Chapter 21

Plantform Bioreactor for Mass Micropropagation of Date Palm

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Abstract

A novel protocol for the commercial production of date palm through micropropagation is presented. This protocol includes the use of a semisolid medium alternation or in combination with a temporary immersion system (TIS, Plantform bioreactor) in date palm micropropagation. The use of the Plantform bioreactor for date palm results in an improved multiplication rate, reduced micropropagation time, and improved weaning success. It also reduces the cost of saleable units and thus improves economic return for commercial micropropagation. The use of the Plantform bioreactor successfully addresses other hindrances that can occur during the scale-up of date palm micropropagation, including asynchrony of somatic embryos, limited maturation of somatic embryos, and highly variable germination frequencies of embryos.

Key words Bioreactor, Commercial micropropagation, Partial desiccation, Plantform, Temporary immersion system

1 Introduction

Date palm (*Phoenix dactylifera* L.) is considered to be one of the oldest fruit trees domesticated by man since the dawn of civilization. Currently, however, date palm cultivation is challenged by various biotic and abiotic factors [1]. Commercial micropropagation is an alternative method for large-scale propagation of disease-free date palm varieties. Micropropagated date palm plants are produced in many countries around the world. The list of available cultivars is expanding as the result of market demands.

Somatic embryogenesis and organogenesis are the two pathways of choice for rapid and large-scale propagation of date palm. They have been successfully used for the micropropagation of elite genotypes using various explants including shoot tips, lateral buds, and inflorescence tissues [2–4]. Several different methods for in vitro propagation of date palm have been developed, but all of these protocols have used semisolid gelled media as a component

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of the system. However, these methods (using only the semisolid gels) are labor intensive and slow, thus increasing production costs [5].

The temporary immersion system (TIS), also known as a RITA bioreactor, is an ideal method for scaling up the micropropagation process. The Plantform bioreactor is a recently developed TIS system. It has higher plant headspace, uses low-pressure fish tank air pumps and digital timers, and allows active aeration to cultures. The Plantform bioreactor can be sterilized with a medium using either an autoclave or a kitchen microwave. The sterile filters are connected to the body afterward, and two timers and two air pumps are used to regulate gas exchange and immersion cycles via silicon tubes. Its modularity and shape allow space savings [6]. This is especially true in the case of somatic embryogenesis, as the bioreactors offer large containers, good aeration for the cultures, and a high degree of control over other culture conditions such as undesirable ethylene buildup and desirable increases in carbon dioxide concentrations [7]. Ibraheem et al. [8] used the TIS for scaling up the production of date palm somatic embryos.

This chapter describes a novel date palm micropropagation (somatic embryogenesis and organogenesis) mixed system (semisolid and liquid medium) using the Plantform bioreactor. Currently, this system is used in Fadak Date Palm Production Laboratory, Basra, Iraq. This mixed system achieved reduction in costs and reduced the time frame for plantlet production to less than 3 years.

2 Materials

2.1 Plant Materials and Sterilization

Explant source: Apical shoot tips of date palms taken from 3-year-old offshoots of cultivar Barhee.

2.2 Culture Medium

1. Basal culture medium: Murashige and Skoog (MS) salts [9] (Table 1).
2. Hormone stock solutions: Auxins including 2,4-dichlorophenoxyacetic acid (2,4-D, 1 mg/mL), naphthaleneacetic acid (NAA, 1 mg/mL), β-naphthoxyacetic acid (NOA, 1 mg/mL), indole-3-acetic acid (IAA, 1 mg/mL), and indole-3-butyric acid (IBA, 1 mg/mL). Cytokinins including benzylaminopurine (BAP, 1 mg/mL), kinetin (KN, 1 mg/mL), 6-(γ,γ-dimethylallylamino) purine (2iP, 1 mg/mL), and thidiazuron (TDZ, 1 mg/mL).
3. Solutions to adjust pH: 0.1 M and 1 M NaOH and 0.1 M and 1 M HCl.
4. Other chemicals: Polyvinylpyrrolidone (PVP, MW 10,000) and activated charcoal (AC).

Table 1
Composition of modified MS medium [9] used for date palm tissue. Hormones, agar, PVP, and activated charcoal are added according to the culture stage as shown in Tables 2 and 3

Composition	Concentration in the stock solution (mg/L)	Concentration in MS culture medium (mg/L)
Stock I: Macronutrients (10× stock) use 100 mL to prepare 1 L of medium		
NH_4NO_3	16,500	1650
KNO_3	19,000	1900
$CaCl_2{\cdot}2H_2O$	4400	440
$MgSO_4{\cdot}2H_2O$	3700	370
KH_2PO_4	1700	170
$NaH_2PO_4{\cdot}H_2O$	1700	170
Stock II: Micronutrients (100× stock) use 10 mL to prepare 1 L of medium		
KI	83	0.83
H_3BO_3	620	6.2
$MnSO_4{\cdot}2H_2O$	2230	22.3
$ZnSO_4{\cdot}7H_2O$	860	8.6
$Na_2.MoO_4{\cdot}2H_2O$	25	0.25
$CuSO_4{\cdot}5H_2O$	2.5	0.025
$CoCl_2{\cdot}6H_2O$	2.5	0.025
Stock III: Iron source		
Fe EDTA Na salt	Added fresh	40
Myoinositol	Added fresh	100
Ca-pantothenate (20 mg/L)	Added fresh	*20*
Stock IV: Vitamins (200× stock) use 5 mL to prepare 1 L of medium		
Nicotinic acid	200	1
Pyridoxine·HCl	200	1
Thiamine·HCl	200	1
Glycine	400	2
Biotin	200	1
Carbon source		
Sucrose	–	30,000
Antioxidants		
Glutamine	–	200
Other additives		
Sodium dihydrogen phosphate dihydrate	Added fresh	187.5
Calcium nitrate	Added fresh	1500

2.3 Media Used for Various Culture Stages (See Tables 2 and 3)

1. Callus induction medium (CIM): Modified MS medium enriched with 100 mg/L 2,4-D medium, 3 mg/L 2iP, sucrose 30 g/L, and 2 g/L activated charcoal.
2. Callus proliferation and maintenance medium (CPM): Modified MS medium supplemented with 30 mg/L NAA, 30 mg/L sucrose, and 2 g/L activated charcoal.
3. Somatic embryo germination medium (SEM1): Modified MS medium fortified with 100 mg/L myoinositol, 0.4 mg/L thiamine-HCL 1 mg/L, biotin, 0.5 mg/L pyridoxine, 0.5 mg/L nicotinic acid, 200 mg/L glutamine, 500 mg/L PVP, and 30 g/L sucrose.
4. Scaling-up medium (SUM): Modified MS liquid medium supplemented with 1.5 mg/L NOA, 1 mg/L NAA, 1 mg/IAA L, and 1 mg/L 2iP.
5. Direct somatic embryogenesis induction medium (DSIM): Modified MS medium supplemented with 50 mg/L NAA, 5 mg/L 2,4-D, and 3 mg/L 2iP; or 50 mg/L NAA, 10 mg/L 2,4-D, and 3 mg/L 2iP; or 50 mg/L NAA, 5 mg/L 2,4-D, and 0.1 mg/L TDZ; or 50 mg/L NAA, 10 mg/L 2,4-D, and 0.1 mg/L TDZ.
6. Somatic embryo germination (SEM2): Modified MS medium fortified with 0.1 mg/L NAA, 4 mg/L BAP, 4 mg/L KN, and 2 g/L activated charcoal.
7. Large-scale embryogenesis medium (LEM): Modified MS medium supplemented with 0.1 mg/L BAP, 0.1 mg/L 2iP, and 0.1 mg/L KN and enriched with a higher level of sucrose (60–90 g/L).
8. Direct organogenesis (DOM): Modified MS medium containing 6 mg/L NAA, 3 mg/L NOA, 0.1 mg/L BAP, 0.2 mg/L 2iP, 500 mg/L PVP, and 6 g/L agar. Sucrose concentration must increase to 50 g/L.
9. Bud growth and proliferation medium (BGPM): Modified MS medium with 0.1 mg/L BAP, 0.1 mg/L Kinetin, and 0.1 mg/L 2iP. Sucrose concentration must increase to 50 g/L.
10. Elongation medium (EM): Modified MS medium supplemented with 80 mg/L adenine sulfate and 1.5 g/L $Ca(NO_3)_2.4H_2O$ and 1 mg/L each of NAA, 2iP, BAP, and KN as well as 0.5 g/L activated charcoal and 0.5 g/L PVP (*see* **Note 2**). Sucrose increased to 80 g/L.
11. Rooting medium (RM1): Modified MS solid medium supplemented with 1 mg/L NAA and 40 g/L sucrose.
12. Rooting medium (RM2): Modified MS liquid medium containing 0.01 mg/L IBA and 40 g/L sucrose.
13. Pre-weaning medium (PWM): Sugar-free MS liquid medium containing 0.01 mg/L IBA and 1 mg/L KN.

Table 2
Different culture stages for somatic embryogenesis and the corresponding hormonal, PVP, and activated charcoal additives supplemented to the MS medium

Culture stage (medium code)	Plant growth regulators, agar, PVP, and activated charcoal additives										
	2,4-D, mg/L	2iP, mg/L	NAA, mg/L	NOA, mg/L	IAA, mg/L	BAP, mg/L	Kinetin, mg/L	Adenine sulfate, mg/L	Agar, g/L	Activated charcoal, g/L	PVP, mg/L
Callus induction medium (CIM)	100	3	–	–	–	–	–	40	7	2	–
Callus proliferation and maintenance medium (CPM)	–	3	30	–	–	–	–	40	7	1	–
Somatic embryo germination medium (SEM1)	–	–	–	–	–	–	–	40	7	–	–
Scaling-up medium (SUM), liquid medium	–	1	1	1.5	1	–	–	40	–		500
Direct somatic embryogenesis induction medium (DSIM)	10	3	50	–	–	–	–	40	7	2	–
Somatic embryo germination (SEM2)	–	–	0.1	–	–	4	4	80	7	2	–
Large-scale embryogenesis medium (LEM), liquid medium	–	0.1	–	–	–	0.1	0.1	40	–	–	500

Table 3
Different culture stages for organogenesis and the corresponding hormonal, PVP, and activated charcoal additives supplemented to the MS medium

Culture Stage	Plant growth regulators, agar, PVP, and activated charcoal additives											
	2,4-D, mg/L	2iP, mg/L	NAA, mg/L	NOA, mg/L	IAA, mg/L	BAP, mg/L	IBA, mg/L	Kinetin, mg/L	Adenine sulfate, mg/L	Agar, g/L	Activated charcoal, mg/L	PVP, mg/L
Callus induction medium (CIM) direct organogenesis medium (initiation stage) (DOM)	–	0.2	6	3	1	0.1	–	–	40	7	–	500
Buds growth and proliferation medium (BGPM)	–	0.1	–	–	–	0.1	–	0.1	80	7	–	500
Elongation medium (EM) Liquid medium	–	1	1	–	–	1	–	1	80	–	500	500
Rooting medium (RM1)	–	–	1	–	–	–	–	–	80	7	–	500
Rooting medium (RM2) Liquid medium	–	–	–	–	–	–	0.01	–	80	–	–	500
Pre-weaning medium (PWM) Sugar-free medium Liquid medium	–	–	–	–	–	–	0.01	1	80	–	–	500

Fig. 1 Bioreactor and its different parts numbered from *1* to *6*. (**a**) Outer container with three inlets/outlets for gas exchange (*4–6*); (*5*) shows the middle filter connected to a plastic tube on the inner chamber. (**b**) (*1*) Inner chamber with connection to the middle filter, (*2*) basket with three rows of small holes, (*3*) frame with four legs. (**c**) Lid with four flaps and an inner silicon ring. (**d**) Filters, plastic tubes, nuts, clamps, and silicon rings to be connected to the three inlets/outlets on the outer container. (**e**) Fish tank pump

2.4 Equipment

1. Tissue culture jars: Standard G7, 250 mL plastic plant tissue culture container.
2. Plantform bioreactor: *see* Fig. 1.

3 Methods

3.1 Explant Preparation and Sterilization

1. Separate healthy offshoots weighing 5–6 kg of selected cultivar Barhee from field-grown trees (Fig. 2a).
2. Remove offshoot leaves one by one from the outer ring toward the center (Fig. 2b).
3. When the soft white tissue area is reached, this part should be handled carefully to avoid breaking the shoot tip, because it is located deep in the soft meristematic area. Remove the outer leaves and expose the shoot tip region, about 3–4 cm in width and 6–8 cm in length (Fig. 2c).

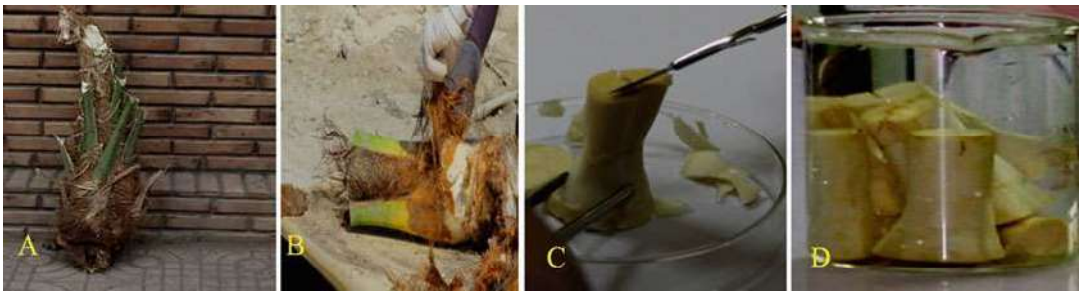

Fig. 2 Explant preparation. (**a**) Excised offshoot weighing 5–6 kg ready for dissection. (**b**) Removing of offshoot leaves. (**c**) Offshoot core or heart. (**d**) Shoot tip dipped in chilled antioxidant solution

4. Transfer the shoot tips with some of the soft white tissues attached to sterilized beakers containing chilled, sterilized antioxidant solution prepared with deionized water supplemented with 150 mg/L citric acid and 100 mg/L ascorbic acid to reduce browning of explant tissues (Fig. 2d).
5. Immerse explants in 0.01% mercuric chloride solution for 80 min with some swirled or inverted by hand every 5 min.
6. In a laminar flow hood, rinse the explants five times with sterilized distilled water.

3.2 Indirect Somatic Embryogenesis

1. For initial callus induction, culture shoot tip explants on CIM solid medium and incubate the cultures in darkness at 27 °C ± 2 for 3 months (*see* **Notes 1–3**).
2. Subculture responsive explants in CPM at 4- to 6-month interval to get sufficient embryonic callus (*see* **Note 4**).
3. For partial desiccation treatment, transfer 500 mg embryogenic callus to sterile bioreactor container containing 550 mL medium, stop immersion cycle, and increase aeration cycle to 3 min/h for 15 days (*see* **Notes 5**).
4. Transfer desiccated callus to SEM1 medium. Keep cultures in light, 100 μmol/m^2/s and 16 h photoperiod for 8 weeks (*see* **Notes 5** and **6**).
5. Transfer embryogenic callus directly without desiccation to the plant basket of the Plantform bioreactor for the scaling-up of somatic embryogenesis. Use liquid SUM medium (550 mL) at an immersion frequency of 3 min every 8 h under the fluorescent lights (100 μmol/m^2/s, 16 h photoperiod) (*see* **Note 7**).
6. To increase the numbers of somatic embryos, shift the germinated embryos obtained in **step 4** to Plantform bioreactors containing LEM liquid medium (*see* **Note 10**).

3.3 Direct Somatic Embryogenesis

1. To induce direct somatic embryogenesis without intervening the callus, culture shoot tip explants on DSIM media for 3–6 months. Cultures should be incubated in the dark at

27 ± 2 °C. Re-culture the explants without cutting them up to the same medium at 8-week intervals (*see* **Note 8**).

2. Transfer the somatic embryos obtained in **step 1** to SEM2 medium (*see* **Note 9**).
3. To increase the numbers of somatic embryos, shift the germinated embryos to Plantform bioreactors containing LEM liquid medium (*see* **Note 10**).

3.4 Direct Organogenesis

1. Culture shoot tip segments on DOM medium, and regularly subculture to fresh medium at 8-week intervals up to 9–10 months. Discard non-responsive cultures and maintain cultures in the dark at 27 °C ± 2 (*see* **Note 11**).
2. Subculture small clumps with up to four initial buds directly onto BGPM (Table 2); divide larger clumps with minimal wounding before subculturing. Incubate cultures under light intensity (7 μmol/m^2/s) for 16 h photoperiod at 27 °C ± 2 (*see* **Note 12**).
3. Transfer actively growing buds to fresh BGPM at 6–8 week intervals until sufficient numbers (30–40) exist for Plantform bioreactor use (*see* **Note 12**).
4. For obtaining elongated shoots in a short period, transfer cultures from **steps 2** and **3** to Plantform bioreactor plant basket, and using EM medium, incubate in the Plantform bioreactor at 27 ± 2 °C, with a light intensity of 7 μmol/m^2/s and with a photoperiod of 16 h. Immersion cycle subculturing of clumps is for 3 min at 8-h intervals (*see* **Note 13**).
5. Repeat **step 4** using a modified Plantform bioreactor by removing the baskets, inner chamber, and frame with four legs and aerate the medium through the polyethylene tube.
6. Alternatively use the stainless steel meshes over the basket to discharge the shoots very easily (*see* **Notes 14–19**).
7. Transfer the separated shoots obtained from **steps 4** and **5** to RM1 solid medium (*see* **Note 20**).
8. Transfer the unrooted shoots from **step 6** to RM2 liquid medium (*see* **Note 20**).
9. Rooting and shoot development: Subculture individual rooted shoots into liquid PWM either using tall glass vessels or the Plantform bioreactor (*see* **Note 21**).

4 Notes

1. Induction and continuous proliferation of embryogenic callus requires 2,4-D or NAA and 2iP, BAP, and kinetin. The issues of low embryo numbers per culture, synchrony of embryos, and

limited maturation and germination frequencies of somatic embryos are major hindrances that increase production costs and add to planning difficulties [10, 11].

2. Callus is induced on shoot tip segments after 1–2 months of culture on CIM medium.
3. Embryogenic callus is induced after 4–6 months by transferring the initial callus to CPM medium.
4. Embryogenic callus is proliferated and maintained on the same medium CPM.
5. Embryogenic callus subjected to desiccation in a sterile bioreactor for 15 days increased somatic embryo formation and germination by transferring to SEM1 medium (Fig. 3a, b) as compared with control. However, the number of somatic embryos decreases with only 5 days of desiccation.
6. The somatic embryogenesis process and plant regeneration are influenced by a number of factors such as genotype, medium composition (auxins, cytokinins, sugar, amino acids, abscisic acid, and retardant), and physical status changes caused by solidifying agents in the medium and desiccation. Partial desiccation has been reported to promote somatic embryo differentiation and development in many plants including date palm [12, 13].
7. Transferring undesiccated embryogenic callus to the Plantform bioreactor containing 550 mL sterilized (SUM) liquid medium (Table 1) at an immersion frequency of 3 min every 8 h increases the formation of somatic embryos in 2 months as compared to culture of this callus to solid medium (Fig. 4).
8. Some shoot tip explants produced direct somatic embryos without intervening callus phase after 16 weeks of culturing

Fig. 3 Using Plantform bioreactor for (**a**) callus desiccation and (**b**) maturation and germination of somatic embryos after subjecting embryogenic callus to desiccation for 15 days in Plantform bioreactor

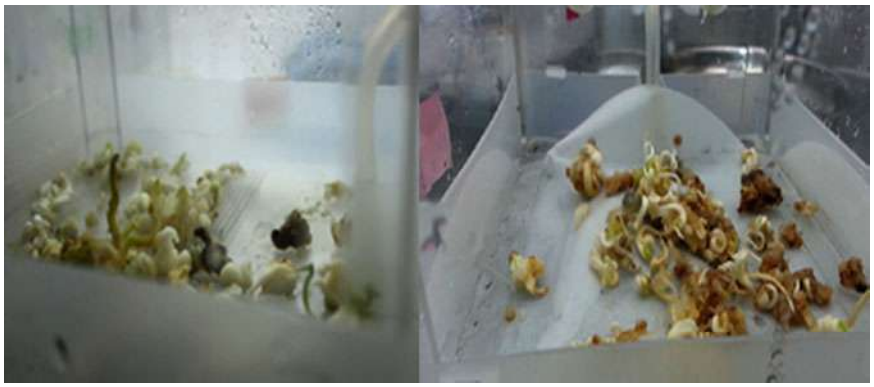

Fig. 4 Using a Plantform bioreactor with temporary immersion system for the induction of somatic embryos from embryogenic callus

Fig. 5 Direct somatic embryogenesis

(Fig. 5) at rates of 15, 25, and 15 and 25% only on DSIM media (Table 1).

9. The efficiency of growth regulator combinations on somatic embryo development is determined based on the somatic embryo germination rate, shoots, and roots (numbers and length). High somatic embryo germination, increasing root and shoot numbers and shoot length are obtained with the treatment of SEM2 (88.88%) (2.57 and 3.25, 3.87 cm and 3.53 cm, respectively).

10. Germinated somatic embryos obtained by direct or indirect somatic embryogenesis mode (Subheadings 3.1 and 3.2) mentioned above are transferred separately to Plantform bioreactors containing LEM (Table 1) liquid medium with a high level of sucrose (90 g/L) for further production of somatic embryos. Secondary embryogenesis is induced within 8 weeks (Fig. 6).
11. Initiation stage: Buds initiated on DOM medium (Table 2) after 8–10 months (Fig. 7).
12. Bud growth stage: Active and slower growing buds are transferred to BGPM (Table 2) for further proliferation. When slower growing buds do not respond, re-culture on the same medium (1–2 times).

Fig. 6 The number of somatic embryos increased by using MS medium supplemented with 0.1 mg/L benzylaminopurine (BAP), 0.1 mg/L 2iP, and 0.1 mg/L kinetin (KN) with 60 g/L of sucrose (LEM)

Fig. 7 Direct organogenesis occurred after 8–10 months on DOM medium

13. Shoot elongation and individualization stage: Use the Plantform bioreactor with a fine mesh (200 holes/in.) in the basket. Fine, delicate shoots should be transferred to (EM) liquid medium. To avoid vitrification or hyperhydricity problems, add high concentration of $Ca(NO_3)_2.4H_2O$ (1–2.5 g/L) in the culture medium (Table 2). Additionally, activated charcoal and PVP must be included in the medium, 0.5 g/L each to avoid browning. Grow shoots under light 7 μmol/m^2/s with a photoperiod of 16 h. Low light aids in shoot individualization and elongation; whereas, higher light intensity does not give good results. Shoots are elongated within 8 weeks.
14. Plantform bioreactor is efficient for both bud and shoot elongation and root development. The main problem is that roots penetrate the small holes of the basket and create difficulties in removing shoots from the basket container (Fig. 8a).
15. Modify Plantform bioreactor by removing the baskets, inner chamber, or frame with four legs and aerate the medium through a polyethylene tube.
16. Alternatively, use the stainless steel meshes over the basket to prevent roots from penetrating the basket holes thus making shoot discharge very easy (Fig. 8b, c).
17. By using both methods, high-quality and easy-to-handle plants are produced, and keep oxygenation of the medium and gas exchange under control through stationary medium. Additionally, LEM with higher levels of sucrose (over 50 g/L) increases shoot length and overcomes slow growth problems. The harvesting and detaching of the shoots from the bioreactor become very simple.
18. Shoot elongation and individualization are important in the in vitro stage of organogenesis of date palm to permit

Fig. 8 In vitro date palm plantlets ready for soil transfer and acclimatization. Roots developed from shoots at elongation phase penetrate the small holes of basket in the TIS bioreactor (**a**). Vessel with successfully developed shoots in stationary liquid medium (**b**, **c**)

conversion of bud shoots to plantlets. This stage depends mainly on the genotype and media composition [14].

19. Detachment of individual shoots from clusters in the multiplication phase is difficult, and attached plantlets need more than 4–6 months in culture to be ready for separation. Liquid medium improves shoot elongation and increases survival rate of date palm plantlets in the greenhouse [15, 16].
20. Rooting stage: Roots are induced in elongated shoots, 15 cm in length, after 8–12 weeks of culturing on (RM1) and (RM2). Smaller shoots less than 10 cm fail to root.
21. The pre-weaning stage is important to increase the percentage of plantlet survival. Main root and branch root numbers increase when cultured on PWM for 4 weeks. Transfer plantlets from this medium to soil.

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