Chapter 25

Somatic Embryogenesis of Date Palm (*Phoenix dactylifera* L.) Through Cell Suspension Culture

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Abstract

Date palm (*Phoenix dactylifera* L.) is the oldest and most economically important plant species distributed in the hot arid regions of the world. Propagation of date palm by seeds produces heterogeneous offspring with inferior field performance and poor fruit quality. Traditionally, date palm is propagated by offshoots, but this method is inefficient for mass propagation because of limited availability of offshoots. Plant regeneration through tissue culture is able to provide technologies for the large-scale propagation of healthy true-to-type plants. The most commonly used technology approach is somatic embryogenesis which presents a great potential for the rapid propagation and genetic resource preservation of this species. Significant progress has been made in the development and optimization of this regeneration pathway through the establishment of embryogenic suspension cultures. This chapter focuses on the methods employed for the induction of callus from shoot tip explants, establishment of cell suspension culture, and subsequent somatic embryogenesis and plant regeneration.

Key words Callus, Cell suspension culture, In vitro regeneration, *Phoenix dactylifera*, Somatic embryogenesis

1 Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious fruit tree native to the hot arid regions of the world, mainly grown in the Middle East and North Africa. Since ancient times this majestic plant has been recognized as the *tree of life* because of its integration into human settlement, well-being, and food security in hot and barren parts of the world, where only a few plant species can flourish [1]. Dates represent a high energy source besides being rich in dietary fibers [2, 3]. Their carbohydrate content range is 44–88 %, while proteins represent 2.3–5.6 % and fats 0.2–9.3 % [4]. Date fruits were found to be rich in phenolics. The phenolics present in dates are known to exhibit various pharmacological activities including antiaging, anticancerous, antioxidant, antiviral, and antimicrobial properties, making them a remedy for certain

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_25,

diseases and prevention of chronic inflammations. They also prevent oxidative damages caused as a result of phagocytosis activity of invasive pathogens and pests by lymphocytes [5]. Phenolics are known to reduce blood pressure and have antithrombotic and anti-inflammatory effects [6, 7]. In addition, phenolics inhibit α-amylase and α-glucosidase activities behind the postprandial increase in blood glucose level, which is often manifested in type-II diabetes [5, 8, 9]. Conventionally, this plant is propagated from offshoots and their availability is often limited. However, it produces only about 20 offshoots during the first 10–15 years of the tree life, but plant regeneration through tissue culture is able to provide technologies for the large-scale propagation of healthy true-to-type plants. Plant tissue culture is also an essential tool for plant breeding programs [10] and the conservation of plant genetic resources [11].

Research in date palm tissue culture has received increasing interest, resulting in plant regeneration protocols for a number of commercial date palm cultivars. The literature indicated that plant regeneration of date palm was achieved via somatic embryogenesis depending upon the genotype and the composition of the culture medium. Date palm somatic embryogenesis was improved using biotin [12], abscisic acid (ABA) supplement [13, 14], and coconut water additive [15, 16]. Moreover, the stimulation of direct somatic embryo regeneration from shoot tip explants using N-phenyl N′-1,2,3-thiadiazol-5-ylurea (TDZ) was achieved by Sidky and Zaid [17]. Somatic embryogenesis, which leads to embryonic differentiation from somatic cells and not from fertilized ovules, is often employed because of its numerous advantages [18]. Embryogenic cells are used for the isolation of protoplasts with a highly regeneration capacity [19], genetic engineering [20], and the cryopreservation of plant genetic resources [21].

Cell suspension culture and somatic embryo genesis provide a number of avenues for the date palm genetic improvement based on mutagenesis and in vitro selection studies [22–26]. For the date palm, Al-Khayri [27] successfully determined the suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis.

The protocol explained here is based on induction of callus from shoot tip explants and somatic embryo genesis via cell suspension culture which has been shown to be widely applicable to numerous commercially important date palm cultivars [12, 15, 27–30]. Procedures including explant preparation, callus induction, cell suspension culture, somatic embryo development, plant formation, and acclimatization are described.

2 Materials

2.1 Explant and Media Preparation

1. Date palm offshoots.
2. Murashige and Skoog (MS) [31] medium stock solutions (MS stock I, II, III and IV) (Table 1). Store in the freezer or cold room at 4 °C (*see* **Note 1**).
3. Beakers, measuring cylinders, and glass rods (*see* **Note 2**).
4. 0.1–10 mL pipettes and/or 0.5–100 μL micropipettes.

Table 1
Chemical composition of the modified MS medium (After dissolving all the stock solutions in the deionized water make up the volume to 1 L, adjust the pH of the medium to 5.8 with 0.1 N HCl/0.1 N NaOH and add 7 g/L agar-agar and autoclave the media for 20 min at 121 °C)

Chemical constituents	Concentration (mg/L)	Volume per liter (mL)
Major inorganic nutrients		
NH_4NO_3	33,000	50
KNO_3	38,000	
$CaCl_2{\cdot}2H_2O$	8800	
$MgSO_4{\cdot}2H_2O$	7400	
KH_2PO_4	3400	
$NaH_2PO_4{\cdot}H_2O$	3400	
Minor inorganic nutrients		
KI	166	5
H_3BO_3	1240	
$MnSO_4{\cdot}2H_2O$	4460	
$ZnSO_4{\cdot}7H_2O$	1720	
$Na_2.MoO_4{\cdot}2H_2O$	50	
$CuSO_4{\cdot}5H_2O$	5	
$CoCl_2{\cdot}6H_2O$	5	
Iron source		
$FeSO_4{\cdot}7H_2O$	5560	5
$Na_2EDTA{\cdot}2H_2O$	7460	
Organic supplements		
myo-Inositol	25,000	5
Glutamine	40,000	
Nicotinic acid	200	
Pyridoxine·HCl	200	
Thiamine·HCl	5000	
Glycine	400	
Carbon source		
Sucrose	30 g/L	

5. 1 N NaOH/1 N HCl.
6. Activated charcoal, used at 1.5 g/L.
7. Agar-agar, used at 7 g/L.

2.2 Induction of Callus from Shoot Tip Explants and Establishment of Cell Suspension Cultures

1. Stock solutions of 2,4-dichlorophenoxyacetic acid (2,4-D) (10 mg/L), 2-isopentenyladenine (2iP) (1 mg/L), and naphthalene acetic acid (NAA) (1 mg/L) are prepared and stored in the freezer at −20 °C.

2.3 Packed Cell Volume (PCV)

1. Graduated centrifuge tube.
2. Centrifuge machine.

2.4 Plating Efficiency (PE)

1. Hemocytometer.
2. Petri dishes.
3. Illuminated colony counter.

2.5 Somatic Embryo Development

1. Hormone-free MS medium.
2. Rotary shaker.

2.6 Plant Formation

1. Embryo.

2.7 Hardening and Acclimatization

1. Peat.
2. Vermiculite.
3. Sand.
4. Dehydrated cow manure.

3 Methods

3.1 Explant Preparation

1. 3–4-year-old date palm offshoots are isolated from mother trees and the outer leaves removed, exposing the shoot tip regions which are excised and immediately placed in a chilled antioxidant solution consisting of ascorbic acid and citric acid, 150 mg/L each, to prevent oxidation-induced browning.
2. Shoot tip tissues of about 8 cm long are surface-sterilized in 70 % ethanol for 1 min followed by 15 min in 1.6 % (w/v) sodium hypochlorite solution (30 % v/v Clorox, commercial bleach) containing three drops of Tween 20 per 100 mL solution.
3. The tissue is rinsed 4 times in sterilized distilled water for 5 min.
4. The tissue surrounding the shoot tips is removed until the shoot tip terminal is exposed, about 1 cm long, which is excised and sectioned longitudinally into 6–12 small sections under the laminar flow bench/clean bench (Fig. 1a).

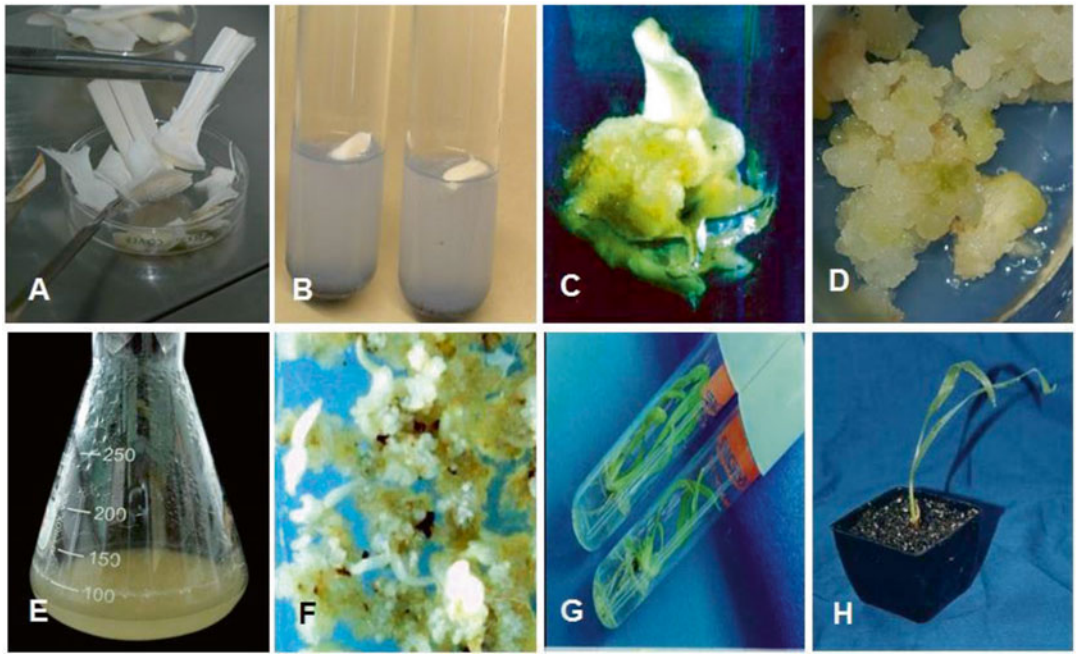

Fig. 1 Stages of date palm cell suspension culture and embryogenesis: (**a**) Explant preparation, (**b**) Culture initiation, (**c**) Callus induction, (**d**) Callus proliferation, (**e**) Cell suspension culture, (**f**) Embryo formation, (**g**) Rooting, (**h**) Transplanted plant. (Source: Part figures (**c**), (**f**), (**g**), (**h**) are taken from Al-Khayri JM (2012) Date palm *Phoenix dactylifera* L. In: Jain SM, Gupta PK (eds) Protocols of somatic embryogenesis in woody plants. Springer, Berlin, pp 309–318)

3.2 Induction of Callus from Shoot Tip Explants

1. These explants are placed on the surface of semisolid MS medium supplemented with 30 g/L sucrose, 100 mg/L 2,4-D, 3 mg/L 2iP, and 1.5 g/L activated charcoal, pH 5.8 for culture initiation (Fig. 1b; Table 2).
2. Incubate cultures at 24 °C ± 3 °C for 12 weeks in the dark. Within 8–10 weeks callus will develop from the shoot tip explants.
3. Culture the resultant callus on semisolid MS medium containing 30 g/L sucrose, 6 mg/L 2iP, 10 mg/L NAA, and 1.5 g/L activated charcoal, pH 5.8 for culture proliferation (Fig. 1c, d; Table 2).
4. Transfer cultures to a fresh medium at 3-week intervals. Use actively growing cell line as explants for the further experiments.

3.3 Proliferation of Cell Suspension in the Liquid Medium

1. Collect actively growing friable cell lines from the semisolid cultures and inoculate 1 g fresh biomass into a 250 mL Erlenmeyer flask containing 50 mL MS liquid medium supplemented with 30 g/L sucrose, 1.5 mg/L 2iP, and 10 mg/L NAA (*see* Table 2). Incubate cultures at 16-h photoperiod of

Table 2
Hormonal and activated charcoal supplements to the culture medium used for date palm callus induction and cell suspension

Media additives	Culture phase		
	Culture initiation	Callus proliferation	Cell suspension
2,4-Dichlorophenoxyacetic acid (2,4-D)	100 mg/L	–	–
2-Isopentenyladenine (2iP)	3 mg/L	6 mg/L	1.5 mg/L
Naphthalene acetic acid (NAA)	–	10 mg/L	10 mg/L
Activated charcoal	1.5 g/L	1.5 g/L	–

cool-white florescent light, 40 μmol/m²/s, and 23 ± 2 °C and agitated at 150 rpm. Maintain cultures by regular subculturing at 2-week interval (Fig. 1e).

3.3.1 Packed Cell Volume (PCV)

1. To estimate the PCV, place 5 mL cell suspension in a sterile graduated centrifuge tube and centrifuge at 2000 × *g* for 5 min.
2. Record the packed cell volume as percentage cell mass of the total centrifuged volume and then the samples are returned to the original cultures.
3. Measurements are taken weekly for 12 weeks. Plot the PCV values in relation to time, to construct a growth curve reflecting various phases of cell growth (Fig. 2).

3.3.2 Plating Efficiency (PE)

1. Dilute cell suspension concentration to give initial cell density of 100, 500, 1000, 5000, 10,000, 50,000, and 100,000 cells/mL with hemocytometer.
2. Mix samples of cell suspensions with melted agar medium after letting the autoclaved medium to cool down to 30–35 °C.
3. The medium used is similar to the cell suspension medium, but contains 7 g/L agar, and is dispensed in 15 × 100 mm petri dishes, at 20 mL per dish (*see* **Note 3**).
4. Mix the cell suspension and molten medium and evenly spread in the plate to solidify, forming a fixed thin layer of cells.
5. To assess the recovery potential of cell suspension to form cell colonies in relation to the initial cell concentration, count the colonies using an illuminated colony counter (Figs. 3 and 4; *see* **Note 4**).

3.4 Somatic Embryo Development

1. Induce cell suspension cultures together with friable callus to undergo somatic embryogenesis by transferring them to a hormone-free medium.

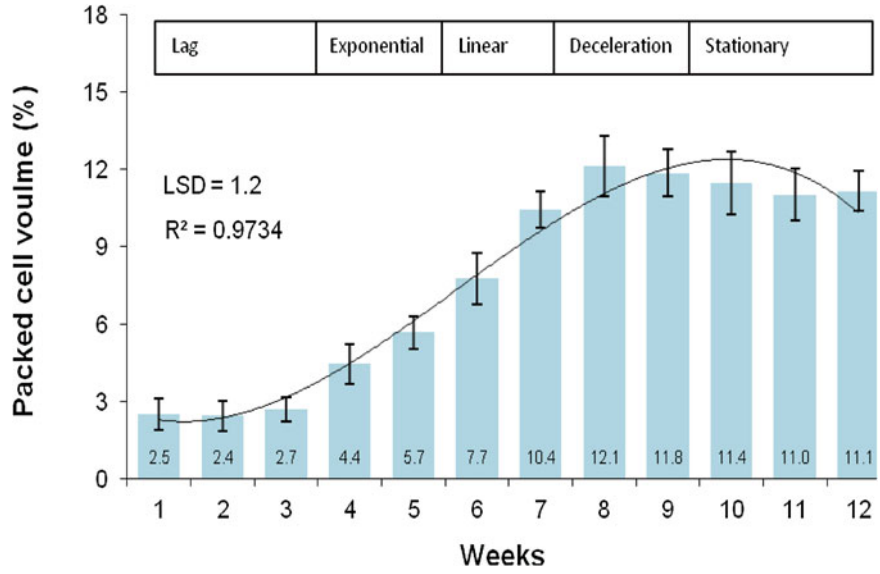

Fig. 2 The cell growth of date palm suspension culture, showing PCV in relation to time as it pertains to each of the growth phases (lag phase, exponential phase, linear phase, progressive deceleration phase, and stationary phase). (Source: This figure is taken from Al-Khayri JM (2012) Determination of the date palm cell suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis. Emir J Food Agric 24(5): 444–455)

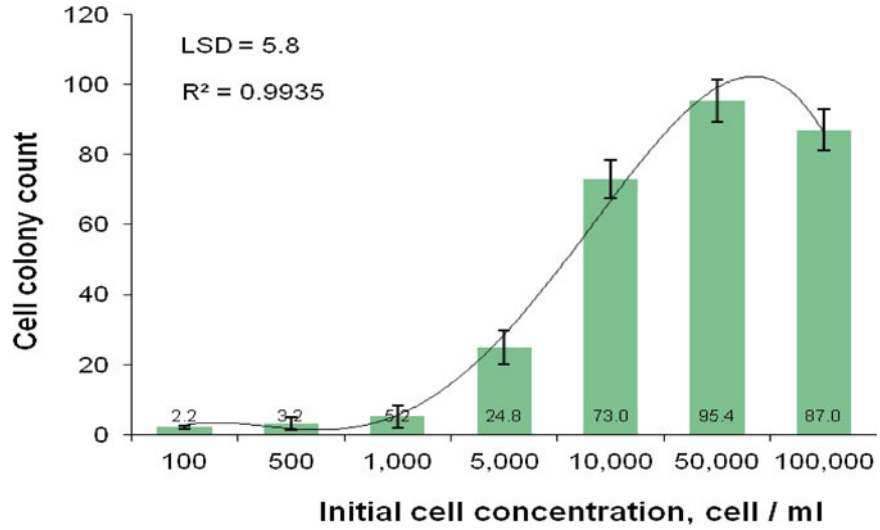

Fig. 3 The cell growth of date palm suspension culture, expressed in the number of colonies, recovered after plating on a semisolid medium at various cell densities. (Source: This figure is taken from Al-Khayri JM (2012) Determination of the date palm cell suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis. Emir J Food Agric 24(5): 444–455)

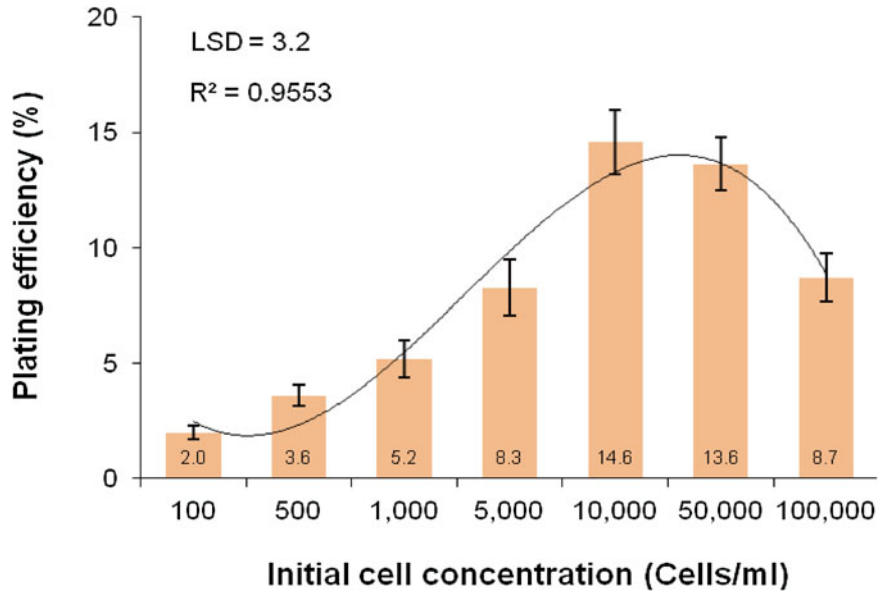

Fig. 4 The cell growth of date palm suspension culture, expressed in plating efficiency, recovered after plating on a semisolid medium at various cell densities. (Source: This figure is taken from Al-Khayri JM (2012) Determination of the date palm cell suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis. Emir J Food Agric 24: 444–455)

2. MS liquid medium supplemented with 30 g/L sucrose
3. Incubate cultures at 16-h photoperiod of cool-white florescent light (40 μmol/m²/s) and 23 ± 2 °C and agitate at 150 rpm. Maintain cultures by regular subculturing at 2-week intervals up to 12 weeks (Fig. 1f).

3.5 Plant Formation

1. To accelerate the complete plant formation, transfer mature or germinating embryos to rooting medium (Fig. 1g; *see* **Note 5**).

3.6 Hardening and Acclimatization

1. Remove the plantlets nearly 5 cm in length with shoot and root from culture tubes and gently rinse under a slow stream of water to remove residual agar media from the root region.
2. Plantlets are transferred to pots with peat–vermiculite mixture of 2:1 ratio in Styrofoam cups of uniform size (Fig. 1h).
3. The plantlets are initially covered with polythene bag to maintain high humidity to prevent the plants from dehydration and are maintained in the plant growth room.
4. Perforate the polythene bags and gradually remove them in a span of 3 weeks.
5. The hardened plants are transferred to potting mixtures consisting of 1:1:1 peat, sand, and dehydrated cow manure in pots (12 in.) and are maintained in the greenhouse under natural light at 27 ± 2 °C and 50–60 % relative humidity.

4 Notes

1. The most efficient way of preparing MS medium (Table 1) is to prepare stock solutions of major, minor inorganic nutrients, iron source, vitamins, and separate growth hormones. The stock solutions are stored at 4 °C except for the vitamins which is stored in small batches at −20 °C. Do not store the stock solutions for longer periods (not more than 2–3 months). It is always recommended to prepare the growth regulators fresh for each batch of media. Any color changes in the stock solutions may be due to precipitation, which can seriously affect the growth of cultures. Alternatively, stock solutions of macroelements, microelements, vitamins, and the ready-to-use MS medium are commercially available.
2. All the glassware should be cleaned with a liquid detergent and be thoroughly washed with tap water. Rinse the glassware with double distilled water and dry in hot air oven at 150 °C for 2 h before use.
3. Autoclave the petri dishes prior to use to avoid any contamination during the procedure as contamination at this stage may affect the results.
4. The plating efficiency is determined using the following equation:

$$\mathrm{PE} = \left(\text{final number of colonies per plate} \,/\, \text{initial number of cellular units per plate}\right) \times 100.$$

5. Half-strength MS medium supplemented with 0.2 mg/L NAA and 0.7 g/L agar.

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