CHAPTER 46

DATE PALM *PHOENIX DACTYLIFERA* L. MICROPROPAGATION

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1. INTRODUCTION

Date palm, *Phoenix dactylifera* L., a monocotyledonous angiosperm belonging to the Palmaceae (Arecacea) family, is native to the tropical or subtropical regions of Africa or Southern Asia. Thousands of years of selection have produced the 3,000 varieties currently cultivated around the world in regions where hot arid climatic conditions favored by date palm prevail (Zaid, 2002).

Date palm is mainly a diploid, 2n = 36, dioecious tree species with separate male and female plants. For fruit setting, fertilization of the female flowers is required which often involves manual or mechanical pollination. The date fruit is a single, oblong, one-seeded berry, consisting of pericarp or fruit skin, fleshy mesocarp, and membranous endocarp around the seed. It is highly nutritious and rich in source of sugar, minerals, and vitamins and is considered the most important economical product of date palm. Because of the great diversity in fruit characteristics, industrial processsing is offered an array of applications utilizing whole dates, date fruit preparations, products derived from date fruit, and by-products. In addition, all the vegetative plant parts of the date palm tree have integrated in traditional or industrial applications (Barreveld, 1993).

Access to modern agricultural practices and commercial knoweledge has encouraged date production. According to FAOSTAT data (2006), world dates production has doubled in the last 15 years as it changed from 3,431,207 metric ton in 1990 to 6,924,975 metric ton in 2005. In a decreasing order, countries produced over 200 thousands metric ton of dates in 2005 were, Egypt, Saudi Arabia, Iran, United Arab Emirates, Pakistan, Algeria, Sudan, and Oman. Other producing countries with yields ranging from 20 thousands to 150 thousands metric ton of dates are Libya, China,

S.M. Jain and H. Häggman (eds.), Protocols for Micropropagation of Woody Trees and Fruits, 509–526.
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Tunisia, Morocco, Yemen and Mauritania. To a limited extent, dates are also produced in the United States of America, Mexico, and Spain among other countries.

Date palm may reach an age of over 100 years and a height of over 20 m. Only about 20 offshoots are produced during the first 10 to 15 years of the tree life. This puts limitations on the efficacy of the traditional propagation method involving separating and planting offshoots. Propagation by seeds is not applicable for cultivation of known cultivars because genetic traits including fruit characteristics are not maintained. Alternatively, micropropagation is gaining increased interest as it provides a rapid mass clonal propagation means.

Date palm micropropagation began a quarter of a century ago (Reuveni, 1979; Reynolds & Murashige, 1979; Tisserat, 1979) and has progressed relatively slowly due to its inherent slow growth nature and limited research resources available to the developing countries where date palm is mostly grown. Several review articles describing early work on plant regeneration through indirect somatic embryogenesis and indirect adventitious organogenesis (Benbadis, 1992; Omar et al., 1992; Tisserat, 1984). Reports demonstrating the ability to regenerate date palm through direct regeneration, without callus stage, are also available (Sudhersan et al., 1993). Micropropagation through direct regeneration is thought to reduce the potential for undesirable somaclonal variants among regenerants while somatic embryogenesis mediated by callus stage is highly efficient and more popular in research laboratories (Al-Khayri, 2005).

In recent years, several studies have examined various components of the culture medium including sucrose (Veramendi & Navarro, 1996), silver nitrate (Al-Khayri & Al-Bahrany, 2001, 2004a), biotin and thiamine (Al-Khayri, 2001), auxins and salt strength (Al-Khayri, 2003), and other tissue culture factors (Bekheet et al., 2001). Cell suspension cultures have been employed to study aspects related to physiology (Al-Khayri, 2002; Al-Khayri & Al-Bahrany, 2004b), and somatic embryogenesis (Fki et al., 2003; Zouine et al., 2005). Expected to revolutionize date palm propagation, development of synthetic seeds is gaining research interest (Bekheet et al., 2002). In addition to offering an effective propagation means, tissue culture proved applicable in genetic improvement of date palm through induced mutations and *in vitro* selection (El Hadrami et al., 2005; Jain, 2005).

The micropropagation protocol described herein is based on indirect somatic embryogenesis using apical shoot tip explant for callus induction and subsequent plant regeneration.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Sterilization and Excision

2.1.1. Separation of Offshoots from Mother Plant

Select 2 to 3-year-old healthy offshoots from the desired cultivars. Note that offshoots separated in summer usually respond poorly to tissue culture procedure which is affected by the amount of phenolics exudates. Using a hatchet and a serrated knife, trim the leaves to about 30 cm above the shoot tip to facilitate handling (Figure 1A–C).

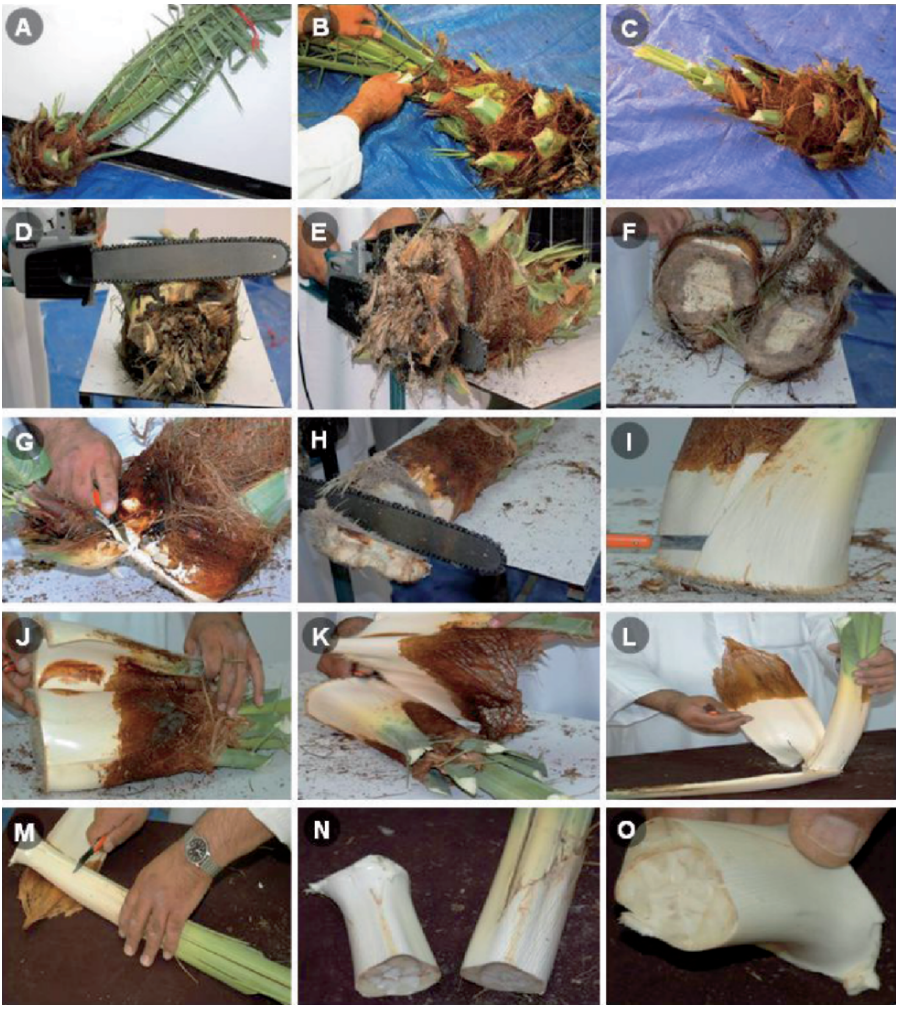

Figure 1. *Procedures for shoot tip isolation from date palm offshoots. A) An offshoot after separation from mother tree. B) Trimming the leaves. C) The remaining stub after leaves trimming. D) Trimming the root region from the truck stub with a chain saw. E) Separating the trimmed root region. F) Note the cut end of the trunk still consists of woody tissues. G) Removal of green older leaves bases and brown fibrous leaf sheath tissue. H) More trimming of the cut base of truck stub with a chain saw. I) Cutting the side with knife to facilitate removal of surrounding tissues. J) Unrolling of the leaf sheath for removal. K) Gradual removal of white young leaves bases and surrounding white fibrous leaf sheath tissue. L) Removal of the last sheath with brown fibrous tissue. M) Cutting the shoot tip region; separation of the shoot tip region. N) Separation of shoot tip region. O) Note the compactness of the immature leaves bases surrounded with a single layer of leaf sheath to exclude disinfectant from reaching the shoot tip.*

2.1.2. Separation of Shoot Tip from Offshoots

Remove outer leaves and surrounding fibrous tissues by cutting at the leaf base in an acropetal fashion until the shoot tip region is exposed. Careful manipulation is necessary to avoid fracturing the characteristically brittle shoot tip and to avoid damaging the axillary buds found at the base of leaf which also can be used as explants source. Continue trimming until the white tissue of the shoot tip region becomes 10-cm high, 4-cm in diameter, cylindrical-shape of white leaf fleshy tissue of young leaves and sheaths protecting the interior containing the shoot tip and the surrounded leaf primordia. Insert a knife about 2 cm away from the circumference of the cylindrical-shaped tip region and cut a circle around the base in a 45° angle. Alternatively, where a chain saw is available, follow the stepwise procedures described in Figure 1A–O. Date palm tissue is known to produce phenolics upon cutting causing browning and tissue deterioration; therefore, keep excised shoot tips in a cooled antioxidant solution (150 mg l^{-1} each, ascorbic acid and citric acid).

2.1.3. Surface Disinfection

Place isolated shoot tip region and axillary buds tissues in a beaker or a jar containing a solution of 70% ethanol for 1 min, then replace with 1.6% w/v sodium hypochlorite (30% v/v commercial bleach) containing two drops of Tween 20 per 100 ml disinfection solution and shake or stir for 15 min. Under aseptic conditions, rinse the tissue with sterile distilled water three times and place in a chilled sterile antioxidant solution through explant manipulation process to minimize browning.

2.1.4. Explants Dissection

Three types of explants can be effectively isolated from offshoots including the axillary buds, leaf primordia, and shoot tip meristem. Section axillary buds into 2 to 4 longitudinal segments, and use them as explants. To isolate leaf primorida and shoot tip meristem explants, follow the steps described in Figure 2A–H. The shoot tip region consists of apical meristematic bud surrounded by a number of leaf primordia, encased by fleshy white tissue of leaf sheaths. In a Petri dish, use a scalpel and forceps to trim the ends exposed to disinfectant, and then remove the outer tissue by cutting longitudinally along the leaf axial and transversely underneath the base of the leaf at the point of attachment. Trim this outer tissue when it is about 5 cm long and 1 to 2 cm wide. Separate leaf primordia and cut each primordium into two transverse sections and use as explants. Section the remaining shoot tip meristematic tissue into 4 to 8 longitudinal sections and use as explants for both somatic embryogenesis and adventitious organogenesis protocols. In addition other explant sources have been utilized including inflorescence parts and leaf tissue (Bhaskaran & Smith, 1992; Fki, et al., 2003).

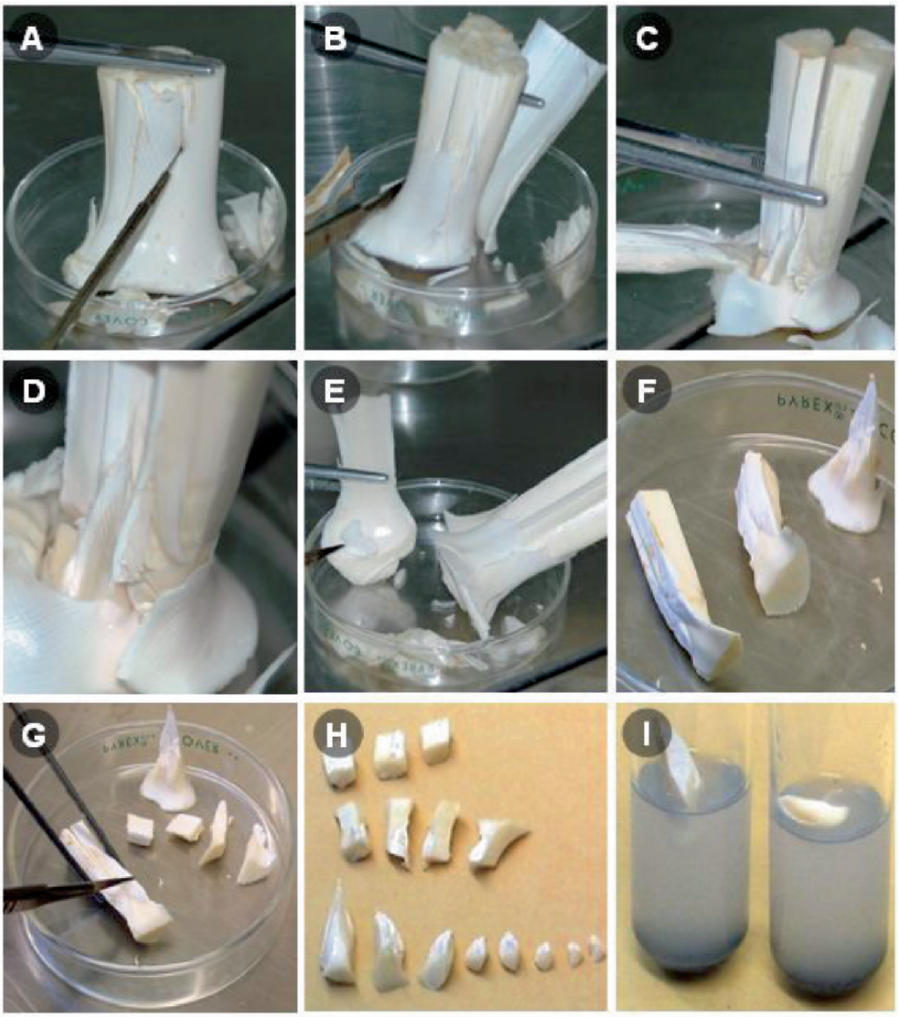

Figure 2. *Date palm shoot tip region dissection stepwise procedures used to excise leaf primordia and tip explants for culture initiation. A) Make a longitudinal cut in the sheath surrounding the immature leaf petioles. B) Remove and discard the leaf petioles surrounding the tip in a whorl fashion until 4 or 5 are remaining. C) Remove the remaining petioles and keep for explanting. D) Exposing the leaf primordia and leaf tip meristematic region. E) Note the bright white region where an axillary bud has formed at the base of the immature interior leaf petioles. F) Selection of tissue with meristematic regions including immature petiole bases and shoot tip shown still surrounded by leaf primordial. G) Dissection of the immature axillary bud regions from the petiole. H) Explants isolated from shoot tip region ready for inoculation. I) Explants on culture initiation medium containing activated charcoal.*

2.2. Culture Medium

2.2.1. Basal Medium Components

For medium used to induce adventitious organogenesis, the reader is referred to other protocols reviewed above since this protocol focuses on indirect somatic embryogenesis. In both cases, however, the basal medium commonly used for date palm tissue culture is based on MS salts formulation (Murashige & Skoog, 1962) with modifications by different authors to achieve genotype-specific and stage-dependent optimizations, by particularly manipulating the hormonal and vitamin content. Either commercially available prepackaged MS salt formulation or in-house prepared stock solutions can be used for medium preparation. Table 1 lists the components of MS medium and specifies other inorganic and organic additives.

2.2.2. Medium Preparation

Prepare the basal medium including all additives except plant growth regulators and activated charcoal which are added according to culture stage as shown in Table 1. Adjust medium to ph 5.7 with 1 M KOH and HCl, and dispense in 150 × 25-mm culture tubes (15 ml per tube), 125-ml culture flasks (30 ml per flask), or GA-7 magenta vessels (50 ml per vessel). These vessels are autoclaved for 15 min at 121°C and 1×10^5 Pa (1.1 Kg cm^{-2}) and kept in a cool place ready for culturing. Culture tubes are more suitable for culture initiation and rooting stages but the other vessels are more appropriate for callus multiplication and regeneration stages.

2.3. Callus Induction and Plant Regeneration

2.3.1. Callus Induction and Multiplication

1. Inoculate explants on culture initiation medium prepared according to Table 1. Place explants vertically with the basal end slightly inserted, 1–2 mm deep, into the medium (Figure 2 I). Because of potential latent internal contamination, a persistent problem in date palm tissue culture, it is advisable to culture explants in individual vessels throughout the procedure to limit cross contamination.
2. Incubate cultures at 23 ± 2°C in complete darkness for 9 weeks, during which transfer at a 3-week interval. Note explants swelling and slight expansion, a useful criterion to determine viability of newly cultured explants (Figure 3A, B).
3. While avoiding cutting the tissue to minimize browning, transfer entire explants to callus induction medium (Table 1) and maintain in darkness for 3 weeks. Callus formation becomes clearly visible. Note that auxin was switched from 2,4-D to NAA to reduce potential mutations. However, it is possible to use the initiation medium (Table 1) throughout callus stages from initiation to maintenance.
4. Again, transfer entire explants to callus proliferation medium (Table 1) and maintain in darkness for 9 weeks. More callus growth occurs that can be easily separated from the original explants at the end of this stage.

Table 1. *Formulation of culture medium used for date palm micropropagation based on modified MS salt augmented with culture stage-specific plant growth regulators.*

Components	Chemical formula	Stock (g/L)	Medium (mg/L)
Major nutrients, 10 × stock, use 100 ml per L medium			
Ammonium nitrate	NH_4NO_3	16.5	1650
Potassium nitrate	KNO_3	19.0	1900
Calcium chloride-2H_2O	$CaCl_2 \cdot 2H_2O$	4.4	440
Magnesium sulfate-7H_2O	$MgSO_4 \cdot 7H_2O$	3.7	370
Potassium orthophosphate	KH_2PO_4	1.7	170
Sodium phosphate	$NaH_2PO_4 \cdot 2H_2O$	1.7	170
Minor nutrients, 100 × stock, use 10 ml per L medium			
Potassium iodide	KI	0.083	0.83
Boric acid	H_3BO_3	0.62	6.2
Manganese sulfate-4H_2O	$MnSO_4 \cdot 4H_2O$	2.23	22.3
Zinc sulfate-7H_2O	$ZnSO_4 \cdot 7H_2O$	0.86	8.6
Sodium molybdate-2H_2O	$Na_2MoO_4 \cdot 2H_2O$	0.025	0.25
Cupric sulfate-5H_2O	$CuSO_4 \cdot 5H_2O$	0.0025	0.025
Cobalt chloride-6H_2O	$CoCl_2 \cdot 6H_2O$	0.0025	0.025
Iron –EDTA, 100 × stock, use 10 ml per L medium			
Iron sulfate-7H_2O	$FeSO_4 \cdot 7H_2O$	2.78	27.8
Ethylenediamine tetraaceticacid disodium	$Na_2EDTA \cdot 2H_2O$	3.73	37.3
Vitamins, 100 × stock, use 10 ml per L medium			
Myo-Inositol		12.5	125
Nicotinic acid		0.1	1
Pyridoxine hydrochloride		0.1	1
Thiamine hydrochloride		0.1	1
Glycine		0.2	2
Calcium pantothenate		0.1	1
Biotin		0.1	1
Other additives			
Glutamine			200
Ascorbic acid			100
Citric acid			100
Sucrose			40000
Agar			7000

Plant growth regulators and activated charcoal, add according to culture stage

	Final concentrations (mg/L)			
Culture stage	2,4-D	2iP	NAA	Charcoal
Culture initiation	100	3	–	1500
Callus induction	—	30	10	1500
Embryogenic callus	—	6	10	1500
Callus multiplication	—	1.5	10	—
Embryogenesis	—	—	—	—
Rooting	—	—	0.2	—

5. Isolate callus growth from original explants and transfer to callus multiplication (or maintenance) medium (Table 1) and maintain in darkness until desired amount of callus is obtained (Figure 3C). Callus maintained over 2 years has preserved embryogenic potential; however, lengthy maintenance periods aught to be avoided to minimize mutations and ensure practical embryogenic capacity.

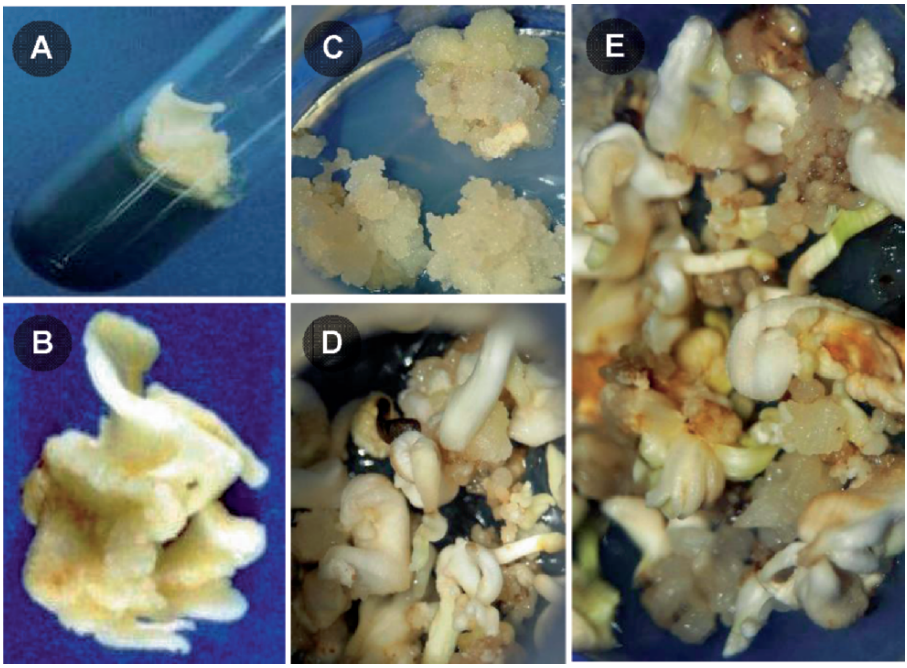

Figure 3. *Date palm callus and regeneration of cultures. A) Explant cultured on initiation medium. B) Expansion of explant and callus induction. C) Callus multiplication culture. D) Various stages of somatic embryogenesis on hormone-free medium. E) Early somatic embryo germination.*

2.3.2. Somatic Embryogenesis

1. Transfer callus from callus multiplication cultures to a hormone-free medium (Table 1) to encourage somatic embryo development, maturation, and germination which normally requires a minimum of 9 to 12 weeks. Incubate cultures at 23 ± 2°C and 16-h photoperiods (50 µmol·m^{-2}·s^{-1}).
2. After 4 to 8 weeks, globular embryos become visible and subsequently elongate forming bipolar-shape (Figure 3D). Mature somatic embryos are transferred individually to a fresh medium for germination and elongation (Figure 3E).

2.3.3. Cell Suspension Culture

1. Cell suspension culture is applicable for efficient mass micropropagation and provides a versatile tool for various *in vitro* studies. To establish cell suspension cultures inoculate 1 g of embryogenic callus into 150-ml culture flask containing 50 ml liquid medium. Use liquid medium for establishing and maintaining cell suspension culture (Table 1).
2. Incubate culture flasks on an orbital shaker set to 100 rpm at 23 ± 2°C and 16-h photoperiods (50 $\mu mol \cdot m^2 \cdot s^{-1}$) provided by cool-fluorescent lamps for somatic embryogenesis or in darkness for callus multiplication.
3. For the first 2 to 3 weeks, replace culture medium every 3 to 5 days to wash out phenolics accumulation. Subsequently, maintain cultures by bi-weekly decanting half of the liquid medium after the suspension has been allowed to settle to the bottom of the flask and adding an equal volume of fresh medium.
4. For somatic embryogenesis, use hormone-free liquid medium. However, the addition of certain compounds were found to stimulate embryo development in some cultivars including 1 mg l^{-1} 2,4-D combined with 300 mg l^{-1}, and 25 µM silver nitrate ($AgNO_3$) combined with 0.5 µM 2iP. Embryo maturation can be enhanced by the addition of 10% PEG or 10 µM ABA. The somatic embryos are germinated on a solid hormone-free medium. Partial desiccation of mature somatic embryos to 80% water content will improve germination.

2.4. Rooting

1. After 3 to 6 weeks on germination medium, the embryos form green shoot growth but root development often requires an additional step in which MS medium containing a rooting auxin such as NAA or IBA is used.
2. Transfer germinating embryos, with 2 to 3 cm long shoot, to rooting medium consisting of half-strength MS medium supplemented with NAA (Table 1) and continues to incubate in the light for a minimum of 9 weeks. Note root formation and elongation, shoot elongation, and development of complete plantlets (Figure 4A,B).

2.5. Hardening

1. When the plantlets are 5 to 10 cm long, gently, remove them from culture vessels and rinse under a slow stream of water to remove residual agar from roots (Figure 4C). Place plantlets upright in a beaker containing enough water to submerge the roots and cover with a transparent plastic bag for 3 to 6 days. To reduce *ex vitro* infestation, submerge plantlets in 500 mg l^{-1} Benlate for 15 min.
2. Plant regenerants individually in 5-cm plastic pots containing moistened potting mix consisting of equal portions of soil, peat moss, and vermiculite (Figure 4D). To prevent desiccation, keep plantlets moistened during the process of soil transfer.

3. Water the transplants with 100 mg l^{-1} N-P-K fertilizer (20-20-20) and keep potted plantlets in clear plastic enclosures under culture room conditions. Gradually reduce humidity by adjusting air flow in the plastic enclosures (Figure 4E). Upon noting any signs of welting, mist with water and readjust opening as necessary.
4. After 1 month transfer plantlets to a cool shaded greenhouse for further growth, after 3 to 6 months transfer to a shade house and maintain for 12 to 24 months depending on cultivar, and then transplant in the field (Figure 5 A–C*)*.

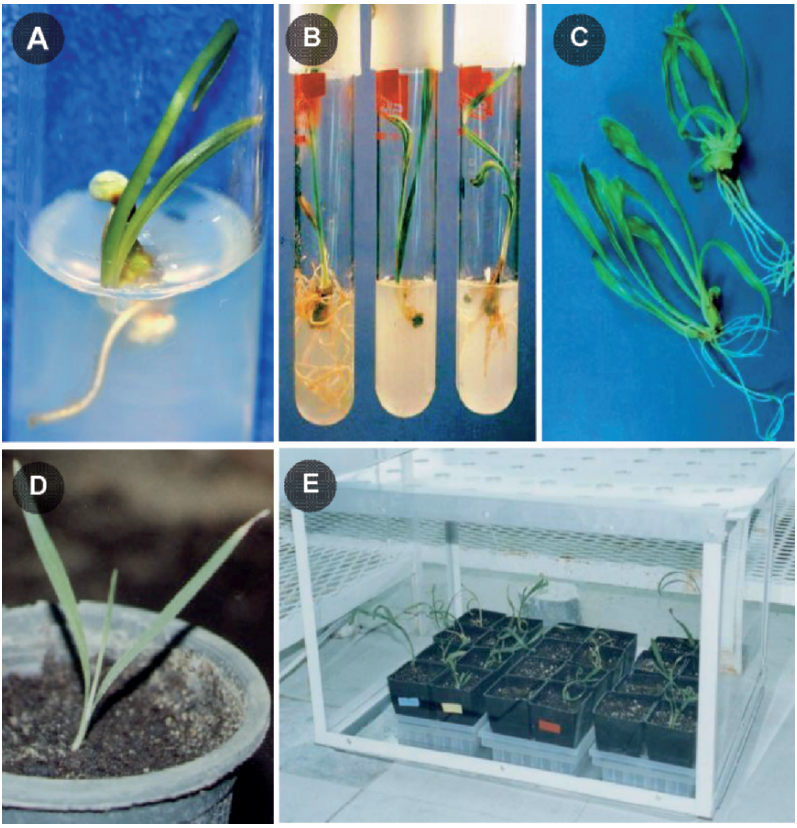

Figure 4. *Rooting and complete date palm plantlets. A) Shoot development and root initiation. B) Shoot and root elongation, from left to right, with 0.2 mg l^{-1} NAA, no hormones, and 0.2 mg l^{-1} IBA, respectively. C) Complete plantlets after rinsing agar residues. D) Soil transfer of plantlets. E) Plantlets in an acclimatization chamber as an alternative to plastic bags or mist systems.*

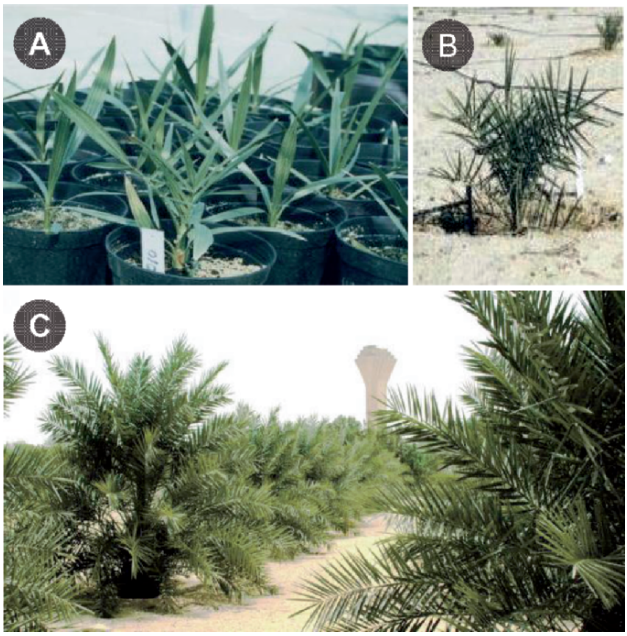

Figure 5. *Field transfer of in vitro-derived date palm plants. A) Formation of a true leaf under greenhouse conditions. B) Plants after 1 year of transfer to field, C) The same field 1 after 4 years showing normal phenotype and vigorous growth.*

2.6. Morphological and Molecular Analysis

The issue of genetic fidelity of date palm plantlets derived from tissue culture has been a controversial subject. Some reports have suggested limited occurrence of somaclonal variants among regenerants based on morphological characteristics and DNA profile, while others observed no variability. Such differences in results could be attributed to the mode of regeneration used, the length of the callus culture maintenance period, the growth regulators augmented to the culture medium, as well as genotypic differences.

Although changes in some of the morphological characteristics and growth parameters of vegetative and fruit stages are visually obvious, correct cultivars identification in date palm is usually not possible until fruit stage which manifests several years after field transplanting. Furthermore, performance characteristics, such as fertility, yield potential, fruit quality or disease susceptibility, are hidden in the juvenile stage. This makes morphological characterization impractical and does not allow either correct cultivar identification or early detection of off-type regenerants at juvenile stage.

Since morphological identification of off-types is unreliable particularly at the juvenile stage, molecular markers describe the internal make-up of a plant and identify the variance based on either the composition of DNA or protein products

expressed from certain regions of the DNA (Kunert et al., 2003). In addition to the commonly used Southern analysis**,** new PCR-based methods namely, randomly amplified polymophic DNA (RAPD) and amplification fragment length polymorphism (AFLP) are gaining interest to assess variation among date palm regenerants (Saker et al., 2006).

2.6.1. DNA Isolation and Purification

Grind 1–2 g plant tissue, young leaves of offshoots or regenerants tissue, in liquid nitrogen with a mortar and pestle, then extract for 30 min at 65°C in 10 ml extraction buffer (0.1M tris-HCl pH 8.0, 50 mM EDTA, 0.5M NaCl, 1% SDS, 150 µg/ml proteinase K and 3% CTAB. Centrifuge lysate for 10 min at 4°C, precipitate DNA with 0.6 vol of cold isopropanol, and then centrifuge to pellet the DNA. Resuspend DNA pellet in 1 ml TE buffer. For further purification of DNA, mix the partially purified DNA with CsCl, then mix in ethidium bromide (EtBr), incubate on ice for 30 min, and then centrifuge at 60,000 × g for 16 h. Collect the DNA band, and remove EtBr and CsCl. Incubate DNA for 1 h at –20°C, centrifuge, and then resuspend pellet in TE buffer. Precipitate DNA by adding 0.1 vol 3 M sodium acetate and 2 vol ethanol. Quantification of DNA can be assessed using UV spectrophotometer at 260 nm.

2.6.2. Restriction Enzyme Digestion and Electrophoresis

Dilute 2 µg DNA in 25 µl TE buffer pH 8, add 10 µl digestion buffer (Pharmacia Biotech), 1 µl of selected restriction enzymes (10 units enzyme per µg DNA) such as EcoRI, BamHI, PstI, HindIII, HinfI, TaqI. Bring final volume to 50 µl with TE buffer and incubate at 37°C for 3 h. Add 5 × gel loading dye and incubate at 65°C for 20 min to terminate digestion. Allow the mixture to cool to room temperature and load samples along with 100 bp ladder DNA marker on 1.8% agarose gel in 0.5 × TBE and electrophoresis for further analysis.

2.6.3. Southern Analysis

Gel preparation and DNA transfer. After restriction digestion of genomic DNA is complete, load 2 µg samples on 1.2% agarose gel in 1 × TAE buffer and electrophorese. Incubate the gel in 200 mM HCl for 10 min to depurinate the DNA, then in denaturation solution (1.5 M NaCl + 0.5 M NaOH) for 25 min, followed by incubation in neutralization solution (1.5 M NaCl + 0.5 M Tris-HCl, pH 7.5) twice for 20 min each at room temperature with gentle shaking and brief wash between different solutions. Transfer DNA to the blot membrane by placing the gel onto a nitrocellulose membrane and position in the capillary blotting assembly using 20 × SSC buffer and following standard procedures. Wash membrane briefly in 2 × SSC, dry between folds of Whatman No.1 filter paper, then bake at 80°C to fix the DNA to the membrane.

Probe labeling and hybridization. Southern procedures require homologous probes which are either randomly selected or specified according to the sequence of the gene of interest. Probes labeling can be preformed using a radioactive or a non-radioactive

random prime labeling kit (Gene Images Southern Analysis Kit, Amersham) following the manufacturer's instructions. The procedures call for taking 100 ng aliquots of purified probe DNA, diluting to 25 µl with TE buffer pH 8.0, and placing in an ice bath. Heating in a boiling water bath for 5 min to denature the DNA then immediately chill on ice. Place another tube on ice and sequentially add 10 µl nucleotide mix, 5 µl primer, 100 ng denatured DNA (25 µl from the previous tube), 1 µl of enzyme (Klenow fragment, 5 unit/reaction), then bring total volume to 50 µl with distilled H_2O. Incubate the reaction mixture at 37°C for 1 h, boil for 5 min to stop the reaction, and immediately place on ice. Pour the labeled probes in the prepared hybridization tube containing the membrane and hybridization buffer (5 × SSC with 0.1% SDS, 5% dextran sulphate, and 1:20 liquid block). Incubate overnight at 60ºC in the hybridization oven.

Washing of blots and detection. Place membrane in a tray and wash with gentle agitation at room temperature firstly with 1 × SSC containing 0.1% (w/v) SDS at 60ºC for 15 min; secondly with 0.5 × SSC containing 0.1% (w/v) SDS at 60°C for 15 min; thirdly with 100 ml of diluted (1:10 in buffer A) liquid blocking agent for 1 h. Incubate blot for 1 h at room temperature in a solution of the anti-fluorescein-AP conjugates (5000 fold dilution prepared in 0.5% BSA in buffer A). Remove unbound conjugates by washing three times in buffer A containing 0.3% Tween 20 for 10 min each wash. Drain buffer from blot and place on a sheet of saran wrap on a flat surface with the sample side up. Pipette the detection reagent on to the membrane (1 ml per blot), allow to spread uniformly cover the surface for 5 min. Drain excess detection reagent and transfer into a film cassette. In the dark room place a sheet of Hyperfilm MP on the sample side, close the cassette, wrap with a cloth, expose for 1 h at –80ºC, and develop using an X-ray processor (Kodak X-Omat 5000 RA processor).

2.6.4. RAPD Analysis

Restriction digestion, electrophoresis, and blotting. Digest 2 µg DNA samples with restriction enzymes, as described above, in the presence of 2 mM spermidine. Separate DNA restriction fragments by electrophoresis on 0.9% agarose gels at 40 V for 23 h in TAE buffer pH 8.3. Denature gel in 0.5 M NaOH, 1.5 M NaCl (30 min), neutralize in 0.5 M TRIS-HCl, pH 7.4, 3 M NaCl (30 min), and transfer DNA to Hybond N^+ membranes (Amersham) by capillarity using 10 × SSC.

Polymerase chain reaction (PCR). Using commercially-available or custom-designed primers (Operon Technologies Inc., Alameda, CA), PCR reaction can be conducted using Perkin-Elmer Gene Amp PCR Kit with AmpliTaq DNA polymerase. Amplification of DNA segments from at least 10^5 to potentially as high as 10^9 fold can be achieved under the specified conditions. Standardization may be required to achieve optimum amplification. Amplification procedures of date palm genomic DNA described for non-stringent or stringent conditions based on the reaction mixtures and PCR conditions as shown in Table 2. Thermocycler devices are commercially available from various vendors such as GeneAmp 2400 PCR system (Perkin Elmer; Amplitron II Thermolyne). To recover the PCR amplified products, increase the volume of the reaction mixture to 200 µl and extract once with 25 phenol: 24 chloroform: 1 isoamyl

alcohol and twice with 24 chloroform: 1 isoamyl alcohol. Precipitate the DNA in 600 µl ethanol with 0.3 M sodium acetate. Centrifuge and wash DNA pellet with 70% ethanol, air dry, and then dissolve DNA in15 µl TE buffer pH 7.4.

Table 2. *Reaction mixture and thermocycler parameters used to amplify date palm genomic DNA under both non-stringent and stringent PCR conditions.*

	Reaction conditions			
	Non-stringent		Stringent	
Reaction components	*Concentration*		*Concentration*	
Amplification buffer, 10 ×	1 ×		1 ×	
$MgCl_2$	4.5 mM		2.5 mM	
Genomic DNA template	100 ng		100 ng	
dNTP's mix	200 µM		200 µM	
Primers, each	50 pM		50 pM	
Taq DNA polymerase	5 U		2.5 U	
Final volume with water	100 µl		100 µl	
Thermocycler program	*Temp.*	*Time*	*Temp.*	*Time*
Pre-denaturation	94ºC	2 min	94ºC	2 min
Denaturation	94ºC	30 sec	94ºC	1 min
Annealing	36ºC	1 min	60ºC	1 min
Extension	72ºC	1 min	72ºC	2 min
Cycles number	50	—	35	—
Post-extension	72ºC	7 min	72ºC	7 min

Probe labeling and hybridization. Label probes, described above, by random priming with [α^{32}P]dCTP (3000 Ci/mmol, Amersham) using Megaprime Labeling Kit (Amersham) to a specific activity of 10^9 counts/min/µg. Prehybridize filters for 2 h at 65ºC in 0.5 M Na_2HPO_4 pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS. Remove unincorporated nucleotides by chromatography using Bio Spin P30 columns (Bio Rad). Heat denature recovered probe by placing in 100ºC water bath for 10 min. Rinse filters at 65ºC twice, 15 min each, in 125 mM Na_2HPO_4 pH 7.2 with 2% SDS, then twice, 20 min each, in 25 mM Na_2HPO_4 pH 7.2 with 1% SDS. At –80ºC, autoradiograph for 24 – 48 h by using X-OMAT (LS or RP) film (Kodak) or Hyperfilm RP (Amersham). After hybridization, strip probes of the membranes, by washing three times, 20 min each, in 0.1 × SSC with 0.1% SDS at 80ºC. Observe film and analyze data after autoradiography and processing.

2.7. Flow Cytometry

Flow cytometry offers a simple, rapid, and accurate method for determining ploidy levels of DNA in plants by estimating nuclear DNA content based on appropriate external reference plant species (Srisawat et al., 2005). Although this technique has not been applied extensively in date palm, flow cytometeric analysis conducted by Fki et al. (2003) showed no variability in the ploidy level of date palm regenerants following a micropropagation protocol based on somatic embryogenesis. Flow cytometry

was also used to assess ploidy level of fruits produced from hormone-treated unpollinated inflorescences (Ben Abdallah & Lepoivre, 2000). This technique provides a convenient method for identification of date palm cultivars and early detection of genetic variation among regenerants that may spontaneously arise by detecting changes in the genome size as a result of alteration in chromosome number or ploidy level.

2.7.1. Tissue Processing

Select date palm tissue to be tested such as callus, cell suspension, somatic embryos, or young leaf. Include proper external reference plant tissue such as soybean (2C = 2.5), tomato (2C = 1.96), or corn (2C = 5.72). With a razor blade, finely chop approximately 25 mg tissue in 1.0 ml extraction buffer (0.2 M Tris, 4 mM MgCl2, 0.5 % w/v Triton X-100 and 3.0 % w/v polyvinylpyrrolidone). Add 50 µl of RNase and propidium iodide then filter through 42 µm nylon mesh.

2.7.2. Measurements and Device

Calculate the nuclear DNA content, according to the following equation:

2C nuclear DNA content (pg) = (2C peak mean of date palm sample)/(2C peak mean of reference).

The number of base pairs per haploid genome (Bp 1C-1 nuclei) is calculated based on the equivalent of 1 pg DNA = 965 Mega base pairs. Measure the PI's at 585 nm following the manufacturer's directions of a flow cytometer device equipped with 488 nm argon iron laser. An example is FACScalibur programmed with CellQuest software (Becton Dickinson Biosciences, San Jose, CA). Check the calibration of the device between samples.

2.7.3. Data Analysis

Include a minimum of replications per sample, and repeat the experiment at least once to maximize accuracy. Data are usually subjected to ANOVA analysis to assess significant differences in the DNA contents. Multiple mean comparison can be performed with LSD or Tukey test.

2.8. Cryopreservation of In Vitro Cultures

Cryopreservation, the storage of biological materials at ultra-cold temperatures, from –79°C to –196°C, while maintaining freeze-thaw damage to sublethal levels, is considered an excellent technique for preservation of plant germplasm. It is applicable to a wide variety of plant tissues including buds, seed, seed parts, twigs, as well as *in vitro* cultures such as callus, cell suspension, adventitious shoots, and somatic embryos. Studies related to the behavior of date palm *in vitro* cultures under cryopreservation conditions are relatively limited. Tisserat et al. (1985) found that date palm tissue survived cryopreservation for 3 months at –69°C in a cryoprotectant solution containing 10% PEG, 8% glycerol, and 10% DMSO. Mater (1987) found that freezing callus cultures at –250°C for 4 months did not affect somatic embryogenesis

potential. Similarly, immature somatic embryos continued normal growth and development after cryopreservation using plunge freezing method in liquid nitrogen, –196°C, when pretreated with a cryoprotectant mixture of 10% glycerol and 10% sucrose followed by partial drying (Mycock et al., 1997).

2.8.1. Tissue Pretreatment

Culture target tissues such as callus, cell suspension, adventitious buds, or somatic embryos, in a pretreatment liquid MS medium supplemented with 0.5 M sucrose. Incubate cultures at 24 ± 3ºC in complete darkness on a gyratory shaker set at 100 rpm for 48 h. Allow tissue to settle to the bottom of the culture flask and decant the liquid medium in preparation for cryopreservation.

2.8.2. Cryoprotectant Treatment

Prepare cryoprotectant solution by supplementing MS medium with 0.5 M sucrose, 10% DMSO, and 2 M glycerol. Cool the solution to 4ºC and mix in plant materials and gently shake on an orbital shaker for 1 h. In the case of cell suspensions or callus tissue, place 1-ml aliquots into 2-ml cryopreservation ampoules (cryovials), cap, and label. Use appropriate vial size for the sample of interest. Gradually cool cryovials by refrigerating at 4°C for 2 h, then at –20°C for 2 h, and finally submerge in liquid nitrogen for storage, or subsequently place in an ultra-freezer where available.

2.8.3. Thawing and Incubation

To thaw the cryopreserved date palm *in vitro* tissues, transfer ampoules to a water bath set at 40°C until samples are completely thawed. Transfer thawed cell suspension, callus, or embryos to a semisolid medium containing 7 g/L agar dispensed in Petri dishes (25 ml culture medium per plate). Seal dishes with a double layer of Parafilm and incubate at 24 ± 3ºC.

2.8.4. Viability Assessment

To assess the effectiveness of cryopreservation treatments, various parameters can be observed including cell colonies re-growth and amount of resultant microcalli, in case of cell suspensions. Callus growth and somatic embryo germination are other indicative parameters. Callus masses recovered from the cryopreserved cells can be transferred to a hormone-free regeneration medium to examine their capacity to develop somatic embryos.

3. CONCLUSION

This chapter has highlighted recent research progress related to date palm tissue culture and described a reproducible micropropagation protocol based on somatic embryogenesis. The availability of reproducible regeneration systems for date palm has paved the way to several biotechnological applications during the current decade including somaclonal variation selection, cryopreservation, synthetic seeds, as well as cell and protoplast cultures. Furthermore, molecular approaches have been utilized

in cultivar identification and assessment of genetic fidelity of regenerants. At the present, researchers are striving to develop a genetic transformation system to enable improving essential characteristics.

Tissue culture has proved effectiveness for date palm propagation; however, current tissue culture protocols utilize high concentrations of 2,4-D as the main auxin for callus induction and proliferation. Because this compound is known to induce mutations, it may be advisable to develop tissue culture protocols utilizing auxins other than 2,4-D or use lower levels of 2,4-D than currently used. Although somaclonal variants can be useful, variants are extremely undesirable for clonal mass production. To eliminate mutants, it is essential to peruse a routine reliable method suitable for early detection of somaclonal variants.

Furthermore, current protocols utilize mainly the shoot tip as explant source, thus sacrificing the tree. This restricts the applicability of tissue culture to cultivars characteristically produce low numbers of offshoots. Although, other explants such as leaf and inflorescent tissue have been reported as explants source, they have not gained popularity because of their limited success. More research is needed to optimize conditions to demonstrate the use of alternative explants in a wide range of genotypes.

Another main challenge for mass production of date palm using tissue culture is to attain an expedited micropropagation protocols since available procedures require over a year in most cultivars. Moreover, some date palm cultivars still remain recalcitrant to current *in vitro* protocols. Responsive cultivars often produce somatic embryos at asynchronous nature. This characteristic is considered undesirable particularly for the purpose of commercial date palm micropropagation and in the case of synthetic seeds production. Synchronization of somatic embryogenesis merits further investigations on the effect of ABA and PEG.

The future appears promising for date palm biotechnology particularly in commercial production. The relatively high sale price of *in vitro* date palm has not met the consumer expectations which may be reduced by research in automation, bioreactor, and synthetic seed technologies. Albeit research progress, achievements and commercial applicability of date palm micropropagation, further research is necessary to maximize the benefits of biotechnology.

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