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In Vitro Multiplication of *Phoenix dactylifera* (L)

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A method of microcloning *via* somatic embryogenesis was established in datepalm (*Phoenix dactylifera L*) cultivars. The method has potential for commercial application. Embryogenic cultures were initiated from soft primordial tissues of 2-3-year-old female suckers. The system was optimized first for the genotype 'Sayar' and then its applicability was tested to other genotypes. A method of pre-acclimation using pre-acclimation chambers has been devised, which improved hardening survival greatly. More than 80 clones obtained from the genotype 'Sayar' have been shifted to field for agronomic evaluation.

***Key words*:** datepalm, *Phoenix dactylifera*, tissue culture, somatic embryogenesis.

Datepalm is a very important plantation crop of Middle East and its cultivation is catching up in semi-arid regions of India, especially in Rajasthan and Gujarat. Although the selected genotypes can be multiplied through offshoot propagation, however, the rate of multiplication is very low. Thus to meet the demand of planting material for newly identified areas for datepalm plantation, it is necessary to obtain true to the type plants through a method of rapid vegetative propagation. In a species where clonal propagation is the norm rather than the exception, cloning through tissue culture could be highly desirable.

Although various *in vitro* protocols of regeneration through somatic embryogenesis in datepalm have been reported, however, there were limitations like non-synchronous plant production or poor field survival (1-5). Also, in most of the protocols very high levels of auxins (mostly 2,4-D, 50-100 mg l^{-1}) have been used, which may lead to induction of somaclonal variation in the regenerated population (6). Apart from this, a large number of other protocols have also been developed, however, majority of these are genotype specific (7). Due to regional differences in varietal demand and strong genotypic influences on *in vitro* regeneration of datepalm, it becomes necessary to optimize regeneration protocol for specific genotypes. In this communication we report a more efficient protocol to clonally multiply plants of genotypes 'Sayar' and 'Jaglool' through somatic embryogenesis from soft primordial tissue of female suckers.

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzyl aminopurine; IAA, indole-3-acetic acid; NAA, α-naphthalene acetic acid; Kn, Kinetin; MS, Murashige and Skoog; ECI, Embryogenic callus induction.

Materials and Methods

***Callus induction* —** Two to three-year-old offshoots of female plants of datepalm genotype Medzool, Sayar, Samran and Jaglool were obtained from Datepalm Research Centre of Rajasthan Agricultural University, Bikaner. After removing the mature leaves, offshoots were trimmed down to the apical bud region. Soft tissue segments measuring about 2-3 cm were cleansed with 3% (v/v) Teepol detergent solution and surface sterilized in 0.1% mercuric chloride solution for 6-7 min, followed by 4-5 rinses in sterile distilled water. Surface sterilized segments were further cut into small pieces (1-3 mm) prior to inoculation on to nutrient culture medium. 20-25 pieces were inoculated per vessel. MS (8) and B5 (9) basal media supplemented with 2,4-D (2.0, 5.0, 10.0, 15.0, 20.0, 25.0 mg l^{-1}) and IAA (0.1, 0.5, 1.0 mg l^{-1}) were used for embryogenic callus induction (ECI medium) in explants. Ascorbic acid (50 mg l^{-1}), citric acid (50 mg l^{-1}), polyvinyl pyrrolidone (100 mg l^{-1}) and adenine sulfate (25 mg l^{-1}) were added in the medium to effectively check the browning of the explants. The pH of the medium was adjusted to 5.8 with 0.1N NaOH or HCl after adding 0.8% agar (Merck, India) and before autoclaving at 1.04 Kg cm^2 for 15 min. All cultures were raised in polystyrene vessels (67x55 mm, Bioplast), each dispensed with 25 ml medium. Cultures were incubated at 26 ± 1°C under continuous dark and were examined at an interval of 15 days.

Embryogenic callus induction — After 40-45 days of dark incubation, the swollen explants with or without callus were transferred to MS medium supplemented with IAA (0.1 mg l^{-1}) + 2,4-D (2.5 mg l^{-1}) + Kn (0.25, 0.5, 1.0 mg l^{-1}) (ECI medium). The cultures were then shifted from dark to 14h photoperiod at a light intensity of 14 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps at 26 ± 1°C. Each treatment was replicated 10 times.

Embryo maturation and germination — White tubular embryoids and associated proembryonic mass (< 0.5 mm) (Fig.1B) were transferred to MS medium supplemented with BAP (0.1, 0.5, 1.0 mg l^{-1}) for maturation. All the cultures were maintained at 26 ± 1°C under 14 h photoperiod at 28 µmol m^{-2} s^{-1}. For germination and growth, 1-2 mm long embryos, which would be easily separable from the clump, were incubated in glass tubes (25 x150 mm, Borosil) capped with plastic caps and dispensed with ½ strength MS medium with or without NAA (0.1, 0.5, 1.0 mg l^{-1}). All the cultures were maintained at 26 ± 1°C under 14 h photoperiod at 68 µmol m^{-2} s^{-1} mixed light. Each subculture was of 25-30 days.

Hardening — Plantlets (5-7 cm long) were transferred to Pre-Acclimation Chambers (PAC, Fig. 2A) with total air volume of 491.5 cm^3. Each chamber consisted of two halves of polystyrene culture vessels (Majenta boxes, Kasablanka™) joined together with a connector ring. Three holes of equal size, placed at an equal distance, were made on top of the upper half of the PAC to permit free but slow air exchange. After filling with a 1:1:1 mixture of Soilrite™, farmyard manure (FYM) and sand, the left over air volume of the PAC was 184.3 cm^3. Three air exchange areas (PAC type I, Type II, Type III, Table 3) were tested for acclimation. Periodically water was poured in PACs. The upper half of the PAC was removed at 1/2/3 week's interval (Fig. 2B). PACs were kept in culture room maintained at 26 ± 1°C, 14 h photoperiod at 68 µmol m^{-2} s^{-1} and 40% RH. After four weeks' acclimation all the plants were shifted to green house maintained at 28 ± 1°C, RH 80 - 40% from pad to fan, 14 h photoperiod with a light intensity of 186 - 232.5 µmol m^{-2} s^{-1}.

Results and Discussion

Embryogenesis — Explants increased in size, became swollen and induced white globular proembryonic mass (Fig. 1A) from within the explants, 40-50 days after incubation on MS medium and fragile snowy callus on B5 medium supplemented with 2,4-D (2.5-15.0 mg l^{-1}) and IAA (0.1 mg l^{-1}). Supplementation of IAA at 0.5 and 1.0 mg l^{-1} enhanced the quantity of callus but made it non-globular. Dark incubation of cultures was found to be necessary, as cultures kept in light did not show any activity. This is in line with the observation made by Dass *et al* (10) and Sharon and Shanker (5). Upon subculturing on ECI medium supplemented with 2,4-D (2.5 mg l^{-1}) + Kn (0.25 mg l^{-1}) + IAA (0.1 mg l^{-1}), embryogenic mass with coleoptile like structures (Fig. 1B) was observed 45-60 days after incubation under 14 h photoperiod. ECI medium containing higher level of Kn (0.5, 1.0 mg l^{-1}) and/or 2,4-D (5-15 mg l^{-1}) were not found conducive for embryo induction. Also it was observed that non-globular callus derived on B5 medium with various levels of 2,4-D did not induce any embryogenesis on ECI medium. This could be due to form of nitrogen and low amount of NH_4^+ present in B5 medium. The form of nitrogen in the induction medium has been reported to significantly affect *in vitro* embryogenesis. Carrot calli initiated on a medium with KNO_3 (B5 medium) as the sole source of nitrogen failed to form embryos after removal of the auxin (11) and absolute and high requirement of ammonium during induction and differentiation of somatic embryogenesis has been reported by various workers (12,13).

Auxins (2,4-D/IBA/IAA) alone or in combination with cytokinins (Kn/BAP/2iP) have been used to induce somatic embryogenesis in datepalm cultures, however, mostly with very high levels (50-100 mg l^{-1}) (2,3,5,14). Such high levels of 2,4-D may, however, induce high degree of somatic variations. High 2,4-D has been reported to induce polyploidy in cell cultures (6), and direct as well as inverse correlation has been reported between polyploidy in tissue cultures and the presence of 2,4-D in the medium (15). Karp (16) has emphasized the need of optimization of plant growth regulators used in culture medium to minimize somaclonal variation through control of rapid disorganized growth and cell division. In our experiments, low level of 2,4-D (2.5 mg l^{-1}) and IAA (0.1 mg l^{-1}) was very effective in inducing competence in the explants for embryogenesis and frequency of embryogenesis on ECI medium was strongly influenced by the level of the 2,4-D in the CI medium (Table1). Maximum frequency (80%) was observed in explants induced on 2.5 mg l^{-1} 2,4-D. Higher level of 2,4-D (5.0-10.0 mg l^{-1}) in CI medium had an inhibitory effect on frequency of embryo induction in ECI medium with complete inhibition at 15.0 mg l^{-1} 2,4-D (Table1).

Table 1. Effect of 2,4-D on the induction of somatic embryogenesis

Level of 2,4-D in ECI medium (mg l^{-1})	Frequency of embryogenesis (%)
02.5	80
05.0	40
10.0	10
15.0	nil

*For the composition of the ECI medium, please see Materials and Methods.

Maturation and germination — Tiny embryos with small coleoptiles did not grow further if left on ECI medium. Upon transfer on MS medium supplemented with BAP (0.1, 0.5,1.0 mg l^{-1}) they turned green and developed into typical monocot embryos, 25-30 days after incubation (Fig. 1C). Occasionally 1-2 embryo precociously germinated (Fig. 1B) on this medium. Supplementation of BAP appeared necessary, as embryos will not develop further on basal medium or medium supplemented with Kn. In somatic embryogenic cultures of datepalm, Mater (17) and Calero *et al* (18) have observed that mature embryos would readily germinate on 0.1 mg l^{-1} BAP than as compared to other BAP levels (0.5, 1.0 mg l^{-1}) and that the presence of BAP was necessary for the normal production of plantlets. Although all the levels of BAP seem to be effective for embryo maturation, concentration of BAP was significantly important for maturation and sustenance of somatic embryogenesis. Embryo maturation and cutting off of the new embryoids will go on at the base of embryonic clump on MS medium supplemented with BAP (0.1 mg l^{-1}, Table 2). However, more and more abnormal embryos would be cut off on 0.5 and 1.0 mg l^{-1} BAP with successive subcultures. Perusal of data in Table 2 would reveal that maximum number (19-20) of normal embryo could be obtained (for more than 1½ year) through regular subculture on 0.1 mg l^{-1} BAP under low light (28 µmol m^{-2} s^{-1}) conditions. The overall embryo maturation frequency (OEM) was comparable at 0.1 and 0.5 mg l^{-1} BAP levels, however, the OEM declined sharply (30 - 42%) at 1.0 mg l^{-1} and number of abnormal embryos were more at 0.5 and 1.0 mg l^{-1} BAP. Statistically, differences between subcultures were non-significant whereas differences between concentrations of BAP were significant (Table 2).

Tiny green embryos (1-2 mm) were needed to be separated early from the clump and needed to be transferred on to ½ strength MS medium with or without NAA (0.1 mg l^{-1}) for germination into healthy plantlets (Fig. 1 D-F). If not separated early, the embryos would become intertwined due to continuous cutting off of new embryos and their growth. It would then be extremely difficult to separate them into individual embryo resulting in a poor harvest. Germination of embryos, with a leaf emerging through white cotyledonary sheath and thick 2-3 roots from the opposite end, took about 25-30 days. Roots emerged little later than shoot emergence and 5-7 cm long plantlets could be harvested in 60-70 days. Although mature embryos germinated on ½ strength MS medium without the supplementation of auxin, NAA/IAA promoted growth

Table 2. Overall frequency of embryo maturation (OEM) and number of normal and abnormal embryos of datepalm regenerated on maintenance and multiplication medium.

Number of subcultures	Level of BAP in the medium								
	0.1 mg l^{-1}			0.5 mg l^{-1}			1.0 mg l^{-1}		
	OEM (%)	N*	AB**	OEM (%)	N	AB	OEM (%)	N	AB
I	59.6	19.3	1.33	55.0	9.0	9.33	33.0	3.0	8.6
II	71.0	19.0	1.33	57.3	8.6	9.0	37.3	2.33	9.33
III	60.0	19.0	2.0	64.0	9.33	9.6	42.0	2.66	11.33
IV	68.6	20.0	1.33	57.3	8.66	8.0	30.0	1.66	9.66

#Data represent mean of three experiments, each consisting of 10 replicates.

CD value : 1.228 at 5% level of significance.

*N : Normal embryos

**AB : Abnormal embryos

Table 3. Effect of air exchange area and duration of acclimation in pre-acclimation chamber (PAC) on final survival of plantlets 15 days after transfer to green house

Air exchange area	Percentage survival#		
	Duration of acclimation (Weeks)		
	One	Two	Three
PAC@ Type I : 3.87 cm^2	75 (09)$	92 (11)	75 (09)
PAC Type II : 7.85 cm^2	58 (07)	75 (09)	67 (08)
PAC Type III : 39.80 cm^2	42 (05)	33 (04)	33 (04)

@Total air volume of each PAC after filling soil mixture : 184.32 cm^3
#12 plants/treatment were used. Size of plants, when shifted to PAC, was 5-7 cm.
$ Values in parentheses show mean number of plants.

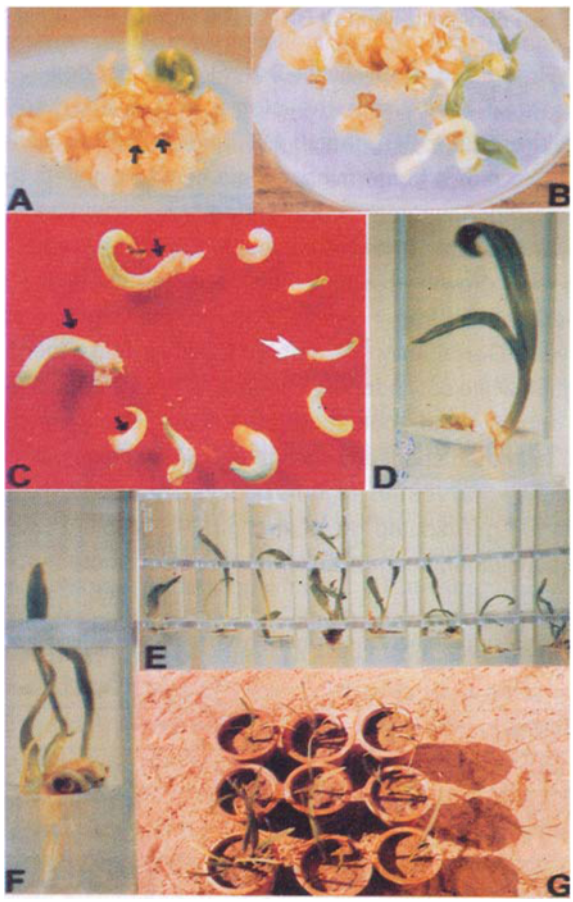

Fig.1. (**A**) Globular callus with proembryonic masses (shown by arrow) on ECI medium; (**B**) Tubular embryos with 1-2 precociously germinated ones on ECI medium; (**C**) Typical mature monocot embryos, both normal and abnormal (black arrow) on MS medium supplemented with 0.1 mg l^{-1} BAP, white arrows indicate a small embryonic mass at the base of embryos which would cut off new embryoids; (**D**) A germinated plantlet on ½ strength MS medium; (**E**) Many germinated embryos; (**F**) A plantlet germinated on ½ strength MS medium supplemented with 0.1 mg l^{-1} NAA having thick root; and (**G**) Hardened plants in pots.

Fig. 2. (**A**) Plantlets in pre-acclimation chambers (PAC type II with an air exchange area of 7.85 cm^2; and (**B**) Healthy plants in PAC (upper half removed) after two weeks of pre-acclimation.

of thicker roots (Fig. 1F). Also the growth of plantlets was better on auxin supplemented medium. NAA at low concentrations (0.1,0.5 mg l^{-1}) has been reported to enhance the root growth during germination of embryos in datepalm (5,10,19).

***Hardening and field survival* —** For hardening, 7-9 cm long plantlets were shifted to green house (RH 80-40% pad to fan, light 186-232 µmol m^{-2} s^{-1}, 16 h photoperiod and temperature 28 ± 1°C) but the mortality was more than 50 per cent (data not shown) in the first week itself. However, the survival rate could be increased effectively (up to 92%, Table 3) if plantlets were passed through a pre-acclimation phase in PACs (Fig. 2A,B). Perusal of data in Table 3 would reveal that maximum survival rate (92%) was obtained if plantlets were pre-acclimated in PAC type I for two weeks with an air exchange area of 3.87 cm^2. Doubling the air exchange area (7.85 cm^2, PAC Type II) decreased the survival rate significantly (75%), whereas further increase (39.80 cm^2) in air exchange area of PAC (Type III) caused severe decline in the survival rate (33%) primarily due to rapid desiccation of plants in the first week of acclimation. Survival of plantlets was also affected if they were left in PAC type I and II for more than 2 weeks due to fungal buildup. Pre-acclimation in PAC also reduced the duration of acclimation significantly in green house (data not shown). In the most recent protocol on datepalm micropropagation, Sharon and Shanker (19) have regenerated plantlets through direct organogenetic pathway, however, field survival of hardened plantlets was poor. In our protocol survival of plantlets is more than 90% and plants are growing in the field since one year without any mortality.

Among the four genotypes tested for regeneration, somatic embryogenesis could be obtained from 'Sayar' and 'Jaglool' only. The other two genotypes did not respond. So far 80 plantlets obtained from 'Sayar' have been hardened and are being evaluated in field for agronomic traits. The results demonstrate efficient cloning of female suckers of two genotypes of datepalm through somatic embryogenesis. The morphogenic potential of cultures could be maintained for more than 1½ year with a high multiplication rate. The protocol under report is highly amenable for scaling up. Further, the novel use of pre-acclimation chambers, not only significantly improved the hardening percentage of the plantlets and their field survival, but it may also significantly cut down the cost of production due to reduction in overall hardening period. Primary RAPD analysis of parents and clonal plants has been attempted which reveals high fidelity of clones (results not shown).

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