Research Article

Factors Influencing Somatic Embryogenesis and Plantlet Regeneration of Date Palm using Immature Floral Buds

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Abstract | Current study explains effective *in vitro* protocols for somatic embryogenesis and plantlet regeneration in commercially important date palm cvs. Begum Jungi and Ajwa. Spikelet explants of spathes (avg. 17, 28, 32 cm) excised at different intervals were used as initial explants. Results revealed that sterilization of spathes with 50% sodium hypochlorite (NaOCl) solution resulted in significantly highest survival, lowest mortality and contamination in spikelet explants. The spikelet explants obtained from immature spathes (avg. 17 cm) resulted in significantly highest callogenesis in cvs. Begum Jungi (83.6%) and Ajwa (75.6%). Significantly highest callus induced in floral buds in cvs. Begum Jungi (87.3%) and Ajwa (84.3%) on medium comprising of 2,4-D (2.0 mg L^{-1}), 2iP (0.5 mg L^{-1}). Medium comprising of 0.05 mg L^{-1} 2,4-D, 2.0 mg L^{-1} 2iP, 3 g L^{-1} activated charcoal induced significantly highest somatic embryos in cvs. Begum Jungi (83.3%) and Ajwa (82.6%). Somatic embryos induced in calli after nine months of initial culture were categorized into repeated and non-repeated. Medium comprising of NAA 0.1 mg L^{-1}, Kin 1.0 mg L^{-1} revealed with significantly highest germination of somatic embryos in cvs. Begum Jungi (55.6%) and Ajwa (52.3%). Findings obtained will support to describe proper spathe size and plant growth regulator (PGR) levels for somatic embryogenesis and plantlet regeneration in commercially important cultivars of date palm worldwide.

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Introduction

Phoenix dactylifera L. is member of family *Arecaceae*, diploid, dioecious and being horticulturally valued crop cultivated in tropical regions in world (Hazzouri *et al*., 2015; Mazri and Meziani, 2015; Abul-Soad *et al*., 2017; Solangi *et al*., 2022). Pakistan holds 6th position in dates production and export with old cultural practices belongs to indus civilization (Marshal, 1931; Jatoi *et al*., 2009; Markhand *et al*., 2010). Seeds and offshoots are two natural propagation methods for date palm, however date

palm propagated through seeds always show variation due to heterozygosity. Desired cultivars of date palm can be multiplied by offshoot propagation, but production of 10-15 offshoots per tree in its whole life is one of the major hindrance. Simultaneously, there is big threat of disease and pests in date palm destroying thousands of trees per year in world (Abul-Soad *et al.*, 2017). Disease and pests-free production of true-to-type plant material on commercial level is possible via micropropagation (Al-Khalifah and Askari, 2011; Jatoi *et al.*, 2015). Effective means of multiplying *in vitro* cultures of date palm is somatic embryogenesis (Quiroz-Figueroa *et al.*, 2006), making possible the availability of required plants in vast number (Zaid and Wet, 2002; Fki *et al.*, 2011). Approximately, increasing per year requirement of date palm plants in international market is 1-2 million (Jain, 2007). Therefore, to fulfil such requirements, the commercial labs establishing effective *in vitro* protocols (Abul-Soad and Mahdi, 2010; Jatoi *et al.*, 2015).

Abul-Soad *et al.* (2002) reported that in 1970, shoot tip explants were utilized in micropropagation, however currently Abul-Soad (2011) and Solangi *et al.* (2020) utilized explants of juvenile inflorescence for *in vitro* propagation of elite and rare date cultivars. Mirani *et al.* (2019) and Mirani *et al.* (2022) observed that *in vitro* propagation via inflorescence explants results in low percentage or no variations during fruiting. Keeping in view genotype, explant age, and auxin-cytokinin responses, the protocols can be exploited for other cultivar (Abul-Soad *et al.*, 2017; Abul-Soad and Al-Khayri, 2018).

Abul-Soad (2011) and Jatoi *et al.* (2015) described that previously shoot tip based *in vitro* propagation was focused, but generally use of novel inflorescence explants in *in vitro* propagation was neglected. Abul-Soad (2012) mentioned that literature describing effective role of auxin-cytokinin interaction in embryogenesis via inflorescence explants is limited. Therefore, current study was carried out for evaluating response of immature inflorescence explants, and to study effects of several auxin-cytokinin levels on *in vitro* propagation of elite date cultivars.

Materials and Methods

Plant material

Spathes of different sizes (avg. 17, 28, 32 cm) were excised from date palm trees Begum Jungi and Ajwa grown in Research Orchard of DPRI. Excised spathes shifted to the laboratory for sterilization and culture process.

Explant preparation

Spathes were immersed in 2 g L^{-1} fungicide for a min and washed gently with tap water. Later spathes were surface disinfected on laminar air flow cabinet using several NaOCl concentrations i.e., 10, 20, 40 and 50% with some drops of Tween-20 for five min and rinsed in distilled water for washing. Spathes were dissected longitudinally from both sides gradually up to inflorescence bunch led to removal of outer hard cover of spathe completely under sterile conditions. Later, intact inflorescence bunch was taken out of spathe. Spikelets (2-3 cm with 8-10 florets) were separated from inflorescence bunch, cultured as primary explants on initiation media.

Media preparation and culture conditions

Murashige and Skoog (1962) medium comprising of various treatments of PGRs specified to callus formation, somatic embryogenesis, germination and plantlet formation described in Table 1. Initial explants after culture in tubes incubated in full dark at 24±2°C, while proliferation, germination of somatic embryos acquired under light.

Table 1: *Media composition for callogenesis, somatic embryogenesis, germination and plantlet formation in cvs. Begum Jungi and Ajwa.*

Growth stage	Medium composition (mg L^{-1})			
	Salts	Additives	Auxins	Cytokinin
Initiation	Micro and Macro salts of MS*	30000 Sucrose+6000 Agar+MS Vitamins+170 KH2PO4+200 Glutamine	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+MS Vitamins+170 KH2PO4+200 Glutamine	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)
Germination and plantlet formation	Micro and Macro salts of MS	30000 Sucrose+6000 Agar+MS Vitamins+170 KH2PO4+200 Glutamine	M1. NAA (0.05) M2. NAA (0.5)	M1. Kin (1.0, 2.0) M2. Kin (1.0, 2.0)

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

Table 2: *Effect of different concentrations of NaOCl on surface sterilization of spathes of cvs. Begum Jungi and Ajwa.*

NaOCl (%)	cv. Begum Jungi			cv. Ajwa		
	Survival %	Contamination %	Mortality %	Survival %	Contamination %	Mortality %
10	19±5.45^{c}	75±0.57^{a}	6±1.15^{b}	21±1.15^{c}	71±1.73^{a}	8±2.30^{ab}
20	23±1.73^{c}	69±2.30^{a}	8±1.15^{a}	24±1.15^{c}	66±1.73^{b}	10±1.73^{a}
40	66±2.30^{b}	22±1.15^{b}	12±1.15^{ab}	67±1.15^{b}	26±1.73^{c}	9±1.73^{ab}
50	87±2.02^{a}	5±1.73^{c}	8±1.73^{c}	89±1.15^{d}	4±1.15^{d}	7±2.30^{b}
LSD *at p<0.05*	0.0000***	0.0000***	0.0647$^{n.s}$	0.0000***	0.0000***	0.7569$^{n.s}$

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

Statistical analysis

Two cultivars were used in study and every treatment comprised of three replicates. Single explant was cultured in tubes. Data analysis done by ANOVA and LSD (*p<0.05*) of obtained means taken using statistix software.

Results and Discussion

Effect of NaOCl concentrations on surface sterilization of spathes

Protocols used in current study were based on different treatments of auxins and cytokinins induced callus and somatic embryos in floral buds on spikelets, keeping in view the impact of spathes' size. Additionally, sterilization of spathes with NaOCl was improved, resulted in highest survival of primary explants.

The results in Table 2 revealed that surface sterilization of spathes with 50% NaOCl solution resulted in significantly highest survival rate in cv. Ajwa (89%) and lowest contamination (4%) and mortality (7%) followed by cv. Begum Jungi with highest survival (87%), contamination (5%) and mortality (8%). On the contrary, significantly lowest survival rate was observed in cv. Begum Jungi (19%) and highest contamination (75%) and mortality (6%) followed by cv. Ajwa (21%), contamination (71%) and mortality (8%). Results showed that survival rate of initial explants (Figure 1d) increased gradually by increasing the NaOCl concentration from 10% to 50%. Spikelets taken from inside immature spathes (Figure 1a) were not sterilized due to complete absence of microbes (Abul-Soad, 2011; Solangi *et al.*, 2020). All spikelet explants contaminated within few days of initial culture if obtained from cracked spathes (Figure 1b) occurred either during excision, transfer or sterilization process. Intact spikelets inside un-cracked spathes of date palm remained free from all types of contaminants, and simultaneously excellent results regarding significantly highest survival rate were obtained by Abul-Soad *et al.* (2007, 2008), Abul-Soad and Mahdi (2010), Solangi *et al.* (2020). Some explants contaminated with fungus via succeeding subcultures due to improper culture process and were discarded immediately to stop further infestation. Furthermore, results showed that 89% of initial explants were contaminants-free, and simultaneously formed callus from floral buds occur on spikelets (Table 2). Similarly, callus induced in primary explants was sub-cultured carefully, similarly remained free from contamination through successive subcultures.

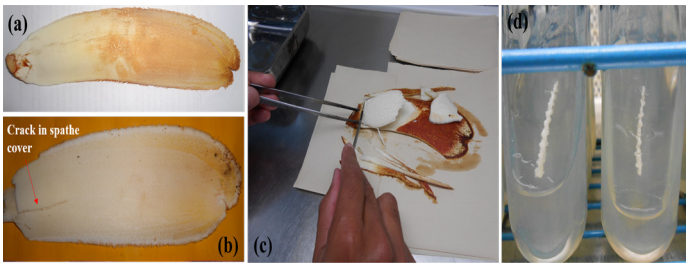

Figure 1: *(a) Spathe without any crack, (b) Crack in spathe cover, (c) Inflorescence bunch, (d) Spikelets cultured on initiation medium.*

Browning of the medium due to phenolic compounds occur in inflorescence explants reduced by transferring spikelets on fresh media after every four weeks. Meanwhile, it was necessary to shift explants to fresh media to keep them away from the effect of oxidized phenolic compounds occurred due to long subculture period and caused the blackening of the media. Moreover, calli multiplied on media lacking antioxidants due to occurrence of least quantity of phenolic compounds in spikelet explants compared to shoot tip explants, which contain more phenolic compounds and cause severe browning (Solangi *et al.*, 2020).

Effect of size and age of spathes on callus induction in floral buds

Callus induction in floral buds depends on effect of size of spathe (proper age of explant containing

meristematic cells). Callus formation in initial explants effected by numerous factors i.e., explant type, genotype, culture period, kind and concentration of PGR described by Mazri and Meziani (2015), Abul-Soad *et al.* (2017), Abul-Soad and Al-Khayri (2018). Data in Table 3 revealed that significantly highest callus induced in spikelets of 17 cm spathes in cvs. Begum Jungi (83.6%) and Ajwa (75.6%) (Figure 2a) excised during starting of February. The spikelet explants of 28 cm spathes excised on 7th February resulted in average callus induction (34% in cv. Ajwa and 31.3% in cv. Begum Jungi), while spikelet explants of 32 cm spathes excised after 15th February formed callus as significantly lowest (11.3% in cv. Begum Jungi and 17% in cv. Ajwa). Spathes excised at different timings vary according to climatic conditions which control growth of primary explants occur inside spathes (Abul-Soad and Al-Khayri, 2018). Spikelet explants of 17 cm spathes resulted in rapid callogenesis occurred in one month, whereas explants of 28 and 32 cm spathes took 2 to 3 months to induce calli and simultaneously with least callus induction percentage (Figure 2b) or in many explants callus could not induced. Furthermore, induced callus could not grow further in spikelets of 32 cm spathes. Size of spathe is genotype-dependent, therefore results obtained can be applied as reference to other date cultivars with similar size of spathes (Solangi *et al.*, 2020). After successful date palm micropropagation using immature spathes (Abul-Soad, 2011), explants from immature spathes were used by other workers (Abahmane, 2013). Zayed and Elbar (2015) used immature spathes (6-7 cm) of cv. Sewi excised at 15th to 30th January. Jatoi *et al.*, (2015) used spikelets of immature spathes of cvs. Gajar, Dedhi, Kashoowari excised during early spring, induced somatic embryos. Hence the current study is strongly supported by the work done previously on juvenile inflorescence for callogenesis, somatic embryogenesis in female date palm.

Table 3: *Effect of spathe size on callus induction in spikelet explants of cvs. Begum Jungi and Ajwa.*

Length of spathe (cm)	Callus induction %	
	Begum Jungi	Ajwa
17	83.6±2.96[a]	75.6±2.96[a]
28	31.3±4.66[b]	34±2.08[b]
32	11.3±2.02[c]	17±2.21[c]
LSD *at p<0.05*	0.0000***	0.0000***

*Means on the same column with different letters are significantly different at p<0.05. n.s. *, *** – nonsignificant or significant at P ≤ 0.05 or 0.001, respectively.*

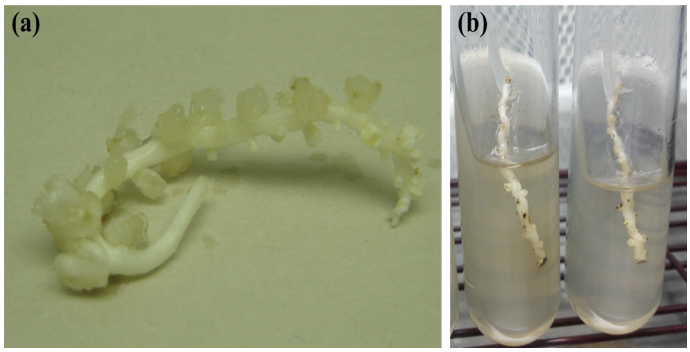

Figure 2: *(a) Callus formation in all floral buds obtained from 17 cm long immature spathe, (b) Spikelet explants obtained from 32 cm long spathe failed to produce callus.*

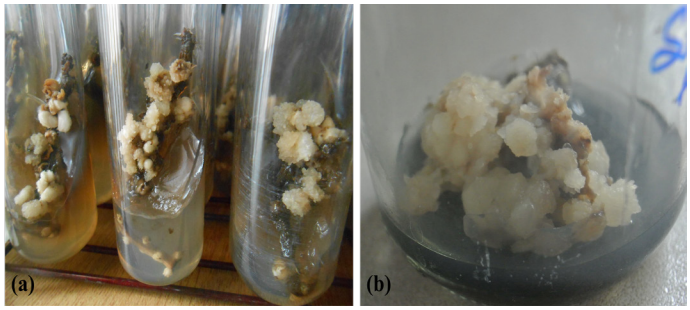

Figure 3: *(a) Callus induction in immature spikelets on the medium consisted of 2.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} 2iP, (b) Somatic embryogenesis on the medium consisted of 0.05 mg L^{-1} 2,4- D + 2 mg L^{-1} 2iP.*

Effect of auxins and cytokinins on callus induction in floral buds

Callus induction in spikelet explants of cvs. Begum Jungi and Ajwa influenced significantly by different auxin-cytokinin combinations. Data in Table 4 revealed with significantly highest callogenesis occurred in cvs. Begum Jungi (84.6%) and Ajwa (75.3%) on medium comprising of 2.0 mg L^{-1} 2,4-D, 0.5 mg L^{-1} 2iP (Figure 3a). Medium comprising of 2.0 mg L^{-1} NAA, 0.5 mg L^{-1} 2iP similarly induced significantly highest callus in primary explants of cvs. Begum Jungi (83.6%) and Ajwa (73.3%) than rest of treatments. Contrary, the significantly lowest callus induction observed in both cultivars on medium comprising of 2, 4-D or NAA (0.1 mg L^{-1}) + 2iP (0.1 mg L^{-1}). Results obtained using 2, 4-D or NAA with 2iP revealed that both auxins required in high quantity for callus formation in primary explants. Hence, auxins favour callus growth actively compared to 2iP, since similar auxin levels with higher levels of 2iP induced calli inadequately. NAA induced comparatively more calli in primary explants compared to 2, 4-D with 2iP. Further observed that 2, 4-D is widely utilized auxin to induce calli in primary explants of date palm (Evans *et al.*, 1981; Solangi *et al.*, 2020). Several other studies done by El-Hadrami

and Baaziz (1995), Fki *et al.* (2003), Eshraghi *et al.* (2005) described effects of different treatments of 2, 4-D concerning embryogenic callus formation in several date palm cultivars.

Table 4: *Effect of different concentrations of 2,4-D + 2iP and NAA + 2iP on callogenesis in floral bud explants.*

Auxin + Cytokinin (mg L^{-1})	Callus formation (%)			
	(2, 4-D+2iP)		(NAA+2iP)	
	Begum Jungi	Ajwa	Begum Jungi	Ajwa
0.1 + 0.1	9.3±1.85^{e}	9.6±1.85^{d}	9.3±2.72^{d}	7.3±3.51^{e}
0.1 + 0.5	20.3±4.25^{d}	20.6±2.96^{cd}	20.6±3.38^{cd}	17.6±2.33^{d}
0.5 + 0.1	27.6±3.92^{cd}	25.3±2.18^{c}	25.6±0.88^{c}	21.3±0.66^{d}
0.5 + 0.5	35.3±2.33^{c}	27.6±1.45^{c}	32.3±2.84^{c}	33.1±2.08^{c}
1.0 + 0.1	53.1±2.88^{b}	50.3±0.57^{b}	51.3±3.78^{b}	51.6±3.00^{b}
1.0 + 0.5	57.6±1.45^{b}	53.6±4.84^{b}	56±4.50^{b}	52±2.84^{b}
2.0 + 0.1	73.1±3.84^{b}	86.3±4.40^{a}	77.3±8.68^{a}	69.2±2.08^{a}
2.0 + 0.5	84.6±3.17^{a}	75.3±7.83^{a}	83.6±6.83^{a}	73.3±3.66^{a}
LSD *at p<0.05*	0.000***	0.0000***	0.0000***	0.0000***

Medium comprising of 2.0 mg L^{-1} 2, 4-D with 2iP formed significantly highest calli and decreased slowly on medium comprised of 0.1 mg L^{-1} 2, 4-D with 2iP concentrations. 2, 4-D at 0.5, 1.0, 2.0 mg L^{-1} with 0.1, 0.5 mg L^{-1} 2iP formed significantly highest callus. Swelling of florets existing on spikelets was first step of callus formation followed by complete conversion into compact callus occurred in four months (Figure 3a). Additionally, calli multiplied rapidly on similar media till induction of proembryos (Figure 3b). Swelling of floral buds on spikelets was enhanced on medium comprising of 2, 4-D (Abul-Soad *et al.*, 2007). Maximum callus induction in inflorescence explants was observed on medium comprising of 2, 4-D 0.5 mg L^{-1}, IBA 0.5 mg L^{-1} and BA 0.2 mg L^{-1} (Drira and Benbadis, 1985).

As discussed earlier the callus formed on medium comprising of 2.0 mg L^{-1} NAA + 0.5 mg L^{-1} 2iP, whereas similar results approximately obtained utilizing 2, 4-D 1.0 and 2.0 mg L^{-1} with 0.1, 0.5 mg L^{-1} 2iP. However, significantly least calli induction percentage obtained on medium consisting of NAA 0.1 and 0.5 mg L^{-1} with 0.1, 0.5 mg L^{-1} 2iP. Additionally, adventive roots formed on base of primary explants on medium comprising of 0.1 mg L^{-1} NAA, 0.1 mg L^{-1} 2iP. 0.1 and 0.2 mg L^{-1} NAA formed direct roots without intervening callus stage in initial explants (Tisserat, 1984; Al-Marri and Al-Ghamdi, 1995), however, using similar treatment of 2, 4-D with 2iP did not induced direct root on the base of initial explants. Subculturing of calli continued on fresh media after every four weeks till formation of proembryos. The time calculated was 7-9 months for differentiation of proembryos from primary callus followed by maturation of somatic embryos induced in initial explants via callus formation (Figure 3b).

Induction and maturation of somatic embryos

Formation of rounded somatic embryos indicate maturation stage of callus occurred in seven months after 1st subculture on media comprising of low 2, 4-D concentration with 2iP (Figure 3b). Somatic embryos as significantly highest induced on medium consisting of 2, 4-D 0.05 mg L^{-1}, 2iP 2.0 mg L^{-1}. In this way, significantly highest induction of somatic embryos obtained in cvs. Begum Jungi (83.3%) and Ajwa (82.6%) on medium comprising of 0.05 mg L^{-1} 2, 4-D and 2.0 mg L^{-1} 2iP. Medium consisting of NAA 0.05 mg L^{-1}, 2iP 2.0 mg L^{-1} similarly induced significantly highest somatic embryos in cvs. Begum Jungi (74.3%) and Ajwa (71.6%). Medium comprised of 0.01 mg L^{-1} NAA and 2.0 mg L^{-1} 2iP induced 7% embryos as significantly lowest values (Table 5). Results indicated that 0.01 mg L^{-1} NAA or 2, 4-D even with higher concentration of 2iP were not sufficient for somatic embryogenesis.

Table 5: *Effect of different concentrations of 2, 4-D + 2iP and NAA + 2iP on somatic embryogenesis from callus.*

Auxin + Cytokinin (mg L^{-1})	Somatic embryogenesis (%)			
	(2, 4-D+2iP)		(NAA+2iP)	
	Begum Jungi	Ajwa	Begum Jungi	Ajwa
0.01 + 1.0	7.5±2.00^{f}	9.4±2.64^{d}	8.6±1.20^{e}	11.8±1.73^{c}
0.01 + 2.0	10.6±2.02^{ef}	11.2±1.52^{d}	11.2±1.76^{e}	11.6±0.66^{c}
0.02 + 1.0	16.6±0.88^{de}	14.6±1.33^{d}	12.4±1.52^{e}	11.2±0.57^{c}
0.02 + 2.0	19.3±2.18^{d}	16.6±4.80^{d}	23.3±3.33^{d}	12.3±0.33^{c}
0.04 + 1.0	49.6±3.17^{c}	51.3±4.17^{c}	47.6±3.84^{c}	45.6±1.85^{b}
0.04 + 2.0	51.6±1.66^{c}	52.7±3.05^{c}	59.3±2.33^{b}	53.3±6.00^{b}
0.05 + 1.0	70.8±2.88^{b}	71.6±1.76^{b}	72.7±1.85^{a}	65.6±5.45^{a}
0.05 + 2.0	83.3±4.17^{a}	82.6±2.84^{a}	74.3±6.00^{a}	71.6±2.02^{a}
LSD *at p<0.05*	0.0000***	0.0000***	0.0000***	0.0000***

Previously, somatic embryos categorized as non-repeated or single, repeated or multiple in a cluster (Abul-Soad, 2011; Solangi *et al.*, 2020). Similarly, in present study both kinds of somatic embryos were observed induced in callus (Figure 4a). Medium comprising of 2, 4-D 0.05 mg L^{-1}, 2iP 2 mg L^{-1}

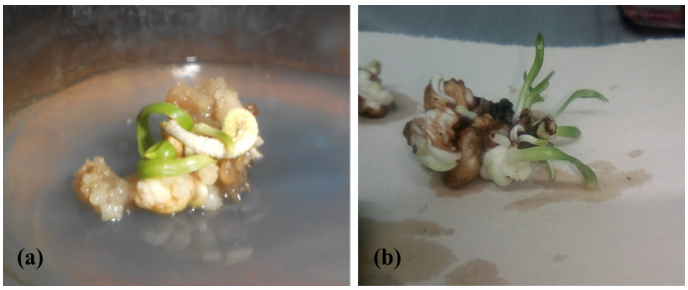

Figure 4: *(a) Formation of normal repeated and non-repeated somatic embryos from callus on the medium consisted of 0.05 mg L^{-1} 2,4-D + 2 mg L^{-1} 2iP under full dark after nine month of initiation, (b) Germination of somatic embryos on the medium consisted of 0.05 mg L^{-1} NAA, 1 mg L^{-1} Kin.*

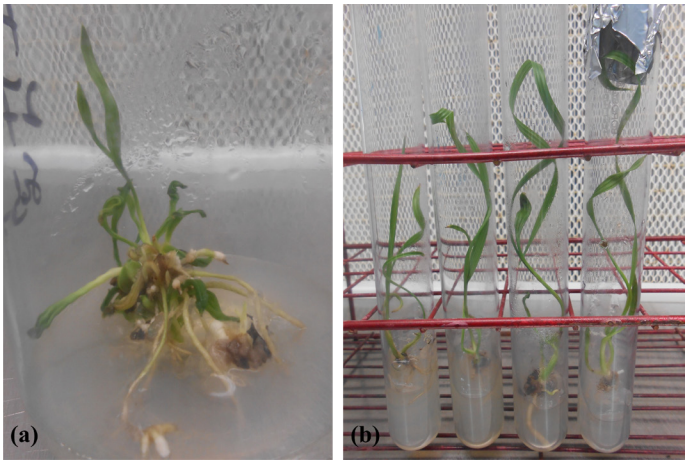

Figure 5: *(a) Cluster of plantlets with roots produced from repeated somatic embryos, (b) Plantlet (10 cm long) of cv. Begum Jungi isolated from cluster and cultured in long tubes for further growth on the medium consisted of 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BA.*

induced repeated and non-repeated embryos in callus after nine months of initial culture. Repeated embryos develop into cluster (Figure 4b) whereas non-repeated embryos developed into single plantlet via germination. Medium devised by Al-Khayri (2018) was without PGRs used for formation, maturation, germination of embryos. Current study utilized PGRs for getting maximum proliferation of somatic embryos. Later, Abul-Soad (2011), Solangi *et al.* (2022) transferred somatic embryos on multiplication media to get plantlets via germination. 2, 4-D and 2iP levels used in callus formation in initial explants similarly utilized in induction of somatic embryos, but 2, 4-D not exceeded to 0.05 mg L^{-1} with 2iP. Medium comprising of 2, 4-D 0.01 mg L^{-1}, 2iP 1.0 mg L^{-1} induced significantly lowest percentage of somatic embryos. Al-Baiz *et al.* (2000) obtained excellent somatic embryos on medium comprised of NAA 0.05 mg L^{-1}, 2iP 1.0 mg L^{-1}. Further observed that percentage of somatic embryos increased based on callus maturation stage, explant age. Nevertheless, medium is in contrast with ideal medium lacking PGRs used for embryogenesis (Abul-Soad, 2011; Solangi *et al.*, 2020). Al-Khayri and Al-Maarri (1997) observed quick multiplication on medium comprising of NAA 10 mg L^{-1}+ 2iP 6 mg L^{-1}. Taha *et al.* (2001) described that 2iP 3 mg L^{-1}, NAA 0.5 mg L^{-1} improved multiplication of embryos.

Somatic embryos germination, multiplication and plantlet formation

Somatic embryos (repeated, non-repeated) were observed after maturation and during multiplication stage (Abul-Soad, 2011) (Figure 4a). Media comprised of NAA and Kin used for multiplication and germination of repeated or non-repeated embryos. Three-way ANOVA exhibited effect of cultivar (0.0032), subculture (< 0.0001), treatment (< 0.0001) and combined effect of treatment and cultivar (0.0351) and subculture (< 0.0001) (Table 6). However, combined effect of cultivar and subculture (0.3211) and cultivar, subculture and treatment (0.6352) collectively showed non-significance. Data in Table 6 exhibited significantly highest germination of somatic embryos in cv. Begum Jungi (55.6%) followed by cv. Ajwa (52.3%) formed little shoot clusters from repeated embryos in 3 months in cv. Begum Jungi on medium comprising of NAA 0.05 mg L^{-1}, Kin 1.0 mg L^{-1} (Figure 4a). Simultaneously non-repeated or single somatic embryos produced single plantlets up on germination. Similar treatment of NAA with 2.0 mgL^{-1} Kin simultaneously induced significantly lowest germination and proliferation of somatic embryos. Results indicate low requirement of NAA with Kin for germination of somatic embryos. Similarly, increasing NAA to 0.5 mg L^{-1} decreased germination and multiplication of somatic embryos. In this way, significantly lowest germination of embryos achieved in cvs. Begum Jungi (7.6%) and Ajwa (8.3%) at the end of third subculture on medium comprising of NAA 0.5 mg L^{-1}, Kin 2 mg L^{-1}. Several studies (Abul-Soad, 2011; Jatoi *et al.*, 2015) obtained maximum multiplication and germination of somatic embryos on the medium consisted of 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} Kin. Solangi *et al.* (2022) obtained high rate of germination in Aseel and Dhakki cvs. on medium comprising of 0.05 mg L^{-1} NAA + 1 mg L^{-1} Kin. Fujimura and Komamine (1975) described requirement of cytokinins for somatic embryos induction and maturation. Ammirato and Steward (1971) observed role of cytokinins in development of cotyledons. Similarly, in this study high germination rate of somatic embryos obtained on medium

Table 6: *Effect of different treatments of NAA + Kin on induction of shoots per little embryos cluster of cvs. Begum Jungi and Ajwa.*

NAA + Kin (mg L^{-1})	Germination of somatic embryos (%)					
	Begum Jungi			Ajwa		
	S1	S2	S3	S1	S2	S3
0.05+1.0	23.3±2.18[a]	34.6±0.88[a]	55.6±2.90[a]	19.3±1.33[a]	29.6±4.37[a]	52.3±3.71[a]
0.05+2.0	13.6±1.85[b]	21.6±0.88[b]	28.6±1.85[b]	13±1.52[b]	18.3±3.66[b]	27.6±1.76[b]
0.5+1.0	6.0±0.57[c]	8.3±1.66[c]	22±0.57[c]	8.6±0.33[c]	7.6±1.45[c]	19.6±1.33[c]
0.5+2.0	2.6±0.66[c]	3.7±0.66[d]	7.6±0.88[d]	3.3±0.33[d]	2.6±0.33[c]	8.3±0.66[d]
LSD at $p<0.05$	0.0000***	0.0000***	0.0000***	0.0000***	0.0009**	0.0000***
Source of variability (3-way ANOVA)						
Cultivar	0.0032					
Subculture (S)	< 0.0001					
Treatment (T)	< 0.0001					
Cultivar*Subculture	0.3211					
Cultivar*treatment	0.0351					
Subculture*treatment	< 0.0001					
Cultivar*Subculture*treatment	0.6352					

comprising of NAA+Kin. After complete germination of repeated embryos, a cluster of plantlet with roots was obtained (Figure 5a). Shoots grown up to 10 cm in height detached from cluster and each isolated plantlet was cultured in long culture tubes for shoot elongation and rooting on medium comprising of NAA 0.1 mg L^{-1}, BA 0.1 mg L^{-1} (Figure 5b).

Conclusions and Recommendations

Protocols established successfully for *in vitro* propagation of two elite date palm cvs. Begum Jungi and Ajwa. Proper time for spathe excision from tree observed based on explant growth on initiation medium utilized in current study. Surface sterilization of spathes carried out successfully using NaOCl solution resulted in highest survival percentage of primary explants. Callus formation occurred in floral buds in spikelet explants using PGRs within short period, subsequently developed into somatic embryos and finally into plantlets through successful germination. The results obtained will help to micropropagate elite date cultivars growing in world.

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Novelty Statement

Current study described the proper age of spathes for getting immature explants, auxin-cytokinin interactions in successful somatic embryogenesis and plantlet regeneration in two commercial cultivars of date palm Begum Jungi and Ajwa.

Author's Contribution

Najamuddin Solangi wrote manuscript as part of his PhD thesis, analyzed data and edited. Mushtaque Ahmed Jatoi helped in data analysis. Adel Ahmed Abul-Soad and Ghulam Sarwar Markhand helped in experimentation, proof reading. Abdul Aziz Mirani and Muhammad Aslam Solangi helped in proof reading and editing.

Conflict of interest

The authors have declared no conflict of interest.

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