D into the inflorescence nutrient medium significantly increased swelling of spike explants. Drira & Benbadis (1985) obtained highest callus percentages on the medium consisted of 0.5 mg L<sup>-1</sup> 2,4-D + 0.5 mg L<sup>-1</sup> IBA and 0.2 mg L<sup>-1</sup> BA using inflorescence explants.

**Table 3. Impact of size of immature inflorescence on callus induction from spikelet explants of date palm (cvs. Aseel and Dhakki).**

Size of inflorescence (length in cm)	Callus induction %	
	Aseel	Dhakki
17	85 a	70 a
28	30.3 b	32.6 b
32	12 c	16 c
LSD at $p < 0.05$	0.001**	0.0001***

Means on the same column with different letters are significantly different at  $p < 0.05$ . n.s., \*\*\*, \* - nonsignificant or significant at  $p \leq 0.05$  or 0.001, respectively

**Table 4. Effect of different combinations of auxins and cytokinins on *in vitro* date palm callogenesis from the spikelets of date palm (cvs. Aseel and Dhakki).**

Auxin + Cytokinin (mg L <sup>-1</sup> )	Callus induction %			
	(2,4-D+2iP)		(NAA+2iP)	
	Aseel	Dhakki	Aseel	Dhakki
0.1 + 0.1	11.6 f	12.3 g	12.6 g	12.6 g
0.1 + 0.5	23.6 e	23.6 f	23 f	20.6 f
0.5 + 0.1	31.3 d	28.3 e	25.6 ef	23.3 f
0.5 + 0.5	33.3 d	29.6 e	29 e	31 e
1.0 + 0.1	52 c	52 d	52.6 d	49.6 d
1.0 + 0.5	59.6 b	57.3 c	60.3 c	54.3 c
2.0 + 0.1	86 a	71.6 b	84 b	73.3 b
2.0 + 0.5	87.3 a	84.3 a	90.3 a	78 a
LSD at $p < 0.05$	0.001**	0.0001***	0.001**	0.001**

Means on the same column with different letters are significantly different at  $p < 0.05$ . n.s., \*\*\*, \* - nonsignificant or significant at  $p \leq 0.05$  or 0.001, respectively

As mentioned above callogenesis occurred on the medium consisting of 2.0 mg L<sup>-1</sup> NAA + 0.5 mg L<sup>-1</sup> 2iP (Fig. 4), same result approximately achieved with 1.0 and 2.0 mg L<sup>-1</sup> NAA along with different treatment of 2iP (0.1, 0.5 mg L<sup>-1</sup>). Whereas the lowest callus% was achieved on NAA treatments at 0.1 and 0.5 mg L<sup>-1</sup>. The formation of adventitious roots at the base of spikelet explants was noticed on the medium provided with NAA and 2iP combination at 0.1 mg L<sup>-1</sup>. The addition of NAA at 0.1 and 0.2 mg L<sup>-1</sup> may induce formation of roots in date palm explants (Tisserat, 1984; Al-Marri & Al-Ghamdi, 1995). The obtained callus cultures were subcultured on fresh media regularly till the appearance of pro-embryos. The formation of pro-embryos was not recognized on initiation medium even after four months of initial culturing on the media contained 2,4-D/NAA and 2iP.

**Somatic embryos induction, maturation and differentiation:** Globular stage of somatic embryos indicates the induction of pro-embryos that induced after seven months of callus formation on the basal medium supplemented with lower levels of 2,4-D along with 2iP (Fig. 5). Highest percentages of somatic embryos (SE) induced on the medium supplemented 0.05 mg L<sup>-1</sup> 2,4-D along with 2.0 mg L<sup>-1</sup> 2iP. Presence of 2iP and reduction in 2,4-D levels was found important for maturation of the developing somatic embryos (Fig. 6a). However, the highest percentages of somatic embryos were recorded in cv. Aseel (88.3%) and cv. Dhakki (86%) using 0.05 mg L<sup>-1</sup> 2,4-D and 2.0 mg L<sup>-1</sup> 2iP. Using 0.05 mg L<sup>-1</sup> NAA and 2 mg L<sup>-1</sup> 2iP highest values of somatic embryos were recorded in cv. Aseel (79.3%) and in cv. Dhakki (76%). Using basal medium supplemented with 0.01 mg L<sup>-1</sup> NAA and 2 mg L<sup>-1</sup> 2iP developed only 7% somatic embryos (Table 5).

According to previous study the normal somatic embryos have been classified into two types: repeated and non-repeated (Abul-Soad, 2011). In the current study the two types of somatic embryos were recognized as well: repeated, i.e. a cluster of somatic embryos (Fig. 6b) and non-repeated somatic embryos, i.e. individual embryos (Fig. 6a). The non-repeated somatic embryos were differentiated from two types of media, containing either NAA or 2,4-D along with 2iP. The non-repeated somatic embryos induced from friable callus after nine months of initiation under full darkness and the medium comprised of 0.05 mg L<sup>-1</sup> 2,4-D + 2 mg L<sup>-1</sup> 2iP. On the other hand, the repeated embryos were induced when the compact callus after nine months of initiation was shifted onto the PGRs free medium under full darkness. The later type of embryos was developed into clusters (produced adventive embryos) under light conditions on multiplication medium. Al-Khayri (2018) in his study conducted on shoot tip explants utilized PGR free medium for development, maturation, and germination of somatic embryos. This is necessary to direct the somatic embryos later into multiplication stage or rooting stage to get quick plantlets (Abul-Soad, 2011). Using higher levels of 2,4-D and 2iP to induce callus found similarly to develop the somatic embryos. while lower number of somatic embryos was obtained on 0.01 mg L<sup>-1</sup> 2,4-D + 1.0 mg L<sup>-1</sup> 2iP medium. This result is in agreement with the findings of Al-Baiz *et al.*, (2000) who mentioned that the medium consisted of 0.05 mg L<sup>-1</sup> NAA and 1.0 mg L<sup>-1</sup> 2iP induced excellent somatic embryos formation. They further noticed an increase in terms of number and size of somatic embryos as per callus maturity stage, type of plant

species and cultivar, and age of explants. But it is in contrary with the preferred medium to induce the somatic embryos which is free from the growth regulators (Abul-Soad, 2011). Also, it was observed the regeneration of date palm explants through somatic embryogenesis process on a medium supplemented with activated charcoal (AC) which was found important to reduce phenolic compounds exuded by cultures during embryo induction and maturation stage. The rapid proliferation of date palm cultures was achieved by Al-Kharyi & Al-Maarri (1997) using 30 g L<sup>-1</sup> sucrose, 1.5 g L<sup>-1</sup> AC, 6 g L<sup>-1</sup> agar, 10 mg L<sup>-1</sup> NAA and 6 mg L<sup>-1</sup> 2iP. Taha *et al.*, (2001) found that 3 mg L<sup>-1</sup> 2iP and 0.5 mg L<sup>-1</sup> NAA enhanced proliferation rate using full strength MS basal medium as compare to half strength.



Fig. 4. Callogenesis in spikelet explant obtained on the medium supplemented with 2.0 mg L<sup>-1</sup> 2,4-D + 0.5 mg L<sup>-1</sup> 2iP



Fig. 5. Somatic embryogenesis on the medium supplemented with 0.05 mg L<sup>-1</sup> 2,4-D + 2 mg L<sup>-1</sup> 2iP.

**Table 5. Effect of different combinations of 2,4-D and 2iP on somatic embryogenesis induction (%) in date palm (cvs. Aseel and Dhakki).**

Auxin + Cytokinin (mg L <sup>-1</sup> )	Somatic embryos induction%			
	(2,4-D+2iP)		(NAA+2iP)	
	Aseel	Dhakki	Aseel	Dhakki
0.01 + 1.0	11 ef	8.6 e	10.3 de	7.6 d
0.01 + 2.0	9.6 f	7 e	8 e	7 d
0.02 + 1.0	14 de	9.6 e	10 e	9.3 d
0.02 + 2.0	16.6 d	13.3 d	16.6 d	12.3 d
0.04 + 1.0	51.6 d	55.6 c	50.3 c	48 c
0.04 + 2.0	54.3 c	56 c	60 b	58.3 b
0.05 + 1.0	71.6 b	70 b	75 a	70.6 a
0.05 + 2.0	88.3 a	86 a	79.3 a	76 a
LSD at $p < 0.05$	0.0001**	0.001**	0.001**	0.015*

Auxin means 2,4-D or NAA. Means on the same column with different letters are significantly different at  $p < 0.05$ . n.s. \*, \*\*\* - nonsignificant or significant at  $p \leq 0.05$  or 0.001, respectively



Fig. 6. (a) Differentiation of normal non-repeated somatic embryos from friable callus on the medium containing 0.05 mg L<sup>-1</sup> 2,4-D + 2 mg L<sup>-1</sup> 2iP under full darkness after nine months of initiation, (b) Differentiation of repeated somatic embryo from compact callus (induced using high auxins) on PGR free medium after nine months of initiation.

## Conclusion

The plant growth regulators are necessary to induce the embryogenic callus and develop the somatic embryos. *In vitro* protocols (up to somatic embryogenesis) found cultivar-independent. Somatic embryogenesis protocol has been optimized in the current study for two of top cultivars of Pakistan namely Aseel and Dhakki in terms of the PGR composition especially the key ones 2,4-D, NAA and 2iP. The appropriate time for inflorescence excision from the mother tree was identified based on explants response on the tested initiation media. Surface sterilization of inflorescence was also carried out successfully using 50% of NaOCl solution (5.25 NaOCl) with the survival rate of explants up to 90%. The viable callus was induced successfully on MS basal medium supplemented with 2 mg L<sup>-1</sup> 2,4-D + 0.5 mg L<sup>-1</sup> 2iP. This callus was encouraged to develop into somatic embryos using 0.05 mg L<sup>-1</sup> 2,4-D/NAA + 2 mg L<sup>-1</sup> 2iP. The findings obtained would pave the way to commercially micropropagate the studied and other cultivars.

## Acknowledgment

The authors would like to acknowledge financial assistance provided by Agricultural Linkages Programme (ALP) of Pakistan Agricultural Research Council (PARC), Islamabad under the project CS020 titled "Micropropagation of Superior National and International Varieties of Date Palm on Commercial Level".

## References

- Abahmane, L. 2013. Recent achievements in date palm (*Phoenix dactylifera* L.) micropropagation from inflorescence tissue. *Emirates J. Food Agri.* 25:863-874.
- Abul-Soad, A. 2011. Micropropagation of date palm using inflorescence explants. In: *Date Palm Biotechnology*. (Eds.): S. Jain, J. Al-Khayri and D. Johnson. Springer, Dordrecht, pp 91-117.
- Abul-Soad, A., I. Ibrahim, N. El-Sherbeny and E. Bakr. 2002. *In vitro* optimization for plant regeneration of date palm (*Phoenix dactylifera* L.). *Minia J. Agric. Res. Dev.*, 22: 25-28.
- Abul-Soad, A., K. Emara, A. Abdallah and S. Mahdi. 2017. Somatic embryogenesis in *Phoenix dactylifera* L. using floral bud explants. In: *IX International Symposium on In vitro Culture and Horticultural Breeding. Acta Hort.*, pp. 13-28.
- Abul-Soad, A., N. El-Sherbeny and S. Baker. 2007. Effect of basal salts and sucrose concentrations on morphogenesis in test tubes of female inflorescence of date palm (*Phoenix dactylifera* L.) cv. Zaghoul. *Egypt J. Agric Res.*, 85: 385-394.
- Abul-Soad, A.A. 2012. Influence of inflorescence explant age and 2,4-D incubation period on somatic embryogenesis of date palm. *Emir. J. Food Agri.*, 24: 434-443.
- Abul-Soad, A.A. and J.M. Al-Khayri. 2018. Date palm somatic embryogenesis from inflorescence explant. In: *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*. (Eds.): S. Jain and P. Gupta. Springer International Publishing, 329-347.
- Abul-Soad, A.A. and S.M. Mahdi. 2010. Commercial production of tissue Culture date palm (*Phoenix dactylifera* L.) by inflorescence technique. *J. Genet. Eng. Biotechnol.*, 8: 39-44.
- Abul-Soad, A.A., G.S. Markhand and S.A. Shah. 2008. Effect of Naphthaleneacetic acid and Indole-3-acetic acid on somatic embryogenesis of female inflorescence explants of date palm (*Phoenix dactylifera* L.) cv. Aseel. In: *3rd International Conference on Date Palm*. Faculty of Agriculture and Environmental Science, Suez Canal University, North Sinai, Egypt., 222-231.
- Abul-Soad, A.A., S.M. Jain and M.A. Jatou. 2017. Biodiversity and conservation of date palm. In: *Biodiversity and Conservation of Woody Plants*. (Eds.): M. Ahuja and S. Jain. Springer International Publishing, pp. 313-353.
- Al-Baiz, A., C. Mouli and F. Al-Oraini. 2000. Suspension cultures from embryogenesis. In: *Date Palm International Symposium*. Food and Agriculture Organization of the United Nations, Windhoek, Namibia, pp. 36-40.
- Al-Khalifah, N. and E. Askari. 2011. Growth abnormalities associated with micropropagation of date palm. In: *Date Palm Biotechnology*. (Eds.): S. Jain, J.M. Al-khayri and D. Johnson. Springer International Publishing, pp. 205-219.
- Al-Kharyi, J. and K. Al-Maarri. 1997. Effect of seasonal variation on the regeneration capacity of date palm. *In vitro*, 33: 22-26.
- Al-Khayri, J.M. 2018. Somatic embryogenesis of date palm (*Phoenix dactylifera* L.) from shoot tip explants. In: *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*. (Eds.): S. Jain and P. Gupta. Springer International Publishing, pp 231-244.
- Al-Marri, K. and A.S. Al-Ghamdi. 1995. Effect of prelevement date on *In vitro* date palm (*Phoenix dactylifera* L.) cv. Hillaly propagation. *Arab. Univ. J. Agri. Sci.*, 3: 151-167.
- Al-Mayahi, A.M.W. 2013. Micropropagation of date palm *Phoenix dactylifera* L. cv. Um Al-Dihin by formation of somatic embryos and the production of adventitious buds from callus induced from flower buds. *J. King Saudi Univ. Agric. Sci.*, 25(2): 89-102.
- Al-Qurainy, F., S. Khan, M. Tarroum, N. Mohammad, S. Alansi, A. Alshameri and A.R. Gaafar. 2020. Comparison of salt tolerance between two potential cultivars of *Phoenix dactylifera* L. growing in Saudi Arabia. *Pak. J. Bot.*, 52(3): 753-761.
- Drira, N. and A. Benbadis. 1985. Multiplication végétative du Palmier dattier (*Phoenix dactylifera* L.) par réversion, en culture *In vitro*, d'ébauches florales de pieds femelles. *J. Plant Physiol.*, 119: 223-235.
- El Hadrami, I. and M. Baaziz. 1995. Somatic embryogenesis and analysis of peroxidases in *Phoenix dactylifera* L. *Biol Plant.*, 37: 197-203.
- Eshraghi, P., R. Zarghami and M. Mirabdulbaghi. 2005. Somatic embryogenesis in two Iranian date palm cultivars. *Afr. J. Biotechnol.*, 4: 1309-1312.
- Evans, D.A., W.R. Sharp and C.E. Flick. 1981. Growth and behavior of cell cultures: embryogenesis and organogenesis. In: *Plant tissue culture: methods and application in agriculture*. (Ed.): T.A. Thorpe. Academic Press, New York, pp. 45-113.
- Fki, L., R. Masmoudi, N. Drira and A. Rival. 2003. An optimised protocol for plant regeneration from embryogenic suspension cultures of date palm, *Phoenix dactylifera* L., cv. Deglet Nour. *Plant Cell Rep.*, 21: 517-24.
- Fki, L., R. Masmoudi, W. Kriaâ, A. Mahjoub, B. Sghaier, R. Mzid, A. Mliki, A. Rival and N. Drira. 2011. Date Palm Micropropagation via Somatic Embryogenesis. In: *Date Palm Biotechnology*. (Eds.): S.M. Jain, J.M. Al-Khayri and D.V. Johnson. Springer International Publishing, Dordrecht, pp 47-68.
- Hazzouri, K.M., J.M. Flowers. H.J. Visser. 2015. Whole genome re-sequencing of date palms yields insights into diversification of a fruit tree crop. *Nat. Commun.*, 6: 8824.
- Jain, S.M. 2007. Recent advances in date palm tissue culture and mutagenesis. In: *III International Date Palm Conference*. (Eds.): A. Zaid, V. Hegarty and H.H. Al-Kaabi. *Acta Hort.*, 736: 205-211.

- Jatoi, M.A., A.A. Abul-Soad, G.S. Markhand and N. Solangi. 2015. Establishment of an efficient protocol for micropropagation of some Pakistani cultivars of date palm (*Phoenix dactylifera* L.) using novel inflorescence explants. *Pak. J. Bot.*, 47: 1921-1927.
- Jatoi, M.A., A.A. Abul-Soad, G.S. Markhand and N. Solangi, 2019. Micropropagation of cv. Dhakki a high value date palm cultivar of Pakistan using offshoot and inflorescence explants. In: (Eds.): Zaid, A. and G. Alhadrami. *Proceedings of the Sixth International Date Palm Conference*, Abu-Dhabi, UAE, pp: 11-16.
- Jatoi, M.A., Z. Markhand and N. Solangi. 2009. Dates in Sindh: Facts and Figures. In: (Eds.): Markhand, G. and A. Abul-Soad. *International Dates Seminar*. Date Palm Research Institute, Shah Abdul Latif University, Khairpur, Pakistan, pp. 59-71.
- Markhand, G.S., A.A. Abul-Soad, A.A. Mirbahar and N.A. Kanhar. 2010. Fruit characterization of Pakistani dates. *Pak. J. Bot.*, 42: 3715-3722.
- Mazri, A.M. and R. Meziani. 2015. Micropropagation of Date Palm: A Review. *Cell Dev. Biol.*, 4: 1-5.
- Mirani, A.A., C.H. Teo, A.A. Abul-Soad, G.S. Markhand, T. Jatt, A.A. Mirbahar and N. Solangi. 2019. Phenotypic reversion of somaclonal variants derived from inflorescence of date palm (*Phoenix dactylifera* L.) in the open field trials. *Sarhad J. Agric.*, 35(3): 719-726.
- Murashige, T. and F. Skoog. 1962. A Revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.
- Quiroz-Figueroa, F.R., R. Rojas-Herrera, R.M. Galaz-Avalos and V.M. Loyola-Vargas. 2006. Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. *Plant Cell Tiss. Organ. Cult.*, 86: 285-301.
- Taha, H.S., S.A. Bekheet and M.M. Saker. 2001. Factors affecting *In vitro* multiplication of date palm. *Biol. Plant.*, 44: 431-433.
- Tisserat, B. 1984. Propagation of date palms by shoot tip cultures (*Phoenix dactylifera*). *Hort. Sci.*, 19: 230-231.
- Zaid, A. and P. de Wet. 2002. Origin, geographical distribution and nutritional values of date palm. In: *Date palm cultivation*. (Ed.): A. Zaid. Food and Agriculture Organization of the United Nations, Rome, Italy, pp. 29-44.
- Zayed, E.M.M. and O.H. Abd Elbar. 2015. Morphogenesis of immature female inflorescences of date palm *In vitro*. *Ann. Agric. Sci.*, 60(1): 113-120.

(Received for publication 18 December 2018)