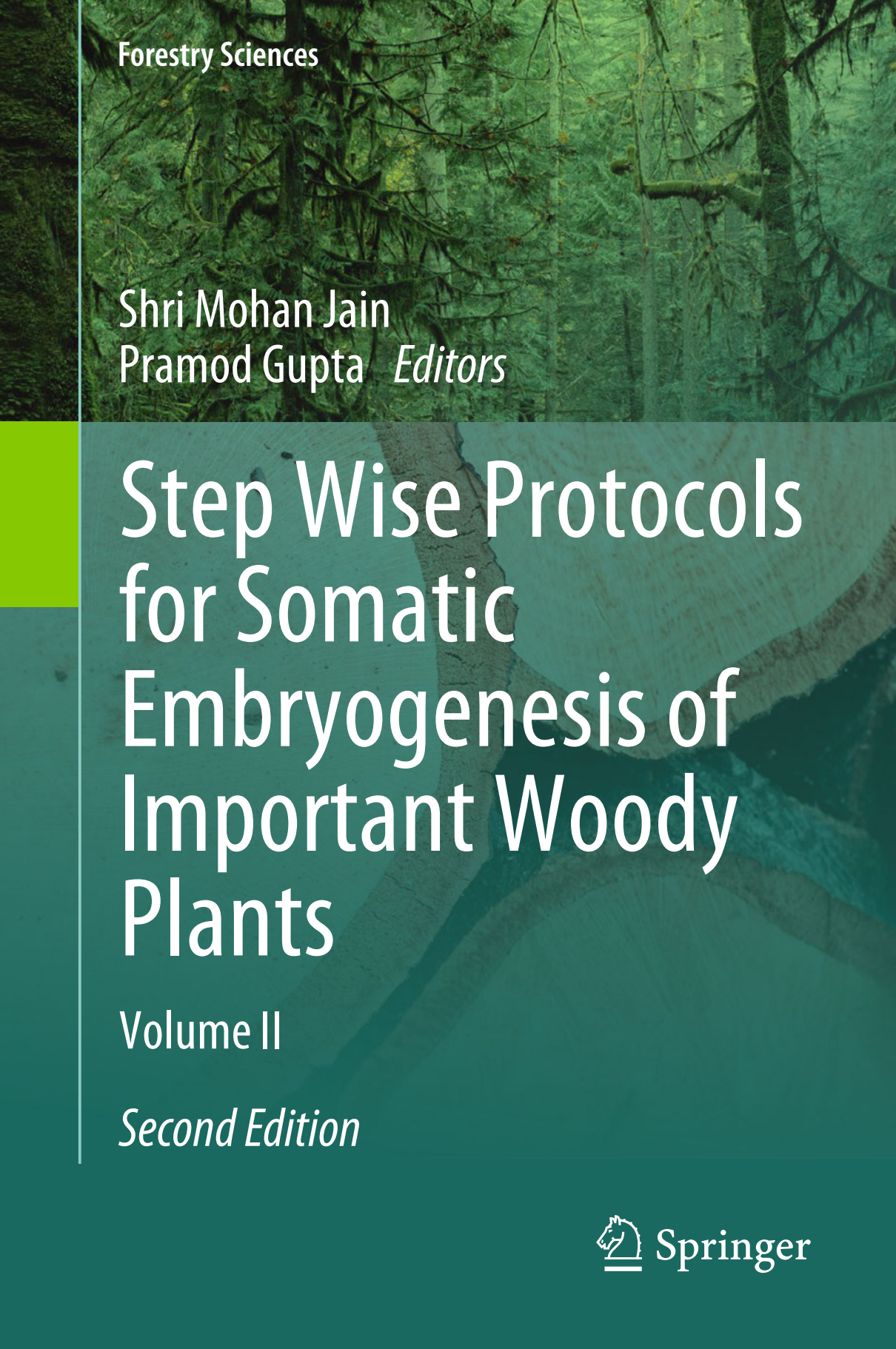
Forestry Sciences
Shri Mohan Jain
Pramod Gupta Editors
Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants
Volume II
Second Edition
Springer

Forestry Sciences

Volume 85

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Shri Mohan Jain · Pramod Gupta
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Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants

Volume II

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ISSN 0924-5480 ISSN 1875-1334 (electronic)
Forestry Sciences
ISBN 978-3-319-79086-2 ISBN 978-3-319-79087-9 (eBook)
https://doi.org/10.1007/978-3-319-79087-9

Library of Congress Control Number: 2018938787

Printed on acid-free paper

This Springer imprint is published by the registered company Springer International Publishing AG
part of Springer Nature
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

There is an increasing demand for various tree products as the world population continues to grow rapidly, leading to slowdown in woody plant product supplies worldwide. To meet the demand of every growing human population, there is a need to maintain continuous supply of woody products by increasing productivity of trees. This can be achieved by improving breeding of trees with better traits; however, conventional breeding methods are slow due to long life cycle of trees.

A basic strategy in tree improvement is to capture genetic gain through clonal propagation. Clonal propagation via organogenesis is being used for the production of selected elite individual trees. However, the methods are labour-intensive and costly and produce low volumes. Genetic gain can now be captured through somatic embryogenesis. Formation of embryos from somatic cells by a process resembling zygotic embryogenesis is one of the most important features of plants. It offers a potentially large-scale propagation system for superior clones. It has several additional advantages such as the ability to produce large numbers of plants, the potential for automation, the opportunities for synthetic seed, long-term storage, packaging, direct delivery systems and genetic manipulation.

Earlier, we edited a series on "Somatic Embryogenesis of Woody Plants", volumes 1–6, which provided reviews on somatic embryogenesis of important angiosperm and gymnosperm tree species. This series has become an excellent source of information for the researchers and students and did not provide "detailed protocols" for inducing somatic embryogenesis. Therefore, researchers may face difficulties in the initiation of somatic embryogenic cultures. The choice of explant is crucial for the initiation of embryogenic cultures.

This book is the second edition of the previously published book entitled Protocol for Somatic Embryogenesis in Woody Plants, 2005, and divided into two volumes. Both volumes include chapters on stepwise protocols of somatic embryogenesis of selected woody plants. This would enable both researchers and students to start somatic embryogenic cultures without too much alteration.

In volume 1, each chapter provides information on initiation and maintenance of embryogenic cultures; somatic embryo development, maturation and germination; acclimatization and field transfer of somatic seedlings. Some chapters include

applications of somatic embryogenic cultures, e.g. *SE Fluidics System*, anther culture, manufactured seeds, cryopreservation and liquid cultures.

Volume 2 contains 27 chapters dealing with similar information on stepwise protocols for somatic embryogenesis as of volume 1. However, this volume covers some major chapters including cacao, coffee, cherry, citrus, coconut, cryopreservation, date palm, guava, microspore embryogenesis, neem, olive, oil palm, passiflora, Vitis, tamarillo and tea.

The invited authors are well known in somatic embryogenesis research, and they belong to industry, universities and research institutes. Each chapter has been extensively reviewed by other expertise before publication. We are grateful to all authors for their contribution to this book, and all reviewers reviewed chapters that have maintained high quality of the book.

Helsinki, Finland — Shri Mohan Jain
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Chapter 1
Somatic Embryogenesis in Guava (*Psidium guajava* L.)

Nasim Akhtar

1.1 Introduction

Guava is derived from the Haitian name guayaba. It has two important species called common guava (*Psidium guajava*) and cattley guava (*Psidium cattleianum*) belongs to family myrtaceae along with *P. guinase*, *P. Chinese*, *P. friedrichsthalia*, *P. arommaticum* and other genera (Pommer and Murakami 2009). Diploid (2n = 22) state is high seed bearing fruit. However, triploids (2n = 3x = 33) guava also exists in some natural and artificial forms and produce seedless fruits. This fruit is highly nutritionally valuable and commercially remunerative fruit in international trade and domestic economy of several countries (Chandra et al. 2010; Kamle et al. 2012; Nimisha et al. 2013). Guava is indemic to tropical America but naturalized throughout the tropics and subtropics from Mexico to Peru to India (Yadav 1996). It is widely exploited commercially in Florida and Hawain islands, Egypt, South Africa, Brazil, Columbia and West Indies (Pommer and Murakami 2009; Nimisha et al. 2013).

Guava is rich in proteins, carbohydrates, minerals, sugars, oils and vitamin-C. The plant is also good source of pectin, several antioxidant poly-phenolic and flavonoid compounds (Singh et al. 2005). Almost all plant parts are used as anti-diarrhoeal, antimicrobial, antimalarial, antitussive, antioxidant, antigenotoxic and antimutagenic etc. agents (Gutierrez et al. 2008).

Conventionally guava is propagated through air layering, cutting, grafting or stooling but only with limited success in development of disease resistant cultivars (Chandra et al. 2004; Pommer and Murakami 2009; Nimisha et al. 2013). Several hybrids have been developed by crossing Seedless x Allahabad safeda; Seedless x Lucknow-49; Allahabad safeda x Patillo; Apple coloured x Kothrud and Apple

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_1

coloured x Red fleshed at Fruit Research Stations, Basti, India. However, retention of their hybrid characteristics during subsequent generations still remained questionable (Mitra and Bose 1985). Similarly, about 26 diploids, 9 trisomics and 5 tetrasomics (2n + 1 + 1) among the 73 plants raised by crossing the diploids and triploids to study the breeding behaviour of aneuploids of guava (Majumdar and Mukherjee 1971, 1972; Mohammed and Majumder 1974). This crop is also seed cultivated, which is heterozygous displaying cast gene pool with genetic variability in both plant and fruit characteristics.

The first in vitro attempt was made to grow the excised tissues of fruit mesocarp for manipulating the somatic cells/tissues is reported by Schroeder (1961). Babbar and Gupta (1986a, b) reported in vitro anthers culture for the induction of callus and androgenesis. Jaiswal and Amin (1986, 1987) regenerated in vitro shoots from somatic tissues and developed a reliable micropropagation for the guava species (Amin and Jaiswal 1987, 1988, 1989a, b). Subsequently, clonal propagation of guava from seedling and grafted plants (Loh and Rao 1989; Singh et al. 2002; Yasseen et al. 1995), nodal and shoot tips culture (Ali et al. 2003, 2007; Fitchet 1989; Meghwal et al. 2003; Papadatou et al. 1990; Rai et al. 2009; Zamir et al. 2007) was reported. Organogenesis from somatic cells was reviewed by Jaiswal and Amin (1992). Encapsulation of shoot tips and nodal segments of guava reported for short-term storage and germplasm exchange (Rai et al. 2008a, b, c; Rai and Jaiswal 2008). Molecular markers such as ISSR marker (Liu and Yang 2012), microsatellite (Herrero et al. 2010) and SSR marker (Rai et al. 2013) was used for assessment of the clonal fidelity of micropropagated guava. In vitro selection of guava for wilt resistance in guava was performed by Kamle et al. (2012).

1.1.1 Progress of Somatic Embryogenesis in Guava

Induction of androgenesis from anthers derived callus was reported by Babbar and Gupta (1986a, b). The first report on somatic embryogenesis as unpublished data appeared in 1992 (see Jaiswal and Amin 1992) followed by studies on induction and factors controlling somatic embryogenesis in guava from the zygotic embryo explants (Jaiswal and Akhtar 1993, 1994). Since then induction of somatic embryogenesis from zygotic embryo culture of guava has been perfected (Akhtar 1996, 1997; Akhtar and Jaiswal 1994, 1995). During this period an overview chapter describing somatic embryogenesis in guava has been published by Ghaffoor and Alderson (1994). The complete protocol for induction of somatic embryogenesis, development, maturation and germination of somatic embryos, production of artificial seeds and improvement of guava species had been achieved with commendable success by the author (Akhtar 1997). Ramirez and Salazer (1998) had followed the protocol and reported the induction and development of somatic embryos from the zygotic embryo explant using MS medium in the presence of 2ip, BAP, KIN, ZEA and ribozeatin. An overview of the somatic embryogenesis in tropical fruit trees and its applications in the improvement of guava and other fruit

species was presented by author (Akhtar et al. 2000; Akhtar and Jain 2000). Subsequently, the progress in guava somatic cell manipulation was overviewed by Jaiswal and Jaiswal (2005). Further, work on somatic embryogenesis in guava was repeated at several locations and some new concepts are being published in the recent years by the author and other groups (Akhtar 2010, 2011, 2013a, b, c; Chandra et al. 2004; Bajpai et al. 2016; Moura and Motoike 2009; Rai and Jaiswal 2008; Rai et al. 2007, 2008a, 2009, 2010b, 2012; Vilchez et al. 2002, 2004). Germination of somatic embryos on a temporary immersion system and solid medium was reported by Kosky et al. (2005). The biotechnological advancement in the improvement of guava was reported in the recent past (Akhtar 2011; Chandra and Mishra 2007; Rai et al. 2010a). Encapsulation of somatic embryos of guava was performed for short-term storage and germplasm exchange (Rai et al. 2008a). A protocol for high efficiency micropraPagation of guava through somatic embryogenesis was published recently by author (Akhtar 2013b). Vilchez et al. (2015) compared the growth between seedlings and in vitro plants of guava cultivar red dwarf Cuban EEA-1840 in nursery.

Various DNA based molecular characterization (RAPD, ISSR, SSR, SRAP, microsatellite etc.) techniques was developed for clonal propagation (Liu and Yang 2012), somatic embryogenesis (Rai et al. 2013; Kamle et al. 2014), parental population (Ahmed et al. 2011; Coser et al. 2012; Padmakara et al. 2015), landraces (Kidaha et al. 2014) in order to ascertain genetic homogeneity and phylogenetic relationship in guava germplasms. The present overview describes various optimization practices followed in recent years on somatic embryogenesis for micropropagation and improvement of guava species.

1.2 Protocol of Somatic Embryogenesis in Guava

1.2.1 Culture Medium

1. The medium used for guava tissue culture is consisted of the normal strength Murashige and Skoog (1962) basal salts. The stock solution is prepared as shown in Table 1.1 and appropriate amount of each one combined to prepare specific volume of medium. This basal medium is used through out the protocol unless and other wise mentioned.
2. Raise the initial pH (4.2 ± 0.05) of the medium to 6.2 ± 0.05 with 0.1 N NaOH. Then the medium is kept on continuous stirring on a magnetic stirrer. The pH of the medium automatically stabilizes to 5.8 ± 0.05 within 1–2 h, or if necessary adjusted with 0.01 N NaOH or HCl, but try to avoid the use of HCl.
3. Add sucrose at the rate of 3% into the medium. Later modify medium with 2.5, 5.0, 7.5, 10.0 and 15.0% to optimize the need for sucrose as carbon source for explants of various physiological ages.

Table 1.1 Constituents of Murashige and Skoog (1962) basal salts for preparation of stock solutions, and final concentration of culture medium

Constituents	Chemical formula	Medium conc. (mg/L)	Stock conc. (mg/L)
Macro-nutrients: Prepare 500 ml *of Stock solution-I (20X) and use* 50 ml *per L medium*			
Ammonium nitrate	NH_4NO_3	1650	33,000
Potassium nitrate	KNO_3	1900	38,000
Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	440	8800
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	370	7400
Potassium dihydrogen orthophosphate	KH_2PO_4	170	3400
Micro-nutrients: Prepare 50 ml *of Stock solution-II (200X) and use* 5 ml *per L medium*			
Potassium iodide	KI	0.83	166
Boric acid	H_3BO_3	6.2	1240
Manganese sulfate tetraydrate	$MnSO_4 \cdot 4H_2O$	22.3	4460
Zinc sulfate hepta hydrate	$ZnSO_4 \cdot 7H_2O$	8.6	1720
Sodium molybdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	0.25	50
Cupric sulfate pentahydrate	$CuSO_4 \cdot 5H_2O$	0.025	5
Cobalt chloride hexahydrate	$CoCl_2 \cdot 6H_2O$	0.025	5
Iron source (chelated): Prepare 50 ml *of Stock solution-III (200X) and use* 5 ml *per L medium*			
Ferrous sulfate heptahydrate	$FeSO_4 \cdot 7H_2O$	27.8	5560
Ethylenediamine tetraaceticacid disodium salt dihydrate	$Na_2EDTA \cdot 2H_2O$	37.3	7460
Organic nutrients: *Prepare* 50 ml *of Stock solution-IV (200X) and use* 5 ml *per L medium*			
Myo-Inositol		100	20,000
Nicotinic acid		0.5	100
Pyridoxine hydrochloride		0.5	100
Thiamine hydrochloride		0.5	100
Glycine		2	400

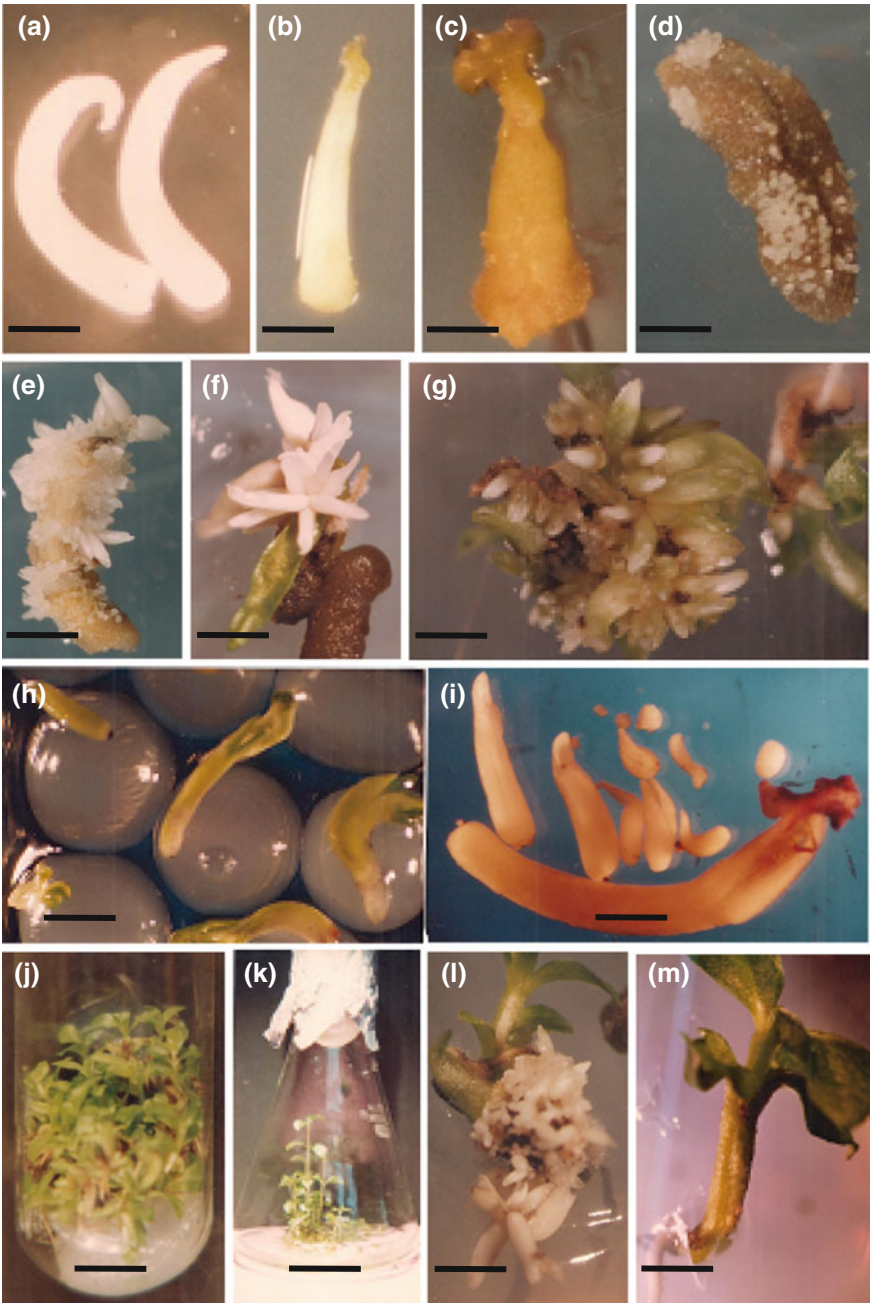
All these constituents are mixed appropriately to prepare a specific volume of normal strength medium. Other medium strength, need for agar, sucrose, growth regulators and other adjuncts are added as per the requirements of the experiments mentioned under different heads

4. Use agar-agar at the rate of 0.8–1.0% (w/v) for gelling of medium. Melt by heating under mild pressure till the boiling but avoid bumping and charing of the sucrose.
5. The medium is modified with broad range concentrations 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 3.0, 5.0, 10.0, 15.0 and 20.0 mg/l of 2,4-D and/or other growth regulators either alone or in various combinations permutations prior to autoclaving. Other modifications to the medium are performed as per the requirement of the experiments.
6. The medium modified with 2,4-D or any growth regulator(s) are referred to as **induction medium** while the one without any growth regulator(s) and used for the development of somatic embryos referred as **development medium** irrespective of sucrose conditions.

7. Dispense about 8–12 ml of medium in 150 × 25-mm culture tubes prelabeled with specific concentrations of sucrose and PGR(s). Plugged with a tight cotton plug. The medium and other miscellaneous requirements are sterilized by autoclave at 121 °C for 15 min at 1.1 kg/cm^2 pressure.

1.2.2 Selection and Preparation of Explant

1. Healthy 5–15 years old high fruit bearing guava cultivars are selected from grower's field or the orchard. Under the mild tropical condition guava flowers through out the year. The time or the periods of collection of fruit for excision of zygotic embryo explant are noted accordingly in order to find out any seasonal trends in the process of somatic embryogenesis.
2. Mature flower buds (prior to calyx cracking) are bagged and tagged properly to ensure the selfing, appropriate age and developmental stage at the time of explants inoculation as well as to ascertain any variations in somatic embryogenesis due to hereditary mechanism.
3. Collect the fruits aged 7–14 weeks after anthesis wash them thoroughly under running tap water. Remove the hard green exo- and greenish white mesocarp with the help of a sharp knife or scalpel. The central ball with pulp and seeds is cut into 4–6 vertical lobes.
4. Surface disinfect the seeds along with pulp lobes by washing under running tap water for 30 min followed by disinfection by gentle shaking in 2% Cetavlon (v/v) with 2–4 drops of Tween-X for about 15-mins. Wash material under running tap water to remove surfactants.
5. Surface sterilization is carried out under the aseptic condition over a Laminar Flow Hood by giving a short rinse in 70% ethanol followed by treatments with 0.05% $HgCl_2$ solution (w/v) for about 20 min. Finally the materials are rinsed 4–5 times with sterile double distilled water to remove any trace of sterilant.
6. Transfer one pulp lobe in sterile water in a petri-plate. Seeds of guava are more or less J or U shaped with one arm slightly longer than the other. Hold the seed with a flame sterilized forceps in left hand over a microscopic slide. Remove a small piece of long arm with the help of a fine and pointed scalpel the turn the seed opposite and remove a small piece of shorter arm similarly. Insert a blunt point needle either through the longer or shorter arm side so that embryo comes out of the other end.
7. The zygotic embryo explant dissected from 10-weeks post anthesis seeds are translucent, milkfish and shining with 4–5 mm long curved axis (Fig. 1.1a).
8. Inoculate the embryo immediately on the induction medium in a test-tube with the help of inoculation needle. Cap the culture tube with a cloth wrapped tight cotton plug and transfer to culture room for incubation.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◂**Fig. 1.1** Somatic embryogenesis in guava (*Psidium guajava* L.). **a** Ten weeks post anthesis zygotic embryo used as explants (*bar:* 1.0 mm), **b** Zygotic embryo explants after 8-days of induction in the presence of 1.0 mg/l 2,4-D in 5% sucrose containing MS medium (*bar:* 1.5 mm), **c** Zygotic embryo explants in development medium after 3-weeks of culture initiation (: 1.5 mm), **d** development of somatic embryos from explants surface at the end of 4th week (*bar*: 1.5 mm), **e** Somatic embryo development after 6 weeks of culture initiation (*bar*: 2.0 mm), **f** Somatic embryo development after 8-weeks of culture initiation (*bar*: 2.0 mm), **g** germinating somatic embryo sub-cultured after 10-weeks of culture initiation on MS basal medium with 3% sucrose (*bar*: 2.0 mm), **h** artificial seeds showing the germination of somatic embryos (*bar*: 2.0 mm), **i** effects of ABA on the growth and maturation of somatic embryos (*bar*: 2.0 mm), **j** growing somatic plantlets after 2-weeks of subculture on agar solidified germination medium (*bar*: 10 mm), **k** Somatic plantlets in 3% sucrose containing MS liquid growth medium (*bar*: 30 mm), **l** Recurrent somatic embryogenesis showing the development of second generation of somatic embryos from the surface of a germinating explants (somatic embryo) (*bar*: 2.0 mm), **m** a somatic plantlets growing for 2-weeks of sub-culture in 75 mM sodium chloride added agar solidified selection media (*bar*: 10 mm)

1.2.3 Culture Environment

1. Perform incubation of all cultures under the uniform conditions in an environmentally controlled culture room maintained at 25 ± 2 °C temperatures, 60–65% relative humidity and 16 h photoperiod. The cultures are illuminated with 40 W white fluorescent tube from a distance of 30–35 cm receiving 30–50 $\mu Em^{-2}\ s^{-1}$ light intensity.
2. The dark treatment is provided in the same culture room on the culture racks covered with double layers of thick black curtail without the tube light fittings.
3. Incubate culture using different BOD incubator for variable temperature treatments.

1.2.4 Observations and Data Analysis

1. Observe the cultures daily and record the change in zygotic embryo explant after an interval of 1-, 2-, 4- days or weekly. Final response for the effects of various treatments on somatic embryogenic is recorded usually 8–10 weeks after culture initiation.
2. Record time dependent developmental course of somatic embryo appearance and growth. Group various stages of somatic embryos into three categories (see Akhtar 1997, 2010, 2011, 2013a, b, c; Akhtar et al. 2000) as they vary in synchronous development, post developmental responses, maturation, germination and in recurrent embryogenesis.
3. Somatic embryos with 1.5 mm or longer axis grouped as elongated torpedo stage (ET); the smaller ones ranging in size between 1.0 and 1.5 mm as short torpedo stage (ST); the rest smaller than 1.0 mm size at any of the cotyledonary, heart and globular stages of development in third category as lower or the CHG stage (see Akhtar 1997, 2010, 2011, 2013a, b, c; Akhtar et al. 2000).

4. Evaluate the effects of various treatments based on six different embryogenic parameters viz. (i) frequency of embryogenesis (FE), (ii) intensity of embryogenesis (IE) i.e. average number of somatic embryos produced per explant per treatment, (iii) frequency of ET stage somatic embryos, (iv) frequency of ST stage somatic embryos, (v) frequency of lower or CHG stage somatic embryos, and vi. efficiency of embryogenesis (EE) (see Akhtar 1997, 2010, 2011, 2013a, b, c; Akhtar et al. 2000). The results are analyzed based on these parameter while the treatment potential is compared for efficiency of somatic embryogenesis (Table 1.2) for presentation and discussion.
5. Keep the sample size contestant at 10 or 12 zygotic embryo explants per treatment. Repeat experiments thrice for each treatment for statistical analysis and data presentation.
6. Represent mean values of three replicates of experiments along with standard deviations or error of means in tabulated presentation. Statistically analyze and compare the mean values of the three replicates of experiments for LSD, univariate or bivariate ANOVA, time series or phylogenetic relationship.

1.3 Plant Regeneration Through Somatic Embryogenesis

1.3.1 Induction of Somatic Embryogenesis

Most efficient somatic embryogenesis in guava had been found in the 8-days treatment from 10-weeks post anthesis zygotic embryo explants with 1.0 mg/l 2,4-D concentrations in 5% sucrose containing MS medium (Table 1.2) (Akhtar 2013a, c; Rai et al. 2007). Though less efficient, development of somatic embryos were also common in continuous presence of much lower concentrations of growth regulator (Table 1.2) (Akhtar 1997, 2010, 2011, 2013b). Other auxins showed varying potential for induction of somatic embryogenesis in guava (Akhtar 1997). Cytokinins alone were not only inefficient but also antagonized the effects of auxins (Akhtar 1997).

1.3.2 Development of Somatic Embryos

1. Small transparent watery white globular somatic embryos were visible under stereozoom microscope at the end or after the third week of culture initiation (Fig. 1.1c, d). In the fourth and fifth week many new globular somatic embryos developed over the same explants while the previous formed one proceeded to the next stage. There was simultaneous change in the colour from watery white transparent to translucent milkfish. In the sixth and seventh week of culture these somatic embryos proceed towards the advanced stages of development. Morphologically normal, mature, hard solid and milky white somatic embryos were ready for germination after eight weeks of culture initiation (Fig. 1.1e, f).

Table 1.2 Optimization response of factors in somatic embryogenesis from zygotic embryo explants of guava (*Psidium guajava* L.) in normal strength Murashige and Skoog's (1962) medium

Sl. No.	Treatments variables	Frequency of Embryogenesis	Intensity of Embryogenesis	Frequency of various stages of somatic embryos			Efficiency of embryogenesis
		FE (%)	IE (ANEPC)	ET (%)	ST (%)	CHG (%)	EE (relative)
1	D.001TP60SI3SD3W10	20.00 ± 4.56	15.79 ± 4.00	23.03 ± 8.78	49.33 ± 3.54	27.64 ± 6.44	2.29 ± 0.84
2	D.005TP60SI3SD3W10	25.42 ± 8.44	15.21 ± 3.89	21.23 ± 6.06	46.63 ± 12.02	32.15 ± 15.28	2.69 ± 1.52
3	D.01TP60SI3SD3W10	68.33 ± 6.97	60.83 ± 3.82	12.89 ± 1.92	41.05 ± 1.90	46.67 ± 2.35	22.21 ± 3.19
4	D.05TP28SI3SD3W10	63.89 ± 4.12	56.30 ± 9.86	13.91 ± 3.49	40.38 ± 2.86	45.70 ± 3.62	19.84 ± 5.80
5	D.1TP14SI3SD3W10	66.67 ± 6.81	60.93 ± 6.13	16.77 ± 1.82	39.20 ± 2.40	43.81 ± 4.05	22.82 ± 3.58
6	D.5TP8SI3SD3W10	68.75 ± 6.35	69.71 ± 14.34	14.99 ± 2.35	42.88 ± 5.77	42.13 ± 5.93	27.72 ± 6.67
7	D1TP8SI3SD3W10	51.39 ± 7.29	45.33 ± 15.39	9.18 ± 2.29	34.56 ± 6.23	56.27 ± 7.09	10.09 ± 3.87
8	D1.5TP8SI3SD3W10	33.34 ± 5.89	30.70 ± 8.96	9.62 ± 1.32	34.44 ± 3.44	54.94 ± 3.63	4.65 ± 1.86
9	D.001TP60SI5SD5W10	26..67 ± 6.70	24.74 ± 4.16	24.50 ± 5.42	46.26 ± 2.33	29.23 ± 6.80	4.73 ± 3.66
10	D.005TP60SI5SD5W10	33.01 ± 16.53	25.54 ± 10.11	19.59 ± 6.21	45.03 ± 11.20	35.39 ± 15.10	6.38 ± 4.34
11	D.01TP60SI5SD5W10	80.31 ± 6.74	187.18 ± 55.61	10.21 ± 1.85	36.72 ± 3.29	53.06 ± 3.65	71.82 ± 13.88
12	D.05TP28SI5SD5W10	75.00 ± 8.33	185.86 ± 37.71	11.14 ± 1.26	36.39 ± 5.15	52.46 ± 3.90	65.55 ± 11.20
13	D.1TP14SI5SD5W10	66.67 ± 8.34	147.67 ± 29.87	9.75 ± 1.38	49.26 ± 1.57	40.99 ± 2.56	59.52 ± 10.95
14	D.5TP14SI5SD5W10	80.55 ± 4.81	247.81 ± 39.90	4.24 ± 0.83	52.56 ± 5.01	43.20 ± 4.59	112.46 ± 12.30
15	D1.5TP8SI5SD5W10	67.50 ± 7.30	166.83 ± 22.38	6.20 ± 4.80	17.74 ± 10.07	76.05 ± 14.59	26.47 ± 17.20
16	D2TP8SI5SD5W10	40.51 ± 9.16	49.75 ± 13.16	4.07 ± 2.09	21.64 ± 6.16	74.30 ± 7.90	5.23 ± 2.52
17	D0.5TP8SI5SD5W8	28.12 ± 3.99	43.33 ± 3.30	18.16 ± 1.0	49.33 ± 6.68	32.52 ± 7.15	8.14 ± 0.77
18	D0.5TP8SI5SD5W9	52.08 ± 4.17	47.29 ± 12.39	29.78 ± 4.44	47.79 ± 4.12	22.43 ± 8.46	19.45 ± 7.33
19	D0.5TP8SI5SD5W10	68.05 ± 9.13	182.95 ± 31.37	17.11 ± 4.30	39.31 ± 5.19	43.58 ± 6.03	69.59 ± 12.48
20	D0.5TP8SI10SD10W11	66.67 ± 6.81	125.79 ± 30.13	7.12 ± 2.71	20.86 ± 4.82	72.02 ± 7.27	23.56 ± 8.60
21	D0.5TP8SI5SD5W12	50.00 ± 11.79	44.53 ± 12.64	12.00 ± 3.05	27.33 ± 2.31	60.67 ± 1.83	9.66 ± 7.26

(continued)

Table 1.2 (continued)

Sl. No.	Treatments variables	Frequency of Embryogenesis	Intensity of Embryogenesis	Frequency of various stages of somatic embryos			Efficiency of embryogenesis
		FE (%)	IE (ANEPC)	ET (%)	ST (%)	CHG (%)	EE (relative)
22	D0.5TP8SI5SD5W14	31.25 ± 7.98	18.22 ± 5.45	5.91 ± 1.74	32.36 ± 5.77	61.74 ± 7.36	2.12 ± 0.73
23	D1TP8SI10SD10 W8	65.00 ± 6.98	118.77 ± 13.82	12.57 ± 2.08	31.93 ± 5.16	55.50 ± 6.34	33.99 ± 4.46
24	D1TP8SI10SD10 W9	65.28 ± 6.28	161.78 ± 26.75	7.64 ± 1.93	27.89 ± 2.96	64.47 ± 4.09	37.16 ± 6.43
25	D1TP8SI5SD5W10	87.50 ± 5.40	491.36 ± 86.84	16.06 ± 2.43	39.46 ± 4.53	44.48 ± 5.63	237.70 ± 44.32
26	D1TP8SI5SD5W11	70.00 ± 4.56	101.21 ± 18.68	14.69 ± 1.61	34.39 ± 5.47	50.92 ± 6.26	34.44 ± 6.26
27	D1TP8SI5SD5W12	65.00 ± 6.97	84.07 ± 9.94	14.48 ± 2.42	39.71 ± 3.10	45.81 ± 5.35	29.67 ± 6.15
28	D1TP8SI5SD5W14	27.08 ± 7.98	12.83 ± 2.91	7.83 ± 1.42	19.38 ± 1.55	72.79 ± 2.81	0.99 ± 0.51

Treatment variables (Numbers immediately followed by letter represents concentrations, treatment period or the age): *D* 2,4-Dichlorophenoxy acetic acid (mg/l); *TP* Treatment period (Days); *SI* Sucrose in induction medium (%); *SD* Sucrose in development medium (%); *W* Age of zygotic embryo explants (weeks after anthesis); *FE* Frequency of Embryogenesis, *IE* Intensity of Embryogenesis (ANEPC—Average number of embryos per culture); *ET* Elongated Torpedo Stage Somatic Embryos; *ST* Short Torpedo Stage Somatic Embryos; *CHG* Cotyledonary, Heart, Globular Stage Somatic Embryos; *EE* Efficiency of Embryogenesis; (for details see Akhtar 1997, 2010, 2011, 2013a, c)

2. Depending upon the nature of treatment the number of somatic embryos developed per explant varied considerably and ranged from a single to usually several hundreds, sometimes more than a thousand (Fig. 1.1d–g). Most of the somatic embryos followed a normal development but sometimes anomalies were also observed as a function of treatment. However, the frequency of anomalous development and the secondary embryogenesis were very low in our case (Akhtar 1997, 2010, 2013a, c). Further there had been asynchrony in somatic embryo with 4–5 discernible stages developing from the same zygotic embryo explants (Fig. 1.1e, g). The complete description and evaluation process had been well represented in Akhtar (1997, 2010, 2011, 2013a, c), Akhtar et al. (2000).

1.4 Protocol for Optimization of Somatic Embryogenesis

1.4.1 Culture Initiation

1. Initiate cultures at first with 10-weeks post anthesis zygotic embryos having 4–5 mm curved axis treating continuously in the presence of broad range of growth regulators.
2. Use full strength MS medium added with 3% sucrose and modified with 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 10.0, 15.0 and 20.0 mg/l 2,4-D.
3. The responsive concentrations range is tested for further optimization of factors. Inefficient treatment and toxic non responsive concentrations are discarded.

1.4.2 Optimizing Treatment Periods

1. Give inductive treatments 10-weeks post anthesis zygotic embryo explants for 2-, 4-, 6-, 8-, 10-, 12-, 14-, 16-, 18-, 20-, 24-, 26-, 28-, 38- and 60-days.
2. Use optimum concentrations of 2,4-D (0.001–5.0 mg/l) for inductive treatments.
3. Transfer treated zygotic embryos to basal medium completely devoid of any growth regulator(s) after respective days of induction periods.
4. The results of these experiments are presented in Fig. 1.2a, b.

1.4.3 Optimizing Physiological Age of Zygotic Embryo Explants

1. Dissect zygotic embryo explants from seeds of 7-, 8-, 10-, 12- and 14-weeks post anthesis fruits.
2. Treat these zygotic embryo explants for 8-days with optimum concentrations (0.5, 1.0 mg/l) of 2,4-D.

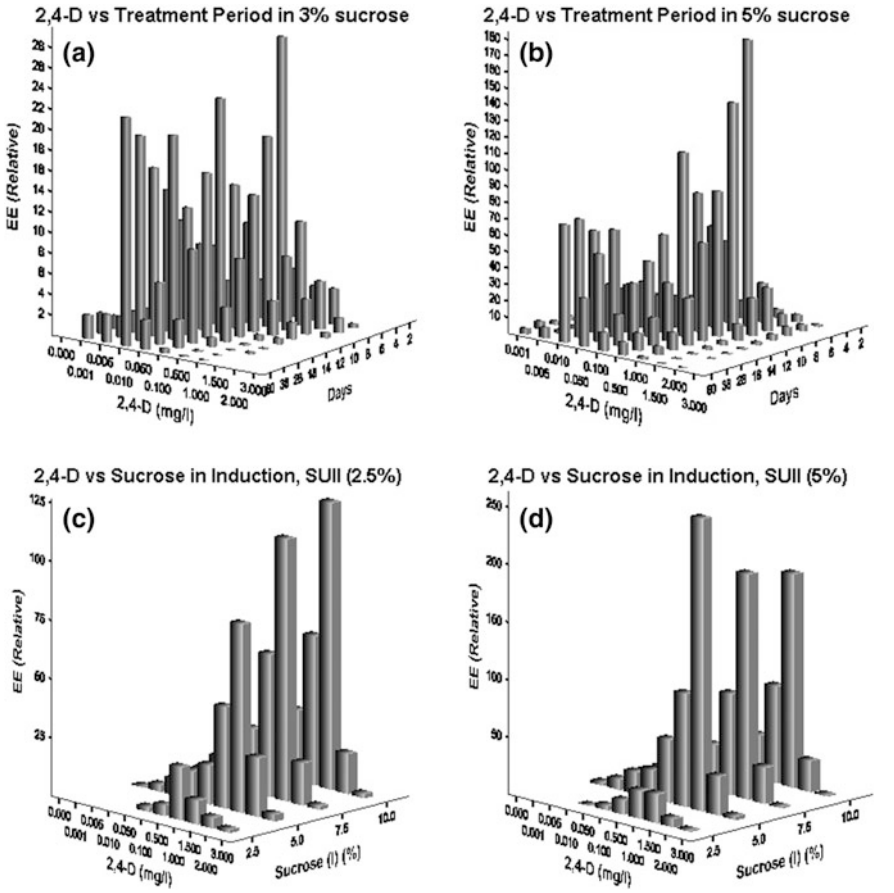

Fig. 1.2 Interactive effects of various factors on the induction and optimization of somatic embryogenesis in guava. **a** Interactions of 2,4-D concentrations and the treatment periods on the process of somatic embryogenesis from 10-weeks post anthesis zygotic embryo explants cultured in full strength MS medium containing 3% (w/v) sucrose, **b** interactions of 2,4-D concentrations and the treatment periods on the process of somatic embryogenesis from 10-weeks post anthesis zygotic embryo explants cultured in full strength MS medium added with 5% (w/v) sucrose, **c** interactive effect of 2,4-D and sucrose concentrations on the induction of somatic embryogenesis from 10-weeks post anthesis zygotic embryo explants when the development medium containing 2.5% (w/v) sucrose, **d** interactions of 2,4-D and sucrose concentrations on the induction of somatic embryogenesis from 10-weeks post anthesis zygotic embryo explants when sub-cultured (following 8-days inductive treatment) to the development medium containing 5% (w/v) sucrose, **e** interactions of 2,4-D and sucrose concentrations on the induction of somatic embryogenesis from 10-weeks post anthesis zygotic embryo explants when sub-cultured (following 8-days inductive treatment) to the development medium containing 7.5% (w/v) sucrose, **f** interactions of 2,4-D and sucrose concentrations on the induction of somatic embryogenesis from 10-weeks post anthesis zygotic embryo explants when sub-cultured (following 8-days inductive treatment) to the development medium containing 10% (w/v) sucrose, **g** interactive effect of physiological age of explants and sucrose concentrations (same in both induction and development media) on the process of somatic embryogenesis in 8-days inductive treatment with 0.5 mg/l of 2,4-D concentrations in full strength MS medium, **h** interactive effect of physiological age of explants and sucrose concentrations (same in both induction and development media) on the process of somatic embryogenesis in 8-days inductive treatment with 1.0 mg/l of 2,4-D concentrations in full strength MS medium

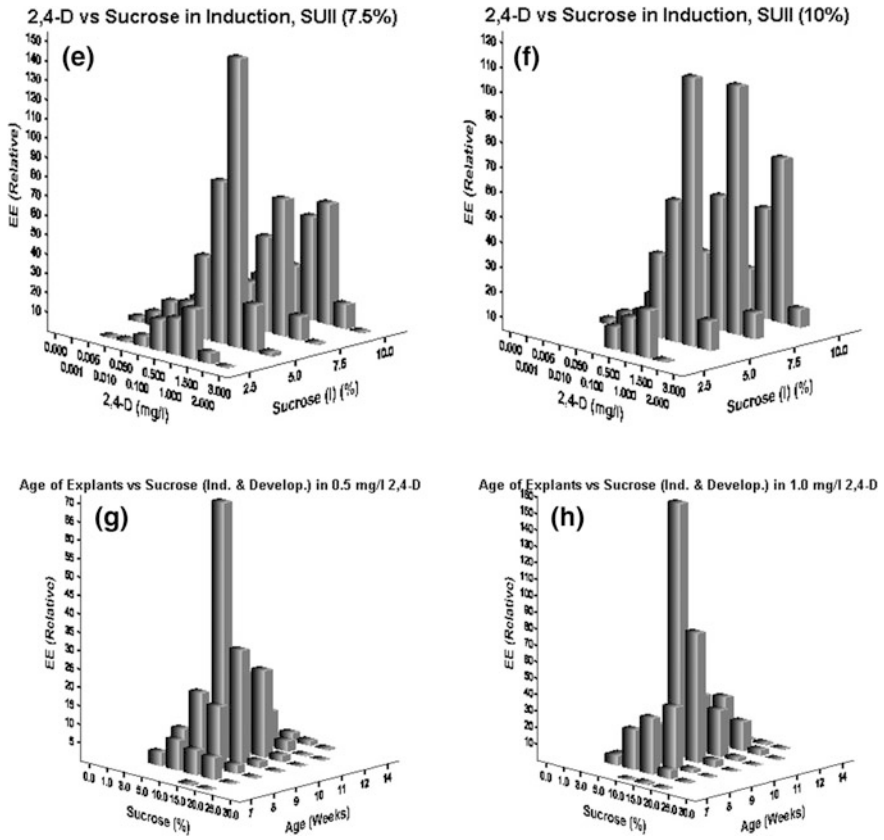

Fig. 1.2 (continued)

3. Supplement full strength MS medium with varying concentrations of sucrose (2.5, 5.0, 7.5, 10.0%).
4. Use complete factorial design to optimize nutritional requirement for carbon source and the age of explants for induction of somatic embryogenesis (Fig. 1.2g, h).

1.4.4 Optimizing Inductive Concentrations and Nutritional Requirements of Carbon Source

1. Treat 10-weeks post anthesis zygotic embryo explants for 8-days.
2. Modify full strength MS medium with 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0, mg/l 2,4-D.

3. Supplements full strength MS medium with 2.5, 5.0, 7.5 and 10.0% sucrose at both induction and development levels.
4. Use complete factorial design to optimize nutritional requirement of carbon source and 2,4-D concentration for induction of somatic embryogenesis.
5. Analyze the result and present as the interactive effects of concentrations of sucrose and the 2,4-D (Fig. 1.2c–f).

1.4.5 Optimizing the Plant Growth Regulators and Their Combinations

1. Prepare normal strength MS medium and apply appropriate conditions as optimized under Sects. 4.1, 4.2, 4.3 and 4.4 above for various plant growth regulators treatments.
2. Plant growth regulators such as auxins (IAA, IBA and NAA), cytokinins (BAP and Kinetin) and Thidiazuron (TDZ) and others are optimized similarly.
3. Treat zygotic embryo explants (10 weeks post anthesis) continuously as well as various treatment periods (8-, 10-, 12- and 14-days).
4. Modify medium with broad range (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 3.0, 5.0, 10.0, 15.0 and 20.0 mg/l) concentrations of a single growth regulator.
5. Supplement medium with 5.0% sucrose containing in the modified MS medium.
6. Use complete factorial design to show the interactions of other growth regulators with the 2,4-D concentrations optimized under Sects. 4.1, 4.2, 4.3 and 4.4 above.

1.4.6 Optimizing the Medium Strength

1. Induction of somatic embryogenesis is highly nutritional intensive process. Macro nutrients usually become limiting in an efficient induction process.
2. One and quarter strength of MS major salt along with normal levels of other salts are tried to enhance the induction process.
3. Half, quarter and one eighth strength of MS medium are tried at both induction and developmental stages in various combinations permutations of sucrose (5, 7.5 and 10%) and 2,4-D concentrations (0.01–2.0 mg/l) for somatic embryogenesis.
4. The best responsive age (10-weeks) of zygotic embryo explants are given 8 days inductive treatment with these combinations to optimize the nutritional need and osmotic potential of the medium during the process of somatic embryogenesis.

1.4.7 Optimizing Other Carbohydrates

1. Equimolar concentration of glucose, fructose, lactose, maltose, mannitol or sorbitol are used either as sole source of carbon or along with the optimum sucrose levels to find out the best possible source or combination of carbohydrates for induction and enhancement of somatic embryogenesis.
2. The zygotic embryo explants from seeds of 10-weeks post anthesis fruit are treated for 8-days with 1.0 mg/l 2,4-D in full strength MS medium with above combinations of carbohydrates.

1.4.8 Optimizing Quality of Sucrose and Purity of Distilled Water

1. Since sucrose was found as an important constituent affecting embryogenesis significantly. Different grades such as high purity sucrose, low quality sucrose, sugar pellets and other sugars are tried for the process economy with their optimum concentrations at both induction and development medium.
2. Various distillation purity (double, single, demineralized) of water combined with different quality of sucrose are tried for process economy.
3. Double distilled water in combinations with pure sucrose proved to be the best, while the single distilled water along with medium grade sucrose found 40% less efficient for the production of normal somatic embryos and the plantlets (Akhtar 1997; Akhtar et al. 2000).

1.4.9 Stress Induced Somatic Embryogenesis

1.4.9.1 Salinity Stress

1. Different combinations permutations of sodium chloride (NaCl) (25–500 mM) and the sucrose (2.5 and 5%) are tired in full strength MS medium.
2. The zygotic embryo explants from 10-weeks post anthesis fruits are incubated in the above media without 2,4-D to study the control of somatic embryogenesis as the stress induced phenomenon caused by NaCl (Akhtar 1997, 2011; Akhtar et al. 2000, 2016).
3. Perform several cycle of 8-days treatment with 1.0 mg/l of 2,4-D are also given to 10-weeks old zygotic embryo explants following Akhtar et al. (2016) by incubating in full strength MS medium with above combinations of sucrose and NaCl at both induction, developmental, maturation and germination phase of somatic embryogenesis.

1.4.9.2 Water Stress

1. Full and half strength MS medium with 2.5% and 5% sucrose are fortified with polyethylene glycol (PEG) (1–10%) for the treatment of zygotic embryo explants (10-weeks post anthesis) in the absence of 2,4-D.
2. Similarly, perform several cycle of zygotic embryo explants (10-weeks post anthesis) treatment for 8 days with 1.0 mg/l of 2,4-D in full strength MS medium with 2.5 and 5% sucrose and fortified with polyethylene glycol (PEG) (1–10%) following Akhtar et al. (2016) to study the effects of water stress on embryogenic induction and the development of somatic embryos.
3. It has been found that the osmotic potential of the medium created by addition of sucrose, NaCl and/or other salts are enough to regulate the somatic embryogenesis and any further disturbance in water potential of the medium would cause deleterious effect on the process (Akhtar 1997, 2011; Akhtar et al. 2000).

1.4.9.3 Other Stresses

1. Guava seeds (10-weeks post anthesis) are treated for 60–120 min with sodium hypochlorite (NaOCl) (4% nascent chlorine) followed by zygotic embryo dissection and incubation in 5% sucrose containing full strength MS medium without any growth regulators to study the role of oxidative stress on the induction of somatic embryogenesis.
2. Similarly, NaOCl treated zygotic embryo explants (10-weeks old) are given 8-days induction with 1.0 mg/l 2,4-D along in 5% sucrose containing full strength MS medium.
3. The results on these experiments on somatic embryogenesis have suggested a partial induction of the process by the stress phenomenon in guava (Akhtar 1997, 2011; Akhtar et al., 2000).

1.4.10 Evaluating the Genotypic Effects on Guava Somatic Embryogenesis

1. Most of the optimization process is conducted on well known cultivar Allahabad Safeda. Very close relative and equally high priced cultivars i.e. Banarsi local as well as Lucknow-49, red skinned, pink fleshed and other cultivars are located grown in the region.
2. The zygotic embryo explants from 10-weeks post anthesis fruits are induced for 8-days in the presence of 1.0 mg/l of 2,4-D in 5% sucrose containing full strength MS medium.

3. The development of somatic embryos is observed by transferring the zygotic embryo explants to 5% sucrose containing full strength MS development medium devoid of any growth regulators.
4. All the six embryogenic parameter described to evaluate the potential of treatments are used in phylogenetic analysis to prepare genetic tree which can be applied as the physiological markers for genotypic identification of guava cultivars (Akhtar 1997, 2011; Akhtar et al. 2000).

1.4.11 Effect of Explanting Season

1. In a mild tropical environment guava bears fruit through out the year. The induction process performed during the different seasons (winter, spring, summer and monsoon) has been recorded properly.
2. Experiments performed under the standard conditions of 8-days treatment of zygotic embryo explants (10-weeks post anthesis) in the presence of 1.0 mg/l of 2,4-D in 5% sucrose containing full strength MS medium are subjected statistically for time series analysis to trace out any seasonal variations in the induction and development of somatic embryos (Akhtar 1997, 2011; Akhtar et al. 2000).
3. The results on all the six embryogenesis parameters for all such experiments performed under the four seasons summer (May–July), monsoon (August–October), winter (November–January), spring (February–April) are pooled for 3–4 consecutive years (2011–2015) and evaluated through time series analysis to find out any seasonal trends in zygotic embryo explants for induction of somatic embryogenesis.

1.4.12 Role of Polyamine in Somatic Embryogenesis in Guava

1. Treat zygotic embryo explants for 8 d with 1.0 mg/l 2,4-D then sub-culture onto PGR-free MS agar medium both containing 5% (w/v) sucrose.
2. Extract different forms (free, conjugated, or bound) of cellular polyamines (putrescine, spermidine, or spermine) in pre-chilled 5% (w/v) trichloroacetic acid (TCA) for analysis using 24 zygotic embryos per 2,4-D treatment at 2, 4, 6, 8, 10, 12, 14, 18, 28, 38, or 60 d after culture initiation.
3. Dansylate polyamines as described by Biondi et al. (1993), and by Gallardo et al. (1994), with slight modifications.

4. Separate dansylated polyamines by thin layer chromatography (TLC) on 7.5 cm 2.5 cm plates (300 μm-thick silica gel 60) using mixture of ethyl acetate: cyclohexane (2:3) (v/v) as mobile phase.
5. Performed chromatography for approx. 15 min and visualise plates under 254 nm UV light. Compare the Rf values of samples with standards and scrap-off respective bands from TLC plante.
6. Re-dissolve the collected bands in acetone, centrifuged and quantify at 365 nm (excitation) and 510 nm (emission) using a high-resolution spectrophotofluorometer (Akhtar 2013a, c).

1.4.13 Recurrent Embryogenesis

1. Somatic embryos at various stages of development (ET, ST and CHG) from different weeks (8-, 10-, 12-, and 14-weeks) old cultures are uses as explant and treated with 0.1, 0.5, 1.0 and 2.0 mg/l of 2,4-D in full strength MS medium containing 5% sucrose at both induction and development phases to study the next generation of embryogenesis.
2. Three to four generation of recurrent embryogenesis are treated similarly to trace the potential of somatic embryo explants for cloning and regeneration.
3. Since no significant variations are noted in the potential of somatic embryo explants and the 2,4-D.
4. Elongated torpedo (ET) stage somatic embryos from eight week old culture proved to be the best explant material, showing induction of somatic embryogenesis very close to zygotic embryo explants in presence of 1.0 mg/l of 2,4-D (Fig. 1.1l).
5. Short torpedo (ST) stage somatic embryos (8-week old cultures) when used as the explants have shown half the embryogenesis response (cf. ET stage) and the best responses are achieved in 0.5 mg/l of 2,4-D in 5% sucrose containing full strength MS medium.
6. Lower stages (CHG) somatic embryos of all the ages have shown almost negligible induction irrespective of the medium modifications (Akhtar 1997; Akhtar et al. 2000).
7. In half strength medium all the stages of somatic embryos, irrespective of their ages and levels of sucrose and 2,4-D, showed significantly reduced efficiency of recurrent embryogenesis.
8. The explanted somatic embryos mostly followed the germination in low sucrose and the callus production in presence of high amount of sucrose in the medium (Akhtar 1997; Akhtar et al. 2000).

1.5 Maturation and Germination of Somatic Embryos

1. Different satges of somatic embryos of various ages (8–10 weeks after culture initiation) when subcultured (8-weeks after culture initiation) to a fresh either full strength or half strength agar as well as in liquid **germination medium** with 1, 3 or 5% sucrose are turned green indicating germination in varying frequencies (Fig. 1.1g, j).
2. It seems that in guava somatic embryos during their developmental progression from globular to torpedo stage followed maturation simultaneously without the need of additional treatments. After eighth weeks of culture initiation, most of the somatic embryos were hard, solid milky white irrespective of their morphology or developmental stage (Fig. 1.1e, f). Almost all of them remained in the same developmental stages if maintained on the same development medium even up to 14 weeks of culture initiation.
3. Hence, there is is no need of separate maturation step in guava as compared to other species.

1.6 Growth of Plantlets and Acclimatization

1.6.1 Growth Medium

1. Somatic plantlets developed in liquid or agar solidified germination medium ceased to grow after two weeks of subculture and showed very little percentage of survival upon transfer and hardening to ex vitro condition.
2. Full strength MS liquid medium with 3% sucrose **(growth medium)** is used to subculture the plantlets after 2-weeks in germination medium for an extended growth of two weeks to enhance survival frequency upon soil transfer and hardening (Fig. 1.1k).

1.6.2 Soil Transfer

1. A mixture of sand, soil and compost in a ratio of 5:1:2 is used for transfer of plantlets initially from liquid growth medium.
2. Once the root system adjusted to this primary mixture, plantlets along with potted soil are transferred to a sand, soil and compost mixture in a ratio of 1:2:3.

1.6.3 Acclimatization

1. Depending upon the regeneration size, a well maintained polyhouse or green house with fogger/sprinkler and exhaust fitting is used for acclimatization process.

2. Initially a low light intensity (covering the polyhouse or green house with shade net) and high humidity (85–90%) is maintained for 1–2 days.
3. Once the new shoots with leaves appeared progressively increase the light intensity with concomitant reduction of humidity in the polyhouse or green house.
4. Water the plantlets at regular interval of 1–2 days till the plant is completely adapted to the external environment.
5. Now the plants are ready for propagation under the filed conditions.

1.7 Conclusion

Zygotic embryo explants at the age of 10-weeks post anthesis, 8-days inductive treatment with 2,4-D (1.0 mg/l) and other growth regulators, development of somatic embryos in hormone free medium without any kind of post-maturation treatment, 5% sucrose at both induction and developmental phase are some the optimum conditions for induction, development, maturation and germination of somatic embryos for high efficiency plantlets recovery in guava. Addition of glutamine (100 mg/l), polyamines (50 mM), NaCl (75 mM), or PEG (1%) at both induction and development phases of the process can facilitate the enhancement of embryogenic efficiency, selection for salinity and water stress tolerant guava somatic plantlets. Application of RAPD, SSR, ISSR, SRAP, micro-satellites and other m molecular techniques have demonstrated the homogeneity amongst plantlet regenerated through somatic embryogenesis from zygotic embryo explants of guava. Micropropagation of guava through both organogenesis and somatic embryogenesis has progressed significantly, however, many aspect of crop improvements have yet to be explored for the production of more clonal materials with commercial applications.

1.8 Research Prospects

Being the product of gamete fusion, use of zygotic embryo as explant for regeneration of guava can create variations among the somatic plantlets. Understanding the molecular mechanisms in acquisition of embryogenic competence by cells of more homogeneous somatic tissue explants involving the reprogramming of gene, their expression patterns, changes in the morphology, physiology, and metabolism leading to induction and development of somatic embryos is the need of future research for commercial exploitation of the process in guava and other species. The regeneration protocol involving all the stages of somatic embryogenesis in cell suspension culture and scale up to produce propagules with low cost is an important issues of future research for commercial production in guava. A bioreactor system

with the automation of the entire process of somatic embryogenesis has to be worked out critically to enhance efficiency of the process for commercialization in future. The genetic transformation is another important aspect to facilitate resistance against diseases, increasing self life of fruits and enhancing the stresses tolerance in guava (*Psidium guajava* L.) for future research.

Acknowledgements The financial support to author by various funding agencies like University Grant Commission, Council of Scientific and Industrial Research, Department of Biotechnology, Department of Science and Technology, Government of India is gratefully acknowledged. The author would like to extend his gratitude to the institutions viz. GITAM University, Visakhapatnam, India, Al-Ameen Arts Science and Commerce College, Bangalore, Jain University, Bangalore for their supports in continuing these researches.

References

Ahmed B, Mannan MA, Hossain SA (2011) Molecular characterization of guava (*Psidium guajava* L.) germplasm by RAPD analysis. Int J Nat Sci 1(3):62–67

Akhtar N (1996) Recurrent embryogenesis as a mean of conservation of guava germplasm. In: Proceedings of the national research seminar in botany: environment, biodiversity and conservation, Balaghat, MP, India, p 17

Akhtar N (1997). Studies on induction of somatic embryogenesis and production of artificial seeds for micropropagation of a tropical fruit tree guava (*Psidium guajava* L.). Unpublished Ph.D. thesis, Banaras Hindu University, Varanasi, 203 p

Akhtar N (2010) Evaluation of the efficiency of somatic embryogenesis in guava (*Psidium guajava* L.). J Hortic Sci Biotechnol 85(6):556–562

Akhtar N (2011) Progress in biotechnology of guava (*Psidium guajava* L.). In: Vyas D, Paliwal GS, Khare PK, Gupta RK (eds) Micribial biotechnology and ecology, vol II. Daya Publication House, New DelhiIndia, pp 501–519

Akhtar N (2013a) Endogenous polyamines: a temporal cellular modulator of somatic embryogenesis in guava (*Psidium guajava* L.) cv. Allahabad Safeda. Res Plant Sci 1(2):4–14. https://doi.org/10.12691/plant-1-2-1

Akhtar N (2013b) Somatic embryogenesis for high efficiency micropropagation of guava (*Psidium guajava* L.). In: Lambardi M, Ozudogru EA, Jain SM (eds) Protocols for micropropagation of selected economically important horticultural plants. Humana Press, Springer Science + Business Media, LLC, New York, USA, pp 161–177

Akhtar N (2013c) Temporal regulation of somatic embryogenesis in guava (*Psidium guajava* L.). J Hortic Sci Biotechnol 88(1):93–102

Akhtar N, Jain SM (2000) Application of somatic embryogenesis for the improvement of tropical fruit trees. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 6. Kluwer Academic Publisher, The Netherland, pp 215–248

Akhtar N, Jaiswal VS (1994) Role of somatic embryogenesis and artificial seed production for micropropagation and improvement of guava (*Psidium guajava* L.). In: Proceedings of the symposium on role of plant tissue culture in biotechnology in India, Varanasi, India, p 2

Akhtar N, Jaiswal VS (1995) Rapid multiplication of *Psidium guajava* L. through recurrent embryogenesis. In: Proceedings of the all India symposium on recent advances in biotechnological application of plant tissue culture, Mysore, India, p 49

Akhtar N, Kumari N, Pandey S, Ara H, Singh M, Jaiswal U, Jaiswal VS, Jain SM (2000) Somatic embryogenesis in tropical fruit trees. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 6. Kluwer Academic Publisher, The Netherland, pp 93–140

Akhtar N, Srivastava RK, Mishra MR (2016) In vitro technique for selection of plants tolerant to environmental stress. In: Gupta RK, Singh SS (eds) Environmental biotechnology: a new approach. Daya Publishing House: A Division of Astral International Pvt. Ltd., New Delhi, India, pp 309–345

Ali N, Mulwa RMS, Norton MA, Skirvin RM (2003) Micropropagation of guava (*Psidium guajava* L.). J Hortic Sci Biotechnol 78:739–741

Ali N, Mulwa RMS, Norton MA, Skirvin RM (2007) Radical disinfestation protocol eliminates in vitro contamination in guava (*Psidium guajava* L.) seeds. Plant Cell Tiss Org Cult 91:295–298

Amin MN, Jaiswal VS (1987) Rapid clonal propagation of guava through in vitro shoot proliferation on nodal explants of mature trees. Plant Cell Tiss Org Cult 9:235–243

Amin MN, Jaiswal VS (1988) Micropropagation as an aid to rapid cloning of a guava cultivar. Sci Hortic 36:89–95

Amin MN, Jaiswal VS (1989a) In vitro propagation of guava (*Psidium guajava* L.): effects of sucrose, agar and pH on growth and proliferation of shoots. Bang J Bot 18:1–8

Amin MN, Jaiswal VS (1989b) Effects of phloroglucinol, sucrose, pH and temperature on in vitro rooting of guava (*Psidium guajava* L.) microcuttings. Bang J Bot. 18:129–139

Babbar SB, Gupta SC (1986a) Induction of androgenesis and callus formation in in vitro cultured anthers of a myrtaceous fruit tree (*Psidium guajava* L.). Bot Mag Tokyo 99:75–85

Babbar SB, Gupta SC (1986b) Embryogenesis in stamen and hypocotyl explants of a myrtaceous fruit tree *Psidium guajva* L. In: VI. international congress on plant tissue and cell culture, Minneapolis, p 133

Bajpai A, Kalim S, Chandra R, Kamle M (2016). Recurrent somatic embryogenesis and plantlet regeneration in *Psidium guajava* L. Braz Arch Biol Technol 59:e16150170. http://dx.doi.org/10.1590/1678-4324-2016150170 ISSN

Biondi S, Hagege D, Rissini P, Bagni N (1993) Polyamine metabolism and ethylene biosynthesis in normal and habituated sugar beet callus. Physiol Plant 89:699–706

Chandra R, Mishra M (2007) Biotechnological interventions for improvement of guava (*Psidium guajava* L.). In: Singh G, Krishnu R, Chandra R (eds) Proceedings of first international guava symposium. International Society for Horticultural Science (ISHS, 2007), Acta Horticulture, p 735

Chandra R, Bajpai A, Gupta S, Tiwari RK (2004) Embryogenesis and plant regeneration from mesocarp of *Psidium guajava* L. (guava). Ind J Biotechnol 3:246–248

Chandra R, Kamle M, Bajpai A (2010) Guava. In: Singh HP, Parthasarathy VA, Nirmal Babu K (eds) Advances in horticultural biotechnology: regeneration systems, vol 1 fruit crops, plantation crops and spices. Westville Publishing House, New Delhi, pp 103–120

Coser SM, Ferreira MFS, Ferreira A, Mitre LK, Carvalho CR, Clarindo WR (2012) Assessment of genetic diversity in *Psidium guajava* L. using different approaches. Sci Hortic 148:223–229

Fitchet M (1989) Citrus and subtropical fruit research institute: information. Bulletin 201:4–5

Gallardo M, Gallardo ME, Mantilla AJ, de Mufioz RP, Sanchez-Calle IM (1994) Inhibition of polyamine synthesis by cyclohexyamine stimulates the ethylene pathway and accelerates the germination of *Cicer arientinum* seeds. Physiol Plant 91:9–16

Ghaffoor A, Alderson PG (1994) Somatic embryogenesis in guava (*Psidium guajava* L.). In: Lumsden PJ, Nicholas JR, Davis WJ (eds) Physiology, growth and development of plants in culture. Kluwer Academic Publishers, The Netherland, pp 272–277

Gutierrez RMP, Mitchell S, Solis RV (2008). *Psidium guajava*: a review of its traditional uses, phytochemistry and pharmacology. J Ethnopharmacol 117:1–27. https://doi.org/10.1016/j.jep.2008.01.025

Herrero JV, Medina NNR, Alor MB, García MMO, Moreno AQ, Teyer LFS, Risterucci AM, Rohde W (2010) Microsatellites developed in guava (*Psidium guajava* L.) and their usefulness in evaluating diversity in the Myrtaceae family. Rev Colomb Biotechnol 12(1):64–76

Jaiswal U, Jaiswal VS (2005) *Psidium guajava*. In: Litz RE (ed) Biotechnology fruits and nut crops. CAB International, Wallingford, UK, pp 394–401

Jaiswal VS, Akhtar N (1993) Studies on induction and factors controlling somatic embryogenesis in guava. In: Proceedings of a symposium on plant tissue culture and its biotechnological application, Jaipur, India, p 48

Jaiswal VS, Akhtar N (1994) Induction of somatic embryogenesis and artificial seed production for micropropagation of guava (*Psidium guajava* L.). In: Proceedings of the VIII International Congress on Plant Tissue Cell Culture, June 12–17, Italy, p 55

Jaiswal VS, Amin MN (1986) In vitro shoot proliferation and plantlet formation from somatic tissues of guava (*Psidium guajava* L.). In: VI international congress on plant tissue and cell culture, Minneapolis, p 279

Jaiswal VS, Amin MN (1987) In vitro propagation of guava from shoot cultures of mature trees. J Plant Physiol 130:7–12

Jaiswal VS, Amin MN (1992) Guava and jackfruit. In: Hammerschlag FA, Litz RE (eds) Biotechnology of perennial fruit crops. Biotechnology in agriculture. No. 8. CAB International, Wallingford, UK, pp 421–431

Kamle M, Kalim S, Bajpai A, Chandra R, Kumar R (2012) In vitro selection for wilt resistance in guava (*Psidium guajava* L.) cv. Allahabad safeda. Biotechnology 11(3):163–171. https://doi.org/10.3923/biotech.2012.163.171

Kamle M, Kumar P, Bajpai A, Kalim S, Chandra R (2014) Assessment of genetic fidelity of somatic embryogenesis regenerated guava (*Psidium guajava* L.) plants using DNA-based markers. NZ J Crop Hortic Sci 42(1):1–9. https://doi.org/10.1080/01140671.2013.814574

Kidaha M, Alakonya AE, Nyende AB (2014) Molecular characterization of guava landraces in Kenya (Western and South Coast) Liharaka. J Biol Agric Healthc 4(15):81–86

Kosky GR, Perozo JV, Penalver DA (2005) Somatic embryo germination of *Psidium guajava* L. in the RITA® temporary immersion system and on semisolid medium. In: Kosky GR, Perozo JV, Penalver DA (eds) Liquid culture system in in vitro micropropagation. Springer, Heidelberg, Germany, vol 1, pp 225–229

Liu X, Yang G (2012) Assessment of clonal fidelity of micro-propagated guava (*Psidium guajava*) plants by ISSR markers. Aust J Crop Sci 6(2):291–295. ISSN: 1835-2707

Loh CS, Rao AN (1989) Clonal propagation of guava (*Psidium guajava* L.) from seedling and grafted plants and adventitious shoot formation in vitro. Sci Hortic 39:31–39

Majumdar PK, Mukherjee SK (1971) Aneuploidy in guava. Nucleus 14(2):119–131

Majumdar PK, Mukherjee SK (1972) Aneuploidy in guava (*Psidium guajava* L.). I. Mechanism of variation in chromosome number. Cytologia 37:541–548

Meghwal PR, Singh SK, Sharma HC (2003) Micropropagation of Aneuploid guava. Indian J Hortic 60:29–33

Mitra SK, Bose TK (1985) Guava. In: Bose TK (ed) Fruits of India: tropical and subtropical. Naya Prokash, Calcutta, pp 277–279

Mohammed S, Majumder PK (1974) Investigations on the breeding behaviour of aneuploids of guava. Euphytica 23:181–185

Moura EF, Motoike SY (2009) Induction of somatic embryogenesis in immature seeds of guava tree cv. Paluma. Rev Bras Frutic Jaboticabal—SP 31(2):507–511

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15:473–497

Nimisha S, Kherwar D, Ajay KM, Singh B, Usha K (2013) Molecular breeding to improve guava (*Psidium guajava* L.): current status and future prospective. Sci Hortic 164:578–588

Padmakara B, Kanupriya C, Lathaa VM, Prashant KS, Dinesh MR, Sailaja D, Aswath C (2015) Development of SRAP and SSR marker-based genetic linkage maps of guava (*Psidium guajava* L.). Sci Hortic 192:158–165

Papadatou P, Pantikis CA, Ephtimiadou E, Lydaki M (1990) Rapid multiplication of guava seedlings by in vitro shoot tip culture. Sci Hortic 45:99–103

Pommer CV, Murakami KRN (2009) Breeding guava (*Psidium guajava* L.). In: Jain SM, Priyadarshan PM (eds) Breeding plantation tree crops: tropical species. Springer Science + Business Media LLC, New York, USA, pp 83–120

Rai MK, Jaiswal VS (2008) Synthetic seeds of guava (*Psidium guajava* L) from somatic embryos and plant regeneration. In: Arya ID, Arya S (eds) Utilization of biotechnology in plant sciences. Microsoft Printech (I) Pvt Ltd., Dehradun, pp 217–222

Rai MK, Akhtar N, Jaiswal VS (2007) Somatic embryogenesis and plantlet regeneration in *Psidium guajava* L. cv. Banarsi Local Sci Hortic 113:129–133

Rai MK, Jaiswal VS, Jaiswal U (2009) Effect of selected amino acids and polyethylene glycol on maturation and germination of somatic embryos of guava (*Psidium guajava* L.). Sci Hortic 121:233–236

Rai MK, Jaiswal VS, Jaiswal U (2008a) Effect of ABA and sucrose on germination of encapsulated somatic embryos of guava (*Psidium guajava* L.). Sci Hortic 117:302–305

Rai MK, Jaiswal VS, Jaiswal U (2008b) Encapsulation of shoot tips of guava (*Psidium guajava* L.) for short-term storage and germplasm exchange. Sci Hortic 118:33–38

Rai MK, Jaiswal VS, Jaiswal U (2008c) Alginate-encapsulation of nodal segments of guava (Psidium guajava L.) for germplasm exchange and distribution. J Hortic Sci Biotechnol 83:569–573

Rai MK, Phulwaria M, Shekhawat NS (2013) Transferability of simple sequence repeat (SSR) markers developed in guava (*Psidium guajava* L.) to four Myrtaceae species. Mol Biol Rep 40:5067–5071. https://doi.org/10.1007/s11033-013-2608-1

Rai MK, Phulwaria M, Harish, Gupta AK, Shekhawat NS, Jaiswal U (2012) Genetic homogeneity of guava plants derived from somatic embryogenesis using SSR and ISSR markers. Plant Cell Tiss Org Cult 111:259–264. https://doi.org/10.1007/s11240-012-0190-1

Rai MK, Asthana P, Jaiswal VS, Jaiswal U (2010a) Biotechnological advances in guava (*Psidium guajava* L.): recent developments and prospects for further research. Trees Struct Funct 24:1–12. https://doi.org/10.1007/s00468-009-0384-2

Rai MK, Jaiswal VS, Jaiswal U (2010b) Regeneration of plantlets of guava (*Psidium guajava* L.) from somatic embryos developed under salt-stress condition. Acta Physiol Plant 32:1055–1062. https://doi.org/10.1007/s11738-010-0496-8

Ramirez VMC, Salazer YEG (1998) In vitro culture of guava (*Psidium guajava* L.) immature embryo. Revista de la Facultad de Agronomia, Universidad del Zulia 15:211–221

Schroeder CA (1961) Some morphological aspects of fruit tissues grown in vitro. Bot Gaz 122 (3):198–204. http://www.jstor.org/stable/2473503

Singh G, Krishnu R, Chandra R (2005) First international guava symposium, organized by society for development of subtropical horticulture and central institute for subtropical horticulture Lucknow in collaboration with international society for horticultural science. Nandi Prisad and Agricultural Processed Food Product Export Authority, at Lucknow. ISHS, ACTA Horticulturae 735 (CDROM)

Singh SK, Meghwal PR, Sharma HC, Singh SP (2002) Direct shoot organogenesis on explant from germinated seedlings of *Psidium guajava* L. cv. Allahabad Safeda. Sci Hortic 95:213–221

Vilchez JA, Albany NR, Gómez KR, Garcia L (2002) Induction of somatic embryogenesis in *Psidium guajava* L. starting at the zygotic embryo stage. Revista de la Facultad de Agronomia (LUZ) 19:284–293

Vilchez JA, Albany NR, Kosky RG, Garcia L (2004) Secondary embryogenesis and plant regeneration in *Psidium guajava* L. Trop Agric (Trinidad) 81(1):41–44

Vilchez J, Martinez L, Albany N (2015) Comparison of growth in nursery between seedlings and in vitro plants of guava cultivar red dwarf Cuban EEA-1840. Interciencia 40(4):270–274

Yadav UL (1996) Guava (*Psidium guajava* L.): an exotic tree fruit with potential in the southeastern United States. Hortic Sci 31(5):789–794

Yasseen YM, Barringer SA, Schnell RJ, Splittstoesser WE (1995) In vitro shoot proliferation and propagation of guava (*Psidium guajava* L.) from germinated seedlings. Plant Cell Rep 14:525–528

Zamir R, Ali N, Shah ST, Muhammad T, Shah SA (2007) In vitro regeneration of guava (*Psidium guajava* L.) from shoot tips of mature trees. Pak J Bot 39:2395–2398

Chapter 2
Olive *Olea europaea* L.

Carolina Sánchez-Romero

Abbreviations

2iP	6-(ɣ,ɣ-dimethylallylamino)purine
BAP	6-benzylaminopurine
IBA	indole-3-butyric acid
MS/2	MS mineral formulation at half strength
NAA	1-naphthaleneacetic acid
SE	somatic embryogenesis
TDZ	thidiazuron

2.1 Introduction

The olive (*Olea europaea* L.) (2n = 2x = 46) is a medium-sized evergreen tree, which produces numerous small fruits, rich in oil (Rapoport et al. 2016). Olive fruits are a very valuable food product, being consumed as oil or table olives. Due to its utility for human feeding, this species was domesticated in the Middle East around 5500 years BP (Rallo et al. 2011). From this area, olive extended to the rest of the Mediterranean basin, where since ancient times it constitutes one of the most important crops, with an enormous impact on the economy, history, culture and environment of this region (Baldoni and Belaj 2009). Although olive cultivation has been traditionally concentrated in the Mediterranean area, flavor and healthy properties of olive oil have provoked its expansion to other countries such as Argentina, Chile, Mexico, USA, Japan, China, New Zealand, Australia and South Africa (Baldoni and Belaj 2009; Rugini and De Pace 2016). Nowadays, the olive is

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_2

the second most important oil tree crop worldwide after oil palm (Baldoni and Belaj 2009) and its growing area reaches 10,272,547 ha, on five continents (FAOSTAT 2014).

Increased economic importance of olive, its recent expansion outside its traditional area of cultivation and requirements of new cultural practices have greatly increased the demand for improved cultivars (Fabbri et al. 2009). According to Fabbri et al. (2009), the main objectives of olive breeding programs include early bearing, resistance to abiotic stresses and pests, limited alternate bearing, suitability to intensive culture and to mechanical harvesting and high-quality productions, in terms of both organoleptic characteristics of fruits and oils, and high contents in substances useful for human health. However, although olive genetic improvement has been repeatedly addressed using classical methodologies, few new cultivars have been obtained compared to other fruit species (Brooks and Olmo 1997). Limited results of conventional breeding in olive can be due to some characteristics of this crop species such as long juvenile period (Acebedo et al. 1997), high level of heterozygosity (Rugini and Gutiérrez-Pesce 2006), male-sterility (Rugini and De Pace 2016), low fruit set and low seed germination (Acebedo et al. 1997).

Biotechnological tools such as genetic transformation, somaclonal variation, in vitro mutagenesis, in vitro selection, protoplast manipulation or somatic hybridization, can be used to increase the efficiency of olive breeding. As in other tree species, somatic embryogenesis (SE) is the regeneration method mostly used in olive (Rugini and Gutiérrez-Pesce 2006; Baldoni and Belaj 2009) and, therefore, it is at the base of biotechnology application. This in vitro technique can also be used for large-scale clonal propagation, application of synthetic seed technologies and germplasm cryopreservation (Pinto et al. 2016). Hence, it can be used as complement of both conventional and unconventional breeding programs (Rugini and De Pace 2016).

Somatic embryogenesis in olive was first reported by Rugini (1988) using immature zygotic embryos as initial explant. Since then, embryogenic cultures have been repeatedly induced utilising different tissues of juvenile origin. However, using this type of explants has the inconvenience of its unproven genetic value (Guan et al. 2016), which limits its utility in breeding programs. As in other woody plant species (Guan et al. 2016), SE initiation from tissues of adult origin remains being a difficult task and, at present, it has only been reported in few cases (Rugini and Caricato 1995; Capelo et al. 2010; Mazri et al. 2013; Toufik et al. 2014). Low rate of embryo germination has also been repeatedly reported as an important drawback limiting application of SE in olive (Benelli et al. 2001; Rugini and Silvestri 2016). Poor germination is mainly due to deficiencies in the embryo development phase, during which embryos with morphological abnormalities or deficient storage products accumulation can be produced. Nevertheless, important improvements in embryo development and maturation have been achieved in the last years (Benzekri and Sánchez-Romero 2012) and acceptable plant productions have been nowadays reported (Bradaï et al. 2016a). Moreover, behavior of regenerated plants during micropropagation, rooting and acclimatization were not significantly different from that of seedlings, thus revealing the high quality of the somatic plants (Bradaï et al. 2016a).

The objective of the present chapter is to describe the procedures currently used in the different phases of olive SE. Due to different requirements for inducing SE from juvenile and mature tissues, protocols for both types of initial explants are detailed.

2.2 Protocol of Somatic Embryogenesis

2.2.1 Culture Media

Culture media used are normally based on modifications of the OM medium (Rugini 1984) carried out by Cañas and Benbadis (1988) and on the MS formulation (Murashige and Skoog 1962). Supplements such as growth regulators, gelling agents and other additives are specifically indicated for each protocol stage (Tables 2.1 and 2.2).

Adjust the pH of the medium to pH 5.7–5.8 with 1 N NaOH or HCl before adding the gelling agent. Sterilize media by autoclaving for 20 min at 121 °C and 0.1 MPa and dispense into 150 × 25 mm test tubes, 90 × 15 mm or 90 × 25 mm Petri dishes or 80 × 85 mm jars, depending on the culture phase.

2.2.2 Induction of Somatic Embryogenesis

Initiation of embryogenic cultures is one of the most limiting steps in the SE systems and the choice of the explant can be of paramount importance for successful induction (Lo Schiavo 1995). In olive, SE have been most times initiated from juvenile tissue explants.

2.2.2.1 Induction from Explants of Juvenile Origin

As in other woody plants (San José et al. 2016), zygotic embryos are the most suitable explants for successful SE initiation. Immature embryos are usually cultured entire and their developmental stage appears to play a key role determining initiation success. According to Rugini (1988), only embryos collected 75 days after full bloom gave SE, independently of the genotype. However, in Tunisian cultivars, Maalej et al. (2006) obtained the highest SE frequency using immature embryos collected 100 days after full bloom. In mature zygotic embryos, radicle and cotyledonary segments are separately cultured (Orinos and Mitrakos 1991; Mitrakos et al. 1992; Trabelsi et al. 2003; Pérez-Barranco et al. 2007; Mazri et al. 2011).

Table 2.1 Components of culture media utilized for somatic embryogenesis induction from juvenile and adult tissues

	Juvenile origin		Adult origin		
	Induction	Expression	Induction	Proliferation	Expression
Macroelements (mM)					
NH_4NO_3	9.00	9.00	10.31	10.31	1.29
KNO_3	9.40	9.40	9.40	9.40	2.72
$CaCl_2{\cdot}2H_2O$	1.13	1.13	1.50	1.50	0.75
$MgSO_4{\cdot}7H_2O$	0.75	0.75	0.75	0.75	1.52
KH_2PO_4	0.50	0.50	0.63	0.63	0.63
$Ca(NO_3)_2{\cdot}4H_2O$	–	–	–	–	0.64
KCl	–	–	–	–	1.68
Microelements (μM)					
H_3BO_3	200.55	200.55	50.14	50.14	25.07
$MnSO_4{\cdot}4H_2O$	100.00	100.00	50.00	50.00	25.00
$ZnSO_4{\cdot}7H_2O$	49.75	49.75	14.96	14.96	7.48
$Na_2MoO_4{\cdot}2H_2O$	1.03	1.03	0.52	0.52	0.26
$CuSO_4{\cdot}5H_2O$	1.00	1.00	0.05	0.05	0.025
$CoCl_2{\cdot}6H_2O$	0.11	0.11	0.06	0.06	0.028
KI	5.00	5.00	2.50	2.50	1.25
$FeSO_4{\cdot}7H_2O$	100	100	50.00	50.00	25.00
Na_2 EDTA	100	100	50.00	50.00	25.00
Vitamins (μM)					
Tiamine.HCl	1.48	1.48	0.74	0.74	0.74
Pyridoxine.HCl	2.43	2.43	1.22	1.22	1.22
Nicotinic acid	40.62	40.62	20.31	20.31	20.31
Glycine	26.64	26.64	13.32	13.32	13.32
Folic acid	1.13	1.13	–	–	–
D-Biotine	0.20	0.20	–	–	–
myo-inositol	554.94	554.94	277.53	277.53	277.53
Growth regulators (μM)					
IBA	25.00	0–2.50	–	–	0.25
NAA	–	–	0.50	–	–
2iP	2.50	–	–	–	0.50
BAP	–	–	–	–	0.44
TDZ			30	–	–
Other additives (mg l^{-1})					
Glutamine	–	–	–	–	–
Caseine hydrolysate	1000	1000	–	–	–
Cefotaxime	–	–	–	–	–
Sugars (g l^{-1})					
Sucrose	20	20	30	30	30
Gelling agents (g l^{-1})					
Phytagel	–	–	–	–	–
Agar	6	6	–	6	6

Table 2.2 Components of culture media utilized in the maintenance, development and germination stages

Components	Maintenance	Development	Germination
Macroelements (mM)			
NH_4NO_3	1.29	1.29	6.87
KNO_3	2.72	2.72	6.26
$CaCl_2{\cdot}2H_2O$	0.75	0.75	1.00
$MgSO_4{\cdot}7H_2O$	1.52	1.52	0.50
KH_2PO_4	0.63	0.63	0.42
$Ca(NO_3)_2{\cdot}4H_2O$	0.64	0.64	–
KCl	1.68	1.68	–
Microelements (μM)			
H_3BO_3	25.07	25.07	100.27
$MnSO_4{\cdot}4H_2O$	25.00	25.00	100.00
$ZnSO_4{\cdot}7H_2O$	7.48	7.48	29.91
$Na_2MoO_4{\cdot}2H_2O$	0.26	0.26	1.03
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.10
$CoCl_2{\cdot}6H_2O$	0.028	0.028	0.11
KI	1.25	1.25	5.00
$FeSO_4{\cdot}7H_2O$	25.00	25.00	100.00
Na_2 EDTA	25.00	25.00	100.00
Vitamins (μM)			
Tiamine.HCl	0.74	0.74	0.30
Pyridoxine.HCl	1.22	1.22	2.43
Nicotinic acid	20.31	20.31	4.06
Glycine	13.32	13.32	26.64
Folic acid	0.57	0.57	–
D-Biotine	0.10	0.10	–
myo-inositol	277.53	277.53	554.94
Growth regulators (μM)			
IBA	0.25	–	–
NAA	–	–	–
2iP	0.50	–	–
BAP	0.44	–	–
TDZ	–	–	–
Other additives (mg l^{-1})			
Glutamine	550	550	–
Caseine hydrolysate	1000	1000	–
Cefotaxime[a]	200	–	–
Sugars (g l^{-1})			
Sucrose	20	20	10
Gelling agents (g l^{-1})			
Phytagel	3	3	–
Agar	–	–	6

[a]Filter-sterilized and added to the cooled sterilized medium

Non-embryogenic tissues, such as roots of germinated embryos (Shibli et al. 2001), have also given rise to embryogenic cultures, although they have been less frequently used.

For induction of SE from zygotic embryos, the protocol of Orinos and Mitrakos (1991), using radicle segments from mature zygotic embryos as initial explant, is suggested.

Explant Preparation

Collect mature fruits and eliminate the exocarp and the mesocarp. Store seeds at 4 °C in darkness. Just before use, break the endocarps and remove the seeds. Sterilize seeds with 70% (v/v) ethanol for 1 min followed by immersion for 20 min in a 0.5% (v/v) sodium hypochlorite solution containing 20 drops l^{-1} of Tween 20. Rinse the seeds three times with sterile distilled water for 5 min each. Keep seeds imbibing in sterile distilled water and darkness for two days and sterilize again with sodium hypochlorite, as previously indicated. Under aseptic conditions, remove the seed coat, isolate the zygotic embryo and excise the radicle (Fig. 2.1a).

Induction Conditions

Place the radicle segments in 90 × 15 mm Petri dishes containing 25 ml of OMc medium (Cañas and Benbadis 1988) supplemented with 25 μM indole-3-butyric acid (IBA), 2.50 μM 6-(γ,γ-dimethylallylamino)purine (2iP) and 6 g l^{-1} agar (Table 2.1). Incubate cultures in a growth chamber under low light intensity at 25 ± 1 °C for three weeks. During this time, radicle fragments increase in size and turn green and development of some friable calli can be observed (Fig. 2.1b).

Expression Conditions

After the induction period, transfer explants to OMc medium without growth regulators or with a low IBA concentration (2.5 μM) (Table 2.1). Incubate cultures under the environmental conditions indicated for induction. Maintain the explants in expression medium, with monthly transference to fresh medium, until proembryogenic masses and/or somatic embryos develop from calli obtained during the induction phase. Isolate embryogenic structures and transfer them to maintenance medium.

2.2.2.2 Induction from Explants of Adult Origin

In cultivated olive (*Olea europaea* var. *europaea*), induction of SE from mature tissues has only been reported by Rugini and Caricato (1995), Mazri et al. (2013)

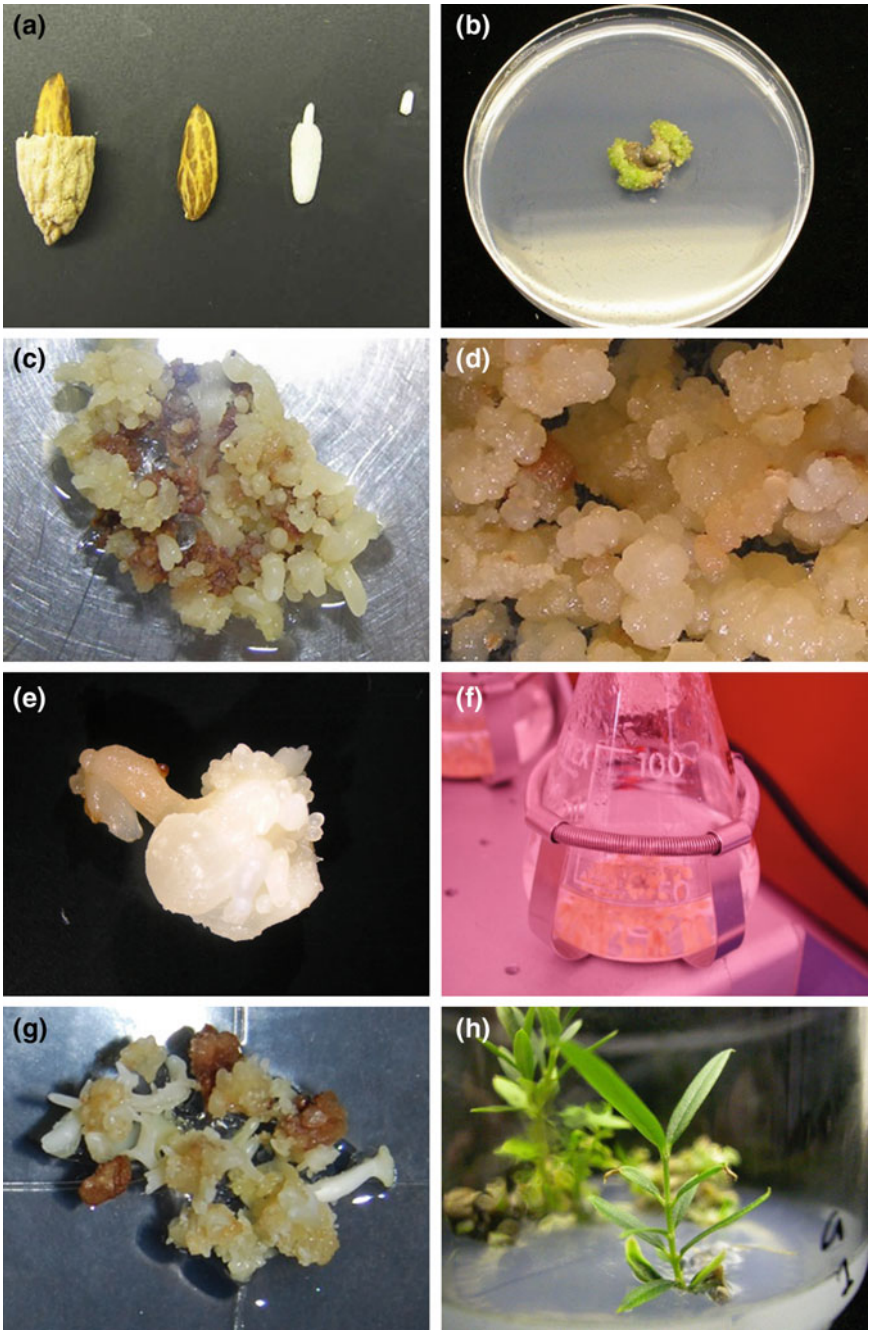

Fig. 2.1 Somatic embryogenesis in olive. **a** Preparation of radicle segments from mature zygotic embryos. **b** Initial step of embryogenic callus induction. **c** Maintenance of embryogenic cultures on solid ECO medium. **d** Proliferation of proembryogenic masses. **e** Development of somatic embryos via secondary embryogenesis. **f** Suspension culture. **g** Development of somatic embryos on basal ECO medium. **h** Shoot development from germinated somatic embryos

and Toufik et al. (2014). Rugini and Caricato (1995) established a cyclic system of SE using as initial explant leaf petioles excised from shoots regenerated via adventitious organogenesis from petioles of plants grown in vitro for 3 and 5 years. Adventitious organogenesis, which normally provokes rejuvenation of regenerants, was considered essential for SE induction (Rugini and Silvestri 2016). However, Mazri et al. (2013) reported SE from mature tissues without any intermediate regeneration step. These authors induced embryogenic cultures from leaf fragments excised from plantlets in vitro rejuvenated by axillary shoot multiplication.

An important inconvenient of protocols for SE induction from mature tissues is their poor reproducibility, probably due to a strong genotype effect (Rugini and Silvestri 2016).

Protocol reported for SE induction from mature tissues refer to leaf sections of in vitro grown plants (Mazri et al. 2013).

Explant Preparation

Excise semi-hardwood cuttings from a plant derived from a self-rooted cutting. Sterilize cuttings by immersion in 70% (v/v) ethanol for 1 min, Mercryl for 1 min and 2% (w/v) calcium hypochlorite for 3 min, followed by three washes of 10 min each with sterile distilled water. Divide semi-hardwood cuttings in micro-cuttings containing a single node. Culture on OM medium supplemented with 30 g l^{-1} sucrose, 13.6 μM zeatin and 6 g l^{-1} agar (Sghir et al. 2005) to establish shoot axillary cultures. Subculture shoots in the same medium at 60-day intervals for prolonged time periods to assure rejuvenation of plant material.

Induction Conditions

Dissect 5 × 5 mm sections from lower half of leaves and culture in liquid induction medium, consisting of MS mineral formulation at half strength (MS/2), modified MS vitamins (277.53 μM myo-inositol, 0.74 μM thiamine-HCl, 13.32 μM glycine, 1.22 μM pyridoxine-HCl and 20.31 μM nicotinic acid), 30 g l^{-1} sucrose, 30 μM thidiazuron (TDZ) and 0.5 μM 1-naphthaleneacetic acid (NAA) (Table 2.1). Maintain cultures in agitation (60 rpm) and darkness at 25 ± 1 °C for 4 days. After this period, transfer explants to proliferation medium, consisting of hormone-free MS/2 medium with 30 g l^{-1} sucrose and 6 g l^{-1} agar (Table 2.1). Incubate in darkness at 25 ± 1 °C for 8 weeks. Calli formation can be observed during culture in proliferation medium.

TDZ appears to be essential for successful induction from mature tissues as substitution of this growth regulator by other adenine-derivative cytokinins, such as 2iP, 6-benzylaminopurine (BAP) or zeatin, did not give rise to positive results (Mazri et al. 2013). Rugini and Silvestri (2016) also consider this hormone essential for inducing SE in this species.

Expression Conditions

Transfer explants to expression medium containing the mineral formulation of ECO medium (Pérez-Barranco et al. 2007, 2009), modified MS vitamins as previously indicated, 0.25 μM IBA, 0.44 μM BAP, 0.5 μM 2iP, 30 g l^{-1} sucrose and 6 g l^{-1} agar (Table 2.1). Maintain cultures in darkness at 25 ± 1 °C for 4 weeks. Somatic embryos develop individually on necrotic calli and nodular structures several weeks after transference to expression conditions.

2.2.3 Culture Maintenance and Proliferation

Different culture media have been used for proliferation of olive embryogenic cultures. Nevertheless, in the last years, ECO medium is the most frequently reported (Pérez-Barranco et al. 2007, 2009; Cerezo et al. 2011; Benzekri and Sánchez-Romero 2012; Bradaï et al. 2016a, b). ECO medium is based in the OMe formulation (Cañas and Benbadis 1988) and consists of ¼ OM macroelements, ¼ MS microelements, ½ OM vitamins, 277.53 μM myo-inositol, 20 g l^{-1} sucrose, 550 mg l^{-1} glutamine and the supplements proposed by Rugini and Caricato (1995), i.e., 0.25 μM IBA, 0.44 μM BAP, 0.5 μM 2iP, 200 mg l^{-1} cefotaxime, 1 g l^{-1} caseine hydrolysate and 3 g l^{-1} phytagel (Table 2.2).

As indicated, cefotaxime is included in ECO medium. This antibiotic has also been used for induction of SE in olive (Rugini and Caricato 1995; Rugini and Silvestri 2016). Although its role is not clear, Rugini and Caricato (1995) proposed that the reduction of growth rate induced allows better cell differentiation, reducing formation of non-morphogenic callus and increasing the percentage of explants producing normal embryos.

For proliferation of olive embryogenic cultures, select embryogenic callus and somatic embryos 1–2 mm long showing no signs of necrosis or tissue degeneration. Transfer the explant to 25 × 150 mm test tubes containing 25 ml of ECO medium. Incubate cultures in darkness at 25 ± 1 °C with subculturing to fresh medium at 6-7-week intervals.

During maintenance of embryogenic cultures in ECO medium, embryogenic callus and somatic embryos can be observed, not being appreciable proliferation of non-embryogenic callus (Fig. 2.1c). Embryogenic callus is cream-coloured and of friable consistency. Somatic embryos are normally beige, translucent and develop isolated or forming small clusters. Olive embryogenic cultures proliferate through both, formation of new proembryogenic masses (Fig. 2.1d) and secondary embryogenesis (Fig. 2.1e), in a proportion clearly dependent on the genotype. Thus, culture appearance during the proliferation step can be very variable, depending on the embryogenic line (Sánchez-Romero et al. 2009).

Olive embryogenic cultures can be maintained in proliferation conditions for prolonged time periods. Although loss of embryogenic capacity has been described after three weeks (Leva et al. 1995), Bradaï et al. (2016a) reported maintenance of

embryogenic capacity in cultures maintained for eight years. Nevertheless, as duration of the proliferation phase increased, the proliferation pattern changed and the regeneration potential decreased.

2.2.4 Suspension Culture

Suspension cultures have important biotechnological applications. However, in olive, embryogenic cultures in liquid medium have normally been used for few weeks, as a previous treatment before inducing embryo maturation. Growth at these conditions followed by filtering through meshes of different pore size allows obtaining of an uniform material to initiate the maturation phase (Pérez-Barranco et al. 2007; Cerezo et al. 2011).

For establishment of olive embryogenic cultures in liquid medium, inoculate 400 mg of friable callus and somatic embryos at early embryogenic stages into 40–50 ml liquid ECO medium in a 100 ml Erlenmeyer flask. Incubate suspension cultures on a rotary shaker (100–120 rpm) at 25 ± 1 °C under low light intensity or darkness (Fig. 2.1f).

2.2.5 Somatic Embryo Development and Maturation

Select embryogenic callus and somatic embryos at early developmental stages from maintenance cultures. Transfer 100 mg to 90 × 25 mm Petri dishes containing 50 ml basal ECO medium, i.e., without growth regulators and cefotaxime (Benzekri and Sánchez-Romero 2012) (Table 2.2). Incubate cultures in darkness at 25 ± 1 °C during 8 weeks. Somatic embryos at different developmental stages are obtained at the end of this period (Fig. 2.1g). Although most of them remain translucent, this circumstance appears not to limit their subsequent conversion into plants.

2.2.6 Somatic Embryo Germination

For germination, isolate embryos equal or larger than 3 mm developed on basal ECO medium and transfer them to 85 × 80 mm jars containing 50 ml of germination medium (Clavero-Ramírez and Pliego-Alfaro 1990). Germination medium consisted of MS medium with 1/3 macroelements, 10 g l^{-1} sucrose and 6 g l^{-1} agar (Table 2.2). Incubate cultures at 25 ± 1 °C under a 16 h light photoperiod and 40 µmol m^{-2} s^{-1} irradiance level. During incubation in germination conditions somatic embryos give rise to shoots and/or roots at acceptable rates (Fig. 2.1h). More than one shoot can be obtained per germinated embryo, especially in embryos

derived from embryogenic lines maintained for prolonged time periods (Bradaï et al. 2016a).

2.2.7 Plant Establishment

Shoots obtained from somatic embryo germination can be multiplied and rooted following the protocol of Revilla et al. (1996).

For shoot multiplication, excise shoots from germinated embryos and cut nodal explants 1.0–1.4 cm long, with two lateral buds. Culture cuttings in 95 × 60 mm jars containing 40 ml of DKW medium (Driver and Kuniyuki 1984) supplemented with 4.4 μM BAP, 0.05 μM IBA and 6 g l^{-1} agar. Maintain cultures at 25 ± 1 °C under light conditions with transference to fresh medium at 6-week intervals (Fig. 2.2a).

For inducing root development, dissect apical shoots minimum 1.5 cm long from axillary shoot cultures. Culture them in test tubes containing 25 ml of half-strength DKW medium lacking vitamins and amino acids, but supplemented

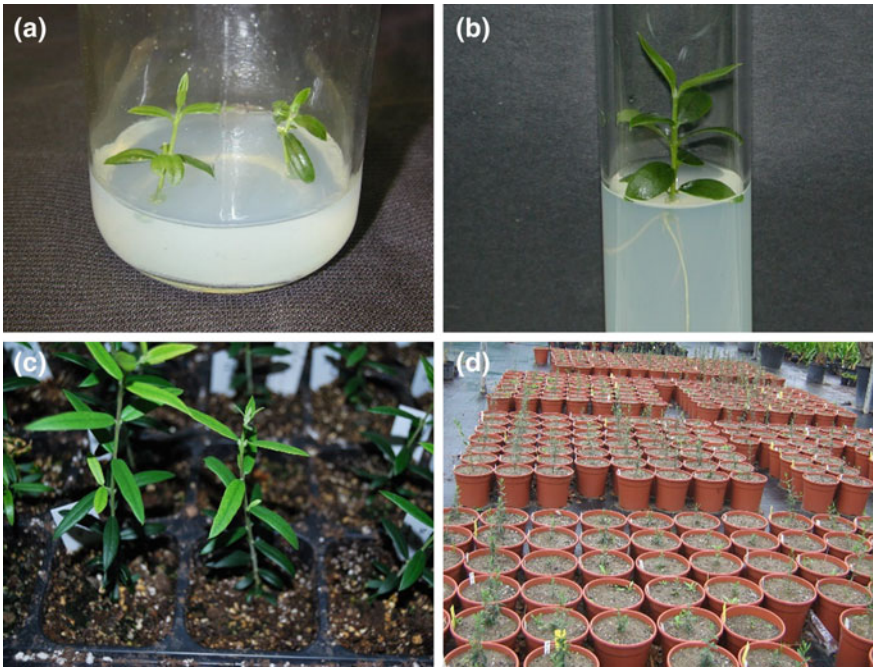

Fig. 2.2 In vivo establishment of olive plants regenerated via somatic embryogenesis. **a** In vitro multiplication of shoots obtained from somatic embryo germination. **b** In vitro shoot rooting. **c** Acclimatization of somatic plants. **d** Somatic embryo-derived plants after transplanting to pots

with 0.5 μM IBA and 6 g l^{-1} agar. Maintain cultures in darkness for 1 week and subsequently under light conditions for 8 weeks (Fig. 2.2b).

2.2.8 *Plant Acclimatization and Field Transfer*

Take out rooted plantlets from culture tubes and rinse under tap water to remove rests of culture medium. Transfer plantlets to 5.5 × 5.5 × 10 cm polyethylene seedling trays containing a mixture of peat and perlite (1:1). Place trays into a beaker containing water and cover with transparent plastic film to maintain high relative humidity conditions. Place plants into a growth chamber under a 16 h light photoperiod (65 μmol m^{-2} s^{-1}), 25 °C and 60% relative humidity. One week after planting, initiate progressive reduction of air humidity by gradually removing the plastic film over 4 weeks (Fig. 2.2c). Maintain plants under growth chamber conditions for additional 4 weeks to calculate the percentage of acclimatized plants. Four months after acclimatization, transfer plants to field (Fig. 2.2d).

2.3 Research Prospects

Although important advances have been achieved in the last years in olive SE, different steps of this process still limit the real applicability of this technique in both, conventional and unconventional breeding programs. SE induction from explants derived from adult trees can be nowadays considered the main drawback. Although obtaining of embryogenic cultures from mature tissues has been reported in *Olea europaea* (Rugini and Caricato 1995; Capelo et al. 2010; Mazri et al. 2013; Toufik et al. 2014), available protocols are hardly reproducible in different genotypes.

Another important aspect to take into account is genetic stability of the regenerated plants. In olive, investigations on somaclonal variation in somatic embryo derived plants are scarce. Phenotypic variation was never observed in field-trials with plants obtained from cv. 'Canino' and an only case showing a different vegetative behavior was reported in plants derived from cotyledons of cv. 'Frangivento' (Rugini and Silvestri 2016). However, in plants regenerated from zygotic embryos of cv. 'Picual', Bradaï et al. (2016b) reported fourteen variant phenotypes, affecting plant vegetative and reproductive development, and intraclonal variation in quantitative traits related to plant architecture. Although somaclonal variation was strongly influenced by the genotype, higher variation was in general observed in plants regenerated from aged cultures (Bradaï et al. 2016b), thus revealing the necessity of utilizing cryopreservation methods to avoid undesirable variation. Nevertheless, capacity of embryogenic cultures of producing variants plants can constitute a source of variation source for breeding programs. Moreover, in vitro selection can be used to select desirable somaclones, particularly when cellular and whole plant responses are correlated (Brar and Jain 1998).

Economic, environmental and cultural importance of olive justify further investigation on SE in order to solve its main limitations and allow the practical application of this technique.

References

Acebedo MM, Lavee S, Liñan J, Troncoso A (1997) In vitro germination of embryos for speeding up seedling development in olive breeding programmes. Sci Hortic 69:207–215

Baldoni L, Belaj A (2009) Olive. In: Vollmann J, Rajcan I (eds) Oil crops. Springer, New York, pp 397–421

Benelli C, Fabri A, Grassi S, Lambardi M, Rugini E (2001) Histology of somatic embryogenesis in mature tissue of olive (*Olea europaea* L.). J Hortic Sci Biotechnol 76:112–119

Benzekri H, Sánchez-Romero C (2012) Maturation of olive somatic embryos. Acta Hortic 961:441–447

Bradaï F, Pliego-Alfaro F, Sánchez-Romero C (2016a) Long-term somatic embryogenesis in olive (*Olea europaea* L.): influence on regeneration capability and quality of regenerated plants. Sci Hortic 199:23–31

Bradaï F, Pliego-Alfaro F, Sánchez-Romero C (2016b) Somaclonal variation in olive (*Olea europaea* L.) plants regenerated via somatic embryogenesis: influence of genotype and culture age on phenotypic stability. Sci Hortic 213:208–215

Brar DS, Jain SM (1998) Somaclonal variation: mechanism and application in crop improvement. In: Jain SM, Brar DS, Ahloowalia BS (eds) Somaclonal variation and induced mutations in crop improvement. Kluwer Academic Publishers, Dordrecht, pp 15–37

Brooks RM, Olmo HP (1997) The Brooks and Olmo register of fruits and nut varieties. ASHS Press, Alexandria, VA

Cañas LA, Benbadis A (1988) In vitro plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L.). Plant Sci 54:65–74

Capelo AM, Silva S, Brito G, Santos D (2010) Somatic embryogenesis induction in leaves and petioles of a mature wild olive. Plant Cell Tiss Org Cult 103:237–242

Cerezo S, Mercado JA, Pliego-Alfaro F (2011) An efficient regeneration system via somatic embryogenesis in olive. Plant Cell Tiss Org Cult 106:337–344

Clavero-Ramírez I, Pliego-Alfaro F (1990) Germinación in vitro de embriones maduros de olivo (*Olea europaea*). Actas de Horticultura 1:512–516

Driver JA, Kuniyuki AH (1984) In vitro propagation of paradox walnut rootstock. Hortic Sci 19:507–509

Fabbri A, Lambardi M, Ozden-Tokatli Y (2009) Olive breeding. In: Jain SM, Priyadarshan PM (eds) Breeding plantation tree crops: tropical species. Springer, New York, pp 423–465

FAOSTAT (2014) Online database available at link. http://fao.org/faostat

Guan Y, Li SG, Fan XF, Su ZH (2016) Application of somatic embryogenesis in woody plants. Front Plant Sci 7:908

Leva A, Muleo R, Petrucelli R (1995) Long-term somatic embryogenesis from immature olive cotyledons. J Hortic Sci 70:417–421

Lo Schiavo F (1995) Early events in embryogenesis. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry. Somatic embryogenesis and synthetic seeds. Springer, Berlin, vol 30, pp 20–29

Maalej M, Chaari-Rkhiss A, Drira N (2006) Contribution to the improvement of olive somatic embryogenesis by mineral and organic analysis of zygotic embryos. Euphytica 151:31–37

Mazri MA, Elbakkali A, Belkoura M, Belkoura I (2011) Embryogenic competence of calli and embryos regeneration from various explants of Dahbia cv, a Moroccan olive tree (*Olea europaea* L.). Afr J Biotechnol 10:19089–19095

Mazri MA, Belkoura I, Pliego-Alfaro F, Belkoura M (2013) Somatic embryogenesis from leaf and petiole explants of the Moroccan olive cultivar Dahbia. Sci Hortic 159:88–95

Mitrakos K, Alexaki A, Papadimitrou P (1992) Dependence of olive morphogenesis on callus origin and age. J Plant Physiol 139:269–273

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473–497

Orinos T, Mitrakos K (1991) Rhizogenesis and somatic embryogenesis in calli from wild olive [*Olea europaea* var. *sylvestris* (Miller) Lehr] mature zygotic embryos. Plant Cell Tiss Org Cult 27:183–187

Pérez-Barranco G, Mercado JA, Pliego-Alfaro F, Sánchez-Romero C (2007) Genetic transformation of olive somatic embryos through biolistic. Acta Hortic 738:473–477

Pérez-Barranco G, Torreblanca R, Padilla IMG, Sánchez-Romero C, Pliego-Alfaro F, Mercado JA (2009) Studies on genetic transformation of olive (*Olea europaea* L.) somatic embryos: I. Evaluation of different aminoglycoside antibiotics for *nptII* selection; II. Transient transformation via particle bombardment. Plant Cell Tiss Org Cult 97:243–251

Pinto G, Correia S, Corredoira E, Ballester A, Correia B, Neves L, Canhoto J (2016) In vitro culture of Eucalyptus: where do we stand?. In: Park YS, Bonga JM, Moon HK (eds) Vegetative propagation of trees. National Institute of Forest Sciences, Korea, pp 441–462

Rallo L, El Riachy M, Rallo P (2011) The time and place for fruit quality in olive breeding. In: Jenks MA, Bebeli PJ (eds) Breeding for fruit quality. Wiley-Blackwell, USA, pp 323–465

Rapoport HF, Fabbri A, Sebastiani L (2016) Olive biology. In: Rugini E, Baldoni L, Muleo R, Sebastiani L (eds) The olive tree genome. Springer, Cham, pp 13–25

Revilla MA, Pacheco J, Casares A, Rodríguez R (1996) In vitro reinvigoration of mature olive trees (*Olea europaea* L.) through micrografting. In Vitro Cell Dev Biol Plant 32: 257–261

Rugini E (1984) In vitro propagation of some olive (*Olea europaea* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Sci Hortic 24:123–134

Rugini E (1988) Somatic embryogenesis and plant regeneration in olive (*Olea europaea* L.). Plant Cell Tiss Org Cult 14:207–214

Rugini E, Caricato G (1995) Somatic embryogenesis and plant recovery from mature tissues of olive cultivars (*Olea europaea* L.) 'Canino' and 'Moraiolo'. Plant Cell Rep 14:257–260

Rugini E, Gutiérrez-Pesce P (2006) Genetic improvement of olive. Pomologia Croatica 12:43–74

Rugini E, De Pace C (2016) Olive breeding with classical and modern approaches. In: Rugini E, Baldoni L, Muleo R, Sebastiani L (eds) The olive tree genome. Springer, Cham, pp 163–192

Rugini E, Silvestri C (2016) Somatic embryogenesis in olive (*Olea europaea* L. subsp. *europaea* var. *sativa* and var. *sylvestris*). In: Germanà MA, Lambardi M (eds) In vitro embryogenesis in higher plants. Methods in molecular biology. Springer, New York, vol 1359, pp 341–349

San José MC, Couselo JL, Martínez MT, Mansilla P, Corredoira E (2016) Somatic embryogenesis in *Camellia japonica* L.: challenges and future prospects. In: Mujib A (ed) Somatic embryogenesis in ornamentals and its applications. Springer, New Delhi, pp 91–105

Sánchez-Romero C, Swennen R, Panis B (2009) Cryopreservation of olive embryogenic cultures. CryoLetters 30:359–372

Sghir S, Chatelet P, Ouazzani N, Dosba F, Belkoura I (2005) Micropropagation of eight Moroccan and French olive cultivars. HortScience 40:193–196

Shibli RA, Shatnawi M, Abu-Ein, Al-Juboory KH (2001) Somatic embryogenesis and plant recovery from callus of 'Nabali' olive (*Olea europaea* L.). Sci Hortic 88:243–256

Toufik I, Guenoun F, Belkoura I (2014) Embryogenesis expression from somatic embryos of olive (*Olea europaea* L.) cv. Picual. Moroccan J Biol 11:17–25

Trabelsi EB, Bouzid S, Bouzid M, Elloumi N, Belfeleh Z, Benabdakkah A, Ghezel R (2003) In-vitro regeneration of olive tree by somatic embryogenesis. J Plant Biol 46:173–180

Chapter 3
Cafe Arabica *Coffea arabica* L.

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3.1 Introduction

Arabica coffee (*Coffea arabica* L.) is a species of the Rubiaceae family that is native to Africa, autogamous and tetraploid with 2n = 44. Although world coffee production is based on two species, *C. arabica* represents 70% and the remaining 30% is *Coffea canephora* (Bertrand et al. 2005). *C. arabica* was introduced in Latin America in the 18th century, and it is there where 80% of the world's export coffee volume is currently produced, as the main source of income in many countries (Bertrand et al. 2011). It is cultivated in equatorial regions from 1500 to 1900 masl, although it is also found at lower elevations near 500 masl (http://ecocrop.fao.org/ecocrop). According to the International Coffee Organization (http://www.ico.org/), world coffee production has grown from 131.6 million 60-kg sacks in the period 1998–2000 to 151.6 million expected for the period 2016–2017; of this, 62.8% corresponds to Arabica coffee, produced mostly in Latin America where the main coffee producers are Brazil, Colombia, Honduras, Peru, Guatemala, Mexico, Nicaragua and Costa Rica.

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_3

The traditional varieties of *C. arabica* in Latin America are mainly propagated by seed and vegetative horticultural propagation has rarely been used. These varieties typically have a very narrow genetic base, since they are derived from genealogical selections based on very few individuals (Bertrand et al. 2005). With the aim of increasing the genetic variability of this species in Central America, the Regional Genetic Improvement Program (PROMECAFE) was begun in 1991 with the technical participation of CATIE, CIRAD and the coffee institutes of the region (Etienne et al. 1999; Bertrand et al. 2005). This program consisted of making controlled crosses of cultivated varieties with recognized cup quality with wild individuals conserved in the CATIE germplasm collection as a source of genetic variability (PROMECAFE 2003; Bertrand et al. 2005). As a result of this work, F1 hybrids were obtained that were more vigorous than the traditional varieties, with optimum agronomic traits as well as disease resistance and good cup quality (Etienne et al. 2002). However, although *C. arabica* is autogamous, it shows high heterosis (Srinivasan and Vishveshvara 1978), so these hybrids have been propagated vegetatively by somatic embryogenesis since their creation (Berthouly and Michaux-Ferriére 1996; Etienne and Bertrand 2001).

The first investigations on somatic embryogenesis of coffee have been known for about 40 years from the work of Staritsky (1970), Sharp et al. (1973), Sondahl and Sharp (1977), Dublin (1984), and Yasuda et al. (1985), who published that coffee SE is obtained from the culture of leaf fragments using two well-defined strategies:—direct or low frequency SE—which in a single phase produces somatic embryos quickly but in limited quantities; and indirect or high frequency SE—with two phases, the induction of primary callogenesis and the regeneration of embryogenic calluses that culminates in the formation of thousands of somatic embryos (Berthouly and Michaux-Ferriére 1996). This route is the one most used for its high yields with different coffee species and varieties (Zamarripa et al. 1991; Van Boxtel and Berthouly 1996; Berthouly and Michaux-Ferriére 1996; Ducos et al. 2007).

In Central America, CATIE and CIRAD carried out research on *C. arabica* and its selected hybrids (Etienne and Bertrand 2001). The most notable technical innovation was the development of two original culture systems, one being the production of somatic embryos in simplified RITA® bioreactors (Berthouly et al. 1995; Etienne et al. 1997) and the other with direct planting in the greenhouse of embryos obtained in a bioreactor for their conversion into plants (Etienne-Barry et al. 1999; Barry-Etienne et al. 2002; Etienne et al. 2012, 2013). Nevertheless, these authors mention that the conversion into plants constitutes the main bottleneck in this technology. Evaluations carried out on 11 genotypes indicated that only 50% of the embryos regenerated plants, indicating a strong genotypic effect among the propagated hybrids. However, it was also shown that plants derived from embryogenic suspensions and secondary embryogenesis are genetically very stable (Bobadilla et al. 2013, 2015); and that the frequency of variant plants (0.1–0.2%) using this methodology is very low (Menéndez-Yuffá et al. 2010).

Because the somatic embryogenesis process is intensive and long lasting, the final cost per plant is very high compared to plants obtained from seed. Technical factors to be resolved, coupled with a lack of promotion and financing policies,

have limited the distribution of these hybrids in Central America for more than 10 years (Aguilar et al. 2017). In an effort to overcome this situation, CATIE implemented a two-phase multiplication strategy: 1—the regeneration of F1 hybrids by somatic embryogenesis (Aguilar et al. 2016, 2017); and 2—the multiplication of the F1 mother plants from mini-cuttings rooted in greenhouses (Mesén and Jiménez 2016).

In this second phase, leafy stem cuttings obtained from non-lignified juvenile shoots are used. The shoots are obtained from plants generated by somatic embryogenesis or from the first cuttings derived from these. These materials, which we call "mother plants", are established at high densities in hydroponic clonal gardens for the continuous and prolonged production of shoots from which cuttings are taken for the rooting process. At present, a rooting percentage higher than 90% is being achieved, with the production of rooted and acclimatized cuttings ready for the nursery stage in approximately 7–8 weeks from the time of the cutting (Mesén and Jiménez 2016).

The rooting of coffee mini-cuttings from somatic plantlets was also reported recently by Georget et al. (2017) as an efficient and more economical way to multiply hybrids. Juvenile shoots can also be generated by bending of branches of adult plants, as described by Duicela et al. (2006) for Robusta coffee.

In our case, the mass production of rooted cuttings and the transfer of plants to the producers will be carried out by the Costa Rica company Gaia Artisan Coffee S.A.

The protocol described is based on obtaining mother plants of F1 Arabica coffee hybrids using high frequency somatic embryogenesis (Van Boxtel and Berthouly 1996); germination and conversion in RITA® bioreactors (Berthouly et al. 1995; Etienne et al. 1997) with some variants (Aguilar et al. 2016, 2017); and the mass multiplication of mother plants using low-cost horticultural propagation in greenhouses (Mesén and Jiménez 2016).

3.2 Protocol for Somatic Embryogenesis in Arabica Coffee

3.2.1 Media and Culture Conditions

1. From a practical point of view, somatic embryogenesis (SE) of coffee is divided into 5 laboratory phases and the greenhouse acclimatization phase described below (Aguilar et al. 2017). Each laboratory phase has a specific culture medium as described in Table 3.1. The basal medium used in most of the process is from Murashige and Skoog (1962), except during the regeneration of embryos in semi-solid culture medium when Yasuda medium is used (Yasuda et al. 1985).
2. Each culture phase has specific requirements in culture medium components (Table 3.1) as well as different photoperiod and culture times (Table 3.2).

Table 3.1 Culture media components used in each phase of Arabica coffee somatic embryogenesis, including mineral salts, vitamins, growth regulators, organic additives and pH

Component	Culture media					
	T1	T2	T3	T4	T5	Yasuda
	%					
MS macroelements	50	50	50	50	100	25
MS microelements	50	50	50	50	100	50[a]
MS FE-EDTA	50	50	50	50	100	50
Morel vitamin					100	
	mg/L					
KH_2PO_4						42.5
Cysteine		40	10			
Thiamine	10	20	5	10		10
Glycine	1	20		2		
Nicotinic acid	1		0.5	1		1
Pyridoxine	1		0.5	1		1
Myo-inositol	100	200	100	200		100
Hydrolyzed casein	100	200	100	400		
Malt extract	400	800	200	400		
Adenine sulfate		60		40		
2,4-D	0.5	1	1			
IBA	1					
2 IP	2					
BAP		4		2	0.3	1
Kinetin			1			
	g/L					
Sucrose	30	30	15	40	40	30
Phytagel[b]	2.2	2.2		2.2	2.2	2.2
pH	5.6	5.6	5.6	5.6	5.6	5.6

[a]Yasuda microelements: 3.1 mg/L H_3BO_3, 11.2 mg/L $MnSO_4.7H_2O$, 4.3 mg/L $ZnSO_4.7H_2O$, 0.125 mg/L $Na_2MoO_4.2H_2O$, 0.05 mg/L $CuSO_4.5H_2O$
[b]Semi-solid culturemedia

3. Cultures placed under light are kept in a photoperiod with 12 h of darkness and 12 h of light supplied by LED lamps. The temperature in all culture rooms is 27 ± 2 °C.
4. Indirect lighting consists of maintaining the culture rooms with 50% of the LED lights switched on, alternating between the different culture racks: the cultures are placed on those racks that are kept with the lamps turned off.

Table 3.2 Phases of coffee somatic embryogenesis, culture conditions, duration and biological responses associated with each phase

Stage	Media and culture vessel				Duration (Months)	Remarks
	Solid	Liquid	Temporary immersion	Photoperiod		
1. Callus induction	Vials 10 ml			Dark	1	Explants form scar calluses
2. Embryogenic calluses proliferation	Vessels 20 ml			Indirect light	6–10	Explants turn black, visible embryogenic callus formation
3. Embryogenic callus multiplication		Erlenmeyer flasks		Light	6	Multiplication of cellular aggregates
4. Somatic embryos regeneration	Petri dishes	Erlenmeyer flasks	RITA®	Indirect light	2.5	White embryos in torpedo state
5. Somatic embryos germination and conversion	Vessels[a] 250 ml		RITA®[b] 1 L	Light	3–5	Embryos elongate, turn green, develop cotyledons and their first true leaves and roots
6. Acclimatization	Plastic trays			Light	4	Development of plantlets, numerous leaves and roots

[a]Somatic embryos germination and conversion on solid medium: 3 months
[b]Somatic embryos germination and conversion into RITA®: 5 months

3.2.2 *Explant Preparation*

1. The induction and proliferation of embryogenic calluses (EC) is performed with immature coffee leaves harvested from mother plants kept in greenhouse (Fig. 3.1a).
2. During disinfection the leaves are washed with running water and soap, then placed in 3% w/v sodium hypochlorite (50% v/v commercial bleach) for 30 min followed by three washes with sterile distilled water (Fig. 3.1b).
3. Under aseptic conditions, the leaves are sectioned into explants of about 1 cm (Fig. 3.1c), margins and the middle vein of the leaf are eliminated.
4. The explants are inoculated (Fig. 3.1d, e) with the adaxial surface in contact with the callus induction medium T1 (Table 3.1).

Fig. 3.1 Harvesting leaves and explant preparation. **a** Coffee mother plan in the greenhouse, **b** Disinfection of leaves inside the laminar flow chamber, **c** Elimination of the middle vein and leaf margins and isolation of the explants, **d**, **e** Inoculation of explants in the culture medium

3.2.3 Callus Induction and Embryogenic Calluses Proliferation

1. After 30 days in the dark on the T1 medium, explants with scar calluses (Fig. 3.2a) are transferred to embryo induction medium T2 (Table 3.1) to initiate the second stage under indirect light conditions; this medium shows significant variation in auxin and cytokinin concentrations, causing a hormonal shock that allows the evolution of a non-embryogenic primary callus into the embryogenic calluses (Etienne et al. 1999).
2. At this stage the explants turn brown and embryogenic, friable callus (EC) is clearly visible (Fig. 3.2b). This process varies among the different hybrids (Aguilar et al. 2017), with 'Centroamericano' as the most recalcitrant since its EC develops only in very small amounts after 8 to 10 months of culture, while in the other genotypes the occurrence of EC can occur in large quantities after 4 or 6 months of culture (Fig. 3.2c).
3. One of the limitations of this EC is that it loses its optimal condition in a short time, so cryopreservation becomes necessary to conserve calluses or ECS of good quality or difficult to obtain, to allow routine management of these cultures and reduce losses due to deterioration (Aguilar et al. 2017).

3.2.4 Embryogenic Callus Multiplication (ECS)

1. Embryogenic callus multiplication is done in a liquid culture medium with the establishment of embryogenic cell suspension (ECS) (Van Boxtel and Berthouly 1996), to make better use of EC mainly in the case of the most recalcitrant hybrids ('Centroamericano').
2. ECS are initiated by inoculating small amounts of EC into the six cavities of multi-well dishes (Fig. 3.2d) containing 7 ml of liquid callus proliferation medium T3 (Table 3.1), or 50 ml Erlenmeyer flasks are used with 12.5 ml of the same medium (Fig. 3.2e).
3. As cell volume increases, the culture is transferred to larger volume Erlenmeyer flasks to finally establish ECS in 250 ml containers with a cell volume of 1.5 ml PCV (Packed Cell Volume) in 50 ml of liquid proliferation medium (Fig. 3.2e).
4. Throughout the entire process, the culture medium is renewed every 15 days during the exponential growth phase; and at 30 days the finest aggregate are filtered using a sterile pipette and then transferred to a larger Erlenmeyer flask. The cultures are kept on rotary shakers at a speed of 100 rpm under indirect light. After 2 months, to establish new ECS cultures.

3.2.5 Somatic Embryos Regeneration

1. Several alternatives have been used to regenerate coffee embryogenic cultures. Etienne et al. (1999) mention that during large-scale micropropagation, the recommended method for initiating ECS regeneration uses liquid medium in 100–250-ml Erlenmeyer flasks according to Van Boxtel and Berthouly (1996).
2. With the development of the RITA® container (Alvard et al. 1993), the system was innovated with the direct transfer of 200 mg of ECS to the bioreactor with 200 ml of regeneration medium. The complete development of the embryo is achieved after 4 months of culture on development medium, with subcultures every two months and using an immersion frequency of two times per day for one minute. This allows regeneration, germination and conversion into plants in the same container, simplifying the process and avoiding the use of a gel medium (Berthouly et al. 1995; Etienne et al. 1999).
3. Using the same methodology at CATIE, we have observed high rates of hyperhydricity, asynchrony and malformations of the embryos mainly in the 'Centroamericano' hybrid. In order to reduce these drawbacks, we have alternatively implemented the regeneration phase in semi-solid medium (Aguilar et al. 2016, 2017).
4. Using forceps, small portions of EC are transferred to Yasuda (Yasuda et al. 1985) medium (Table 3.1), in Petri dishes (100 × 15 mm-diameter) containing 20 ml of semisolid medium and kept under indirect light. With many of the EC, hundreds of thousands of very good quality white embryos in torpedo state are regenerated after 2–2.5 months of culture (Fig. 3.2f). ECS are also regenerated on Yasuda or T4 semisolid medium (Table 3.1), placing 1 ml of cell mass on 90-mm diameter filter paper placed over Petri dishes (Fig. 3.2g).
5. Some hybrids such as 'Milenio' or 'Esperanza' produce large masses of EC in the embryo induction medium, so we consider the establishment of ECS unnecessary; this reduces a possible source of variability because of the reduction in time the culture spends in suspension (Aguilar et al. 2017).

3.2.6 Somatic Embryos Germination and Conversion

3.2.6.1 Temporary Immersion Culture (RITA®)

1. In our case, germination of coffee somatic embryos and their conversion into plants is normally carried out in RITA® bioreactors after regeneration in semi-solid medium according to the aforementioned procedure (Aguilar et al. 2016, 2017).

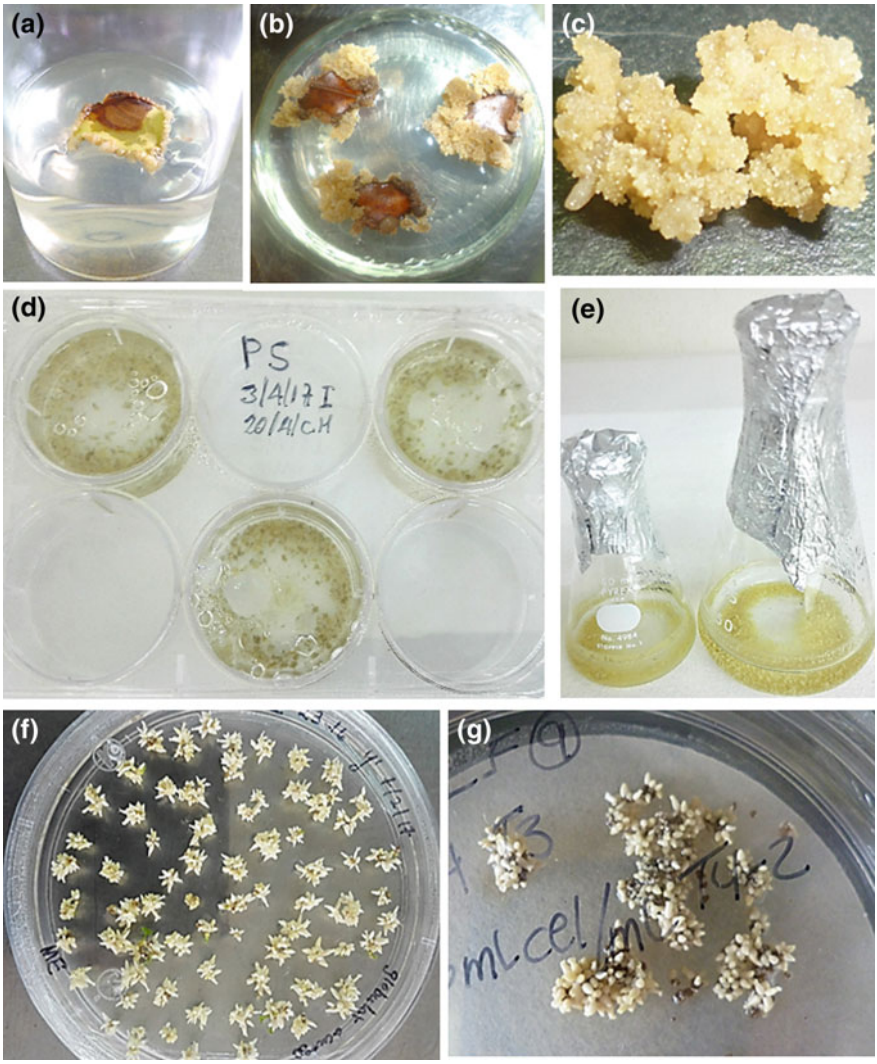

Fig. 3.2 Induction and multiplication of EC and regeneration of somatic embryos. **a** Explant after one month of culture in darkness in the T1 medium, showing callus cicatrization, **b** Brown color explants showing the EC, **c** Mass of friable EC, **d** Initiation of cellular suspensions in multi-cavity dishes with liquid T3 medium, **e** Establishment of ECSs in Erlenmeyer flasks, **f** Regeneration of EC on semi-solid Yasuda medium, **g** Regeneration of ECSs on filter paper in semi-solid T4 medium

2. A density of 1.25 g of regenerated white embryos per container is used with 200 ml of development medium T5 (Table 3.1) with two one-minute immersions per day.
3. Each culture usually has a duration of five months and during that period the T5 culture medium is renewed every month; the content of some containers can be divided according to the density of the culture.
4. In the first month of culture in a RITA® container, the embryos lengthen, they begin to develop cotyledons and turn green (Fig. 3.3a).

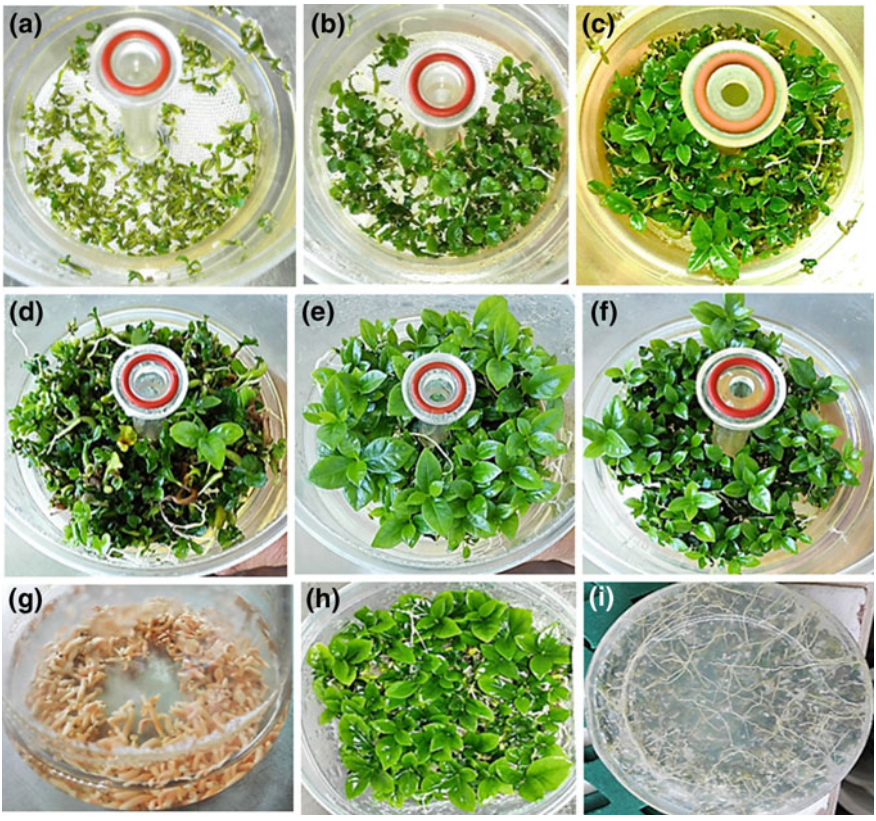

Fig. 3.3 Germination of somatic embryos and conversion into plants in liquid T5 culture medium in RITA® bioreactors and in semi-solid T5 medium. **a** Green embryos after the first month of culture in RITA® containers, **b** Embryos in the cotyledon stage in the second month of culture in RITA® containers, **c** Plants with one or two pairs of leaves in the third month of culture in RITA® containers, **d** Control culture, with a frequency of two daily immersions of one minute; observe the asynchrony in the development and hyperhydricity of the culture, **e** Good quality plants cultured using 2 one-minute immersions every 2 days, **f** Plants cultured using 1 immersion every 2 days. **g** Pre-germination of embryos regenerated in liquid T5 medium in Erlenmeyer flasks, **h** Conversion into plants in semi-solid T5 medium, **i** Plants showing root development

5. In the second month, most of the embryos have reached the cotyledon stage, some have formed roots, and other embryos remain in earlier stages of embryonic development (Fig. 3.3b). Therefore, despite the initial control of culture density, the embryos of some RITA® containers must be divided after 2 or 3 months to reduce density and favor embryo development.
6. Many embryos achieve plant conversion in the third month of culture in RITA® containers, developing one or two pairs of true leaves and roots (Fig. 3.3c). During this period, the harvesting of plants for acclimatization begins. A secondary embryogenesis often develops, especially if a high density of embryos is maintained in the RITA® container.
7. Many advantages are attributed to the use of RITA® containers during coffee SE, since they facilitate handling during different phases, large numbers of embryos are accommodated in a single container, and the use of liquid medium is less labor intensive and reduces production costs (Berthouly et al. 1995; Etienne et al. 1999). However, in practical terms, certain limitations are often observed in the system (Aguilar et al. 2016, 2017), mainly the vulnerability of the cultures to hyperhydricity causing the loss of numerous embryos (Fig. 3.3d).
8. In order to reduce this drawback, we repeatedly compared the control condition of 2 immersions per day for 1 min (Fig. 3.3d) with the use of one daily immersion; and 1 and 2 immersions per day for 1 min every two days (Aguilar et al. 2016, 2017). The highest number of plants in good condition was observed in cultures subjected to 1 (Fig. 3.3e) or 2 (Fig. 3.3f) immersions every two days. It was observed that when immersion frequency is reduced, plant growth is slower but hyperhydricity is less and secondary embryogenesis is reduced. Therefore, the increase in biomass is visually inferior, favoring synchronization in development and the conversion of embryos into plants (Fig. 3.3e). These results indicate that two daily immersions may be excessive since it appears to cause the high hyperhydricity observed (Aguilar et al. 2017).

3.2.6.2 Pre-germination of Embryos in Liquid Medium and Conversion Plants in Semi-solid Medium

1. An alternative method of conversion into plants was put into practice in semi-solid medium to obtain plants rapidly (3 months) without excessive cost (Aguilar et al. 2016, 2017). In this case, 1.25 g of regenerated embryos were pre-germinated in a 250-ml Erlenmeyer flask, containing 50 ml of liquid development medium T5 (Table 3.1) and cultured in the dark on a rotary shaker (100 rpm) for 15 days.
2. As a result, elongated embryos were obtained with evident cotyledons, some roots and a strong pink coloration (Fig. 3.3g). Subsequently, pre-germinated embryos were rapidly transferred by spatula to culture vessels of 250 ml

volume, containing 75 ml of semi-solid development medium T5 (Table 3.1) and kept under a 12 h light/12 h dark photoperiod for 8 or 10 weeks. This process has allowed us to generate 50 good quality plants (Fig. 3.3h) with root per vessel (Fig. 3.3i) on average in only two steps.

3. The system is easy to handle, requiring the transfer of the batch of embryos only once and there are no changes in the culture medium or the sub-cultures over time; secondary embryogenesis is reduced and therefore the synchronization in embryo development is better, the hyperhydricity of the plants is eliminated and the resulting plant quality is better, which favors the response at the acclimatization (Aguilar et al. 2017).

3.2.7 Acclimatization

An efficient plantlet acclimatization protocol was implemented at CATIE as detailed below (Aguilar et al. 2016, 2017):

1. The selection of plants with at least one pair of leaves (Fig. 3.4a), and improvement of the acclimatization protocol enabled survival to rise from 45–50% to 75–85% or more, depending on the hybrid.

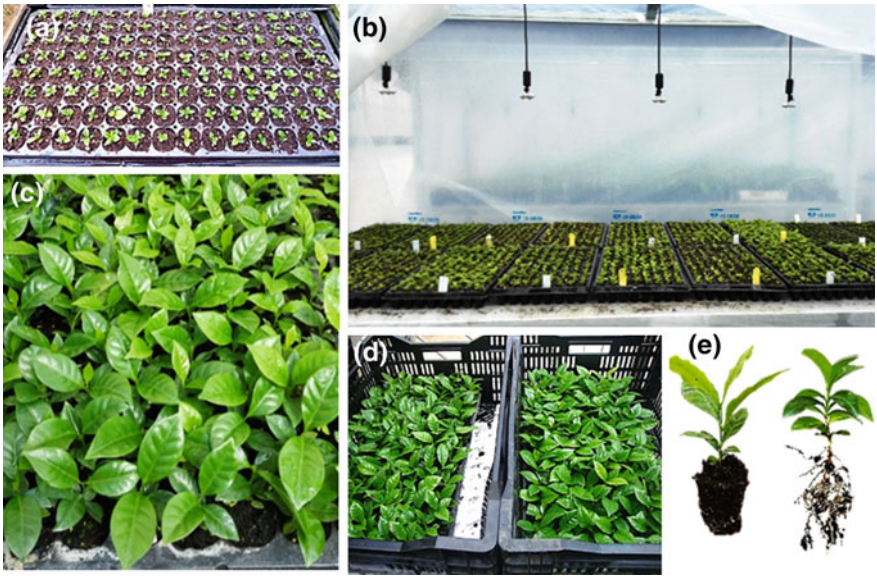

Fig. 3.4 Acclimatization of coffee plants produced by somatic embryogenesis. **a** Plants with at least one pair of leaves planted in a tray, **b** Plastic tunnels for acclimatization, **c** Plants acclimated after 4 months in the greenhouse, **d** Mother plants ready for transfer to nurseries for horticultural propagation, **e** Morphology of an acclimatized mother plant; observe the good root system

2. Acclimatization is carried out in plastic trays (128 cells) with a peat moss substrate (Canadian sphagnum 65%, horticultural perlite, horticultural vermiculite) mixed with Osmocote slow release fertilizer (14-14-14) at a rate of 21 g per tray (Fig. 3.4a).
3. The trays are placed in the greenhouse inside plastic tunnels (Fig. 3.4b) with a relative humidity close to 100%. An irrigation regimen of 15 s, 3 or 4 times per day is used, depending on weather conditions.
4. Fertilizers and pesticides for pest control are applied regularly. Foliar fertilization beginning one week after planting in trays. Alternate applications of Multiminerals + Maxibost or Bayfolán until the end of the growth cycle, depending on plant development and the appearance of nutritional deficiencies.
5. Application every two weeks of systemic fungicide (Benomil-2 g/L) and systemic bactericide (Agrimicin-2 g/L), during the growth cycle in the greenhouse and alternately with other products. These applications may vary depending on infection and weather conditions.
6. After 4 months in the greenhouse the plants are acclimated (Fig. 3.4c) and ready for the horticultural multiplication stage by rooting of cuttings (Fig. 3.4d, e).
7. The direct planting in horticultural substrate of embryos germinated in RITA® bioreactors was developed (Etienne-Barry et al. 1999) and evaluated (Barry-Etienne et al. 2002) to simplify the process and reduce production costs associated with the plant conversion phase under in vitro conditions. At CATIE, the acclimatization of germinated embryos was also put into practice. However, many of the germinated embryos remained in this state and never developed, or they showed mortality above 40% for all the hybrids (Aguilar et al. 2017); similar to what was found by Etienne-Barry et al. (1999).

3.3 Clonal Propagation of Arabica Coffee by Rooting of Leafy Stem Cuttings

3.3.1 Rooting Protocol

3.3.1.1 Clonal Gardens

1. The clonal garden (Fig. 3.5a) is one of the main elements of the clonal propagation system. It is an area where the mother plants are established at high densities for the continuous and prolonged production of cuttings by frequently harvesting shoots.
2. In our case, the harvests are made every 15 days, and the gardens have managed to maintain their cutting production and rooting rates for more than three years.
3. The clonal garden is an intensive crop that requires an appropriate technological package in terms of maintenance practices and adequate nutritional and phytosanitary management in particular.

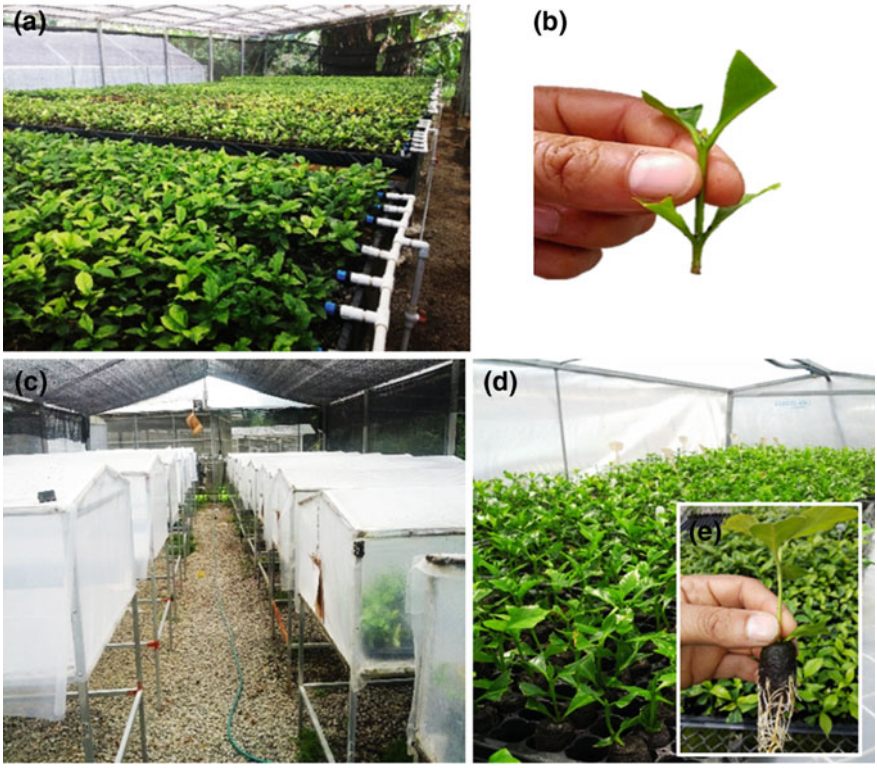

Fig. 3.5 Clonal propagation of coffee mother plants from rooted cuttings. **a** Hydroponic garden with mother plants from somatic embryogenesis, established in a 10 × 10 cm, **b** Prepared cutting 7 cm long, with two nodes and four pruned leaves, **c** Exterior of the tunnels for rooting cuttings, **d** Interior of the tunnels for rooting, **e** Cutting rooted in 'Jiffy®' pellets after 40 days in the rooting tunnels

4. CATIE uses hydroponic beds that require intensive management and increased technification, but maximize the number of mother plants per unit area and the production of optimum quality shoots for rooting.
5. The hydroponic beds are 18 cm deep, 1.5 m wide and variable length, placed at 80 cm from the ground to facilitate handling.
6. A mixture of gravel (0.5–1 cm diameter) and coconut fiber in a 60:40 ratio respectively is used as a substrate. The mother plants are established at a density of 10 × 10 cm.
7. Drip irrigation is used to water and fertilize the gardens; foliar and substrate fertilizer applications are also made according to the scheme detailed in Table 3.3.

Table 3.3 Fertilization program in the hydroponic beds

Timing of application	Product[a]	Chemical composition	Dose x liter of water
Irrigation			
First week of the month	Bloomtasctic	3% N; 14% P; 3% K; minerals and biological stimulants	5 ml
	Sal Wax Max	3% N; 15% K_20; 14% CaO; 50% carboxylic acids	1 g
Week 2	Tricho elements	3.8% Fe; 2.5% Zn; 2.5% Mn, 0.1% Mo; 0.2% B	5 ml
	Boric acid	B	0.2 g
Week 3	Alcaplant	35% CaO	2 ml
Foliar			
First day of the week	Bayfolan	11.47% N; 6% K_2O; 0.036% B; 0.04% Cu; 0.05% Fe; 0.005% Mo; 0.08% Zn; 0.004% thiamine hydrochloride; 8% P_2O_5; 0.23% S; 0.025% CaO; 0.002% Co; 0.036% Mn; 0.025% MgO; 0.003% AIA	5 ml
	Clawmag	13.3% Mg	3 ml
	Citomastic	3% Zn	2 ml
Day 3	Fortaleza	0.3% B; 10% Ca	5 ml
	Sitco	10% P; 24.31% K; 5.72% salicylic acid; 7.15% silica	6 ml
	Borycal	7% CaO; 1% B; 0.4% Mn; 0.2% Mo; 0.4% Zn	3 ml
Day 5	Tricho elements	3.8% Fe; 2.5% Zn; 2.5% Mn, 0.1% Mo; 0.2% B	3 ml
	Iron	4% Fe	1 ml
	Moli-blue	11.7% Mo	0.5 ml
For the substrate			
Every 3 months	Osmocote	14% N; 14% P; 14% K	3–5 g/ liter of substrate

[a]Commercial products are mentioned for informational purposes only and it does not imply an express recommendation by CATIE or the author

8. To optimize rooting, the environment of the clonal garden should be very similar to what the cuttings will subsequently have in the propagators, in order to minimize the physiological shock involved in cutting the shoot (Mesén et al. 1997a). For this reason, the clonal gardens are established in closed greenhouses with 60% saran shade fabric. The shoots are cut with scissors every 15 days just above the third node, and these are immediately transferred to the propagation area submerged in containers with water.

3.3.2 Length and Leaf Area of the Cuttings

1. The preparation of the cuttings is done in a cool and shaded place and during the whole process, the shoots are kept in containers with water. The prepared cuttings are 5–7 cm long with two nodes and two pairs of leaves (Fig. 3.5b).
2. The rooting of succulent cuttings is dependent on the presence of leaves because photosynthesis products are needed, but at the same time they are very prone to water loss through transpiration (Mesén et al. 1997a, b, 2001; Leakey 2014). The works of Davis (1988), Hoad and Leakey (1994, 1996), and Mesén et al. (1997a, 2001) illustrate the importance of photosynthesis on the percentage of cuttings rooted, the number of roots and the speed of rooting. Successful rooting, therefore, requires an optimum leaf area to balance the negative effects of transpiration and the beneficial effects of photosynthesis, which is important in the production of assimilates for root formation (Leakey 2004).
3. In our case (Mesén and Jiménez 2016), leaves are pruned to leave an area of 2.5–3 cm^2 in each one (Fig. 3.5b).
4. Prior to the application of auxin and transfer to the rooting tunnels, the cuttings are deposited in a container with water to which is added a fungicide ("Proplant", fungicide propyl-3-propylcarbate 72.5%) and stimulators ("Sprintene", 1% Zn; 1% Mo; 0.1% Co and "Fortaleza", 0.3% B; 10% CaO).

3.3.3 Application of Auxin

1. We use powder Indole-3-butyric acid (IBA) at a dose of 0.3%.
2. The wet base of the cutting is dipped in the powder and any excess is shaken off, since too much auxin may cause rotting at the cutting base.
3. Subsequently, the cutting is inserted about 2 cm deep into the substrate, which is then slightly compacted around the cutting to ensure its vertical position.

3.3.4 The Rooting Substrate

1. The rooting substrate must remain moist and drain easily to prevent water accumulation. It should also be kept as sterile as possible to prevent the development of diseases.
2. At CATIE, we use 'Jiffy'® pellets (2 cm in diameter by 4.5 cm in height) or peat moss plus a slow-release fertilizer ("Osmocote" 14-14-14) at a dose of 7.5 g per liter of substrate, in multi-cell trays with 200 cavities.
3. Prior to the insertion of the cutting, the substrates are sprayed with a "Carbendazine" fungicide (Benzimidazol-2-ylcarbamate, methyl, 1.2 ml/L).

3.3.5 Rooting Tunnels

1. The trays with the cuttings are put into plastic tunnels for rooting. These tunnels are 3 m long, 1 m wide and 60 cm high, with the base of the tunnel 80 cm from the ground (Fig. 3.5c, d). The advantage of using tunnels is to have better control of possible pathological problems during the rooting phase.
2. Saran shade fabric (50–60%) or another type of material is also used in the propagator area to reduce luminosity, temperature and water loss in the cuttings while also allowing some photosynthetic activity for rooting (Mesén et al. 1997a, b, 2001).
3. Inside the tunnels, relative humidity is higher than 80% and the temperature ranges between 30 and 35 °C during the day.
4. During the rooting period in the tunnels, foliar fungicides and fertilizers are applied weekly as detailed in Table 3.4.
5. Mist irrigation is done through sprinklers located in the roof of the tunnels, at a frequency of 15 s every two hours during the day, controlled by a timer; however, the duration and frequency of irrigation should be evaluated and adjusted according to the area's season and climate.
6. Depending on the climate, type of cutting and other factors, the time of root emission varies between 7 and 8 weeks (Fig. 3.5e).

3.3.6 Acclimatization

1. Because the tunnels provide a shaded environment with high humidity, the newly rooted plants require a period of acclimatization to prepare the plant for the external environment.
2. In our case, this period is approximately two weeks and it begins once the roots are visible through the pellets or the bottom of the trays. At that time, the curtains of the tunnels are raised and the plantlets are left in that condition for a week.

Table 3.4 Application program for cuttings in the tunnels

Timing of application	Product[a]	Chemical composition	Dose x liter of water
First day of the week	Cicozinc	Thiophanate-methyl fungicide 50%	1 g
	Hormovit	4% N; 8% P; 0.3% humics; 0.3% auxins	5 ml
Day 5	Bellis	Boscalid fungicide 25.2% + Pyraclostrobin 12.8%	1 g
Biweekly	Fosnutren	6% P; 39.2% free amino acids	1.5 ml

[a]Commercial products are mentioned for informational purposes only and it does not imply their express recommendation by CATIE or the author

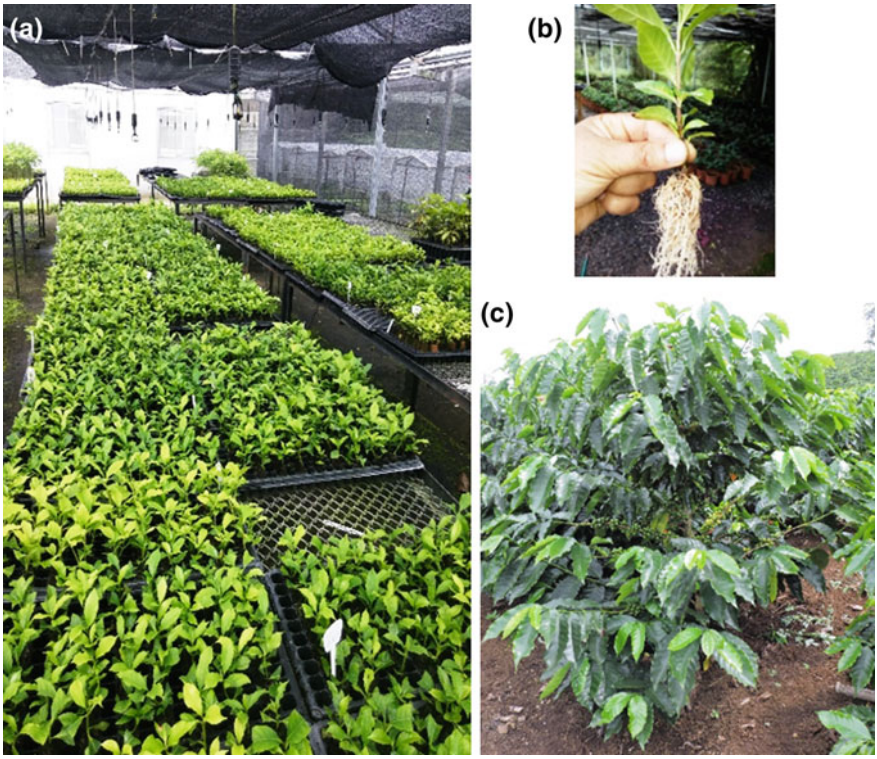

Fig. 3.6 Acclimatization of rooted cuttings. **a** Greenhouse for acclimatization of newly rooted plants before their transfer to the nursery, **b** Root system of a rooted cutting, **c** Plant derived from a rooted cutting, 1 year of age, CATIE, Costa Rica

3. They are then transferred for another week to a common greenhouse (Fig. 3.6a) with 60% shade and more spaced irrigations (2–3 per day).
4. After acclimatization, the plants are transferred to the nursery where they will complete their development prior to planting out in the field. Once in the nursery, the management of the cloned plants is the same as for plants produced from seed and the time they remain in the nursery is similar, 3–6 months, depending on the desired size for the plants.
5. If the rooting and nursery process is done properly, the root system of the rooted plants will be vigorous and profuse (Fig. 3.6b), which will guarantee its good start and development in the field.

3.4 Industrial Multiplication of Arabica Coffee F1 Hybrids

To facilitate the transfer of F1 hybrids to coffee producers, CATIE established a partnership with the Costa Rican company Gaia Artisan Coffee S.A. which specializes in vegetative propagation on an industrial scale. This company uses the plantlets produced by somatic embryogenesis to carry out the mass multiplication of the F1 hybrids through the rooting of cuttings and the development of plants in bags for marketing. The cost of a finished plant for planting will be lower ($\leq$ \$0.75) than if the plants came directly from the Laboratory. This company has approximately 2 hectares of greenhouses and it has developed a three-year pilot production plan that began in 2016 with the multiplication of 240,000 plants; 640,000 plants will be produced in 2017 and for 2018, production is projected to be 1,000,000 plants. After that period, plant production will be increased according to demand in the region.

3.4.1 Simplified Production Protocol

1. Plantlets from somatic embryogenesis are used to establish propagation lots or mother plants in a 1:1 ratio, meaning one pot per each unit in vitro (Fig. 3.7a). This ensures health as well as genetic purity, as it allows making selections and the elimination of any defective units.
2. The fertilization of the mother plants plays an important role in multiplication rates and these vary from traditional coffee production formulas, since the objective is the production of foliage and not fruit. A complete liquid formula (total Nitrogen 90 g/L, Ammoniacal nitrogen 10%, Ca/Mg 4:1, Fe 1 g/L and Electrical conductivity 1.8–2.2) is applied twice a week using auto-compensated drip irrigation. This ensures that nutrition is uniform throughout the lot.
3. Three months after the mother plants are established, cuttings or microcuttings are harvested. This is done manually with the aid of a scalpel to make the cut as clean as possible. At this stage, sanitation becomes more important and the tools must be disinfected every 100 cuts at a minimum.
4. Root formation can last up to 8 weeks depending on the hybrid used. This is done inside plastic tunnels (Fig. 3.7b) conditioned with automated micro sprinklers to keep the temperature at 30 °C and relative humidity close to 100%. The cuttings are kept under these conditions for callus formation during the first two weeks of the process. From the third week to the sixth week (maximum), the rooting medium is kept moist. In the last few weeks, humidity is reduced and the degree of luminosity is increased (up to 40%) to "harden off" the foliage.
5. Once the cutting and its root are ready (Fig. 3.7c), it is planted in a bag for the development of the plant (Fig. 3.7d). Bags larger than conventional ones are used (10 cm $\times$ 30 cm) to protect the root system.

Fig. 3.7 Industrial multiplication of coffee F1 hybrids. **a** Cutting producer mother plants at the Paraiso-Gaia Artisan Coffee farm, **b** Plastic tunnels for rooting cuttings, **c** Rooted cutting, **d** Planting of rooted cuttings in bags, **e** Plants developed over 5 months at Gaia Artisan Coffee, **f** Aerial development of plants in bags, **g** Development of the root system in 5-month old plants

6. Pasteurized soil plus chaff is used as a substrate with drip ferti-irrigation. The bags are kept in nurseries covered with plastic (Fig. 3.7e).
7. The average time needed to have a developed plant that shows a balance between the aerial part (Fig. 3.7f) and the root system (Fig. 3.7g) that is commercially acceptable is 5 months.
8. By maintaining the health of the mother plants during the entire process, the company ensures that the plants have a production for 24 months at a rate of 1900 cuttings per m^2 per year. In this way, it is possible to dilute the cost of the material in vitro and achieve much larger numbers of plants.

3.5 Research Prospects

Taking into account the importance of Arabica coffee cultivation in Central America, where the economies of some of these countries revolve around coffee production, it is necessary to strengthen this activity with more efficient varieties in terms of their productivity, new cup quality demands, resistance to diseases and pests, and traits that favor adaptability to the challenges of climate change. Although the F1 hybrids have been selected for such characteristics and have been available for more than a decade, they have not been distributed to farmers in the

quantities required and at competitive prices due to the complexity of the propagation technology and the lack of investment. New hybrids resulting from the breeding programs will be released in the medium term (https://worldcoffeeresearch.org/work/annual-report-2016), so efficient vegetative propagation techniques will be required for the cloning of new materials while adjustments and improvements in some phases of coffee somatic embryogenesis are necessary for this tool to be applicable to the greatest number of genotypes. Promoting embryogenic calluses production and establishing cryogenic cultures of these calluses or even ECS is urgent for the storage and better management of embryogenic material, mainly in the most recalcitrant genotypes. Improving the conversion of embryos into plants and increasing success during acclimatization is another challenge to be addressed. CATIE has implemented a more efficient way of expanding the production of these coffee hybrids by combining somatic embryogenesis technology for the production of mother plants in the laboratory with the rooting of cuttings produced in the greenhouse from these mother plants. In a trial conducted in 2014 on CATIE grounds in Costa Rica, plants derived from rooted cuttings showed normal and vigorous behavior and started production one year after planting (Fig. 3.6c). Moreover, in a study by Georget et al. (2017), no significant differences were found five years after planting between plants derived from cuttings and somatic embryogenesis, except for a larger basal diameter in plants from cuttings, which indicates even greater vigor in the latter.

Results at both experimental and commercial levels indicate that the technique of rooted cuttings from juvenile mother plants produced by SE is a reliable way to produce plantable material on a large scale and in a short time, using simple, low cost technology. Nevertheless, although this technology helps reduce plant costs for the producer, other factors in the production chain also require attention. The region's coffee sector requires financial support to invest in coffee plantation renovation using genetic materials that are more appropriate for confronting climate vulnerability and increasingly demanding market needs. The technical accompaniment of the institutions governing coffee in each country is also necessary to guide producers on the technical rigors, advantages and challenges involved in the cultivation of high productivity hybrid materials.

Acknowledgements This work was made possible thanks in part to the support provided by the United States Department of Agriculture´s Foreign Agricultural Service under the terms of Award No. 59-314-4-016, and support from the National Institute of Forest Science (NIFoS) of the Republic of South Korea. The authors are also grateful to the research staff of the Biotechnology Laboratory and the Forest Seed Bank of CATIE for their invaluable work.

References

Aguilar ME, Ortiz JL, Mesén F, Altmann F (2016) Embriogénesis somática para la multiplicación clonal de híbridos F1 de café (*Coffea arabica*) y escalamiento industrial por técnicas hortícolas: experiencia del CATIE. In: abstracts of the IX Encuentro REDBIO 2016-PERÚ, Biotecnología para el Desarrollo y Uso Sostenible de la Biodiversidad, Lima, 27 June–1 July 2016

Aguilar ME, Ortiz JL, Kim YW, Kim JA, Moon HK (2017) Contributions of somatic embryogenesis and other *in vitro* propagation techniques to the genetic improvement of tropical woody species: *Coffee arabica, Tectona grandis* and *Gmelina arborea*. In Proceedings of the Fourth International Conference of the IUFRO Unit 2.09.02 Somatic Embryogenesis and Other Vegetative Propagation Technologies, La Plata, Buenos Aires, Argentina, 19–23 September. pp 59–78

Alvard D, Cote F, Teisson C (1993) Comparison of methods of liquid medium culture for banana micropropagation. Effects of temporary immersion of explants. Plant Cell Tissue Organ Cult 8:185–196

Barry-Etienne D, Bertrand B, Vásquez N, Etienne H (2002) Comparison of somatic embryogenesis-derived Coffee (*Coffee arabica* L.) plantlest regenerated *in vitro* or *ex vitro*: morphological, mineral and water characteristics. Ann Bot 90:77–85

Berthouly M, Michaux-Ferriére N (1996) High frequency somatic embryogenesis in *Coffea canephora*: induction conditions and histological evolution. Plant Cell Tissue Organ Cult 44:169–176

Berthouly M, Dufour M, Alvard D, Carasco C, Alemano L, Teisson C (1995) Coffee micropropagation in liquid medium using temporary immersión system. In: ASIC (ed) 16th International science colloquium coffee, ASIC, Vevey, Switzerland, pp 514–519

Bertrand B, Etienne E, Cilas C, Charrier A, Baradat P (2005) *Coffea arabica* hybrid performance for yield, fertility and bean weight. Euphytica 141:255–262

Bertrand B, Etienne E, Alpizar E, Lara L, SantaCreo R, Hidalgo H, Quijano JM, Montagnon C, Georget F (2011) Performance of *Coffea arabica* F1 hybrids in agroforestry and full-sun cropping systems in comparison with American pure line cultivars. Euphytica 181:147–158

Bobadilla R, Cenci A, Georget F, Bertrand B, Camayo G, Dechamp E, Herrera JC, Santoni S, Lashermes P, Simpsom J (2013) High genetic and epigenetic stability in *Coffea arabica* plants derived from embryogenic suspensions and secondary embryogenesis as revealed by AFLP, MSAP, and the phenotypic variation rate. PLoS One 8:e56372. https://doi.org/10.1371/journal.pone.0056372

Bobadilla R, Cenci A, Guyot R, Bertrand B, Georget F, Dechamp E, Herrera JC, Aribi J, Lashermes P, Etienne H (2015) Assessment of genetic and epigenetic changes during cell culture ageing and relations with somaclonal variation in *Coffea arabica*. Plant Cell Tissue Organ Cult 122:517–531

Davis T (1988) Photosynthesis during adventitious rooting. In: Davis TD, Haissig BE, Sankhla N (eds) Adventitious root formation in cuttings. Advances in Plant Sciences Series, vol 2. Disocorides Press, Portland, Oregon, USA, pp 79–87

Dublin P (1984) Techniques de reproduction vegetative *in vitro* et amelioration genetique chez le cafeiers cultives. Café Cacao Thé 28:231–244

Ducos JP, Labbe G, Lambot C, Petiard V (2007) Pilot scale process for the production of pre-germinated somatic embryos of selected robusta (*Coffea canephora*) clones. In vitro Cell Dev Biol_Plant 43:652–659

Duicela LA, Corral R, Fernández F, Macías A, Muñoz R, Shiguango D (2006) Reproducción de plantas clonales de café Robusta. Cofernac/Pronorte/USAID, Ecuador, p 17

Etienne H, Bertrand B (2001) Trueness-to-type and agronomic characteristics of *Coffea arabica* trees micropropagated by the embryogenic cell suspension technique. Tree Physiol 21:1031–1038

Etienne E, Solano W, Pereira A, Barry-Etienne D, Bertrand B, Anthony F, Cote F, Berthouly M (1997) Utilización de la embriogénesis somática en medio líquido para la propagación masal de los híbridos F1 de *Coffea arabica*. In: Bertrand B, Rapidel B (eds) Desafíos de la caficultura centroamericana. IICA Publishers, San José Costa Rica, pp 253–261

Etienne H, Barry-Etienne D, Vásquez N, Berthouly M (1999) Aportes de la biotecnología al mejoramiento genético del café: el ejemplo de la multiplicación por embriogénesis somática de híbridos F1 en América Central. In: Bertrand B, Rapidel B (eds) Desafíos de la caficultura centroamericana. IICA Publishers, San José Costa Rica, pp 457–496

Etienne H, Anthony F, Dussert S, Fernández D, Lashermes P, Bertrand B (2002) Biotechnological applications for the improvement of coffee (*Coffea arabica* L.). In vitro Cell Dev Biol_Plant 38:129–138

Etienne H, Bertrand B, Montagnon C, Bobadilla R, Dechamp E, Jourdan I, Alpizar E, Malo E, Georget F (2012) Un exemple de trasfert de technologie reussi dans le domaine de la micropropagation: la multiplication de *Coffea arabica* par embryogenese somatique. Cah Agric 21:115–124

Etienne H, Bertrand B, Georget F, Lartaud M, Montes F, Dechamp E, Verdeil JL, Barry-Etienne D (2013) Development of coffee somatic and zygotic embryos to plants differs in the morphological, histochemical and hydration aspects. Tree Physiol 33:640–653

Etienne-Barry D, Bertrand B, Vásquez N, Etienne H (1999) Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and regeneration of plants. Plant Cell Rep 19:111–117

Georget F, Courtel P, Malo Garcia E, Hidalgo M, Alpízar E, Breitler JC, Bertrand B, Etienne H (2017) Somatic embryogenesis-derived coffee plantlets can be efficiently propagated by horticultural rooted mini-cuttings: a boost for somatic embryogenesis. Scientia Horticulturae 216:177–185

Hoad SP, Leakey RRB (1994) Effects of light quality on gas exchange and dry matter partitioning in *Eucalyptus grandis* W. Hill ex Maiden. Forest Ecol Manag 70:265–273

Hoad SP, Leakey RRB (1996) Effects of pre-severance light quality on the vegetative propagation of *Eucalyptus grandis*. Cutting morphology, gas exchange and carbohydrate status during rooting. Trees 10:317–324

Leakey RRB (2004) Physiology of vegetative propagation. In: Burley J, Evans J, Youngquist JA (eds) Encyclopedia of forest sciences. Academic Press, London, pp 1655–1668

Leakey RRB (2014) Plant cloning: macropropagation. In: Van Alfen N (ed) Encyclopedia of agriculture and food systems, vol 4. Elsevier, San Diego, pp 349–359

Menéndez-Yuffá A, Barry-Etienne D, Bertrand B, Georget F, Etienne H (2010) A comparative analysis of the development and quality of nursery plants derived from somatic embryogenesis and from seedlings for large-scale propagation of coffee (*Coffea arabica* L.). Plant Cell Tissue Organ Cult 102:297–307

Mesén F, Jiménez LD (2016) Producción de clones de café por miniestacas. CATIE, Turrialba, Costa Rica. Serie Técnica, Manual Técnico No. 130, 27 p

Mesén F, Newton AC, Leakey RRB (1997a) The effects of propagation environment and foliar areas on the rooting physiology of *Cordia alliodora* (Ruiz & Pavon) Oken cuttings. Trees 11:401–411

Mesén F, Newton AC, Leakey RRB (1997b) Vegetative propagation of *Cordia alliodora* (Ruiz & Pavon) Oken: the effect of IBA concentration, propagation medium and cutting origin. Forest Ecol Manage 92:45–54

Mesén F, Leakey RRB, Newton AC (2001) The influence of stockplant environment on morphology, physiology and rooting of leafy stem cuttings of *Albizia guachapele*. New Forests 22:213–227

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plantarum 15:473–497

PROMECAFE (2003) Avances en mejoramiento genético. Antecedentes. In: Boletín PROMECAFE Nº 99 October 2003–January 2004. IICA/ PROMECAFE. 20 p

Sharp WR, Caldas LS, Crocomo OJ, Monaco LC, Carvalho A (1973) Production of *Coffea arabica* callus of three ploidy levels and subsequent morphogenesis. Phyton 31:67–74

Sondahl MR, Sharp WR (1977) High frequency induction of somatic embryos in cultured leaf explants of *Coffee arabica* L. Z Plazenphysiol 81:395–408

Srinivasan CS, Vishveshvara S (1978) Heterosis and stability for yield in Arabica coffee. Indian J Genet Plants Breed 38:416–420

Staritsky G (1970) Embryoid formation in callus tissues of coffee. Acta Bot 19:509–514

Van Boxtel J, Berthouly M (1996) High frequency somatic embryogenesis from coffee leaves. Plant Cell Tissue Organ Cult 44:7–17

Yasuda T, Fujii Y, Yamaguchi T (1985) Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. Plant Cell Physiol 26:595–597

Zamarripa A, Ducos JP, Bollon H, Dufour M, Petiard V (1991) Production d'embryons somatiques de cafeier en millieu liquide: effets densite d'inoculation et renouvellement du millieu. Café Cacao The 35:233–244

Chapter 4
Microspore Embryogenesis in Almond (*Prunus dulcis* Mill.)

Giuseppe Cimò and Maria Antonietta Germanà

4.1 Introduction

Almond is the most important tree nut crop in terms of commercial production. Its production is limited to areas characterized by a Mediterranean climate, including regions in the Mediterranean countries, the Central Valley of California, Central Asia, the Himalayan slopes and some equivalent areas in the Southern Hemisphere, including Argentina, Australia and South Africa (Kester et al. 1975).

Cultivated almonds, often resulted from traditional seed propagation, show high levels of genetic variability due, in part, to their self-incompatibility, that makes them obligate out-crossers (Kester et al. 1991). Commercial cultivars within individual production areas, however, often show a limited genetic base due to their origin from few founder genotypes selected for their desirable regional value (Socias i Company and Felipe 1992). The range of almond species is extensive with a wide diversity of traits (Gradziel et al. 2001; Kester and Gradziel 1996). Controlled crosses of *Prunus dulcis* with other almond species in sections *Euamygdalus* and *Spartiodes* have been often carried out (Gradziel et al. 2001; Gradziel 2003).

One of the oldest almond germplasm collections was established at the Nikistki Botanical Garden in Yalta (Crimea), at the end of the XIX century (Rikhter 1969). Greater genetic variability and so increased breeding options for desired traits are being pursued through the incorporation of breeding material from other regions (Martìnez-Gòmez et al. 2003). Basic objectives of most almond breeding programs target increased yields, improved quality and decreased production costs. These traits have been found to be largely inherited in a quantitative manner (Spiegel-Roy

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_4

and Kochba 1981). Inheritance of important breeding traits have been recently reviewed by Socias i Company et al. (2007).

Plant breeders use traditional and biotechnological techniques to create and use novel genetic variation, aimed at selecting new elite and suitable varieties, with improved traits to satisfy both farmers and consumers. Biotechnologies provide powerful tools for plant breeding, and among these ones, haploid (H) and doubled haploid (DH) technology, can effectively help to select superior plants (Seguí-Simarro 2010). Hs are sporophytes with the gametophytic chromosome number (n instead of 2n), originated from a single, male or female, immature gamete (Germanà 2011a). When spontaneous or induced chromosome duplication of a H occurs, a DH is obtained. Therefore, DHs are homozygous at all loci and can represent a new variety (in self-pollinated crops) or parental inbred line for the production of hybrid varieties (in cross-pollinated crops). Haploid plants arouse interest in the fields of genetic and developmental studies, as well as of plant breeding. Using DH technology, completely homozygous plants can be established in one step, saving thus several generations of selfing in comparison to conventional methods, by which also only partial homozygosity is obtained (Germanà 2011b). Indeed, this technique is the most rapid route to achieve homozygosity and, for self incompatible species, dioecious species and species that suffer from inbreeding depression due to self-pollination, gametic embryogenesis may be the only way to develop inbred lines (Murovec and Bohanec 2011). Particularly, microspore embryogenesis is an indispensable tool to quickly obtain homozygosis in woody plants, since these are characterized by long juvenility, high levels of heterozygosis and, often, by self-incompatibility (Germanà 2009). Unfortunately, many woody species, as well as fruit crops, are still recalcitrant to this process, generally carried out using various methods, mainly including in vitro culture of anthers (Germanà 2011a, b).

Moreover, haploid technology can be particularly effective in accelerating breeding if combined with other biotechnologies, such as 'marker-assisted selection' (MAS), providing a shortcut in backcross conversion to select elite lines. In fact, molecular markers are very useful for planning new crosses, predicting novel gene combinations (Tuvesson et al. 2007). DHs are also very useful for genome mapping, including the construction of genetic linkage maps and gene tagging, providing reliable information on the location of major genes for economically important traits. Nowadays, several genome sequencing programs are using haploid genome because of its simplified assembly, such as in many fruit crops like peach, pear, apple and citrus (Dunwell 2010). Obviously, the availability of an efficient and cost-effective protocol for Hs and DHs production is necessary to take advantages of combining different techniques with DH systems. There is not a universal suitable protocol and the development of new techniques is still required for many recalcitrant genotypes (Germanà 2011a, b; Cimò et al. 2017).

This chapter describes a protocol to regenerate microspore-derived embryo via microspore embryogenesis, through in vitro almond anthers culture.

4.2 Materials

1. Immature flower buds of *Prunus dulcis* Mill., cvs. Filippo Ceo, Lauranne and Genco, at different developmental stages.
2. Laminar-flow hood with ultraviolet light.
3. Petri dishes (60 × 15 mm), forceps, scalpels, glass bead sterilizer, beakers, 1–20 ml serological pipettes, and 10–1000 μl air-displacement pipettes, capped Pyrex bottles, Parafilm, magnetic stirrers.
4. Ethyl alcohol 70% (v/v), sodium hypochlorite solution (0.5% active chlorine), tween-20, sterile distilled water.
5. 1 mg/ml of 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI).
6. Microscope slides and cover glasses.
7. Stereo microscope and fluorescent microscope.
8. Tissue culture chamber.
9. pH meter, autoclave.
10. Culture media (see Tables 4.1 and 4.2).

Basic culture induction medium composition is listed in Table 4.1. Embryo germination medium is listed in Table 4.2. pH was adjusted to 5.8 with 0.5 M KOH or 0.5 M HCl and growth regulators were added to culture medium prior to autoclave at 121 °C for 20 min. Pour 10 ml culture induction medium into 60 × 15 mm Petri dishes.

4.3 Methods

The microspore embryogenesis procedure includes three steps: (1) flower bud size and microspore stage correlation, (2) flower bud sterilization and anther culture, (3) embryo production and maturation.

Table 4.1 Microspore embryogenesis induction medium

Chemicals	Quantity	Chemicals	Quantity
Chu salts	1X	2,4-D	0.5 mg
N&N vitamins	1X	Kinetin	0.5 mg
Galactose	18 g	Zeatin	0.5 mg
Lactose	36 g	Gibberellic acid	0.5 mg
Myoinositol	0.5 g	Thidiazuron	0.5 mg
Ascorbic acid	500 mg	Benzyladenine	0.5 mg
Malt extract	500 mg	Coconut water	100 mL
Biotin	500 mg	Thiamine	5 mg
Casein	500 mg	Pyridoxine	5 mg
Glutamine	800 mg	Glycine	2 mg
Serine	100 mg	Plant agar	7.5 g

All units are in g/L or mg/L (coconut water mL/L); **Chu salts** (Chu 1978), **N&N vitamins** (Nitsch and Nitsch 1969), (Germanà et al. 1996)

Table 4.2 Embryo germination medium

Chemicals	Quantity
MS basal salt mixture	4.3 g
Glycine	2 g
Thiamine	1 g
Nicotinic acid	1 g
Pyridoxine	0.5 g
Ascorbic acid	0.5 g
Sucrose	20 g
Myoinositol	0.2 g
L-Cysteine	2 mg
Zeatin	0.05 mg
1-Naphthaleneacetic acid	0.018 mg
Indole-3-butyric acid	0.02 mg
Plant agar	7.5 g

All units are in g/L or mg/L, **MS** (Murashige and Skoog 1962), (Li et al. 2008)

4.3.1 Flower Bud Size and Microspore Stage Correlation

The microspore development stage is a key factor influencing their ability to turn totipotent. Therefore, selection of buds with the maximum proportion of competent microspores (late uninucleated-vacuolated stage) is essential for efficient microspore-derived embryo yield. For this reason, collect one year almond shoots bearing flower buds at different developmental stages to determine the microspore developmental stage.

1. Select flower buds of different sizes (Fig. 4.1a1–d1) and divide them into groups.
2. Excise anthers separately from each bud size group and squash them in a few drops of DAPI staining solution to determine the microspore developmental stage.
3. Observe the different microspore developmental stages under a fluorescent microscope (Fig. 4.1a2–d2).
4. For the in vitro anthers culture, use only flower buds of the correct size, generally 7–9 mm in length depending on the cultivar (Fig. 4.1b1), containing anthers mostly with microspores at the polarized uninucleated-vacuolated stage (Fig. 4.1b2).

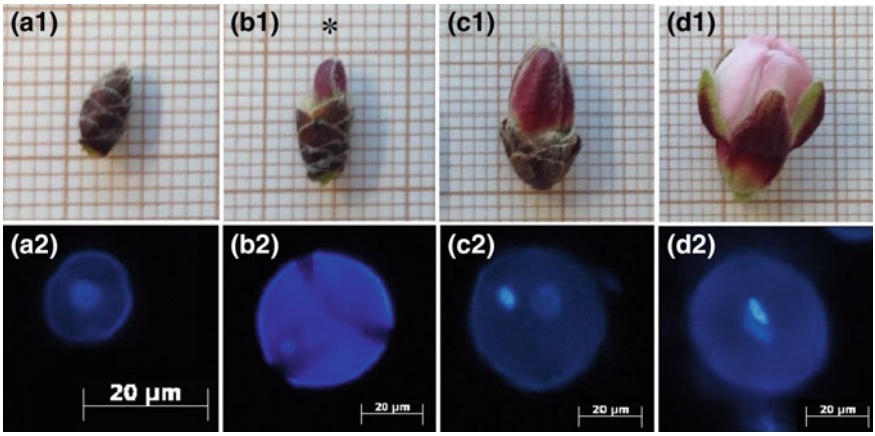

Fig. 4.1 Bud size and microspore development stage correlation. **a1** Inflorescence bud swelling: bud closed, light brown scales visible (BBCH 5.1); **b1** Bud burst: scales separated, light green bud sections visible (BBCH 5.3) *(best flower stage); **c1** Sepals open: white or pink petal tips visible (BBCH 5.7); **d1** Flowers with petals forming a hollow ball (BBCH 5.9); **a2** Middle microspore; **b2** Polarized uninucleated-vacuolated microspore; **c2** Early bicellular pollen; **d2** Mature pollen grain. Growth Stage numbering system is in accordance to the extended BBCH scale (www.agvita.com.au/pdf/sampling/Almond_GS_V2.pdf)

4.3.2 Flower Bud Sterilization and Anther Culture

Store selected flower buds at 4 °C for one week before use (cold-pretreatment).

1. Perform sterilization under a laminar flow hood by immersion of the flower buds in 70% (v/v) ethyl alcohol for 5 min, followed by immersion in a sodium hypochlorite solution (0.5% active chlorine), with a few drops of Tween-20 for 20 min and finally rinse three times in sterile distilled water.
2. Aseptically remove sepals, petals and carefully excise anthers using small forceps.
3. Place immediately the anthers in sterilized Petri dishes containing about 10 mL of solidified culture induction medium (Table 4.1).
4. Incubate the Petri dishes in a grow chamber at 25 ± 1 °C in the dark for 30 days.
5. After one month in the dark, transfer the Petri dishes under cool white fluorescent lamps, with a photosynthetic photon flux density of 35 µmol m^{-2} s^{-1} and a photoperiod of 16 h light.

4.3.3 Embryo Production and Maturation

Different features have been described in almond anther culture as first signs of a change in the developmental pathway (from gametophytic to sporophytic) and of a

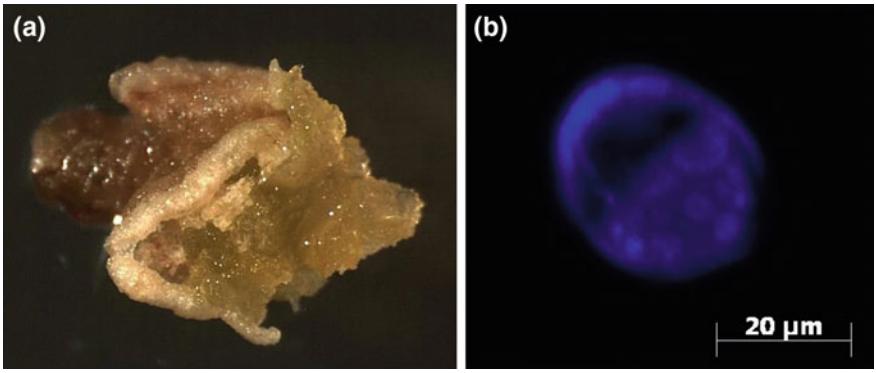

Fig. 4.2 Indications of the morphogenic response in anther culture and of the change of developmental pathway in microspore: **a** Anther producing callus after 2 weeks of culture; **b** Multinucleated structure after one month of culture

morphogenic response. After two weeks of in vitro culture, anthers start to increase their dimension and produce calli (Fig. 4.2a) and, after one month, it will be already possible to observe the presence of multinucleated structures (Fig. 4.2b). In addition, when transferred under cool white light, embryo production (Fig. 4.3) will be also achieved.

To obtain the germination of the microspore-derived embryos, they have to be transferred to a fresh medium containing Murashige and Skoog basal salt and vitamins, sucrose, zeatin (Z), naphthaleneacetic acid (NAA) and indolbutyric acid (IBA) (Table 4.2).

Transfer globular (Fig. 4.3a) or early cotyledonary embryos (Fig. 4.3b, c) into the germination medium reported in Table 4.2, to induce their maturation and development (Fig. 4.2d).

4.3.4 Ploidy Analysis and Detection of Homozygosity

Through anther culture, also somatic and heterozygous embryos can be obtained. For this reason, after successful embryo regeneration, their evaluation is needed to determine their origins (assessing homozygosity), and to distinguish between spontaneously doubled haploids (obtained through microspore embryogenesis) and heterozygous diploids (resulted from somatic embryogenesis). Many direct and indirect approaches are available for determining the ploidy level of regenerated plants. Direct methods are more reliable and include conventional cytological techniques, such as counting the chromosome number (Maluszynska 2003) or measurement of DNA content using flow cytometry (Fig. 4.4), that allows the analysis of a large number of target plants in a shorter period of time (Ochatt 2008).

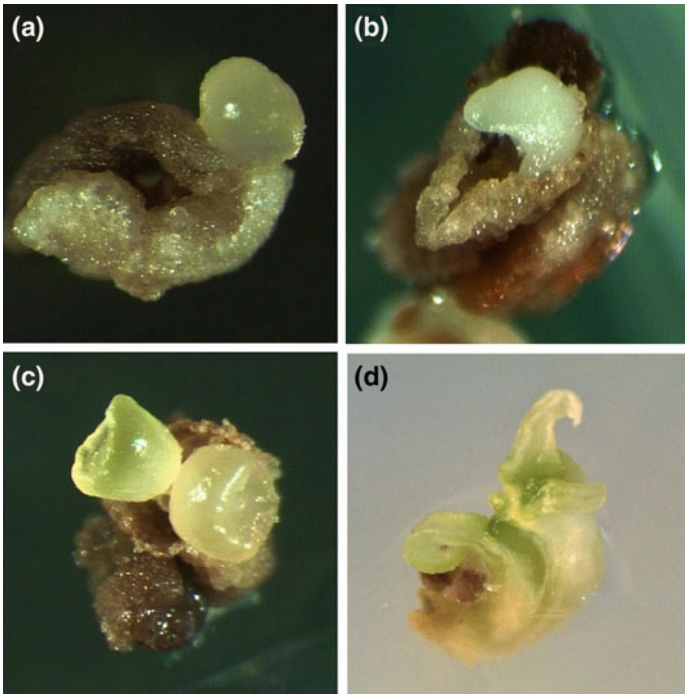

Fig. 4.3 Embryo maturation and germination **a** Direct embryogenesis from almond anther culture; **b**, **c** Early cotyledonary embryos coming out from almond anthers; **d** Embryo developing cotyledons

Moreover, because of the possible spontaneous chromosome doubling occurring during the microspore embryogenesis, ploidy level analysis cannot always identify pollen-derived plants. To detect homozygosity and distinguish between gametic and somatic diploids, DNA molecular markers and microsatellites can be employed (De Vienne 2003). Simple Sequence Repeat (SSR) markers can be adopted to assess the homozygosity, to determine embryo origin (gametic or somatic) and to discriminate homozygous from heterozygous individuals (Fig. 4.5).

SSR polymorphic microsatellites, selected for being heterozygous in the donor plant and because they amplified in almond (i.e. PaCITA-21, MA031a, BPPCT-007, UDAP-468, EPPCU-5990 and PaCITA-23) can be employed for the analysis (Cimò et al. 2017).

The polymerase chain reaction (PCR) is carried out with three primers: the specific forward primer of each microsatellite with M13(-21) tail at its 5' end, the sequence specific reverse primer and the universal fluorescent-labeled M13(-21) primer (Schuelke 2000). PCR reactions performed in a GeneAmp® PCR System 9700 thermal cycler (Perkin-Elmer Corp, Freemont, CA) in a final volume of 25 μL, containing 2.5 μL of 10X PCR buffer, 0.2 μL MgCl2, 2 μL dNTPs, 0.5 μL of the forward primer (5 μM), 0.5 μL of the M13(-21) primer, 1 μL of the reverse

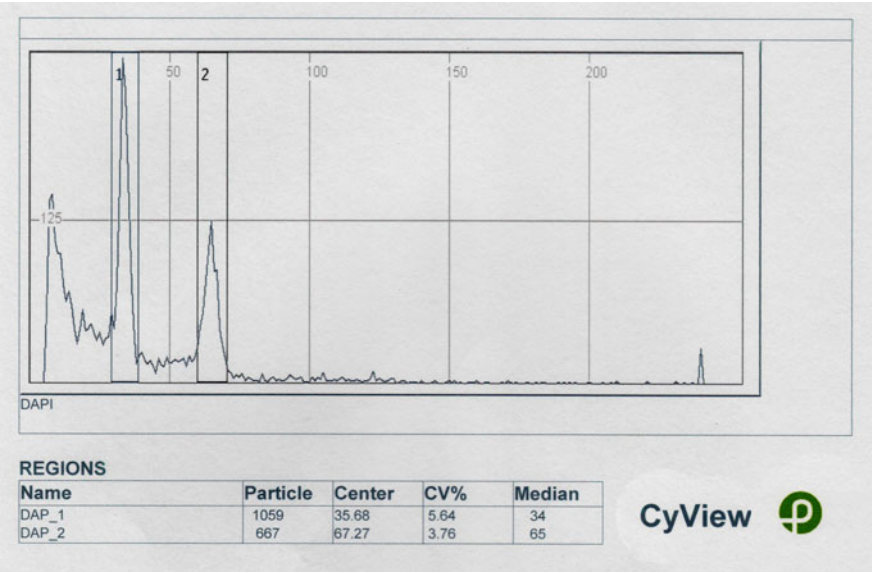

Fig. 4.4 Flow cytometry histogram. The relative fluorescence of the nuclear mixture is 2-fold lower (≈34) in haploids when compared to diploid (≈65) controls

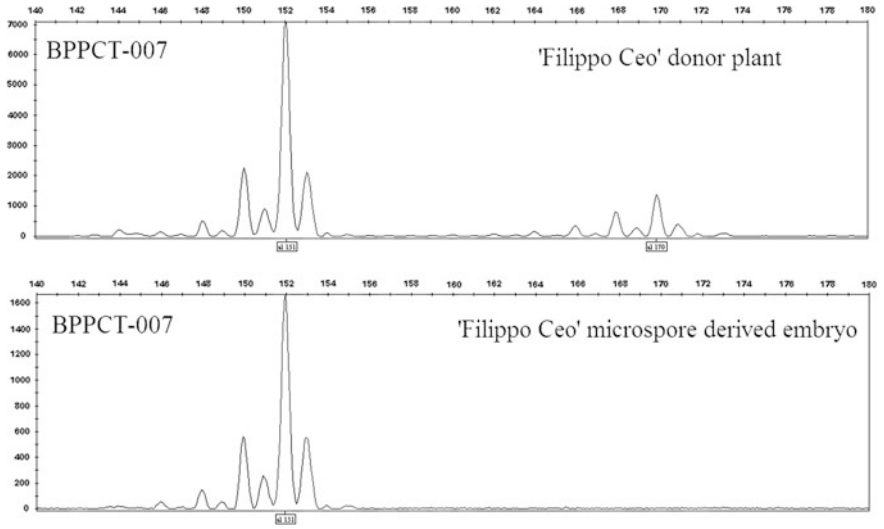

Fig. 4.5 Characterization of anther culture regenerants. Microsatellite analysis: Pherograms of the microsatellite markers BPPCT-007 profiles of the mother plant (top) and of one 'Filippo Ceo' microspore-derived embryo. The mother plant is heterozygous and carries two alleles, the embryo shows only one of the mother plant alleles, considered as support for the gametic origin of the embryo

primer, 0.2 μL Taq polymerase (Invitrogen) and 20 ng of genomic DNA. PCR thermal profile as follows: an initial denaturation step at 95 °C for 60 s, followed by the annealing for 20 cycles at 60 °C (−0.5 °C/cycle) for 60 s, and extension at 72 °C for 90 s; followed by a second thermal profile of one cycle at 95 °C for 60 s; 40 cycles at 50 °C for 60 s, 72 °C for 90 s, finishing with 72 °C for 30 min. Three μL of desalted PCR product mixed with 12 μL of loading solution (70% formamide and 1 mM EDTA), 0.3 μL of LIZ dye, denatured at 95 °C for 5 min, and cooled on ice. Electrophoresis performed on a ABI PRISM 3130 Genetic Analyzer capillary system (Applied Biosystems, Warrington, UK). Allele lengths determined using an ABI Prism 3130 Genetic Analyzer with the GeneMapper software, version 4.0 (Applied Biosystems).

4.4 Identify Steps Required Further Protocol Modifications

In this chapter, microspore-derived embryo regeneration in almond, through in vitro anther culture, is described. However, further protocol modifications are needed. These include: (1) increase of the frequency of embryo recovery; (2) achievement of embryo conversion into plantlets; (3) optimization of embryo maturation and germination and (4) enlargement of the number of respondent almond cultivars.

References

Chu C (1978) The N6 medium and its applications to anther culture of cereal crops. In: Proceedings of symposium on plant tissue culture. Science Press, Peking, pp 43–50

Cimò G, Marchese A, Germanà MA (2017) Microspore embryogenesis induced through *in vitro* anther culture of almond (*Prunus dulcis* Mill.). Plant Cell Tiss Org 128(1):85–95

De Vienne D (2003) Molecular markers in plant genetics and biotechnology. Science Publishers, Inc., Enfield, NH

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol J 8:377–424

Germanà MA (2009) Haploids and doubled haploids in fruit trees. In: Advances in haploid production in higher plants. Springer, Netherlands, pp 241–263

Germanà MA (2011a) Anther culture for haploid and doubled haploid production. Plant Cell Tiss Org 104:283–300

Germanà MA (2011b) Gametic embryogenesis and haploid technology as valuable support to plant breeding. Plant Cell Rep 30:839–857

Germanà MA, Scarano MT, Crescimanno FG (1996) First results on isolated microspore culture of *Citrus*. Proc Int Soc Citricul 2:882–885

Gradziel TM (2003) Interspecific hybridizations and subsequent gene introgression within *Prunus* subgenus *Amygdalus*. Acta Hort 622:249–255

Gradziel TM, Dicenta F, Kester DE (2001) The utilization of related almond species for almond variety improvement. J Amer Pomol Soc 55:100–108

Growth Stage numbering system is in accordance to the extended BBCH, a uniform coding of phenologically similar growth stages for all plant species. Almond: Sampling & Growth Stages. www.agvita.com.au/pdf/sampling/Almond_GS_V2.pdf

Kester DE, Asay R, Almonds. in: Janick J, Moore, JN (eds) (1975) Advances in fruit breeding. Purdue Univ. Press, West Lafayette, IN, USA. pp 387–419

Kester DE, Gradziel TM, Grasselly C (1991) Almonds (*Prunus*). In: Moore JN, Ballington HJ (eds) Genetic resources of temperate fruit and nut crops. International Society for Horticultural Science, The Netherlands, pp 701–758

Kester DE, Gradziel TM (1996) Almonds. In: Janick J, Moore JN (eds), Fruit breeding, vol 3. John Wiley & Sons, New York, USA. pp 1–97

Li J, Wang Y, Lin L, Zhou L, Luo N, Deng Q, Xian J, Hou C, Qiu Y (2008) Embryogenesis and plant regeneration from anther culture in loquat (*Eriobotrya japonica* L.). Sci Hortic-Amsterdam 115:329–336

Maluszynska J (2003) Cytogenetic tests for ploidy level analyses - chromosome counting. In: Doubled haploid production in crop plants: a manual. Kluwer Academic Publishers, Dordrecht, pp 391–395. ISBN 1-4020-1544-5

Martìnez-Gòmez P, Sozzi GO, Sánchez-Pérez R, Rubio M, Gradziel TM (2003) New approaches to *Prunus* tree crop breeding. J Food Agr Env 1:52–63

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15:473–497

Murovec J, Bohanec B (2011) Haploids and doubled haploids in plant breeding. Plant breeding. InTech, Rijeka, Croatia. pp 87–106

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85

Ochatt SJ (2008) Flow cytometry in plant breeding. Cytometry 73A:581–598

Rikhter AA (1969) Ways and methods of almond breeding. Tr Gos Nikit Bot Sad 43:81–94

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234

Seguí-Simarro JM (2010) Androgenesis revisited. Bot Rev 76:377–404

Socias i Company R, Felipe AJ (1992) Self-compatibility and autogamy in 'Guara'almond. J Hortic Sci 67(3):313–317

Socias i Company R, Kodad O, Alonso JM, Gradziel JTM (2007) Almond quality: a breeding perspective. In: Janick J (ed), Horticultural reviews, vol 33, pp 1–33

Spiegel-Roy P, Kochba J (1981) Inheritance of nut and kernel traits in almond (*Prunus amygdalus* Batsch). Euphytica 30:167–174

Tuvesson S, Dayteg C, Hagberg P, Manninen O, Tanhuanpaa P, Tenhola-Roininen T, Kiviharju E, Weyen J, Forster J, Schondelmaier J, Lafferty J, Marn M, Fleck A (2007) Molecular markers and doubled haploids in European plant breeding. Euphytica 158:305–312

Chapter 5
Somatic Embryogenesis in Elite Indonesian Cacao (*Theobroma cacao* L.)

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5.1 Introduction

Theobroma cacao L. tree is the most widely cultivated among twenty two species reported in the genus *Theobroma* and its importance as a major cultivated cash crop is still growing. Also known as chocolate tree as it is the only principal source of cacao beans, the essential ingredient for the production of chocolate. Cacao butter is also used for manufacture cosmetic products.

The cacao tree is native to the humid tropical regions of the northern parts of South America (Motamayor et al. 2008) even though it was domesticated in Mesoamerica (Coe and Coe 1996). By the end of 16th century it had reached Indonesia, introduced by Spaniards from Venezuela (Toxopeus and Geisberger 1983). Although Indonesia has a long cacao history, until the 1980s Indonesian cocoa did not play a major role at world markets (Durand 1995). In the late 1980s cocoa growing began seriously in the regions of Sulawesi island, and considerably lifted the fortune of cocoa-growing communities over the next two decades. Indonesia is nowadays the third largest cacao beans producer in the world after Ivory Coast and Ghana, supplying around 16% of the world demand. Most of cacao farms in Indonesia are small family owned, 0.5–5 ha. When harvest season begins, the entire family, their family, friends and neighbours help collect the colour-full pods. Smallholders from Sulawesi island produce nearly 75% of the national cocoa bean output, providing the main source of income for over 400,000 farming households (Panlibuton and Meyer 2004). Therefore, small farmers are important partners in the future production of Indonesia's supply of cocoa.

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_5

However, in recent years, Indonesian cacao bean production has been steadily declining while demand in local and international markets is growing. Aging trees (planted in the 1980s), increasing exposure to biotic and abiotic stress, insufficient quality of young plants and lack of recent elite plant material are several causal factors. Thus, the renewal of ageing plantations as well as enhancing the quality of cacao by planting elite plant material are required to improve the productivity.

Some farmers still propagate cacao through seeds from the previous harvest. Anyhow, because of their heterozygous nature, cacao elite cultivars must be propagated vegetatively. Attempts to root cuttings of mature cacao trees commonly failed, due to aberrant plant architecture (Niemenak et al. 2008). Cacao is currently propagated by bud grafting on seedling rootstocks. Drawbacks are the high cost and variability. Indeed, grafted plants can be considered as half clones, as the heterogeneous seedling rootstocks cause a lot of growth variation. Thus, selected clones for planting material must be available to distribute to the farmers, and it is therefore crucial to have alternative mass clonal propagation of selected elite Indonesian cacao.

As with many other woody species, somatic embryogenesis has been found being a powerful tool for mass clonal propagation of cacao. In the last 30 years, cacao research in vegetative propagation through somatic embryogenesis has gained much prominence. The initiation of somatic embryo in cacao by using zygotic embryo explant was informed for the first time by Esan in 1975. Thereafter Pence et al. (1979) developed somatic embryos from the zygote derived explants nevertheless plantlet regeneration could not be obtained. Since then somatic embryo induction on mother plant derived tissues of a number of cacao genotypes was achieved in several laboratories in the world. Encouraging results were obtained mostly using floral explants such as staminodes and petals (Alemanno et al. 1997; Li et al. 1998; Tan and Furtek 2003; Masseret et al. 2009). Research was also initiated to enhance the formation of somatic embryo in cacao tissue using the homologous BABYBOOM transcription factor (Florez et al. 2015).

Li et al. (1998) reported the successful plant regeneration of cacao from primary somatic embryos. This group further optimized the procedure for secondary somatic embryogenesis (Maximova et al. 2005). Later on, Guiltinan and Maximova (2010) published the protocol that is most applied for cacao somatic embryogenesis. The nutrient media and concentration of growth substances presented in this protocol have worked well for several cacao genotypes. Nevertheless, since somatic embryogenesis in cacao is highly genotype-dependent (Traore 2006; Minyaka et al. 2008; Quainoo and Dwomo 2012), these protocols are not generally applicable but have to be fine-tuned. Recent results indicate that somatic embryo expression in elite Indonesian cacao ‘Sulawesi 2’ could not be observed in staminode and petal cultures following the Guiltinan and Maximova (2010) protocol (Bustami and Werbrouck 2017). Another approach was necessary. Thus, it was combined with the protocol of Fontanel et al. (2002). This protocol was only reported on a conference, and has been tested on Equadorian cacao clones. A study of Masseret et al. (2009) confirmed that cacao trees produced by somatic embryogenesis using this protocol performed excellently in the field.

Until recently, somatic embryogenesis for cacao genotypes presently used in Sulawesi island of Indonesia have not been developed. Here, we provide a protocol for somatic embryogenesis in 'Sulawesi 2' cultivated mainly in Sulawesi island of Indonesia.

5.2 Materials

1. Growth chamber
2. Binocular microscope
3. Laminar flow hood, forceps, scalpel
4. Immature flower bud of cacao and 50 ml Falcon tubes
5. Ethanol 70%, commercial sodium hypochlorite, Tween-20, sterile distilled water
6. Petri dishes, culture tubes, glass jars, Erlenmeyer flasks, polyethylene plastic, aluminium foil
7. 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kn), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) abscisic acid (ABA), naphthalenacetic acid (NAA), gibberellic acid (GA_3), adenine, glucose, sucrose, gelrite
8. Arginine, glycine, leucine, lysine, tryptophane (Table 5.3)
9. Activated charcoal
10. Media (Tables 5.1 and 5.2)
11. Soil mix for potting.

5.3 Methods

5.3.1 Plant Material

Immature flower bud of cacao (Fig. 5.1a) have to be harvested early in the morning (before anthesis). In Indonesia this means before 9 a.m. Once open, the risk of contamination will be much higher.

5.3.2 Transport

Flowers can easily be transported. Each 50 ml Falcon tube can contain 25 flowers. Cold storing conditions are not necessary. 24 h at room temperature does not affect their freshness.

Table 5.1 Medium composition used for somatic embryogenesis of elite Indonesian cacao

Solutions	Primary embryogenesis		Secondary embryogenesis	Conversion into plantlet		
	Induction	Expression	Induction	Maturation	Germination	Plantlet development
DKW basal[a] (g)	5.585	5.585	–	–	–	–
MS macro[a] (g)	–	–	1.652	0.826	0.826	0.826
MS micro[a] (g)	–	–	–	0.5	0.5	–
DKW micro 100X (ml)	–	–	10	–	–	5
DKW vitamins[a] 100X (ml)	1	1	1	1	1	1
Amino acids 1000X (ml)	1	1	1	1	1	–
2,4-D (mg/ml)	2	–	–	–	–	–
2,4,5-T (mg/ml)	–	–	1	–	–	–
Kinetin (mg/ml)	0.25	0.25	–	–	–	–
NAA (mg/ml)	–	–	–	–	0.01	–
ABA (mg/ml)			–	1	–	–
GA_3 (mg/ml)	–	–	–	–	0.02	–
Adenine (mg/ml)	–	–	0.025	0.5	0.5	–
Glucose (g)	30	30	30	40	40	10
Sucrose (g)	–	–	–	–	–	5
Activated charcoal (g)	–	–	–	–	1 g	–
pH	5.8	5.8	5.8	5.8	5.8	5.8
Gelrite[a] (g)	3	3	3	3	3	2

DKW Driver and Kuniyuki (1984); *MS* Murashige and Skoog; *2,4-D* 2,4-Dichlorophenoxyacetic acid; *2,4,5-T* 2,4,5-Tricholophenoxyacetic acid; *NAA* napthaleneacetic acid; *GA_3* gibberellic acid

[a]Duchefa Biochemie

Table 5.2 Amino acid 1000X stock solution (Guiltinan and Maximova 2010)

Solution	100 ml (mg)
Arginine	43.55
Glycine	18.76
Leucine	32.80
Lysine	45.65
Tryptophane	51.05

Use 1 ml for 1 L of medium

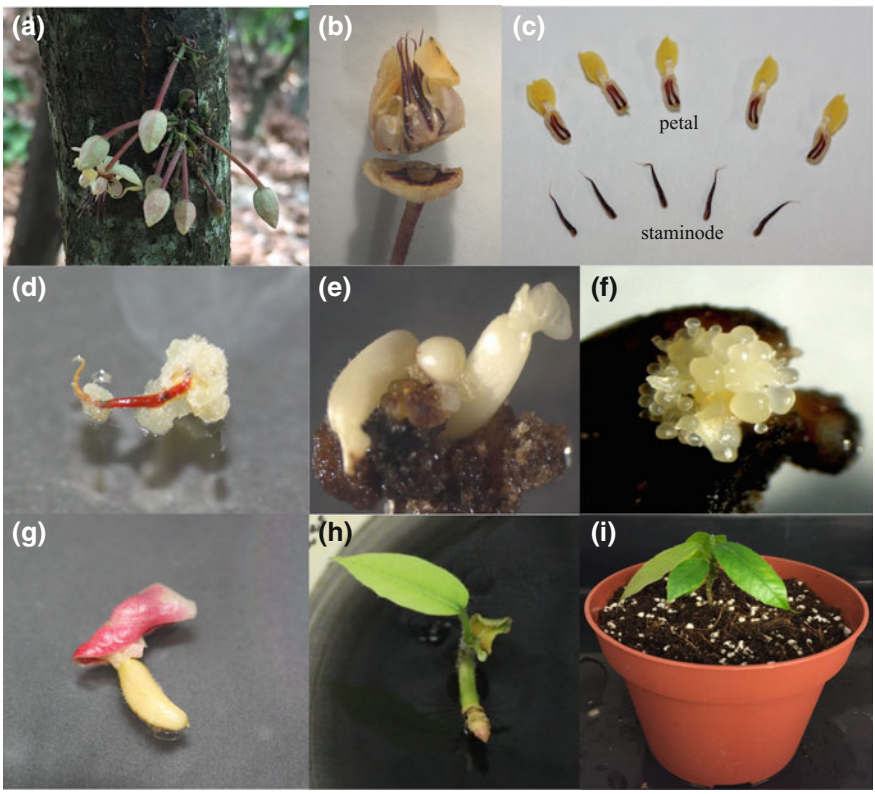

Fig. 5.1 Somatic embryogenesis and plant regeneration in elite Indonesian cacao clone. **a** Immature cacao flower bud; **b** Flower bud is sliced at the distal end; **c** Petal and staminode; **d** Callogenesis 2 weeks after culture in callus induction medium; **e** White globular and cotyledonary of primary somatic embryos from staminode explant 3 weeks after culture; **f** Secondary somatic embryos from cotyledon explant of primary somatic embryo 3 months after incubation on secondary embryogenesis induction medium; **g** Developing embryo with pink cotyledon on maturation medium with ABA; **h** Embryo on germination medium with GA_3; **i** Somatic embryo regenerated plant 8 days after transfer to ex vitro condition

Table 5.3 DKW 100X micro elements solutions (Guiltinan and Maximova 2010)

Solutions	1 L (g)
$Zn(NO3)_2.6H_2O$	1.700
$MnSO_4.H_2O$	3.340
$CuSO_4.5H_2O$	0.025
H_3BO_3	0.480
$Na_2MoO_4.2H_2O$	0.039
$FeSO_4.7H2O$	3.380
Na_2EDTA	4.540

Use 10 ml for 1 L of medium

5.3.3 Explant Preparation

Upon arrival, the whole, closed flower buds are surface-disinfected as follows:

1. Immerse flower bud briefly in 70% ethanol in a glass jar, gently shake by hand for 60 s and decant the ethanol.
2. Transfer the flowers in the 0.8% sodium hypochlorite solution containing 1 drop of Tween 20, for 10 min.
3. In a horizontal laminar flow hood, decant the solution and wash the flower thoroughly three times with autoclaved distilled water to remove any trace of sodium hypochlorite.
4. The flower buds are sectioned at the distal end to isolate the staminode and petal (Fig. 5.1b). The remaining part is discarded.

5.3.4 Medium Preparation

The culture mediums are described in Table 5.1, mainly based on the Fontanel et al. (2002) protocol. DKW micro elements (Table 5.3) and amino acid solution are based on Guiltinan and Maximova (2010) protocol. Heat-labile compound such as ABA and GA_3 should be filter sterilized and added to autoclaved media. Adjust the pH of the medium to 5.8 before adding gelrite, by means of 1M KOH or HCl whichever necessary. Petri dishes are used for Step 1–4. Baby food jars can be used for germinating media to provide more room for plant development. All media are sterilized by autoclaving at 121 °C for 15 min.

5.3.5 Culture Conditions

All cultures are incubate at 26 ± 2 °C in an environmentally controlled growth chamber unless otherwise stated.

5.3.6 Steps in Somatic Embryogenesis

It takes six culture stages to produce acclimatizable somatic embryos, each with a specific medium (Table 5.1).

1. **Callus induction**
   Staminode and petal are placed horizontally on the surface of callus induction media and the Petri-dishes then sealed with polyethylene plastic strips. Cultures are incubated in the dark for 3 weeks by covering Petri-dishes in aluminium foil in an environmentally controlled growth chamber. The staminodes will enlarge, most callus initiates from the cut surface within 1–2 weeks and continuous to proliferate over the staminode for 3 weeks (Fig. 5.1d). Then these cultures are transferred to fresh medium for another 3 weeks under the same condition as before.
2. **Primary somatic embryos**
   Staminode with callus are subcultured onto expression medium. A further subculture on a liquid expression medium enhances the number of globular embryos. Compact white callus will remain non embryogenic. Embryos are produced from brownish friable callus (Fig. 5.1e) about 6 weeks after transfer to the plant growth regulator-free medium. They are globular shaped and appear translucent and white. These embryos are regularly subcultured onto fresh medium every 3 weeks and transferred to maturation medium after reaching the cotyledonary stage.
3. **Multiplication of embryogenic tissue**
   To produce large amounts of somatic embryos, cyclic embryogenic cultures are possible, as was describe for other cacao genotypes. The cotyledons of primary somatic embryos are excised and cut into approximately 4 mm pieces with a scalpel and are placed on secondary somatic embryo induction medium (Table 5.1). These explants are subcultured every 3 weeks on the same medium. When the callus is covering the explant (after 6 weeks), they are transferred to expression medium. Cultures are still kept in the dark at 26 °C.
4. **Maturation and germination of somatic embryos**
   When the somatic embryos reach the cotyledonary stage, they are transferred to maturation medium which contains ABA for 2 weeks. At the mature stage, the colour of cotyledon sometimes turns to pink and the radicle has shown up (Fig. 5.1g). Mature somatic embryos are collected individually and placed vertically on germination medium in tubes. This will stimulate leaf and root growth. They are grown at 26 °C under 16 h light conditions.

5. **Conversion of somatic embryos into plants**
   For conversion to plantlets the germinated embryos are put onto plantlet development medium in baby glass jars and are grown at 26 °C under 16 h light conditions. These explants are subcultured onto fresh medium every 3 weeks. After 9–18 weeks, growing plantlets need to be acclimatized, since their root system and shoot are completely developed.
6. **Transfer of plantlets to soil**
   Well-rooted plantlets are carefully removed from the solidified medium and the gelrite is gently rinsed away using running water. Plantlets are placed in a potting mix with high grade commercial standard white peat substrate and are grown initially under low light (shade) and high humidity (mist, humid chamber) condition. The plantlets are covered with either plastic container immediately after transfer to soil. The plantlets are kept for the first 7–14 days under low light intensity until a new leaf develops. After this period, the humidity is gradually reduced and the plantlets can be grown outside the chamber.

5.3.7 Conclusion and Future Prospective

Somatic embryogenesis in elite Indonesian cacao clone can provide adequate quality planting material to the farmers. Anyhow, if applied in genetic transformation protocols, it may play a crucial role in developing new cultivars more tolerant against abiotic and biotic factors such as Vascular Streak Dieback resistance. Because the production of somatic embryos as well as plant regeneration takes a relatively long time, and germination rates are low, future experiments have to focus on achieving higher rates of germination. Also the browning problem, sometimes occurring at the germination stage, should be studied further.

Acknowledgements This work was supported by a Ph.D. research grant from Directorate General of Resources for Science, Technology and Higher Education Ministry of Research, Technology and Higher Education of Indonesia.

References

Alemanno L, Berthouly M, Michaux-Ferriere M (1997) A comparison between *Theobroma cacao* L. zygotic embryogenesis and somatic embryogenesis from floral explants. In Vitro Cell Dev Biol Plant 33(3):163–172

Bustami MU, Werbrouck S (2017) Comparison of two protocols for somatic embryo induction in a Sulawesi elite *Theobroma cacao* L. clone. In: Beruto M and Ozudogru EA (eds) Proc. VI int symp on production and establishment of micropropagated plants. Acta Hortic. 1155. ISHS 2017

Coe SD, Coe MD (1996) The true history of chocolate, 1st edn. Thames and Hudson Ltd., New York

Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut rootstock. HortScience 19:507–509

Durand F (1995) Farmer strategies and agricultural development: the choice of cocoa in Eastern Indonesia. In: Ruf F, Siswoputanto PS (eds) Cocoa cycles: the economics of cocoa supply. Woodhead Publishing, Cambridge, pp 315–338

Esan ES (1975) Tissue culture studies on cacao (*Theobroma cacao* L.). In: Proceedings of 5th int. cocoa research conf., pp 116–125. Ibadan, Nigeria

Florez SL, Erwin RL, Maximova SN, Guiltinan MJ, Curtis WR (2015) Enhanced somatic embryogenesis in *Theobroma cacao* using the homologous BABY BOOM transcription factor. BMC Plant Biol 15:121

Fontanel A, Gire-Bobin S, Labbé G, Favereau P, Álvarez M, Rutte S, Pétiard V (2002) In vitro multiplication and plant regeneration of *Theobroma cacao* L. via stable embryogenic calli. In: 10th IAPTC congress, plant biotechnology 2002 and beyond, Orlando, Florida, USA

Guiltinan MJ, Maximova S (2010) Integrated system for vegetative propagation of cacao. Protocol Book. Version 2.1. November 17, 2010. American Cacao Research Institute. United States Department of Agriculture. The Pennsylvania State University

Li Z, Traore A, Maximova S, Guiltinan M (1998) Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. In Vitro Cell Dev Biol Plant 34(4):293–299

Masseret B, Gianforcaro M, Bouquet JF, Brulard E, Florin B (2009) Somatic embryogenesis applied to the creation of a cacao collection. Malays Cocoa J 5:1–10

Maximova SN, Young A, Pishak S, Miller C, Traore A, Guiltinan MJ (2005) Integrated system for propagation of *Theobroma cacao* L. In: Jain SM, Gupta PK (eds) Protocol for Somatic Embryogenesis in Woody Plants, Series: Forestry Sciences, vol 77, pp 209–227, Springer, Berlin

Minyaka E, Niemak N, Sangare FA, Ndoumou OD (2008) Effect of $MgSO_4$ and K_2SO_4 on somatic embryo differentiation in *Theobroma cacao* L. Plant Cell Tissue Organ Cult 94 (2):149–160

Motamayor JC, Lachenaud P, da Silva e Mota JW, Loor R, Kuhn DN, Brown JS, Schnel RJ (2008) Geographic and genetic population differentiation of the Amazonian chocolate tree (*Theobroma cacao* L.). PLoS One 3(10):e3311

Niemenak N, Saare-Surminski K, Rohsius C, Ndoumou OD, Lieberei R (2008) Regeneration of somatic embryos in *Theobroma cacao* L. in temporary immersion bioreactor and analyses of free amino acids in different tissues. Plant Cell Rep 27:667–676

Panlibuton H, Meyer M (2004) Value chain assessment: Indonesia cocoa. Accelerated microenterprise advancement project (AMAP) microREPORT #2 (June). Prepared by action for enterprise and ACDI/VOCA for USAID, Washington, D.C.

Pence VC, Hasegawa PM, Janick J (1979) Initiation and development of asexual embryos *Theobroma cacao* L. J Amer Soc Hort Sci 104:145–148

Quainoo AK, Dwomo BI (2012) The effect of TDZ and 2,4-D concentrations on the induction of somatic embryo and embryogenesis in different cocoa genotypes. J Plant Stud 1(1):72–78

Tan CL, Furtek DB (2003) Development of an in vitro regeneration system for *Theobroma cacao* from mature tissues. Plant Sci 164(3):407–412

Toxopeus H, Giesberger G (1983) History of cocoa and cocoa research in Indonesia. In: Toxopeus H, Wessel PC (eds) Cocoa research in Indonesia 1900–1950. American Cocoa Research Institute, International Office of Cocoa and Chocolate, Washington D.C., Wageningen, pp 7–32

Traore A (2006) Effect of carbon source and explant type on somatic embryogenesis of four cacao genotypes. J Hortic Sci 41(3):753–758

Chapter 6
Black Cohosh (*Actaea racemosa* L.)

Ina Pinker and Regina Schenk

6.1 Introduction

Black cohosh (*Actaea racemosa* L., syn. *Cimicifuga racemosa* (L.) NUTTALL) native to eastern North America, is member of the plant family Ranunculaceae. The perennial plants are growing in deciduous woodlands. Products of dried plant rhizomes (*Cimicifugae racemosae rhizoma*) and lateral roots (*Radix Cimicifugae racemosae*) are used in phytotherapy. Traditionally the herbal products are applied against rheumatic pains, female complains and nowadays as alternatives to estrogen therapy to treat climacteric symptoms (Bradley 2011). As major secondary compounds triterpene glycosides and phenylpropanoids are considered (Bradley 2011; Jiang et al. 2011). The mostly wild-grafted plants are problematic in some respects. There are reports on adulteration and substitutions of black cohosh in herbal samples with other species (Jiang et al. 2006; Bittner et al. 2016). Moreover, Vickers et al. (2015) reported significant differences in some secondary metabolic compounds among black cohosh accessions from different physiogeographic regions and different years. Therefore, defined varieties with specific patterns of secondary compounds should be available to produce homogenous standardized raw material for herbal products (Popp et al. 2003). Recently, first results on mass content of six phenolic compounds in black cohosh clones were released indicating a homogenous quality within the clones (Bittner et al. 2015).

Furthermore, for field cultivation also homogenous plant material is required, but seed progenies are genetically and phenotypically heterogeneous. Probably the secondary compounds are different between the plants too (Bittner et al. 2015). Therefore, vegetative propagation of selected, high-value plants should be preferred. The standard method for cloning is currently the division of root stocks into

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_6

pieces with at least one well developed shoot bud (Fischer et al. 2006). For producing thousands of plants for field production this method is too slow. Alternatively, in vitro propagation techniques were developed to accelerate the young plant production. There are few reports on micropropagation of *Actaea* using shoot multiplication (Prakash 2009) and adventitious shoot formation on leaf segments (Lata et al. 2002; Piagnani et al. 2003). As many genera of Ranunculaceae with ornamental or medicinal value *Actaea* exhibits some problems for micropropagation (Prakash 2009). For other members of Ranunculaceae, some papers were published e.g. on shoot multiplication in *Anemone* and *Delphinium* (Prakash 2009), axillary budding in *Helleborus* (Beruto et al. 2012), adventitious shoot regeneration (Pugliesi et al. 1992) or axillary shoot multiplication (Beruto et al. 1996) in *Ranunculus.*

Somatic embryogenesis is a powerful technique employed for multiplication of some genera of Ranunculaceae as *Aconitum* (Giri et al. 1993; Hanato et al. 1987), *Clematis* (Luttman et al. 1994; Mandegaran and Sieber 2000; Weber et al. 1994), *Hepatica* (Szewczyk-Taranek and Pawłowska 2015), *Nigella* (Elhag et al. 2004), *Paeonia* (Teixeira da Silva et al. 2012), and *Ranunculus* (Beruto and Debergh 1992; Beruto et al. 1996; Meynet and Duclos 1990). In the 90th of the last century large scale propagation of somatic embryos in bioreactors was already impressively demonstrated with *Clematis tangutica* (Luttman et al. 1994; Weber et al. 1994). They reported on 1200 embryogenic clusters, 500 globular and 700 heart or torpedo embryos yielded per gram initiated cell mass after four weeks of cultivation. Around half a million of embryos were theoretically expected per litre suspension. However, this multiplication rate could not be reproduced with other *Clematis* species.

As basal culture medium the Murashige and Skoog (1962) medium with some modifications was used in all these protocols. Special media compositions were reported for stages as embryogenic mass induction, maintenance and propagation of embryogenic mass, embryo maturation, embryo conversion, and rooting.

The embryogenic mass (embryogenic callus) was induced by application of auxins as 2,4-dichlorophenoxyacetic acid (2,4-D) in *Actaea*, *Nigella* and *Ranunculus* (Beruto and Debergh 1992; Elhag et al. 2004; Meynet and Duclos 1990; Pinker and Schenk 2007; Pinker et al. 2016) or 1-naphthaleneacetic acid (NAA) in *Hepatica* (Szewczyk-Taranek and Pawłowska 2015). While in *Aconitum* and *Clematis,* auxins (2,4-D or NAA) together with cytokinins were applied for the induction of friable callus and somatic embryos (Giri et al. 1993; Hanato et al. 1987; Mandegaran and Sieber 2000). In tree peony, the embryogenic callus along with embryos was induced by application of 6-benzyladenine (BA) (Teixeira da Silva et al. 2012). For the embryo histo-differentiation and maturation of somatic embryos, plant growth regulator free media supplemented with active charcoal were used (Pinker and Schenk 2007; Szewczyk-Taranek and Pawłowska 2015).

Somatic embryogenesis in Ranunculaceae was initiated on different explants as leaf segments or nodal sections from in vitro cultures of *Aconitum* and *Clematis* (Giri et al. 1993; Mandegaran and Sieber 2000) or hypocotyl segments of *Actaea* and *Hepatica* (Pinker and Schenk 2007; Szewczyk-Taranek and Pawłowska 2015). Some reports were found using explants from flowers of in situ plants as anthers of

Aconitum (Hanato et al. 1987) and *Ranunculus* (Meynet and Duclos 1990), stamens of *Actaea* (Pinker et al. 2016) and thalamus segments of *Ranunculus* (Beruto and Debergh 1992). From these explants, regenerants with the same genetic constitution as the donor plants are obtained. However, anther cultures were also applied to produce haploid plants of *Hepatica* (Nomizu et al. 2004), *Helleborus*, and *Paeonia* (Zenkteler et al. 1974) for breeding purposes.

The developmental sequence of the somatic embryogenesis follows the common developmental pattern of dicots. It was described by many authors as white, friable callus in the beginning exhibiting on its surface globular embryos developing later into heart, torpedo and cotyledonary stage embryos. All embryo stages are often observed on the same embryogenic mass (Elhag et al. 2004; Pinker et al. 2016; Pinker and Wernicke 2017). Histological studies proved the embryo character of the propagules (Aréne et al. 2006; Beruto and Debergh 1992; Giri et al. 1993; Pinker and Schenk 2007; Szewczyk-Taranek and Pawłowska 2015) whereas Aréne et al. (2006) observed differences in cell and nucleus sizes of zygotic and somatic embryos of *Clematis*.

This chapter describes a protocol to propagate black cohosh plantlets from embryogenic cultures derived of stamens.

6.2 Materials

1. Immature stamens of flower buds of 3–5 mm in diameter
2. Laminar-flow hood
3. Autoclave
4. 55 × 15 mm sterile, polystyrene Petri dishes with ventilating cam
5. 100 ml Magenta™ vessels with B-cup
6. 100 ml Erlenmeyer flasks
7. Forceps
8. Scalpels
9. 0.3% (m/v) mercuric chloride solution with 2 droplets of Tween 20®
10. Sterile deionized water
11. pH-meter
12. Tissue culture chamber
13. Rotary shaker
14. Dissecting microscope
15. Media (see Tables 6.1 and 6.2): MS-medium, 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA), agar (SERVA Kobe I), activated charcoal, and sucrose.

Basal medium composition is listed in Table 6.1. Required modifications for different culture stages are listed in Table 6.2. The pH has to be adjusted to 5.8 with 1N KOH or 1N HCl prior to autoclaving at 121 °C for 15 min.

Table 6.1 Basal culture medium for *Actaea* cultivation

Chemicals	Quantity in mg/l	Chemicals	Quantity in mg/l
KNO_3	1900	$CoCl_2.6H_2O$	0.025
NH_4NO_3	1650	$CuSO_4.7H_2O$	0.025
$CaCl_2.2H_2O$	440	$FeSO_4.7H_2O$	27.85
$MgSO_4.7H_2O$	370	$Na_2EDTA.2H_2O$	37.25
KH_2PO_4	170		
$MnSO_4.H_2O$	16.89	Myo-inositol	100
$ZnSO_4.7H_2O$	8.6	Thiamine HCl	2.5
HBO_3	6.2	Pyridoxin HCl	0.2
KI	0.83	Biotin	0.2
$Na_2MoO_4.2H_2O$	0.25		

The pH was adjusted to 5.8 with 1N KOH or 1N HCl prior to autoclaving at 121 °C for 15 min

Table 6.2 Formulations of *Actaea* media

Chemicals	AR-1	AR-2	AR-3	AR-4	AR-5
	Stage I Initiation	Stage II Proliferation and maintenance	Stage III Development and maturation	Stage IV Conversion	Stage V Plant development
2,4-D[a]	1	0.5	–	–	–
BA[a]	–	–	–	0.2	–
Activated charcoal[a]	–	–	1000	–	1000
Agar[a]	7000	7000[b]	7000	7000	7000
Sucrose[a]	3000	2000	2000	2000	3000
Macro nutrients[c]	100%	50%	50%	50%	100%

[a]All units are in mg/l
[b]Agar, not used for liquid media
[c]Referred to Table 6.1

Pour 10 ml medium to Petri dishes and 25 ml medium to 100 ml Magenta™ vessels or 20 ml liquid medium to 100 ml Erlenmeyer flasks.

6.3 Method

The regeneration procedure includes five stages: (1) embryogenic mass initiation from explants, (2) maintenance and proliferation of embryogenic cultures, (3) embryo development and maturation, (4) embryo conversion and (5) plant development in vitro. After this the gained plantlets can be acclimatized and transferred to the field.

6.3.1 Embryogenic Mass Initiation

Use immature stamens isolated from closed flower buds for embryogenic mass initiation. Collect the green or just turning to white flower buds from mid-June to early July.

1. Isolate the closed flower buds (3–5 mm in diameter) from the flower stalk.
2. Sterilize the flower buds with 0.3% (w/v) mercuric chloride supplemented with 2 droplets of Tween 20® for 3 min.
3. Rinse the buds 3 times with sterile deionized water in the laminar-flow hood, for one min each time.
4. Transfer the sterile flower buds into a Petri dish.
5. Open the flower buds with sterile scalpels and forceps to isolate aseptically the stamens (Fig. 6.1a).
6. Place the isolated stamens horizontally on the surface of semi-solid AR-1 medium (Table 6.2) in Petri dishes for callus induction. Try to distribute the stamens evenly.
7. Incubate the stamens in darkness at 24 °C for three months.
8. First callus formation on filaments can be observed in dependence on the genotype after 2–8 weeks on AR-1medium (Table 6.2). Translucent, glossy callus develops from the filaments (Fig. 6.1b) becoming opaque and exhibiting nodular structures after three months.
9. Transfer the embryogenic mass to AR-2 medium after three months (Fig. 6.2, Table 6.2).

6.3.2 Maintenance and Proliferation of Embryogenic Cultures

After three months on AR-1 medium, white to yellowish, friable, nodular embryogenic mass with first globular embryos is developed.

1. Transfer small parts of the embryogenic mass (size of less than 4 mm) to the AR-2 medium (Table 6.2) for proliferation and maintenance.
2. The nodular mass can be grown on agar-solidified medium or in liquid medium (see Sect. 6.3.3).
3. On the embryogenic mass, rather opaque proliferating clusters (Fig. 6.1c) and all stages of embryo development till the torpedo stage giving the embryogenic mass its white and friable appearance can be observed around six months after culture initiation. This asynchronous embryo development was already reported for *Nigella* (Elhag et al. 2004) and *Actaea* (Pinker and Schenk 2007).

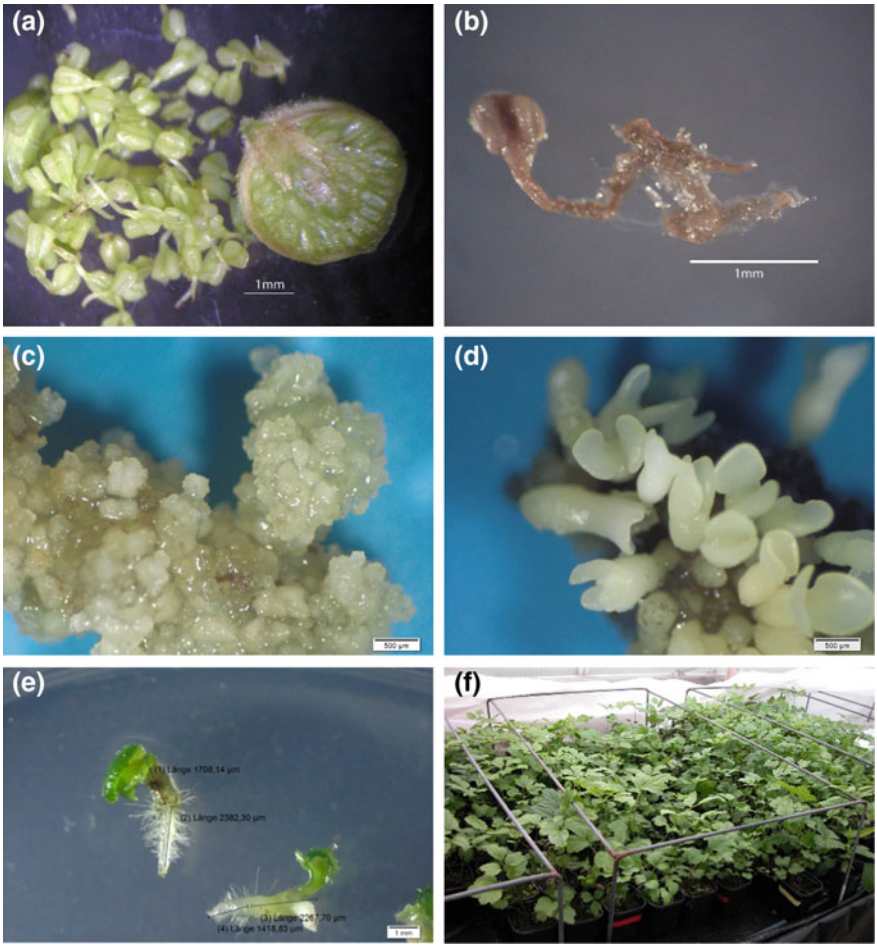

Fig. 6.1 Plant regeneration via somatic embryogenesis in *Actaea racemosa*. **a** Halved Flower bud and isolated stamens; **b** early stages of embryogenic callus obtained from filaments; **c** clusters of embryogenic mass with globular and heart stage somatic embryos on AR-2 medium; **d** clusters of cotyledonary somatic embryos on AR-3 medium; **e** bipolar conversion of somatic embryos on AR-4 medium, **f** acclimatized plantlets end of April just before transplanting into the field

4. Incubate the embryogenic mass in darkness at 24 °C and propagate it by subculturing every 6 weeks on fresh AR-2 medium.

After 6 weeks, the embryogenic mass increases in size at least to the double. The embryonic cultures can be maintained and propagated for at least 3 years.

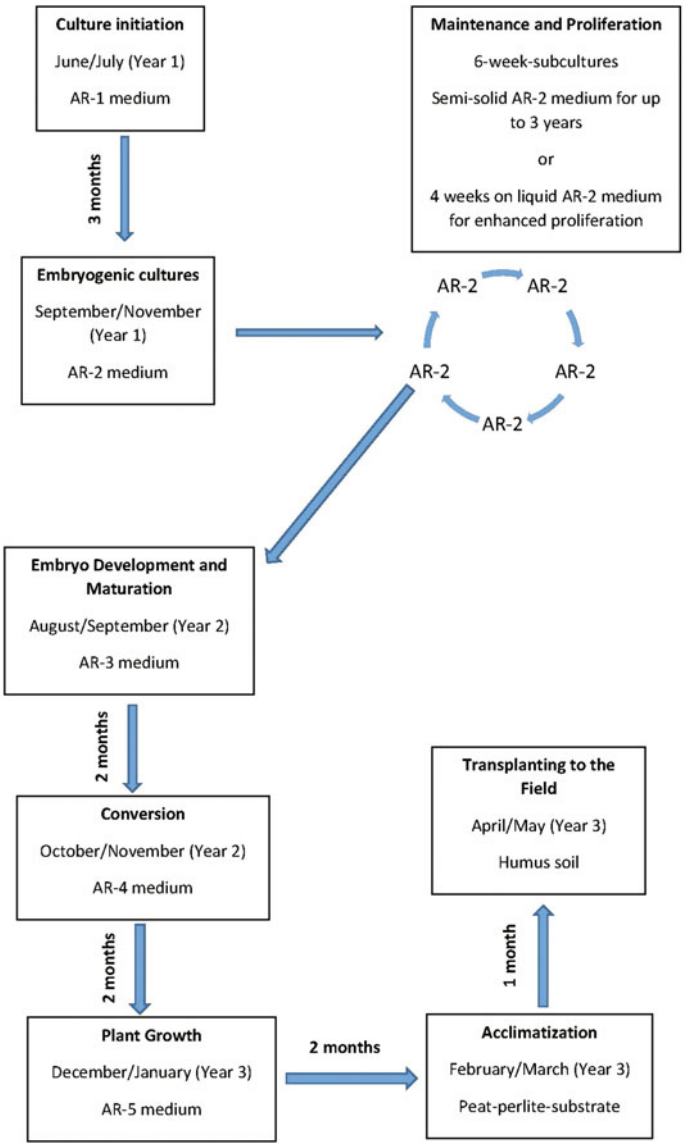

Fig. 6.2 Scheme for *Actaea* plant production via somatic embryogenesis. The mentioned time periods indicate the minimum of time necessary for this stage

6.3.3 Making of Cell Suspension Cultures

1. Chop the embryogenic mass with a scalpel.
2. Suspend 150 mg of the small clusters of the friable mass to 20 ml of liquid proliferation medium supplemented with 0.5 mg/l 2,4-D and 2% sucrose (AR-2, Table 6.2) in Erlenmeyer flasks.
3. Place the Erlenmeyer flasks on a rotary shaker with shaking at 60 rpm in darkness at 24 °C. The resulting liquid suspension culture consists of small embryogenic clusters with globular and heart stage embryos on their surfaces.
4. Transfer the embryogenic clusters to semi-solid AR-3 medium for maturation (Table 6.2) after four weeks. The suspension cultures are not further subcultured in liquid medium.

After four weeks the cell mass can increase 10 times.

6.3.4 Embryo Development, Maturation, and Conversion

The treatment at this stage is dependent on the developmental stage of the somatic embryos on AR-2 medium. Treat embryos up to heart stage differently than embryos of torpedo and cotyledonary stage.

1. Transfer small embryogenic clusters (maximum 3 mm in diameter) with mainly globular and heart stage embryos from semi-solid or liquid AR-2 medium to AR-3 medium (Table 6.2) for maturation (Fig. 6.2).
2. Incubate the clusters in darkness for at least two months.
3. Secondary embryogenesis will occur on AR-3 medium and the embryo clusters increase in size by producing more embryos of globular stage. At the same time somatic embryos of the cluster mature and reach torpedo or cotyledonary stage. Well-developed cotyledonary embryos even in clusters (Fig. 6.1d) are available for conversion after at least 2 months.
4. Separate the mature embryos of torpedo or cotyledonary stage from the clusters under the microscope and transfer them onto AR-4 medium with cytokinin (Table 6.2) in Petri dishes for conversion.
5. If on AR-2 medium already mature embryos of torpedo or cotyledonary stage are available, separate and transfer them immediately from AR-2 to AR-4 medium in Petri dishes for conversion.
6. Maintain the cultures on AR-4 medium in deem light for 16 h daily (photosynthetic active radiation: 10 µmol m^{-2} s^{-1}, fluorescent tubes Osram 35) at 24 °C for 8–10 weeks.

Nearly all embryos of cotyledonary stage will exhibit bipolar conversion (Fig. 6.1e) while from embryos of torpedo stage only around 40% may convert (Pinker and Wernicke 2017).

6.3.5 *Plant Growth in Vitro, Acclimatization, and Field Transfer*

1. Transfer bipolar converted embryos of 5–8 mm in size to Magenta jars containing 25 ml AR-5 medium (Table 6.2).
2. Cultivate the converted embryos under light for 16 h a day (photosynthetic active radiation: 35 µmol m^{-2} s^{-1}, fluorescent tubes Osram 35) at 24 °C for two months.
3. Use for acclimatization well-developed plantlets of at least 2 cm in size.
4. Transplant the morphologically normal plantlets into plastic pots (5 cm in diameter) containing a perlite-peat-substrate (1:3 v/v).
5. Cultivate them for 1–2 weeks at a photosynthetic active radiation of 83 µmol m^{-2} s^{-1} (fluorescent tubes Osram 35) at 21 °C and an air humidity above 85% in the beginning gradually reduced to 70%.
6. For the next step of acclimatization, transplant the well-rooted plantlets into pots of 7 cm diameter and cultivated them in the greenhouse under a plastic foil at photosynthetic active radiation of less than 150 µmol m^{-2} s^{-1} sunlight.
7. Expose the plantlets to greenhouse conditions for 2–3 weeks by stepwise removing the covering.
8. Transplant plants at a size of 8–10 cm (Fig. 6.1f) to the field in a humus soil in May or June.

Morphological features of the plants generated by somatic embryogenesis as plant size, plant architecture and leaf shape are not to distinguish from seed progenies but they exhibit a higher homogeneity in phenotype than the seed propagated plants.

6.4 Identify Steps Required Further Protocol Modifications

Plant regeneration via somatic embryogenesis in *Actaea racemosa* is described in this protocol. The whole process takes from culture initiation to acclimatization at least one year to produce some plants. But for plant production in field a high number of young plants of the same quality should be available in springtime or latest in early summer. Therefore, the process should be timed accordingly (Fig. 6.2).

Further protocol modifications are needed. These include: (1) accelerating and synchronization of somatic embryo induction, development and maturation; (2) minimizing the genotype effect; (3) increasing the frequency of somatic embryo germination from embryos of the torpedo stage; (4) improving the liquid culture system; (5) developing cultures in bioreactors which can be used for large-scale production of somatic embryos.

References

Aréne L, Chevalier M, Cognée N, Tellier M, Cadic A (2006) A comparative histological study between 'embryogenic structures' and zygotic embryos of *Clematis* hybrids cultivated in vitro. Acta Hortic 725:227–229. https://doi.org/10.17660/ActaHortic.2006.725.28

Beruto M, Debergh P (1992) Somatic embryogenesis in *Ranunculus asiaticus* L. hybr. thalamus cultivated in vitro. Plant Cell Tiss Org Cult 29:161–165. https://doi.org/10.1007/BF00034348

Beruto M, Giampiero C, Debergh P (1996) Field performance of tissue-cultured plants of *Ranunculus asiaticus* L. Scientia Hort 66:229–239. https://doi.org/10.1016/S0304-4238(96)00916-8

Beruto M, Viglione S, Bisignano A (2012) Micropropagation of *Helleborus* through axillary budding. In: Lambardi M, Ozudogru EA, Jain SM (eds) Protocols for micropropagation of selected economically-important horticultural plants, vol. 994 ser. Methods in molecular biology. Springer, Berlin, pp 259–267

Bittner M, Schenk R, Melzig MF (2015) Qualitative and quantitative analysis of polyphenolic compounds from clone plants of *Actaea racemosa* L. by high-performance thin layer chromatography and reversed-phase high-performance liquid chromatography. In: Poster in Planta Medica 81(16) August 2015, https://doi.org/10.1055/s-0035-156585808/2015

Bittner M, Schenk R, Melzig MF (2016) Alternative approach to species identification of *Actaea racemosa* L. (syn. *Cimicifuga racemosa* (L.) Nutt., black cohosh) herbal starting material: UV spectroscopy coupled with LDA. Phytochem Lett 18:220–225. https://doi.org/10.1016/j.phytol.2016.10.001

Bradley P (ed) (2011) Cimicifugae rhizome – Black cohosh. ESCOP Monographs, online series, ISSN 2221-9021, p 24

Elhag H, El-Olemy MM, Al-Said MS (2004) Enhancement of somatic embryogenesis and production of developmentally arrested embryos in *Nigella sativa* L. HortScience 39:321–323

Fischer S, Berti M, Wilckens R, Del Pozo A (2006) Development of vegetative propagation for *Actaea racemosa* Nutt. Ind Crops Prod 24:244–252

Giri A, Ahuja PS, Kumar PVA (1993) Somatic embryogenesis and plant regeneration from callus cultures of *Aconitum heterophyllum* Wall. Plant Cell Tiss Org Cult 32:213–218. https://doi.org/10.1007/BF00029845

Hatano K, Shoyama Y, Nishioka I (1987) Somatic embryogenesis and plant regeneration from the anther of *Aconitum carmichaeli* Debx. Plant Cell Rep 6:446–448. https://doi.org/10.1007/BF00272779

Jiang B, Kronenberg F, Nuntanakorn P, Qiu MH, Kennelly EJ (2006) Evaluation of the botanical authenticity and phytochemical profile of black cohosh products by high-performance liquid chromatography with selected ion monitoring liquid chromatography–mass spectrometry. J Agric Food Chem 54:3242–3253

Jiang B, Ma Ch, Motley T, Kronenberg F, Kennelly EJ (2011) Phytochemical fingerprinting to thwart black cohosh adulteration: a 15 *Actaea* species analysis. Phytochem Anal 22: 339–351, (wileyonlinelibrary.com) https://doi.org/10.1002/pca.1285

Lata H, Bedir E, Hosick A, Ganzera M, Khan I, Moraes RM (2002) In vitro plant regeneration from leaf derived callus of *Cimicifuga racemosa*. Planta Med 68:912–915. https://doi.org/10.1055/s-2002-34933.PubMed

Luttman R, Florek P, Preil W (1994) Silicone-tubing aerated bioreactors for somatic embryo production. Plant Cell Tiss Org Cult 39:157–170. https://doi.org/10.1007/BF00033923

Mandegaran Z, Sieber VK (2000) Somatic embryogenesis in Clematis integrifolia × C. viticella. Plant Cell Tiss Org Cult 62:163–165. https://doi.org/10.1023/A:1026514707169

Meynet J, Duclos A (1990) Culture in vitro de la renoncule des fleuristes *(Ranunculus asiaticus* L.). II. Production de plantes par culture d'antheres in vitro. Agron 10:213–218

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco cultures. Physiol Plant 15:473–479

Nomizu T, Niimi Y, Han DS (2004) Haploid plant regeneration via embryogenesis from anther cultures of *Hepatica nobilis*. Plant Cell Tiss Org Cult 79:307–313

Piagnani C, Pirola E, Pardossi A (2003) In vitro culture of *Cimicifuga racemosa*. Agr Med 133:113–116

Pinker I, Schenk R (2007) Somatic embryogenesis in *Cimicifuga racemosa.* Acta Hortic 764:265–270. http://dx.doi.org/10.17660/ActaHortic.2007.764.35

Pinker I, Brosowski S, Schenk R (2016) Somatic embryogenesis from stamens of *Actaea racemosa* L. Acta Hortic 1113:13–20. https://doi.org/10.17660/ActaHortic.2016.1113.2

Pinker I, Wernicke M (2017) Development, maturation and conversion of somatic embryos of *Actaea racemosa* L. Acta Hortic 1155:135–140. https://doi.org/10.17660/ActaHortic.2017.1155.18

Popp M, Schenk R, Abel G (2003) Cultivation of *Cimicifuga racemosa* (L.) Nuttal and quality of CR extract BNO 1055. Maturitas 44:1–7

Prakash J (2009) Micropropagation of ornamental perennials: progress and problems. Acta Hortic 812:289–294

Pugliesi C, Rabaglio M, Cecconi F, Baroncelli S (1992) Plant regeneration from tissue cultures of Persian buttercup (*Ranunculus asiaticus* L.). Plant Cell Tiss Org Cult 28:125–128. https://doi.org/10.1007/BF00055506

Szewczyk-Taranek B, Pawłowska B (2015) Recurrent somatic embryogenesis and plant regeneration from seedlings of *Hepatica nobilis* Schreb. Plant Cell Tiss Org Cult 120:1203–1207. https://doi.org/10.1007/s11240-014-0661-7

Teixeira da Silva JA, Shen M, Yu XN (2012) Tissue culture and micropropagation of tree peony (*Paeonia suffruticosa* Andr.). J Crop Sci Biotech 15:159–168. https://doi.org/10.1007/s12892-012-0040-z

Vickers A, Brosi SL, Howell J, Kaur B, Puthoff D, Eisenstein E (2015) Ecological and chemotypic analysis for improved growth and management of naturally occurring black cohosh (*Actaea racemosa* L.) populations in Western Maryland. Amer J Plant Sci 6:3272–3281. Published online Dec 2015 in SciRes. http://www.scirp.org/journal/ajps, http://dx.doi.org/10.4236/ajps.2015.619319

Weber J, Preil W, Lieberei R (1994) Somatic embryogenesis in bioreactor cultures of *Clematis tangutica*. In: Abstracts VIIIth intern congress plant tissue and cell culture, Abstr No S11-111; Firenze

Zenkteler M, Misiura E, Ponitka A (1974) Induction of androgenetic embryoids in the in vitro cultured anthers of several species. Experienta 32:289–291

Chapter 7
Cryopreservation of Tamarillo (*Solanum betaceum* Cav.) Embryogenic Cultures

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7.1 Introduction

Plant germplasm preservation is crucial to store genetic resources for both breeding and conservation programs to minimize biodiversity losses. Biotechnological resources, and in vitro cultures in particular, are progressively becoming an alternative for germplasm conservation (Engelmann 2011). Although several methods can be used, cryopreservation is the only method that ensures long-term storage (Ozudogru and Lambardi 2016).

Two commonly techniques used for cryopreservation are slow cooling and vitrification. While slow cooling (Withers and King 1980; Chen et al. 1984) uses controlled freezing rates and has mainly been used for temperate plants (Barun 2015), Plant Vitrification Solution 2 (PVS2) (Sakai et al. 1990) uses fast freezing rates and is effective for both temperate and tropical plants (Reed et al. 2004).

Tamarillo, *Solanum betaceum* Cav. (Syn *Cyphomandra betacea* (Cav.) Sendtn.) is a small solanaceous tree from the Andes, with special interest for the food industry. The Laboratory of Plant Biotechnology of the University of Coimbra in collaboration with IVALSA in Italy developed an effective protocol of somatic embryogenesis, where different explants from tamarillo have the ability to initiate embryogenic cultures (Correia and Canhoto 2012) (Fig. 7.1). This is an advantage when compared to many other species, in particular trees, that have been reported to have problems to be cloned do to their recalcitrance for somatic embryogenesis induction (Thorpe and Stasolla 2001; Bonga 2012). However, previous work have shown that embryogenic masses of tamarillo obtained from somatic embryogenesis

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_7

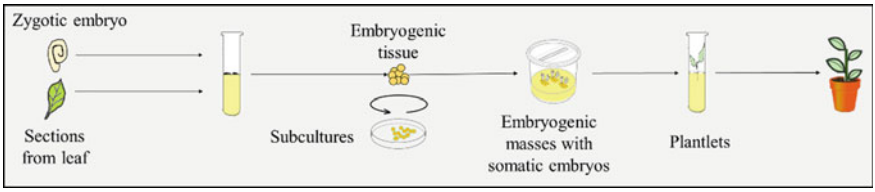

Fig. 7.1 Two-step somatic embryogenesis in tamarillo. In a first step, embryogenic masses are obtained by culturing zygotic embryos and leaf sections in appropriate induction media. These embryogenic masses may be subcultured and in a second step, the embryogenic tissues are set to develop somatic embryos and subsequently plantlets

are unstable after two years in culture, and changes in the karyotype of those cells have been observed (Currais et al. 2013). Thus, it is important to develop a strategy to maintain these tissues without modifications in the chromosome number to assure a true-to-type cloning whereas plants from the embryogenic calli can be tested in the field.

The strategical approach was to cryopreserve the tamarillo's embryogenic masses. The objective of this chapter is to describe the cryopreservation protocol for tamarillo's embryogenic masses preservation.

7.2 Cryopreservation by PVS2 Vitrification of Tamarillo Embryogenic Masses

7.2.1 Embryogenic Masses Induction and Proliferation

Tamarillo's embryogenic masses are obtained using a two-step somatic embryogenesis protocol (Canhoto et al. 2005, Fig. 7.1).

1. Somatic embryogenesis induction media depends on the origin of the explants. The media is based on MS salts (Murashige and Skoog 1962) supplemented with sucrose, hormones and a jellifying agent (Table 7.1).
2. Distribute 12 mL portions of culture media in glass test tubes (2.2 cm diameter, 15 cm high).

Table 7.1 Tamarillo induction media according to the origin of the explant

Induction medium	Origin	Basal medium	Sucrose	Plant growth regulator	Jellifying agent	pH
TD	Zygotic embryo	MS	0.25 M	9 μM 2.4D (2.4-dichlorophenoxyacetic acid)	2.5 g/L Phytagel	5.7
TP	Leaf explant	MS	0.25 M	20 μM of Picloram	2.5 g/L Phytagel	5.7

3. Expose tamarillo's zygotic embryos or young leaves explants to the appropriate induction media. Incubate in the dark at 25 °C for 10–12 weeks (after this time induced embryogenic masses are visible and easily separated from surrounding non-embryogenic masses).
4. To subculture the embryogenic masses, use the same induction media (Table 7.1). Distribute 25 mL portions of media in Petri dishes plates (100 × 20 mm).
5. Transfer the embryogenic tissues to the petri dishes and incubate in the dark at 25 °C for continuous proliferation of the tissues.

7.2.2 Cold Hardening and Preculturing

1. Obtain embryogenic masses as described above.
2. Induce cold adaptation by exposing the embryogenic masses at 4 °C in the dark for 5 days, in the same culture media in which they were subcultured (TP/TD) (Fig. 7.2a).
3. To promote cell dehydration, submit the tissues to a 3-day treatment where the cells are exposed to media with a gradual increase in sucrose concentration. Use

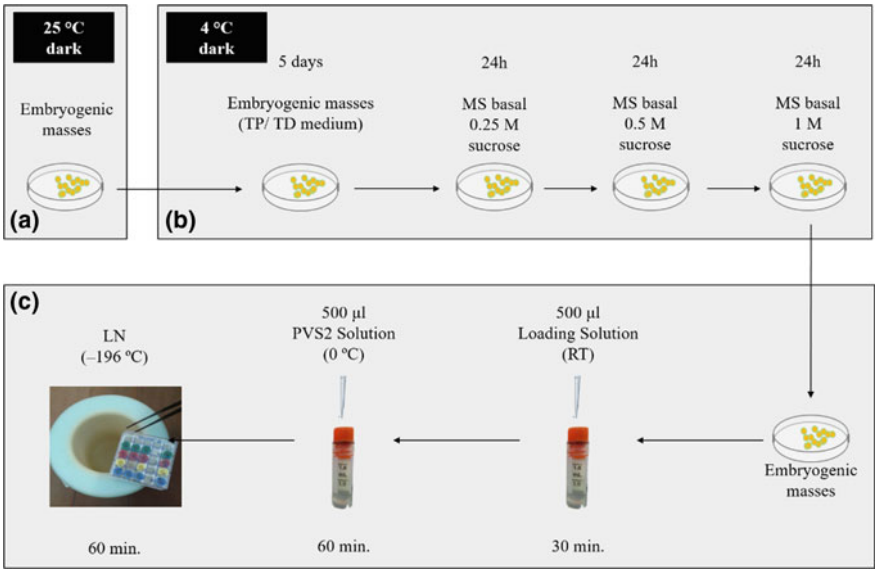

Fig. 7.2 Preparation of the embryogenic masses for cryopreservation. **a** Cold hardening, where cells are placed in the dark at 4 °C during 5 day. **b** Preculturing, a dehydration process achieved by placing the cells in MS salts with an increasing sucrose concentration. **c** PVS2 vitrification, where embryogenic masses are submitted to a loading solution for 30 min, prepared for the cryoprotective treatment with PVS2 for 60 min and submerged in liquid nitrogen

Petri dishes (100 × 20 mm) with 25 mL of a basal MS medium (pH 5.7 with 2.5 g/L of Phytagel, autoclaved 20 min at 120 °C) supplemented with: 0.25 M, 0.5 M and 1 M sucrose (Fig. 7.2b).
4. Day 1: Transfer the embryogenic masses to the Petri dishes with MS 0.25 M sucrose. Incubate 24 h in the dark at 4 °C.
5. Day 2: Remove the tissues from the MS 0.25 M sucrose media and incubate them in the Petri dishes with MS 0.5 M sucrose, 24 h in the dark at 4 °C.
6. Day 3: Remove the tissues from the MS 0.5 M sucrose media and place them in the Petri dishes with MS 1 M sucrose. Incubate 24 h in the dark at 4 °C.

7.2.3 PVS2 Vitrification

1. After preculture, transfer the embryogenic masses to cryovials (200 mg/cryovial) for the application of cryoprotectors.
2. Add 0.5 mL of loading solution [2 M glycerol (w/v) + 0.4 M Sucrose (w/v), autoclaved] to each cryovial and leave at room temperature for 30 min.
3. Remove the loading solution from cryovials and add 0.5 mL of PVS2 solution [30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% DMSO (w/v) + 0.4 M sucrose in MS salts, pH 5.7, autoclaved] to each vial and incubate at 0 °C (in ice) for 1 h.
4. Replace the PVS2 solution from cryovials with 0.5 ml of new PVS2 solution.
5. Place the vials in appropriate cryostorage equipment (for example, Nalgene® CryoBox™ Boxes) and submerge in liquid nitrogen (Fig. 7.2c).

7.2.4 Tissues Thawing and Recovery After Liquid Nitrogen Exposure

1. When in need to use the cryopreserved embryogenic masses, the cryovials are removed from the liquid nitrogen and placed immediately in a 40 °C bath for approximately 1 min. This is a very important step for the success of the technique once the defrost may damage the cells, therefore the embryogenic masses must be thawed rapidly.
2. Remove the PVS2 solution from cryovials.
3. Add 0.5 mL of washing solution (MS salts with 1.2 M sucrose, pH 5.7, autoclaved) and leave it for 30 min at room temperature.
4. Transfer the embryogenic masses to the appropriate induction media (TP and TD) and incubate in the dark at 25 °C.
5. The time for tissues recovery is 2–4 months (Fig. 7.3a, b, c). During this period, change the culture media at least at one-month intervals.
6. After the embryogenic masses recovery they are subcultured as described in Sect. 2.1.

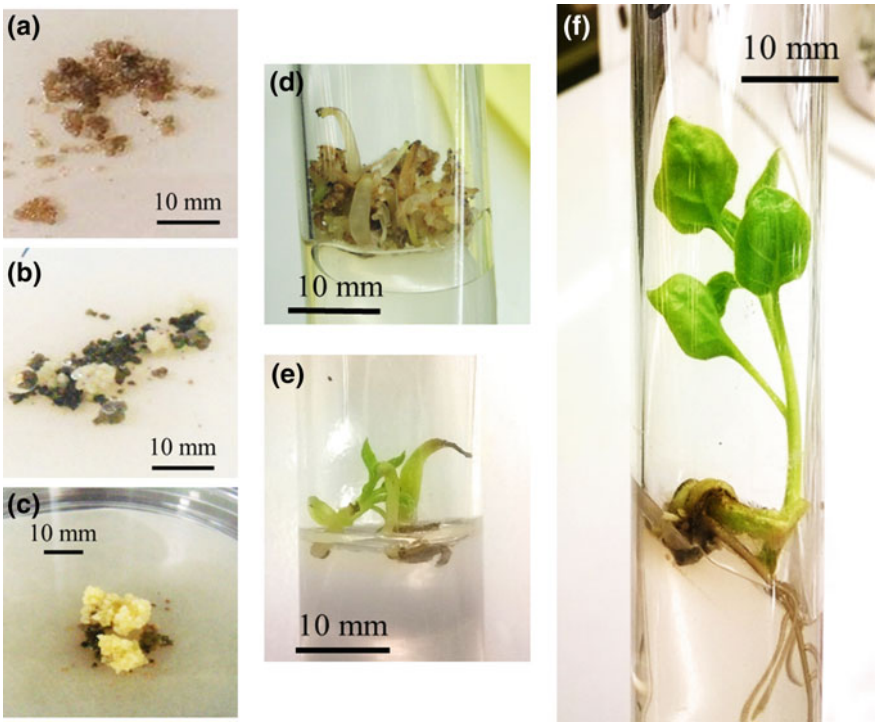

Fig. 7.3 Tamarillo's embryogenic masses in recovery after cryopreservation: **a** In the first week there is a high level of cellular death; **b** After two months the surviving cells begin to proliferate; **c** Cells with normal proliferation rates after 4 months. Final stages of the "two-step" somatic embryogenesis for the cryopreserved embryogenic tissues: **d** Somatic embryos in germination medium; **e** Embryo initializing germination observable by the green tone and by the small root; **f** Plantlet development

7.2.5 *Somatic Embryos Development and Plant Acclimatization*

1. Embryogenic masses development into somatic embryos enables the validation of the cryopreservation methods for embryogenic tissues when normal and functional embryos are obtained from the embryogenic masses after liquid nitrogen exposure. To do so, the embryogenic tissues are set to continue the somatic embryogenesis process from the subcultures (Fig. 7.3a, b, c).
2. Place the embryogenic masses into polypropylene Combiness© microboxes containers (210 mL of capacity, L filter lids) with 50 mL of MS medium with 0.11 M sucrose, 2.5 g/L of Phytagel, pH 5.7 in the dark at 25 °C.
3. After embryos maturation transfer to glass test tubes with 12 mL of MS medium with 0.08 M sucrose, 2.5 g/L of Phytagel, pH 5.7 and place them in a controlled growth chamber (25 °C, 16 h/8 h photoperiod) (Fig. 7.3d, e).

4. When with well-developed roots(Fig. 7.3f), remove from the glass test tubes, wash to eliminate all residual medium and transfer to plastic vases, with a 3:2 ratio of universal substrate and perlite, in a controlled climatic chamber (25 °C, 70% humidity and a 16 h/8 h photoperiod).

7.3 Future Research

There is still space of optimization of the cryopreservation protocol. For instance, cell dehydration may be improved by preculturing the embryogenic masses in a different sucrose gradient. There is also room to test for other exposure periods. Finally, variations of the tissues incubation periods in the vitrification solutions, particularly in PVS2, could be used to decrease the toxicity effects.

Although cryopreservation techniques are already being successfully used in several plant species and structures (Engelmann 2011; Ozudogru and Lambardi 2016), there is a need for increase efficiency (Popova et al. 2015). Research in this area is mostly performed in universities, institutes, botanical gardens and gene banks, and it is expected that new findings, especially about physiological mechanisms, will contribute expressively in the development of the techniques.

References

Barun S (2015) A review on applications & advantages of cryopreservation in different fields of science. The Beats Nat Sci 2:1–6

Bonga JM (2012) Recalcitrance in the in vitro propagation of trees. In: Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management (IUFRO, ed), p 37, Brno, Czech Republic

Canhoto J, Lopes M, Cruz G (2005) Protocol of somatic embryogenesis: tamarillo (*Cyphomandra betacea* (Cav.) Sendt). In: Jain S, Gupta P (eds), Protocol for somatic embryogenesis in woody plants, pp 379–389. Springer, Berlin

Chen TH, Kartha KK, Leung NL, Kurz WG, Chatson KB, Constabel F (1984) Cryopreservation of alkaloid-producing cell cultures of periwinkle (*Catharanthus roseus*). Plant Physiol 75:726–731

Correia SI, Canhoto JM (2012) Biotechnology of tamarillo (*Cyphomandra betacea*): from in vitro cloning to genetic transformation. Scientia Hort 148:161–168

Currais L, Loureiro J, Santos C, Canhoto JM (2013) Ploidy stability in embryogenic cultures and regenerated plantlets of tamarillo. Plant Cell Tissue Org Cult 114:149–159

Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. Vitro Cell & Dev Biology - Plant 47:5–16

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plant 15:473–497

Ozudogru EA, Lambardi M (2016) Cryotechniques for the long-term conservation of embryogenic cultures from woody plants. In: Germanà M, Lambardi M (eds) In vitro embryogenesis in higher plants. Springer, Berlin, pp 537–550

Popova E, Shukla M, Kim HH, Saxena PK (2015) Plant cryopreservation for biotechnology and breeding. In: Al-Khayri JM, Jain SM, Johnson DV (eds) Advances in plant breeding strategies: breeding, biotechnology and molecular tools. Springer, Berlin, pp 63–93

Reed BM, Kovalchuk I, Kushnarenko S, Meier-Dinkel A, Schoenweiss K, Pluta S, Straczynska K, Benson EE (2004) Evaluation of critical points in technology transfer of cryopreservation protocols to international plant conservation laboratories. CryoLetters 25:341–352

Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. brasiliensis Tanaka) by vitrification. Plant Cell Rep 9:30–33

Thorpe TA, Stasolla C (2001) Somatic embryogenesis. In: Thorp TA (ed) Current trends in the embryology of angiosperms. Kluwer Academic Publishers, Dordrecht, pp 279–336

Withers LA, King P (1980) A simple freezing unit and routine cryopreservation method for plant-cell cultures. Cryo-letters 1:213–220

Chapter 8
Coconut (*Cocos nucifera* L.) Somatic Embryogenesis Using Immature Inflorescence Explants

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8.1 Introduction

Coconut (*Cocos nucifera* L.) is a high value perennial crop, cultivated in several countries, contributing considerably to the improvement of nutrition, food security, employment and income generation (Sáenz-Carbonell et al. 2016). The commercial importance of coconut has been growing very rapidly over the last fifteen years, with high value products such as packed coconut water, virgin oil, coconut milk products, coco-biodiesel, fiber derivatives for the automobile industry and horticulture (Roolant 2014; Lao 2009). The loss of coconut trees, due to old age, phytosanitary threats, in particular phytoplasma diseases such as lethal yellowing (LY), has led to a reduction in coconut production (Batugal et al. 2005). In order to maintain the growing markets and increasing demand for coconut products, replanting of most of the cultivated land worldwide as well as the establishment of new cultivated land, is urgently needed. This immense task cannot be accomplished by traditional propagation through seed alone and would require in vitro propagation by somatic embryogenesis (SE) given its exceptional propagation capacity. This method has been applied for the development of highly efficient and commercially viable protocols in different laboratories worldwide. Different types of coconut explants have been tested, but most of the research carried out has focused on rachilla explants from immature inflorescence (Hornung and Verdeil 1999) and plumule explants from zygotic embryos (Sáenz et al. 1999).

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_8

In the case of rachilla, in studies carried out during the nineties, only limited success was achieved (Blake and Hornung 1995). Formation of callus and somatic embryos was observed, but subsequent germination to form clonal plantlets was scarce and random (Blake 1990; Blake and Hornung 1995). Further research using rachilla facilitated the development of a reproducible regeneration protocol, but the efficiency for the formation of somatic embryos and plantlet conversion was not determined (Verdeil et al. 1994).

During the nineties, collaborative research involving Wye College and CICY showed that plumular explants were more responsive than rachilla explants for the formation of embryogenic callus (Blake et al. 1994; Hornung 1995). Further studies with plumule explants allowed the development of a protocol that was efficient and reproducible for the formation of embryogenic callus, somatic embryos and conversion into plantlets (Chan et al. 1998). CICY continued to work on the improvement of coconut micropropagation from plumule explants and developed a process based on the incorporation of multiplication of embryogenic callus and secondary somatic embryogenesis, with which it is possible to produce tens of thousands of somatic embryos from a single plumule explant (Perez-Núñez et al. 2006; Sáenz et al. 2006).

With the use of plumule explants it is possible to clone the progeny of selected parents, but at the stage of zygotic embryos. However, if the aim is to propagate adult palms, already producing nuts, whose performance has been established (e.g. productivity or resistance to diseases), a different source of explants from these palms must be used. Hence, recent studies have tested floral tissue explants other than rachillae such as anther (Perera et al. 2008) and unfertilized ovaries isolated from immature female flowers of coconut (Perera et al. 2007) with promising results for the formation of somatic embryos and plantlets. Floral tissues were tested in CICY also. Results showed that explants obtained from rachilla of immature inflorescences (Figs. 8.1 and 8.2a) formed calluses with necrosis or spongy tissue; however, some also showed pearly white structures resembling embryogenic structures (Sandoval-Cancino et al. 2016), similar to those formed in embryogenic calluses previously reported by Pérez-Núñez et al. (2006) and Sáenz et al. (2006). Isolated pearly white structures were subcultured repeatedly until embryogenic calluses (similar to those reported by Pérez-Núñez et al. 2006; Sáenz et al. 2006) were obtained (Fig. 8.2b). These embryogenic calluses were capable of forming somatic embryos (Fig. 8.2b, c) that germinated (Fig. 8.2d), formed shoots (Fig. 8.2e) and converted into plantlets (Fig. 8.2f, g). In addition, the embryogenic structures of the calluses and the somatic embryos formed, could be used as explants to form new embryogenic callus. These options open up the possibility of developing a massive propagation process similar to that developed for plumule and currently being scaled up in México (Oropeza et al. 2016).

Fig. 8.1 Collection of inflorescences from a coconut palm. Select a palm of interest (**a**). Detach leaves to obtain a cylinder above the stem apex that contains the immature inflorescences (**b**, **c**). Collect inflorescences at different stages of development (**d**). Rinse them with ethanol and water (**e**, **f**), wrap them with paper (**f**), place them in a plastic bag and keep in an icebox (**g**), for transportation to the laboratory

Here we described the protocol for coconut micropropagation by somatic embryogenesis, using rachilla explants from immature inflorescence, that was developed at CICY, Mérida, México, based on our research (Pérez-Núñez et al. 2006; Sáenz et al. 2006; Sandoval-Cancino et al. 2016).

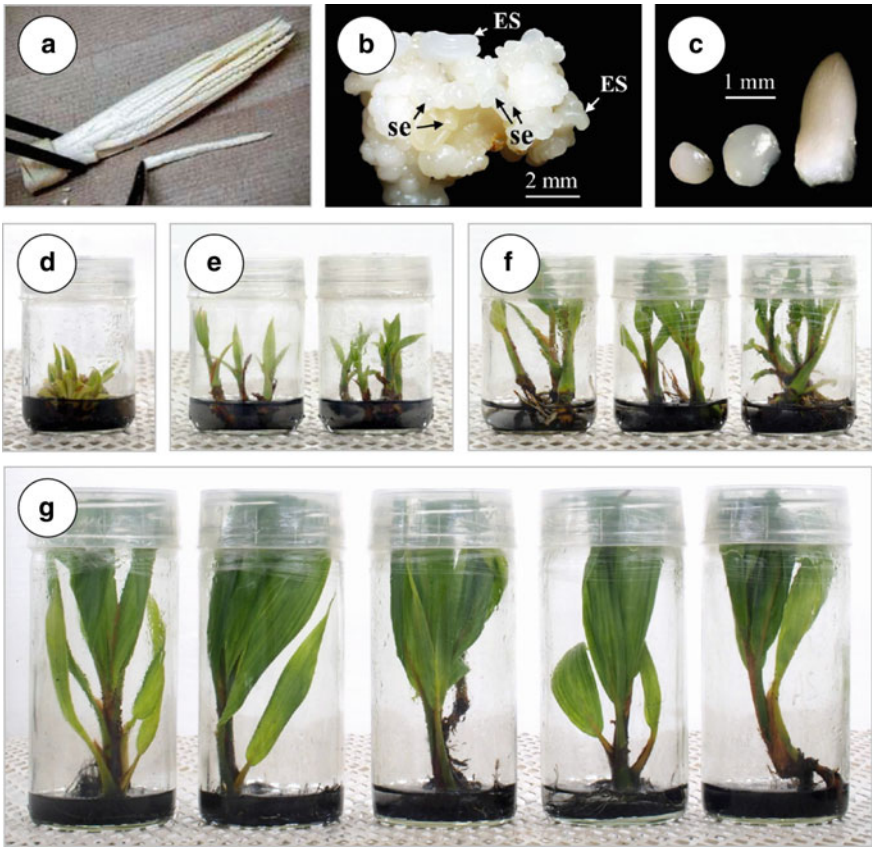

Fig. 8.2 Plantlet production from coconut rachilla explants. An immature inflorescence (without spathe) shown during rachillae dissection (**a**). Embryogenic callus at 90 days of culture, with embryogenic structures (ES) and somatic embryos (SE) growing on the embryogenic structures (**b**). Isolated somatic embryos shown at different stages of development (**c**). Embryogenic callus with growing shoots (**d**). Shoots isolated from callus cultured for development (**e**, **f**). Plantlets ready for acclimatization (**g**)

8.2 Materials

a. Rachillae from immature inflorescences of coconut at different developmental stages −4 to −11, with 0 corresponding to the youngest opened inflorescence (Perera et al. 2010).
b. Culture media based on Y3 medium (see Tables 8.1 and 8.2).
c. Ethanol 70% (v/v), sterile distilled water, commercial bleach 30 g L^{-1} NaOCl.
d. Antioxidant Solution: ascorbic acid (0.15 g L^{-1}), citric acid (0.1 g L^{-1}), and sucrose (30 g L^{-1}).
e. Growth rooms, laminar flow hoods, glass containers of different sizes, Petri dishes, tweezers, scalpels and other assorted materials.

Table 8.1 Formulation for the preparation of Y3 medium (basic culture medium)

Stock solution	Reactive	Amount (gr/l)	Volume used (ml/l)
1	NH_4Cl	26.8	20
	KNO_3	101.2	
2	KCl	149.6	10
	H_2NaPO_4	27.56	
	H_3B0_3	0.3092	
3	$MgS0_4{\cdot}7H_2O$	24.8	10
	$MnSO_4$	0.848	
	KI	0.832	
	$CuSO_4$	0.0248	
	$ZnSO_4{\cdot}7H_2O$	0.72	
4	$NiCl_2{\cdot}6H_2O$	0.0024	10
	$CaCl_2{\cdot}2H_2O$	29.4	
	$CoCl_2$	0.024	
	$Na_2MoO_4{\cdot}2H_2O$	0.024	
5	$C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$	3.724	10
	$Fe_2SO_4{\cdot}7H_2O$	1.5	
6	Thiamine	0.1012	10
	Pyridoxine	0.1028	
	Nicotinic acid	0.1	
	Myo-Inositol	10	
	L-Aspargine	8.8	
	L-Arginine	10	
	L-Glutamine	10	

Table 8.2 Chemicals added to Y3 medium for the preparation of the different media used. EC, embryogenic callus; SE, somatic embryos

Chemicals	I[a]	II[a]	III[a]	IV[a]	V[a]
	Induction of EC	Formation of SE	Germination of SE	Formation of shoots	Plantlet growth
Basic culture medium	Y3	Y3	Y3	Y3	Y3
Gel rite	0.3%	0.3%	0.3%	0.3%	–
Charcoal	0.25%	0.25%	0.25%	0.25%	0.25%
2,4-Dichlorophenoxyacetic acid (2,4-D)	0.6 mM	0.325 mM	0.006 mM	0.006 mM	–
6-Benzylamino-purine (6-BAP)	–	–	0.3 mM	0.3 mM	–
Gibberellic acid (GA_3)	–	–	0.0046 mM	–	–
Sucrose	5%	5%	5%	5%	5%

[a]Once all medium components are mixed, adjust pH to 5.75 and sterilize

8.3 Methods

8.3.1 Collection of Explant Source Tissues

1. Select mature palms according to your interests (e.g. disease resistance, productivity, etc.) (Fig. 8.1a). Cut the plant down and detach the leaves to expose the immature inflorescences, including those that have already emerged and those that have not yet emerged (Fig. 8.1b, c).
2. Select the unopened immature inflorescences of developmental stages −4 to −11 (according to Perera et al. 2010) (Fig. 8.1d).
3. Immediately after collection, the unopened inflorescences (still covered with the outer spathe) are rinsed with 70% (v/v) ethanol/water (Fig. 8.1e).
4. Wrap each inflorescence with moisture absorbent paper (Fig. 8.1f), place them in a plastic bag and store inside an ice box for safe transportation (Fig. 8.1g).

8.3.2 Media Preparation

1. For the preparation of the basic culture medium Y3 (Eeuwens 1976), prepare the corresponding stock solutions as shown in Table 8.1.
2. Then, for each of the different media used (I–VI) supplement the basic Y3 formulation with growth regulators and other components as shown in Table 8.2.
3. Once all components are mixed for each medium, adjust pH to 5.75 before autoclaving for 20 min at 120 °C.

8.3.3 In Vitro Culture

1. Under aseptic laboratory conditions, in laminar a flow hood, the immature inflorescences with inner spathe are washed in 70% (v/v) ethanol/water for 3 min, rinsed three times with sterile distilled water, washed again in commercial bleach (30 g L^{-1} NaOCl) for 20 min, and finally rinsed three times with sterile water.
2. The inner spathe is removed and the rachillae are separated from the exposed inflorescences and placed in a sterile antioxidant solution. The rachillae are then dissected from the inflorescences (Fig. 8.2a) and 0.3–0.5 mm rachilla slices are obtained and placed directly on culture medium I for 90 days, under complete darkness at 27 ± 2 °C and without subculturing. At the end of this culture period a callus is formed consisting of spongy tissue and pearly white structures.

3. The pearly white structures are subcultured twice, each time in medium I for 90 days, under complete darkness at 27 ± 2 °C. At the end of this period, embryogenic callus is formed (Fig. 8.2b).
4. The embryogenic callus is transferred to medium II for 30 days, under complete darkness at 27 ± 2 °C and without subculturing. During this period somatic embryos are formed (Fig. 8.2c) and start germinating (Fig. 8.2d).
5. Subculture in medium III for 90 days, under a 16-h photoperiod (45–60 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density [PPFD] provided by Tri-Phosphor (F32T8, 6500 K, 32 W) daylight tubes (MAGG^{MR}, Tlatilco, Mexico) at 27 ± 2 °C. During this culture period shoots start growing (Fig. 8.2e).
6. Transfer to medium IV for 120 days, under a 16-h photoperiod (45–60 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density [PPFD] provided by Tri-Phosphor (F32T8, 6500 K, 32 W) daylight tubes (MAGG^{MR}, Tlatilco, Mexico) at 27 ± 2 °C, subculturing every 2 months. During this time shoots continue growing and at the end of the period they are separated from the callus for individualized growth and further development (Fig. 8.2f).
7. Transfer to medium V for 180 days, under a 16-h photoperiod (45–60 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density [PPFD] provided by Tri-Phosphor (F32T8, 6500 K, 32 W) daylight tubes (MAGG^{MR}, Tlatilco, Mexico) at 27 ± 2 °C, subculturing every 2 months. During this period plantlets develop and by the end of the period they are ready for ex vitro acclimatization (Fig. 8.2g).

8.4 Conclusions and Future Prospects

A protocol for the micropropagation of coconut by somatic embryogenesis from rachilla explants is described. This protocol is based on the formation of embryogenic callus (Sandoval-Cancino et al. 2016) through a stepwise process of subculturing the white pearly structures that grow on the explants. Initially, these structures are scarce, but they are progressively more abundant in the new calluses formed, until it is possible to obtain fully embryogenic calluses. These are able to form somatic embryos that germinate and develop into plantlets in a reproducible fashion. With this protocol it is possible to clone fruit-bearing palms of interest due to their productivity, disease resistance, etc. Further improvement of the protocol is necessary, which includes: (a) higher efficiency rates in the formation of embryogenic callus and somatic embryos and their germination, (b) in vitro hardening of the plantlets so they can be more easily transferred to ex-vitro conditions, (c) testing new approaches for somatic embryo formation such as suspension culture, and (d) scaling up of the process for commercial production.

References

Batugal P, Rao RV, Oliver J (2005) Coconut genetic resources. International Plant Genetic Resources Institute—Regional Office for Asia, the Pacific and Oceania (IPGRI-APO). Serdang, Selangor DE, Malaysia, pp XII–XIV

Blake J (1990) Coconut (*Cocos nucifera* L.): micropropagation. In: Bajaj YPS (ed) Legumes and oilseed crops I. Biotechnology in agriculture and forestry, vol 10. Springer, Berlin, pp 538–554

Blake J, Hornung R (1995) Somatic embryogenesis in coconut (*Cocos nucifera* L.). In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants, vol 2. Kluwer Academic Publishers, Dordrecht, pp 327–340

Blake J, Robert M, Taylor F, Navarro C, Hornung R, Oropeza C (1994) Studies on the *in vitro* propagation and cloning of elite and disease resistant coconut palms. Final Report (The European Commission, Project C11.0764.M)

Chan JL, Sáenz L, Talavera C, Hornung R, Robert M, Oropeza C (1998) Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis. Plant Cell Rep 17:515–521

Eeuwens CJ (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut (*Cocos nucifera*) and date (*Phoenix dactylifera*) palms cultured *in vitro*. Physiol Plant 36:23–28

Hornung R (1995) Micropropagation of *Cocos nucifera* (L.) from plumular tissue excised from mature zygotic embryos. Plant Rech Dev 2:38–41

Hornung R, Verdeil JL (1999) Somatic embryogenesis in coconut from immature inflorescence plumular explants. In: Oropeza C, Verdeil JL, Ashburner GR, Cardeña R, Santamaría J (eds) Current advances in coconut biotechnology. Kluwer Academic Publishers, The Netherlands, pp 297–308

Lao DA (2009) Coco-biodiesel in the Philippines. In: Coconut Philippines published by Asia Outsourcing

Oropeza C, Sáenz L, Chan L, Sandoval G, Pérez-Núñez G, Narvaez M, Rodríguez G, Borroto C (2016) Coconut micropropagation in México using plumule and floral explants. Cord 32:21–26

Perera PIP, Hocher V, Verdeil JL, Doulbeau S, Yakandawala DMD, Weerakoon LK (2007) Unfertilized ovary: a novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis. Plant Cell Rep 26:21–28

Perera PIP, Perera L, Hocher V, Verdeil JL, Yakandawala DMD, Weerakoon LK (2008) Use of SSR markers to determine the anther-derived homozygous lines in coconut. Plant Cell Rep 27:1697–1703

Perera PIP, Hocher V, Weerakoon LK, Yakandawala DMD, Fernando SC, Verdeil JL (2010) Early inflorescence and floral development in Cocos nucifera L. (Arecaceae: Arecoideae). S Afr J Bot 76(3):482–492

Pérez-Núñez MT, Chan JL, Sáenz L, González T, Verdeil JL, Oropeza C (2006) Improved somatic embryogenesis from *Cocos nucifera* (L.) plumule explants cultured in vitro. *In Vitro* Cell Develop Biol—Plant 42:37–43

Roolant L (2014) https://transferwise.com/blog/2014-05/why-coconut-water-is-now-a-1-billion-industry/[02/11/2015]. Why coconut water is now a one billion industry

Sandoval-Cancino G, Sáenz L, Chan JL, Oropeza C (2016) Improved formation of embryogenic callus from coconut immature inflorescence explants. *In vitro* Cell Develop Biol—Plant 52:367–378

Sáenz L, Oropeza C, Chan JL, Hornung R, Rillo E, Verdeil JL (1999) The use of plumular explants for the regeneration of coconut. In: Oropeza C, Verdeil JL, Ashburner GR, Cardeña R, Santamaría J (eds) Current advances in coconut biotechnology. Kluwer Academic Publishers, The Netherlands, pp 309–319

Sáenz L, Azpeitia A, Chuc-Armendariz B, Chan JL, Verdeil JL, Hocher V, Oropeza C (2006) Morphological and histological changes during somatic embryo formation from coconut plumule explants. *In vitro* Cell Develop Biol—Plant 42:19–25

Sáenz-Carbonell L, Montero-Cortés M, Pérez-Nuñez T, Azpeitia-Morales A, Andrade-Torres A, Córdova-Lara I, Chan-Rodríguez JL, Sandoval-Cancino G, Rivera-Solis G, Oropeza-Salín C (2016) Somatic Embryogenesis in *Cocos nucifera* L. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Somatic embryogenesis: fundamental aspects and applications. Springer International Publishing, Switzerland, pp 297–318

Verdeil JL, Huet C, Grosdemange F, Buffard-Morel J (1994) Plant regeneration from cultured immature inflorescences of coconut (*Cocos nucifera* L.): evidence for somatic embryogenesis. Plant Cell Rep 13:218–221

Chapter 9
Somatic Embryogenesis in Cherry (*Prunus* sp.)

Kaouther Ben Mahmoud, Yordan Muhovski, Fabienne Delporte, Ahmed Jemmali and Philippe Druart

9.1 Introduction

Cherry is a member of the *Rosaceae* family, subfamily *Prunoideae*, subgenus *Cerasus.* It is the common name of several *Prunus* species such as *P. avium*, *P. cerasus*, *P. mahaleb*, *P. serotina*, *P. serrulata*, *P. incisa* and many interspecific hybrids (*P. canescens* x *P. incisa, P. avium* x *P. cerasus*, *P. incisa* x *serrula*, etc.). Cherry is one of the economically important species used for direct fruit production or as rootstock for many cherry varieties (Brown et al. 1996). The estimated world annual production of cherries is over 3 million tons, and has increased steadily since 1990. Turkey is the leading producer of cherries, followed by the USA and Russia (Jayasankar and Kappel 2011).

Cherry can be propagated by seeds only for breeding, as this leads to genetic segregation due to its heterozygous nature. Therefore, for better homogeneity, this species can only be multiplied by vegetative means. Although vegetative propagation by conventional methods does not guarantee the phytosanitary status of the results, in vitro tissue culture could be considered as an alternative method. Among tissue culture techniques, somatic embryogenesis is preferred, especially for woody plants that have a long life cycle and are difficult to propagate by conventional methods (Isah 2016). Compared to organogenesis, somatic embryogenesis may offer many advantages for breeding programs thanks to the single-cell origin of the regenerated embryos, as well as for large-scale production of embryos in

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_9

bioreactors (Giri et al. 2004). Moreover, somatic embryos have a bipolar structure (stem and root) that allows them to regenerate directly as rooted plantlets (Von Arnold et al. 2002).

However, the application of somatic embryogenesis in a wide range of woody plants is limited by many difficulties. First of all, the ability of cells to acquire embryogenic capacity depends on whether the genotype is competent or recalcitrant (Verdeil et al. 2007; Isah 2016). Complete recalcitrance to somatic embryogenesis is encountered in various groups, families and genera such as *Prunus* (Druart 1999). Even competent genotypes may show some limitations during the different steps of the embryogenic process. Among these limitations, the poor conversion rate of induced embryos to plantlets constitutes the major problem. These embryos tend to show a variety of morphological abnormalities, including variation in size, in shape and in number of cotyledons in later stages. These abnormalities hamper their further development in the used culture media, as there generation of somatic embryos depends on induction conditions, among which hormonal balance in culture media has a primary role (Feher et al. 2003). Moreover, other medium components such as carbohydrates are known to influence somatic embryo induction and expression. In this regard, it is well documented that specific carbohydrates may have differential effects on morphogenesis in *Prunus* genera, such as *Prunus persica* (Raj Bhansali et al. 1990), *Prunus avium* (Reidiboym-Talleux et al. 1999) and *Prunus incisa* x *serrula* (Druart 1999). Also, modification of MS salt solution has been reported to influence embryogenic induction and embryo quality (Lee et al. 2001; Samson et al. 2006).

This chapter focuses on the study of differential reactions to somatic embryogenesis in recalcitrant genotype CAB 6P and competent genotype No 131 at the morphological and molecular levels. CAB 6P (*Prunus cerasus*) is a dwarfing cherry rootstock. Genotype 131 (*Prunus incisa*) is a parental genotype of cherry dwarfing rootstock 'Inmil' (*P. incisa* x *serrula*) and is known for its embryogenic capacity. It has therefore been used as a positive control.

9.2 Somatic Embryogenesis Protocols

9.2.1 Plant Material and Culture Conditions

Genotype CAB 6P belongs to the *Prunus cerasus* collection located in the Emilia Romagna region of Italy. It was introduced by the Laboratory of Plant Biotechnology and Physiology of the National Agronomic Research Institute of Tunisia (INRAT) as a dwarfing cherry rootstock. Genotype 131 (*Prunus incisa*) belongs to a *Prunus* collection established by the Wallon Agricultural Research Centre (CRA-W) in Gembloux (Belgium). Plant materials from these two genotypes CAB 6P and 131 were introduced in vitro through meristem culture and proliferated through axillary branching according to methods and culture conditions

described elsewhere for fruit tree micropropagated (Druart 2003). Cultures of mother shoots were obtained in a growth chamber under a 16-hour photoperiod provided by a fluorescent light (Sylvania Grolux F36W) and at a constant temperature of 23 ± 1 °C. Roots and leaves derived from in vitro plantlets were used as explants for induction of somatic embryogenesis and incubated in Petri dishes (9 cm Ø) containing 25 ml of culture media. Before culturing, leaf explants were wounded at three equidistant sites on the adaxial blade surface across the midrib.

9.2.2 Somatic Embryogenesis in Genotype CAB 6P

9.2.2.1 Protocol 1

Explants consisting of roots and leaves were cultured in the dark for 4 weeks, at a temperature of 23 ± 1 °C, on MS (Murashige and Skoog 1962) medium containing 87.6 mM sucrose with added AIA or 2,4-D at different concentrations (1, 2 or 5 μM) combined with 0.2 μM BAP. These cultures were then transferred to light on MS medium containing 0.4 μM BAP and 0.05 μM ANA.

No embryogenic reaction was obtained. These explants were limited to forming amorphous compact calli without any morphogenic reaction.

9.2.2.2 Protocol 2

In the absence of any embryogenic reaction under the preceding conditions, we tested the following protocol inspired by the work of Druart (1999), which has proved favourable for other species such as *Prunus incisa*. Root and foliar explants were subjected to a pretreatment for 15 days at a low temperature (4 °C) in a solution which was poor in nutrients but highly concentrated in sugar (2.5 mM NH_4NO_3, 2 μM 2.4-D and 175.2 mM sucrose). These explants were then transplanted onto a medium containing 87.6 mM sucrose with the addition of 1 μM ANA in combination with BAP at different concentrations (2.2, 4.4 or 8.8 μM). The genotype never expressed any embryogenic reaction and then displayed recalcitrant behaviour which consists in the formation of the compact callus. However, some calli that had neoformed from root explants gave rise to budding nodules (Fig. 9.1).

9.2.2.3 Protocol 3

The recalcitrant behaviour of CAB6P to somatic embryogenesis was confirmed during this last trial based on the comparison of CAB 6P with the embryogenic control cherry genotype 131 of *Prunus incisa*, both cultured under the same conditions. In this experiment, leaves were used as explants, and cultured for 10, 20, 30 and 40 days in the darkonto MS medium with added picloram at different

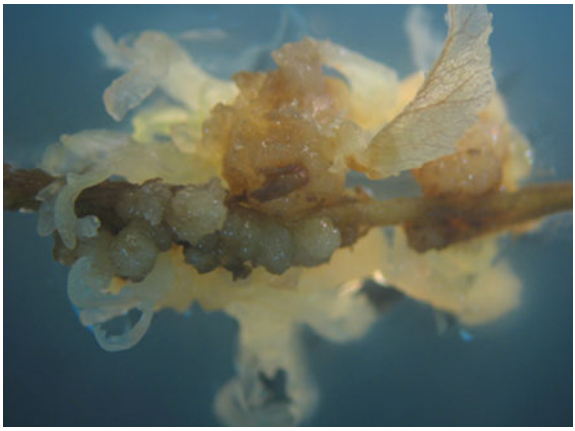

Fig. 9.1 Nodules observed on root explants of genotype CAB 6P (*Prunus cerasus*) pretreated at 4 °C in a solution composed of 2.5 mM NH_4NO_3, 2 μM 2.4-D and 175.2 mM sucrose for 15 days, and then cultured onto MS medium containing 87.6 mM sucrose with the addition of 1 μM ANA in combination with BAP at different concentrations (2.2, 4.4 or 8.8 μM)

concentrations (0, 2, 4 and 6 μM). Picloram, which is known for its auxin-like activity, could be more effective than other auxins such as 2,4-D at inducing somatic embryogenesis (Steinmacher et al. 2007). After the induction phase, these explants were transplanted onto MS expression medium supplemented with 0.4 μM BAP and 0.05 μM ANA.

The results confirmed those of the previous protocols concerning the recalcitrance of the CAB 6P genotype to somatic embryogenesis process; this genotype was unable to express any embryogenic reaction. Conversely, genotype 131 confirmed its embryogenic ability and started to produce embryogenic callus after approximately four weeks of culture (Fig. 9.2). The highest rate of embryogenesis was registered in explants cultured for 30 days (D3) with the addition of 4 μM picloram (Table 9.1). Moreover, the results highlighted a tendency for the duration of induction to be inversely correlated with the concentration of picloram.

9.2.3 Protocols of Somatic Embryogenesis in Genotype 131

9.2.3.1 Protocol 1

In this first protocol, leaf explants were cultured for 30 days on inductive medium consisting of MS base medium supplemented with 4 μM picloram but varying carbohydrate sources. We used sucrose (S), glucose (G), fructose (F) or maltose (M) at different concentrations: 58.4 (C_1), 87.6 (C_2) or 116.85 (C_3) mM. Explants were incubated in the same conditions as described above. The expression step of somatic embryogenesis was realized by the transfer of the obtained calli into MS medium supplemented with 0.44 μM BAP and 0.005 μM NAA, and their exposure to a 16-hour photoperiod provided by a fluorescent light (Sylvania Grolux F36W) at 23 ± 1 °C.

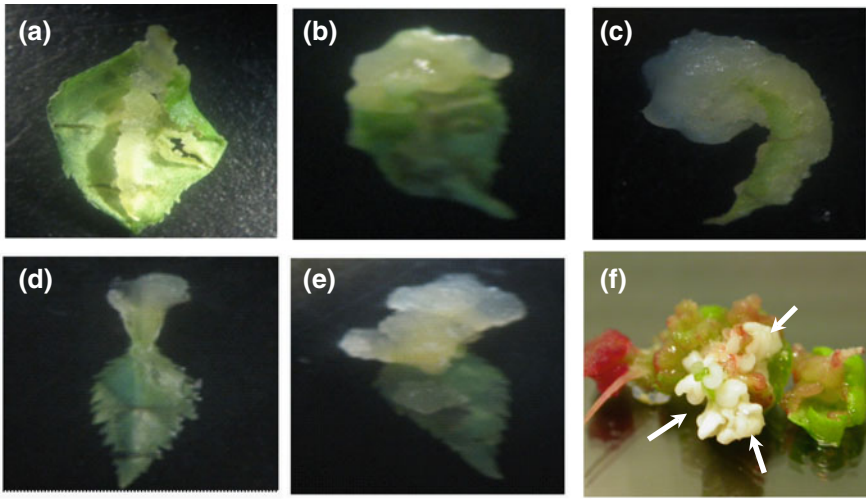

Fig. 9.2 Morphogenic responses of CAB 6P (*P. Cerasus*) (**a**, **b**, **c**) and 131 (*P. incisa*) (**d**, **e**, **f**) leaf explants cultured on MS medium containing 4 μM picloram: **a** swelling of the midrib vein of leaf on 10th day of culture, **b** callus formation at petiolar site of leaf on 15th day, **c** amorphous callus invades leaf on 25th, **d** swelling of the midrib vein of leaf, **e** callus formation at wounded sites of leaf on 15th day, **f** differentiation of proembryos (arrows) on 25th day of culture

Table 9.1 Effect of the interaction between duration of induction and picloram concentration on the frequency of embryogenic leaves in two genotypes CAB 6P and 131

	Frequency of embryogenic leaves (%)	
Interaction duration of induction*picloram concentration	CAB 6P	131
D_1*P_1	–	–
D_1*P_2	–	0.8^{e}
D_1*P_3	–	5^{e}
D_1*P_4	–	5^{e}
D_2*P_1	–	–
D_2*P_2	–	3.3^{e}
D_2*P_3	–	6.7^{e}
D_2*P_4	–	22.5^{b}
D_3*P_1	–	–
D_3*P_2	–	2.5^{e}
D_3*P_3	–	32.5^{a}
D_3*P_4	–	15.8^{c}
D_4*P_1	–	–
D_4*P_2	–	$13.3^{c, d}$
D_4*P_3	–	$7.5^{d, e}$
D_4*P_4	–	$7.5^{d, e}$

D_1: 10 days; D_2: 20 days; D_3: 30 days; D_4: 40 days; P_1: pic (0 μM); P_2: pic (2 μM); P_3: pic (4 μM) and P_4: pic (6 μM)

Means in the same column followed by the same letter are not significantly different at $P < 0.05$ according to the Newman-Keuls test. Different letters (a, b, c, d and e) denote significant differences

The results showed that callogenesis was the first response of the leaves cultured in picloram-containing medium supplemented with sucrose, glucose and fructose. However, no callus appeared in the presence of maltose. Somatic embryogenesis has shown to be affected by various carbohydrate kinds and concentrations. It was induced in picloram-containing medium supplemented with all the sugars used, but at different rates. Frequencies (%) of embryogenic explants were significantly affected by carbohydrate sources and concentrations (Table 9.2). Two interactions (F^*C_1 and F^*C_2) were significantly superior to all other combinations for this parameter. This result is in agreement with findings in *Citrus* (Tomaz et al. 2001) and *Coffea canephora* (Fuentes et al. 2000), where fructose has also been found efficient for somatic embryogenesis. Control represented by the interaction S^*C_2 recorded 39% embryogenic leaves. Sucrose has also induced embryogenesis in other plant species such as *Olea europea* (Shibli et al. 2001), *Glycin max* (Omid et al. 2008), *Tylophora indica* (Dennis 2006). Sucrose is the most commonly used sugar for somatic embryogenesis and it partially acts as an essential source of energy supply in tissue-cultured cells and partly as an osmoticum (Lou and Kako 1995). These comparable effects of sucrose and fructose are not intriguing because fructose is a product of sucrose hydrolysis. The worst result was obtained with maltose at all concentrations and particularly at 58.4 mM (C_1), where embryogenesis was completely inhibited. It has been found to be inhibitory to somatic embryogenesis in *Coffea* (Fuentes et al. 2000). Its metabolism produces glucose but more slowly than sucrose (Fuentes et al. 2000), and it may be suggested that this sugar was not easily available for cells during the induction period.

In our culture conditions, the morphology of somatic embryos seemed to be also affected by carbohydrate source and concentration (Table 9.3). Regenerated

Table 9.2 Effect of interaction carbohydrate source*concentration on the frequency (%) of embryogenic leaves in genotype 131

Interaction carbohydrate source*concentration	Frequency of embryogenic explants (%)
S*C1	35.71[b]
S*C2	39.28[b]
S*C3	32.09[b]
G*C1	28.90[b]
G*C2	42.85[b]
G*C3	38.95[b]
F*C1	57.14[a]
F*C2	53.57[a]
F*C3	39.28[b]
M*C1	0[c]
M*C2	4.76[c]
M*C3	4.16[c]

S: sucrose; G: glucose; F: fructose; M: maltose; C_1: 58.4 mM; C_2: 87.6 mM; C_3: 116.85 mM
Means in the same column followed by the same letter are not significantly different at $P < 0.05$ according to the Newman-Keuls test. Different letters (a, b and c) denote significant differences

Table 9.3 Effect of interaction carbohydrate source*concentration on the frequency (%) of abnormality and developmental stage of somatic embryos in genotype 131

Interaction carbohydrate source*concentration	Frequency of abnormality (%)	Developmental stage	
		% Glo-Heart	% Tro-Cot
S*C1	19.73^{e}	65.00$^{a, b}$	35.00$^{d, e}$
S*C2	43.19$^{c, d}$	62.75$^{a, b, c}$	37.25$^{c, d, e}$
S*C3	34.21^{d}	44.04$^{d, e}$	55.96$^{a, b}$
G*C1	33.42^{d}	46.88$^{c, d, e}$	53.13$^{a, b, c}$
G*C2	63.02^{a}	55.92$^{b, c, d}$	44.08$^{b, c, d}$
G*C3	43.90$^{c, d}$	49.56$^{b, c, d, e}$	50.44$^{a, b, c, d}$
F*C1	41.82^{d}	78.64^{a}	21.36^{e}
F*C2	51.91$^{b, c}$	73.50^{a}	26.50^{e}
F*C3	53.68^{b}	34.76^{e}	65.48^{a}

S: sucrose; G: glucose; F: fructose; C_1: 58.4 mM; C_2: 87.6 mM; C_3: 116.85 mM
Means in the same column followed by the same letter are not significantly different at $P < 0.05$ according to the Newman-Keuls test. Different letters (a, b, c, d and e) denote significant differences

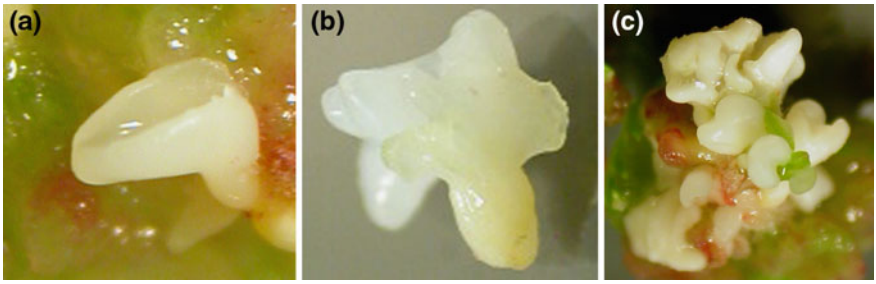

Fig. 9.3 Morphological variability within somatic embryos regenerated from *P. incisa* leaves cultured on MS medium containing 4 µM picloram: **a** abnormal embryo showing one cotyledon, **b** abnormal embryo showing several cotyledons, **c** cluster of fused embryos with thickened cotyledons

embryos showed some teratological abnormalities such as mono (Fig. 9.3a) or polycotyledonary embryos (Fig. 9.3b). In some cases, abnormal embryos showed fused or thickened cotyledons (Fig. 9.3c). It may be noted that the interaction G*C_2 registered a significantly higher rate of abnormal embryos (Table 9.3). Conversely, the interaction S*C_1 reduced the frequency of abnormality. Likewise, qualitative variation in somatic embryos has been observed in other *Prunus* species such as *P. incisa* x *serrula* (Druart 1999) and *P. avium* (Reidiboym-Talleux et al. 1999). Inadequate culture conditions were presumed to be the origin of these abnormalities.

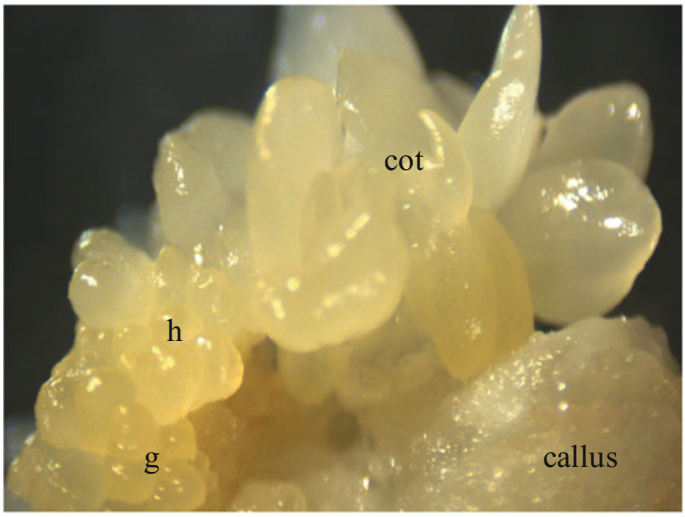

Fig. 9.4 Somatic embryos at different developmental stages: globular (g), heart-shaped (h) and cotyledonary (cot) arising on callus

Concerning the development stage of embryos, the initiation of somatic embryos was not synchronous, and embryos could simultaneously be found at different stages ranging from the globular to the cotyledonary stage (Fig. 9.4). This morphological variability was more or less affected by the interaction carbohydrate source*concentration.

Data recorded after 30 days of the expression phase revealed that the interactions $S*C_1$, $S*C_2$, $F*C_1$ and $F*C_2$ were less favourable to the development of embryos into the ultimate cotyledonary stage than $S*C_3$ and $F*C_3$ (Table 9.3). We found that increasing the fructose or sucrose concentration in the culture medium helped embryos to reach the cotyledonary stage. Similar results have been reported for *Prunus avium* (Reidiboym-Talleux et al. 1999) and *Daucus carota* (Lee et al. 2001). This could be attributed to the increase in medium osmolarity which is required for production of cotyledonary-stage embryos (Stasolla et al. 2002).

9.2.3.2 Protocol 2

In this protocol, we carried out modifications in the macro-elements concentration of MS medium. Full strength (MS), double strength (MS × 2) and diluted strength (MS/2, MS/4) of MS macro-elements were tried. The results showed significant variability in the embryogenic response of leaf explants. All tested media improved the percentage of embryogenic leaves of genotype 131, except MS × 2 (Table 9.4). MS/2 medium allowed us to obtain a higher frequency of embryogenes is than the control. Double strength MS salts (MS × 2) inhibited the embryogenic process and

Table 9.4 Effect of MS macro-elements concentration on the frequency (%) of embryogenic leaves in genotype 131

Medium	Frequency of embryogenic leaves (%)
MS	37.33[b]
MS/2	50.67[a]
MS/4	41.00[b]
MSX2	29.67[c]

Means in the same column followed by the same letter are not significantly different at $P < 0.05$ according to the Newman-Keuls test. Different letters (a, b and c) denote significant differences

gave the lowest level. Similar results have been found with different *Coffea* species (Samson et al. 2006) and carrot (Lee et al. 2001), in which embryogenic response increases with the dilution of the MS salt solution. This could be attributed to the reduction of total nitrogen content (Lee et al. 2001) or nitrate content (Das et al. 2001). The reduction in nitrate concentration may result in the accumulation of reserves and then trigger induction of embryogenesis (Tapan et al. 2001).

Concerning the effects of MS macro-elements concentration on quality of somatic embryos, we found that MS/2 and MS/4 media lead to an increase in typical embryos (Table 9.5). This beneficial effect on embryo quality could be correlated to stress caused by manipulations in MS media. It has been reported that stress is an important factor for the increased production of high-quality somatic embryos (Lee et al. 2001).

In addition, differences in the developmental stages of embryos were detected among different MS culture media (Table 9.5). MS/2 produced more embryos at advanced stages than at early stages. About Lee et al. (2001), MS dilution increased the percentage of cotyledonary embryos. Lowering total nitrogen by diluting the MS medium favoured the development of induced somatic embryos. The highest frequency of cotyledonary embryos was obtained with MS/2 medium. No cotyledonary embryos were formed with MS × 2 medium. High concentration of nitrogen seems to hamper the subsequent development of somatic embryos.

Table 9.5 Effect of MS macro-elements concentration on the frequency (%) of abnormality and developmental stage of somatic embryos in genotype 131

Medium	Frequency of abnormality (%)	Developmental stage	
		% Glo-Heart	% Tro-Cot
MS	36[a]	44.83[c]	55.17[b]
MS/2	15.17[b]	33.14[d]	66.87[a]
MS/4	23[b]	55.43[b]	44.57[c]
MSX2	32[a]	84.25[a]	15.75[d]

Means in the same column followed by the same letter are not significantly different at $P < 0.05$ according to the Newman-Keuls test. Different letters (a, b, c and d) denote significant differences

9.3 Molecular Study

In this section, we aim to discover the relationship between morphological reactions and molecular events that occur during the embryogenesis process in competent (131) and recalcitrant (CAB 6P) genotypes. Molecular investigations were based on the expression of genes known for their involvement in cellular response to auxin (*PiABP19*) (Tromas et al. 2010), cell cycle regulation (*Picdc2*) (Malumbres 2014) and somatic embryogenesis process (*PiSERK3*) (Salvo et al. 2014).

For molecular analyses, samples consisted of leaf explants of these two genotypes cultured for 30 days on MS medium supplemented with 4 μM picloram. The samples were taken every 5 days of culture and kept at −80 °C for subsequent total RNA (ribonucleic acid) extraction according to the protocol described by Ben Mahmoud et al. (2013). These samples consisted of leaves (on the 5th and 10th days), derived calli(on the 15th and 20th days), embryogenic calli in the case of genotype 131 or non-embryogenic calli in the case of genotype CAB 6P (on the 25th and 30th days) (Fig. 9.2). The specific primers were based on expressed sequence tags (EST) available from *Prunus persica*, as well as *ABP*, *cdc2* and *SERK* genes functionally characterized in other plant species (Ben Mahmoud et al. 2013). The *18S rRNA* gene was used as a control for RNA loading and normalization. PCR (polymerase chain reaction) amplifications were carried out with 30 cycles of the following conditions: denaturation at 94 °C for 30 s, annealing for 45 s (at 55 °C for *SERK* and *cdc2*, 58 °C for *ABP19* and 55 °C for *18S rRNA*), elongation at 72 °C for 1 min and final extension at 72 °C for 7 min. All reactions were performed with iCycler (Bio-Rad). The amplified cDNA (complementary deoxyribonucleic acid) fragments were of the expected sizes based on sequence information data from homologous sequences. Each PCR amplification was repeated three times. The amplified PCR products were electrophoresed on a 1% (w/v) agarose gel and visualized by staining with ethidium bromide. *ABP19*, *cdc2* and *SERK* partial cDNAs were cloned, sequenced and submitted to GenBank as newly identified partial transcripts named *PiABP19*, *Picdc2* and *PiSERK3* (Ben Mahmoud et al. 2013).

Semi-quantitative RT-PCR was used to monitor the relative expression of *PiABP19*, *Picdc2* and *PiSERK3* in freshly excised leaf explants (day 0) and throughout the culture period of 30 days in competent (131) and recalcitrant (CAB 6P) cherry genotypes.

In the case of gene *PiABP19*, similar expression patterns were registered in the two genotypes (Fig. 9.5). The highest transcript levels were recorded in samples before culture induction (day 0). This gene has been postulated to mediate cell expansion (Chen et al. 2001; Tromas et al. 2009). During the culture period, *PiABP19* transcripts recorded a significant decrease that could be attributed to the cell divisions, which leads to subsequent callogenesis observed from the 5th day of culture.

In contrast, *Picdc2* (Fig. 9.6) and *PiSERK3* (Fig. 9.7) showed differential and distinctive expression profiles between embryogenic and recalcitrant genotypes.

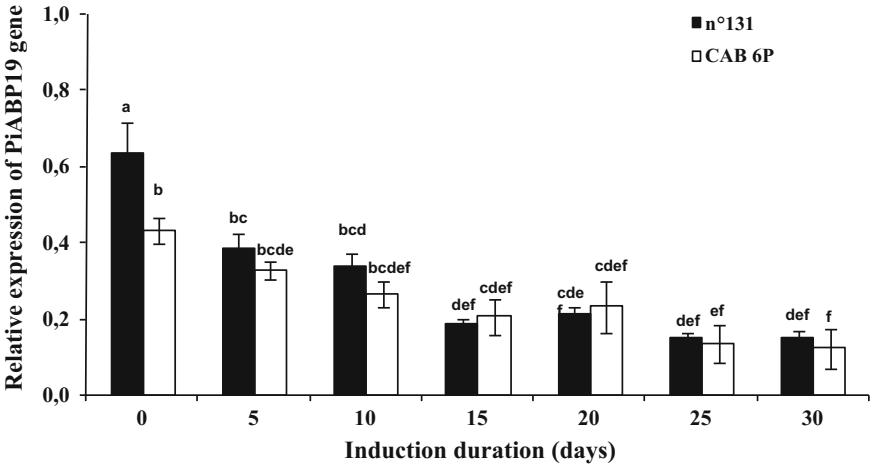

Fig. 9.5 Expression profile of *PiABP19* during induction period of somatic embryogenesis in embryogenic genotype 131 (*P. incisa*) and recalcitrant genotype CAB 6P (*P. cerasus*) from leaf explants cultured in picloram-containing medium for 30 days. RT-PCR amplification data were normalized to the housekeeping gene, 18S rRNA. Data are reported as mean values ± SDM, n = 3. Different letters denote significant differences ($P < 0.05$) according to the Newman-Keuls test

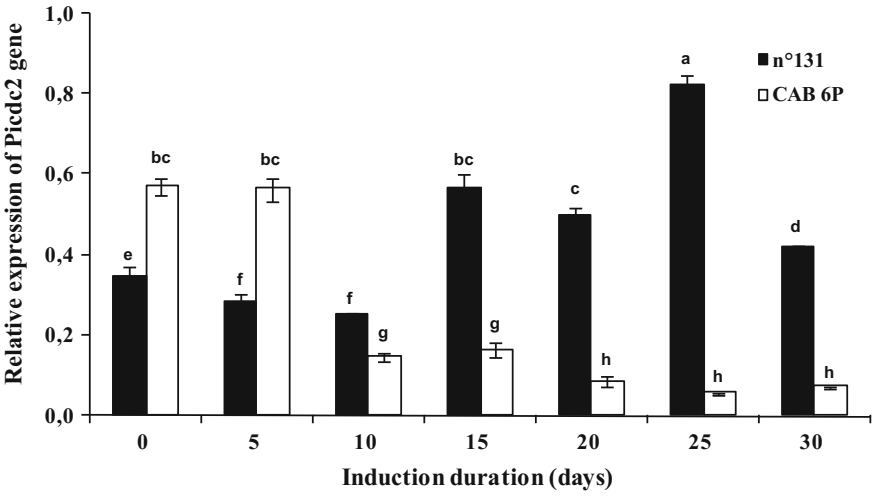

Fig. 9.6 Expression profile of *Picdc2* during induction period of somatic embryogenesis in embryogenic genotype 131 (*P. incisa*) and recalcitrant genotype CAB 6P (*P. cerasus*) from leaf explants cultured in picloram-containing medium for 30 days. RT-PCR amplification data were normalized to the housekeeping gene, 18S rRNA. Data are reported as mean values ± SDM, n = 3. Different letters denote significant differences ($P < 0.05$)

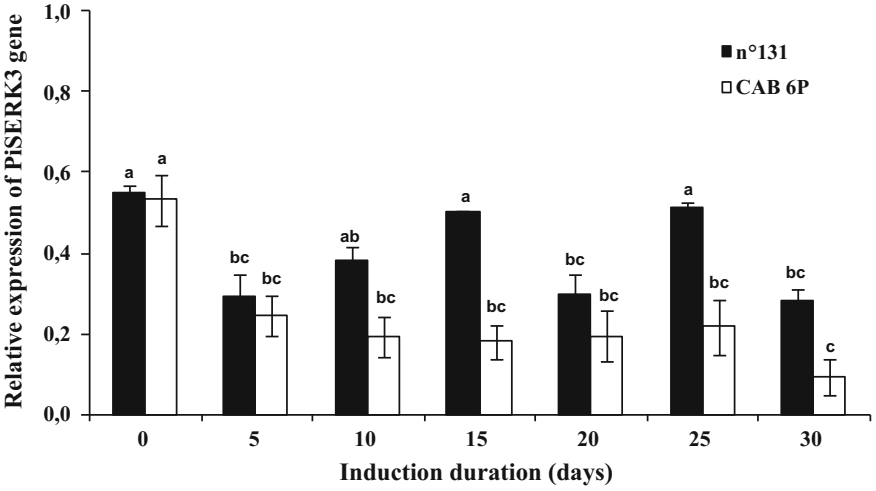

Fig. 9.7 Expression profile of *PiSERK3* during induction period of somatic embryogenesis in embryogenic genotype 131 (*P. incisa*) and recalcitrant genotype CAB 6P (*P. cerasus*) from leaf explants cultured in picloram-containing medium for 30 days. RT-PCR amplification data were normalized to the housekeeping gene, 18S rRNA. Data are reported as mean values ± SDM, n = 3. Different letters denote significant differences ($P < 0.05$)

Regarding *Picdc2*, transcript levels recorded in genotype 131 were stable at the beginning of the culture up to the 10th day and then increased gradually to reach a peak at day 25 (Fig. 9.6) when embryogenic structures could be observed (Fig. 9.2). They then decreased nearly twofold at the end of the culture period (Fig. 9.6). During the first ten days, declining transcript levels of this gene seem to correspond to the cell dedifferentiation process confirmed by anatomical observations (Ben Mahmoud 2012). After this, transcripts showed a considerable increase, reaching a peak at day 25, that could be associated with activation of cell proliferation and callogenesis observed from the 15th day and formation of embryogenic structures detected since day 25 of culture (Fig. 9.2). In this context, a previous study has confirmed that CDK activity is a major factor underlying cell divisions (Joubès et al. 2001; Lin et al. 2014; Malumbres 2014), callus induction and in vitro organogenesis (Cheng et al. 2015). However, in the case of CAP 6P, transcript levels of *Picdc2* that were higher than in cv. No 131 until day 5 significantly decreased from the 10th day and remained low during the whole culture period (Fig. 9.6). According to these observations, accumulation of *Picdc2* transcripts on the 5th day could stimulate mitotic activity leading to callus formation in leaves from the 15th day (Fig. 9.2); but the decline of these transcripts could hamper cell dedifferentiation, as proved by histological study (Ben Mahmoud 2012), and consequently the embryogenic process.

Concerning gene *PiSERK3*, transcript levels were detected in these two genotypes (131 and CAB 6P) and were relatively similar on day 0. During the induction period, the expression profile of gene *PiSERK3* in genotype 131 showed a biphasic pattern marked by two peaks (Fig. 9.7). A first peak was detected on the 15th day when calli were formed in different wounded sites of the leaves. A second peak was recorded on day 25 in explants developing embryogenic structures (Fig. 9.2). We suggest that this *PiSERK3* biphasic pattern coincided with the cellular dedifferentiation and differentiation steps occurring through the embryogenic process. The first peak recorded on the 15th day of culture appears to coincide with the acquisition of embryogenic competence as confirmed in other plant species (Nolan et al. 2003; De Oliveira Santos et al. 2005; Shimada et al. 2005). The second peak registered on the 25th day could be associated with the differentiation of embryogenic structures and proembryo formation (Ben Mahmoud 2012). The decline of the *SERK* transcript level at the end of the culture period is also observed in carrot, where *DcSERK* expression is characteristic of embryogenic development up to the globular stage and stops thereafter (Schmidt et al. 1997). In contrast, *PiSERK3* transcript levels in CAB 6P significantly declined from the beginning of the culture and remained low throughout the whole culture period (Fig. 9.7). This reduction of *PiSERK3* transcript levels was accompanied by the formation of amorphous and compact calli without any embryogenic reaction (Fig. 9.2).

9.4 Research Prospects

Despite the progress achieved during the last few years in understanding the mechanisms involved in somatic embryogenesis, there are still many aspects that are not well understood and need to be elucidated.

In the case of cherry, the recalcitrant behaviour of genotype CAB 6P to somatic embryogenesis has handicapped the exploitation of this biotechnological tool especially for its propagation and genetic improvement. Deeper investigations at the molecular and cytological levels may provide more information about the relationship between the molecular and the cellular process in this recalcitrant cherry genotype. Genes associated with somatic embryogenesis could serve as biomarkers to determine embryogenic competence. In addition, data provided by proteomics and transcriptomics may lead to abetter understanding of the molecular mechanisms underlying somatic embryogenesis.

Regarding the competent genotype, the results showed that the major problem consisted of the production of a relatively high number of abnormal embryos that failed to convert into whole plantlets. Future research should therefore focus especially on culture media composition in order to improve both the quantity and quality of somatic embryos.

References

Ben Mahmoud K (2012) Etude de l'aptitude à l'embryogenèse somatique du porte-greffe de cerisier CAB 6P (*Prunus cerasus* L.) et des mécanismes histologiques et moléculaires associés. Ph.D., National Agronomic Institute of Tunisia, Tunis, Tunisia

Ben Mahmoud K, Delporte F, Muhovski Y, Elloumi N, Jemmali A, Druart Ph (2013) Expression of *PiABP19*, *Picdc2* and *PiSERK3* during induction of somatic embryogenesis in leaflets of *Prunus incisa* (Thunb.). Mol Biol Rep 40:1569–1577

Brown SK, Iezzoni AF, Fogle HW (1996) Cherries. In: Janick J, Moore JN (ed) Fruit breeding: tree and tropical fruits, 1st edn. Wiley, New York, USA, pp 213–255

Chen JG, Shimomura S, Sitbon F, Stanberg G, Jones AM (2001) The role of auxin-binding protein 1 in the expansion of tobacco leaf cells. Plant J 28:607–617

Cheng Y, Liu H, Cao L, Wang S, Li Y, Zhang Y, Jiang W, Zhou Y, Wang H (2015) Down regulation of multiple CDK inhibitor ICK/KRP genes promotes cell proliferation, callus induction and plant regeneration in *Arabidopsis*. Front Plant Sci 6:1–12

Das S, Ray S, Dey S, Dasgupta S (2001) Optimisation of sucrose, inorganic nitrogen and abscisic acid levels for *Santalum album* L. somatic embryo production in suspension culture. Process Biochem 37:51–56

De Oliveira Santos M, Romano E, Yotoko KSC, Tinoco MLP, Dias BBA, Araga FJL (2005) Characterization of the cacao somatic embryogenesis receptor-like kinase (*SERK*) gene expressed during somatic embryogenesis. Plant Sci 168:723–729

Dennis TT (2006) Effect of sugars, gibberellic acid and abscisic acid on somatic embryogenesis in *Tylophora indica* (Burm. f.) Merrill. Chin J Biotech 22:465–471

Druart Ph (1999) Somatic embryogenesis in *Prunus* species. In: Gupta PK, Newton RJ, Mohain JS (eds) Somatic embryogenesis in woody plants, 1st edn. Kluwer Academic, Dordrecht, Netherlands, pp 215–235

Druart Ph (2003) Micropropagation of apples (*Malus* sp.). In: Jain SM, Ishii K (eds) Micropropagation. Kluwer Academic, The Netherlands, pp 433–463

Feher A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tiss Org Cult 74:201–228

Fuentes SRL, Calheiros MBP, Manetti-Filho J, Vieira LGE (2000) The effects of silver nitrate and different carbohydrates sources on somatic embryogenesis in *Coffea canephora*. Plant Cell Tiss Org Cult 60:5–13

Giri C, Shyamkumar B, Anjaneyulu C (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to tress: an overview. Trees 18:115–135

Isah T (2016) Induction of somatic embryogenesis in woody plants. Acta Physiol Plant 38:1–22

Jayasankar S, Kappel F (2011) Recent advances in cherry breeding. Fruit Vegetable Cereal Biotechnol 5:63–67

Joubès J, Lemaire-Chamley M, Delmas F, Walter J, Hernould M, Mouras A, Raymond P, Chevalier C (2001) A new C-type cyclin-dependent kinase from tomato expressed in dividing tissues does not interact with mitotic and G1 cyclins. Plant Phys 126:1403–1415

Lee EK, Cho DY, Soh WY (2001) Enhanced production and germination of somatic embryos by temporary starvation in tissue cultures of *Daucus carota*. Plant Cell Tiss Org Cult 20:408–415

Lin HY, Chen JC, Wei MJ, Lien YC, Li HH, Ko SS, Liu ZH, Fang SC (2014) Genome-wide annotation, expression profiling and protein interaction studies of the core cell-cycle genes in *Phalaenopsis Aphrodite*. Plant Mol Biol 84:203–226

Lou H, Kako S (1995) Role of high sugar concentrations in inducing somatic embryogenesis from cucumber cotyledons. Sci Hort 64:11–20

Malumbres M (2014) Cyclin-dependent kinases. Genome Biol 15:122–131

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Nolan KE, Irwanto RR, Rose RJ (2003) Auxin up-regulates MtSERK1 expression in both *Medicago truncatula* root-forming and embryogenic cultures. Plant Phys 133:218–230

Omid K, Deljou A, Kordestani GK (2008) Secondary somatic embryogenesis of carnation (*Dianthus caryophyllus* L.). Plant Cell Tiss Org Cult 92:273–280

Raj Bhansali R, Driver JA, Durzan DJ (1990) Rapid multiplication of adventitious somatic embryos in peach and nectarine by secondary embryogenesis. Plant Cell Rep 9:280–284

Reidiboym-Talleux L, Diemer F, Sourdioux M, Chapelain K, De March G (1999) Improvement of somatic embryogenesis in wild cherry (*Prunus avium*). Effect of maltose and ABA supplements. Plant Cell Tiss Org Cult 55:199–209

Salvo SAGD, Hirsch CN, Buell CR, Kaeppler SM, Kaeppler HF (2014) Whole transcriptome profiling of maize during early somatic embryogenesis reveals altered expression of stress factors and embryogenesis-related genes. PLoS One 9:1–17

Samson NP, Campa C, Le Gal L, Noirot M, Thomas G, Lokeswari TS, de Kochko A (2006) Effect of primary culture medium composition on high frequency somatic embryogenesis in different *Coffea* species. Plant Cell Tiss Org Cult 86:37–45

Schmidt ED, Guzzo F, Toonen MA, de Vries SC (1997) A leucine rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124:2049–2062

Shibli RA, Shatnawi M, Abu-Ein, Al-Juboory KH (2001) Somatic embryogenesis and plant recovery from callus of 'Nabali' Olive (*Olea europea* L.). Sci Hort 88:243–256

Shimada T, Hirabayashi T, Endo T, Fuji H, Kita M, Omura M (2005) Isolation and characterization of the somatic embryogenesis receptor-like kinase gene homologue (*CiSERK1*) from *Citrus unshiu* Marc. Sci Hort 103:233–238

Stasolla C, Kong L, Yeung EC, Thorpe TA (2002) Maturation of somatic embryos in conifers: morphogenesis, physiology, biochemistry and molecular biology. In vitro Cell Dev Biol-Plant 38:93–105

Steinmacher DA, Clement CR, Guerra MP (2007) Somatic embryogenesis from immature peach palm inflorescence explants: towards development of an efficient protocol. Plant Cell Tiss Org 89:15–22

Tapan KM, Bhattacharya A, Ahuja PS (2001) Induction of synchronous secondary somatic embryogenesis in *Camellia sinensis* (L.) O. Kuntze. J Plant Physiol 158:945–951

Tomaz ML, Januzzi Mendes BM, Mourao Filho DAA, Demétrio CGB, Jansakul N, Rodriguez APM (2001) Somatic emrbyogenesis in citrus spp.: carbohydrate stimulation and histodifferentiation. In vitro Cell Dev Biology-Plant 37:446–450

Tromas A, Braun N, Muller P, Khodus T, Paponov IA, Palme A, Liung K, Lee JY, Benfey P, Murray JAH, Scheres B, Perrot-Rechenmann C (2009) The auxin binding protein 1 is required for differential auxin responses mediating root growth. PLoS One 4:1–11

Tromas A, Paponov I, Perrot-Rechemmann C (2010) Auxin binding protein 1: functional and evolutionary aspects. Trends Plant Sci 8:436–446

Verdeil JL, Alemanno L, Niemenak N, Tranbarger TJ (2007) Pluripotent versus totipotent plant stem cells: dependence versus autonomy? Trends Plant Sci 12:245–252

Von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tiss Org Cult 69:233–249

Chapter 10
Somatic Embryogenesis in *Gomortega keule*

Diego Muñoz-Concha

10.1 Introduction

The tree *Gomortega keule* is an endemic and endangered species which occurs exclusively in the coast of central-south Chile and is the only representative in the family Gomortegaceae. This evergreen tree produces edible fruits (Fig. 10.1a, b) that can be eaten fresh or processed (Hechenleitner et al. 2005). The wood and its ornamental attributes are also of interest. However, it is not presently cultivated mainly due to propagation difficulties and the lack of knowledge of agricultural practices. This neglected fruit plant clearly deserves more efforts in order to exploit its potential (Muñoz-Concha and Davey 2011a).

The species can be propagated sexually by seeds, although the genetic characteristics are not preserved. The soft seed is protected by a strong endocarp (Fig. 10.1c) that delays germination. Traditional vegetative propagation has not been successful but in vitro propagation is possible. Active buds and seed embryos can be used as a source of explants. Shoot proliferation and elongation must be obtained before rooting and acclimatization of plantlets (Muñoz-Concha and Davey 2011b).

Somatic embryogenesis has been observed in *Gomortega keule* (Muñoz-Concha et al. 2012) and, as for a number of other tree species, represents a promising alternative for mass plant production. Embryogenic tissues are the only tissues successfully cryopreserved, and a potential target for transformation (Muñoz-Concha 2010). Somatic embryogenesis also seems to be at the base of the process to obtain triploid plants in *Gomortega keule*, which opens new alternatives to the genetic improvement and domestication of the species (Muñoz-Concha 2016).

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_10

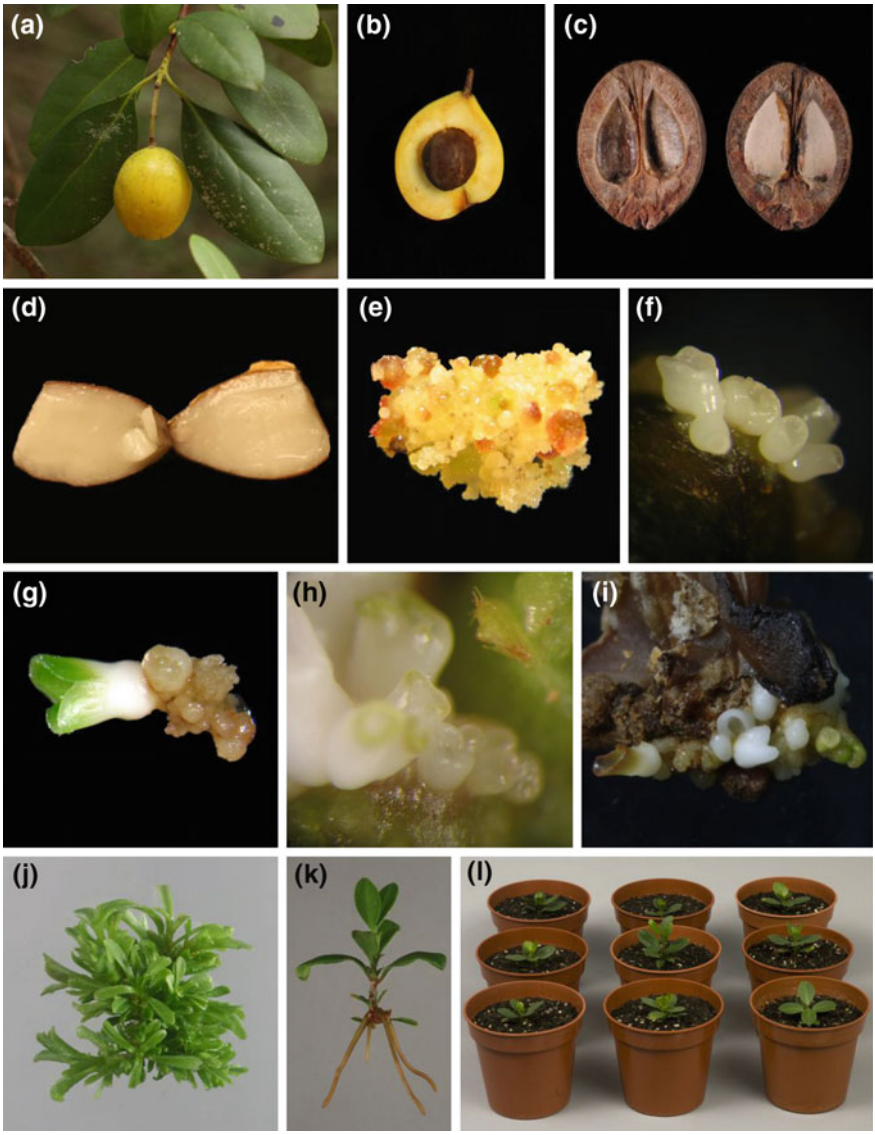

Fig. 10.1 Somatic embryogenesis in *Gomortega keule*. **a** Fruits are collected in autumn. **b** The pulp is removed. **c** Seeds are extracted from the endocarp. **d** The seed is sterilized and dissected. **e** Embryogenic callus. **f** Somatic embryos from the radicle of a zygotic embryo. **g** Somatic embryos developing from callus. **h** Somatic embryos from an in vitro cultured shoot. **i** Somatic embryos from the endosperm (seed). **j** In vitro cultured shoots. **k** Regenerated plantlet with roots. **l** Acclimatized plantlets

10.2 Somatic Embryogenesis

Somatic embryogenesis has been achieved in several tree species of different plant families and has been regarded as a potential way for the production of high numbers of plants. The occurrence of somatic embryogenesis is highly species dependent and requires specific conditions. There are species where this process is very difficult to achieve or not possible, while in some species or groups it is fairly easy to obtain (Guan et al. 2016).

In *Gomortega keule*, somatic embryogenesis occurs in low rates from zygotic embryos, but once an embryogenic line has been established, it is possible to maintain its embryogenic capacity for a long time. Although somatic embryos have been induced in shoots (Muñoz-Concha et al. 2012), no further development or recurrent production of somatic embryos have been observed from this type of explant. In a very interesting line of work, cultures of endosperm can produce shoot primordia that may correspond to somatic embryos and are eventually able to produce triploid shoots and plantlets (Muñoz-Concha 2016). Generally, somatic embryos of *Gomortega keule* have failed to produce roots or radicle development, but shoots do develop roots and plants can be regenerated.

10.2.1 Source of Explants

Fruits of field trees ripe in autumn (April–June in Chile) and are collected from the forest floor. After removing the pulp, the endocarps can be stored for several months. If the endocarps have been stored for more than one month, they must be soaked in water for a week before breaking them with a bench vise. Care is to be taken to avoid damaging the seed. The seeds are surface sterilized by immersion for 20 min in 5% (v/v) sodium hypochlorite and rinsed with sterile water three times under sterile conditions. Seeds are carefully dissected using forceps and scalpel, cutting the edges and then opening the two halves (Fig. 10.1d). The small embryo (1–3 mm) is located in the pointed end of the seed and must remain undamaged. The embryos are placed on the culture medium (Table 10.1) to allow germination and shoot production. Alternatively, if the endosperm is to be cultured, the seed is placed on the medium (Table 10.1), exposing the embryo and the endosperm upwards, including the internal tissue.

When shoots from field trees are the desired source of material, actively growing shoots (November–January) are surface sterilized by immersion for 10 min in 5% (v/v) sodium hypochlorite.

Table 10.1 Media composition for in vitro culture of *Gomortega keule* tissues

Component	Concentration (mg l^{-1})			
	MS[a]	WP[b]	OM[c]	GM[d]
NH_4NO_3	1650	400	412	100
KNO_3	1900	–	1100	–
$Ca(NO_3)_2 \cdot 4H_2O$	–	556	600	1000
$CaCl_2 \cdot 2H_2O$	440	96	440	190
$MgSO_4 \cdot 7H_2O$	370	370	1500	1500
KH_2PO_4	170	170	340	340
KCl	–	–	500	1310
K_2SO_4	–	990	–	–
KH_2PO_4	0.83	–	0.83	0.83
KI	6.2	6.2	12.4	12.4
H_3BO_3	22.3	22.3	22.3	22.3
$MnSO_4 \cdot 4H_2O$	8.6	8.6	14.3	14
$ZnSO_4 \cdot 7H_2O$	0.25	0.25	0.25	0.25
$Na_2MoO_4 \cdot 2H_2O$	0.025	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	–	0.025	0.025
$CoCl_2 \cdot 6H_2O$	27.8	27.8	27.8	27.8
$FeSO_4 \cdot 7H_2O$	37.3	37.3	37.5	37.3
Na_2 EDTA	100	100	100	100
Myo-inositol	0.5	0.5	5	5
Nicotinic acid	0.5	0.5	0.5	0.5
Pyridoxine—HCl	0.1	1	0.5	0.5
Thiamine—HCl	2	2	2	2
Glycine	1650	400	412	100

[a]MS medium (Murashige and Skoog 1962)
[b]Woody Plant Medium (Lloyd and McCown 1981)
[c]Olive Medium (Rugini 1984)
[d]Medium for *Gomortega keule*, modified from OM (unpublished work)

10.2.2 Culture Media

Culture conditions differ depending on the tissue and the desired response. From the several media that have been tested for tissues of *Gomortega keule*, the composition of those showing better tissue growth is detailed in Table 10.1. Medium, hormones and supplements to maintain embryogenic tissues (Fig. 10.1e, g), induce shoot primordia from endosperm (Fig. 10.1i), and regenerate plantlets are detailed in Table 10.2. To maintain embryogenic tissues in culture, semi-solid or liquid medium can be used, the latter producing small and actively growing cell aggregates (Muñoz-Concha et al. 2012).

The development of somatic embryos is rarely observed using non-seed explants. However, shoots of at least one genotype cultured on medium with some

Table 10.2 Culture conditions and responses in *Gomortega keule* tissues

Stage	Basal medium	Supplements (mg l^{-1})	Response	References[a]
Induction				
Induction on zygotic embryo	MS	NAA (0.01), BA (0.1)	SE from radicle after 3–7 months	B
Induction on endosperm	MS	2,4D (0.1), 2iP (0.1)	Callus formation after 1.5 months, shoot primordia after 9 months	C
Induction on shoots	MS	2,4D (1.0), 2iP (1.0)	SE after 6.5 months (only some genotypes)	B
Proliferation				
Embryogenic callus	MS	NAA (0.01), BA (1.0)	Embryogenic callus with constant growth	B
Cell aggregates growth	MS, OM liquid	NAA (0.01), 2iP (0.1)	Growth of small embryogenic cell aggregates and big aggregates	B, C
Shoot	WP, OM, GM	NAA (0.1), BA (1.0)	Shoot growth (genotype dependent)	A, C, D
Regeneration				
Shoot elongation	WP, OM, GM	AC[b] (2000)	Long shoot growth with big leaves after 1 month (genotype dependent)	C
Root induction	WP, OM	IBA (20)	Long shoot induced after 1–2 weeks	A, C
Root development	WP, OM	AC[b] (2000)	Root growth after 1 month	A, C

[a]*Sources of reference* A = Muñoz-Concha and Davey (2011b); B = Muñoz-Concha et al. (2012); C = Muñoz-Concha (2016); D = unpublished work
[b]Activated charcoal

supplements (Table 10.2) produced embryogenic tissues (Fig. 10.1h). The use of putrescine may further stimulate the development of embryogenic tissues in other genotypes (Marchant 2015). Future research may achieve somatic embryogenesis using in vitro cultured shoots from a range of genotypes.

10.3 Plant Regeneration

10.3.1 Shoot Production

Shoots of *Gomortega keule* grow in vitro on formulations of WP, OM, and GM basal media (Table 10.1) supplemented with NAA (0.1 mg l^{-1}) and BA (1.0 mg l^{-1}), 30 g l^{-1} sucrose at pH 5.7. Medium selection will depend on the genotype, with

some genotypes growing well on several media (Fig. 10.1j), while others show poor growth and even in some cases growth has not been achieved. Subculture can be done every 3–4 weeks.

10.3.2 Shoot Elongation

Actively growing shoots are cultured on the chosen medium supplemented with 2 g l^{-1} activated charcoal, without the addition of hormones. Usually one long shoot with big leaves will develop after 1 month from each initial shoot. Shoots with active growth will have a better response to produce a new long shoot. Small or old shoots should be discarded.

10.3.3 Rooting

Rooting is induced on the chosen medium supplemented with IBA (20 mg l^{-1}) during 1–2 weeks followed by transfer to the same conditions as for shoot elongation. After 1 month, 2–4 roots (usually thick and lacking hairy roots) develop from the base of the shoot. Only long shoots with minimum 2 big leaves must be induced (Fig. 10.1k).

10.3.4 Acclimatization

Plantlets can be transferred to small plastic pots with compost or a mix of peat and perlite (1:1), covered with a transparent plastic plant sleeve. Fourteen days after transfer, a cut is made in a corner of the plastic bag, then at day 18 a second cut is made on the opposite corner. Along the top of the bag, a third cut is done at day 20 and a final cut around the middle of the bag at day 22, removing the bag at day 25 (Muñoz-Concha and Davey 2011b). Alternatively, plant pots can be covered with a transparent plastic pot placed bottom-up. Also, plantlets can be placed in transparent plastic boxes with substrate and maintained closed for a month before gradually opening.

The stage of acclimatization is a critical process in micropropagation (Fig. 10.1l). Vital for *Gomortega keule* plantlet survival are the use of rooted shoots of good quality (long, several big leaves), and a controlled (gradual) decrease of air humidity over at least a month. After acclimatization, direct sun or excessive water in the substrate must be avoided.

10.4 Ploidy Manipulation

The changes in the level of ploidy in plants are an important part of the genetic variability that supports genetic crop improvement and the domestication of plants. One of the traditional approaches to double the number of chromosomes is the use of colchicine. But the production of a triploid plant is not directly possible from a diploid tissue. Instead, a previously obtained tetraploid plant can be manually crossed with a diploid. This process is especially long and difficult in tree species. However, in recent years, the regeneration of triploid plants through in vitro culture of endosperm has been reported in several species, including trees such as *Gomortega keule* (Muñoz-Concha 2016).

Tetraploid tissues of *Gomortega keule* can be induced with the use of colchicine. For this, in vitro grown shoots are exposed to 1 mg l^{-1} colchicine in liquid culture medium with the same supplements used for shoot growth (Table 10.2). After 4 days of incubation on an orbital shaker, shoots are washed with sterile water and cultured on semi solid-medium. Within a period of 3 months of culture the shoots recover their proliferation ability. During subsequent cultures, the shoots can be selected by their morphology (abnormal leaf shape, bigger size, dark colour and more apparent hair presence).

Regardless the methodology employed, polyploid tissues, shoots or regenerated, plants must be evaluated in order to verify the level of ploidy. A direct method, although time consuming, is chromosome observation and counting. A more recent and easier technique is to estimate the size of the nuclei by flow cytometry.

10.4.1 Chromosome Observation

Chromosomes can be observed in embryogenic tissues of *Gomortega keule*, such as those cultured in liquid medium. It is very difficult to observe and count chromosomes from other types of tissues (Muñoz-Concha 2016). It is possible to observe a few cells with chromosomes from root tips.

The tissues are placed in a solution containing 2 mM 8-hydroxyquinoline. After 24 h at room temperature, the tissues are changed to a fixative solution of ethanol: acetic acid (3:1 v/v) for a minimum of 16 h. The tissues are hydrolyzed at 65 °C for 20 min in 1.0 M hydrochloric acid, and washed three times in distilled water. The tissues are then transferred to 200 µl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) followed by a digestion in 200 µl of enzyme mixture for 30 min at 37 °C. The enzyme mixture (cellulase Onozuka R10:pectolyase Y23, 4:1 w/w) is dissolved (5%, w/v) in citrate buffer (10 mM citric acid, 10 mM sodium citrate, pH 4.6) in a 1.5 ml-centrifuge tube. After the digestion, distilled water (400 µl) is added and the tube is centrifuged for 1 min at 1400 × g. After discarding the supernatant, the pellet is resuspended in 600 µl of water. The tube is centrifuged for 1 min at 1400 × g and the supernatant is discarded. The pellet is resuspended in 600 µl of a

mixture of methanol and acetic acid (4:1, v/v). After centrifugation for 1 min at 1400 × g, the supernatant is discarded. The pellet is resuspended in 150 µl of a mixture of methanol:acetic acid (4:1, v/v).

Drops of the suspension are placed on glass slides and allowed to air-dry. The material is stained using 0.6 mM DAPI (2,4-diamidino-2-phenylindole) in the dark for 5 min, and washed with distilled water. Cells are observed and chromosomes counted using an oil immersion 100 × objective lens.

10.4.2 Flow Cytometry

A variety of tissues of *Gomortega keule* can be assessed using this technique, such as callus, cell aggregates, shoots, and leaves obtained in vitro. Fully developed leaves of field trees are used for comparison as a diploid control (Muñoz-Concha 2016).

Analyses are performed using a flow cytometer equipment such as Partec PA (Partec GmbH, Münster, Germany) equipped with an HBO 100 W/2 lamp. Tissues are chopped with a razor blade and DAPI is added for nuclei detection. Data can be analyzed using a software such as Flowing Software Version 2.5.0 (P. Teho, University of Turku, Finland).

10.5 Research Prospects

Gomortega keule is a potential fruit tree for mild temperate regions that may help satisfy the increasing interest for new food products and fruits. However, the development of crop techniques and field plot evaluations are much needed. Propagation procedures that use in vitro techniques are of great interest and have proven their potential for the production of plants in this species. This will help agricultural interests and, given the endangered status of the species, will also permit the availability of plants for conservation purposes.

For the propagation of selected genotypes (i.e. adult trees), the induction of somatic embryogenesis from differentiated tissues must be further researched. Also, the processes of maturation and germination of somatic embryos have to be improved, in particular the direct development of the root system from the somatic embryo. Finally, there are some limitations through the in vitro growth of shoots and plantlets that are attributed to the suitability of the culture media. The formulation of media allowing proper growth of tissues will sustain mass propagation of a wide range of genotypes within this species.

References

Guan Y, Li S-G, Fan X-F, Su Z-H (2016) Application of somatic embryogenesis in woody plants 7:938. https://doi.org/10.3389/fpls.2016.00938

Hechenleitner P, Gardner MF, Thomas PI, Echeverría C, Escobar B, Brownless P, Martínez C (2005) Plantas amenazadas del centro-sur de Chile. Universidad Austral de Chile and Royal Botanic Garden of Edinburgh, Chile

Lloyd G, McCown B (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by the use of shoot-tip culture. Comb Proc Intl Plant Prop Soc 30:421–427

Marchant P (2015) Evaluación de la embriogénesis somática en tejidos de *Gomortega keule* (Mol.) Baillon, usando 2,4 D y otras sustancias. Undergraduate thesis, Universidad Católica del Maule, Chile, 61 p

Muñoz-Concha D (2010) *Gomortega keule*: micropropagation and germplasm characterization. Ph.D. thesis, University of Nottingham, UK, 212 p

Muñoz-Concha D (2016) Culture of triploid tissue from the endosperm of an endangered Chilean tree species *Gomortega keule*. J Hortic Sci Biotech 91:79–86

Muñoz-Concha D, Davey MR (2011a) *Gomortega keule*, the neglected and endangered Chilean fruit tree. Eur J Forest Res 130:677–693

Muñoz-Concha D, Davey MR (2011b) Micropropagation of the endangered Chilean tree, *Gomortega keule*. In Vitro Cell Dev Biol-Plant 47:170–175

Muñoz-Concha D, Mayes S, Ribas Davey MR (2012) Somatic embryogenesis from zygotic embryos and shoot-tips of the Chilean tree *Gomortega keule*. Plant Cell Tiss Org Cult 109:123–130

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Rugini E (1984) *In vitro* propagation of some olive (*Olea europaea* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Sci Hort 24:123–134

Chapter 11
Turkish Cyclamen sps.

Tolga İzgü, Başar Sevindik and Yeşim Yalçın Mendi

11.1 Introduction

Cyclamen is an economically important ornamental plant used commonly as a pot plant or outdoor plant. In addition, wild cyclamen species possess a enormous potential as medicinal plants (Seyring et al. 2009; İzgü et al. 2016). Twenty cyclamen taxa belonging to Myrsinaceae family originate from the Mediterranean region and grow under trees and bushes. Ten cyclamen species grow naturally in Turkey and six are being endemic (*C. pseudibericum* Hildebr., *C. trochopteranthum* O. Schwarz, *C. parviflorum* Pobed., *C. cilicium* Boiss. et Heldr., *C. cilicium* var. *intaminatum* Meikle, *C. mirabile* Hildebr.) (Grey-Wilson 2002; Takamura 2006; Çürük et al. 2016). These species have different blooming season such as *C. persicum, C. pseudibericum, C. coum, C. trochopteranthum* and *C. parviflorum* in spring, while *C. hederifolium* Aiton., *C. graecum, C. cilicium*, *C. cilicium* var. intaminatum and *C. mirabile* in autumn. Chromosome numbers and ploidy levels vary across these species; *C. persicum* (2n = 48), *C. hederifolium* Aiton (2n = 2x = 34, 4x = 68 or 6x = 102), *C. graecum* (2n = 84), *C. coum*, *C. pseudibericum*, *C. cilicium, C. mirabile*, *C. parviflorum* (2n = 30), or *C. repandum* (2n = 20) (Çürük et al. 2016).

Somatic embryogenesis is a very effective method for clonal propagation of cyclamen plants. The success of which somatic embryogenesis in cyclamen depends on genotype, source of explant, medium content, plant growth regulators, and their concentrations and culture conditions (Tagipur et al. 2016). Somatic

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_11

embryogenesis has been determined to be an efficient system for the clonal propagation of *C. persicum* (Kiviharju et al. 1992; Motoyasu and Takiko 1991; Kreuger et al. 1995; Schwenkel and Winkelmann 1998; Ruffoni et al. 1998; Hohe et al. 1999; Winkelmann et al. 2003, 2006; Winkelmann and Serek 2005; Naderi et al. 2012; Koçak et al. 2014). Only few reports have came to grips with somatic embryogenesis/embryo-like structures (ELSs) in wild cyclamen species, such as *C. cilicium* and *C. mirabile* (Furukawa et al. 2001, 2002; Seyring et al. 2009; Prange et al. 2010; İzgü et al. 2016), *C. pseudibericum* and *C. parviflorum* (İzgü et al. 2016). It has been also showed that ELS formation in *C. africanum* Boiss. and Reut., *C. cilicium* Boiss. and Heldr., *C. coum* Mill., *C. hederifolium* Ait., *C. persicum* and *C. purpurascens* Mill. (Seyring et al. 2009). In another researched somatic embryogenesis were optimized on *C. coum, C. alpinum, C. mirabile* and *C. graecum* described by Prange et al. (2010). In addition, somatic embryogenesis was investigated in other cyclamen species (*C. cilicium, C. coum, C. graecum, C. hederifolium, C. persicum, C. purpurascens*, and *C. rohlfsianum*) (Furukawa et al. 2001). İzgü et al. (2016) described ELS induction from ovule, ovary, leaf and petiole explants and have proved applicable protocol for endemic Turkish cyclamen species (*C. cilicium, C. mirabile, C. pseudibericum* and *C. parviflorum*). Different explants resources including tubers, petioles, peduncles, leaves and ovules of *C. persicum* have been used to initiate in vitro culture in cyclamen(Hawkes and Wainwright 1987; Schwenkel and Grunewaldt 1988; Kiviharju et al. 1992; Kreuger et al. 1995; Takamura et al. 1995; Schwenkel and Winkelmann 1998; Winkelmann et al. 1998a, b; Al-Majathoub 1999; Karam and Al-Majathoub 2000; Savona et al. 2007; Borchert et al. 2007; Seyring et al. 2009; Jalali et al. 2010a, b; Yamaner and Erdağ 2008; Rode et al. 2011, 2012; Naderi et al. 2012; Nhut et al. 2012; Koçak et al. 2014).

The protocol described here is based on ELSs/somatic embryogenesis (SE) from different explant types (ovule, ovary, leaf and petiole) on wild Turkish cyclamen species (Koçak et al. 2014; İzgü et al. 2016). This procedure including explant preparation/surface disinfection, callus formation, ELS/somatic embryo development, plantlet formation and acclimatization is explained in detailed.

11.2 Material

Wild (*C. persicum*) and endemic (*C. cilicium, C. mirabile, C. pseudibericum* and *C. parviflorum*) cyclamens at the flowering stage with their intact tubers were collected from native habitats in different locations around Turkey. Collected plant samples were placed in 13 cm diameter pots in a polycarbonate greenhouse in a substrate mixture contained turf, sand and perlite (1:1:1, v/v).

11.3 Protocol of ELS/Somatic Embryogenesis in Cyclamen Species

11.3.1 Culture Media

1. To obtain efficient callus formation and embryo like structures (ELS) from endemic *Cyclamen* species and somatic embryos (SE) from wild *C. persicum*, modified half strength MS (Murashige and Skoog 1962) basal medium is used. Powdered stock MS contents are presented at Table 11.1 (for 1 L).
2. Half strength modified MS basal medium is mixed with other supportive chemicals and plant growth regulators (PGRs) in erlenmayer flask filled with distillated water.
3. Medium is adjusted to pH 5.7 with 1 N KOH and petri dishes (90 × 15 mm) containing 30 ml of medium are used for initiation stages then magenta boxes (76 × 76 × 100 mm) containing 40 ml of medium are used in the final stage in order to develop plantlets.

Table 11.1 Modified MS medium composition

Macro nutrients	mg/L
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \times 2H_2O$	440
$MgSO_4 \times 7H_2O$	370
KH_2PO_4	170
Micro nutrients	mg/L
H_3BO_3	6.2
$MnSO_4 \times 1H_2O$	16.9
$ZnSO_4 \times 7H_2O$	10.6
KI	0.830
$Na_2MoO_4 \times 2H_2O$	0.25
$CuSO_4 \times 5H_2O$	0.025
$CoCl_2 \times 6H_2O$	0.025
Amino acid and vitamins	mg/L
Glycine	2
Nicotinic acid	0.1
Thiamine HCl	0.5
Pyridoxine HCl	0.5
Other additives	
Gelrite	3.5 g/L
Fe EDTA	5 ml/L
Sucrose	30 g/L
MES	1 g/L
Glucose	2 g/L
pH	5.7–5.8

11.3.2 Surface Disinfection and Explant Preparation

1. Unopened flower buds are harvested in autumn season for *C. cilicium* and *C. mirabile*, and in spring season for *C. persicum*, *C. pseudibericum* and *C. parviflorum* about 3–4 days before anthesis (Fig. 11.1a). Fresh leaves and petioles are also collected from healthy plants in the flowering stage.
2. Unopened flower buds are washed under tap water for 20 min, dipped in 70% ethanol for 1 min, washed three times with sterile distilled water (SDW) and then immersed in 20% sodium hypochlorite solution (commercial bleach solution with 4–5% active chlorine, v/v, NaOCl; Domestos) with 1–2 drops of Tween-20 for 20 min. They are washed three times with SDW (Fig. 11.1b). The leaves and petioles are also washed under tap water for 20 min, immersed in 0.1% mercuric chloride ($HgCl_2$) solution for 10 min, washed with SDW three times, dipped in 70% ethanol for 1 min, washed three times with SDW and in 20% NaOCl solution with 1–2 drops of Tween-20 for 20 min. Finally, explants are rinsed three times with SDW (Fig. 11.1b).

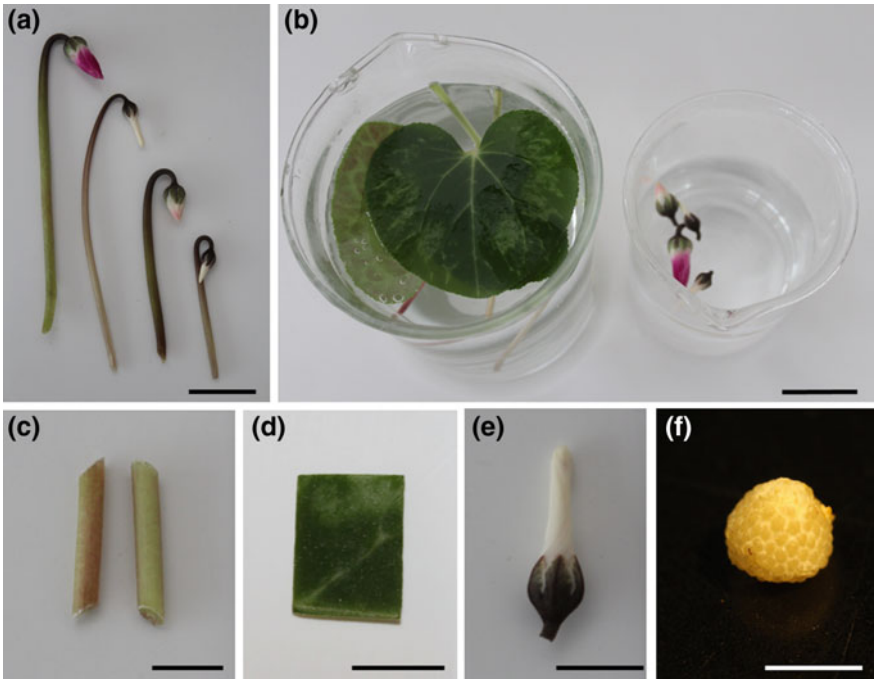

Fig. 11.1 Explant preparation of cyclamen, **a** harvest unopened flower buds before anthesis 3–4 days, **b** surface disinfected of leaves, petioles and flower buds, **c** petiole explant, **d** leaf explant, **e** and **f** Ovary (including ovules) region removed from flower bud. Bars, **a**, **b**: 3 cm. **c**: 0.5 cm, **d**: 1 cm, **e**: 1 cm, **f**: 3 mm

3. After surface disinfection, ovule and ovary explants are removed from flower buds under a stereo binocular microscope at 5x magnification. Ovaries are divided into four equally sized pieces and ovules are removed with a surgical blade from ovaries (Fig. 11.1e, f). Leaf ($\sim$50 mm^2) and petiole ($\sim$7 mm length) segments are sectioned aseptically (Fig. 11.1c, d).
4. All explants are placed onto callus induction medium (CIM) (25 ovules and 8 pieces of ovary/petri dish (90 × 15 mm) 15 leaf segments (abaxial side down) and petiole segments).

11.3.3 Callus Induction

1. This protocol is formed from three different stages, induction of callus production, maturation of SE or ELS and plantlets formation. The final step is acclimatization. Explants are cultured in CIM until callus formation, ELS or SE and microtuber structures are shown in Fig. 11.2.
2. Callus induction medium (CIM) for *C. persicum* contains ½MS macro and micronutrients, except for full strength Fe-EDTA, 250 mg l^{-1} peptone, 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} thiamine HCl, 0.5 mg l^{-1} pyridoxin-HCl, 30 g l^{-1} sucrose, 2 g l^{-1} glucose, 3.7 g l^{-1} gelrite, 2.0 mg l^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid) and 0.8 mg l^{-1} 6-(6-(γ,γ-Dimethylallylamino)purine) purine (2iP) (Koçak et al. 2014).
3. For endemic cyclamen species, same CIM and plant growth regulators (PGRs) are used but different concentration of 2iP and 2,4-D described by Izgü et al. (2016) are added (Table 11.2).
4. All explants are incubated at 16 weeks in constant darkness at 25 ± 2 °C and then subcultured every 4 weeks until sufficient callus formation (approximately 3–4 mm diameter).

11.3.4 Embryo like Structure/Embryogenesis and Plant Establishment

1. Embryogenic callus structures are transferred to PGR free CIM when callus structures turned into ELS or SE that are globular-and torpedo shaped and when callus is 3–4 mm in diameter. Calli structures are incubated at 25 ± 2 °C in constant darkness and subcultured every 4 weeks.
2. To convert ELSs into plantlets, mature ELSs from different explant types are transferred to magenta boxes containing PGR-free CIM and maintained for 2 months in growth chamber.

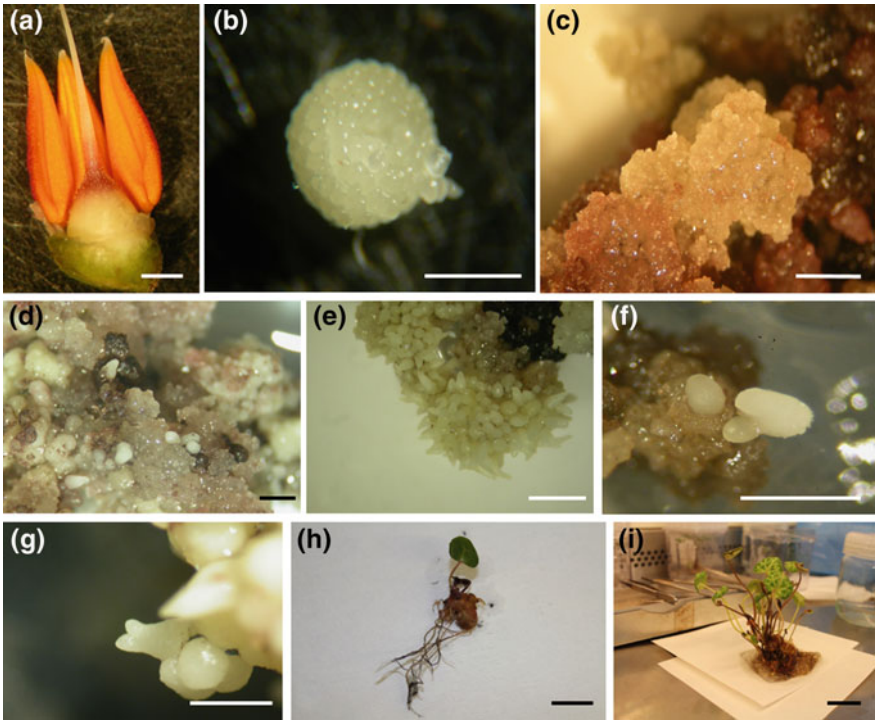

Fig. 11.2 Callus induction and somatic embryogenesis in *C. persicum*. **a** Flower structure including anther and ovarium, **b** Naked ovarium explant before adding the medium, **c** Embryogenic callus formation, **d**, **e** and **f** Globuler, early and late torpedo stages from embryogenic callus, **g** Torpedo turns microtuber formation, **h**, **i** Plantlet formation. Bars **a**, **b**, **c**, **e**: 3 mm, **d**: 2 mm, **f**, **g**: 4 mm, **h**, **i**: 3 cm

11.3.5 Plantlet Formation and Acclimatization

1. The caps of magenta boxes with plants are opened slowly and fractionally in ten days. Plantlets carried healthy shoots, roots and leaves are taken off gently from the magenta boxes containing half strength PGR free CIM.
2. Roots are washed carefully under tap water to remove the medium cling to the roots and dipped in a solution containing 50% (w/v) fungicide (Captan 50WP) at 250 g/100 l for 10 s (Fig. 11.3b). The different size plantlets are transferred to the pots (13 × 10 cm) including peat, soil and pearlite (1:1:1 v/v/v).
3. Plantlets cultured in pots are placed in a greenhouse under natural light at 95–98% relative humidity (Fig. 11.3d, e, f).

Table 11.2 Stages of ELS/SE system, specific PGRs, culture conditions, durations, and responses associated with each stage in cyclamen species

Stage	Species	Explants and PGRs	Duration (wk)	Remarks
Callus induction	*C. persicum*	**Leaf, petiole ovule and ovary** 2.0 mg l^{-1} 2,4-D + 0.8 mg l^{-1} (2iP)	8	Explants turn callus formed are generally light brown, soft and separable
	C. cilicium	**Leaf, petiole and ovule** 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP **ovary** 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP or 2 mg l^{-1} 2,4-D + 1 mg l^{-1} 2iP		Callus formation are, light or dark brown friable callus from leaves, yellow-brown compact callus from petioles while yellow-dark brown compact callus formation from ovules and ovaries, dark-brown compact callus inducing from ovules while producing friable callus from petioles
	C. mirabile	**Leaf and petiole** 2 mg l^{-1} 2,4-D + 2 mg l^{-1} 2iP or 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP **ovule and ovary** 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP.		Callus structure is generally dark-brown or yellowish, soft, friable and separable also are dark brown but turned light brown over time
	C. pseudibericum	**Leaf** 2 mg l^{-1} 2,4-D + 2 mg l^{-1} 2iP **Petiole** 2.5 mg l^{-1} 2,4-D + 1 mg l^{-1} 2iP, **ovule and ovary** 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} 2iP.		Callus formation first brown then dark yellow
	C. parviflorum	**Leaf, petiole and ovule** 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP		Callus structure are mostly light brown, soft, friable and separable. yellowish-white from ovule and ovary, brown,

(continued)

Table 11.2 (continued)

Stage	Species	Explants and PGRs	Duration (wk)	Remarks
		ovary 2 mg l^{-1} 2,4-D + 1 mg l^{-1} 2iP.		dark-brown from leaf, friable and compact from petiole
Differentiation of ELSs or somatic embryos	*C. persicum*	**Leaf, petiole ovule and ovary** 2.0 mg l^{-1} 2,4-D + 0.8 mg l^{-1} (2iP)	9	Embryogenic callus and ELS/SE development
	C. cilicium	**Leaf, petiole, ovule** 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP **ovary** 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP or 2 mg l^{-1} 2,4-D + 1 mg l^{-1} 2iP		
	C. mirabile	**Leaf** 2 mg l^{-1} 2,4-D + 2 mg l^{-1} 2iP **Petiole** 2 mg l^{-1} 2,4-D + 2 mg l^{-1} 2iP or 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP **Ovule and ovary** 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP		
	C. pseudibericum	**Leaf** 2 mg l^{-1} 2,4-D + 2 mg l^{-1} 2iP **Petiole** 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP **Ovule** 2.5 mg l^{-1} 2,4-D + 0.5 mg l-1 2iP		

(continued)

Table 11.2 (continued)

Stage	Species	Explants and PGRs	Duration (wk)	Remarks
		or 2.5 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP **Ovary** 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} 2iP or 2 mg l^{-1} 2,4-D + 1 mg l^{-1} 2iP		
	C. parviflorum	**Leaf, petiole, ovule** 2 mg l^{-1} 2,4-D + 1.5 mg l^{-1} 2iP **Ovary** 2 mg l^{-1} 2,4-D + 1 mg l^{-1} 2iP		
Germination and Plantlet Formation of ELSs	*C. persicum*	PGRs free CIM	9	ELS/SE maturation, microtuberization and germinate
	C. cilicium			
	C. mirabile			
	C. pseudibericum			
	C. parviflorum			

11.4 Research Prospects

Somatic embryogenesis is one of the most important techniques of cyclamen biotechnology for cryopreserving and synthetic seed production of genetic resources such as endemic cyclamens, micropropagation of these species for ornamental and industrial sectors and breeding strategy. The success of somatic embryogenesis depends on genotype, season, explant type, medium content, different PGRs and their concentration and culture condition.

Physiological disorders associated with a large amount of SEs as well as asynchronous development limit commercial application (Rode et al. 2012). In endemic cyclamens, in order to reduce these limitations such as arrested torpedo stages or germination problems, different additives including sucrose, raffinose, proline, GABA, and/or glutamate can be used to optimize regeneration protocol (Winkelmann et al. 2015).

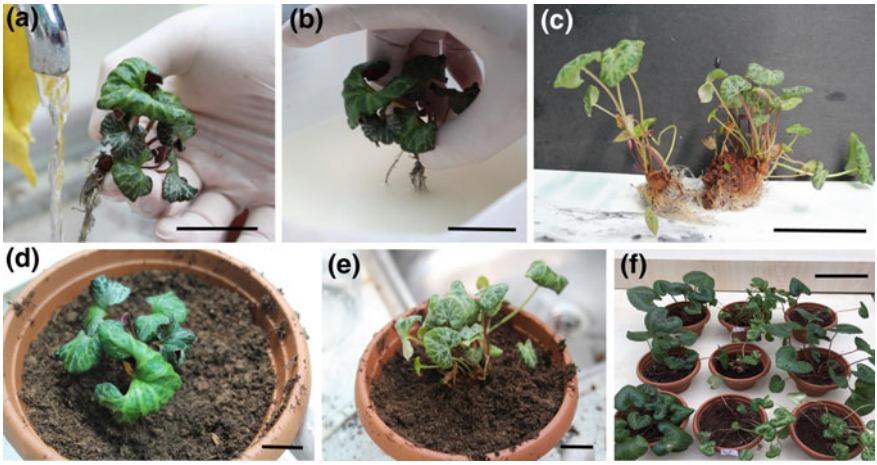

Fig. 11.3 *C. persicum* plantlets about 190 days after transfer of SE to PGR-free CIM prior to the acclimatization in the greenhouse. **a**, **b** and **c** Under tap water washing and fungicide application of plantlet, **d**, **e** Plantlets during acclimatization, **f** Ex vitro plantlet in potted. Bars, **a**: 5 cm, **b**: 4 cm, **c**, **f**: 10 cm, **d**, **e**: 2 cm

Acknowledgements We would like to thanks to Dr. Jaime A. Teixeira da Silva (Kagawa University) for supporting us our biotechnology knowledge in cyclamen. We thank also to Dr. Pembe Çürük to help us improving our knowledge on endemic cyclamen species. This research was supported by TUBITAK (The Scientific and Technical Research Council of Turkey) (Project No. TOVAG 110O102) project.

References

Al-Majathoub M (1999) In vitro propagation of wild cyclamen (*Cyclamen persicum* Mill.). M.Sc. thesis. Jordan University of Science and Technology, Irbid

Borchert T, Fuchs J, Winkelmann T, Hohe A (2007) Variable DNA content of *Cyclamen persicum* regenerated via somatic embryogenesis: rethinking the concept of long-term callus and suspension cultures. Plant Cell Tissue Organ Cult 90(3):255–263

Çürük P, Söğüt Z, İzgu T, Sevindik B, Tagipur EM, da Silva JAT, Serçe S, Solmaz İ, Aka Kaçar Y, Mendi NYY (2016) Morphological characterization of Cyclamen sp. grown naturally in Turkey: Part II. Acta Scientiarum Polonorum-Hortorum Cultus 15(5):205–224

Furukawa K, Kakihara F, Kato M (2001) Somatic embryogenesis in seven wild Cyclamen species. J SHITA 13:270–278

Furukawa K, Kakihara F, Kato M (2002) Somatic embryos produced from aseptic seedlings of wild Cyclamen species. J SHITA 14:71–80

Hawkes HY, Wainwright H (1987) In vitro organogenesis of *Cyclamen persicum* Mill. tissue. Acta Hortic 212:711–714

Hohe A, Winkelmann T, Schwenkel HG (1999) CO_2 accumulation in bioreactor suspension cultures of *Cyclamen persicum* Mill. and its effect on cell growth and regeneration of somatic embryos. Plant Cell Rep 18:863–867

Grey-Wilson C (2002) Cyclamen a guide for gardener, horticulturists and botanists, New edn. Batsford, London, p 192

İzgü T, Sevindik B, Çürük P, Şimşek Ö, Kaçar YA, da Silva JAT, Aka Kaçar Y, Mendi YY (2016) Development of an efficient regeneration protocol for four Cyclamen species endemic to Turkey. Plant Cell Tissue Organ Cult (PCTOC) 127(1):95–113

Jalali N, Naderi R, Babalar M, Mirmasoumi M (2010a) Somatic embryogenesis in Cyclamen with two explants and combinations of plant growth regulators. Hortic Environ Biotechnol 51:445–448

Jalali N, Naderi R, Teixeira da Silva JA, Babalar M, Mirmasoumi M (2010b) Influence of salt concentration of media and plant growth regulator combination on callus formation and somatic embryogenesis of *Cyclamen persicum* Mill. Floricult Ornam Biotechnol 4(1):84–87

Karam NS, Al-Majathoub M (2000) Direct shoot regeneration and microtuberization in wild *Cyclamen persicum* Mill. using seedling tissue. Sci Hortic 86:235–246

Kiviharju E, Tuominen U, Törmala U (1992) The effect of explant material on somatic embryogenesis of *Cyclamen persicum* Mill. Plant Cell Tissue Organ Cult 28:187–194

Koçak M, İzgü T, Sevindik B, Tütüncü M, Çürük P, Şimşek Ö, Aka Kaçar Y, Teixeira da Silva JA, Mendi YY (2014) Somatic embryogenesis of Turkish *Cyclamen persicum* Mill. Sci Hortic 172:26–33

Kreuger M, Postma E, Brouwer Y, Van Holst GJ (1995) Somatic embryogenesis of *Cyclamen persicum* in liquid medium. Physiol Plant 94:605–612

Motoyasu O, Takiko S (1991) Somatic embryogenesis and plant regeneration from *Cyclamen persicum* Mill. leaf cultures. Plant Tissue Cult Lett 8:121–123

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15, 473–497

Naderi R, Jalali N, Babalar M, Mirmasoumi M (2012) Estimate of callus induction and somatic embryogenesis in Cyclamen. Int J Agric Crop Sci 4(11):699–702

Nhut DT, Tran H, Thu M, Van B (2012) Thin cell layer technology in regeneration and micropropagation of *Cyclamen persicum* Mill. Propag Ornam Plants 12(2):89–95

Prange ANS, Bartsch M, Serek M, Winkelmann T (2010) Regeneration of different Cyclamen species via somatic embryogenesis from callus, suspension cultures and protoplasts. Sci Hortic 125:442–450

Rode C, Gallien S, Heintz D, Van Dorsselaer A, Braun, HP and Winkelmann T (2011) Enolases: storage compounds in seeds? Evidence from a proteomic comparison of zygotic and somatic embryos of Cyclamen persicum Mill. Plant Mol Biol 75(3):305–319

Rode C, Lindhorst K, Braun HP, Winkelmann T (2012) From callus to embryo: a proteomic view on the development and maturation of somatic embryos in *Cyclamen persicum*. Planta 235(5):995–1011

Ruffoni B, Semeria L, Profumo P, Bisio A (1998) *Cyclamen persicum* Mill. somatic embryos developed in suspension cultures: Histological analysis and conversion to plants. Acta Hortic 520:83–90

Savona M, Ruffoni B, Giovannini A, Altamura MM (2007) *Cyclamen persicum* Mill. cv. 'Halios': somatic embryogenesis and phenotypic analysis of somatic embryo-derived plants. Acta Hortic 743:91–98

Schwenkel HG, Grunewaldt J (1988) In vitro propagation of *Cyclamen persicum* Mill. Acta Hortic 226:659–662

Schwenkel HG, Winkelmann T (1998) Plant regeneration via somatic embryogenesis from ovules of *Cyclamen persicum* Mill. Plant Tissue Cult Biotechnol 4:28–34

Seyring M, Ewald A, Muller A, Haensch KT (2009) Screening for propagation suitability in vitro of different Cyclamen species. Electron J Biotechnol 12:4–7

Tagipur EM, Şeker G, Teixeira da Silva JA, Mendi YY (2016) Somatic embryogenesis, cryopreservation, and in vitro mutagenesis in Cyclamen. In: Mujib A (ed) Somatic embryogenesis in ornamentals and its applications. Springer, Berlin, pp 155–167

Takamura T, Miyajima I and Matsuo E (1995) Somatic embryogenesis of Cyclamen persicum Mill.'Anneke' from aseptic seedlings. Plant Cell Rep 15(1–2):22–25

Takamura T (2006) Cyclamen. In: Anderson NO (ed) Flower breeding and genetics. Springer, Berlin, pp 459–478

Winkelmann T, Serek M (2005) Genotypic differences in callus formation and regeneration of somatic embryos in *Cyclamen persicum* Mill. Euphytica 144:109–116

Winkelmann T, Sangwan RS, Schwenkel HG (1998a) Flow cytometric analyses in embryogenic and non-embryogenic callus lines of *Cyclamen persicum* Mill.: relation between ploidy level and competence for somatic embryogenesis. Plant Cell Rep 17(5):400–404

Winkelmann T, Hohe A, Schwenkel HG (1998b) Establishing embryogenie suspension cultures in *Cyclamen persicum* 'Purple Flamed'. Adv Hortic Sci 12(1):25–30

Winkelmann T, Meyer L, Serek M (2003) Maturation and desiccation of somatic embryos of *Cyclamen persicum*. Acta Hortic 612:27–34

Winkelmann T, Specht J and Serek M (2006) Efficient plant regeneration from protoplasts isolated from embryogenic suspension cultures of Cyclamen persicum Mill. Plant Cell, Tissue Organ Cult 86(3):337–347

Winkelmann T, Ratjens S, Bartsch M, Rode C, Niehaus K and Bednarz H (2015) Metabolite profiling of somatic embryos of Cyclamen persicum in comparison to zygotic embryos, endosperm, and testa. Front Plant Sci 6:597

Yamaner Ö, Erdağ B (2008) Direct shoot formation and microtuberization from aseptic seedlings of *Cyclamen mirabile* Hildebr. Biotechnology 7:328–332

Chapter 12
Grapevine (*Vitis* spp.)

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12.1 Introduction

Somatic embryogenesis, i.e. the initiation of embryos from plant somatic tissues, is employed as a multiplication method for several plant species. Conversely, with regard to grapevine somatic embryogenesis is mostly used in breeding programs, embryo tissues being the best sources for regeneration of genetically modified plants (Franks et al. 1998; Reustle and Buchholz 2009; Gambino and Gribaudo 2012; Gray et al. 2014). In addition, somatic embryogenesis has been proposed as a strategy aimed at inducing somaclonal variation (Kuksova et al. 1997) and as a tool for additional aims such as the separation of periclinal chimeras (Franks et al. 2002), while improvements in setting up grapevine cryopreservation protocols have been obtained (González-Benito et al. 2009; Vasanth and Vivier 2011). Its interest as a tool in functional genomics studies has recently increased (Gambino et al. 2014) thanks to the availability of the reference genome in grapevine (Jaillon et al. 2007) and the consequent need of characterization of the putative genes identified in silico. Somatic embryogenesis is also a precious tool for studying embryo development as somatic embryos follow a developmental pathway very similar to that of their zygotic counterparts (Dodeman et al. 1997).

In this context, a reliable technique for the production of somatic embryos and regenerated plantlets from an ample number of *Vitis* genotypes is an essential pre-requisite for any application of somatic embryogenesis. The large genetic variability occurring in the genus *Vitis* may strongly influence the results. During the years, a broad number of protocols and media composition has been proposed. Details can be found in reviews such as those of Martinelli and Gribaudo (2009), and more recently Dhekney et al. (2016). From the available literature some

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_12

common, main developmental phases can be identified: (a) Induction of callogenesis and embryogenic competence in cultured explants; (b) Culture of embryogenic calli and expression of the embryogenic program; (c) Long term culture of embryogenic callus and preservation of the embryogenic ability of the cultures or re-initiation of embryogenic calli from somatic embryos; (d) Development of somatic embryos into plantlets.

12.2 Materials

a. Grapevine inflorescences
b. Laminar-flow hood, forceps, scalpels, glass bead sterilizer, sterile cutting pads
c. Autoclave, pH meter, balances
d. Tissue culture chambers
e. Stereomicroscope
f. Sodium hypochlorite, wetting agent, sterile distilled or deionized water
g. Culture media (see Table 12.1), also available on the market as products for micropropagation

Table 12.1 Basal composition of culture media

Chemicals (μM)	Basal medium NN	Basal medium MS
$CaCl_2$	1500	1500
KH_2PO_4	500	625
KNO_3	9400	9400
NH_4NO_3	9000	10305
$MgSO_4$	750	750
$ZnSO_4{\cdot}7H_2O$	34.8	14.95
$Na_2MoO_4{\cdot}2H_2O$	1.03	0.51
$CuSO_4{\cdot}5H_2O$	0.1	0.05
$MnSO_4{\cdot}H_2O$	112	50
H_3BO_3	162	50
$CoCl_2{\cdot}6H_2O$	–	0.05
KI	–	2.5
FeNaEDTA	100	50
Myo-inositol	555	555
Nicotinic acid	4	4
Pyridoxin HCl	2.43	2.43
Thiamine HCl	0.3	0.3
Glycine	26.64	26.64

Basal medium NN has macro and microelements from Nitsch and Nitsch (1969), and vitamins from Murashige and Skoog (1962). Basal medium MS is from Murashige and Skoog (1962), with mineral salts at half concentration

h. Plant growth regulators (PGRs): 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA), naphtoxy acetic acid (NOA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA)
i. Tissue culture agar, gellan gum (Gelrite™, Phytagel™, etc.), sucrose
j. Generic laboratory glassware and plasticware (beakers, Erlenmeyer flasks, cylinders, glass autoclavable bottles)
k. Sterile Petri dishes (for media A, B, C and G: see below), Parafilm®
l. Single-use or re-usable containers for plant tissue cultures (for media D, E and F: see below)
m. Peat pellets (Jiffy7®)

Basic media composition is listed in Table 12.1. Required composition and modifications for different culture stages are listed in Table 12.2.

12.3 Method

The regeneration procedure includes:

(a) Embryogenic culture initiation (acquisition of embryogenic competence): culture of initial explants and induction of callogenesis
(b) Embryo differentiation (expression of the embryogenic program)
(c) Embryo germination and development into plantlets
(d) Long term culture of embryogenic callus and preservation of the embryogenic ability
(e) Micropropagation and acclimatization of the resulting plantlets.

12.3.1 Embryogenic Culture Initiation

Stamens (anthers plus filaments) have been the most widely used starting material for culture, and successful initiation of regenerable embryogenic calli has been obtained from stamens of a considerable number of grapes. Immature pistils (ovaries plus styles, stigmas and receptacles) also represent a good starting explant, with the advantage of being collectible easily and simultaneously with anther excision. Below we refer to the described explants simply as anthers and ovaries. In some cultivars the efficiencies obtained from ovaries can be notably higher than compared to anthers, ovaries being more responsive to embryogenic induction (Nakano et al. 1997; Martinelli et al. 2001; Kikkert et al. 2005; Vidal et al. 2009).

Even whole flowers proved to be suitable material for initiating embryogenic cultures for some cultivars: their collection from the inflorescence is easier and faster than excision of anthers and ovaries from the flower itself; it can be done without the use of a stereomicroscope and damage to the explant is unlikely. No morphological difference was noted among embryogenic cultures originated from ovaries, flowers, or anthers (Gambino et al. 2007).

Table 12.2 Formulations of Grapevine Media

Chemicals	A	B	C	D	E	F	G
	Callus induction	Embryo induction	Embryo germination	Shooting	Rooting	Micro propagation	Long-term culture
Basal medium	NN	NN	NN	MS	MS	MS	NN
Sucrose (g/L)	60	60	30	15	20	20	60
2,4-D (μM)	4.5	–	–	–	–	–	5
BA (μM)	8.9	1	–	10	–	–	1
NOA (μM)	–	10	–	–	–	–	–
IAA (μM)	–	20	–	–	–	–	–
IBA (μM)	–	–	–	–	2.5	–	–
NAA (μM)	–	–	–	–	2.5	–	–
Activated charcoal (g/L)	–	2.5	2.5	–	–	–	–
Gellan gum (g/L)	3	–	–	–	–	–	5
Agar (g/L)	–	10	10	9	8	8	–
pH	5.8	5.8	6.2	5.7	5.6	5.6	5.7

The pH is adjusted with 1 N NaOH or 0.5 N HCl prior to autoclaving at 121 °C for 10 min. IAA is filtered and aseptically added to autoclaved medium when the medium temperature has decreased to about 55 °C. Pour 25 ml medium to 90 mm Petri dishes (media A, B, C, G) or an appropriate volume to plant tissue culture containers (media D, E, F)

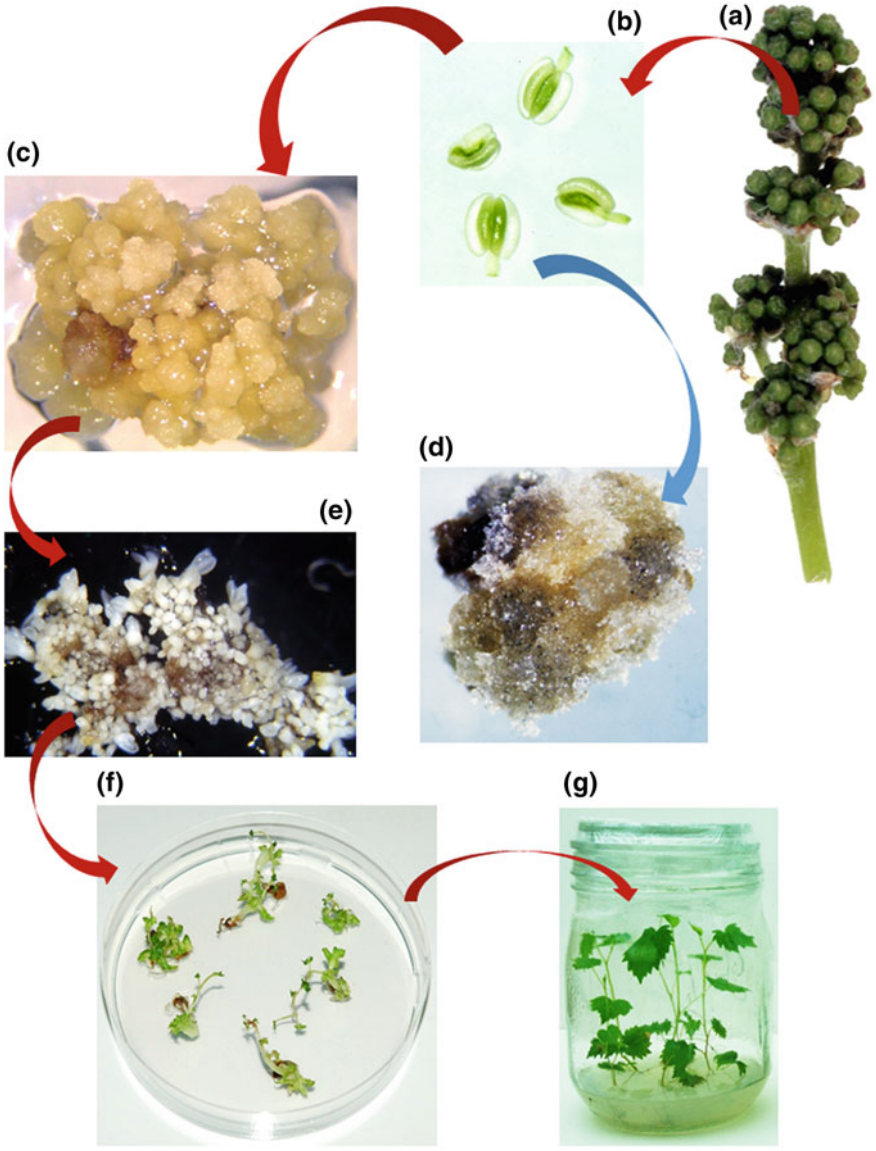

Fig. 12.1 Plant regeneration via somatic embryogenesis in grapevine. **a** Immature inflorescence suitable for explant collection; **b** immature stamens (anthers plus filaments); **c** embryogenic callus; **d** non-embryogenic callus; **e** embryo development from embryogenic callus; **f** embryo germination; **g** micropropagation of plantlets derived from somatic embryos

As for the best developmental stage to initiate anther culture, here we describe the stage that is most often reported best for *Vitis vinifera* (Faure et al. 1996; Gribaudo et al. 2004; Vidal et al. 2009), although in the case of some cultivar or

species of the genus *Vitis* somatic embryogenesis occurred more frequently if explants were collected at later developmental stages (Gribaudo et al. 2004; Bouamama et al. 2007; Prado et al. 2010).

(i) Use immature inflorescences for embryogenic culture initiation. Collect flower in the vineyard 10–14 days before full bloom, when: inflorescence are still compact but flowers gradually separate from each other (Fig. 12.1a), calyptra is dark green and gradually lengthens, anthers are green and translucent (Fig. 12.1b), the pollen mother cells are in late pre-meiotic phase (corresponding to the active synthesis of DNA and proteins needed for meiosis)
(ii) Surface-sterilize the inflorescences for 15 min with a solution of sodium hypochlorite (1.5% available chlorine) containing a few drops of a wetting agent (for instance Tween 20®), and rinse several times with sterile distilled or deionized water.
(iii) Store inflorescences in sterile Petri dishes or other closed container at 4 °C for 2 up to a maximum of 5 days.
(iv) Perform a second surface sterilization before use as described in (ii)
(v) Using a stereomicroscope and under a laminar-flow hood carefully open the flowers, excise intact stamens (anthers plus filaments) and pistils (ovaries plus styles, stigmas and receptacles) by separating them from the calyptra. Place stamens and pistils in Petri dishes containing the medium A.
(vi) Seal Petri dishes with Parafilm® and place in the dark at 26 °C.

12.3.2 Embryo Differentiation

After three months of culture on medium A, most explants have produced callus. However, callus type is seldom homogeneous and can be classified in embryogenic (generally granular white or slightly yellow callus, sometimes associated to dark callus) (Fig. 12.1c) or non-embryogenic (dry and compact or watery and soft callus; colours can vary from yellow to brown) (Fig. 12.1d) (Gambino et al. 2006; Vidal et al. 2009; Prado et al. 2010). Initial embryogenic cultures are generally asynchronous. Transfer the cultures onto medium B is needed to allow embryo differentiation and development (Fig. 12.1e).

(i) Transfer the explants that have produced embryogenic callus onto medium B.
(ii) Seal Petri dishes with Parafilm® and place in the dark at 26 °C.
(iii) Every 4 weeks transfer the cultures onto fresh B medium.

12.3.3 Embryo Germination and Development into Plantlets

From about 5 months after culture initiation onwards, single large embryos can be isolated from the embryogenic callus (Fig. 12.1e) and transferred to medium C, at 24 °C under light (photoperiod 16 h). Conversion of somatic embryos into plants is accompanied by development of the primary root, greening of hypocotyls and cotyledons and formation of the shoot apex with one or two foliar primordia (Redenbaugh et al. 1986) (Fig. 12.1f).

However, as germination and plant recovery can vary considerably depending on the genotype and the embryo morphological and physiological state, various dormancy-breaking treatments have been proposed (Martinelli and Gribaudo 2009; Larrouy et al. 2017) and can be applied in case of germination failure. The following simple strategy is based on the one proposed by Franks et al. (1998):

(i) Remove the basal part of the germinating embryo cutting it at the hypocotyl
(ii) Subculture the cut upper part of the embryo on medium D for shoot growth
(iii) Culture the resulting shoot for 2 weeks on medium E for root induction
(iv) Transfer to medium F for further growth.

All the steps are at 24 °C under light (photoperiod 16 h).

12.3.4 Long-Term Culture of Embryogenic Callus and Preservation of the Embryogenic Ability

During the subsequent subcultures, the embryogenic competence of the callus can be maintained through subcultures performed monthly, alternating every two months medium A and medium B (Franks et al. 1998; Gambino et al. 2005).

However, this strategy is not always advisable for a long-term culture as several grapevine cultivars tend to reduce their embryogenic ability if subjected to many cycles of callogenesis/differentiation. In addition, in some cultivars those culture conditions may impair the successive germination of developed embryos. In these cases, the embryogenic callus obtained from floral explant cultured on medium A can be transferred onto medium G and subcultured every month on the same medium. It is important to subculture only the pro-embryogenic masses, carefully selecting the finely granular, friable callus and discarding the black parts and the developed embryos that can be present.

12.3.5 Micropropagation and Acclimatization

(i) Micropropagate plantlets derived either from direct embryo germination or from the embryo germination protocol above described by periodically

culturing apical cuttings (3–4 cm long) on the PGRs-free medium F (Fig. 12.1g) in suitable plant tissue culture containers

(ii) When transfer to *ex-vitro* conditions is required, collect apical cuttings (4–5 cm long) from the micropropagated plants and place them in soaked, autoclaved peat pellets (Jiffy7®) for 2–4 weeks

(iii) For acclimatization, replace the container lid with plastic film and gradually remove the film within the space of 8–10 days
All the previous steps are at 24 °C under light (photoperiod 16 h).

(iv) Transfer the acclimatized plantlets to greenhouse and, after a suitable period of growth, to soil in the field.

12.4 Steps Requiring Further Protocol Refinements

The protocol for plant regeneration via somatic embryogenesis in grapevine described in this chapter proved efficient for many cultivars during a twenty-year experience in our laboratory (Gribaudo et al. 2017). However, further protocol refinements or modifications will be useful, particularly concerning the embryo conversion into plant and the extension of the range of regenerating genotypes.

Germination step is particularly troublesome, as it can impair the success of the whole procedure. In principle, germination may occur in white, well-shaped, well-polarized embryos with root and shoot axes, a hypocotyl and two cotyledons. Germination is hindered by physiological anomalies such as endodormancy, and/or by morphological abnormalities. Abnormal, missing or non-functional apexes have been observed as well as other embryo teratologies (Goebel-Tourand et al. 1993; Faure et al. 1998; Larrouy et al. 2017). The pronounced anomalous behavior where the embryo exhibits continuous growth leading to abnormal structure and function of the shoot meristem has been connected with the 'precocious germination', an event already described for zygotic embryos during in vitro culture (Finkelstein and Crouch 1984). Indeed the morphological and physiological state of embryos and the culture conditions are crucial aspects in promoting embryo conversion and ensuring the success of the culture. A number of strategies and protocols have been proposed (Martinelli and Gribaudo 2009) but their efficiency must be tested in specific culture conditions.

The strong genotype influence on the performance of in vitro cultures in general, and of grapevine embryogenic culture in particular, makes difficult to set up a unique protocol optimal or even efficient for all cultivars and species of the genus *Vitis* (Oláh et al. 2009; Vidal et al. 2009; Gribaudo et al. 2017). Undeniably the embryogenic response in a grapevine cultivar involves a complex interaction of the genotype with explants, culture medium and culture conditions (Dhekney et al. 2016). Therefore, cultivar or species not yet tested for their embryogenic capacity or having a low response to the described protocol may be evaluated on alternative induction media, gleaning suggestions from the substantial available literature.

References

Bouamama B, Ben Salem-Fnayou A, Ben Jouira H, Ghorbel A, Mliki A (2007) Influence of the flower stage and culture medium on the induction of somatic embryogenesis from anther culture in Tunisian grapevine cultivars. J Int Sci Vigne Vin 41:185–192

Dhekney SA, Li ZT, Grant TNL, Gray DJ (2016) Somatic embryogenesis and genetic modification of *Vitis*. In: Germanà MA, Lambardi M (eds) *In vitro* embryogenesis in higher plants, methods in molecular biology, Springer Science + Business Media, USA, pp 263–277

Dodeman VL, Ducreux G, Kreis M (1997) Zygotic embryogenesis versus somatic embryo-genesis. J Exp Bot 48:1493–1509

Faure O, Aarrouf J, Nougarède A (1996) Ontogenesis, differentiation and precocious germination in anther-derived somatic embryos of grapevine (*Vitis vinifera* L.): proembryogenesis. Ann Bot 78:23–28

Faure O, Dewitte W, Nougarède A, VanOnckelen H (1998) Precociously germinating somatic embryos of *Vitis vinifera* have lower ABA and IAA levels than their germinating zygotic counterparts. Physiol Plant 102:591–595

Finkelstein RR, Crouch ML (1984) Precociously germinating rapeseed embryos retain characteristics of embryogeny. Planta 162:125–131

Franks T, He DG, Thomas M (1998) Regeneration of transgenic *Vitis vinifera* L. Sultana plants: genotypic and phenotypic analysis. Mol Breed 4:321–333

Franks T, Botta R, Thomas M, Franks J (2002) Chimerism in grapevines: implications for cultivar identity, ancestry and genetic improvement. Theor Appl Genet 104:192–199

Gambino G, Gribaudo I (2012) Genetic transformation of fruit trees: current status and remaining challenges. Transgenic Res 21:1163–1181

Gambino G, Gribaudo I, Leopold S, Schartl A, Laimer M (2005) Molecular characterization of grapevine plants transformed with GFLV resistance genes: I. Plant Cell Rep 24:655–662

Gambino G, Bondaz J, Gribaudo I (2006) Detection and elimination of viruses in callus, somatic embryos and regenerated plantlets of grapevine. Eur J Plant Pathol 114:397–404

Gambino G, Ruffa P, Vallania R, Gribaudo I (2007) Somatic embryogenesis from whole flowers, anthers and ovaries of grapevine (*Vitis* spp.). Plant Cell Tiss Organ Cult 90:79–83

Gambino G, Pagliarani C, Gribaudo I (2014) Functional genomics in fruit trees. In: Ramawat KG, Mérillon JM, Ahuja MR (eds) Tree Biotechnology. CRC Press, USA, pp 583–613

Goebel-Tourand I, Mauro MC, Sossountazov L, Miginiac E, Deloire A (1993) Arrest of somatic embryo development in grapevine: histological characterization and the effect of ABA, BAP and zeatin in stimulating plantlet development. Plant Cell Tiss Org Cult 33:91–103

González-Benito ME, Martín C, Vidal JR (2009) Cryopreservation of embryogenic cell suspensions of the Spanish grapevine cultivars 'Albariño' and 'Tempranillo'. *Vitis* 48:131–136

Gray DJ, Li ZT, Dhekney SA (2014) Precision breeding of grapevine (*Vitis vinifera* L.) for improved traits. Plant Sci 228:3–10

Gribaudo I, Gambino G, Vallania R (2004) Somatic embryogenesis from grapevine anthers: the optimal developmental stage for collecting explant. Am J Enol Vitic 55:427–430

Gribaudo I, Gambino G, Boccacci P, Perrone I, Cuozzo D (2017) A multi-year study on the regenerative potential of several *Vitis* genotypes. Acta Hortic 1155:45–50

Jaillon O et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463–468

Kikkert JR, Striem MJ, Vidal JR, Wallace PG, Barnard JB, Reisch BI (2005) Long-term study of somatic embryogenesis from anthers and ovaries of 12 grapevine (*Vitis* sp) genotypes. *In vitro* Cell Dev Biol-Plant 41:232–239

Kuksova VB, Piven NM, Gleba YY (1997) Somaclonal variation and *in vitro* induced mutagenesis in grapevine. Plant Cell Tiss Organ Cult 49:17–27

Larrouy J, Jaksons P, Bicknell R (2017) Response interactions in grape somatic embryogenic cultures to cold and gibberellic acid treatments to overcome embryo dormancy. Plant Cell Tiss Organ Cult 129:45–52

Martinelli L, Gribaudo I (2009) Strategies for effective somatic embryogenesis in grapevine (*Vitis spp*.). An appraisal. In: Roubelakis-Angelakis KA (ed) Grapevine molecular physiology & biotechnology, Springer Science + Business Media, NL, pp 461–493

Martinelli L, Gribaudo I, Bertoldi I, Candioli E, Poletti V (2001) High efficiency somatic embryogenesis and plant germination in grapevine cultivars Chardonnay and Brachetto a grappolo lungo. *Vitis* 40:111–115

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nakano M, Sakakibara T, Watanabe Y, Mii M (1997) Establishment of embryogenesis cultures in several cultivars of *Vitis vinifera* and *V. labruscana*. *Vitis* 36:141–145

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87

Oláh R, Zok A, Pedryc A, Howard S, Kovács LG (2009) Somatic embryogenesis in a broad spectrum of grape genotyes. Sci Hortic 120:134–137

Prado MJ, Grueiro MP, González V, Testillano PS, Domínguez C, López M, Rey M (2010) Efficient plant regeneration through somatic embryogenesis from anthers and ovaries of six autochthonous grapevine cultivars from Galicia (Spain). Sci Hortic 125:342–352

Redenbaugh K, Paasch BD, Nichol JW, Kossler ME, Viss PR, Walker KA (1986) Somatic seeds: encapsulation of asexual plant embryos. Bio/Technol 4:797–801

Reustle GM, Buchholz G (2009) Recent trends in grapevine genetic engineering. In: Roubelakis-Angelakis KA (ed) Grapevine molecular physiology & biotechnology. Springer Publisher, The Netherlands, pp 495–508

Vasanth K, Vivier MA (2011) Improved cryopreservation procedure for long term storage of synchronised culture of grapevine. Biol Plant 55:365

Vidal JR, Rama J, Taboada L, Martin C, Ibañez M, Segura A, González-Benito ME (2009) Improved somatic embryogenesis of grapevine (*Vitis vinifera*) with focus on induction parameters and efficient plant regeneration. Plant Cell Tiss Organ Cult 96:85–94

Chapter 13
Somatic Embryogenesis in *Rosa hybrida*

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13.1 Introduction

Roses are among the most economically important cut flowers throughout the world. Improving a number of rose traits, including resistance to pests and diseases, flower color and form, vase life and fragrance are some of the major goals of rose breeding programs (Bao et al. 2012; Ludwig et al. 2000; Zakizadeh et al. 2008). Traditional breeding methods for roses, including sexual hybridization and subsequent selection are restricted to some limiting factors such as the hybrid nature of roses, their narrow gene pool, the cross incompatibility and the unequal ploidy level of the potential parents (Marchant et al. 1998; Azadi et al. 2016). Therefore, the use of genetic engineering technologies including gene transformation approaches are required to improve the rose species and cultivars.

Somatic embryogenesis is an efficient technique for plant regeneration during which somatic cells are induced to differentiate to embryo. It is a valuable technique for large-scale production, genetic improvement and long term storage of plant materials. Furthermore, it provides an attractive model for in depth studies of

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_13

developmental process during embryogenesis in plants (Mahdavi-Darvari et al. 2015).

Establishment of somatic embryogenesis for roses dates back to 1990 when, for the first time, the embryogenic callus obtained from leaves of *Rosa hybrida* (Roberts et al. 1990; Wit et al. 1990). Since then somatic embryogenesis has frequently been described either as a technique for mass propagation or as a perquisite for genetic improvement of its genotypes for several species and cultivars of roses. However, it should be noted that based on the existing documents, the efficiency of somatic embryogenesis in roses is low and often limited to specific genotypes.

Somatic embryogenesis for roses has been reported from various explants including leaf (Azadi et al. 2013; Chen et al. 2014; Vergne et al. 2010), petiole (Estabrooks et al. 2007), stem (Rout et al. 1991), immature seed (Asano and Tanimoto 2002; Kim et al. 2003b), petal (Jang et al. 2016), root (Kim et al. 2009a; Marchant et al. 1996), anther (Arene et al. 1993) and zygotic embryo (Kim et al. 2009b). Furthermore, somatic embryogenesis was proved to be successful from protoplast of certain species and cultivars of roses (Kim et al. 2003a; Matthews et al. 1994). Among different types of explants that have been used for somatic embryogenesis of roses, in vitro derived leaves are the most frequently employed ones in various protocols. They are also known to be the most efficient tissues for rose embryogenesis.

Plant growth regulators (PGRs) have been shown to be major factors in plant regeneration via somatic embryogenesis technique in roses. However, types and concentrations of the potential PGRs are different depending on specific genotypes. The common plant growth regulators employed for somatic embryogenesis are auxin and cytokinins, however, in many studies 2,4-Dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, has been frequently applied to initiate somatic embryogenesis in roses. Some documents demonstrated that the preincubation of explants in higher level of 2,4-D improves somatic embryo formation in roses. Hsia and Korban (1996) reported the significant increase in organogenesis and regeneration of embryogenic callus of *Rosa hybrida* when the leaves were cultured on Murashig and Skoog (MS) medium supplemented with 100 μm 2,4-D for 6 weeks. Pourhosseini et al. (2012) showed that 2,4-D promoted the highest frequency of callus production for indirect regeneration in *Rosa hybrida* cv. Apollo. Despite the wide use of 2,4-D in somatic embryogenesis, there are some reports which indicate the inefficiency of 2,4-D compared to other forms of auxins. For example, 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) was found to be more efficient compared to 2,4-D in embryogenic callus induction in *Rosa hybrida* cv Livin Easy (Estabrooks et al. 2007). In another study on somatic embryogenesis of *Rosa chinensis*, leaf explants were not responsive to 2,4-D while they produced embryogenic callus in the presence of Naphthaleneacetic acid (NAA) (Vergne et al. 2010).

It has been shown that during somatic embryogenesis in roses, presence of some specific substances has stimulatory effect in different stages of this process. For instance, addition of L-proline in 400–800 mg/l during embryogenic callus induction in *Rosa hybrida* cv. Landora enhanced the frequency of induced embryogenic tissue (Das 2010). Zeinipour et al. (2015) also confirmed the stimulatory effect of Proline for induction of embryogenic callus and subsequent primary

and secondary embryogenesis of *Rosa hybrida* cv. Ocean Song. Furthermore they demonstrated that Sodium chloride as a stress factor could encourage embryogenesis in the same cultivar. In another study, addition of Cyctein and increasing the level of copper in culture medium was reported to be beneficial in embryogenic callus induction of *Rosa hybrida* cv. Soraya (Kintzios et al. 2000).

Maturation and further development of somatic embryo to a normal plant is a critical step in rose somatic embryogenesis. Usually, in this phase the concentration of auxin is reduced or it is completely removed from culture medium to encourage further development and germination of somatic embryos (Matthews et al. 1994). The highest frequency of regeneration was obtained by Pourhosseini et al. (2012) when calli of *Rosa hybrida* cv. Apollo were transferred to MS medium supplemented with 2.5 μM Thidiazuron (TDZ) and 2 μM Gibberellic acid. Silver nitrate (58.8 μm) was reported to promote embryo germination of two rose cultivars when combined with 9.8 μm Benzyl Adenine (BA) during embryo maturation phase (Kim et al. 2004). Addition of Methyl laurate, a chemical pruning substance for agricultural crops, in combination with BA improved germination and development of bipolar growth of *Rosa hybrida* as well (Sarasan et al. 2001). Application of Abscisic acid (ABA) also proved to have stimulatory effect in embryo maturation of rose cultivars and species in several documents (Azadi et al. 2013; Chen et al. 2014; Murali et al. 1996).

In this chapter an efficient protocol for rose regeneration via somatic embryogenesis from immature leaf explant has been described which is applicable to a wide range of rose genotypes.

13.2 Materials

1. Rose stem cuttings of 15 cm length excised during the spring months from the middle sections of the stem
2. Laminar air flow cabinet with ultraviolet light, forceps, scalpel, Petri dishes, 125 ml Erlenmeyer flasks, 1–20 ml serological pipettes, and 10–1000 μl air-displacement piston pipettes, autoclave, pH meter
3. Ethanol, Sodium hypochlorite, sterile distilled water,
4. Murashige and Skoog medium (MS) (Table 13.1), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (BAP) α-naphthalene acetic acid (NAA), Indole-butyric acid (IBA), Thidiazuron (TDZ), Zeatin, Gibberellic acid (GA_3), Proline, Sodium chloride (NaCl), Plant agar, Gelrite and sucrose
5. Growth chamber with 22 ± 2 °C and 16-h photoperiod with cool-white fluorescent light and a PPFD of 60 mol $\mu M^{-2}s^{-1}$ Light microscope

Basic medium composition is listed in Table 13.1. Modifications are required when the shoots are transferred to proliferation stage. At proliferation stage VS medium (Van der Salm et al. 1994) in which FeNaEDTA is replaced by FeEDDHA is used. In all culture media the pH is adjusted to 5.8 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 18 min.

Table 13.1 Chemical composition of MS culture medium (Murashige and Skoog 1962)

Chemicals (macroelements)	Quantity in mg/l	Chemicals (microelements)	Quantity in mg/l
NH_4NO_3	1650.00	KI	0.83
KNO_3	1900.00	H_3BO_3	6.20
$CaCl_2$	332.02	$MnSo_4{\cdot}H_2O$	16.90
KH_2PO_4	170.00	$ZnSo_4{\cdot}7H20$	8.60
$MgSO_4$	180.54	$Na_2MoO_4{\cdot}2H_2O$	0.25
		$CuSo_4{\cdot}5H_20$	0.025
		$CoCl_2{\cdot}6H_2O$	0.025
		Fe Na-EDTA	36.70

13.3 Method

Somatic embryogenesis in roses is usually through indirect embryogenesis of in vitro leaves. The in vitro leaves are achieved by performing two steps: (1) Establishment stage of micropropagation and (2) Shoot proliferation stage of micropropagation. The Indirect somatic embryogenesis includes four main steps: (1) Explant preparation for embryogenic callus induction, (2) Embryogenic callus induction and somatic embryo initiation, (3) Embryo development and maturation and (4) Embryo germination and proliferation. Below these steps are explained.

13.3.1 Establishment Stage of Micropropagation

To establish roses at in vitro conditions, incise rose stem cutting of 10–15 cm length in the spring during the months of April and May, when the buds begin to open. Store them in wet paper and wrap them in plastic bags. Keep them at 4 °C and use them as soon as possible. They can be stored for a maximum period of only 48 days.

1. Cut nodal 10–15 mm segments from stems of rose plants and place them in jam jars
2. Wash them thoroughly with running tab water for half an hour
3. Surface sterilize for 30 s in 70% ethanol, make sure to use sterilized jam jars and work under the laminar air flow cabinet from now
4. Soak the nodal segments in 2.5% sodium hypochlorite solution for 15 min with a few drops of Tween-20 as a wetting agent
5. Rinse them three times with sterile distilled water, every time add water to the jam jar and empty it
6. Culture them on Murashige and Skoog (1962) (MS) medium supplemented with 30 g/l sucrose and 7 g/l plant agar with pH adjusted to 5.8.
7. Incubate them at 22 ± 2 °C for under a 16-h photoperiod with cool-white fluorescent light and a PPFD of 60 mol $\mu M^{-2}s^{-1}$ phytotron.

13.3.2 Shoot Proliferation Stage of Micropropagation

The established shoots are proliferated on the rose proliferation culture medium, which consists of cytokinins and very low levels of auxins.

1. Detach the growing buds from the stem and culture them on Van der Salm et al. (1994) (VS) medium supplemented with 0.45 mg/l 6-Benzylaminopurine (BAP) or 0.9 mg/l BAP plus 0.1 mg/l NAA, 30 g/l sucrose and 7 g/l plant agar with pH adjusted to 5.8.
2. Incubate them at 22 ± 2°C for under a 16-h photoperiod with cool-white fluorescent light and a PPFD of 60 mol $\mu M^{-2}s^{-1}$ phytotron.
3. Sub-culture the explants every four weeks.

According to our previous work (Khosravi et al. 2007) a moderate BAP level (4 μM) in combination with 0.5 μM NAA resulted in the highest growth rate, which is attained only when the concentrations of endogenous or exogenous auxins and cytokinins are balanced.

13.3.3 Explant Preparation for Embryogenic Callus Induction

To induce embryogenic callus, lateral leaflets from the third to fifth leaves are excised from plantlets and are used as the embryogenic explants.

1. Either keep the intact leaflet (L1) or cut the leaflet into three sections: basal section of the leaflet (L2), upper section of the leaflet (L3), half of the leaflet cutting along the midrib (L4) (Fig. 13.1)
2. Make three scratches (dashes), perpendicular to the midrib, using a scalpel on the underside of each explant.

13.3.4 Embryogenic Callus Induction and Somatic Embryo Initiation

1. Put the explants with their abaxial side (lower surface) up, on MS media supplemented with 3 mg/l NAA, 300 mg/l Proline (Stage I medium 1) or 2 mg/l 2,4-D, 100 mg/l NaCl (Stage I medium 2) plus 30 g/l sucrose solidified by 7 g/l Plant agar (Table 13.2).
2. Incubate the cultures in the dark at 22 ± 2 °C for up to six weeks.

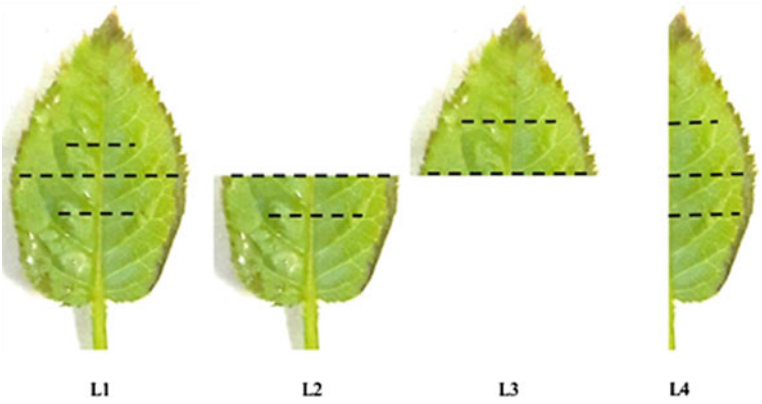

Fig. 13.1 Explants excised from lateral leaflets (third to fifth leaves) of in vitro rose

Table 13.2 Formulation of each stage in somatic embryogenesis of *Rosa hybrida*

Chemicals	Stage I embryogenic callus induction medium 1	Stage I embryogenic callus induction medium 2	Stage II development and maturation medium 1	Stage II development and maturation medium 2	Stage III secondary embryo formation and proliferation
MS	Full	Full	Full	½ MS	Full
Sucrose	30,000[a]	30,000	30,000	30,000	30,000
2,4-D	_	2.0	_	_	_
NAA	3.0	_	_	_	_
Proline	300	_	_	_	_
NaCl	_	100	_	_	_
Zeatin	_	_	3.99	_	_
TDZ	_	_	_	0.5	_
GA3	_	_	_	1	_
Agar	7000	7000	_	_	_
Gelrite	_	_	2500	2500	2500

[a]All units are in mg/l

Callogenesis initiates from cut surfaces of explants after two weeks. Calli may have different sizes depending on the type of cultivar or they may have different morphology depending on the applied treatment. However, all calli formed in the darkness would be soft and yellow in color. At this stage, primary embryos form (Fig. 13.2a).

For induction of somatic embryo a combination of NAA and proline is effective for rose somatic embryogenesis. Proline as a source of NADP+ which is necessary for rapidly growing embryo, acts as a stimulatory agent in this protocol (Pasternak et al. 2002).

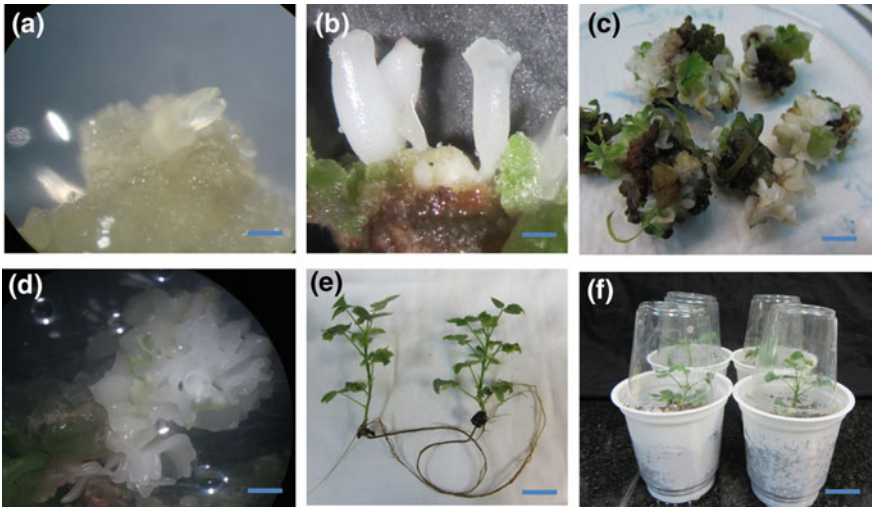

Fig. 13.2 Somatic embryogenesis and plant regeneration in *Rosa hybrida*. **a** Embryogenic callus induction and somatic embryo initiation from in vitro leaf explants (bar = 0.4 mm), **b** embryo development and maturation (bar = 0.5 mm), **c** and **d** Secondary somatic embryos and proliferation (**d**: bar = 4 mm **e**: bar = 1 mm), e: plantlet regenerated from somatic embryos (bar = 2 cm), **f** acclimatized plants at in vivo conditions (bar = 2 mm). Figure 13.2b adopted from Zeinipour et al. 2015; used by permission of international journal of biosciences

13.3.5 Embryo Development and Maturation

1. Transfer the embryogenic calli and primary embryos to the MS medium supplemented with 3.99 mg/l Zeatin, (Stage II, medium 1) or ½ MS medium containing 0.5 mg/l TDZ, 1 mg/l GA_3 (Stage II, medium 2), 30 g/l sucrose solidified with 2.5 g/l Gelrite (Table 13.2).
2. Incubate the cultures at 22 ± 2 °C for 12 weeks until the maturation and production of cotyledonary-stage embryos are observed (Fig. 13.2b).

13.3.6 Secondary Embryo Formation and Proliferation

1. Transfer the matured embryos to PGR free MS medium (Stage III, Table 13.2).
2. Incubate the cultures at 22 ± 2 °C for 8 weeks in the darkness.
3. In this medium, germination of primary embryo and secondary somatic embryogenesis will occur (Fig. 13.2c, d).

13.3.7 Confirming the Embryogenic Nature of the Regenerants

1. Fix embryogenic calli at different developmental stages in FAA (formalin, acetic acid, ethanol: 1,2,17 v/v) for 24 h.
2. Dehydrate them by serial grades of ethanol-xylol and then embed them in paraffin
3. Cut serial sections of 8 μM thick and stain them with hematoxylin and eosin.
4. Visualize the sections under a light microscope (Zeiss, Axiostar Plus, Germany) (Fig. 13.3).

13.3.8 Acclimatization of the Plantlets

Sub-culture the regenerated shoots derived by somatic embryogenesis onto hormone-free MS medium solidified with 2.5 g l-1 Gelrite for a 1 month. The roots will appear and plantlets would be ready to be transferred to the greenhouse (Fig. 13.2e).

- Transfer the plantlets to a 1:1:1 (v/v/v) of soil, peat, and perlite in 4 cm diameter plastic pots
- Cover the plantlets with a transparent plastic cup for 2 weeks (Fig. 13.2f).
- Make one hole onto the plastic cup every two days.
- Remove the plastic, the well-developed plantlets are ready to be transferred to the greenhouse at 23 ± 2 °C.
- Water the plants daily using a drip-irrigation system, and fertilize once every 2 weeks with 250 mg l-1 of a 20–20–20 Crystalon NPK solution.

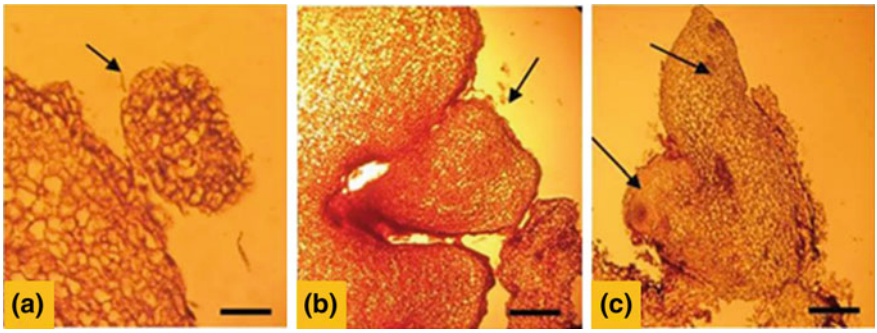

Fig. 13.3 Confirming the embryogenic nature of the regenerants via histological study. **a**, **b** Longitudinal section of transient stage to heart-shaped embryo (**a**: bar = 100 μm; **b**: bar = 50 μm); **c** longitudinal section of somatic embryo in cotyledonary stage (bar = 50 μm). Adopted from Zeinipour et al. 2015; used by permission of international journal of biosciences

13.4 Conclusions and Future Prospects

Somatic embryogenesis in rose is described in the present protocol. However, since rose embryogenesis is highly genotype dependent, some minor protocol optimizations for each specific cultivar are needed. Also future researches are necessary to increase the frequency of somatic embryo initiation and maturation. Somatic embryogenesis protocol which has been reported in this study, could be used in future modern breeding of roses such as in vitro mutation and gene transformation.

References

Arene L, Pellegrino C, Gudin S (1993) A comparison of the somaclonal variation level of *Rosa hybrida* L. cv Meirutral plants regenerated from callus or direct induction from different vegetative and embryonic tissues. Euphytica 71:83–90

Asano G, Tanimoto S (2002) Plant regeneration from embryogenic calli derived from immature seeds in miniature rose cultivar. 'Shortcake'; somaclonal variation, cytological study and RAPD analysis. Plant Biotechnol 19:271–275

Azadi P, Beyrami ZE, Otang NV (2013) A simple protocol for somatic embryogenesis in *Rosa hybrida* L. cv. Apollo. J Hortic Sci Biotechnol 88:399–402

Azadi P, Bagheri H, Nalousi AM, Nazari F, Chandler SF (2016) Current status and biotechnological advances in genetic engineering of ornamental plants. Biotechnol Adv 34:1073–1090

Bao Y, Liu G, Shi X, Xing W, Ning G, Liu J, Bao M (2012) Primary and repetitive secondary somatic embryogenesis in *Rosa hybrida* 'Samantha'. Plant Cell Tissue Organ Cult 109: 411–418

Chen JR, Wu L, Hu BW, Yi X, Liu R, Deng ZN, Xiong XY (2014) The influence of plant growth regulators and light quality on somatic embryogenesis in China rose (*Rosa chinensis Jacq.*). J Plant Growth Regul 33:295–304

Das P (2010) Mass cloning of rose and mussaenda, popular garden plants, via somatic embryogenesis. Hortic Sci 37:70–78

Estabrooks T, Browne R, Dong Z (2007) 2, 4, 5-Trichlorophenoxyacetic acid promotes somatic embryogenesis in the rose cultivar "Livin'Easy" (Rosa sp.). Plant Cell Rep 26:153–160

Hsia CN, Korban SS (1996) Organogenesis and somatic embryogenesis in callus cultures of *Rosa hybrida* and *Rosa chinensis minima*. Plant Cell Tissue Organ Cult 44:1–6

Jang HR, Lee HJ, Park BJ, Pee O-J, Paek K-Y, Park S-Y (2016) Establishment of embryogenic cultures and determination of their bioactive properties in *Rosa rugosa*. Hortic Environ Biotechnol 57:291–298

Khosravi P, Kermani MJ, Nematzadeh GA, Bihamta MR (2007) A protocol for mass production of *Rosa hybrida* cv. Iceberg through *in vitro* propagation. Iranian J Biotechnol 5(1):100–104

Kim C, Oh J, Chung J, Burrell A, Byrne D (2004) Somatic embryogenesis and plant regeneration from in-vitro-grown leaf explants of rose. HortScience 39:1378–1380

Kim SW, Oh MJ, Liu JR (2009a) Plant regeneration from the root-derived embryonic tissues of *Rosa hybrida* L. cv. Charming via a combined pathway of somatic embryogenesis and organogenesis. Plant Biotechnol Rep 3:341–345

Kim SW, Oh MJ, Liu JR (2009b) Somatic embryogenesis and plant regeneration in zygotic embryo explant cultures of rugosa rose. Plant Biotechnol Rep 3:199–203

Kim SW, Oh SC, In DS, Liu JR (2003a) Plant regeneration of rose (*Rosa hybridia*) from embryogenic cell-derived protoplasts. Plant Cell Tissue Organ Cult 73:15–19

Kim SW, Oh SC, Liu JR (2003b) Control of direct and indirect somatic embryogenesis by exogenous growth regulators in immature zygotic embryo cultures of rose. Plant Cell Tissue Organ Cult 74:61–66

Kintzios S, Drossopoulos J, Lymperopoulos C (2000) Effect of vitamins and inorganic micronutrients on callus growth and somatic embryogenesis from young mature leaves of rose. J Plant Nutr 23:1407–1420

Ludwig C, Nehring K, Debener T, Dohm A (2000) Somatic embryogenesis in roses. III Int Symp Rose Res Cult 547:341–347

Mahdavi-Darvari F, Noor NM, Ismanizan I (2015) Epigenetic regulation and gene markers as signals of early somatic embryogenesis. Plant Cell Tissue Organ Cult 120:407–422

Marchant R, Davey MR, Lucas JA, Power JB (1996) Somatic embryogenesis and plant regeneration in Floribunda rose (*Rosa hybrida* L.) cvs. trumpeter and glad tidings. Plant Sci 120:95–105

Marchant R, Davey MR, Lucas JA, Lamb CJ, Dixon RA, Power JB (1998) Expression of a chitinase transgene in rose (*Rosa hybrida* L.) reduces development of blackspot disease (Diplocarpon rosae Wolf). Mol Breeding 4:187–194

Matthews D, Mottley J, Yokoya K, Roberts A (1994) Regeneration of plants from protoplasts of rosa species (Roses). Springer, Plant Protoplasts and Genetic Engineering V, pp 146–160

Murali S, Sreedhar D, Lokeswari T (1996) Regeneration through somatic embryogenesis from petal-derived calli of *Rosa hybrida* L. cv Arizona (hybrid tea). Euphytica 91:271–275

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Pasternak TP, Prinsen E, Ayaydin F, Miskolczi P, Potters G, Asard H, Onckelen HAV, Dudits D, Fehér A (2002) The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiol 129:1807–1819

Pourhosseini L, Jafarkhani Kermani M, Habashi AA, Khalighi A (2012) Efficiency of direct and indirect shoot organogenesis in different genotypes of *Rosa hybrida*. Plant Cell Tissue Organ Cult 112:101–108

Roberts A, Horan I, Matthews D, Mottley J (1990) Protoplast technology and somatic embryogenesis in Rosa, integration of in vitro techniques in ornamental plant breeding. In: Proceedings, symposium, 10–14 Nov 1990. Eucarpia, pp 110–115

Rout G, Debata B, Das P (1991) Somatic embryogenesis in callus cultures of *Rosa hybrida* L. cv. Landora. Plant Cell Tissue Organ Cult 27:65–69

Sarasan V, Roberts A, Rout G (2001) Methyl laurate and 6-benzyladenine promote the germination of somatic embryos of a hybrid rose. Plant Cell Rep 20:183–186

Van der Salm TP, Van der Toorn CJ, ten Cate CHH, Dubois LA, De Vries DP, Dons HJ (1994) Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. 'Moneyway'. Plant Cell Tissue Organ Cult 37:73–77

Vergne P, Maene M, Gabant G, Chauvet A, Debener T, Bendahmane M (2010) Somatic embryogenesis and transformation of the diploid *Rosa chinensis* cv old blush. Plant Cell Tissue Organ Cult 100:73–81

Wit JC, Esendam HF, Honkanen JJ, Tuominen U (1990) Somatic embryogenesis and regeneration of flowering plants in rose. Plant Cell Rep 9:456–458

Zakizadeh H, Debener T, Sriskandarajah S, Frello S, Serek M (2008) Regeneration of miniature potted rose (*Rosa hybrida* L.) via somatic embryogenesis. European J Hortic Sci 73:111–117

Zeinipour M, Azadi P, Majd A, Kermani MJ, Irian S (2015) Effect of stress factors on somatic embryogenesis of rose. Int J Biosci 6:255–265

Chapter 14
Somatic Embryogenesis of Tamarillo (*Solanum betaceum* Cav.)

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14.1 Introduction

Tamarillo, *Solanum betaceum* Cav. *syn Cyphomandra betacea* (Cav.) Sendt, is a solanaceous tree also known by other common names such as tree tomato or 'tomate de la paz' (Bois 1927) depending on the region where it grows. Originated from the Andean region of Peru, Chile, Ecuador and Bolivia it has spread to several other world areas (Dawes and Pringle 1983; Meadows 2002) and nowadays it is produced mainly in New Zealand and Colombia. The main economic interest in this species is related to the high nutritional value of its fruits, which can be eaten fresh or processed, and which have a relatively high content of proteins, vitamin C, vitamin E, provitamin A and minerals, such as potassium and iron (McCane and Widdowson 1992). More recent studies have shown that tamarillo fruits are also rich in anthocyanins and carotenoids and may be used as a source of antioxidants (Hurtado et al. 2009; Kou et al. 2008).

In vitro propagation by axillary shoot proliferation, induction of organogenesis or somatic embryogenesis (SE) and genetic transformation techniques are valuable biotechnological tools that have been applied to tamarillo breeding (reviewed by Correia and Canhoto 2012). SE is of particular interest because it has a great potential for the improvement of tree species and for rapid large-scale clonal propagation, genetic transformation, and cryopreservation of desirable selected lines (Corredoira et al. 2014, 2015; Ozudogru and Lambardi 2016). Moreover,

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_14

somatic embryo induction and development serve as model to understand genetic regulation of embryo development (Rose et al. 2010; Yang and Zhang 2010; Hand et al. 2016).

Since 1988, different aspects related with SE induction and somatic embryo development of tamarillo have been studied at the Laboratory of Plant Biotechnology in the University of Coimbra, Portugal (Guimarães et al. 1988; Lopes et al. 2000; Canhoto et al. 2005; Correia et al. 2011, 2012a, b) making it a suitable model to understand the cytological and molecular mechanisms involved in somatic embryo formation and development, a morphogenic process with important applications both for plant cloning and genetic transformation and also to better understand embryo formation and development (Correia and Canhoto 2012).

As in most other woody species (Bonga et al. 2010) immature or mature zygotic embryos were found to be the most suitable and successful explants, followed by seedling explants, such as cotyledons and hypocotyls (Lopes et al. 2000; Canhoto et al. 2005). The recalcitrance of adult material to SE induction is well known and is one of the major problems preventing wider use of SE technology in both angiosperms and conifers (Klimaszewska et al. 2011; Lelu-Walter et al. 2013). Effective protocols of SE induction from adult trees permit a faster propagation of the elite trees, hence increasing genetic gains in each breeding generation.

In spite of the difficulties in cloning adult trees through SE there have been recent reports on the induction of SE and plant development from explants of adult trees of both angiosperms (Mauri and Manzanera 2003; Sriskandarajah and Lundquist 2009; San-José et al. 2010; Corredoira et al. 2015; Maillot et al. 2016) and conifers (Klimaszewska et al. 2011).

In the present work a methodology for the propagation of adult tamarillo trees through SE is presented, by following an indirect approach in which an adult tree was first established in vitro through axillary shoot proliferation and then leaves from this material were used for SE induction (Fig. 14.1).

14.2 Protocol of Somatic Embryogenesis in Tamarillo

14.2.1 Establishment and Multiplication of Axillary Shoots from an Adult Tree

1. Initiate shoot cultures of *S. betaceum* from lignified branches (1–2 cm thick) with visible undeveloped new buds, collected from the adult plant (Fig. 14.2a).
2. To allow the development of axillary shoots under controlled conditions, treat branch segments (20–25 cm) as follows: wash in running water, spray with a fungicide solution (e.g., Mancozebe, 1% w/v) and set them upright in jars containing water, in a climatic growth chamber at 23 ± 2 °C and 70–80% relative humidity, under a 16 h photoperiod at 15–20 μmol $m^{-2}s^{-1}$ (cool white fluorescent lamps), during 2 weeks.

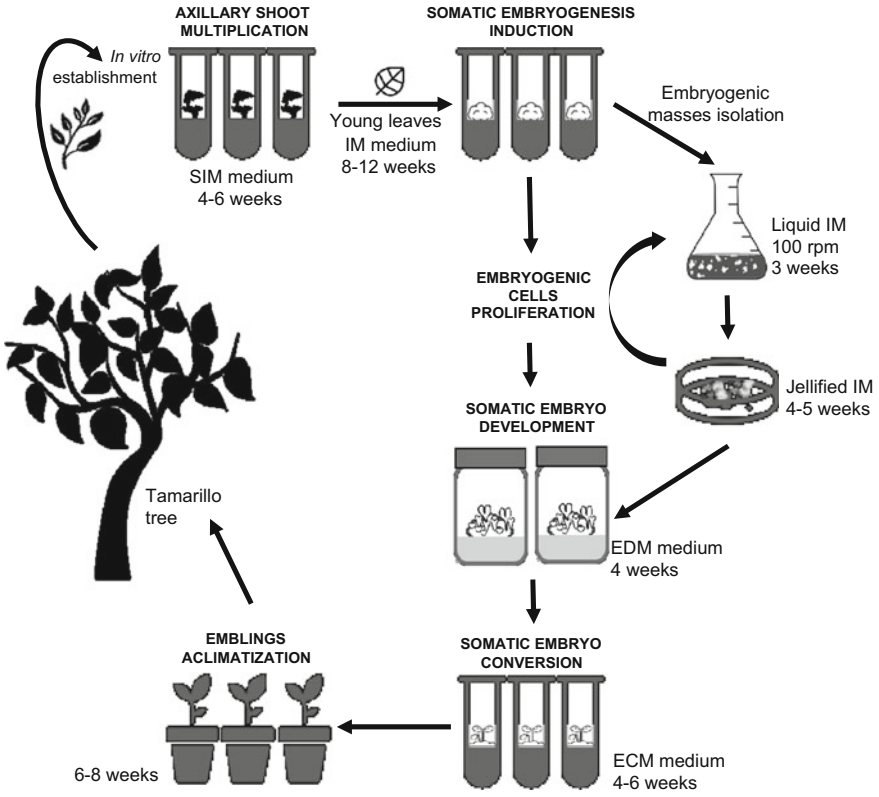

Fig. 14.1 Schematic representation of the process of propagation of and adult tamarillo tree through somatic embryogenesis induction on leaf explants of in vitro established axillary shoots

3. After axillary shoot development (circa 1 cm) remove the leaves from the braches and cut them into nodal segments (1.5–2 cm long) possessing only one axillary bud.
4. Wash the nodal segments with detergent (2–3 drops of Tween 20) in a volume of 100 ml of water, and surface disinfect them by a brief immersion (30 s) in 70% (v/v) ethanol followed by a 15 min. immersion in 5% (w/v) calcium hypochlorite solution containing 2–3 drops of Tween 20 under stirring. After disinfection, wash nodal segments three times in sterile pure water.
5. Excise the axillary shoot buds, with small amount of stem tissues, from each nodal segment and inoculate them individually in test tubes (15 × 2.2 cm) with 12 ml/tube of shoot induction medium (SIM, Table 14.1): MS medium (Murashige and Skoog 1962) supplemented with 0.8 μM benzyladenine (BA) and 0.07 M sucrose, pH adjusted to 5.7 (before autoclaving at 121 °C for 20 min, and jellified with 7 $g.l^{-1}$ Agar (Duchefa, Netherlands).

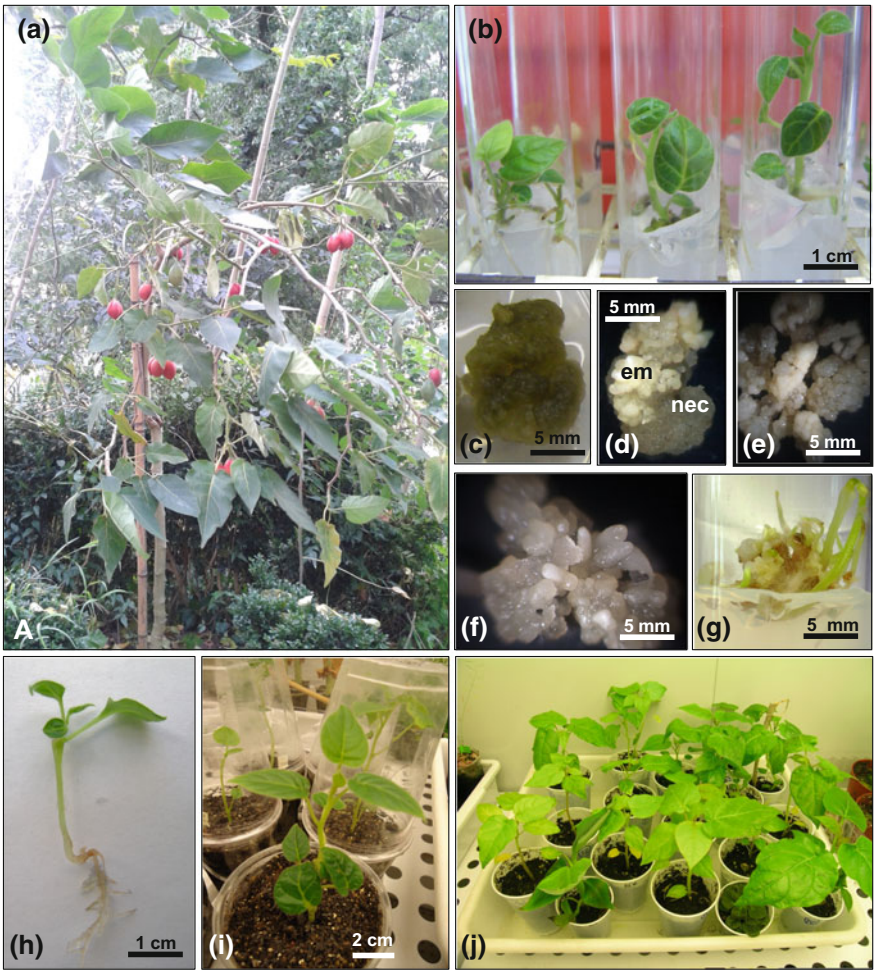

Fig. 14.2 In vitro cloning of an adult tamarillo tree (**a**) using axillary shoot establishment and micropropagation (**b**) before SE induction. Leaf explant after 1 month in the induction medium (IM)—MS supplemented with picloram (**c**). Embryogenic masses (em) and non-embryogenic callus (nec) zones formed on a leaf explant after 10–12 weeks of culture (**d**). Embryogenic mass (**e**). Somatic embryos at different developmental stages after 2 weeks in EDM medium—MS basal medium without auxins and lower sucrose conditions (**g**). Note the abnormal morphology of some of the embryos in (**g**), showing fused or undifferentiated cotyledons. Somatic embryo conversion (**h**) and plantlet development, with well developed root system (**h**), after 6 weeks on in ECM medium. Acclimatization of the rooted plants (**i**, **j**)

Table 14.1 Tamarillo explants culture media and culture conditions according to the stage of the somatic embryogenesis process

Process stage	Culture media	Basal medium	Sucrose (M)	Plant growth regulators	Jellifying agent	Culture vessel	Culture conditions
Shoot induction and multiplication	SIM	Full strenght MS	0.07	0.8 μM BA	7 $g.l^{-1}$ Agar	Test tubes (12 ml SIM)	16 h daily illumination/ 24 ± 2°C
SE Induction	IM	Full strenght MS	0.25	20 μM picloram	2.5 $g.l^{-1}$ Gellan gum	Test tubes (12 ml IM)	Dark/24 ± 2 °C
Embryogenic cells proliferation	IM (liquid)	Full strenght MS	0.25	20 μM picloram	–	Erlenmeyers (20 ml IM)	Dark/100 rpm/24 ± 2 °C
	IM (jellified)	Full strenght MS	0.25	20 μM picloram	2.5 $g.l^{-1}$ Gellan gum	Petri dishes (25 ml IM)	Dark/24 ± 2 °C
Somatic embryo development	EDM	Full strenght MS	0.11	–	7 $g.l^{-1}$ Agar	Flasks (25 ml EDM)	Dark/24 ± 2 °C
Somatic embryo conversion	ECM	Full strenght MS	0.07	–	7 $g.l^{-1}$ Agar	Test tubes (12 ml ECM)	16 h daily illumination/ 24 ± 2 °C

SIM Shoot induction medium; *IM* Induction Medium; *EDM* Embryo Development Medium; *ECM* Embryo Conversion medium

6. Allow in vitro shoot development in the growth chamber at 24 ± 2 °C under a 16 h photoperiod at 15–20 μmol $m^{-2}s^{-1}$ (cool white fluorescent lamps), during 4–8 weeks, while evaluating contamination rates.
7. Subculture the shoot cultures (Fig. 14.2b), in six weeks' periods, by sectioning elongated shoots (2.5–3 cm) into 1–1.5 cm nodal sections and inoculating them onto fresh SIM.

14.2.2 Somatic Embryogenesis Induction from Leaf Explants of the Established Shoots

1. Excise the most apical expanding leaves from proliferating cultures (Sect. 2.1) and used them as initial explants for SE induction.
2. Cut each leaf blade into 4 sections (avoiding the midrib) and make superficial random wounds in the abaxial side with the tip of a scalpel.
3. Immediately place each wounded leaf section (with the abaxial side down) in test tubes containing 12 ml of induction medium (IM, Table 14.1): MS medium plus 0.25 M sucrose and 20 μM picloram, pH adjusted to 5.7 (before autoclaving at 121 °C for 20 min, and jellified with 2.5 $g.l^{-1}$ gellan gum (Phytagel, Sigma).
4. Incubate cultures at 24 ± 2 °C, in the dark, during 12 weeks. By the 8–10th week of culture whitish clusters of embryogenic cells are visible in the explants (Fig. 14.2c, d).
5. Evaluate the percentage of explants forming embryogenic tissue after the 12 weeks of incubation.
6. Separate the embryogenic cell clumps from the non-embryogenic surrounding callus and transfer them to fresh induction medium.
7. Proliferate embryogenic cell masses (Fig. 14.2e) by subculturing them in test tubes (50–100 mg FW per tube) on the same medium, each 4–5 weeks or in liquid suspension cultures (Sect. 2.3).

14.2.3 Embryogenic Cell Proliferation in Suspension Cultures

1. Inoculate 40 mg of callus into 50 ml culture flask containing 20 ml liquid induction medium (IM, Table 14.1), without gellan gum.
2. Incubate cultures at 24 ± 2 °C, in the dark, on an orbital shaker set to 100 rpm, during 3 weeks.
3. After the proliferation period, transfer the embryogenic cells to petri dishes (100 × 20 cm) with 25 ml of jellified IM. Remove the embryogenic cells from the liquid IM by vacuum filtration, with qualitative filter paper (grade 3), under sterile conditions.

14.2.4 Somatic Embryo Development

1. Remove white opaque clusters of embryogenic tissue (80–100 mg FW) from the induction medium, after the proliferation stage, and transfer them to 100 ml flasks containing 25 ml of solidified embryo development medium (EDM, Table 14.1)—full strength MS basal medium supplemented with 0.11 M sucrose, pH adjusted to 5.7 (before autoclaving at 121 °C for 20 min, and jellified with 7 $g.l^{-1}$ Agar (Duchefa, Netherlands).
2. Incubate cultures at 24 ± 2 °C, in the dark, during 4 weeks. By the 4th week of culture somatic embryos at different development stages (globular, heart-shaped, torpedo and cotiledonary) are observed, since embryo development is not a synchronous process (Fig. 14.2f). Also several morphologically abnormal embryos usually develop. However, most of the embryos are at the cotyledonary stage and ready to conversion. Evaluate the efficiency of the development conditions by counting the number of mature cotyledonary and anomalous somatic embryos after the culture period.

14.2.5 Somatic Embryo Conversion and Plant Acclimatization

1. Following somatic embryo development, transfer mature cotyledonary somatic embryos to test tubes (5–6 embryos/tube) containing embryo conversion medium (ECM, Table 14.1): MS basal medium with 0.07 M sucrose, pH adjusted to 5.7 (before autoclaving at 121 °C for 20 min, and jellified with 7 $g.l^{-1}$ Agar (Duchefa, Netherlands).
2. Culture somatic embryos in ECM, at 24 ± 2 °C, under a 16 h daily illumination regime of 15–20 μmol $m^{-2}s^{-1}$ photosynthetically active radiation provided by cool-white fluorescent lamps, during 4–6 weeks, to achieve plantlet formation.
3. Following root formation (Fig. 14.2g, h), gently remove the agar from roots with tap water and transfer the rooted plantlets to pots containing a mixture of sand, loam and peat (1:1:1) (covered with plastic sheet to maintain high humidity environment) and placed in a growth cabinet chamber. Allow plant acclimatization in the climatic growth chamber at 23 ± 2 °C and 70–80% relative humidity, under a 16 h photoperiod at 15–20 μmol $m^{-2}s^{-1}$ (cool white fluorescent lamps), during 6–8 weeks. Gradually reduce humidity by raising the plastic sheets (Fig. 14.2i).
4. After 8 weeks of acclimatization plants are ready to grow under standard greenhouse conditions (Fig. 14.2j).

14.3 Future Reserch

This protocol was successfully applied to clone red tamarillo cultivar (Correia et al. 2011), allowing to overcome the lack of potential of the adult tissues for SE. By this indirect approach it was possible to obtain relatively low rates of embryogenic tissue formation, but still in the range of initiation rates from leaf explants of in vitro cloned plantlets of other different tamarillo cultivars. However, differences may appear in trees of different cultivars as has been observed with the material of seedling origin. Further studies should be directed towards specific optimizations of the protocol regarding genotype dependent variations and towards increasing the frequency of embryogenic tissue formation and the number of regenerated plantlets per mass of embryogenic tissue.

The presence of a high number of anomalous embryos, characteristic of several SE systems, do not significantly affect plant conversion, indicating that shoot apical meristem development is not affected in anomalous somatic embryos. Nevertheless, further analysis should focus on the analysis of the effect of different maturation treatments tested in relation to the storage compounds accumulated in the embryo cells, since the improvement of maturation conditions could be very important to the yield of the somatic embryogenesis process in this species.

References

Bois D (1927) Les plantes alimentaires. In: Le chevalier P (Ed), vol 1, pp 365–366

Bonga JM, Klimaszewska K, von Aderkas P (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tiss Org Cult 100:241–254

Canhoto J, Lopes M, Cruz G (2005) Protocol of somatic embryogenesis: tamarillo (*Cyphomandra betacea* (Cav.) Sendt). In: Jain S, Gupta P (ed), Protocol for somatic embryogenesis in woody plants. Springer, Berlin, pp 379–389

Corredoira E, Ballester A, Ibarra M, Vieitez A-M (2015) Induction of somatic embryogenesis in leaf and shoot apex explants of shoot cultures derived from adult *Eucalyptus globulus* and *Eucalyptus saligna* x *E. maidenii* trees. Tree Physiol 35:663–677

Corredoira E, Toribio M, Vieitez A-M (2014) Clonal propagation via somatic embryogenesis in *Quercus* spp. In: Ramawhat KG, Mérillon JM, Ahuja MR (eds), Tree biotechnology. CRC Press, Boca Raton, FL, pp 262–302

Correia SI, Canhoto JM (2012) Biotechnology of tamarillo (*Cyphomandra betacea*): From in vitro cloning to genetic transformation. Scientia Hort 148:161–168

Correia SI, Lopes L, Canhoto JM (2011) Somatic embryogenesis induction system for cloning an adult *Cyphomandra betacea* (Cav.) Sendt. (tamarillo). Trees 25:1009–1020

Correia SI, Cunha AE, Salgueiro L, Canhoto JM (2012a) Somatic embryogenesis in tamarillo (*Cyphomandra betacea*): approaches to increase efficiency of embryo formation and plant development. Plant Cell Tiss Org Cult 109:143–152

Correia SI, Vinhas R, Manadas B, Lourenço AS, Veríssimo P, Canhoto JM (2012b) Comparative proteomic analysis of induced embryogenic and non-embryogenic tissues of the solanaceous tree *Cyphomandra betacea* (tamarillo). J Proteome Res 11:1666–1675

Dawes SN, Pringle GL (1983) Subtropical fruit from south and central America. In: Wratt GS et al (eds), Plant breeding in New Zealand. Butterworths, Wellington, pp 123–138

Guimarães ML, Cruz GS, Montezuma-de-Carvalho JM (1988) Somatic embryogenesis and plant regeneration in *Cyphomandra betacea* (Cav.) Sendt. Plant Cell Tiss Org Cult 15:161–167

Hand ML, Vries S, Koltunow AMG (2016) A comparison of *In Vitro* and *In Vivo* Asexual Embryogenesis. In: Germanà M, Lambardi M (ed), *In Vitro* embryogenesis in higher plants. Springer and Humana Press, Berlin, pp 3–23

Hurtado NH, Morales AL, González-Miret ML, Escudero-Gilete ML, Heredia FJ (2009) Color, pH stability and antioxidant activity of anthocyanin rutinosides isolated from tamarillo fruit (*Solanum betaceum* Cav.). Food Chem 117:88–93

Klimaszewska K, Overton C, Stewart D, Rutledge RG (2011) Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression proles of 11 genes followed during the tissue culture process. Plant 233:635–647

Kou M, Yen J, Hong J, Wang C (2008) *Cyphomandra betacea* Sendt. phenolics protect LDL from oxidation and PC12 cells from oxidative stress. LWT – Food Science Technol. 42:458–463

Lelu-Walter MA, Thompson D, Harvengt L, Sanchez L, Toribio M, Pâques LE (2013) Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction. Tree Genetics & Genomes 9:883

Lopes ML, Ferreira MR, Carloto JM, Cruz GS, Canhoto JM (2000) Somatic embryogenesis induction in tamarillo (*Cyphomandra betacea*). In: Jain SM, Gupta PK, Newton RJ (eds), Somatic embryogenesis in woody plants, vol 6. Kluwer Academic Publishers, Dordrecht, pp 433–455

Maillot P, Deglène-Benbrahim L, Walter B (2016) Efficient somatic embryogenesis from meristematic explants in grapevine (*Vitis vinifera* L.) cv. Chardonnay: an improved protocol. Trees 30:1377

Mauri PV, Manzanera JA (2003) Induction, maturation and germination of holm oak (*Quercus ilex* L.) somatic embryos. Plant Cell Tiss Org Cult 74:229–235

McCane J, Widdowson DA (1992) Fruit and nut. In: Suppl. to the composition of foods, 5th edn. Unwin & Buss., London, Holland, pp 74–77

Meadows LR (2002) Growing tamarillo relatives. In: The New Zealand home garden, pp 74–77. www.naturalhub.com. Accessed June 2017

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plant 15:473–497

Ozudogru EA, Lambardi M (2016) Cryotechniques for the long-term conservation of embryogenic cultures from woody plants. In: Germanà M, Lambardi M (eds), *In Vitro* embryogenesis in higher plants. Springer and Humana Press, Berlin, pp 537–550

Rose RJ, Mantiri FR, Kurdyukov S, Chen S-K, Wang X-D, Nolan KE, Sheahan MB (2010) Developmental biology of somatic embryogenesis. In: Pua, EC, Davey MR (eds), Plant developmental biology – biotechnological perspectives, vol 2. Springer, Berlin, pp 3–26

San-José MC, Corredoira E, Martínez MT, Vidal N, Valladares S, Mallón T, Vieitez AM (2010) Shoot apex explants for induction of somatic embryogenesis in mature *Quercus robur* L. trees. Plant Cell Rep 29:661–671

Sriskandarajah S, Lundquist P (2009) High frequency shoot organogenesis and somatic embryogenesis in juvenile and adult tissues of seabuckthorn (*Hippophae rhamnoides* L.). Plant Cell Tiss Org Cult 99:259–268

Yang X, Zhang Y (2010) Regulation of somatic embryogenesis in higher plants. Critical Rev Plant Sci 29:36–57

Chapter 15
Somatic Embryogenesis in Micropropagation System of Hybrid Aspen (*P. tremuloides* × *P. tremula*)

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15.1 Introduction

Growing demand for biomass revealed the advantages of fast-growing hybrid aspen trees under North European conditions (Luoranen et al. 2006; Tullus et al. 2009). The term "hybrid aspen" usually indicates a cross between *Populus tremula* L. which is widespread in Eurasia (being native also to the northernmnost parts of continental Europe) and *Populus tremuloides* Michx. originating from North America. Besides being economically promising, these trees are recognized as a suitable model to study plant morphogenesis in vitro and hormonal interactions alongside such well-known model plants as Arabidopsis (Mauriat et al. 2014).

Several technologies have been developed for the fast propagation of selected aspen genotypes, varying from root cuttings (Stenvall et al. 2004) to laboratory (in vitro) micropropagation which provides the opportunity to obtain a large number of new plants in a relatively short period of time (Malá et al. 2006). An originally-modified technology for hybrid aspen propagation through tissue culture has been developed also in Forest Plant Biotechnology Laboratory of Lithuanian Research Centre for Agriculture and Forestry. The testing of the efficiency of this technology included ex vitro acclimatization of microshoots and production of saplings with closed root system, revealing the rate of rooted and vigorously growing clones to be as high as 90–98%. Obtained hybrid aspen plants were used not only to establish experimental clonal stands but also, through the cooperation with a private enterprise, to establish several industrial-scale plantations.

Among other technological suggestions, such as culturing explants under the conditions of restricted gas exchange, that led to an increased rate of organogenesis and subsequent shoot production (Žiauka et al. 2013), induction of somatic

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_15

embryogenesis at certain culture stages also contributed significantly to the micropropagation success of hybrid aspen. As the scientists reviewing the use of somatic embryogenesis in forest tree biotechnologies readily agree, the possibility to obtain somatic embryos from the plant material other than zygotic embryos is of particular importance (Hazubska-Przybył and Bojarczuk 2016). This makes a place for somatic embryogenesis in the propagation of selected elite trees. Accordingly, the present protocol describes the use of somatic embryogenesis in the micropropagation process of hybrid aspen with shoot parts of an adult tree taken as primary explants.

15.2 Materials

1. Rejuvenated shoots of hybrid aspen selected trees.
2. Laminar-flow hood with ultraviolet light, Petri dishes, forceps, scalpel, fixed volume pipettes from 200 to 1000 µl, 10 ml syringes, sterile acrodisc syringe filters 0.22 µm, parafilm.
3. Ethanol, sterile distilled water, 50% commercial bleach, Tween 80, 125 ml Erlenmeyer flasks, plastic vessels 200 ml, plastic Petri dishes (15 mm × 90 mm).
4. Dissecting microscope.
5. 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA), indole-acetic-acid (IAA), gibberellic acid (GA_3), tissue culture agar, sucrose, L-maltose.
6. Myo-inositol, polyethylene glycol (PEG).
7. Tissue culture chambers.
8. Nutrient media (see Tables 15.1, 15.2, 15.3 and 15.4).

Table 15.1 Woody plant medium (WPM)

Chemicals	Quantity in mg/l	Chemicals	Quantity in mg/l
$CaCl_2$	72.50	$MnSO_4 \cdot H_2O$	22.30
$Ca(NO_3)2 \cdot 4H_2O$	471.26	$Na_2MoO_4 \cdot 2H_2O$	0.25
KH_2PO_4	170.00	$ZnSO_4 \cdot 7H_2O$	8.60
K_2SO_4	990.00	Glycine	2.00
$MgSO_4$	180.54	Myo-inositol	100.00
NH_4NO_3	400.00	Nicotinic acid	0.50
$CuSO_4 \cdot 5H_2O$	0.25	Pyridoxine HCl	0.50
FeNaEDTA	36.70	Thiamine HCl pH 5,6	1.00
H_3BO_3	6.20		

(McCown and Lloyd 1980)

Table 15.2 Murashige and Skoog medium (MS)

Chemicals	Quantity in mg/l	Chemicals	Quantity in mg/l
$CaCl_2$	332.02	KI	0.83
KH_2PO_4	170.00	$MnSO_4 \cdot H_2O$	16.90
KNO_3	1900.00	$Na_2MoO_4 \cdot 2H_2O$	0.25
$MgSO_4$	180.54	$ZnSO_4 \cdot 7H_2O$	8.60
NH_4NO_3	1650.00	Glycine	2.00
$CoCl_2 \cdot 6H_2O$	0.025	Myo-inositol	100.00
$CuSO_4 \cdot 5H_2O$	0.025	Nicotinic acid	0.50
FeNaEDTA	36.70	Pyridoxine HCl	0.50
H_3BO_3	6.20	Thiamine HCl pH 5,6	0.10

(Murashige and Skoog 1962)

Table 15.3 The composition of media for hybrid aspen direct somatic embryogenesis

Chemicals	Quantity in mg/l	Chemicals	Quantity in mg/l
$CaCl_2$	72.50	KI	0.83
$Ca(NO_3)2 \cdot 4H_2O$	471.26	$MnSO_4 \cdot H_2O$	16.90
KH_2PO_4	170.00	$Na_2MoO_4 \cdot 2H_2O$	0.25
K_2SO_4	990.00	$ZnSO_4 \cdot 7H_2O$	8.60
$MgSO_4$	180.54	myo-Inositol	100.00
NH_4NO_3	400.00	Nicotinic acid	1.00
$CuSO_4 \cdot 5H_2O$	0.025	Pyridoxine HCl	1.00
FeNaEDTA	36.70	Thiamine HCl	10.00
H_3BO_3	6.20	pH 5,6	

Makro WPM (Table 15.1); mikro MS (Table 15.2), vitamins by Gamborg et al. (1968)

The composition of supplementary organic compounds of the modified HASP medium L-maltose 10,000 mg/l, Myo-Inositol 50 mg/l. The pH was adjusted to 5.6 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 20 min.

Basic medium composition is listed in Tables 15.1, 15.2 and 15.3. Required modifications for different culture stages are listed in Table 15.4. The pH is adjusted to 5.6 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 20 min. Filter 2,4-D, BA, IAA, GA_3, to sterilize and add to sterile media aseptically. Pour 35 ml medium into plastic vessels 200 ml, 40 ml medium into 125 ml Erlenmeyer flasks, 20 ml medium into 15 mm × 100 mm Petri dishes.

Table 15.4 Modifications of media at different stages of somatic embryogenesis process

Chemicals	HASP-1	HASP-2	HASP-3	HASP-4	HASP-5
	Stage I Treatment of leaves	Stage II Initiation somatic embryogenesis	Stage III Germination, organogenesis	Stage IV Regeneration shoots	Stage V Long time stable shoot culture
Base	MS	WPM+MS+G	WPM+MS+G	WPM	WPM
Myo-inositol	–	–	–	50.00	–
L-maltose	–	10,000	10,000	–	–
Sucrose	30,000	15,000	15,000	25,000	30,000
PEG 6000	–	–	12,500	12,500	–
2,4-D	1	–	–	–	–
IAA	–	0.01	0.01	0.01	0.01
BA	0.2	0.5	0.5	0.125	0.125
GA_3	–	1.00	1.00	–	–
Gellrite[a]	–	2700	3000	3000	3000

All units are in mg/l

[a]Tissue culture solidifier, not used for liquid media

15.3 Method

The procedure of plant regeneration via somatic embryogenesis includes five basic steps: (1) embryogenic culture initiation from explants, (2) maintenance and proliferation of embryogenic cultures, (3) embryo development and germination, (4) shoot development in larger in vitro vessels, (5) sapling acclimatization and field transfer.

Embryogenic Culture Initiation

Tree branches for the primary in vitro culture that would eventually serve as an explant source for somatic embryogenesis were collected in February from selected hybrid aspens (30 years old). Branch segments (30–40 cm long) containing apical and axillary buds were soaked in a vessel filled with tap water for new shoot sprouting. Afterwards the procedures were conducted in the following order:

1. Separate new shoots (1–4 cm) from a primary branch.
2. Treat the shoots with 70% v/v ethanol for 1 min.
3. Wash shoots four times with sterile distilled water, 3 min each time.
4. Sterilize the shoots with 50% commercial bleach (containing sodium hypochlorite) supplemented with Tween 80 (two drops) for 3 min, slowly shaking by hand under laminar-flow environment.
5. Rinse the seeds five times with sterile distilled water in the laminar-flow environment, 3 min each four times and 4 min the last (fifth) time.

6. Treat the shoots with silver nitrate ($AgNO_3$) 0.1% for 1 min.
7. Place the sterile shoots vertically into gelled shoot induction medium HASP-4 (35 ml of medium in a plastic 200 ml-vessel for five explants).
8. Incubate the explants in photoperiod 16/8 at 24 °C for three weeks.
9. Take leaves from new sterile shoots in vitro and place them into induction liquid medium HASP-1 in 125 ml Erlenmeyer flasks.
10. Incubate the leaves in darkness at 24 °C, shaking the liquid medium at 80 rpm/min.

Maintenance and Proliferation of Embryogenic Cultures

1. The explants (leaves) after 20 days treatment in the medium HASP-1 with basal MS supplemented by 1 mg/l 2,4-D and 0.2 mg/l BA were anatomically changed and friable (Fig. 15.1a).
2. The leaves after incubation in medium HASP-1were divided into smaller fragments and inserted into the solidified medium HASP-2 in Petri dishes for direct somatic embryogenesis. The process of direct somatic embryogenesis was observed on solidified HASP-2 medium (Fig. 15.1b) formulated of WPM macro salts, MS micro salts, Gamborg vitamins and supplemented with 1 mg/l GA_3, 0.5 mg/l BA, 0.01 mg/l IAA, 10 g/l L-maltose, 15 g/l sucrose, and gelrite 3 g/l. The culture was incubated in photoperiod 16/8 at 24 °C for three weeks.

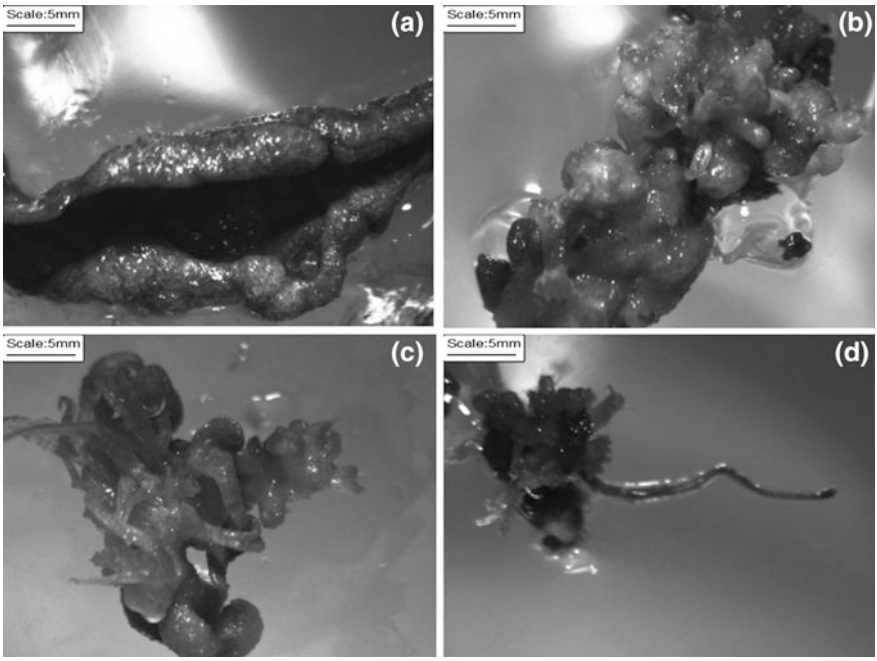

Fig. 15.1 Plant regeneration via somatic embryogenesis in hybrid aspen. **a** Anatomically changed and friable explant (leaf); **b** Somatic embryogenesis on a leaf fragment; **c** Developing somatic embryos; **d** Root emerging from a somatic embryo cluster

3. Embryogenic processes of somatic cells continued through different stages during three weeks of culture on the HASP-2 medium (Fig. 15.1c, d). The induced clumps (clusters) of somatic structures were transferred on the fresh medium HASP-3 (the same composition as previous medium, only supplemented with PEG 12.5 g/l). The culture was incubated in photoperiod 16/8 at 24 °C for three weeks.
4. The clumps of germinated somatic embryos were transplanted into plastic vessels 200 ml with medium HASP-4 (WPM medium supplemented with 50 mg/l myo-inizitol, 0.001 mg/l IAA, 0.125 mg/l BA, 25 g/l sucrose) for shoot regeneration. The culture was incubated in photoperiod 16/8 at 24 °C for three weeks.

Plant Regeneration, Stabilization, Acclimatization, and Field Transfer

Transfer of the plantlets, obtained from somatic embryogenesis, directly to ex vitro conditions could be associated with certain risk. Therefore, additional steps were taken in the micropropagation process that led to the production of strong shoots for ex vitro acclimatization.

1. The regenerated shoots of hybrid aspen from somatic embryos are separated and transferred to plastic vessels with 35 ml WPM medium incorporated with 0.125 mg/l BAP, 0.01 mg/l IAA, 30 g/l sucrose, for physiological state stabilization. The culture is incubated in photoperiod 16/8 at 22 °C for three weeks.
2. The morphologically normal rhizogenic plantlets (2.5–4 cm in height) are transferred to mini greenhouse with moistened Jyffy tablets and located in growth room for three weeks under photoperiod 16/8 GreenPower LED light.
3. After 14 days of acclimatization, the covers from mini greenhouses are periodically removed for gradually increasing periods of time each day. In order to avoid drying, plants must be sprayed with water regularly.
4. The acclimatized plantlets with developed roots are transferred to greenhouse conditions in the nursery garden. The plants are transplanted to pots with turf for the establishment of closed root system. Once the saplings reach $\sim$20 cm in height, they can be moved to the nursery yard.
5. Once the saplings reach 60–80 cm in height, they can be planted in the plantation site

Micropropagation Scheme

A summarising scheme for hybrid aspen micropropagation, which includes somatic embryogenesis stage, is given in Fig. 15.2.

15.4 Proposed Steps for Further Protocol Modifications

Large number of somatic embryos obtained in hybrid aspen *Populus tremuloides* × *P. tremula* tissue culture, obviating callus phase, provides an opportunity for increased rates of clonal multiplication of selected genotypes.

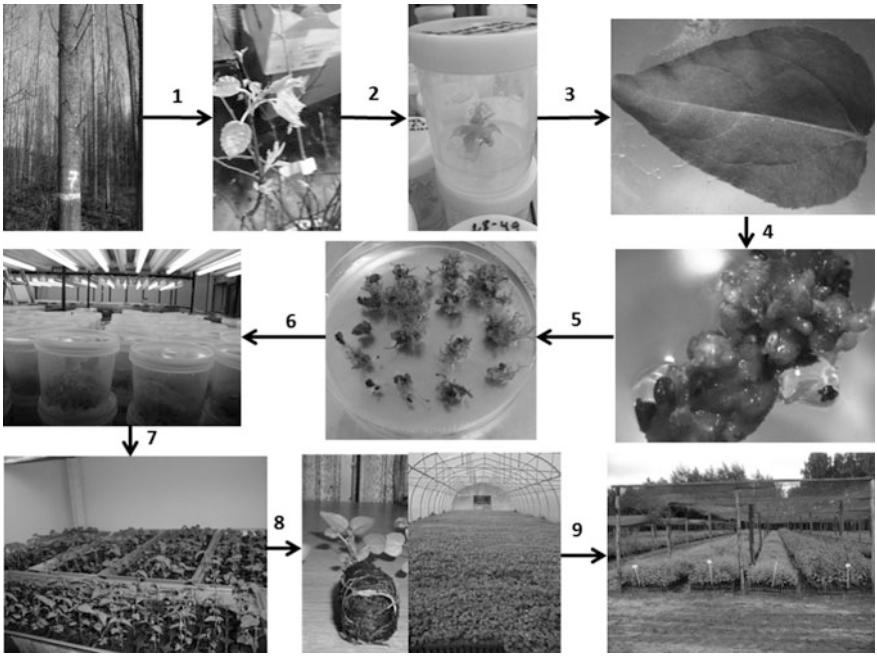

Fig. 15.2 Steps in hybrid aspen micropropagation: 1—Fresh shoot development on branches that were cut from a donor tree and brought to a laboratory; 2—Shoot segments disinfected and put on nutrient medium in vitro; 3—In vitro-developed leaves are taken as explants for the following induction of somatic embryogenesis; 4—Somatic embryogenesis on leaf segments; 5—Microplant development from somatic embryo clusters; 6—Transfer of microplants to more spacious vessels for further shoot development; 7—In vitro-grown shoots are transferred and acclimatized to ex vitro conditions; 8—Successfully acclimatised saplings with well-developed root system are transferred to a greenhouse; 9—After reaching ~20 cm of height in a greenhouse, saplings are transferred to a nursery garden

However, the stages of proliferation and embryo development still need optimization in order to achieve synchronic development of uniform embryos that could be exploited also for the technology of synthetic seed production.

References

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspensions cultures of soybean root cells. Exp Cell Res 50:151

Hazubska- T, Bojarczuk K (2016) Tree somatic embryogenesis in science and forestry. Dendrobiol 76:105–116

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia,* by use of shoot tip culture. Acta Hortic 30:42–427

Luoranen J, Lappi J, Zhang G, Smolander H (2006) Field performance of hybrid aspen clones planted in summer. Silva Fenn 40(2):257–269

Malá J, Máchová P, Cvrčková H, Čižková L (2006) Aspen micropropagation: use for phytoremediation of soil. J For Sci 52(3):101–107

Mauriat M, Petterle A, Bellini C, Moritz T (2014) Gibberellins inhibit adventitious rooting in hybrid aspen and Arabidopsis by affecting auxin transport. Plant J 78:372–384

Murasige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473

Plant cell and tissue culture. Phytopathology. Biochemicals. Edited by Kors F.T.M. Duchefa. Catalogue; 2010–2012

Stenvall N, Haapala T, Pulkkinen P (2004) Effect of genotype, age and treatment of stock plants on propagation of hybrid aspen (*Populus tremula* × *Populus tremuloides*) by root cuttings. Scand J For Res 19(4):303–311

Tullus A, Tullus H, Soo T, Pärn L (2009) Above-ground biomass characteristics of young hybrid aspen (*Populus tremula* × *P. tremuloides* Michx.) plantations on formal agricultural land in Estonia. Biomass Bioenergy 33(11):1617–1625

Žiauka J, Kuusienė S, Šilininkas M (2013) Fast growing aspens in the development of a plant micropropagation system based on plant-produced ethylene action. Biomass Bioenergy 53:20–28

Chapter 16
Somatic Embryogenesis of Alpataco (*Prosopis alpataco* L.)

Patricia Boeri and Sandra Sharry

16.1 Introduction

Prosopis alpataco Phil. (Fabaceae, Mimosoideae) is a native woody species from the arid region. It is normally found in the central *Monte* desert, *Argentina* (Vega Riveros et al. 2011; Catalano et al. 2008; Villagra and Roig 2002; Kiesling 1994) where great part of Patagonia steppe is included. It is commonly known as "alpataco", quechua word that means "tree with subterraneous branches". It is a multifunction species with great importance for soil support and wildlife refuge. The alpataco is also an economically valued species, being used as food, fuel wood and medicinal resource by the indigenous communities of the arid Patagonian (Ragonese and Martínez-Crovetto 1947; Ruiz Leal 1972; Portal 1996; Steibel 1997; Rapoport et al. 2003; Ladio and Lozada 2009; Boeri et al. 2016). Its wood is used for fuel and charcoal production. Currently, overgrazing and logging are degrading this important genetic resource.

The genus *Prosopis* is sexually produced, which is a disadvantage in the establishment of the seedlings, depending on the sites and the specie. The vegetative propagation of this genus has a great interest to advance the domestication, improvement, restoration and conservation of these multi-purpose plants. There are some reports on the micropropagation of different *Prosopis* species including

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_16

P. tamarugo and *P. chilensis* (Jordan and Balboa 1985; Jordan 1987), *P. alba* (Jordan et al. 1985; Tabone et al. 1986; Castillo de Meier and Bovo 2000), *P. juliflora* (Nandwani and Ramawat 1991), *P. cineraria* (Goyal and Arya 1984; Shekhawat et al. 1993), *P limensis* (Minchala-Patiño et al. 2014), *P. glandulosa* (Rubluo et al. 2002) and *P. laevigata* (Buendía-González et al. 2007, 2012). However, there is only one protocol reported for plant regeneration via somatic embryogenesis in the genus *Prosopis* (which comprises about 44 species) (Buendía-González et al. 2007). There is no protocol available for in vitro plantlet regeneration of alpataco. This chapter describes the embryogenic callus induction, somatic embryo development and germination of *P. alpataco* from cotyledonary explants.

16.2 Materials and Methods

All the cultures steps must be incubated in a plant growth chamber under controlled conditions of temperature (25 ± 2 °C), under a 16 h light: 8 h dark photoperiod (provided by fluorescent tubes) and average photosynthetic photon flux of 50 μmol $m^{-2}s^{-1}$.

Basic medium composition is listed in Table 16.1. Required modifications for different culture stages are listed in Table 16.2. The pH is adjusted to 5.8 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 18 min.

Table 16.1 Chemical composition of *Prosopis alpataco* basic culture medium (Murashige and Skoog media)

Ingredients	mg/L
Ammonium nitrate	1650.000
Boric acid	6.200
Cobalt chloride.$6H_2O$	0.025
Copper sulphate.$5H_2O$	0.025
EDTA disodium salt.$2H_2O$	37.300
Ferrous sulphate.$7H_2O$	27.800
Glycine (free base)	2.000
Magnesium sulphate	180.690
Manganese sulphate.H_2O	16.900
Molybdic acid (sodium salt).$2H_2O$	0.250
Myo-inositol	100.000
Nicotinic acid (free acid)	0.500
Potassium iodide	0.830
Potassium nitrate	1900.000
Potassium phosphate monobasic	170.000
Pyridoxine hydrochloride	0.500
Thiamine hydrochloride	0.100
Zinc sulphate.$7H_2O$	8.600

The pH was adjusted to 5.8–6.2 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 18 min

Table 16.2 Formulations of *Prosopis alpataco* media

	Stage I Initiation explant preparation	Stage II Induction SE culture medium	Stage III SE proliferation	Stage IV Maturation	Stage V Germination
Sucrose	30,000	30,000	30,000	30,000	30,000
2,4-D	–	2	–	–	–
IBA	–	–	–	–	
BA	–	1.5	–		–
Activated charcoal	–	1000	–	–	–
Agar	8000	–	8000	8000	8000
Phytagel	–	2000	–	–	–
Basal salt mixture	1.0 × MS salts	1.0 × MS salts	1.0 × MS salts	0.5 × MS salts	0.5 × MS salts

All units are in mg/l
Basal salt mixture containing micro and macro elements with vitamins as described by Murashige and Skoog (1962)

16.2.1 Explant Preparation

Mature pods (containing the seeds) should be collected from adult *P. alpataco* trees growing in natural field conditions, during January and March (Argentina).

1. Place the collected seeds at −20 °C to prevent attack of coleopteran (Bruchidae).
2. Store the seeds in paper envelopes under controlled conditions of humidity, light and temperature (0–5 °C).
3. Scarify the seeds mechanically by removing a small lateral fragment of the seed coat and disinfested according to Villagra and Roig-Juñent (1997) method modified as follows: immersion in ethanol 70% (v/v) for 5 min, followed by immersion in sodium hypochlorite 20% (v/v) (27 g Cl*/l).
4. Rinse the seeds five times with sterile double-distilled water.
5. Transfer the sterile seeds into the Petri dish with semisolid Murashige and Skoog (1962) medium (MS) supplemented with 3% sucrose (w/v), 0.8% (w/v) agar without plant growth regulators (Table 16.2).
6. The pH of the medium is adjusted to 5.8 before autoclaving at 120 °C for 25 min.
7. Incubate the seeds in a plant growth chamber under controlled conditions of temperature, humidity, and light (mixed light provided by fluorescent tubes and incandescent lamps). The germination events began on the second day.
8. Remove the cotyledon explants from 2 week-old seedlings as shown in Fig. 16.1.

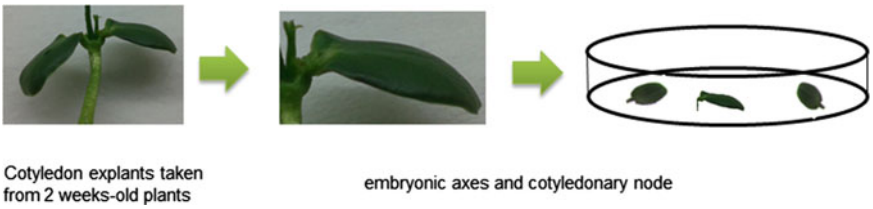

Fig. 16.1 Cotyledonary explants used for the induction of somatic embryogenesis of *P. alpataco*

16.2.2 Callus Culture

Both cotyledonary nodes and embryonic axes can be used as explants for callus induction.

1. Put the explants onto a 10 cm plastic Petri dish (100 × 15 mm) with *Induction SE culture medium* (30 ml) (Table 16.2).
2. The onset of callogenesis is from the embryonic axis and the cut surface (Fig. 16.2).

16.2.3 Proliferation and Maturation of Somatic Embryos

1. After 60 days in the *Induction medium culture* embryogenic callus should be transferred into MS medium without plant growth regulators (Table 16.2).
2. Initially, the embryogenic callus looks dark brown and after 10 days localized greenish callus appears.
3. Globular somatic embryos appear first on the surface of callus, and then, embryos at different stages of development form (Fig. 16.3a–d).

The essential characteristics of the development of somatic embryos of alpataco, especially after the globular stage, were comparable to zygotic embryos of the specie (Fig. 16.4).

Fig. 16.2 Callogenesis from cotyledonary explants of alpataco

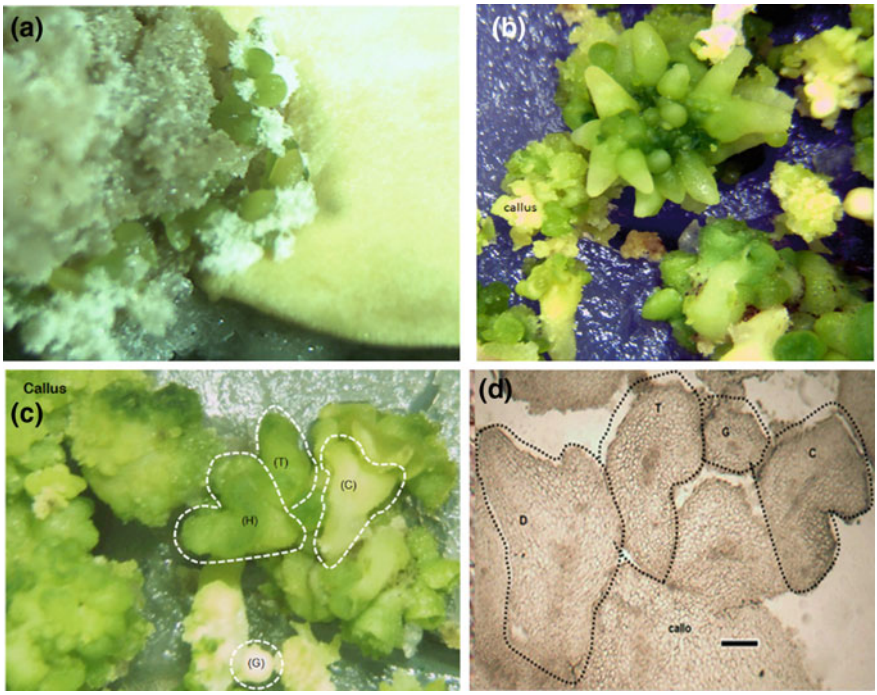

Fig. 16.3 **a** Somatic globular embryos from embryogenic callus. **b** Somatic embryos obtained at different developmental stages. **c** globular (G), heart (H), torpedo (T), cotyledonary (C). **d** Microscopy of callus: globular state (G), heart (C), torpedo (T) and cotyledonary, early phase (D)

16.2.4 Germination and Conversion of Somatic Embryos

1. Transfer the somatic embryos to the *Germination culture medium* (Table 16.2) under the same environmental conditions (temperature and photoperiod). Somatic embryos germinate normally (Fig. 16.5). Separate somatic embryos from the callus without any further delay for preventing formation of secondary SE.

1. In order to obtain plants, place the germinated somatic embryos in Petri dishes (60 × 15 mm) with sterile vermiculite, moist with culture liquid half strength MS medium or on bridge filter paper in test tubes filled with 25 ml same culture medium.

16.2.5 Plant Establishment and Acclimatization

1. Rinse roots of the seedlings with sterile water to remove agar.
2. Transfer the normal plantlets to sterile vermiculite as substrate in glass containers.

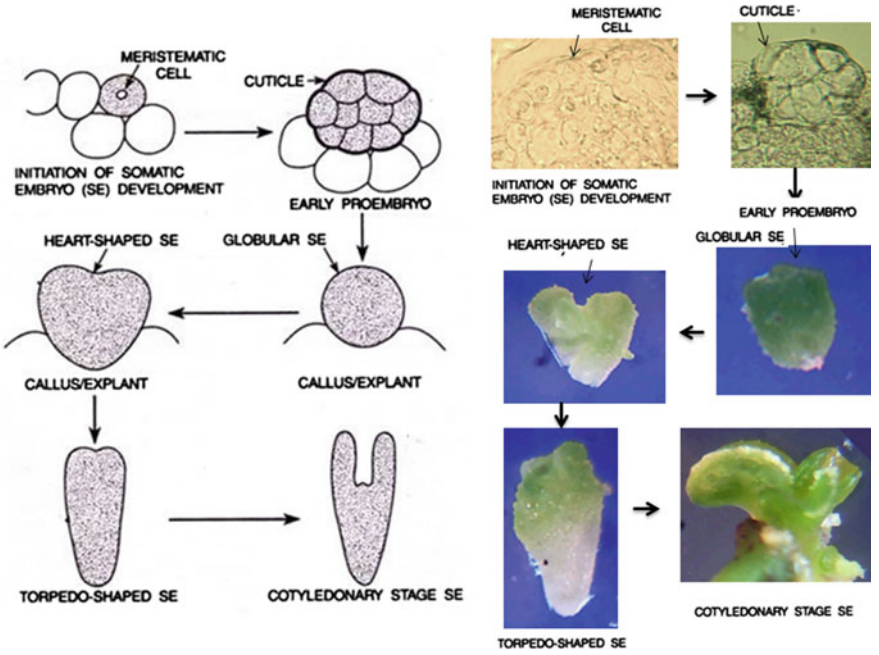

Fig. 16.4 Complete process of somatic embryogenesis from a surface cell of the explant. Figure left taken from S. Pujari (*Source* http://www.yourarticlelibrary.com/biotechnology/plant-tissues/regeneration-of-plants-shoot-regeneration-and-somatic-embryogenesis/33234/); right: somatic embryogenesis in *P. alpataco*

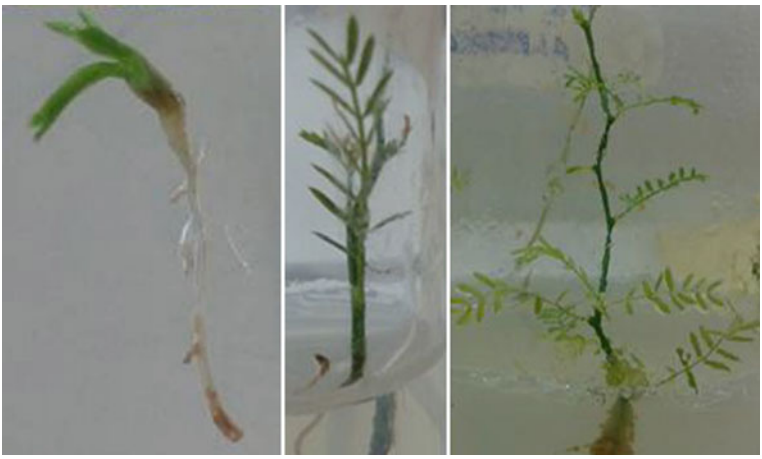

Fig. 16.5 Embling conversion from SE

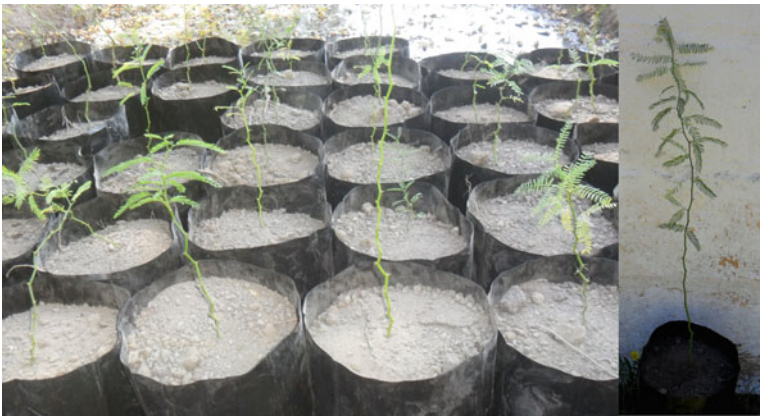

Fig. 16.6 Plants from somatic embryos of *Prosopis alpataco* under greenhouse conditions

3. Cover the plantlets with nylon and keeps under the same environmental conditions.
4. After a week, open slowly the nylon wrapping. Transfer the plants to the growth chamber in controlled conditions of temperature (25 ± 2 °C), under a 16 h light: 8 h dark photoperiod (provided by fluorescent tubes) and average photosynthetic photon flux of 50 μmol $m^{-2}s^{-1}$.
5. Once acclimatized, transplant the plantlets to the containers with a mixture of 1:1 vermiculite–perlite under greenhouse.
6. During hardening, plantlets defoliate which is maintained during the first ten days in the greenhouse, and form new leaflets.
7. After 3 months, plants are transferred to natural conditions (Fig. 16.6).

16.3 Conclusions and Future Prospects

The present report describes the first protocol (Fig. 16.7) plantlet regeneration via somatic embryogenesis of *Prosopis alpataco*, which is one of the most important native woody species in Patagonia. However some protocol modifications are being developed. The somatic embryos must be separated manually, which is very difficult. Poor germination has to be improved. This shows that focus is needed to increase the rate of conversion of somatic embryos for field deployment. Without this, the somatic embryogenesis technology will have a very limited impact. Automation of synthetic seed production is our final goal. Including advanced tissue culture technology, bioreactors, somatic embryo encapsulation, and development of appropriate synthetic seed coating material requires further study to meet the goal of producing millions of synthetic seeds in a short time and cutting the cost of seed production.

Propagation of alpataco through seeds has limitations, because of hard seed coat. The seeds hardly germinate without any treatment under nursery conditions. The development and implementation of in vitro methodologies for the culture of *P. alpataco* not only allows the ex situ conservation but also massive production for this native species with regional interest.

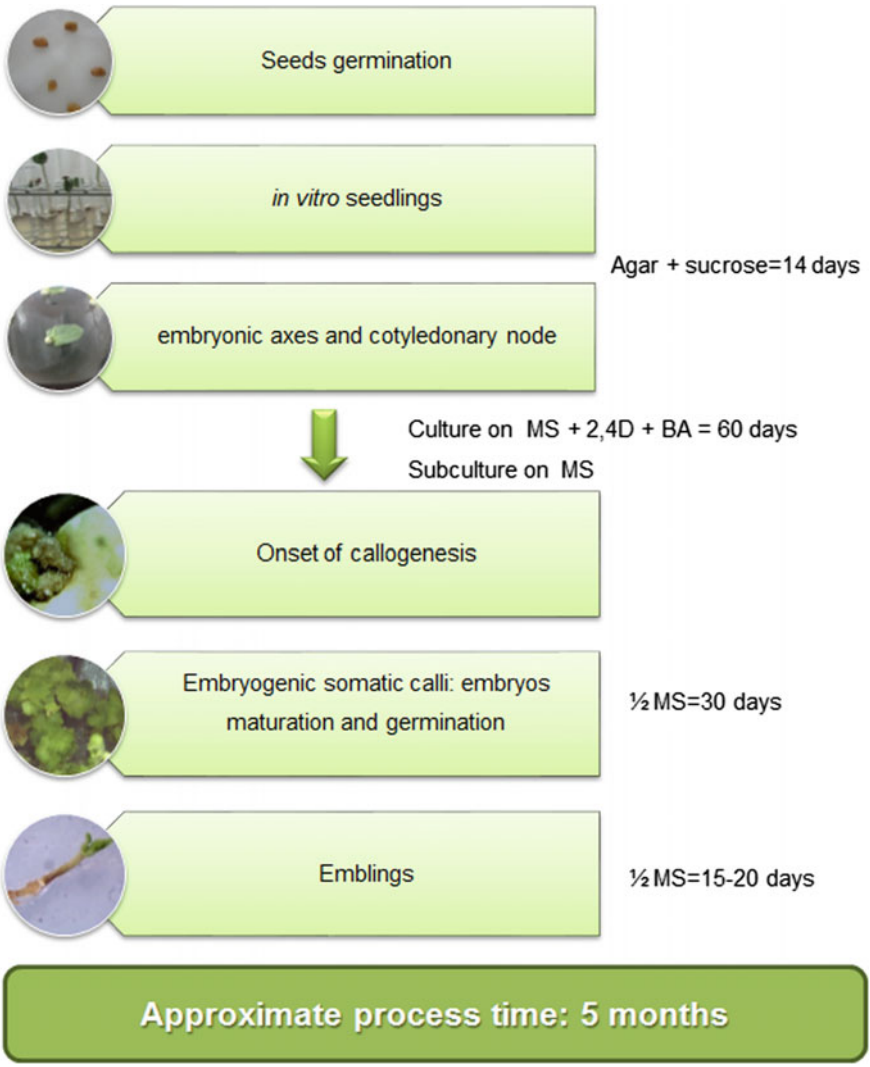

Fig. 16.7 Complete process for obtaining *P. alpataco* seedlings via somatic embryogenesis. Stages of alpataco in vitro regeneration system, specific PGR and time

References

Boeri P, Piñuel L, Sharry S, Barrio D (2016) Caracterización nutricional de la harina integral de algarroba (*Prosopis alpataco*) de la norpatagonia Argentina. Rev de la Facultad de Ciencias Agrarias y Forestales, UNLP In press

Buendía-González L, Orozco-Villafuerte J, Cruz-Sosa F, Chávez-Ávila VM, Vernon-Carter EJ (2007) Clonal propagation of mesquite tree (*Prosopis laevigata* Humb. & Bonpl. ex Willd. MC Johnston). I. via cotyledonary nodes. In Vitro Cell Dev Biol-Plant 43(3):260–266

Buendía-González L, Estrada-Zúñiga ME, Orozco-Villafuerte J, Cruz-Sosa F, Vernon-Carter EJ (2012) Somatic embryogenesis of the heavy metal accumulator *Prosopis laevigata*. Plant Cell Tiss Org Cult 108:287–296

Catalano SA, Vilardi JC, Tosto D, Saidman BO (2008) Molecular phylogeny and diversification history of *Prosopis* (Fabaceae: Mimosoideae). Biol J Lin Soc 93(3):621–640

de Meier Castillo GC, Bovo OA (2000) Plant regeneration from single nodal-stem explants of legume tree *Prosopis alba* (Griseb). Biocell 24(2):89–95

Goyal Y, Arya HC (1984) Tissue culture of desert trees I. Clonal multiplication of *Prosopis cineraria* by bud culture. J Plant Physiol 115:183–189

Jordan M, Balboa O (1985) In vitro regeneration of *Prosopis tamarugo* Phil. and *P. chilensis* (Mol.) Stuntz from nodal sections. Gartenbauwissenschaft 50:138–142

Jordan M (1987) *In vitro* culture of *Prosopis* species. In: Bonga JM, Durzan DJ (eds), Cell and tissue culture in forestry, vol 3. Martinus Nijhoff Publishers, Boston, pp 370–384

Jordan M, Pedraza J, Goreux A (1985) *In vitro* propagation studies of three *Prosopis* species (*P. alba*, *P. chilensis* and *P. tamarugo*) through shoot tip culture. Gartenbauwisenschaft 50:265–267

Kiesling R (1994) Flora de San Juan Volumen I. Pteridofitas, Gimnospermas. Dicotiledóneas Dialipétalas (Salicáceas - Leguminosas). In: Vázquez Mazzini (eds), 348 pp

Ladio AH, Lozada M (2009) Human ecology, ethnobotany and traditional practices in rural populations inhabiting the Monte region: resilience and ecological knowledge. J Arid Environ 73(2):222–227

Minchala-Patiño J, Poma-Angamarca R, Muñoz-Chamba L, Yaguana-Arévalo M, González-Zaruma D, Eras-Guamán VH, Rojas-Idrogo C, Delgado-Paredes GE (2014) Propagación *in vitro* de *Prosopis limensis* Benth. In: Hook (Fabaceae-Mimosoideae). Quebracho, Revista de Ciencias Forestales, vol 22, issue 1–2. Universidad Nacional de Santiago del Estero, Argentina, pp 88–89

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol 15:473–497

Nandwani D, Ramawat KG (1991) Callus culture and plantlets formation from nodal explants of *Prosopis juliflora* (Swartz) DC. Indian J Exp Biol 29:523–527

Portal R (1996) La vegetación del Monte en el Yacimiento Aguada de la Pichana. Total Austral S. A., Argentina. 43 pp

Pujari S (2016) Disponible en: http://www.yourarticlelibrary.com/biotechnology/plant-tissues/regeneration-of-plants-shoot-regeneration-and-somatic-embryogenesis/33234/. Ultimo acceso: febrero 2016

Ragonese AE, Martínez Crovetto RN (1947) Plantas indígenas de la Argentina con frutos y semillas comestibles. Rev Invest Agric 1:147–246

Rapoport EH, Ladio A, Sanz EA (2003) Plantas nativas comestibles de la Patagonia andina argentina-chilena, parte II. Ed. Imaginaria, Bariloche. Programa de Extensión, Universidad Nacional del Comahue. 79 pp

Rubluo A, Arriaga E, Brunner I (2002) Shoot production from cotyledons of *Prosopis glandulosa* var t*orreyana* cultures *in vitro*. In Inst Biol (UNAM) Universidad Nacional Autónoma de Mexico Ser. Bot. 73(1):83–87

Ruiz Leal A (1972) Flora popular mendocina. Deserta, 3

Shekhawat NS, Rathore TC, Singh RP, Deora NS, Rao SR (1993) Factors affecting in vitro clonal propagation of *Prosopis cineraria*. Plant Growth Regul 12:273–280

Steibel PE (1997) Nombres y usos de las plantas aplicados por los Indios Ranqueles de La Pampa, Argentina. Rev. Fac. Agron. Universidad Nacional de La Pampa 9:14–15

Tabone TJ, Felker P, Bingham RL, Reyes I, Loughrey S (1986) Techniques in the shoot multiplication of the leguminous tree *Prosopis alba* clone BV. For Ecol Manage 16:191–200

Vega Riveros C, Meglioli PA, Villagra PE (2011) *Prosopis alpataco* Phil. Fabaceae, Mimosoideae. Kurtziana. Tomo 36(2):53–64

Villagra PE, Roig FA (2002) Distribución geográfica y fitosociología de *Prosopis argentina* y *P. alpataco* (Fabaceae, Mimosoidea). Boletín de la Sociedad Argentina de Botánica 37(1–2):99–106

Villagra PE, Roig-Juñent FA (1997) Wood structure of *Prosopis alpataco* and *P. argentina* growing under different edaphic conditions. IAWA J 18(1):37–51

Chapter 17
Rough Lemon (*Citrus Jambhiri* Lush.)

Savita, Pratap Kumar Pati and Avinash Kaur Nagpal

17.1 Introduction

The genus *Citrus* is an important group of fruit crops worldwide; belongs to family Rutaceae comprising 140 genera and 1300 species distributed throughout the world. Some of the well known fruits of citrus group are oranges, lemons, grapefruits and limes. They are long-lived perennial crops grown in more than 100 countries all across the world (Saunt 1990). Citrus fruits are known for their distinctly pleasant aroma, arising due to terpenes present in the rind. The genus derives its commercial importance from its fruits, which are of great economic and health value and are consumed fresh or pressed to obtain juice (Talon and Gmitter Jr. 2008). Citrus peels can be candied, used as livestock feed, in perfumeries, bakeries and in soap industry. Essential oils obtained from citrus leaves have insecticidal property. Lemon oil obtained from lemon peels is extensively used in furniture polish. The rind of citrus fruits is slightly bitter in taste and can be added to baked products to impart a distinct flavour. Citrus has been utilized in a number of medicinal preparations for the remedy of scores of ailments ranging from toothache, diarrhoea, constipation, insomnia to vomiting (Singh and Rajam 2010).

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_17

Now-a-days, *Citrus* species are almost universally propagated by budding on to seedling rootstocks. Since early 1950s extensive rootstock trials on citrus have been conducted under different environmental conditions (Bhattacharya and Dutta 1952; Rangacharlu et al. 1958; Singh 1962). The best performing rootstocks included Sour orange (*Citrus aurantium),* rough lemon (*Citrus jambhiri)*, cleopatra mandarin (*Citrus reticulata)*, trifoliate orange (*Poncirustrifoliata)*, citrange (*Citrus sinensis* x *Poncirustrifoliata),* rangpur mandarin lime, volkamer lemon etc. Sour orange was considered to be the major rootstock because of its tolerance to different soil conditions, cold and foot rot but after the appearance of the new citrus threat by *Citrus tristeza virus* (CTV), the dominant sour orange rootstock has been replaced by rough lemon rootstock which was tolerant to CTV. Rough lemon (*Citrus jambhiri* Lush.) is native to Northeastern India. Locally in Punjab it is known as "JattiKhatti". It is probably a natural hybrid because of its high degree of polyembryony as compared with other lemon species. In Punjab and nearby states, rough lemon has been considered to be the most important rootstock for lemons, oranges, mandarins, grapefruits and knows because of its high vigour, well adaptation to warm-humid areas with deep sandy soils and resistance to *Citrus tristeza virus*. It produces high yielding trees with large fruits.

The potential of conventional methods of improvement of citrus rootstocks is limited by biological factors such as heterozygosity, inbreeding depression, nucellar polyembryony and juvenility. Under such circumstances tissue culture techniques offer best possible alternative for improvement and inducing variations and selection of variants for different needs. Plant tissue culture provides reliable and economical method of maintaining pathogen free plants that allows rapid multiplication and international exchange of germplasm.

Somatic embryogenesis is the process of a single cell or group of cells initiating the developmental pathway that leads to reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants. A remarkable homology is known to exist between somatic and zygotic embryos (Redenbaugh et al. 1988). In *Citrus* species, this phenomenon has been studied in detail by Chaturvedi et al. (2001), Ricci et al. (2002), Guo and Grosser (2005), Savita et al. (2015).

Somatic embryogenesis from a wide range of organs and tissues has been employed to produce plants having some specific required characteristics, such as virus-free, vigorous, disease resistant and genetically true-to-type (Singh et al. 2006; Duan et al. 2007; Iyer et al. 2009). The technique of growing isolated citrus embryos in artificial media was described by Hurosvilli (1957). Rangan et al. (1968) reported culture of nucellar tissues in *C. grandis*. Nucellar tissue isolated from monoembryonic varieties gave rise to 10–15 embryos per culture, which developed into complete plantlets. The plants arising from embryogenesis of nucellus in vitro were found to be free from most of the pathogenic viruses (Bitters et al. 1970). This chapter describes the protocol to regenerate rough lemon plantlets via somatic embryogenesis from ovaries and nucellar tissues.

17.2 Materials

1. Ovaries excised from unopened flower buds and nucellar tissues isolated from seeds.
2. Autoclave, laminar—flow hood with ultraviolet light, tissue culture chambers, petri dishes, test-tubes, 250 ml Erlenmeyer flasks, measuring cylinders, dissecting microscope, forceps, scalpels, 1–20 ml serological pipettes, 10–1000 µl micro-pipettes.
3. 5% (*v/v*) Teepol® solution, 0.1% (*w/v*) $HgCl_2$ (Mercuric chloride), ethanol, sterile distilled water.
4. MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and various concentrations of plant growth regulators (PGRs)/supplements viz. 2,4-dichlorophenoxy acetic acid (2,4-D), 6-benzylaminopurine (BAP), kinetin (KN), malt extract (ME) and abscisic acid (ABA) for culture initiation.
5. Polyethylene glycol (PEG) and activated charcoal.

Preparation of MS medium

MS medium is prepared by using 4 stock solutions and the composition is given in Table 17.1.

Stock solution I

Prepare the stock solution-I by dissolving NH_4NO_3 (3.300 g), KNO_3 (3.800 g), $CaCl_2.2H_2O$ (0.880 g), $MgSO_4.7H_2O$ (0.740 g) and KH_2PO_4 (0.340 g) in double distilled water to make 100 ml solution.

Stock solution II

Prepare the Stock solution II by dissolving KI (0.0166 g), H_3BO_3 (0.1240 g), $MnSO_4.4H_2O$ (0.4460 g), $ZnSO_4.7H_2O$ (0.1720 g), $Na_2MoO_4.2H_2O$ (0.0050 g), $CuSO_4.5H_2O$ (0.0005 g) and $CoCl_2.6H_2O$ (0.0005 g) in double distilled water to make 100 ml solution.

Stock solution III

Prepare Stock solution III afresh by dissolving $FeSO_4.7H_2O$ (0.556 g) and $Na_2EDTA.2H_2O$ (0.746 g) separately in 40 ml double distilled water by constant stirring. Mix the solutions together and adjust the pH to 5.5 using 1 N HCl or 1 N NaOH. Use the standard buffer of pH 7.0. Makethe final volume up to 100 ml by adding more distilled water. Filter the stock solution three times by using Whatman No. 1 filter paper and store in an amber coloured bottle.

Table 17.1 Composition of MS medium

Sr. No.	Constituent	Amount (g/100 ml)	Amount (g/1000 ml)
Stock solution-I			
1.	NH_4NO_3	3.300	33.000
2.	KNO_3	3.800	38.000
3.	$CaCl_2.2H_2O$	0.880	8.800
4.	$MgSO_4.7H_2O$	0.740	7.400
5.	KH_2PO_4	0.340	3.400
Stock solution-II			
6.	KI	0.0166	0.166
7.	H_3BO_3	0.1240	1.240
8.	$MnSO_4.4H_2O$	0.4460	4.460
9.	$ZnSO_4.7H_2O$	0.1720	1.720
10.	$Na_2MoO_4.2H_2O$	0.0050	0.050
11.	$CuSO_4.5H_2O$	0.0005	0.005
12.	$CoCl_2.6H_2O$	0.0005	0.005
Stock solution-III			
13.	$FeSO_4.7H_2O$	0.556	5.560
14.	$Na_2EDTA.2H_2O$	0.746	7.460
Stock solution-IV			
15.	Inositol 2.000 g	2.000	20.000
16.	Nicotinic acid	0.010	0.100
17.	Pyridoxine-HCl	0.010	0.100
18.	Thiamine-HCl	0.010	0.100
19.	Glycine	0.040	0.400

Stock solution IV

Prepare the Stock solution IV by dissolving meso-inositol (2.000 g), nicotinic acid (0.010 g), pyridoxine-HCl (0.010 g), thiamine-HCl (0.010 g) and glycine (0.040 g) in double distilled water to make 100 ml solution (Table 17.2).

Note-*Store all the above stock solutions in plastic reagent bottles in refrigerator at* 4 °C.

17.3 Method

Plantlet regeneration through somatic embryogenesis in *Citrus* includes five steps: (1) callus induction, (2) callus multiplication (3) somatic embryo induction, (4) somatic embryo germination and (5) acclimatization and field transfer.

Table 17.2 Summary table showing the composition of different media[a] used at various stages of culture

Stage of culture	Conc. of sucrose (% w/v)	Conc. of agar (% w/v) (%)	Conc. of PGR/supplement (mg/l) used alone/in combination
Callus induction and multiplication	3%	0.8	BA (4 mg/l)
Somatic embryo induction and maturation	3%	0.8	BA (4 mg/l) + ME (500 mg/l)
Embryo germination	1. 8% sucrose alone 2. 8% sucrose + 1 (g/l) of Activated charcoal (AC) 3. 8% sucrose + 3% of Polyethylene glycol (PEG)	0.8	–

[a]MS medium was used as basal medium

Note Filter all the plant growth regulators (PGRs) being used in the protocol to sterilize and add to sterile media aseptically. Pour 25 ml medium into 25 × 150 mm culture tubes for nucellar tissues and 50 ml medium into 250 ml Erlenmeyer flask for ovaries

Sterilization

1. Excise the ovaries from unopened flower buds (5–6- year- old plant of *Citrus jambhiri* Lush.) and isolate the nucellar tissues from seeds (isolate from mature and fresh fruits).
2. Wash the flower buds and seeds under running tap water.
3. Transfer both type of explants separately to one-liter flasks containing 5% (*v/v*) Teepol® solution (active ingredients: Linear alkyl benzene sulfonate, potassium salt 10% *w/v* and sodium lauryl ether sulfate 7.2% *w/v*) and swirled for 15 min.
4. Further sterilize the explants with 0.1% (*w/v*) $HgCl_2$ for 5 min, rinse 4–5 times with autoclaved (cooled) double-distilled water.
5. After sterilization excise ovaries and nucellar tissues under aseptic conditions and inoculate them on callus induction medium.
6. Incubate the cultures at 25 ± 1 °C under 40 μmole m^{-2} s^{-1} cool white fluorescent light for 16 h photoperiod.

Callus induction and multiplication

1. Inoculate the ovaries and nucellar tissues on MS medium supplemented with BA 4 mg/l. Ovaries start to increase in size (swelling) after 7–8 days of incubation on MS medium supplemented with BA 4 mg/l but shows proliferation only after 60–90 days of culture.
2. Multiply the callus every three weeks by subculturing on freshly prepared medium containing same supplements which were used for callus induction.

Somatic embryo induction

The embryogenic potential of citrus varied with genotype and type of explant, but better results were usually obtained from regeneration protocols involving the use of explants of ovular origin. BA and ME have shown somatic embryogenesis in other *Citrus* species like *C. sinensis* (Grosser et al. 1988; Carimi et al. 1998; D'Onghia et al. 2001; *C. grandis* (Yang et al. 2000); *C. reticulata* (Madhav et al. 2002); *C. macroptera* (Miah et al. 2002); *C. aurantifolia* and *C. sinensis* (Mukhtar et al. 2005); *C. madurensis* (Siragusa et al. 2007); *C. jambhiri* (Altaf et al. 2008; Savita et al. 2015).

1. Inoculate the calli raised from ovaries and nucellar tissues on somatic embryo induction medium (MS medium supplemented with BA 4 mg/l + ME 500 mg/l).
2. Somatic embryos develop from the friable callus originated from the walls of ovaries and the callus obtained from nucellar tissues.
3. Multiply the somatic embryos by subculturing on same medium for 3–4 weeks. After proliferation, the somatic embryos are further transferred on the same medium for maturation. The mature somatic embryos are transferred to somatic embryo germination medium (Basal-MS medium supplemented with 8% sucrose).

Somatic embryo germination

Basal MS-medium supplemented with higher concentrations of sucrose (8%) favours somatic embryo germination in *Citrus jambhiri* Lush. This is also been demonstrated by several researchers in different *Citrus* species (Carimi et al. 1999; Pérez et al. 1999; Wu and Mooney 2002; Moiseeva et al. 2006). Activated charcoal (AC) and Polyethylene glycol (PEG) can also be used in combination with this medium (MS medium with 8% sucrose), but 8% sucrose alone is sufficient for somatic embryo germination in *Citrus jambhiri* Lush. If we use 1 g/l AC and 3% PEG in combination with MS medium supplemented with 8% sucrose, then somatic embryo germination takes place but the response is lower than when sucrose 8% alone is used. In other plant species, addition of AC in the medium has enhanced the maturation of normal somatic embryos. Charcoal reduces the salt and growth hormone concentrations of the medium. It substantially lowers the levels of phenylacetic and p-OH benzoic acids which are inhibitory for somatic embryogenesis (Fridborg et al. 1978). PEG is a non-plasmolysing osmoticum and stimulates the maturation and germination of somatic embryos by regulating their osmotic potential (Table 17.3; Figs 17.1 and 17.2).

Acclimatization and hardening of regenerated plants

1. Wash the regenerated plantlets with water to remove adhering agar, and transfer to autoclaved plastic pots containing a mixture of garden soil, sand, and vermiculite (3:1:1).

Table 17.3 Effect of supplements on callus induction, somatic embryo production, germination and plantlet formation

Supplements	Callus induction (%)		Somatic embryo production (%)		Somatic embryo germination (%)		Plantlet formation (%)	
	Ovary	Nucellar Tissue	Ovary	Nucellar Tissue	Ovary	Nucellar Tissue	Ovary	Nucellar Tissue
BA (4 mg/l)	75.00	71.66	–	–	–	–	–	–
BA (4 mg/l) + ME(500 mg/l)	–	–	89.33	83.50	–	–	–	–
Basal MS medium + 8% Sucrose	–	–	–	–	98.85	81.38	98%	97%

Adopted from Savita et al. (2015)

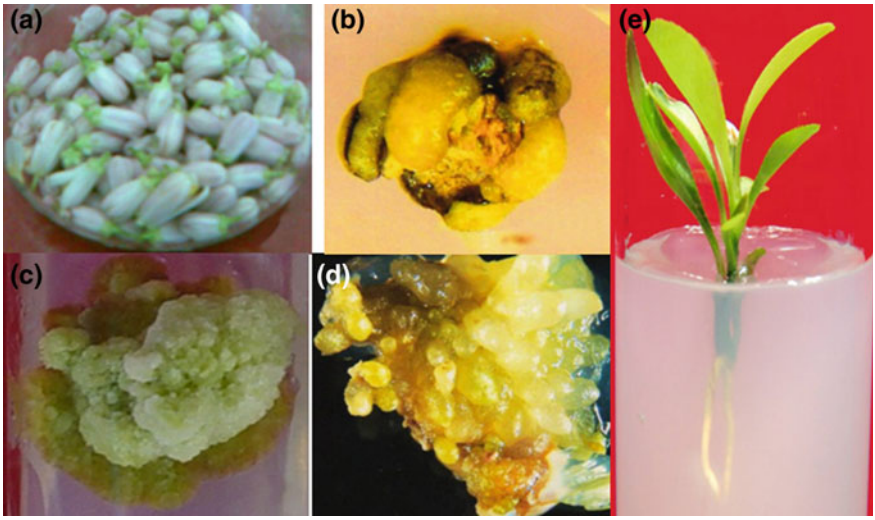

Fig. 17.1 Plantlet regeneration via somatic embryogenesis from ovaries; **a** Unopened flower buds; **b** Ovary showing swelling after 60–90 days of inoculation; **c** callus induction from the wall of ovary; **d** somatic embryo induction and **e** somatic embryo germination

2. For hardening place the potted plantlets in a culture room set at 26 ± 2 °C, 16 h day-length (40 µmol m^{-2} s^{-1}).
3. Cover the plantlets with polythene bags to maintain high humidity.
4. After 12–15 days, remove the polythene bags daily initially for a short duration (15–30 min) for about one week.

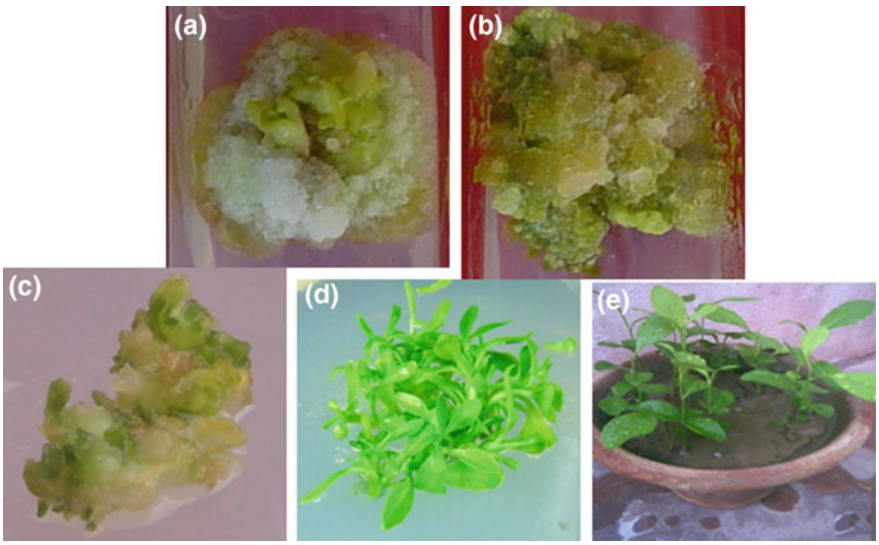

Fig. 17.2 Plantlet regeneration via somatic embryogenesis from nucellar tissues; **a** callus induction from nucellar tissue; **b** callus multiplication; **c** somatic embryo induction; **d** somatic embryo germination; **e** plantlets transferred to earthen pot for hardening in polyhouse

5. Gradually increase the daily exposure time by 30 min each day. Remove the polythene bags after 20 days, and subsequently transfer the plantlets to earthen pots containing only garden soil and kept in the polyhouse (to maintain high humidity up to 90–100%) for one month before transfer to the field (Savita et al. 2011).

17.4 Conclusion and Future Prospects

In the present investigation, it has been shown that ovary wall tissue from unpollinated ovaries and nucellar tissues isolated from seeds of *Citrus jambhiri* produced somatic embryos and successfully germinated into plantlets. A successful application of gene transformation requires an efficient regeneration method that allows both transformation and regeneration into plantlets. Somatic embryos have been shown to be an optimal cell source for application of genetic transformation.

References

Altaf N, Khan AR, Ali L, Bhatti IA (2008) Propagation of rough lemon (*Citrus jambhiri*Lush.) through *in vitro* culture and adventitious rooting in cuttings. Elect J Environ Agric Food Chem 7:3326–3333

Bhattacharya SC, Dutta S (1952) A preliminary observation on rootstocks for Citrus in Assam. Indian J Horticulture 9:1–11

Bitters WP, Murashige T, Rangan TS, Nauer E (1970) Investigations on established virus-free plants through tissue culture. California Citrus Nursery Soc 9:27–30

Carimi F, De Pasquale F, Crescimanno FG (1999) Somatic embryogenesis and plant regeneration from pistil thin cell layers of *Citrus*. Plant Cell Rep 18:935–940

Carimi F, Tortorici MC, De Pasquale F', Somatic GF (1998) embryogenesis and plant regeneration from undeveloped ovules and stigma/style explant of sweet orange navel group [*Citrus sinensis* (L.) Qsb.]. Plant Cell Tissue Organ Culture 54:183–189

Chaturvedi HC, Singh SK, Sharma AK, Agnihotri S (2001) Citrus tissue culture employing vegetative explants. Indian J Exp Biol 39:1080–1095

D'Onghia AM, Carimi F, De Pasquale F, Djelouah K, Martelli GP (2001) Somatic embryogenesis from style culture: a new technique for the sanitation, conservation and safe exchange of citrus germplasm. In: Proceedings ix international society of citriculture

Duan YX, Liu X, Fan J, Li DL, Wu RC, Guo WW (2007) Multiple shoot induction from seedling epicotyls and transgenic citrus plant regeneration containing the green fluorescent protein gene. Botanical Stud 48:165–171

Fridborg G, Pedersen M, Landstrom LE, Eriksson T (1978) Effects of activated charcoal on tissue cultures: adsorption of metabolites in plant tissue cultures. Physiol Plant 43:104–106

Grosser JW, Gmitter FG Jr, Chandler JL (1988) Intergeneric somatic hybrid plants of *Citrus sinensis* CV. Hamlin and *Poncirustrifoliata* CV. Flying Dragon. Plant Cell Rep 7:5–8

Guo WW, Grosser JW (2005) Somatic hybrid vigor in *Citrus*: Direct evidence from protoplast fusion of an embryogenic callus line with a transgenic mesophyll parent expressing the GFP gene. Plant Sci 168:1541–1545

Iyer RI, Jayaraman G, Ramesh A (2009) Direct somatic embryogenesis in *Myristicamalabarica* Lam., an endemic, threatened medicinal species of Southern India and detection of phytochemicals of potential medicinal value. Indian J Sci Technol 7:11–17

Madhav MS, Rao RN, Singh S, Deka PC (2002) Nucellar embryogenesis and artificial seed production in *Citrus reticulata*. Plant Cell Biotechn Molecular Biology 3:77–80

Miah MN, Islam S, Hadiuzzaman S (2002) Regeneration of plantlets through somatic embryogenesis from nucellus tissue of *Citrusmacroptera*Mont. var. a*nammensis*('Sat Kara'). Plant TissueCulture 12:167–172

Moiseeva NA, Serebryakova VN, Nardi L, Lucretti S, Butenko RG (2006) Organization of initial stages of somatic embryogenesis in tissue culture of *Citrus sinensis* cv. Tarocco at the organismal level. Russian J Plant Physiol 53:548–555

Mukhtar R, Khan MM, Fatima B, Abbas M, Shahid A (2005) *In vitro* regeneration and multiple shoots induction in *Citrus reticulata* Blanco. Int J Agric Biology 7:414–416

Pérez RM, Mas O, Navarro L, Duran-Vila N (1999) Production and cryoconservation of embryogenic cultures of mandarin and mandarin hybrid. Plant Cell Tissue and Organ Culture 55:71–74

Rangacharlu VS, Naidu N (1958) Anjaneyulu; Narsimharao, T.L. Performance of Sathgudi orange (*C.sinensis*Osbeck) on different rootstocks at Kodur (South India). Indian J Horticulture 15:35–58

Rangan TS, Murashige T, Bitters WP (1968) *In vitro* initiation of nucellar embryos in monoembryonate *Citrus*. Hort Sci 3:226–227

Redenbaugh K, Fujii J, Slade D (1988) Encapsulated plant embryos. In: Green C, Somers D, Hackett W, Biesboer D (eds) Advances in biotechnological processes, vol 9. Liss, New York, pp 473–493

Ricci AP, Filho MFAA, Mendes BMJ, Piedade SM (2002) Somatic embryogenesis in *Citrus sinensis, C.reticulata*and *C. nobilis* × *C. deliciosa*. Scientia Agricola 59:41–46

Saunt J (1990) *Citrus varieties of the world*. Sinclair International Ltd.

Savita, Pati PK, Nagpal A, Virk GS (2015) An somatic embryogenesis protocol for Citrus jambhiri and assessment of clonal fidelity of plantlets using RAPD markers. J Plant Growth Regulation 34(2):309–319

Savita, Singh B, Virk GS, Nagpal A (2011) Efficient micropropagation protocol for regeneration of *Citrus jambhiri* Lush.using cotyledon as explant. Physiol Molecular Biology Plants 17:161–169

Singh B, Sharma S, Rani G, Zaidi AA, Virk GS, Nagpal A (2006) *In vitro* flowering in embryogenic cultures of kinnow mandarin (*Citrus nobilis*Lour × *C. deliciosa*Tenora). Afr J Biotech 5:1470–1474

Singh LB (1962) Studies on the rootstocks for sweet orange in the web tropics I Variety Rangtra. Hort Adv 6:19–27

Singh S, Rajam MV (2009) Citrus biotechnology: Achievements, limitations and future directions. Physiol Molecular Biology Plants 15:3–22

Siragusa M, Carra A, Salvia L, Puglia AM, De Pasquale F, Carimi F (2007) Genetic instability in calamondin (*Citrus madurensis*Lour.) plants derived from somatic embryogenesis induced by diphenylurea derivatives. Plant Cell Reports 26:1289–1296

Talon M, Gmitter GF Jr (2008) Citrus Genomics. Int J Plant Genomics 1–17:2008

Wu J-H, Mooney P (2002) Autotetraploidtangor plant regeneration from *in vitro Citrus* somatic embryogenic callus treated with colchicine. Plant Cell Tissue Organ Cult 70:99–104

Yang XL, Kitajima A, Hasegawa K (2000) Callus induction and embryoid regeneration from the endosperm culture of 'Tosa-Buntan' pummelo (*Citrus grandis*[L.] Osb.). Environ Control Biology 38:241–246. (in Japanese)

Chapter 18
Oil Palm (*Elaeis guineensis Jacq.*) Somatic Embryogenesis

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18.1 Introduction

The oil palm (*Elaeis guineensis*) originated from West Africa. The species is monoecious, producing separate male and female inflorescences on the same palm, in cycles of varying duration. Oil palm has no distinct types or races, the fruit forms (dura, pisifera and tenera) just being the internal fruit structure, fruit morphology (normal, mantled) and pigments in the exocarp (nigrescens, virescens) (Kushairi et al. 2011). The effectiveness of oil palm breeding from seed remains the basis for long term genetic process. However, there is still considerable unexploited genetic variability within the selected crosses, thus inducing interest in vegetative propagation via tissue culture. This method enables true-to-type reproduction of the best genotypes. Through this, further oil yield improvements can be expected, amongst other advantages (Baudouin and Durand-Gassellin 1991).

Cloning or vegetative propagation is advantageous in the multiplication of elite plants. Many plants are cloned via cuttings, bud grafting, marcotting, etc. However, there are plants that cannot be cloned using these methods. These plants do not produce axillary buds or suckers that can be used for cloning. The oil palm, coconut and some rattan species are examples of such plants. Advances in tissue culture or in vitro methods has led to the cloning of these plants. In the past, monocots have been categorized as recalcitrant to the tissue culture process. Through dedicated research and perseverance, it is now possible to clone monocots such as the date palm (Tisserat et al. 1979), coconut (Thanh-Tuyen and Apurillo 1992), rattan (Aziah 1989) and oil palm (Jones 1974; Rabechault and Martin 1976; Paranjothy

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_18

and Othman 1982). The success in producing oil palm clones has spurred oil palm organizations to set up their own tissue culture laboratories. With an average estimated projection of 20% increase in yield by clones compared to commercial seedlings (Zamzuri 2004), making planting clones a potentially good investment.

In the initial attempts at developing tissue culture protocols for oil palm, extensive studies were carried out using excised zygotic embryos as the starting material (Rabechault et al. 1970; Smith and Thomas 1973; Jones 1974). Subsequently, leaf tissues from polybag plants and mature palms were utilized. The tissue culture protocol for cloning oil palm was established in the 1970s (Jones 1974; Rabechault and Martin 1976; Paranjothy and Othman 1982). Since then, most laboratories in the oil palm industry today, have established their own tissue culture media and protocols (Rajanaidu et al. 1997). Owing to the commercial interests of the laboratories, unfortunately details of the culture media has never been revealed despite the availability of the information through published articles and personal contacts. When the floral abnormality, also known as mantling, was first highlighted in 1986, this triggered all the laboratories to review their media and culture protocols to ensure abnormality free cloning. This led to a reduction in the hormone levels used or a total avoidance of them at certain culture stages (Duval et al. 1988), or the replacement of the chlorinated auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) with α-naphthaleneacetic acid (NAA) (Sogeke 1998). Corley et al. (1986) reported that prolonged maintenance of polyembryoid cultures in vitro showed a higher frequency and degree of abnormality, hence many laboratories set a duration limit for maintaining cultures in vitro either by reducing the number of subculture cycles or the number of plantlets produced.

18.2 Explant Preparation

18.2.1 Field Activities

18.2.1.1 Ortet Sampling

An ortet is a source palm from which leaf, inflorescence and root explants are sampled. In this chapter, only the sampling of leaf explants, which is routinely performed, is described. MPOB selects elite ortets from breeding trials based on the palm performance over a four-year period. The main criteria for selecting an ortet are the yield and other genetic traits, such as disease resistance, palm compactness, oil quality, etc. For yield, the minimum standards required for the selection are 50 kg/palm/year oil yield and 27% oil to bunch ratio, recorded over four consecutive years and five analyses, respectively. This is in accordance with the MS 2099:2008, a Malaysian Standard (MS) specification established for oil palm ortet selection for cloning.

Selected ortets are first marked in the field. During sampling, several steps are taken to ensure workers' safety and that the ortets and samples are maintained in good condition. The young leaves or spears are contained within a cylinder of older leaf petioles or leaf cabbage at the centre of the palm canopy. Climbers have to remove thorns at the petiole bases followed by hewing a small wedge underneath each petiole to ease flattening of the frond. To avoid excessive injury to the palm, only several fronds, of about half whorl of the palm canopy, are flattened as a working platform. The older fronds and fruit bunches are removed, leaving only the cabbage or cylinder of younger frond petioles. The exposed fronds above the cabbage are trimmed off and the youngest leaf with fully opened leaflets, conventionally numbered as 1 (Corley and Gray 1976), is identified and labelled using marker pen. Cutting of the cabbage is a critical operation. The oil palm shoot apex is hidden inside the base of the leaf cabbage at the region where the cylinder bulges. The cabbage is cut at ≥ 10 cm above the bulge ends to ensure that the shoot apex within remains intact. The apex is usually located 6–7 cm below this point. The cabbage is cut towards frond number 1. The trimmed cabbage is about 40–70 cm long depending on the ortet age and the number of times the leaf cabbage had been sampled. The cabbage is placed in a bag and tied with a rope before it is lowered to the ground. The cabbage stump left after sampling needs to be protected from pest attack and fungal infection. If the stump surface is flat, a small sunken cut is made as a precautionary step to discharge any accumulated water or moisture during rainy days before covering the stump with a net pegged by several nails (Fig. 18.1).

Cabbage Handling

Proper handling and prompt transportation of the cabbage from field to the laboratory is very important to maintain its freshness. Both cut ends are wrapped with cling film to prevent dehydration. High humidity, high temperature, improper wrapping and dirty condition of the container or vehicle can spoil the cabbage.

18.2.2 Tissue Culture Laboratory Activities

18.2.2.1 Cabbage Processing

Once the cabbage reaches the laboratory, the wrapping material is removed. The cabbage is inspected for freshness, discoloration, breakage or odor. A sour odor usually indicates the cabbage is contaminated. A cabbage in good condition is whitish in color. Before bringing the leaf cabbage into the laminar airflow cabinet (LAC), some cleaning is required. Outer petioles are removed and only petiole of either frond number 1 or 0 (marked earlier at the sampling site) is maintained. Both ends of the cabbage are sawn or trimmed to expose fresh and clean surfaces. All surfaces (petioles and exposed ends) are surface sterilized by swabbing with absolute alcohol before placing the cabbage in the LAC. Since internal leaf spears

Fig. 18.1 Leaf explant sampling. **a** Removing thorns from leaf petioles. **b** Preparing a working platform. **c** Cabbage before cutting. **d** Cabbage stump after cutting. **e** Lowering of cabbage sample to the ground (arrow indicates cabbage). **f** Covering the exposed cut stump with netting

are considered naturally sterile, the leaf explants can be cultured directly on to the media. The cabbage then undergoes frond marking as each leaf explant is tracked throughout the tissue culture process. Basically, frond marking involves numbering the frond in a descending order, from frond number 1, 0, −1, −2, −3 and so forth using labeled pinheads or thumb-tacks (Fig. 18.2a). A longitudinal cut is made to remove the outermost petiole followed by the subsequent petiole until all the internal fronds or leaf spears comprising stacks of young leaflets are exposed (Fig. 18.2b). The uppermost exposed leaf spear is excised first followed by the next exposed spear. The leaf spears are cut into three or four segments of 9 ± 1 cm in length. Each segment is referred to as a zone. Zone 1 is located nearest to the base of the spear. Since the lower zones generate lower callusing rates than the upper zones (Rohani 2001), zone 1 is usually not cultured. This portion is often deposited with the MPOB Molecular Laboratory for DNA banking. The leaf explants at about

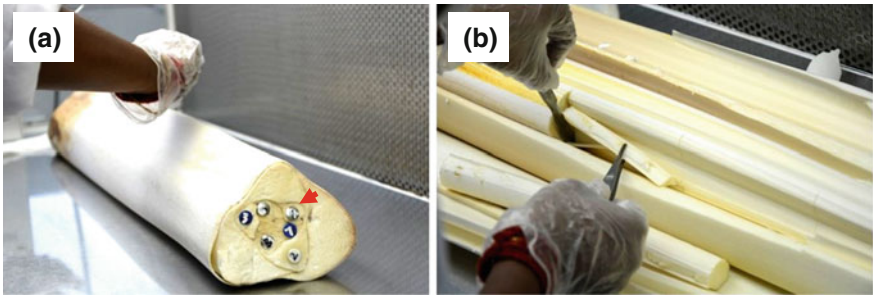

Fig. 18.2 Leaf cabbage. **a** The basal end of the cabbage is marked with labelled pinheads (as arrowhead) (frond −2 to −7). **b** The cabbage is cut longitudinally to expose the leaf spears that are cut into three segments

5 cm from the distal end, is also not cultured to avoid contamination. The leaf spear segments (according to zone and frond number) are placed separately in sterile petri dishes, sealed with cling film and then distributed to the operators for explant cutting and inoculation. At MPOB, fronds −4 to −9 are often used.

18.3 Culture Medium and Condition

18.3.1 Solid Culture

The nutrient formulations were based on modified Murashige and Skoog (1962) as shown in Table 18.1. Nutrient and agar solutions are prepared separately. For both portions, volumes are halved while concentrations of the constituents are maintained. The pH of the nutrient solutions is adjusted to 5.7 while the pH of agar is adjusted to 7.0 before mixing. All media are sterilized by autoclaving at 121 °C under 1.05 kg cm^{-2} pressure for 15 min. The explant and callus cultures are cultured in petri dishes containing 30 ml of callus and embryoid initiation media respectively and incubated under dark condition. Polyembryoid (PE) and shoot development cultures are cultured in 250 ml conical flasks containing 85 ml each of PE proliferation and shoot development medium incubated under 12/12 h light and dark photoperiod. Cultures are maintained at 28 ± 1°C with ± 60% relative humidity.

Callus Induction and Proliferation Media

The nutrient medium composed of components listed in sections A–D, and G–I (Table 18.1) and supplemented with;

(a) 8–10 mg $litre^{-1}$ NAA; or
(b) 5–10 mg $litre^{-1}$ NAA + 0.1–1.0 mg 2,4-D

Table 18.1 Modified Murashige And Skoog (MS) media

Constituents	Concentrations (mg l^{-1})
A. Macronutrients	Modified Murashige and Skoog (1962)
NH_4NO_3	1650
KNO_3	1900
$CaC1_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_2	170
B. Micronutrients	
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
KI	0.83
$ZnSO_4.7H_2O$	8.6
$CuSO_4.5H_2O$	0.025
$CoC1_2.6H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.25
NaFeEDTA	37.5
C. Vitamins	
Thiamine.HCl	0.1
Pyridoxine.HCl	0.5
Nicotinic acid	0.5
D. Myo-inositol	100
E. Amino acids (Eeuwens and Blake 1977)	
L-Arginine	100
L-Asparagine	100
F. L- Glutamine	100
G. Glycine	2
H. Sucrose (local refined table sugar)	30,000
I. Agar	7000
J. Gelrite	2500

Embryoid induction and polyembryoid multiplication medium

Calli is placed in embryoid induction medium consisting of the basal nutrients of sections A–D, G–I (Table 18.1) with half amounts of hormone used. This is followed by a gradual decrease in hormone usage in subsequent PE subcultures.

Shoot development (SD) and root initiation (RI) medium

The medium constitutes components from sections A–D, G, H and J (Table 18.1), supplemented with 0.02 mg $litre^{-1}$ NAA.

Root elongation (RE) medium

The medium constitutes components from sections A–D, G and H (Table 18.1) and 0.2 mg litre^{-1} NAA. A volume of 10–12 ml of the RE medium is dispensed into each test tube.

18.3.2 Liquid Culture

18.3.2.1 Liquid Media for Culture Maintenance

The liquid medium consists of components from sections A–C, E and G (Table 18.1) supplemented with 1 mg litre^{-1} 2,4-D and 0.1 mg litre^{-1} NAA. A volume of 20 ml media is dispensed into each 100 ml flask.

18.3.2.2 Liquid Media for Culture Maturation

The liquid medium for maturation contains nutrients listed in sections A–E and G (Table 18.1), a basal medium without plant hormones. A volume of 20 ml media is dispensed into each 100 ml flask.

18.4 In Vitro Culture Protocol

18.4.1 Explant Cutting

To prepare the explant strips, sub-stacks of six leaflets are peeled from the 9 ± 1 cm segment stack and transferred to another sterile petri dish for explant cutting. These leaflets are cut into strips of about 2 mm in width (Fig. 18.3a). Each strip (six-layer stack) is inoculated with one side of the cut edges having direct contact with the callus induction medium (Fig. 18.3b). A 9 cm diameter petri dish can accommodate 18 explants. This process is repeated until all the explants are inoculated. The petri dishes are sealed with cling film and labelled with information such as ortet identification, operator identity, frond number, zone number, medium type and inoculation date. The petri dishes are piled up in a petri dish holder before placing them on racks under continuous darkness for three months. A three-piece petri dish holder can accommodate 42 petri dishes, while a rack-shelf (151.5 × 75.5 cm) can accommodate 24 petri dish holders. Thus, a rack with six shelves can hold 6048 petri dishes (42 × 24 × 6) in total. A cabbage can produce up to ca. 3000 strips (six-layer stacks) in ca. 1000 petri dishes. Therefore, a six-shelf rack can generally accommodate ca. six ortet cabbages.

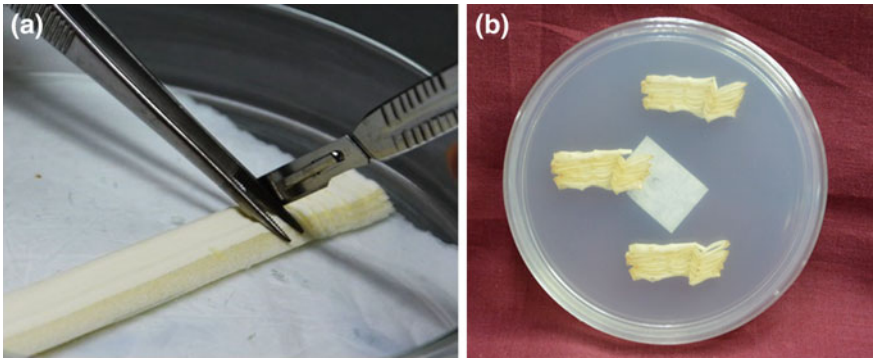

Fig. 18.3 Leaf explants. **a** Six layers of leaf segments are cut into 2 mm explant slices. **b** Three bands of explants comprising of six layers each were placed in a petri dish

18.4.2 Callus Culture

After three months of incubation, the explants are examined for callus formation. Leaf strips with calli are transferred to petri dishes containing fresh embryoid induction medium, while non-callusing explants are subcultured onto fresh callus induction medium at three-monthly intervals for one year before discarding. The callogenic cultures (Fig. 18.4a) are incubated in continuous darkness and subcultured at three monthly intervals for bulking and primary embryoid initiation (Fig. 18.4b). The callogenic cultures are also maintained for one year before discarding.

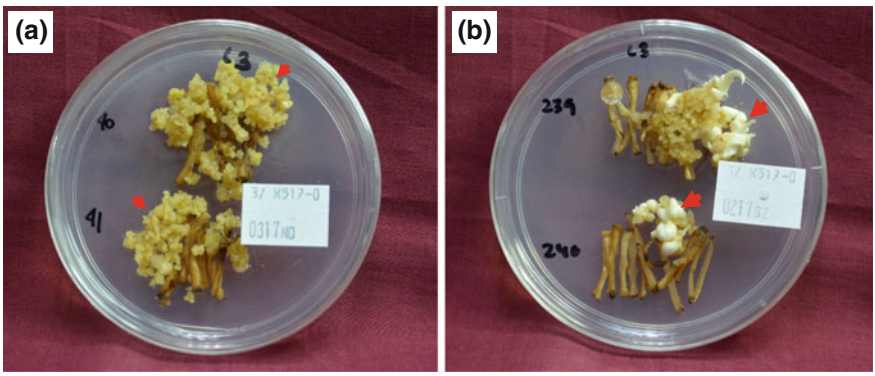

Fig. 18.4 Proliferation of callus. **a** Highly proliferating callus (as arrowheads). **b** Primary embryoids (as arrowheads)

18.4.3 Liquid Culture

Establishment of liquid cultures start with an initial 20 flasks with approximately 0.5 g of cultures each. The duration for establishment of liquid cultures may vary between clones and usually requires more than three subcultures. Weight increment for established clones was about 2 to 4-fold after four subcultures in maintenance media and this varied between clones. After about two to four weeks in maturation media, a weight increment of 2 to 6-fold has been observed. Although MPOB's liquid culture protocol is routinely used, certain genotypes required some modifications of the process. Generally, selection of the suitable callus morphotype, hormone concentration and duration in culture were found to be crucial for the development of liquid cultures at all stages.

The liquid culture method can be divided into three main steps: initiation of liquid culture, maintenance and maturation of liquid culture leading to embryoid regeneration. After these steps, the cultures are transferred back into the solid culture process.

Initiation of Liquid Suspension Culture

All culturing work is carried out in the LAC. For initiation of liquid cultures, friable calli (Fig. 18.5a) and media supplemented with plant hormones are required. Flasks containing the media are weighed prior to subculturing of selected friable calli into the liquid media. The opening of the flask is then briefly flamed before covering with a square plastic sheet and sealed with cling film. The flasks are then usually labelled with Experiment Number, Date of Culture, Weight of cell aggregates (g) and culture number. Similar information is recorded on the record-sheet. The flasks of cultures are incubated on a rotary shaker (Fig. 18.5b) in the dark at 27–30 °C.

Maintenance and Maturation of Liquid Culture

Before subculturing, pre-sterilized sieves with pore sizes of 300 µm and 1 mm, sterile 250 ml jars, liquid media for culture maintenance, liquid media for culture maturation and solid media for formation of embryoids are prepared. All these

Fig. 18.5 Liquid culture. **a** Friable callus used for initiation of liquid cultures. **b** Rotary shakers in dark room for liquid cultures. **c** Smaller aggregates for maintenance of liquid cultures. **d** Larger aggregates (>300 µm) for maturation

apparatus are surface sterilized before placing them into the laminar airflow cabinet. The cultures in flask are poured onto the 300 μm sieve for maintenance process of liquid cultures (Fig. 18.5c) while 1 mm sieve is used for selection of cell aggregates for maturation stage (Fig. 18.5d). Suitable cell aggregates are filtered from cultures trapped by the sieve, then carefully transferred onto fresh respective media. Selected cell aggregates collected from maturation cultures are carefully transferred onto solid media for regeneration. The liquid cultures are incubated in the dark room on a rotary shaker while those that have been transferred onto solid media are incubated in the light room. Both the rooms are maintained at 27–30 °C.

Regeneration of Embryoids

First phase

Prior to conducting this procedure, the LAC is UV-sterilized for 15 min and all the apparatus are surface sterilized. Petri dishes containing solid media, precut sterile filter papers that fit the petri dishes, 1 mm sieves, sterile jars and flasks containing cell aggregates in maturation media are prepared and placed in a trolley next to the LAC. Firstly the petri dishes containing media are single-layered with precut filter papers using forceps. The cell aggregates in the flasks are poured onto the sieves and the flow-through is captured in the jars. The cell aggregates collected on the sieves are weighed and separated into batches of approximately 0.5 g and transferred into the pre-prepared petri dishes using a spatula, while the flow through is discarded. The petri dishes are then sealed with parafilm and appropriately labelled with experiment number, date, weight and the flask number of its origin. The plates are then transferred into the dark room with the temperature set between 27 and 30 °C.

Second phase (approx. after 1 month)

After one month, the plates from the dark room are prepared for harvesting. Only embryogenic calli/primary embryoids are selected and transferred into flasks containing fresh media. The flasks are then sealed and appropriately labelled. The remaining non-embyogenic calli in the plates are returned back to the dark room for another month for further growth. The same procedure is repeated the next month. After the second harvest, all plates are discarded. Primary embryoids obtained from both harvests are further maintained and multiplied on hormone-free media for another 14-18 months. Subculture is performed every two months. Shoots obtained during the multiplication process are transferred to shoot development media. At this stage, the protocol is similar to the solid culture system.

18.4.4 Polyembryogenic Culture

Primary embryoids (Fig. 18.6) that are white to pale yellow in colour with smooth surfaces are detached from the callus mass before being transferred into conical

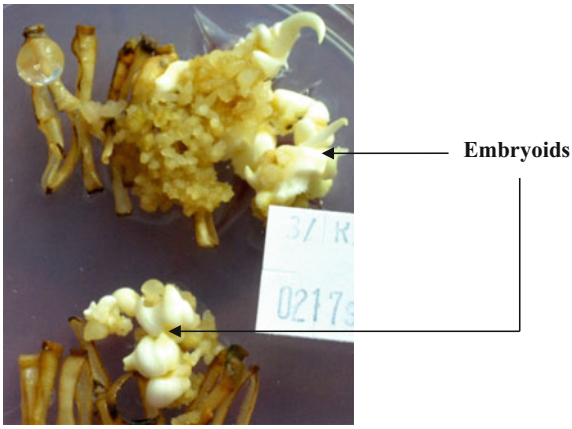

Fig. 18.6 Whitish embryoids with smooth surfaces

flasks containing PE multiplication medium (Table 18.1). These are incubated under a 12/12 h light and dark photoperiod. The flasks are plugged with non-absorbent cotton bungs. The embryoids multiply slowly into bigger compact tissue masses known as polyembryoids (PE) (Fig. 18.7a). Subculture of the PE is carried out every two months. Generally, after the fourth or fifth subculture, multiplication of PE occurs concurrently with shoot formation. The PE masses are cleaned off agar residue, dead or soggy tissues and then cut into smaller clumps of 1.5–2 cm in diameter. Each clump is transferred into a flask for further proliferation, while shoots from PE masses are collected for the next SD stage.

For shoot production, PE cultures are maintained till the maximum 15 subcultures. Upon reaching subculture 11 and thereafter, the PE mass is cut into 0.5 cm clumps and transferred to fresh medium at five clumps per flask. These cultures will be maintained for more than two months to increase shoot formation.

18.5 Shoot Regeneration and Maintenance

18.5.1 Shoot Production and Selection

Quality and quantity are important to attain a substantial number of shoots. Rohani et al. (2000) described that nodular embryoids are more prolific than other embryoid phenotypes. Based on previous records, clones with low number of shoots have either only one or very few embryoid lines. Embryoid phenotypes such as globular or torpedo-shaped did not proliferate readily. As described by Wong et al. (1996), some PE cultures only produce shoots without increasing their embryogenic mass. In some clones, PE cultures did not proliferate up to the maximum 15th subculture

(Table 18.2). Other factors contributing to the low PE culture quantity include contamination, sogginess and poor development of embryoids. At times, shoots derived from the same palm but originating from different explant type (L210 and M210) can lead to significantly different shoot production (Table 18.2).

18.5.2 Shoot Development

Shoots germinated from PE clumps that have attained a height of 2 cm and above are isolated and each shoot base is cut into a V-shape tip before transferring to SD medium. The V-cut was to avoid wounding if the shoot apex which is located at the center of the base and to provide a fresh contact surface to the medium. Usually, ten shoots are transferred into a 250 ml conical flask containing 85 ml SD medium. Each flask is covered with a non-absorbent cotton bung. The flasks are placed directly on a rack in the light room. After 2–3 months, most of the shoots would have attained a height of 6–7 cm with emerging roots (Fig. 18.7b) and are ready for the root elongation stage. Shoots without roots will be subcultured into fresh SD medium.

18.6 Root Induction and Elongation

18.6.1 Root Development in Test Tube

Shoots with roots or emerging roots are transferred into test tubes containing 10 ml liquid RE medium each (Fig. 18.7c).

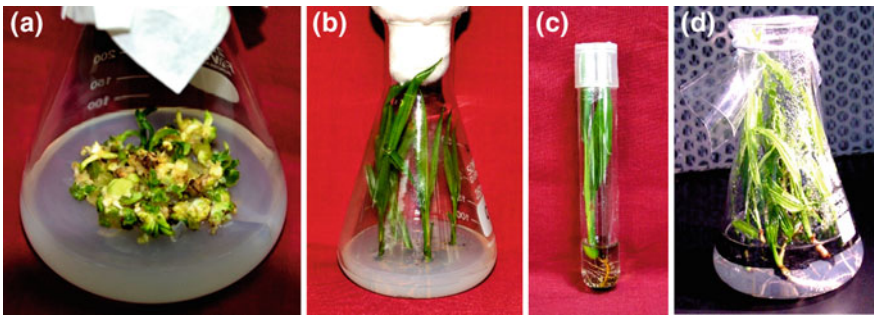

Fig. 18.7 **a** Polyembryoids **b** Shoot Development **c** Root development in test tube. **d** Root development using Double Layer technique

Table 18.2 Clonal shoot production

Clone	Explant type	Ortet ID*	Last subculture number	No. of shoots produced
L207	Leaf	0.827/133	8	45
L208	Leaf	0.827/115	15	393
L210	Leaf	0.189/141	13	160
L233	Leaf	0.827/107	15	1897
L234	Leaf	0.827/65	15	1590
L235	Leaf	0.827/25	13	2750
L236	Leaf	0.187/12	15	29,115
L238	Leaf	0.827/221	13	282
L240	Leaf	0.827/62	13	420
L241	Leaf	0.179/128	15	374
L242	Leaf	0.179/3	15	860
L245	Leaf	0.189/396	8	120
L246	Leaf	0.189/3443	11	40
M210	Male inflorescence	0.189/141	15	17,519
M224	Male inflorescence	0.184/466	14	164
F223	Female inflorescence	0.189/289	6	19

Note *Elite *tenera* ortets

18.6.2 Root Development via Double Layer (DL) Technique

This alternative method is simple and quick. About 50 ml of liquid RE medium, in a dispensing flask is aseptically poured into each flask containing intact shoots, overlaying the solid SD medium (Fig. 18.7d). The cultures are incubated in the light. Two to three months after the addition of the liquid medium, most ramets would have generally exhibited shoot and root elongation. The growth varies among plantlets due to competition for nutrients, space and light. Poor growing plantlets are either culled or subcultured into fresh medium in test tubes for further elongation. This DL method is recommended as a means to mitigate labour shortage when processing high volume of cultures at the shoot development stage. Zamzuri (1998, 2000) showed that by using the DL rooting method, work output could be increased by 18-fold.

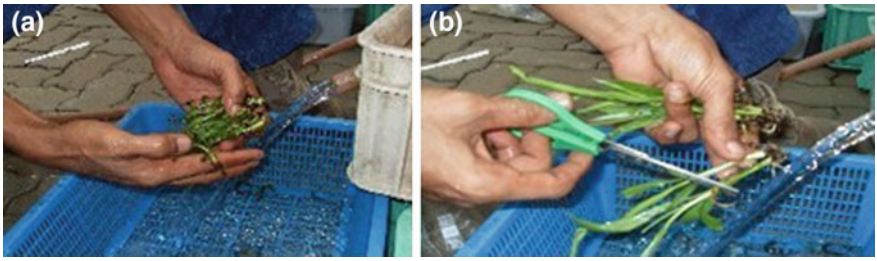

Fig. 18.8 **a** Cleaning and **b** removing wilted leaves of hardened shoots

18.6.3 In Vitro Acclimatization

Ramets with a height of more than 12 cm (at the two- to three-leaf stage) and good rooting system are ready for in vitro hardening in the nursery. Rooted cultures still under sterile condition in culture vessels are transferred to pre-nursery with 80% shade for acclimatization for four days. Their vessel caps are opened on the third day and transplanting commences on the fifth day.

18.7 Nursery

18.7.1 Transplanting Stage

The hardened shoots are removed from their test tubes, washed (Fig. 18.8a and b) and soaked in water before transplanting into compacted compost clump covered with netted cloth known as Jiffy-7 pots that are pre-soaked in water. Transplanted ramets are labeled, arranged neatly in perforated trays and placed on a raised platform in the pre-nursery with ca. 75% shade. General maintenance involve irrigation, weekly application of foliar fertilizer, weeding, pest control, culling etc. After four to five months, the ramets are sent to the main nursery and transferred into larger polybags under temporary shading conditions before exposing them to direct sunlight. General nursery maintenance is applied as recommended for DxP seedlings (Kushairi and Rajanaidu 2000). The whole protocol for solid and liquid culture systems is summarized in Fig. 18.9.

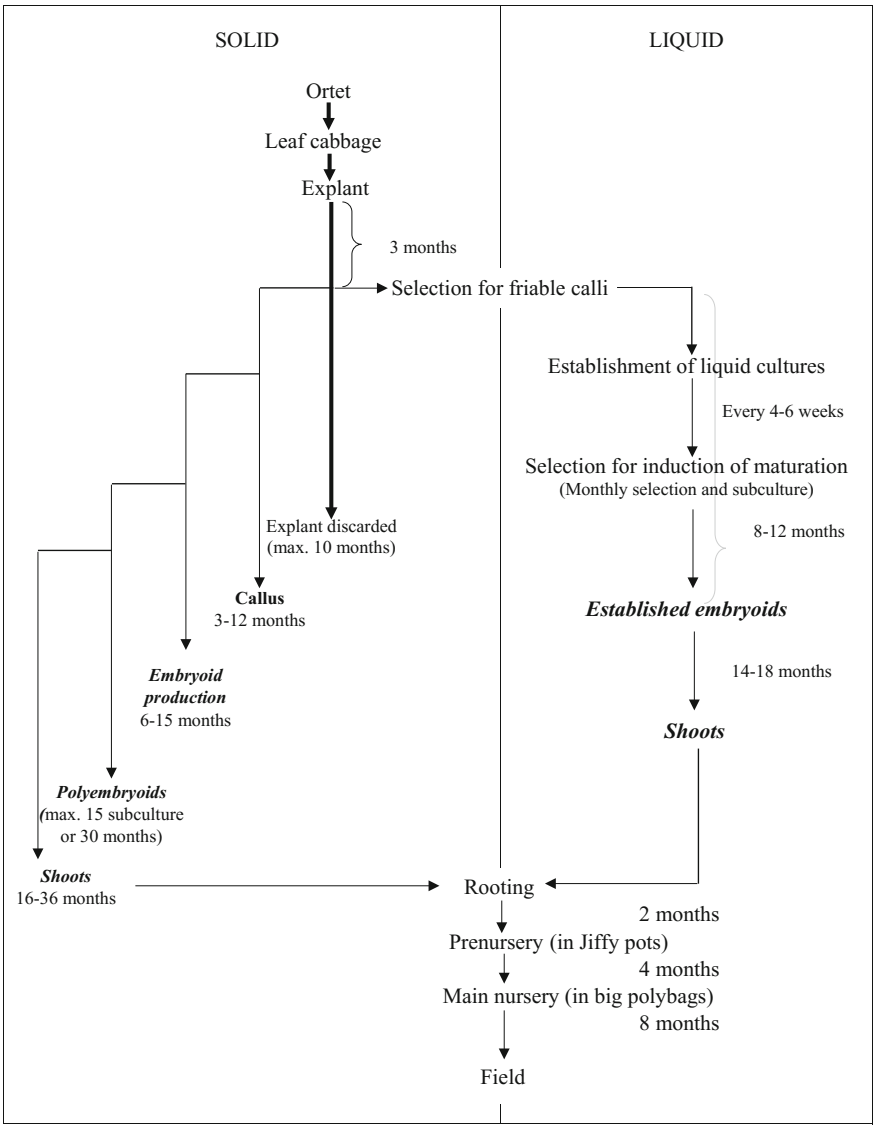

Fig. 18.9 MPOB protocol with time scale

18.7.2 Quality Control

Physical control (Culling)

Culling is carried out at both the in vitro and ex vitro stages. At the in vitro stage, callus cultures that appear flaccid and brownish are discarded. It is quite common to obtain various types of embryoids. They can be nodular, torpedo-shaped (single or fused), globular, wavy, etc. It is not known whether the different morphotypes will give rise to abnormality. From our experience, the nodular, torpedo-shaped (fused) and wavy types are generally more prolific. Most of the culling is carried out at the shoot and rooting stages. A normal DxP seedling derived from the embryo rescue method is used as a reference. Stunted erect shoots and shoots producing terminal inflorescences are discarded. Shoots with fleshy bases and poorly developed laminae are also discarded (Tarmizi 1997; Rohani et al. 2000; Ong-Abdullah et al. 2005). At the ex vitro stage (pre-nursery), abnormal shoots tend to be stunted, remain juvenile for prolonged periods and show nutrient deficiency or diseased symptoms. Such ramets are discarded.

Molecular control (*SureSawit*^{TM} KARMA)

The mantling abnormality affects fruit development and thus oil yields from clonal palms. Although the mantling rate has been reported to be at an average of 5%, it varies widely and unpredictably depending on genotypes or culture conditions (Jaligot et al. 2011). A diagnostic marker to screen clonal planting material at an early age would be favorable to reduce the risk of field planting abnormal plants. This was made possible by the recent discovery that hypomethylation of a *Karma* retrotransposon at the MANTLED locus was associated with this abnormal phenotype (Ong-Abdullah et al. 2015). A diagnostic assay was subsequently developed for screening palms using leaf samples.

The *SureSawit*^{TM} KARMA assay is a PCR-based method to detect the level of methylation at the Karma region (Ong-Abdullah et al. 2016a, b). A leaf punch measuring 0.5 cm in diameter is sampled using the proprietary leaf puncher in the kit. DNA is then extracted from the sample through an automated process in the laboratory. Bisulfite conversion followed by desulfonation is then carried out on the DNA to convert unmethylated cytosines to uracil bases, while methylated cytosines on the DNA will remain unconverted. After having gone through several rounds of optimization, the final converted DNA, which is used as the template undergoes subsequent probe-based PCR assay. Various controls are incorporated into the analysis to determine the methylation levels of the target site in the DNA and finally, predict the mantling risk of the sampled plant (Figs. 18.10 and 18.11). This diagnostic test can be utilized as a decision making tool for the industry. Tissue culture laboratories can then plan and refine their cloning strategies and subsequent clonal plantings.

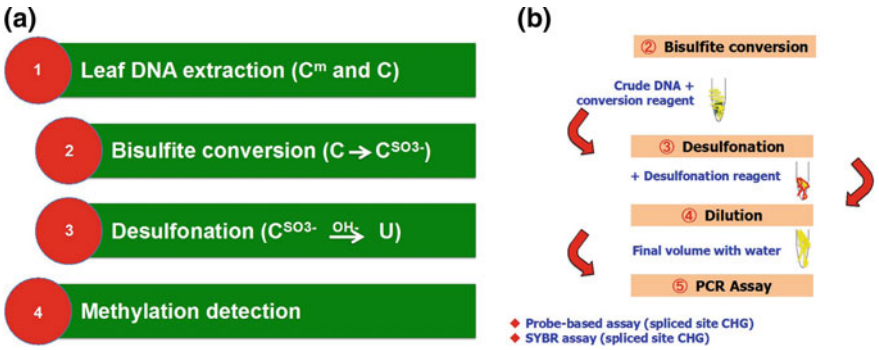

Fig. 18.10 **a** The general process flow of the KARMA assay and **b** Steps involved in preparation of DNA for the PCR assay

18.8 Conclusion and Future Technologies

To ensure a successful venture into the production of clonal oil palm materials, a good established breeding programme is essential for the identification of superior palms or ortets. In relations to this, MPOB, having the largest oil palm germplasm collection in the world, has continuously evaluated its collection for traits of economic interest and value (Mohd Din et al. 2005). Acknowledging the fact that conventional breeding is time consuming, tissue culture has now become an appropriate strategy for expediting the development of commercial planting materials. However, requirement of a high number of ortets for tissue culture remains a setback.

The liquid culture system was demonstrated to have significant effects on multiplication rates, morphology of shoots, somatic embryo development, microtubers or bulblets produced in vitro (Preil 2005). Liquid cultures were also proven to be a good source of starting material for transformation and protoplast isolation studies (Xiao et al. 2009; Sallets et al. 2015) as well as for secondary metabolite production (Zare et al. 2010; Boonsnongcheep et al. 2010). However, the productivity of cultures in the conventional liquid culturing system is limited due to the size of vessels used. Various innovative technologies have been developed to further improve the efficiency of the liquid culture system. Technologies such as the MPOB Fast Transfer Technique (MoFaTT) (Tarmizi and Zaiton 2007) in liquid culture system, the 2-in-1 MoSlim (MPOB Simple Impeller for liquid culture, the Simple Impeller with Fast Transfer Technique (SLIM-FaTT), the MPOB Modified Vessel (MoVess), MultiVessel (MV) bioreactor, Motorized Vessel (MotoVess) and Motorized Vessel with Fast Media Transfer (MoVeFast) (Tarmizi et al. 2009, 2016) systems allow rapid and convenient propagation of liquid cultures at a larger scale compared to the conventional shake flasks system. These innovations can be applied to any fluidic platforms even in other crops and can be a catalyst for future semi- or fully-automated liquid culture systems.

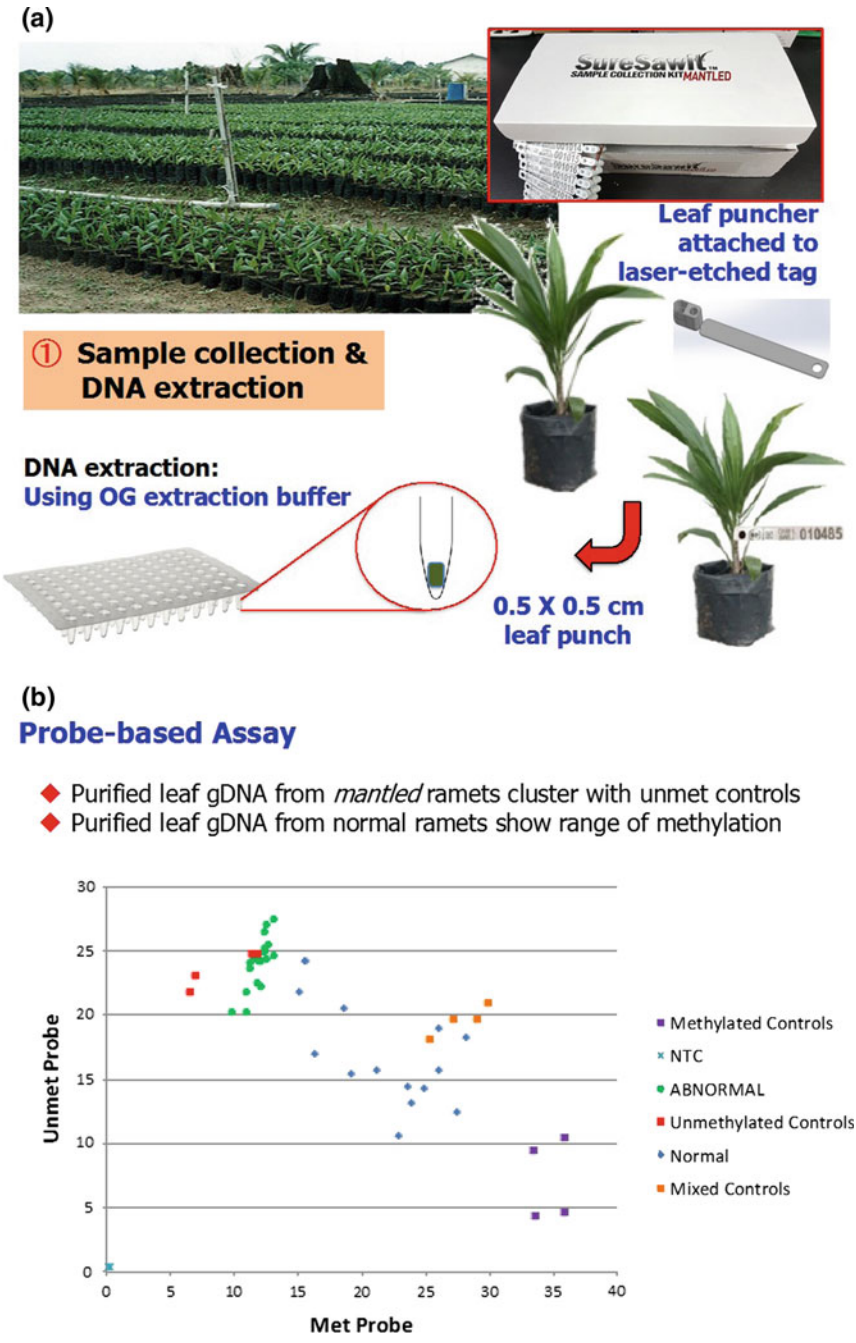

Fig. 18.11 **a** Sampling procedure made-easy with the SureSawit^{TM} KARMA collection kit and OG DNA extraction buffer and **b** Methylation detection Adapted from Ong-Abdullah (2016)

Due to the slow process of oil palm in vitro propagation, which takes two to five years from the start of the explant stage to acquiring nursery ramets, oil palm clones cannot meet the entire demand for improved planting materials in the near future. However, while moving ahead with the production of clones, high-performing duras and pisiferas with good combining abilities are also cloned for use in the mass production of "clonal seeds". Clonal seeds can be in the form of either a semi-clone, whereby only one of the parents is cloned (often is the dura) or a bi-clone, when both parents are tissue culture-derived. One of the setbacks in tissue culture is the high number of ortets required to meet production demands. Therefore, the proposed establishment of ortet gardens would certainly aid in overcoming the limited source of explants.

Essentially, Malaysia being limited in arable land is not in the position to stay competitive based on planted area. Therefore maximizing productivity through increasing oil yield by utilizing the best planting materials and possibly reconfiguring estates should be emphasized. In parallel, policies and regulations may need to be re-examined. This requires close cooperation between the various agencies and strengthening the support mechanisms towards realizing the aspirations set by the government.

Clonal planting material is expected to create the "second wave" in yield improvement. Clones tested with the *SureSawit*™ KARMA assay will assist in minimizing mantling to further boost yield productivity. With tissue culture labs imposing higher than the minimum selection standards and coupled with implementing Good Agricultural Practices in plantations, oil yield is expected to substantially increase the overall national productivity.

Acknowledgements The authors wish to thank Datuk Dr. Ahmad Kushairi Din, Director-General of MPOB for permission to publish the paper. Support and encouragement from Dr. Ahmad Parveez Ghulam Kadir, Deputy Director-General and Dr. Mohamad Arif Abd Manaf, Director of ABBC and technical assistance from the Tissue Culture staff are gratefully acknowledged.

References

Aziah MY (1989) Shoot formation in *Calamus manan* under *in vitro*. In: Rao AN, Aziah MY (eds) Proceedings of the seminar on tissue culture of forest species, June 15–18 1987. Kuala Lumpur, Malaysia, pp 45–49

Baudouin L, Durand-Gasselin T (1991) Genetic transmission of characters linked to oil yields in oil palm by cloning results for young palms (1). Oléagineux 49:313–320

Boonsnongcheep PP, Korsangruang SS, Soonthornchareonnon NN, Chintapakorn Y, Saralamp P, Prathanturarugand PS (2010) Growth and isoflavonoid accumulation of *Pueraria candollei var. candollei* and *P. candollei var. mirifica* cell suspension cultures. Plant Cell Tissue Organ Cult 101:119–126

Corley RHV, Gray BS (1976) Yield and yield components. In: Corley RHV, Hardon JJ, Wood BJ (eds) Oil Palm Research. Elsevier, Amsterdam, pp 77–86

Corley RHV, Lee CH, Law IH, Wong CY (1986) Abnormal flower development in oil palm clones. The Planter Kuala Lumpur 62:233–240

Duval Y, Durand-Gasselin T, Konan K, Pannetier C (1988) *In vitro* vegetative micropropagation of oil palm *(Elaeis guineensis* Jacq.) strategy and results. In: Proceedings of the 1987 international oil palm/palm oil conference—agriculture, June 23-July 1 1987, PORIM, Bangi, pp 191–198

Eeuwens CJ, Blake J (1977) Culture of coconut and date palm tissue with a view to vegetative propagation. Acta Hort 78:277–281

Jaligot E, Adler S, Debladis E, Beule T, Richaud F, Ilbert P, Finnegan EJ, Rival A (2011) Epigenetic imbalance and the floral developmental abnormality of the in vitro-regenerated oil palm Elaeis guineensis. Ann Bot 108:1453–1462

Jones LH (1974) Propagation of clonal palms by tissue culture. Oil Palm News. 17:1–8

Kushairi A, Rajanaidu N (2000) Breeding populations, seed production and nursery management. In: Yusof B, Jalani BS, Chan KW (eds) Advances in oil palm research, vol 1. MPOB, Bangi. pp 39–96

Kushairi A, Mohd Din A, Rajanaidu N (2011) Forging ahead with clones In: Wahid MB, Choo YM, Chan KW (eds) Further advances in oil palm research (2000–2010), vol 1. MPOB, pp 47–101

Mohd Din A, Rajanaidu N, Kushairi A (2005) Exploitation of genetic variability in oil palm. IN: Proceedings MOSTA best practices workshops: agronomy and crops management, Malaysian Oil Sci Technol Assoc., March 27-August 14. Malaysia, pp 19–42

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473–497

Ong-Abdullah M, Rohani O, Zamzuri I, Tarmizi AH, Sharifah SA (2005) Handbook: photographic perspective on the anomalies of oil palm. Published by the Malaysian Palm Oil Board

Ong-Abdullah M, Ordway JM, Jiang N, Ooi S-E, Kok S-Y, Sarpan N, Azimi N, Hashim AT, Ishak Z, Rosli SK, Malike FA, Abu Bakar NZ, Marjuni M, Abdullah N, Yaakub Z, Amiruddin MD, Nookiah R, Singh R, Low E-TL, Chan K-L, Azizi N, Smith SW, Bacher B, Budiman MA, Van Brunt A, Wischmeyer C, Beil M, Hogan M, Lakey N, Lim C-C, Arulandoo X, Wong C-K, Choo C-N, Wong W-C, Kwan Y-Y, Syed Alwee SSR, Sambanthamurthi R, Martienssen RA (2015) Loss of Karma transposon methylation underlies the mantled somaclonal variant of oil palm. Nature 525:533–537

Ong-Abdullah M, Ordway JM, Jiang N, Ooi S-E, Mokri A, Nookiah R, Singh R, Low E-TL, Sachdeva M, Smith SW, Lakey N, Sambanthamurthi R, Martienssen RA (2016a) Tissue culture and epigenetics. The Planter. Kuala Lumpur. 92:741–749

Ong-Abdullah M, Ordway JM, Jiang N, Ooi S-E, Mokri A, Kok SY, Sarpan N, Azimi N, Hashim AT, Ishak Z, Rosli SK, Nookiah R, Singh R, Low E-TL, Sachdeva M, Smith SW, Lakey N, Martienssen RA, Sambanthamurthi R (2016b) *SureSawit*^{TM} KARMA—a diagnostic assay for clonal conformity. MPOB Information Series No. 156, Malaysia

Paranjothy K, Othman R (1982) *In vitro* propagation of oil palm. In: Fujiwara A (ed) proceedings of the fifth congress plant tissue and cell cult. July 11–16 1982, Maruzen, Tokyo. pp 747–748

Preil W (2005) General introduction: a personal reflection on the use of liquid media for *in vitro* culture. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for *in vitro* plant propagation. Springer, Berlin, pp 1–18

Rabéchault H, Martin J-P (1976) Multiplication vegetative du palmier a huile *(Elaeis guineensis* Jacq.) al'aide de cultures de tissus foliares. C.R Acad Sci Paris 283:1735–1737

Rabechault H, Ahee J, Guenin G (1970) Colonies cellulaires et formes embryos *in vitro* á partir de cultures d' embryons de palmier a huile (*Elaeis guineensis* Jacq. Var. dura Becc.). C.R. Acad Sci Paris 270:3067–3070

Rajanaidu N, Rohani O, Jalani S (1997) Oil Palm clones: current status and prospects for commercial production. Planter 73(853):163–184

Rohani O (2001) Positional effect of leaf explant on callusing and embryogenesis in Oil Palm *(Elaeis guineensis).* In: Proceedings of the 2001 PIPOC international palm oil congress—agriculture, MPOB, Bangi, pp 677–685

Rohani O, Sharifah SA, Mohd Rafii Y, Ong M, Tarmizi AH, Zamzuri I (2000) Tissue culture of oil palm. In: Yusof B, Jalani BS, Chan KW (eds) Chapter 7, advances in oil palm research, vol 1. MPOB, Bangi. pp 238–283

Sallets AA, Delimoy D, Boutry M (2015) Stable and transient transformation of *Artemisia annua* suspension cells. Plant Cell Tissue Organ Cult 120:157–164

Smith WK, Thomas JA (1973) The isolation and *in vitro* cultivation of cells of *Elaeis guineensis*. Oleagineux 28:123–127

Sogeke AK (1998) Stages in the vegetative propagation of Oil Palm, *Elaeis guineensis* Jacq. through tissue culture. J Oil Palm Res 10(2):1–9

Tarmizi AH (1997) Assessment of variability in tissue culture-derived buds of Oil Palm (*Elaeis guineensis* Jacq.). Ph.D. thesis. University of Liverpool, England, 234 pp

Tarmizi AH, Zaiton R (2007) Development of the MPOB Fast Transfer Technique (MoFaTT) for maintenance and maturation of oil palm culture aggregates. J Oil Palm Res. 19(2):435–439

Tarmizi AH, Zaiton R, Rosli MY (2009) MPOB Motorized Vessel (MPOB-MotoVess) for liquid tissue culture system. MPOB Information Series TT No. 454

Tarmizi AH, Zaiton R, Rosli MY (2016) Simple impeller system for maintenance of Oil Palm culture aggregates. J Oil Palm Res 1928(2):184–190

Thanh-Tuyen NT, Apurillo DI (1992) Plant regeneration through somatic embryogenesis and cultured zygotic embryos of coconut. Pakistan J Contemp Sci 17(1):1–7

Tisserat B, Foster G, de Mason D (1979) Plantlet production *in vitro* from *Phoenix dactylifera L.* Date Growers' Institute Rep 54:19–23

Wong G, Tan CC, Soh AC (1996) Large-scale propagation of oil palm clones - experience to date. Paper presented at the International symposium on *in vitro* culture and horticultural breeding, June 16-21 1996. Jerusalem, Israel. pp 649–658

Xiao W, Huang X, Gong QDX, Zhao JT, Wei YR, Huang XL (2009) Somatic hybrids obtained by asymmetric protoplast fusion between Musa Silk cv. Guoshanxiang (AAB) and Musa acuminate cv. Mas (AA). Plant Cell Tissue Organ Cult 97:313–32

Zamzuri I (1998) Efficient rooting of Oil Palm *in vitro* plantlets using double-layer technique. PORIM Bulletin No. 36:23–36

Zamzuri I (1999) Using double-layer technique in rooting of Oil Palm *in vitro* plantlets. In: Proceedings of the 1999 PORIM international palm oil congress-agriculture, February 1–6 1999. MPOB, Bangi. pp 223–229

Zamzuri I (2004) Clonal trial report. Biology Division, MPOB. 26-3-2004

Chapter 19
Somatic Embryogenesis of Date Palm (*Phoenix dactylifera* L.) from Shoot Tip Explants

Jameel M. Al-Khayri

19.1 Introduction

Date palm (*Phoenix dactylifera* L.) is a diploid, dioecious tall evergreen fruit tree belonging to the monocot family Arecaceae. This majestic tree has been acknowledged for contributing to the development of ancient civilizations and human settlements in the arid hot regions of the Middle East and North Africa, where it is predominantly cultivated and remains of significant social and economic values (El Hadrami and Al-Khayri 2012). Currently, date palm cultivation has expanded to various countries in five continents, Africa and the Americas (Al-Khayri et al. 2015a) as well as Asia and Europe (Al-Khayri et al. 2015b).

Despite the harsh desert environmental conditions, where this species mostly thrives, this tree is a bountiful source for food, shelter, furniture, handcrafts, fuel and numerous other uses (Johnson 2016; Abd Rabou and Radwan 2017). The average economic life of the tree is 40–50 years but productive life can reach up to 150 years in some cases (Chao and Krueger 2007). Female trees bear nutritious fruits rich in sugar, minerals, vitamins, and numerous pharmaceutical compounds utilized in traditional medicine (Vayalil 2012; Ahmed 2013; Omran 2017; Selmani et al. 2017). Date fruit is a suitable raw material for manufacturing numerous food products and functional healthy ingredients (Di Cagno et al. 2017). Date palm leaves, pruning waste residues, hold a great potential for ruminant feeding (Rajaee Rad et al. 2015; Ghorbani et al. 2017).

Date palm seeds are abundant and readily available and serve well as genetic resources for breeding as well as ornamental planting (El Hadrami et al. 2011; Johnson et al. 2013). However, seeds are not suitable for propagating known cultivars because they produce heterogeneous offsprings with variable characteristics

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_19

and inferior dates. Traditionally, date palm is propagated vegetatively by offshoots to ensure production of true-to-type trees. This method is restrained by the limited number of available offshoots which are produced during the early life of the tree. Presently, tissue culture is an effective alternative for large-scale propagation of date palm. This approach is increasingly attracting commercial interests (Zaid et al. 2011). Currently, several commercial tissue culture laboratories are producing date palm plantlets for international distribution (Al-Khayri et al. 2015a, b).

To initiate the date palm cultures, researchers have examined various explant sources including zygotic embryos, inflorescence tissues and in vitro-derived leaf segments. Although it requires sacrificing the plant, the most commonly used explants are apical shoot tips and lateral buds isolated from young offshoots which proved most responsive and practical for date palm micropropagation (Al-Khayri 2005, 2007). Research progress of date palm micropropagation was the focus of several reviews (Tisserat 1984; Omar et al. 1992; Benbadis 1992; Al-Khayri 2013; Mazri and Meziani 2015). Plant regeneration in date palm has been demonstrated through somatic embryogenesis (Fki et al. 2011) as well as adventitious organogenesis (Abahmane 2011) by manipulating the hormonal supplements to the culture medium. Somatic embryogenesis provides the ability of producing large numbers of plantlets of synchronized growth, a desired attribute for commercial micropropagation. Researchers have optimized various in vitro factors to achieve improved somatic embryogenesis of several commercially important date palm cultivars (Al-Khayri 2013).

The present communication focuses on indirect somatic embryogenesis. It describes a micropropagation protocol based on callus induction from apical shoot tip explants. This protocol has been demonstrated suitable for in vitro regeneration of several commercially important date palm cultivars including the commercially important cultivars Khalas, Barhi, Hillali, Naboot Saif, Ruzaiz, Khusab and Shishi (Al-Khayri and Al-Bahrany 2001, 2004). Fully-detailed step-wise procedures are described including explant isolation and disinfection, callus induction and maintenance, development of somatic embryos, rooting and plantlet formation, and finally acclimatization and field establishment.

19.2 Protocol of Somatic Embryogenesis

19.2.1 Culture Medium

1. The basal medium used for date palm tissue culture is based on MS salts (Murashige and Skoog 1962) with some modifications. Prepare the stock solutions of the MS medium as specified in Table 19.1. Combine adequate volumes of stock solutions, as described in Table 19.1, in a flask containing half the final volume of double distilled water. For preparing the rooting medium, use half-strength MS salts (major and minor nutrients and Fe-EDTA) and full

Table 19.1 Constituents of date palm in vitro culture medium including salt components and organic additives, preparation of stock solutions, and final concentration of culture medium

Constituents	Chemical formula	Stock conc. (g l^{-1})	Medium conc. (mg l^{-1})
Major nutrients[a] 10X *stock, use* 100 ml per L *medium*			
Ammonium nitrate	NH_4NO_3	16.5	1650
Potassium nitrate	KNO_3	19.0	1900
Calcium chloride-$2H_2O$	$CaCl_2.2H_2O$	4.4	440
Magnesium sulfate-$7H_2O$	$MgSO_4.7H_2O$	3.7	370
Potassium orthophosphate	KH_2PO_4	1.·7	170
Sodium phosphate	$NaH_2PO_4.2H_2O$	1.7	170
Minor nutrients[1] 100X *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-$4H_2O$	$MnSO_4.4H_2O$	2.23	22.3
Zinc sulfate-$7H_2O$	$ZnSO_4.7H_2O$	0.86	8.6
Sodium molybdate-$2H_2O$	$Na_2MoO_4.2H_2O$	0.025	0.25
Cupric sulfate-$5H_2O$	$CuSO_4.5H_2O$	0.0025	0.025
Cobalt chloride-$6H_2O$	$CoCl_2.6H_2O$	0.0025	0.025
Iron–EDTA[1] 100X *stock, use* 10 ml per L *medium*			
Iron sulfate-$7H_2O$	$FeSO_4.7H_2O$	2.78	27.8
Ethylenediaminetetraaceticacid disodium salt dihydrate	$Na_2EDTA.2H_2O$	3.73	37.3
Vitamins 100X *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			30,000
Agar			7000

(continued)

Table 19.1 (continued)

Constituents	Chemical formula	Stock conc. (g l^{-1})	Medium conc. (mg l^{-1})
Hormones and charcoal			
Hormones	According to stage as specified in Table 19.2		
Activated charcoal	According to stage as specified in Table 19.2		
pH			5.7

Note that hormonal supplements and activated charcoal are specified elsewhere according to culture stage

[a]These constituents (major and minor nutrients and Fe-EDTA) are used at half strength in preparation of medium used for rooting stage

strength of the other ingredients listed in Table 19.1. Commercially available prepackaged formulations of MS salt as well as other media are preferred alternatives among researchers for their user convenience as compared to stock solution preparation and mixing. However, to reduce culture cost, in-house prepared stock solutions are more feasible for medium preparation particularly for commercial purposes.

2. Prepare the plant growth regulators (PGR) stock solutions separately by dissolving 5 g 2,4-dichlorophenoxyacetic acid (2,4-D) or 0.1 g naphthalene acetic acid (NAA) in 95% ethanol and 0.1 g 2-isopentenyladenine (2iP) in 1 N HCl. Use 2–5 ml of solvent to dissolve the PGR powder and place in a 100-ml volumetric flask. Bring the final volume to 100 ml by adding double distilled water. The resultant concentrations of the stock solutions are: 50 mg ml^{-1} 2,4-D, 1 mg ml^{-1} NAA and 1 mg ml^{-1} 2iP. Store the hormonal stock solutions in the refrigerator at 4 °C until use but limit the storage to 3 months to avoid crystallization and decrease in hormonal activity.
3. This protocol requires six media differing in hormones and activated charcoal content as described in Table 19.2. Add these additives to MS medium accordingly and adjust the mixture to final volume.
4. Adjust medium to pH 5.7 with 1 *N* KOH, and dispense in 150 × 25-mm culture tubes (15 ml medium per tube) for initiation stage or in 125-ml culture flasks or GA-7 Magenta vessels (50 ml per vessel) or 200-ml culture jars (20 ml per jar) for subsequent sages. Sterilize the medium by autoclaving for 15 min at 121 °C and 1 × 10^5 Pa (1.1 kg cm^{-2}).

19.2.2 Explant Preparation

1. Select healthy 2–3-year-old offshoots and separate from mother plants. Explants isolated from offshoots separated during the warm months, May through

Table 19.2 Stages of date palm regeneration system, specific hormones and activated charcoal additives, culture conditions, durations, and responses associated with each stage

Stage	Medium additives[a] (mg l^{-1})	Light[b]	Duration[c] (wk)	Remarks
1. Culture initiation	2,4-D (100), 2iP (3) Act. charcoal (1500)	Dark	9	Explants swell, small tissue/ callus protrusions
2. Callus induction	NAA (10), 2iP (30) Act. charcoal (1500)	Light	3	Explants turn green, visible callus formation
3. Embryogenic callus proliferation	NAA (10), 2iP (6) Act. charcoal (1500)	Light	9	Marked explants expansion, embryogenic callus growth
4. Callus multiplication	NAA (10), 2iP (1.5)	Dark	9	Callus multiplication and long-term maintenance
5. Embryogenesis	None	Light	12	Somatic embryos develop, mature, and germinate
6. Rooting	NAA (0.2)	Light	12	Roots form, plantlets ready for acclimatization in soil

[a]Full-strength MS salt except for rooting where half-strength MS salt is used
[b]Incubation at 16-h photoperiods (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or complete darkness at 23 ± 2 °C
[c]Transfer at 3-wk intervals throughout culture duration

September, often turn brown, in response to the accumulation of phenolic compounds, leading to decreased viability and eventual death; whereas, explants established in the cool months, November through March, showed limited browning and higher frequencies of somatic embryogenesis. Using a hatchet and a serrated knife, trim the leaves (Fig. 19.1a) and remove outer leaves acropetally to expose the shoot tip region (Fig. 19.1b). Careful manipulation is necessary to avoid fracturing of brittle shoot tip. Cut around the base in a 45° angle, about 2 cm away from the circumference of the cylindrical-shaped tip region and remove it; note the conical shape base of the isolated tissue (Fig. 19.1c). Further trim the shoot tip region to a 10-cm high, 4-cm in diameter, cylindrical-shaped shoot tip tissue surrounded with white fleshy tissue of young leaves sheaths. Immediately place excised shoot tips in a chilled antioxidant solution consisting of ascorbic acid and citric acid at 150 mg l^{-1} each. This is necessary to reduce browning of the explant tissue.

2. Surface sterilize the shoot tip (Fig. 19.1d) in 70% ethanol for 1 min, followed by shaking or stirring for 15 min in 1.6% w/v sodium hypochlorite (30% v/v

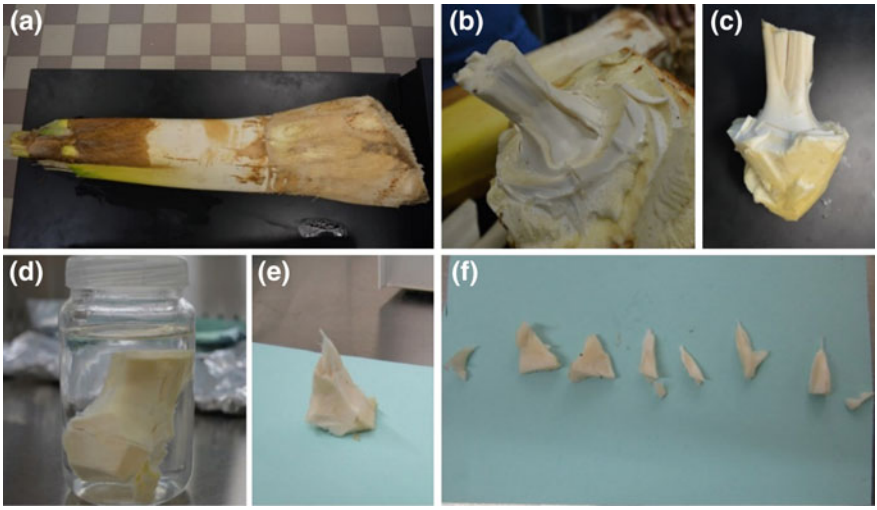

Fig. 19.1 Date palm explant preparation. **a** Trimmed offshoot, **b** Shoot tip after acropetically removing the surrounding leaves and leaf sheaths, **c** Isolated shoot tip region, **d** Shoot tip region in the disinfection solution, **e** Trimmed shoot tip which is used as the explant source, **f** Isolated leaf primordia and shoot tip explants

commercial bleach) containing two drops of Tween 20 per 100 ml solution. Rinse tissue with sterile distilled water three times and keep in a chilled sterile antioxidant solution all through explant manipulation process.

3. The shoot tip region consists of apical meristematic bud surrounded by a number of leaf primordia encased by fleshy white tissue of leaf sheaths. Only the meristematic regions and leaf primordia are suitable for embryogenic callus induction. Under aseptic conditions, using scalpel and forceps trim the ends and further remove the outer tissue to expose leaf primordia and shoot terminal, leaving about 5 cm in length and 1 cm in diameter tip tissue (Fig. 19.1e). Separate leaf primordia, surrounding the terminal shoot tip, at the point of attachment. Section the terminal tip into 4–8 longitudinal sections, approximately 0.5–1 cm^3. A single offshoot may yield 10–20 explants, depending on offshoot size, including terminal tip sections and leaf primordia (Fig. 19.1f). Explants isolated from young offshoots, one year old or less, exhibit less browning; however, they provide a fewer explants. Older offshoots contain axillary buds within the leaves clustered around the shoot tip. These buds can be isolated during the removal of the offshoot leaves and processed as explant sources in a similar manner to the shoot tip explants.

19.2.3 Callus Induction and Maintenance

1. It is advisable to immerse the explants in ascorbic acid and citric acid solution prior to culturing in order to prevent severe browning in response to the oxidation of phenolic exudates that can be detrimental to the explant tissue. Culture the explants isolated from the shoot tip on the initiation medium as specified in Table 19.2.
2. Position whole leaf primordia explants vertically with the cut end inserted into the medium (Fig. 19.2a). 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 minimize potential explants losses due to culture contamination.

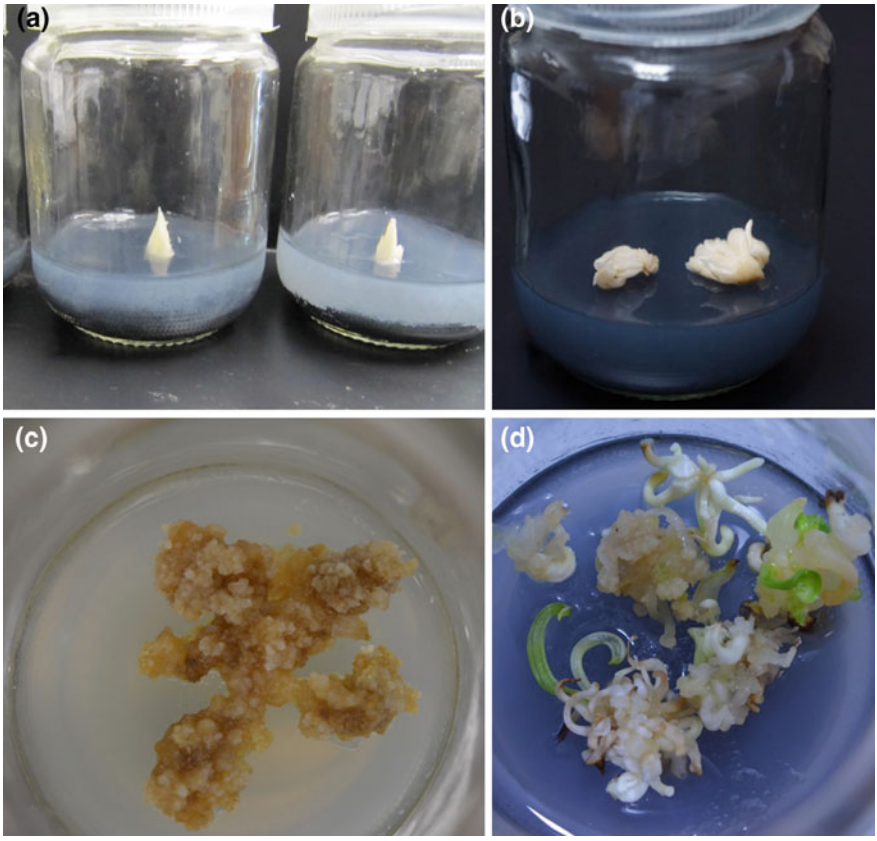

Fig. 19.2 Date palm callus induction and somatic embryogenesis. **a** Newly cultured explant, **b** Explant swelling and callus initiation, **c** Embryogenic callus multiplication and maintenance culture, **d** Somatic embryos development on hormone-free medium

3. Incubate cultures as described in Table 19.2. Note that Table 19.2 also describes the duration and conditions for each culture stage. Despite the addition of activated charcoal to the medium, browning often persists in date palm in vitro cultures. Frequent transferring to a fresh medium at 3-wk intervals is recommended throughout the system to reduce accumulation of phenolic compounds.
4. Transfer entire explants to callus induction medium (Table 19.2). Although, the explant would significantly enlarge, avoid sectioning the explants at this point to prevent re-exudation of phenolics. Light incubate these cultures these cultures at 23 ± 2 °C and 16-h photoperiods (50 μmol·m^{-2}·s^{-1}) provided by cool-fluorescent lamps. This stimulates greening of explants and callus formation becomes obvious (Fig. 19.2b).
5. Again, transfer entire explants with the attached callus to callus proliferation medium (Table 19.2). More callus growth occurs that can be easily separated from the original explants. Portions of explants tend to turn brown to black during this stage; however, upon maintaining, these explants often produce whitish spots of embryogenic callus growth on their surfaces. The callus spots can be cut and placed directly on the culture medium.
6. Separate callus growth from explants and transfer to callus multiplication medium (Table 19.2). Note that date palm callus cultures can be maintained under these conditions for over 2–3 years; however, it is recommended to start new cultures yearly to avoid potential spontaneous mutation and loss of embryogenic capacity. Nonetheless, the lengthy duration necessary to reach this stage, long-term maintenance of viable callus cultures for future experiments and embryo production is a feasible procedure. These callus multiplication cultures are suitable for establishment of cell suspension as described below.

19.2.4 Regeneration of Somatic Embryos

1. Transfer callus from callus multiplication cultures (Fig. 19.2c) to hormone-free MS medium (Table 19.2) to encourage the development, maturation, and germination of somatic embryos.
2. After 3–6 wk, globular embryos begin to appear which subsequently elongate forming bipolar-shaped embryos and eventually germinate on the same medium forming shoots only (Fig. 19.2d).
3. Significant increases in callus weight, expedited development of somatic embryos and higher numbers of resultant embryos can be obtained by augmenting the culture medium with organic additives, such as 10–15% v/v coconut water (Al-Khayri 2010) and 0.5–1 g l^{-1} yeast extract or casein hydrolysate (Al-Khayri 2011).

19.2.5 Cell Suspension Culture

1. An alternative route for inducing somatic embryogenesis is to use cell suspension culture which provides a faster rate of callus growth and somatic embryo formation (Al-Khayri 2012; Naik and Al-Khayri 2016). Callus from callus multiplication cultures (described above) is used to establish cell suspension cultures. Inoculate 1 g of macerated callus into 150-ml culture flask containing 50 ml liquid medium. For cell culture establishment use callus multiplication medium (Table 19.2) but without agar. Cap the culture flasks with double layers of aluminum foil.
2. Incubate callus multiplication cultures in the dark at 23 ± 2 °C on an orbital shaker set to 100 rpm (Fig. 19.3a).
3. During the first 2 wk of cell suspension culture, replace the medium at 3–5 day intervals to overcome browning that often occurs during initial weeks of culture.
4. Maintain cultures by decanting half of the liquid medium and adding an equal volume of liquid medium. After 2–3 months, each culture can be divided to establish two new suspension cultures (Fig. 19.3b). After dividing, add an equal volume of fresh liquid medium. To induce somatic embryogenesis, embryos development, decant the callus multiplication liquid medium and replace with hormone-free liquid medium. Adding 50–100 μM silver nitrate to this medium encourages synchronized development of the somatic embryos (Al-Khayri and Al-Bahrany 2001, 2004; Alwael et al. 2017).
5. Incubate the these cultures at 23 ± 2 °C on an orbital shaker set to 100 rpm and 16-h photoperiods (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) provided by cool-fluorescent lamps. Somatic embryos form and mature within 2 months (Fig. 19.3c). Transfer the resultant embryos to solidified hormone-free medium for germination.
6. Transfer the germinating somatic embryos to solidified rooting medium (Table 19.2) and then follow the same procedures described for acclimatization and plant establishment.

19.2.6 *Acclimatization and* Ex Vitro *Establishment*

1. Transfer germinating embryos to rooting medium (Table 19.2). This stimulates root induction and shoot elongation forming complete plantlets (Fig. 19.4a). Maintaining these plantlets in vitro until they reach 8–10 cm long increases their survival rate ex vitro.
2. Somatic embryo germination and rooting can be achieved in a single step by culturing 1–2-cm long embryos on half-strength MS medium containing 0.2–0.4 mg l^{-1} indole-3-butyric acid (IBA). This approach accelerates the process of obtaining whole plantlets in a shorter time, an attractive criterion for commercial micropropagation (Al-Khayri 2003).

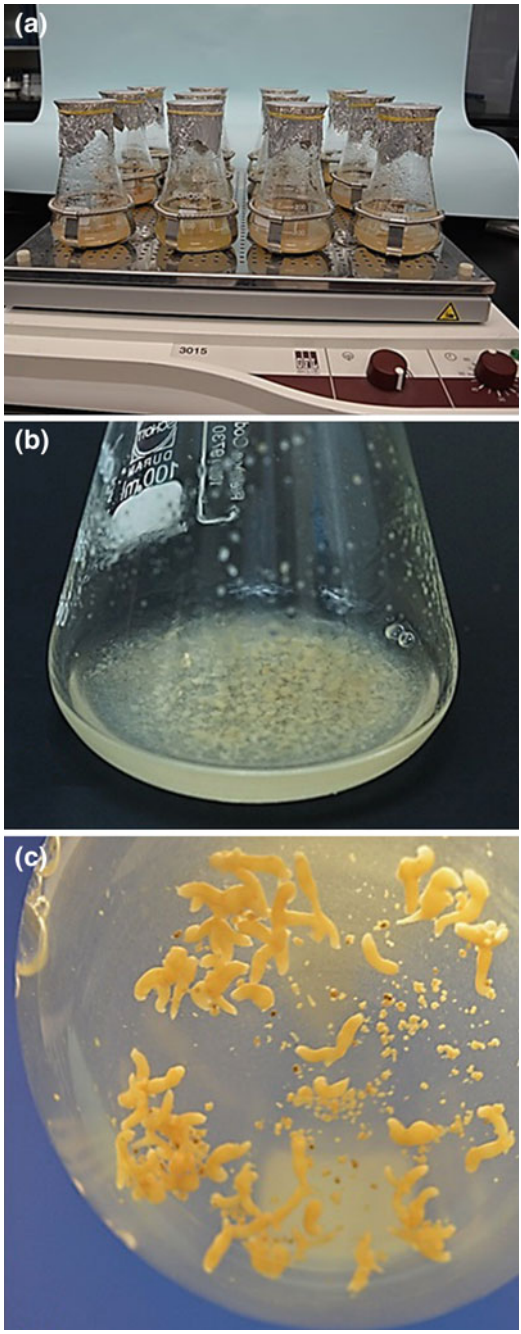

Fig. 19.3 Somatic embryogenesis of date palm from cell suspension. **a** Culture flasks incubated on an orbital shaker, **b** Cell suspension culture in multiplication liquid medium containing plant growth regulators, **c** Somatic embryo formation in a hormone-free liquid medium

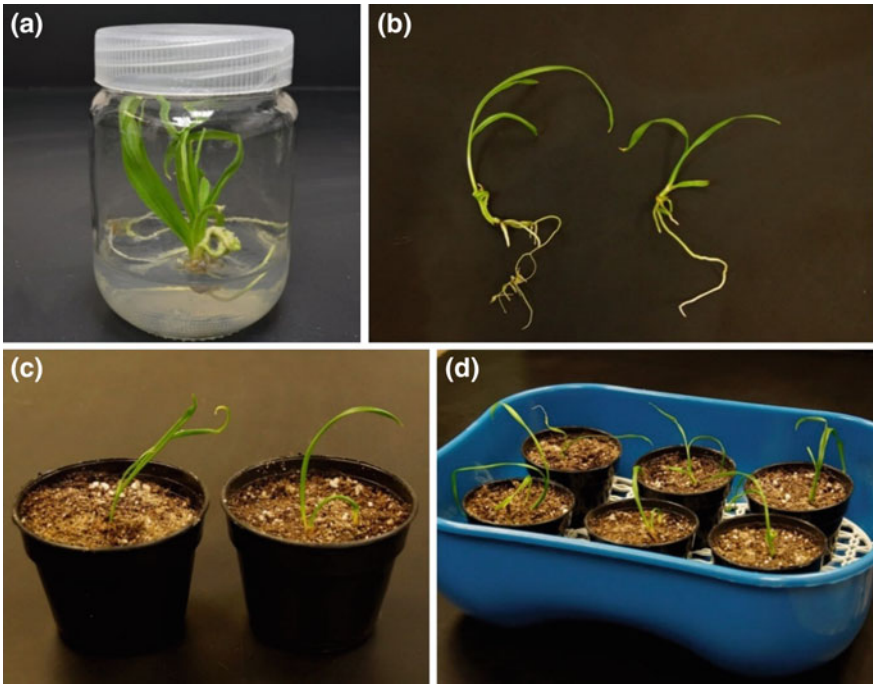

Fig. 19.4 Date palm in vitro plant establishment. **a** In vitro plantlets on rooting medium, **b** Complete plantlets ready for hardening, **c** Ex vitro plantlets transferred to potting mixture, **d** Plantlets in the acclimatization container

3. Using forceps, carefully remove the rooted plantlets from culture vessel and gently rinse under a slow stream of water to remove residual agar residues adhering to the roots (Fig. 19.4b).
4. Place the plantlets upright in a beaker containing enough water to submerge the roots and cover with a transparent plastic bag for 3d. This pre-acclimatization step enhances survival rate.
5. Immerse the plantlets in a fungicide solution, 500 mg l^{-1} Benlate, and plant in 5-cm plastic pots containing potting mixture (1 soil: 1 peat moss: 1 vermiculite) (Fig. 19.4c). Water the transplants with 100 mg l^{-1} N-P-K fertilizer (20-20-20) and subsequently as needed. During the process of soil transfer, occasionally mist the plantlets with water to prevent desiccation.
6. Keep potted plantlets inside clear plastic enclosures under culture room conditions for 3 wk during which gradually reduce humidity by adjusting air flow in the plastic enclosures (Fig. 19.4d). Transfer the transplants to a shaded area of a greenhouse. Alternatively, the plantlets can be maintained under a shaded plastic tent in a greenhouse immediately after transplanting to soil. The key for successful acclimatization is the gradual exposure of the plantlets to ambient conditions by gradually uncovering the plastic enclosure over a period of 3 wk.

Note that in the event the plantlets show signs of water stress, immediately mist with water and close the acclimatization enclosures. After 2 days try again to gradually expose the plantlets to the ambient relative humidity. Date palm growth vigor varies among cultivars. After 3–6 months, and subsequently as often as necessary, transfer the plantlets to larger pots and maintain in a greenhouse. Transfer the plants to a shade house and maintain for 12–24 months before field planting.

19.3 Research Prospects

Despite the socio-economic value of date palm, in many parts of the world the natural habitat has been under destruction because of the increasing needs for housing and other services for the rapidly growing human population in the date palm natural oases. There is dire needs for germplasm conservation efforts and the establishment a gene bank as many of date palm cultivars are no longer maintained by farmers in pursue of cultivating consumer-desired cultivars. On the other hand, large date palm orchards are being established in new areas requiring large numbers of uniformed offshoots which can be a limiting factor because of limited availability and high prices. Optimizing the procedures and the expenses involved in date palm tissue culture is essential to contribute to meeting this demand. Recently, date palm has wittiness an increasing interest of both the research institutions as well as the private sector. A number of date palm micropropagation laboratories have been established. However, the price of the tissue culture-derived plants still expensive due to the high production cost and limited competition. Most date palm countries import the needed expensive chemicals aggravated by taxes and importer profits. Research is needed to identifying alternatives for the composition of the culture medium and replacement with natural substances and less expensive alternative components. Research in automation and bioreactor technologies have not been yet exploited in date palm. Ways to expedite the existing micropropagation protocols are needed as the currently available long tissue culture cycle contributes greatly to production cost. Commercial production of synthetic seeds may offer a way to reduce production cost per plant. Utilizing biotechnological approaches such as somaclonal variation and mutagenesis, somatic hybridization, as well as genetic transformation for trait improvement are important in modern breeding. Research in these areas is extremely scarce; at least in part, due to technical limitations in countries where date palm is of importance. Biotechnological approaches are essential to overcome the devastating current threats imposed by the red palm weevil (*Rhynchophorus ferrugineus* Oliver) and bayoud disease caused by *Fusarium oxysporum* f. sp. *albedinis.* Other challenges that require further research are associated with the impact of the global climate change particularly under the current water limitations.

References

Abahmane L (2011) Date palm micropropagation via organogenesis. In: Jain SM, Al-Khayri JM, Johnson DV (eds) Date palm biotechnology. Springer, Dordrecht, pp 69–89

Abd Rabou AFN, Radwan ES (2017) The current status of the date palm (*Phoenix dactylifera*) and its uses in the Gaza Strip. Palestine Biodiversitas 18(3):1047–1061

Ahmed J, Al-Jasass FM, Siddiq M (2013) Date fruit composition and nutrition. In: Siddiq M, Aleid SM, Kader AA (eds) Dates: postharvest science, processing technology and health benefits. Wiley, Chichester, United Kingdom, pp 261–283

Al-Khayri JM (2001) Optimization of biotin and thiamine requirements for somatic embryogenesis of date palm (*Phoenix dactylifera* L.). In vitro Cell Dev Biol Plant 37:453–456

Al-Khayri JM (2003) *In vitro* germination of somatic embryos in date palm: effect of auxin concentration and strength of MS salts. Curr Sci 84:680–683

Al-Khayri JM (2005) Date palm *Phoenix dactylifera* L. In: Jain SM, Gupta PK (eds) Protocols of somatic embryogenesis in woody plants, forestry science, vol 77. Springer, The Netherlands, pp 309–319

Al-Khayri JM (2007) Date palm *Phoenix dactylifera* L. micropropagation. In: Jain SM, Haggman H (eds) Protocols for micropropagation of woody trees and fruits. Springer, Berlin, pp 509–526

Al-Khayri JM (2010) Somatic embryogenesis of date palm (*Phoenix dactylifera* L.) improved by coconut water. Biotechnology 9:477–484

Al-Khayri JM (2011) Influence of yeast extract and casein hydrolysate on callus multiplication and somatic embryogenesis of date palm (*Phoenix dactylifera* L.). Sci Hortic 130:531–535

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

Al-Khayri JM (2013) Factors affecting somatic embryogenesis in date palm (*Phoenix dactylifera* L.) In: Aslam J, Srivastava PS, Sharma MP (eds) Somatic embryogenesis and genetic transformation in plants. Narosa Publishing House, New Delhi, pp 15–38

Al-Khayri JM, Al-Bahrany AM (2001) Silver nitrate and 2-isopentyladenine promote somatic embryogenesis in date palm (*Phoenix dactylifera* L.). Sci Hortic 89:291–298

Al-Khayri JM, Al-Bahrany AM (2004) Genotype-dependent *in vitro* response of date palm (*Phoenix dactylifera* L.) cultivars to silver nitrate. Sci Hortic 99:153–162

Al-Khayri JM, Jain SM, Johnson DV (eds) (2015a) Date palm genetic resources and utilization, vol 1. Africa and the Americas, Springer, Dordrecht, p 546

Al-Khayri JM, Jain SM, Johnson DV (eds) (2015b) Date palm genetic resources and utilization, vol 2. Asia and Europe, Springer, Dordrecht, p 566

Alwael HA, Naik PM, Al-Khayri JM (2017) Synchronization of somatic embryogenesis in date palm suspension culture using abscisic acid. In: Al-Khayri JM, Jain SM, Johnson DV (eds) Date Palm biotechnology protocols, vol 1, tissue culture applications, methods in molecular biology, vol 1637. Humana Press, New York, pp 215–226

Benbadis AK (1992) Coconut and date palm. In: Hammerschlag FA, Litz RE (eds) Biotechnology of perennial fruit crops. CAB International, Wallingford, pp 383–400

Chao CT, Krueger RR (2007) The date palm (*Phoenix dactylifera* L.): overview of biology, uses, and cultivation. HortScience 42(5):1077–1082

Di Cagno R, Filannino P, Cavoski I, Lanera A, Mamdouh BM, Gobbetti M (2017) Bioprocessing technology to exploit organic palm date (*Phoenix dactylifera* L. cultivar Siwi) fruit as a functional dietary supplement. J. Funct. Food 31:9–19

El-Hadrami A, Al-Khayri JM (2012) Socioeconomic and traditional importance of date palm. Emir J Food Agric 24(5):371–385

El Hadrami A, Daayf F, El Hadrami I (2011) Date palm genetics and breeding. In: Jain SM, Al-Khayri JM, Johnson DV (eds) Date palm biotechnology. Springer, Dordrecht, pp 479–512

Fki L, Masmoudi R, Kriaâ W, Mahjoub A, Sghaier B, Mzid R, Mliki A, Rival A, Drira N (2011) Date palm micropropagation via somatic embryogenesis. In: Jain SM, Al-Khayri JM, Johnson DV (eds) Date palm biotechnology. Springer, Dordrecht, pp 47–68

Ghorbani M, Ahmadi F, Rajaee Rad A, Zamiri M, Cone J, Polikarpov I (2017) The effect of lime pre-treatments of date palm leaves on delignification and *in vitro* rumen degradability. J Agric Sci 155(1):184–190

Johnson DV (2016) Unusual date palm products: prayer beads, walking sticks and fishing boats. Emir J Food Agric 28(1):12–16

Johnson DV, Al-Khayri JM, Jain SM (2013) Seedling date palms (*Phoenix dactylifera* L.) as genetic resources. Emir J Food Agric 25(11):809–830

Mazri MA, Meziani R (2015) Micropropagation of date palm: a review. Cell Dev Biol 4:160. https://doi.org/10.4172/2168-9296.1000160

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Naik PM, Al-Khayri JM (2016) Somatic embryogenesis of date palm (*Phoenix dactylifera* L.) through cell suspension culture. In: Jain SM (ed) Protocols for *in vitro* cultures and secondary metabolite analysis of aromatic and medicinal plants, 2nd edn., Methods in Molecular Biology, vol 1391. Humana Press, New York, pp. 357–366

Omar MS, Hameed MK, Al-Rawi MS (1992) Micropropagation of date palm (*Phoenix dactylifera* L.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, protoplast and genetic engineering, vol 18. Springer-Verlag, Berlin, pp 471–492

Omran R, Al-taee Z, Hashim H, Al-Jassani M (2017) Preventive effects of *Phoenix dactylifera* polyphenols against 7,12-dimethylbenz(a)anthracene-induced mammary cancer. Asian J Pharm Clin Res 10(7):172–181

Rajaee Rad A, Ahmadi F, Mohammadabadi T, Ziaee E, Polikarpov I (2015) Combination of sodium hydroxide and lime as a pretreatment for conversion of date palm leaves into a promising ruminant feed: an optimization approach. Waste Biomass Valor 6:243–252

Selmani C, Chabane D, Bouguedoura N (2017) Ethnobotanical survey of *Phoenix dactylifera* L. pollen used for the treatment of infertility problems in Algerian oases. Afr J Tradit Complement Altern Med 14(3):175–186

Tisserat B (1984) Date palm. In: Sharp WR, Evans DA, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture, vol 2. Macmillan Publishing Company, New York, pp 505–545

Vayalil PK (2012) Date fruits (*Phoenix dactylifera* Linn): an emerging medicinal food. Crit Rev Food Sci Nutr 52(3):249–271

Zaid A, El-Korchi B, Visser HJ (2011) Commercial date palm tissue culture procedures and facility establishment. In: Jain SM, Al-Khayri JM, Johnson DV (eds) Date palm biotechnology. Springer, Dordrecht, pp 137–180

Chapter 20
Tea (*Camellia sinensis* L.)

Behzad Kaviani

20.1 Introduction

Tea (*Camellia sinensis* L.) is an important species; especially its leaves are used in medicinal and pharmaceutical industries, also as a drink everywhere around the world. It is proven that biomolecules like flavonoids and antioxidants in tea possess medicinal properties (Ghanati and Ragmati Ishka 2009).

Somatic embryogenesis is a proper and efficient method for micropropagation of important crops and has the potential of commercial mass propagation and plant regeneration allowing for rapid production of a large number of healthy plantlets within a short period. This method offers advantages in improving species over other in vitro propagation methods. Somatic embryogenesis acts as a key component of in vitro propagation and provides a precious implement to boost the pace of genetic enhancement of commercial crop species (Stasolla and Yeung 2003). Plant regeneration through embryogenesis often gave variant plants (Diettrich et al. 1991). True-to-type clonal fidelity is one of the most important pre-requisites in the in vitro propagation of crop species (Faisal et al. 2012). Somatic embryogenesis is considered profitable over other in vitro propagation systems as it curtails the proliferation time duration and proves to be potential as an efficient regeneration system with high genetic integrity (Kothari et al. 2010). Somatic embryogenesis is a proper morphogenetic system for investigating the cellular and molecular process, also growth and differentiation (Benelli et al. 2001). In addition, this approach also provides the possibility to produce artificial seeds and valuable tools for genetic engineering and germplasm storage by cold preservation and cryopreservation (Litz and Gray 1995; Merkle 1997). Somatic embryogenesis as a suitable method of micropropagation offers many advantages as compared to the conventional methods

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_20

such as mass propagation, lower probability of genetic variation, production of synthetic (artificial) seeds for short-, medium- and long-term storage and valuable tools for biochemical studies, genetic engineering and germplasm conservation by cryopreservation (Wang and Bhalla 2004; Yaacob et al. 2012).

Somatic embryogenesis is carried out as direct and indirect. Direct somatic embryogenesis has a lower probability of genetic variation than indirect somatic embryogenesis and other propagation methods (Merkle 1997) and is the most desirable approach because of genetic stability of regenerated plantlets (Pedroso and Pais 1995). Induced somatic embryogenesis from callus (indirect somatic embryogenesis) (often in suspension cultures) can result in variation among seedlings. Direct somatic embryogenesis is generally rare compared with indirect somatic embryogenesis. More studies are focused on indirect somatic embryogenesis using vegetative organs particularly leaf and nodal segments (Akula et al. 2000; Mondal et al. 2001).

Auxins especially 2,4-D play the most important role in induction of somatic embryogenesis (Pierik 1987; Jain and Ochatt 2010). There are some studies on induction of somatic embryos using cytokinins like TDZ and BAP (Mondal et al. 1998). Many studies on in vitro somatic embryos regeneration have involved the cultures of excised sexual tissues (Germana and Chiancone 2003). Study on tea micropropagation has recently concentrated on somatic embryogenesis as a more efficient means of plant manipulation and regeneration (Begum et al. 2015). Reports on tea tissue culture including somatic embryogenesis are available, however, rapid multiplication is very poor as the plant is relatively recalcitrant (Akula et al. 2000; Mondal et al. 2001; Suganthi et al. 2012). Thus, the present study was carried out to obtain direct somatic embryos from embryonic axes explants of tea (*Camellia sinensis* L.) cultured in media containing 2,4-D and BAP.

20.2 Materials

1. Tea ripe capsules and seeds
2. Laminar-flow hood with ultraviolet light, Petri dishes, beaker, forceps, scalpels, micropipettes and test tubes
3. Ethanol, Peroxide hydrogen (H_2O_2), Sodium hypochlorite (NaClO), sterile distilled water, 125 ml Erlenmeyer flasks
4. 2,4-dichlorophenoxyacetic acid (2,4-D), 6-Benzyl aminopurine (BAP), tissue culture agar and sucrose
5. Casein hydrolysate and mannitol
6. Tissue culture chamber
7. Media (see Tables 20.1 and 20.2).

Basal MS (Murashige and Skoog 1962) medium composition is listed in Table 20.1. Required modifications for different culture stages are listed in Table 20.2. The pH is adjusted to 5.7 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 20 min. Pour 30 ml medium to 100 ml test tubes.

Table 20.1 Tea basic culture medium (MS medium)

Chemicals	Quantity (mg/l)	Chemicals	Quantity (mg/l)
NH_4NO_3	1650	$CuSO_4 \cdot 5H_2O$	0.025
KNO_3	1900	$CoCl_2 \cdot 6H_2O$	0.025
$CaCl_2 \cdot 2H_2O$	440	$FeSO_4 \cdot 7H_2O$	27.8
$MgSO_4 \cdot 7H_2O$	370	$Na_2EDTA \cdot 2H_2O$	37.3
KH_2PO_4	170	Myo-inositol	100
KI	0.83	Nicotinic acid	0.5
H_3BO_3	6.2	Pyridoxin HCl	0.5
$MnSO_4 \cdot 4H_2O$	22.3	Thiamine HCl	0.5
$ZnSO_4 \cdot 7H_2O$	8.6	Glycine	2
$Na_2MoO_4 \cdot 2H_2O$	0.25	pH	5.7

The composition of basal MS medium was similar at initiation, development, maturation and germination stages. The pH was adjusted to 5.7 with 1 N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 20 min

Table 20.2 Formulations of tea media

Chemicals	Stage I: initiation	Stage II: development	Stage III: maturation	Stage IV: germination
Myo-Inositol	100[a]	100	100	100
Mannitol	30,000	30,000	3000	–
Casein hydrolysate	200	200	200	–
Sucrose	30,000	30,000	3000	3000
2,4-D	1	1	1	–
Agar-agar	7000	7000	7000	7000

[a]All units are in mg/l except for 2,4-D which is in μM
The pH of all media are adjusted to 5.7

20.3 Method

Somatic embryogenesis includes five steps: (1) embryo initiation, (2) embryo maintenance, (3) embryo development, (4) maturation, and (5) embryo germination and acclimatization. In this protocol, these five steps were carried out in two culture media during four months. In first medium, embryo initiation and production, and in second medium, embryo maturation and germination was done.

20.3.1 Embryo Initiation and Production

Collect mature capsules in September–October from tea fields. Keep capsules with seeds in a dry place for 1–2 months before use.

1. Remove the seeds from the mature capsules.
2. Remove the seed coat.
3. Rinse the seeds without coat under tap water and agitate for 20 min.
4. Treat the seeds with 96% v/v ethanol for 2 min.
5. Wash the seeds 3 times with sterile distilled water, 3 min each time.
6. Sterilize the seeds with 10% (v/v) H_2O_2 and shake for 10 min followed by 15% (w/v) NaClO for 15 min.
7. Rinse the sterilized seeds 3 times with sterile distilled water in the laminar-flow hood, 3 min each time.
8. Put the seeds in a beaker containing sterile distilled water in the laminar-flow hood for 3–4 h.
9. Transfer the seeds into a Petri dish.
10. Remove the thin coat with sterile forceps and aseptically isolate embryonic axes from the cotyledons with scalpel.
11. Place the isolated embryonic axes on the surface of MS solidified medium in test tubes containing 0, 1 and 5 μM 2,4-D (Fig. 20.1a).
12. Incubate the embryos in a growth chamber set for 16-h photoperiod, a light flux of 50 μmol $m^{-2}s^{-1}$ and day/night temperatures of 25/20 °C.
13. Callus is formed on the surface of embryonic axes after 2 months on medium enriched with 5 μM 2,4-D and remained constant in this statement after 4 months (Fig. 20.1b).

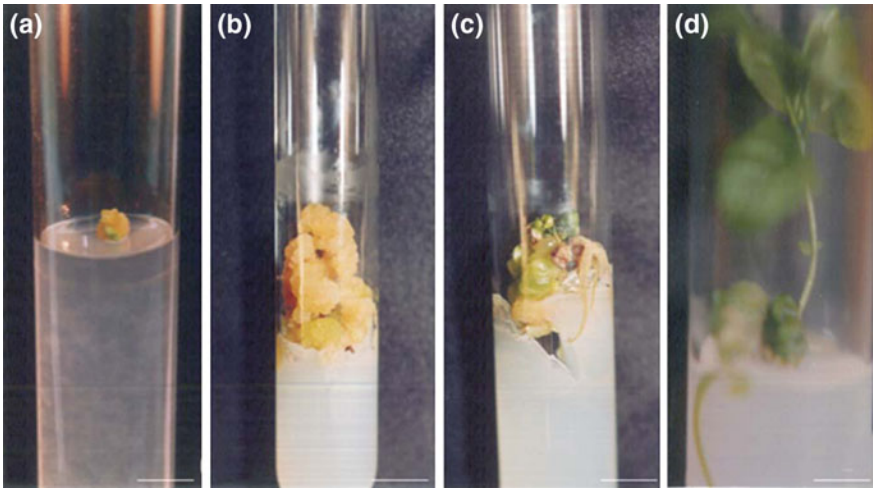

Fig. 20.1 The process of callus induction, direct somatic embryogenesis and plant regeneration from embryonic axes explants of tea (*Camellia sinensis* L.). **a** The start of callus formation on the explant in MS medium containing 2,4-D; **b** mass callus formation in medium supplemented with 5 μM 2,4-D; **c** induction of direct somatic embryogenesis on the explant cultured in MS medium enriched with 1 μM 2,4-D and **d** plantlet regeneration from somatic embryos (scale bar = 10 mm)

14. After 2–3 months of incubation of explants on the medium containing 1 μM 2,4-D, somatic embryos emerged around embryonic axes explants without apparent callus formation (Fig. 20.1c).
15. No callus and embryos was formed on explants cultured on medium without 2,4-D and with 5 μM BAP.

20.3.2 Embryo Maturation and Germination

To obtain the maturation and germination of tea somatic embryos, these embryos need to be transferred to a medium without plant growth regulators. Germination of these embryos is stimulated in this medium.

1. Prepare a basal MS medium without plant growth regulators, casein hydrolysate and mannitol and pour them into the test tubes.
2. Dissect somatic embryos formed on MS medium supplemented with 1 μM 2,4-D with a sterilized sharp razor blade carefully.
3. Transfer each somatic embryo into each test tube (Fig. 20.1d).
4. Incubate the embryos in a growth chamber set for 16-h photoperiod, a light flux of 50 μmol $m^{-2}s^{-1}$ and day/night temperatures of 25/20 °C.

20.3.3 Acclimatization and Field Transfer

1. Prepare some small plastic pots and fill them with garden soil, compost and sand in the ratio of 2:1:1.
2. The morphologically normal plantlets with both shoots and roots that develop from somatic embryos are transferred to these plastic pots located in a greenhouse.
3. For acclimatization, plantlets are covered with polythene sheet to maintain humidity for one week. These plantlets were exposed to natural environment for one hour daily and then again placed in greenhouse for another week.
4. After 60 days, the plants are transplanted to soil in the field. Forty five days after planting, the survival rate of regenerated plants can be determined.
5. Following in vitro culture, regenerated plantlets need a gradual decrease in relative humidity to acclimatize to greenhouse conditions prior to planting in the field. Without acclimatization, plantlets often fail to form functional organs.

20.4 Identify Steps Required Further Protocol Modifications

Induction of somatic embryos and plant regeneration via somatic embryogenesis in tea (*Camellia sinensis* L.), the most popular beverage in the world, is described in this protocol. However, more studies are required. These studies focus on improvement of

all five steps of somatic embryogenesis in tea, synthetic seed production, genetic manipulation and secondary metabolites production. The use of other auxins and cytokinins, singularly or in combination, other explants, also other culture media such as woody plants medium (WPM) in somatic embryogenesis of tea is proposed.

References

Akula A, Becker D, Bateson M (2000) High-yielding repetitive somatic embryogenesis and plant recovery in a selected tea clone, 'TRI-2025', by temporary immersion. Plant Cell Rep 19 (12):1140–1145

Begum A, Ahmad I, Prodhan SH, Azad AK, Sikder MBH, Ara MR (2015) Study on *in vitro* propagation of tea [*Camellia sinensis* (L.) O. Kuntze] through different explants. J Glob Biosci 4(7):2878–2887

Benelli C, Fabbri A, Grassi S, Lambardi M, Rugini E (2001) Histology of somatic embryogenesis in mature tissue of olive (*Olea europaea* L.). J Hortic Sci Biotechnol 76:112–119

Diettrich B, Schneider V, Luckner M (1991) High variation in cardenolide content of plants regenerated from protoplasts of the embryogenic cell strain VII of Digitalis lanata. J Plant Physiol 139:199–204

Faisal M, Alatar A, Ahmad N, Anis M, Hegazy AK (2012) Assessment of genetic fidelity in Rauvolfia serpentina plantlets grown from synthetic (encapsulated) seeds following in vitro storage at 4 °C. Mol 17:5050–5061

Germana MA, Chiancone B (2003) Improvement of Citrus clementine Hort. ex Tan. Microspore-derived embryoid production and regeneration. Plant Cell Rep 22:181–187

Ghanati F, Ragmati Ishka M (2009) Investigation of the interaction between abscisic acid (ABA) and excess benzy-adenine (BA) on the formation of shoot in tissue culture of tea (*Camellia sinensis* L.). J Plant Prod 3(4):735–743

Jain SM, Ochatt SJ (2010) Protocols for *in vitro* propagation of ornamental plants. Humana Press, Springer Protocols

Kothari SL, Joshi A, Kachhwaha S, Ochoa-Alejo N (2010) Chilli peppers—a review on tissue culture and transgenesis. Biotech Adv 28:35–48

Litz RE, Gray DJ (1995) Somatic embryogenesis for agricultural improvement. World J Microbiol Biotechnol 11:416–425

Merkle SA (1997) Somatic embryogenesis in ornamentals. In: Geneve RL et al (eds) Biotechnol ornam plants. CAB International, Wallingford, pp 13–33

Mondal TK, Bhattacharya A, Sood A, Ahuja PS (1998) Micropropagation of tea (*Camellia sinensis* (L.) O. Kuntze) using thidiazuron. Plant Growth Reg 26:57–61

Mondal TK, Bhattacharya A, Ahuja PS (2001) Induction of synchronous secondary somatic embryogenesis in *Camellia sinensis* (L.) O. Kuntze. J Plant Physiol 158(7):945–951

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:73–497

Pedroso CM, Pais MS (1995) Factors controlling somatic embryogenesis. Plant Cell Tiss Org Cult 43:147–154

Pierik RLM (1987) *In vitro* propagation of higher plants. Martinus Nizhoof Publishers, Boston

Stasolla C, Yeung EC (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tiss Org Cult 74:15–35

Suganthi M, Arvinth S, Raj Kumar R (2012) Impact of osmotic and abscisic acid on direct somatic embryogenesis in tea. Int J Plant Res 2(2):22–27

Wang YH, Bhalla PL (2004) Somatic embryogenesis from leaf explants of Australian fan flower, *Scaevola aemula* R. Br Plant Cell Rep 22:408–414

Yaacob JS, Yussof AIM, Taha RM, Mohajer S (2012) Somatic embryogenesis and plant regeneration from bulb, leaf and root explants of African blue lily (Agapanthus praecox ssp. Minimus). Aus J Crop Sci 6(10):1462–1470

Chapter 21
Protocol for Somatic Embryogenesis in *Passiflora cincinnata* Mast. (Passifloraceae)

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21.1 Introduction

The Passifloraceae family comprises around 700 species, including the 520+ species that belong to *Passiflora* L., which is the largest genus in this family and presents a broad distribution over tropical and subtropical regions (Ulmer and MacDougal 2004; Simpson 2010). These species have a recognized economic importance for the food, pharmaceutical, and cosmetic industries due to the nutritional value of their fruits as well as to the medicinal properties and ornamental value of their flowers, the latter have historically attracted people by their impressive and exotic array of colours and forms (Dhawan et al. 2004; Ulmer and MacDougal 2004; Zerbini et al. 2008; Yockteng et al. 2011; Tiwari et al. 2016, Chitwood and Otoni 2017). Those aspects have inspired and motivated research groups worldwide to establish biotechnology tools and in vitro regeneration systems for different *Passiflora* species, allowing the sophistication of cloning practices as well as their large-scale multiplication and the genetic manipulation.

Tissue culture in the genus *Passiflora* was pioneered by Nakayama (1966) by culturing stem segments of mature *Passiflora caerulea* plants. Since then, much progress has been made on in vitro morphogenesis, germplasm preservation, polyploidization, genetic transformation, micrografting, and *in vitro* propagation of *Passiflora* species (Otoni et al. 1995, 2007, 2013; Vieira and Carneiro 2004;

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https://doi.org/10.1007/978-3-319-79087-9_21

Trevisan et al. 2006; Zerbini et al. 2008; Rêgo et al. 2011; Ozarowski and Thiem 2013; Ribeiro et al. 2015; Rocha et al. 2015; Pacheco et al. 2016; Faria et al. 2018), to mention a few.

According to Xu and Huang (2014), higher plants show three main types of regeneration systems: tissue regeneration, de novo organogenesis, and somatic embryogenesis. Somatic embryogenesis refers to the developmental process through which a single somatic cell or a group of somatic cells is reprogrammed and grow into embryos under suitable conditions. These somatic embryos can differentiate into whole plants by passing through the typical stages of zygotic embryogenesis (Zimmerman 1993; Yang and Zhang 2010; Rocha and Dornelas 2013; Smertenko and Bozhkov 2014). The formation of somatic embryos can occur directly from explant tissues, in a process known as direct embryogenesis, or indirectly through the establishment of a callus before the somatic embryo formation, i.e., indirect embryogenesis (Yang and Zhang 2010; Rocha and Dornelas 2013; Rocha et al. 2018).

Somatic embryogenesis has become an essential asset as it enables plant regeneration and large-scale propagation. Furthermore, it provides the means for understanding molecular and biochemical events that occur during plant embryogenesis (Rocha and Dornelas 2013; Rocha et al. 2018). Numerous studies have focused on unravelling molecular, anatomical, and histochemical aspects of somatic embryogenesis in *Passiflora*. As a result, *PeSERK1* gene was suggested to play a role in *Passiflora* somatic embryogenesis, apparently associated with differentiation processes and the maintenance of a cellular-competent state (Rocha et al. 2016). Rocha et al. (2012) characterized cellular changes during somatic embryogenesis and described anatomical and ultrastructural changes involved in the acquisition of embryogenic competence and embryo differentiation in *P. cincinnata.*

Despite the economic and ecological importance of *Passiflora* species, somatic embryogenesis studies are still limited (Ferreira et al. 2015). However, there has been a considerable effort by research groups to provide an approach as close as possible to the state of art in somatic embryogenesis in *Passiflora*. Based on embryogenic leaf-derived cultures initiated in picloram-supplemented medium, *P. gibertii* was the first species used to successfully establish somatic embryogenesis (Otoni 1995; Anthony et al. 1999). In another study with three different *P. edulis* genotypes, Paim Pinto et al. (2011) reported the highest somatic embryogenesis frequency in mature somatic embryos with 72.4 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.4 μM 6-benzyladenine (BA). However, no conversion of somatic embryos into plantlets occurred. Rosa et al. (2015) evaluated *in vitro* morphogenic responses of five different *Passiflora* species, namely: *P. alata*, *P. crenata*, *P. edulis*, *P. foetida*, and *P. gibertii.* After cultivating zygotic embryos in MS medium supplemented with 4.5 μM BA and different 2,4-D concentrations, somatic embryos were verified in all species, except for *P. foetida*. Recently, Ferreira et al. (2015) established a protocol for the wild species *P. miniata*

and *P. speciosa*, in which immature zygotic embryos (IZE) were inoculated in 2,4-D-containing and 2,4-D-free media. Interestingly, the percentage of regenerants was superior in the treatment lacking regulator, demonstrating that *P. miniata* and *P. speciosa* IZE possess sufficient levels of endogenous phytohormones to trigger a high rate of indirect somatic embryogenesis.

Our research team has pioneered a reproducible somatic embryogenesis protocol using mature *P. cincinnata* zygotic embryo (Silva et al. 2009). *P. cincinnata* Mast. is a non-commercial perennial vine widely distributed throughout tropical America. Besides its vigorous and rustic growth (Silva et al. 2009), this species has aroused attention for its desirable traits, such as high yield and quality of fruits, disease resistance (Meletti et al. 2005), and genetic variability (Silva et al. 2009). Especially for its reliable levels of in vitro morphogenic responses (Lombardi et al. 2007), *P. cincinnata* stands out as a source of genetic diversity. Therefore, considering the hurdles associated with passion fruit cultivation, these beneficial characteristics can be handled by breeding programs and tissue culture techniques in order to improve commercial cultures. To date, for *P. cincinnata*, there are reports on de novo shoot organogenesis (DNSO) on leaf- (Lombardi et al. 2007), hypocotyl- (Dias et al. 2009, 2010) and root-derived (Lombardi et al. 2007; Silva et al. 2011) explants, protoplast isolation (Dornelas and Vieira 1993), somatic hybridization (Dornelas et al. 1995), somatic embryogenesis, and synthetic seeds (Silva et al. 2009, 2015; Paim Pinto et al. 2010; Rocha et al. 2012; Silva and Carvalho 2014).

Here, we describe a reliable, reproducible protocol in which *P. cincinnata* explants were exposed to a medium supplemented with 2,4-D (18.1 μM/4 mg L^{-1}) and BA (4.44 μM/1.0 mg L^{-1}). Interestingly, when compared to the treatments with regulators, the authors observed the conversion of well-formed somatic embryos in the treatments lacking regulators and also high frequencies of acclimatized plants. The current protocol provides an alternative to previous organogenesis-related procedures. It is based on the induction of somatic embryogenesis from zygotic embryos of *P. cincinnata* on 2,4-D- and BA-enriched medium. The methods described here may be adjusted to regenerate other *Passiflora* species via somatic embryogenesis.

21.2 Protocol for Somatic Embryogenesis in *Passiflora cincinnata* Mast

21.2.1 Culture Media

1. The culture media used for inducing somatic embryos and regenerating plants via somatic embryogenesis in *P. cincinnata* are listed in Table 21.1.
2. The culture conditions and duration of each step of the somatic embryogenesis process in *P. cincinnata* are described in Table 21.2.

Table 21.1 Constituents of Induction Medium (IM), Maturation Medium (MM), and Germination/Elongation Medium (GM) used for *Passiflora cincinnata* plant regeneration via somatic embryogenesis

Constituents	IM (mg L^{-1})	MM (mg L^{-1})	GM (mg L^{-1})
Basal salts			
NH_4NO_3	1650	1650	1650
KNO_3	1900	1900	1900
$CaCl_2.2H_2O$	440	440	440
KH_2PO_4	170	170	170
$MgSO_4.7H_2O$	370	370	370
Na_2EDTA	37.3	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8	27.8
H_3BO_3	6.2	6.2	6.2
$MnSO_4.7H_2O$	16.9	16.9	16.9
$ZnSO_4.7H_2O$	8.6	8.6	8.6
Kl	0.83	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025
$CoCl_2.6H2O$	0.025	0.025	0.025
Modified B5 vitamins			
Thiamine	1000	1000	1000
Pyridoxine	100	100	100
Nicotinic acid	100	100	100
Plant Growth Regulators			
2,4-dichlorophenoxyacetic acid	4	–	–
6-benzyladenine	1	–	–
Gibberellic acid	–	–	0.5
Others			
Sucrose	30,000	30,000	30,000
Myo-inositol	100	100	100
Activated charcoal	–	15,000	15,000
Phytagel	2500	2500	2500

3. All culture media used have their pH values adjusted to 5.7 ± 0.1 and are autoclaved at 121 °C and 1.1 atm for 20 min.
4. Growth regulators (i.e., 2,4-D and BA) and activated charcoal are added to the media and autoclaved.
5. Gibberellic acid (GA_3) is filter-sterilized and added to the media after autoclaving.

Table 21.2 Culture conditions for plant regeneration via somatic embryos in *Passiflora cincinnata*

Media[a]	Culture Conditions	Duration
IM	60 cm × 15 cm plates with 15 mL of medium in the absence of light at 27 ± 2 °C	30 days
MM	90 cm × 15 cm plates with 30 mL of medium maintained under photoperiod of 16/8 h at 27 ± 2 °C	30 days
GM	90 cm × 15 cm plates maintained under photoperiod of 16/8 h at 27 ± 2 °C	15 days

[a]Culture media: *IM* (Induction Medium), *MM* (Maturation Medium), *GM* (Germination/Elongation Medium)

21.2.2 Seed Extraction

1. *P. cincinnata* seeds are obtained from mature fruits from the open pollinated orchard.
2. After pulp extraction, the seeds are washed in running water and have the aryl removed manually. Seeds are then placed in trays with absorbent paper and dried in the shade at room temperature for 3 days.

21.2.3 Seed Surface Disinfection

1. Seed coat is removed with the aid of a mini-vise (Fig. 21.1I).
2. In a laminar flow hood and after coat extraction, seeds are surface-sterilized by immersion in 70% (v/v) ethanol for 1 min, followed by immersion in 2.5% (v/v) commercial sodium hypochlorite added by three Tween 20 drops per 100 mL of solution as well as five washings in autoclaved distilled water (Fig. 21.1II).
3. After disinfestation, seeds remain immersed in autoclaved distilled water for about 12 h for rehydration and zygote embryo excision.

21.2.4 Initiation of the Embryogenic Culture

1. Zygotic embryos used as explants are removed in a laminar flow hood with the aid of tweezers and scalpel.
2. With tweezers, seeds are horizontally fixed and, with the scalpel, seeds are sectioned on the opposite side of the embryo. With the scalpel, the embryonic axes are removed (embryo and cotyledons) (Figs. 21.1III and 21.2a).
3. Approximately ten zygotic embryos are arranged horizontally in 60 cm × 15 cm plates containing 15 mL of induction medium (Fig. 21.1III). The plates are sealed with non-woven viscose rayon tape with hypoallergenic acrylic adhesive (Nexcare MICROPORE® 3M, Brazil) and incubated under the conditions described in Table 21.2.
4. Zygote embryos are evaluated at 10-day intervals for the presence of embryogenic areas and somatic embryos (Fig. 21.2b, c).

21.2.5 Differentiation/Maturation of Somatic Embryos

1. After the induction phase, the calli obtained in the IM are transferred to the maturation medium (MM).

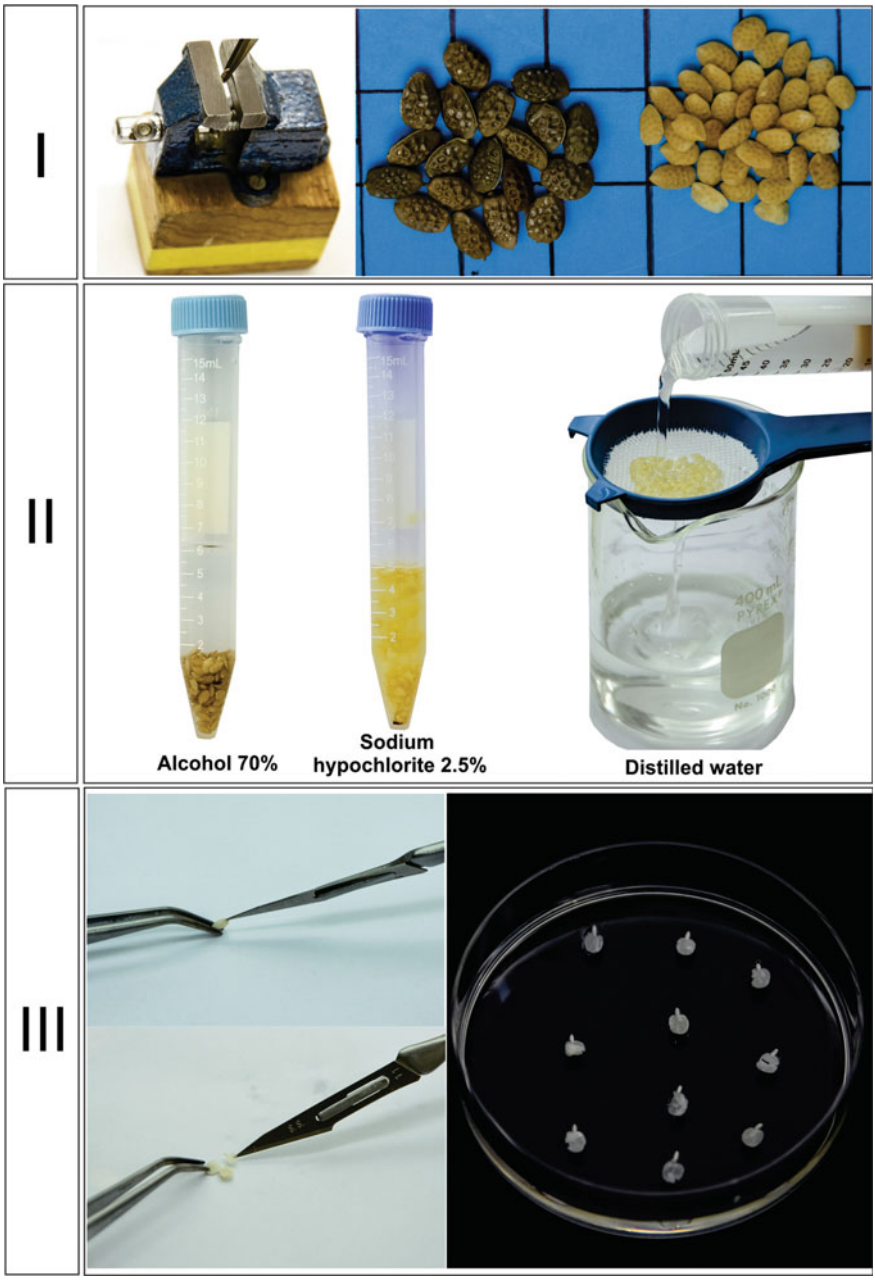

Fig. 21.1 Procedure for seed surface-sterilization and zygotic embryo excision from mature *Passiflora cincinnata* Mast seeds

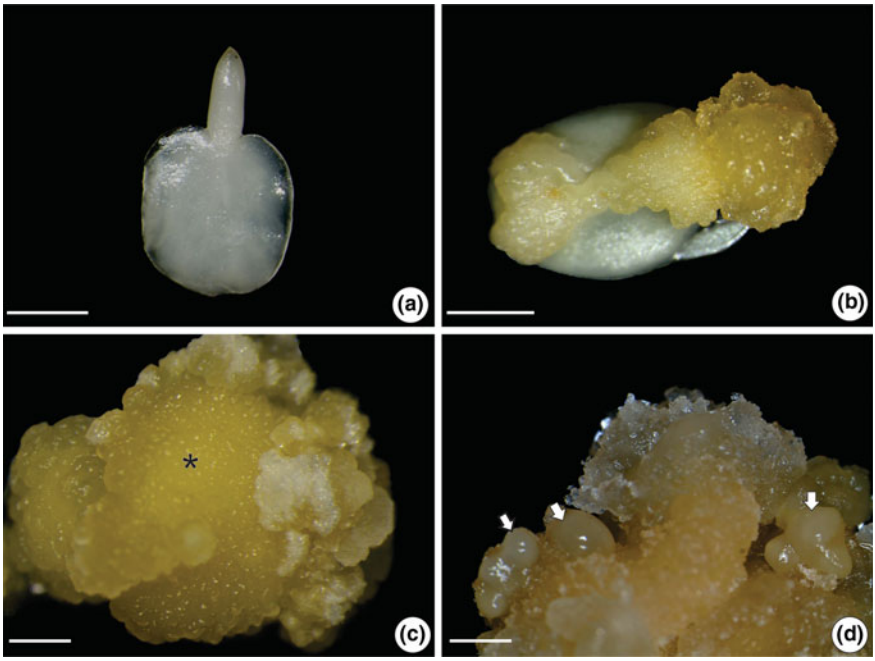

Fig. 21.2 Induction of *Passiflora cincinnata* somatic embryos. **a** Zygote embryo used as initial explant; **b** Callogenesis after 10 days in the induction medium (IM); **c** Embryogenic callus after 20 days in IM (*); **d** Somatic embryos formed after 30 days in IM (white arrows). Bars = 1 mm

2. With the aid of tweezers, calli presenting embryogenic regions are transferred to 90 cm × 15 cm plates with 30 mL of MM. The selected embryogenic calli have characteristics of friable and yellowish-brown cells (Fig. 21.2d).
3. Plates are sealed with polyvinylchloride film strips (Goodyear, São Paulo, Brazil) and incubated under the conditions described in Table 21.2.

21.2.6 Germination and Elongation of Somatic Embryos

1. After 30 days in the MM (Fig. 21.3a, b), somatic embryos in cotyledonary stage (Fig. 21.3c) are individually transferred to plates containing germination/elongation medium.
2. The germination/elongation medium is the same as that for maturation, except for the addition of 1.45 μM GA_3. The culture conditions are described in Table 21.2.

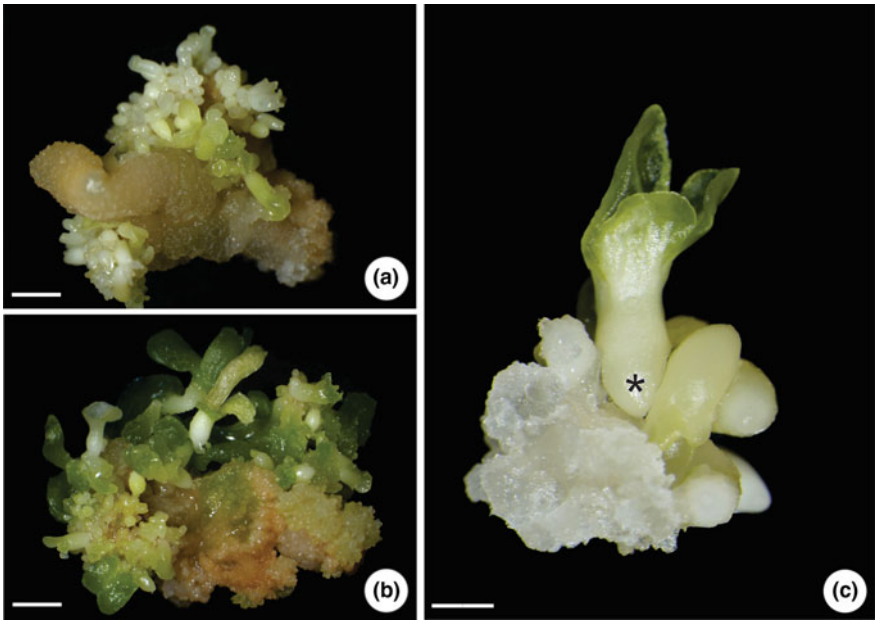

Fig. 21.3 *Passiflora cincinnata* somatic embryos at various stages of development after 20 (**a**) and 40 (**b**) days in maturation medium, respectively; **c** Somatic embryo at cotyledonary stage, evidencing the absence of connection with the original tissue (*). Bars = 2 mm (**a** and **b**) or 500 μm (**c**)

3. The somatic embryos remain in the germination/elongation medium until reach a height of approximately 5 cm and are then submitted to the acclimatization stage (Fig. 21.4a).

21.2.7 Induction of Secondary Somatic Embryogenesis

1. Cotyledon somatic embryos that are not selected for the germination/elongation medium are cultured horizontally in the MM in relation to the embryonic axis.
2. After the induction of secondary somatic embryogenesis, the embryos are individually transferred to GM.
3. The same procedure performed for acclimatization of regenerated plantlets (emblings) via primary somatic embryos is applied to plantlets regenerated from secondary somatic embryos.

Fig. 21.4 Acclimatization process of somatic embryogenesis-derived *Passiflora cincinnata* plantlets. **a** Cotyledonary somatic embryos germinating in the germination/elongation medium; **b** Acclimatization stage of regenerated plants in plastic containers; **c** Acclimatized plants in a greenhouse

21.2.8 Acclimatization of Regenerated Plants

1. The regenerated plantlets (average height: 5 cm) in GM are transferred to plastic containers comprising a mixture of autoclaved Plantmax® HT substrate (Eucatex, Curitiba, Brazil) and coconut fiber (3:1), covered by a plastic bag for humidity maintenance (Fig. 21.4b).
2. The plantlets are kept under these acclimatization conditions for about 2 to 3 weeks. The plastic bag is gradually withdrawn until plantlets are moved to the greenhouse in order to complete the acclimatization cycle.
3. The acclimatization time is around 15 days, period which varies among plants (Fig. 21.4c).

21.3 Conclusions and Future Prospects

The *Passiflora cincinnata* Mast. propagation system via somatic embryogenesis was established using zygotic embryos as explants, which were exposed to an induction medium supplemented with 2,4-D and BA which was further enriched with activated charcoal and GA_3 in the maturation and germination stages, respectively (Silva et al. 2009). Also, due to the efficacy and reproducibility of this regeneration protocol, new perspectives arise as it can be extended to other Passifloraceae species that arouse agronomic, ornamental, and commercial interest. Moreover, this is an alternative for genetic transformation protocols that traditionally rely upon the organogenic system.

The establishment of a propagation system via somatic embryogenesis in *P. cincinnata* allows studies involving molecular aspects, such as the isolation, characterization, and dynamics of gene expression throughout the morphogenic route. In addition, genetic, histocytological and histochemical analyses enable the understanding of mechanisms that rule embryogenic cellular competence acquisition, establishment of polarity, differentiation, reserve mobilization dynamics. and the transition from somatic to embryogenic cells. Understanding the major cellular and molecular events in somatic embryogenesis will simplify processes aimed to induce this regeneration pathway in other plants, allowing the advance of new techniques to favor mass propagation and the improvement of recalcitrant species.

Acknowledgements The Brazilian sponsoring agencies *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (FAPEMIG, Belo Horizonte, MG, Brazil; Grant no. CBB - APQ-01131-15) and *Conselho Nacional de Desenvolvimento Científico e Tecnológico (*CNPq, Brasília, DF, Brazil: Grant no. 459.529/2014-5) are acknowledged for the financial support. P.O.S. was recipient of a PhD scholarship from *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES, Brasília, DF, Brazil). A.M.F. is currently recipient of a MSc scholarship from FAPEMIG, and L.M.V. a post-doctoral scholarship from CAPES/PNPD). Authors are also grateful to Dr. Daniela L. Paim-Pinto for generously making available Figs. 21.4B and 21.4C, and to Mr. Herculano José de Freitas, for kindly making available the photos in Fig. 21.1. Our gratitude to Caio G. Otoni for the English revision.

References

Anthony P, Otoni WC, Power JB, Lowe KC, Davey MR (1999) Protoplast isolation, culture, and plant regeneration from *Passiflora*. In: Hall RD (ed) Plant cell culture protocols. Humana Press, Totowa, pp 169–181

Chitwood DH, Otoni WC (2017) Morphometric analysis of *Passiflora* leaves: the relationship between landmarks of the vasculature and elliptical Fourier descriptors of the blade. GigaScience 6:1–13

Dhawan K, Dhawan S, Sharma A (2004) *Passiflora*: a review update. J Ethnopharmacol 94:1–23

Dias LLC, Santa-Catarina C, Ribeiro DM, Barros RS, Floh EIS, Otoni WC (2009) Ethylene and polyamine production patterns during *in vitro* shoot organogenesis of two passion fruit species as affected by polyamines and their inhibitor. Plant Cell Tiss Organ Cult 99:199–208

Dias LLC, Ribeiro DM, Santa-Catarina C, Barros RS, Floh EIS, Otoni WC (2010) Ethylene and polyamine interactions in morphogenesis of *Passiflora cincinnata*: effects of ethylene biosynthesis and action modulators, as well as ethylene scavengers. Plant Growth Reg 62:9–19

Dornelas MC, Vieira MLC (1993) Plant regeneration from protoplast cultures of *Passiflora edulis* var. flavicarpa Deg., *P. amethystina* Mikan and *P. cincinnata* Mast. Plant Cell Rep 13:103–106

Dornelas MC, Tavares FCA, Oliveira JC, Vieira MLC (1995) Plant regeneration from protoplast fusion in *Passiflora* spp. Plant Cell Rep 15:106–110

Faria RB, Carvalho IF, Rossi AAB, Matos EM, Rocha DI, Paim Pinto DL, Otoni WC, Silva ML (2018) High responsiveness in de novo shoot organogenesis induction of Passiflora cristalina (Passifloraceae), a wild Amazonian passion fruit species. Vitro Cell Dev Biol-Plant 54:166–174

Ferreira DAT, Sattler MC, Carvalho CR, Clarindo WR (2015) Embryogenic potential of immature zygotic embryos of *Passiflora*: a new advance for *in vitro* propagation without plant growth regulators. Plant Cell Tiss Organ Cult 122:629–638

Lombardi SP, Passos IRDS, Nogueira MCS, Appezzato-da-Glória B (2007) *In vitro* shoot regeneration from roots and leaf discs of *Passiflora cincinnata* mast. Braz Arch Biol Technol 50:239–247

Meletti LMM, Soares-Scott MD, Bernacci LC, Passos IR (2005) Passion fruit breeding: past and future. In: Faleiro FG, Junqueira NTV, Braga MF (eds) Passion fruit: germplasm and breeding. Embrapa Cerrados, Planaltina, DF, Brazil, pp 55–78 (in Portuguese)

Nakayama F (1966) *In vitro* tissue culture of *Passiflora caerulea*. Rev Fac Agric Nac La Plata 42:63–74 (in Spanish)

Otoni WC (1995) Somatic embryogenesis, somatic hybridization, and genetic transformation in *Passiflora* species. Ph.D. Dissertation, Universidade Federal de Viçosa, Viçosa, MG, Brazil (in Portuguese)

Otoni WC, Blackhall NW, d'Utra Vaz FB, Casali VWD, Power JB, Davey MR (1995) Somatic hybridization of the *Passiflora* species, *P. edulis* f. flavicarpa Degener and *P. incarnata* L. J Exp Bot 46:777–785

Otoni WC, Silva ML, Lima ABP, Paim Pinto DL, Lani ERG, Reis LB (2007) Transformação genética de maracujazeiros. In: Torres AC, Dusi AN, Santos MDM (eds) Transformação genética de plantas via *Agrobacterium*: Teoria e prática, 1ª ed, vol 1. Embrapa Hortaliças, Brasília, pp 121–137 (In Portuguese)

Otoni WC, Paim-Pinto DL, Rocha DI, Vieira LM, Dias LLC, Silva ML, Silva CV, Lani ERG, Silva LCD, Tanaka FAO (2013) Organogenesis and Embryogenesis in Passionfruit (*Passiflora* sp.). In: Aslam J, Srivastava PS, Sharma MP (eds) Somatic embryogenesis and gene expression. Narosa Publishing House, New Delhi, pp 1–17

Ozarowski M, Thiem B (2013) Progress in micropropagation of *Passiflora* spp. to produce medicinal plants: a mini-review. Braz J Pharmacogn 23:937–947

Pacheco G, Simão MJ, Vianna MG, Garcia RO, Vieira MLC, Mansur E (2016) In vitro conservation of Passiflora—a review. Sci Hortic 211:305–311

Paim Pinto DL, Barros BA, Viccini LF, Campos JMS, Silva ML, Otoni WC (2010) Ploidy stability of somatic embryogenesis-derived *Passiflora cincinnata* Mast. plants as assessed by flow cytometry. Plant Cell Tiss Organ Cult 103:71–79

Paim Pinto DL, Almeida AMR, Rêgo MM, Silva ML, Oliveira EJ, Otoni WC (2011) Somatic embryogenesis from mature zygotic embryos of commercial passionfruit (*Passiflora edulis* Sims) genotypes. Plant Cell Tiss Organ Cult 107:521–530

Rêgo MM, Rêgo ER, Bruckner CH, Finger FL, Otoni WC (2011) *In vitro* induction of autotetraploids from diploid yellow passion fruit mediated by colchicine and oryzalin. Plant Cell Tiss Organ Cult 107:451–459

Ribeiro LM, Nery LA, Vieira LM, Mercadante-Simões MO (2015) Histological study of micrografting in passionfruit. Plant Cell Tiss Organ Cult 123:173–181

Rocha DI, Dornelas MC (2013) Molecular overview on plant somatic embryogenesis. CAB Rev 8:1–17

Rocha DI, Vieira LM, Tanaka FAO, Silva LC, Otoni WC (2012) Somatic embryogenesis of a wild passion fruit species *Passiflora cincinnata* Masters: histocytological and histochemical evidences. Protoplasma 249:747–758

Rocha DI, Monte-Bello CC, Dornelas MC (2015) Alternative induction of *de novo* shoot organogenesis or somatic embryogenesis from *in vitro* cultures of mature zygotic embryos of passion fruit (*Passiflora edulis* Sims) is modulated by the ratio between auxin and cytokinin in the medium. Plant Cell Tiss Organ Cult 120:1087–1098

Rocha DI, Paim Pinto DL, Vieira LM, Tanaka FAO, Dornelas MC, Otoni WC (2016) Cellular and molecular changes associated with competence acquisition during passion fruit somatic embryogenesis: ultrastructural characterization and analysis of *SERK* gene expression. Protoplasma 253:595–609

Rocha DI, Vieira LM, Koehler AD, Otoni WC (2018) Cellular and morpho-histological foundations of *in vitro* plant regeneration. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Plant cell culture protocols, methods in molecular biology, 4th edn. Springer, Heidelberg (In press)

Rosa YBCJ, Bello CCM, Dornelas MC (2015) Species-dependent divergent responses to *in vitro* somatic embryo induction in *Passiflora* spp. Plant Cell Tiss Organ Cult 120:69–77

Silva TCR, Carvalho CR (2014) Vertical heterogeneity of DNA ploidy level assessed by flow cytometry in calli of *Passiflora cincinnata*. In Vitro Cell Dev Biol Plant 50:158–165

Silva ML, Paim Pinto DL, Guerra MP, Floh EIS, Bruckner CH, Otoni WC (2009) A novel regeneration system for a wild passion fruit species (*Passiflora cincinnata* Mast.) based on somatic embryogenesis from mature zygotic embryos. Plant Cell Tiss Organ Cult 99:47–54

Silva CV, Oliveira LS, Loriato VAP, Silva LC, Campos JMS, Viccini LF, Oliveira EJ, Otoni WC (2011) Organogenesis from root explants of commercial populations of *Passiflora edulis* Sims and a wild passionfruit species, *P. cincinnata* Masters. Plant Cell Tiss Organ Cult 107:621, 407–416

Silva ML, Paim Pinto DL, Guerra MP, Lani ERG, Carvalho IF, Rossi AAB, Otoni WC (2015) Synthetic seeds of a wild passionfruit species with ornamental potential. Ornam Hortic 21:331–338 (In Portuguese)

Simpson MG (2010) Plant systematics, 2nd edn. Academic Press, San Francisco, pp 322–323

Smertenko A, Bozhkov PV (2014) Somatic embryogenesis: life and death processes during apical-basal patterning. J Exp Bot 65:1343–1460

Tiwari S, Singh S, Tripathi S, Kumar S (2016) A pharmacological review: *Passiflora* species. Int J Pharmacogn 3:10–18

Trevisan F, Mendes BMJ, Maciel SC, Vieira MLC, Meleti LMM, Rezende JAM (2006) Resistance to *Passion fruit woodiness virus* in transgenic passionflower expressing the virus coat protein gene. Plant Dis 90:1026–1030

Ulmer T, MacDougal JM (2004) *Passiflora:* passionflowers of the world. Timber Press, Portland Oregon, p 430

Vieira MLC, Carneiro MS (2004) *Passiflora* spp., passionfruit. In: Litz RE (ed) Biotechnology of fruit and nut crops. CABI Publishing, pp 435–453

Xu L, Huang H (2014) Genetic and epigenetic controls of plant regeneration. Curr Top Dev Biol 108:1–33

Yang X, Zhang X (2010) Regulation of somatic embryogenesis in higher plants. Crit Rev Plant Sci 29:36–57

Yockteng R, d'Eeckenbrugge GC, Souza-Chies TT (2011) *Passiflora*. Wild crop relatives: genomic and breeding resources, tropical and subtropical fruits. Springer, Heidelberg, pp 129–171

Zerbini FM, Otoni WC, Vieira MLC (2008) Passionfruit. In: Kole C, Hall TC (eds) A compendium of transgenic crop plants, Tropical and subtropical fruit and nuts, 1st edn, vol 5. Wiley, Hoboken, pp 213–234

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5:1411–1423

Chapter 22
Somatic Embryogenesis in Açaí Palm (*Euterpe oleracea* Mart.)

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22.1 Introduction

The açaí palm (*Euterpe oleracea* Mart.) is a species that belongs to the family Arecaceae (Henderson and Galeano 1996) and supplies one of the most popular "superfruits" of the Amazon rainforest, commonly known as "açaí-do-pará" (Yamaguchi et al. 2015; Fig. 22.1). Due to the many health benefits, continuously elucidated by the scientific community, this species has become the target of several consumer markets around the globe.

Naturally distributed on floodplains of the Amazon River estuary (in the North of Brazil), as well as Guyana, Venezuela (Lorenzi et al. 1996; Oliveira 2002), Ecuador (Ferreira et al. 2016), and Colombia (Ferreira et al. 2016; Garzón et al. 2017), it presents different uses beyond the borders of regional communities, with an emphasis on the food and cosmetics industries (Gordon et al. 2012) as well as the pharmaceutical industry (Yamaguchi et al. 2015).

Although it is potentially exploited by the palm heart agroindustry, its use as food is mainly focused on the fruit (Costa et al. 2001), considered one of the most widely studied in the world (Yamaguchi et al. 2015). The fruit, especially the pulp (mesocarp), rich in bioactive compounds such as polyphenols, is a traditional constituent of the diet of native populations. Additionally, in recent decades it has

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_22

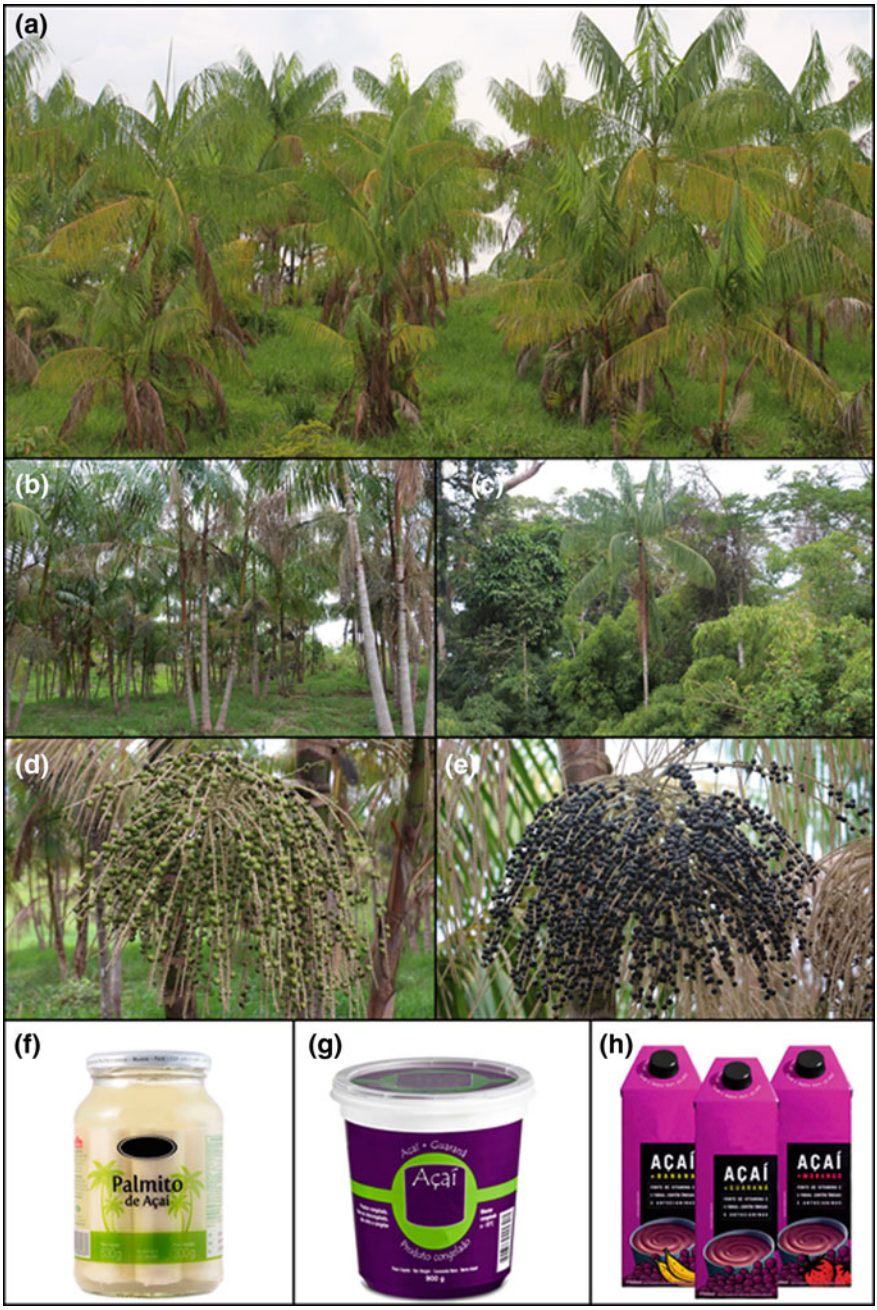


Fig. 22.1 Morphological aspects of *Euterpe oleracea* and *E. precatoria* and commercial products derived from them. **a**, **b** *Euterpe oleracea* in the fiel (note the tillering). **c** *Euterpe precatoria* in the field. **d** Unripe fresh fruit of *E. oleracea*. **e** Ripe fresh fruit of *E. oleracea*. **f** Heart of palm. **g** Frozen fruit pulp. **h** Juice

gained international attention due to its nutritional/energetic potential, as well as the various health benefits, of which the following are mentioned: antioxidant effects (Kang et al. 2010, 2012; Bem et al. 2014; Barbosa et al. 2016) and anti-inflammatory effects (Heinrich et al. 2011; Xie et al. 2012; Kang et al. 2012; Carey et al. 2015; Machado et al. 2016) related to the reduction of the incidence of age-related neurodegenerative disorders (Poulose et al. 2012) and to the reduction of cigarette-induced lung damage (Moura et al. 2012); antiproliferative (Hogan et al. 2010; Ribeiro et al. 2010; Heinrich et al. 2011; Fragoso et al. 2012); arteroprotetor (Sun et al. 2010; Feio et al. 2012); antigen (Machado et al. 2016); vasodilatory effects (Bem et al. 2014) associated with reduced risk of coronary disease (Rocha et al. 2007); and probiotic effects (Costa et al. 2017), among others.

Due to the aforementioned benefits, the demand for products derived from *E. oleracea*, in Brazil and internationally, has increased over the last years. According to the Brazilian Institute of Geography and Statistics (IBGE), in 2015, Brazil produced 216,071 metric tons of açaí fruit, especially in the state of Pará, considered the nation's largest producer (54%) (Conab 2016). Brazilian production, in addition to supplying the domestic demand, is also exported to countries such as the United States (main importer), the Netherlands, Japan and Australia, and it is noteworthy that Brazil is responsible for 100% of worldwide demand (Carvalho 2015; Garzón et al. 2017).

Among the products derived from *E. oleracea* that are marketed globally are frozen açaí pulp, açaí powder (used as an ingredient in the manufacture of dietary supplements) (Mulabagal et al. 2012), and beverages (Sabbe et al. 2009; Taylor 2010) (Fig. 22.1). It is also worth mentioning that the dissemination of açaí products as a global commodity is due in large part to the dissemination of information via the Internet (Heinrich et al. 2011) and to food companies that constantly seek different forms of fruit industrialization, such as instant capsules and powders (Silva et al. 2017).

This growing consumption of açaí fruits on an increasingly global scale requires not only the development of regional agroindustries that use more modern methods and equipment that meet the criteria demanded by the market, but also a transition from an extractive production system to a system based on large commercially farmed areas. According to Silvestre et al. (2017), the expansion of *E. oleracea* to new croplands (non-floodplain areas), aside from promoting an increase in fruit production, is an alternative to reforest degraded areas, either as monoculture or (preferably) as a component of agroforestry systems. In this context, such expansion will demand large scale production of plantlets, as well as selection of genotypes adapted to the different edaphoclimatic conditions and that produce fruits with high pulp yield and quality, as well as efficient methods of propagation of these genotypes, aimed at accelerating these very selection cycles.

The açaí palm can be propagated via seeds (sexual propagation) and via tillering (asexual propagation). However, due to the low rates of production and survival of the plants in the field obtained by tillers, commercial-scale production of plantlets is limited to sexual propagation (Scherwinski-Pereira et al. 2012; Freitas et al. 2016), which presents drawbacks such as the slowness and unevenness of the germination

process (Nascimento 2008), aside from the resulting genetic heterogeneity. These barriers are unfavorable to commercial-scale production (Saldanha and Martins-Corder 2012), as well as to the propagation of elite individuals selected from natural populations (Scherwinski-Pereira et al. 2012) and the consequent development of breeding programs. Additionally, *E. oleracea* seeds are considered recalcitrant, which makes it difficult to maintain them for long periods (Martins et al. 2004; Nascimento et al. 2010) and restricts the period of usage of the seeds. It is also noteworthy that palm trees present unique growth habits, with exclusive primary growth (Tomlinson and Huggett 2012), which limits the application of traditional vegetative propagation methods. In this setting, in vitro cultivation techniques—especially somatic embryogenesis—are seen as a promising alternative to clonal propagation of *E. oleracea*.

Somatic embryogenesis, apparently reported for the first time in palm trees by Staritsky (1970) and after by Rabechault and collaborators in 1970 for *Elaeis guineensis* (African oil palm), is the most widely used tissue culture technique in the vegetative propagation of palm trees (Steinmacher et al. 2007b), as well as the most promising and efficient (Ree and Guerra 2015). This technique has been generally described as a multifactorial process, involving the transition from somatic cells to structures similar to zygotic embryos (Emons 1994) (bipolar structures with no vascular connection to the source tissue) (Schumann et al. 1995; Von Arnold et al. 2002) and no fusion of gametes (Williams and Maheswaran 1986), which germinate and thereby give rise to plants genetically identical to the source that supplied the initial cells.

The initiation of the technique has been based on exposing cells or tissues from different explants (zygotic embryos, inflorescences, shoot tips, immature leaves) to controlled in vitro conditions that include, in addition to environmental conditions, concentrations of mineral salts, organic compounds (Dudits et al. 1995; Fehér et al. 2003; Zavattieri et al. 2010), and growth regulators such as auxins and cytokinins (Jiménez 2005; Elhiti et al. 2013), as well as several stressors (Féher 2015).

The type of explant and its stage of development are probably the most important factors in determining the embryogenic capacity of a culture (Gaj 2004). In this regard, zygotic embryos exhibit the following set of advantages for usage: high responsiveness to in vitro culture, presumably because they present many genes expressed during zygotic embryogenesis that are still active (Isah 2016), especially when immature; they are generally free of endophytes and pathogens; they are abundant in many species, and they can be harvested with little harm to the mother plant. However, protocols of regeneration from zygotic embryos present the disadvantage of unpredictability of the nature of the regenerants (Jayanthi et al. 2015), given the cross-pollination. On the other hand, the use of this explant may allow for the reproduction of materials obtained from controlled pollination between selected commercial genotypes.

It is also mentioned that the degree of maturity of the tissues used influences the responses to somatic embryogenesis (Freitas et al. 2016), in such a way that, according to Isah (2016), the potential for induction and obtainment of a significant number of somatic embryos is inversely proportional to the degree of maturity of the explant used, mainly in monocots (Fehér 2006).

In the *Euterpe* genus, relatively few studies of somatic embryogenesis have been developed, limited to *E. edulis* (Guerra and Handro 1988, 1991; Saldanha and Martins-Corder 2012) and *E. oleracea* (Ledo et al. 2002; Scherwinski-Pereira et al. 2012; Freitas et al. 2016), the vast majority using zygotic embryos as initial explants. Immature inflorescences have also been studied as potential explants for palm trees, due to the high embryogenic capacity of the calli originating therefrom (Fki et al. 2011), as well as the additional advantages of presenting low fungal and bacterial contamination (Teixeira et al. 1994; Abul-Soad 2011), harvest with little damage to the mother plant (Teixeira et al. 1994; Jayanthi et al. 2015), and abundant production of inflorescences by many species. In addition to the explants mentioned above, immature leaves are also seen as possible explants to be used in the initiation of the technique, mainly because they—not unlike inflorescences—allow for the cloning of phenotyped individuals in the field without the influence of the segregation of the genetic material after cross-fertilization.

Currently, in addition to the aforementioned studies on açaí palms, studies with different sources of explants have been conducted by Embrapa Genetic Resources and Biotechnology (Cenargen)—Brazil, Tissue Culture Laboratory II, also for *Euterpe precatoria*, popularly known as "açaí-do-amazonas" (Yamaguchi et al. 2015). This other potential species of *Euterpe* presents a similar and complementary economic profile to that reported for *E. oleracea*, but different from the açaí palm, has a solitary stipe (with no tillering), which further substantiates the application of somatic embryogenesis to this genus.

22.2 General/Initial Aspects of Somatic Embryogenesis

22.2.1 Explants

22.2.1.1 Zygotic Embryos

For induction of somatic embryogenesis in *E. oleracea*, zygotic embryos from unripe fruits collected from adult plants, characterized by green epicarp and dark purple epicarp (Fig. 22.2), respectively, can be used. The centers where *E. oleracea* fruits are available are located in the North region of Brazil, such as Açaí Palm Germplasm Active Bank, operated by Embrapa Eastern Amazon, located in Belém, Pará. Mature zygotic embryos can be used for the induction of somatic embryogenesis of *E. oleracea* because they do not differ significantly from immature embryos in the percentage of embryogenic calli and embryogenic calli with plant somatic embryos (Fig. 22.3).

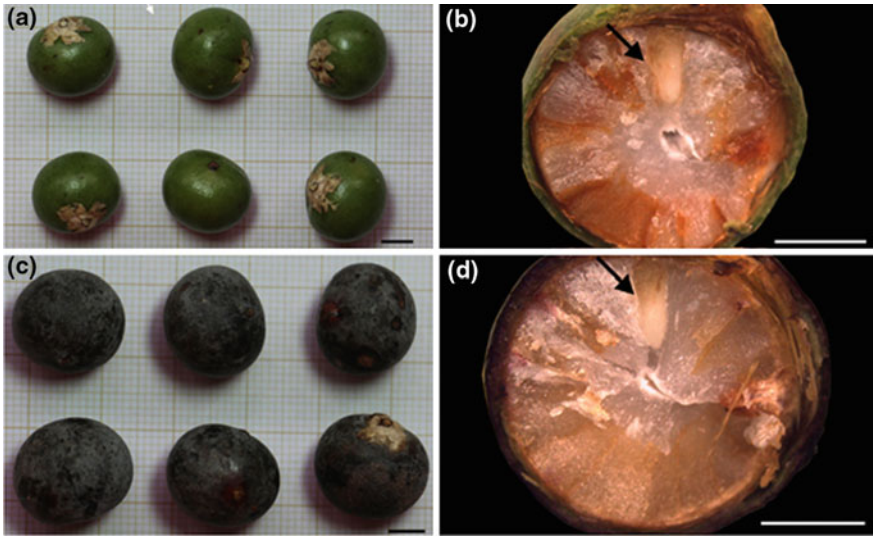


Fig. 22.2 Unripe (**a**, **b**) and ripe (**c**, **d**) fruits of *Euterpe oleracea*, with zygotic embryos exposed. Bars = **a**, **b**: 5 mm e **b**, **d**: 4 mm

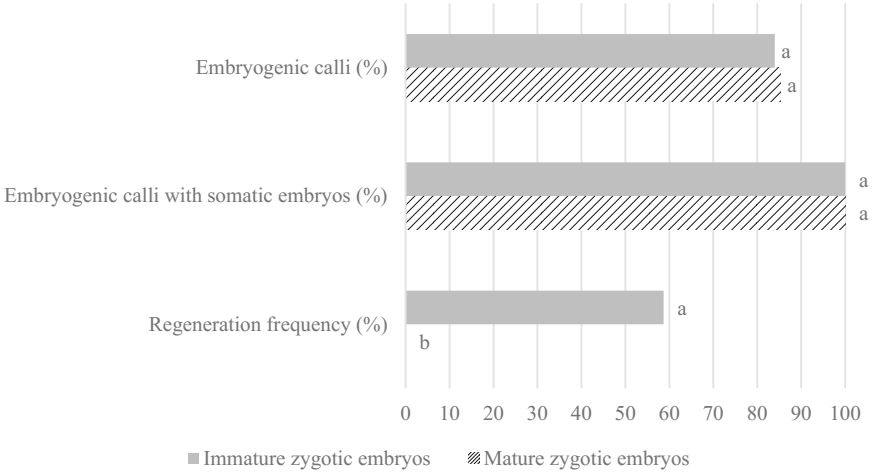


Fig. 22.3 Percentage of embryogenic calli, embryogenic calli with somatic embryos, and frequency of plant regeneration from somatic embryos obtained from mature and immature zygotic embryos of *Euterpe oleracea*. Different lowercase letters differ from each other by the Tukey test at 5% significance. Adapted from Freitas et al. (2016)

22.2.1.2 Inflorescences and Immature Leaves

In addition to zygotic embryos, inflorescences and leaves can be used as sources of explants for the induction of somatic embryogenesis in *E. oleracea*. The centers where such tissues are available, as well as post-harvest transportation and processing thereof, are the same as those cited for zygotic embryos.

Immature inflorescences, located inside closed and achlorophyllous spathes are used as sources of explants. The ideal size of the spathes collected from adult plants can vary between 6 and 12 cm, and are located between the innermost young and achlorophyllous leaves (palm heart) of the plant. The same plant that supplies the spathe can also supply the palm heart used as the explant source.

22.2.2 Preparation of Culture Media and Cultivation Conditions

The basic medium used throughout the somatic embryogenesis protocols from the different sources of *E. oleracea* explants consists of salts and vitamins of the MS medium (Murashige and Skoog 1962), supplemented with 30 g L^{-1} sucrose and 2.2 g L^{-1} Phytagel gelling agent (Sigma, St. Louis, MO, USA). Addition of other organic and inorganic compounds to the culture medium, besides growth regulators, are specific for each cultivation stage, depending on the type of explant used (Table 22.1). The pH of the culture media is always adjusted to 5.8 ± 0.1, with 0.1 N HCl or 0.1 N NaOH, after addition of all components except the gelling agent, then the media is subjected to sterilization for 20 min in an autoclave at 121 °C and a pressure of 1.5 kgf/cm^2. The solutions of 2-isopentenyladenine (2-iP) (Sigma, St. Louis, MO, USA) and gibberellic acid (GA_3) (Sigma, St. Louis, MO, USA) are inserted by filtration (0.22 μm filter) in the culture medium cooled in a laminar flow chamber after the autoclaving process. Specifically, stock solutions of 4-amino-3, 5, 6-trichloropicolinic acid growth regulators (Picloram) (Sigma, St. Louis, MO, USA), 1-naphthaleneacetic acid (NAA) (Sigma, St. Louis, MO, USA), and 2i-P are obtained by dilution in 1 N NaOH; 6-benzylaminopurine (BAP) in 1 N HCl, and GA_3 in absolute ethyl alcohol P.A. The addition of activated charcoal (Sigma, St. Louis, MO, USA) in the culture medium requires prior cooling thereof to approximately 45 °C, before being poured into the Petri dishes, in order to avoid the formation of agglomerates.

In the stages of induction of somatic embryogenesis and differentiation and maturation of somatic embryos, the explants are kept under dark conditions, while in the regeneration phase, the explants are subjected to light, with a photoperiod of 16 h. In all stages of the process, the temperature of the growth chamber is 25 ± 2 °C.

Table 22.1 Basic composition of the culture media employed in the different stages of somatic embryogenesis in açaí palms (*Euterpe oleracea* Mart.)

Components	Zygotic embryos			Inflorescences			Immature leaves		
	CIN	DIF/MAT	GE	CIN	DIF	MAT	CI	DIF	MAT
Picloram (μM)	450	–	–	450	0.41	–	450	0.41	–
NAA (μM)	–	0.6	–	–	–	0.6	–	–	0.6
2–iP* (μM)	–	12.3	–	–	–	12.3	–	–	12.3
BAP (μM)	–	–	1.0	–	–	–	–	–	–
GA_3[a] (μM)	–	–	0.5	–	–	–	–	–	–
L–Glutamine (g L^{-1})	0.5	–	–	0.5	0.5	0.5	0.5	0.5	0.5
Cysteine	–	–	–	–	–	–	0.1	0.1	0.1
Sucrose (g L^{-1})	30	30	30	30	30	30	30	30	30
Phytagel (g L^{-1})	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Activated charcoal (g L^{-1})	2.5	0.3	1.5	2.5	–	–	2.5	–	–
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Average time (days)	210	84	60	224	168	56	252	84	56

Abbreviations Calli induction (CIN), *differentiation* (DIF), *maturation* (MAT) and *germination* (GE)

[a]Filter–sterilized stock solution was added after autoclaving

22.2.3 Disinfestation and Preparation of Explants

Initially, under laboratory conditions, the explants (fruits, closed spathes and palm heart) are submitted to a surface washing process with running water and commercial detergent for five minutes. In the case of the fruits, they need to be manually pulped and taken to the laminar flow chamber for disinfestation before removing the zygotic embryos for cultivation. For disinfestation, all of the explants are immersed in ethyl alcohol 70% (v/v) for 3 min in a laminar flow chamber, followed by immersion in sodium hypochlorite (NaOCl) (containing 2.5% active chlorine) for 30 min and, subsequently, triple washing in distilled autoclaved water to remove any residue of the disinfecting agents used.

After disinfestation, the pulped fruits are placed to dry in Petri dishes containing sterile disks of filter paper for 20–30 min, and then sectioned with the aid of tweezers and scalpels to extract the zygotic embryos. The spathes are carefully opened, exposing the rachillae that lie within. The rachillae are sectioned transversely into segments approximately 5 mm long. The palm heart, in turn, has the tips excluded, and the remaining central portion is divided into three regions of approximately 10 cm each, which facilitates the inoculation process. The immature leaves are carefully isolated and sectioned transversely into rectangles measuring roughly 0.5 by 1.0 cm.

22.3 Induction of Somatic Embryogenesis

22.3.1 Zygotic Embryos

Once excised, the zygotic embryos are cultured in Petri dishes (90 × 15 mm) sealed with parafilm, containing 20 mL of MS medium, plus 2.5 g L^{-1} activated charcoal, 0.5 g L^{-1} L-glutamine, and supplemented with 450 μM Picloram (Fig. 22.4) (Table 22.1). The explants are maintained in this culture medium for up to 210 days, with monthly subcultures.

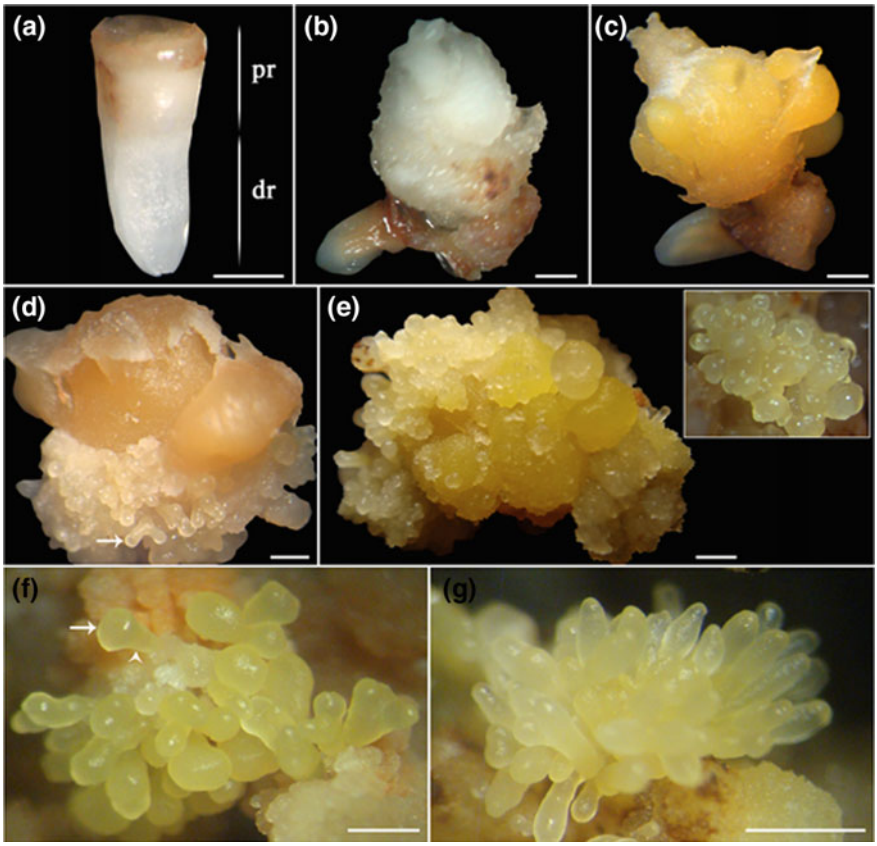


Fig. 22.4 Morphological stages of the induction of embryogenic calli starting from zygotic embryos of *Euterpe oleracea*. **a** Aspect of the zygotic embryo used in the experiments. **b** Zygotic embryos with early callus formation after 60 days of culture on an induction medium. **c** Development of yellow embryogenic callus, after 90 days of culture in the induction medium. **d**, **e** Development of globular somatic embryos (arrow) on the surface of embryogenic callus at 180 days of culture in the induction medium. **f** Detail of the development of globular somatic embryos (arrow) and suspensor (arrowhead) 210 days after culture in an induction medium. **g** Cluster of somatic embryos with onset of elongation. Abbreviations: (dr) distal region and (pr) proximal region. Bars = **a**, **d**, **e**, **f**, **g**: 1 mm and **b**, **c**: 2 mm

Incipient callogenic formations are observed after 60 days of culture, primarily in the proximal region of the zygotic embryo, which evidences the indirect model of somatic embryogenesis. Starting at 90 days, these calli develop into embryogenic calli, morphologically characterized by yellow coloration, nodular shape, and compact consistency. At 180 days in a medium with high concentration of Picloram, globular somatic embryos begin to be observed on the surfaces of the calli, whether isolated or in clusters. Some somatic embryos are shown connected to the callogenic mass by suspensor-like structures; others exhibit onset of elongation (Fig. 22.4).

22.3.2 *Inflorescences and Immature Leaves*

Once excised, the segmented rachillae are cultured in Petri dishes (90 × 15 mm) sealed with parafilm, containing 20 mL of MS medium plus the same components used for zygotic embryos (Fig. 22.5). Inflorescences are maintained in this culture medium for up to 250 days, with monthly subcultures. Initial calli, whitish and with an irregular surface, are observed at 140 days of culture. Portions of embryogenic calli covered by somatic embryos with clearly yellow coloration are evident starting at 220 days of culture in the induction medium.

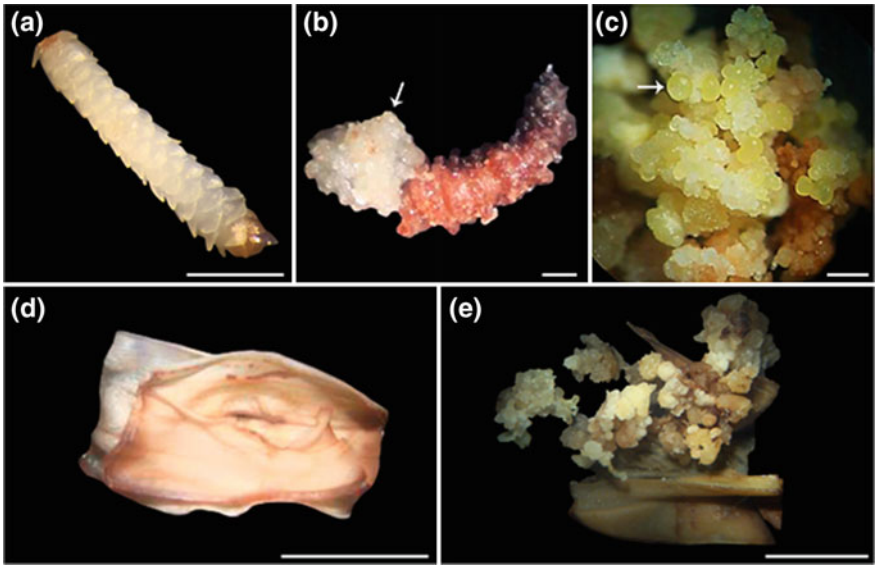


Fig. 22.5 Morphological stages of the induction of embryogenic calli from inflorescences and immature leaves of *Euterpe oleracea*. **a** Rachilla segment. **b** Immature inflorescence with onset of callus formation (arrow), after 140 days of culture in the induction medium. **c** Development of globular somatic embryos (arrow) on the surface of embryogenic callus, at 220 days of culture in the induction medium. **d** Immature leaf with early callus formation after 84 days of culture on an induction medium. **e** Development of embryogenic callus, after 252 days of culture in the induction medium. Bars = **a**, **b**: 1 mm; **d**, **e**: 5 mm

Leaf segments, in turn, are cultured in the MS medium plus the same components used for zygotic embryos and inflorescences, in addition to 0.1 g L^{-1} cysteine. The leaves are kept in this culture medium generally for up to 250 days, with monthly subcultures. Initial callogenic formations are observed after 90 days in the induction medium, usually compact, elongated and yellowish, from the edges and from the center of the leaves. After 250 days, progression of the primary calli to embryogenic stages is observed (Fig. 22.5).

22.4 Differentiation and Maturation of Somatic Embryos

22.4.1 Zygotic Embryos

For the differentiation of new somatic embryos and maturation of somatic embryos already differentiated in the previous phase, the same culture medium of the induction phase is used, however, without Picloram, supplemented with 0.3 g L^{-1} activated charcoal, 12.3 μM 2iP, and 0.6 μM NAA (Table 22.1). Embryogenic cultures are inoculated in Petri dishes (90 × 15 mm), containing 20 mL MS medium, and remain in these conditions until the appearance of somatic embryos in the torpedo stage, with a milky appearance (Fig. 22.6), characterized onset of maturity (around 90 days).

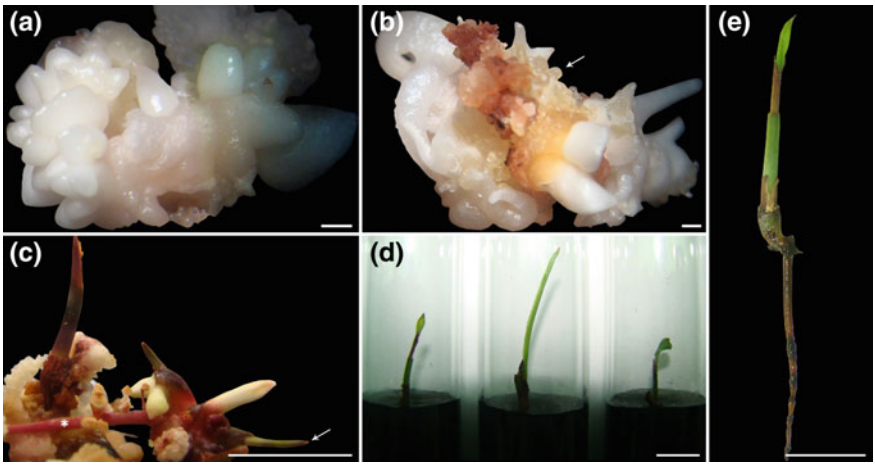


Fig. 22.6 Morphological stages of the maturation and regeneration of somatic embryos of *Euterpe oleracea* from zygotic embryos. **a** Somatic embryos in the torpedo stage with a milky aspect (mature for germination), 80 days after culture in a differentiating and maturing medium. **b** Cluster with somatic embryos at different stages of development (globular—arrow). **c** Somatic embryos germinating; detail of aerial part (arrow) and the root system (*). **d** Plants originating from the germination of somatic embryos. **e** Detail of plant originated from somatic embryo germination. Bars = **a**, **b**: 1 mm; **c**: 5 mm and **d**, **e**: 1 cm

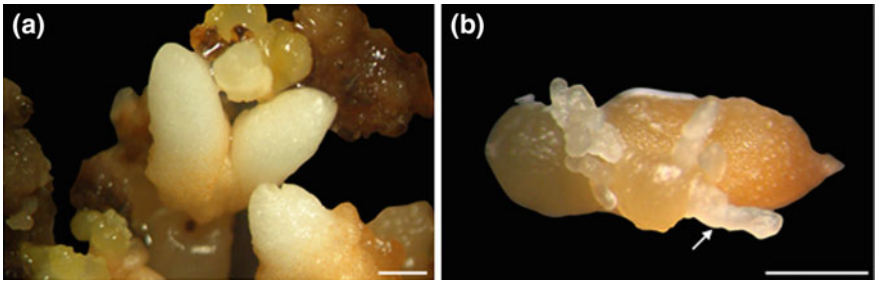


Fig. 22.7 Morphological stages of the development of somatic embryos of *Euterpe oleracea* obtained from inflorescences and immature leaves. **a** Mature somatic embryos from immature inflorescences, after 56 days in the maturation medium. **b** Somatic embryos in the initial stage of development originating from immature leaves after 84 days in the differentiation medium. Bars: **a**, **b**: 1 mm

22.4.2 Inflorescences and Immature Leaves

For the differentiation of somatic embryos, the explants containing embryogenic calli from sectioned rachillae and from immature leaves are transferred to the same formulation of salts as the culture medium of the induction stage, but with no activated charcoal and with the concentration of Picloram reduced to 0.41 μM (Table 22.1). After up to 180 days for inflorescences and 90 days for leaves, in the differentiation medium, the explants with differentiated somatic embryos are transferred to a new culture medium, supplemented with 0.6 μM NAA and 12.30 μM 2i-P, aimed at maturation and continuation of differentiation. Explants remain in this condition for around 60 days.

For immature inflorescences, after 90 days of cultivation in the differentiation medium, progression of the development of the somatic embryos is observed, becoming more elongated. At 60 days in the maturation medium, the development of somatic embryos in the torpedo phase is observed, already with white coloration and ready to commence germination, some of which still have a translucent appearance, possibly indicating the occurrence secondary embryogenesis. For immature leaves, somatic embryos were observed after 90 days in the differentiation medium, although they may require more time to progress to later developmental stages (Fig. 22.7).

22.5 Germination and Acclimatization of the Plants

Once mature, the somatic embryos of *E. oleracea* are transferred to a regeneration medium basically containing BAP and GA_3 at concentrations of 1.0 and 0.5 μM, respectively, aimed at conversion into plants (Table 22.1). The explants are inoculated in the culture media, where they remain for at least 60 days, until the

complete development of apical meristems (aerial part and root system) (Fig. 22.6). Although there is still no fully defined protocol for starting from somatic materials, it is possible that, for these materials, the time required for regeneration is longer because of the later nature of the responses when using these types of explants. In a study by Freitas et al. (2016), 58.7% of somatic embryos from immature zygotic embryos were converted into plants (Fig. 22.3).

After regeneration, plants between 5 and 8 cm high are placed in recipients, filled with a mixture of commercial substrate (Bioplant®) and washed sand (3:1 v/v, respectively) and covered with transparent plastic cups or bags, with small openings (up to 5 mm in diameter) at the top. Under these conditions, the plants are placed in incubation chambers (Percival I-60) with low luminosity (20 $\mu mm^{-2}\ s^{-1}$) and photoperiod of 12/12 h (light/dark) at 25 ± 1 °C for up to 3 weeks, when the upper protection of the recipients is then removed. After one week under the foregoing conditions, the plants are transferred to greenhouse conditions.

22.6 Morphological and Anatomical Aspects of Somatic Embryogenesis

The monitoring of all morphological modifications of the explants during the process of dedifferentiation (callus formation) and development of somatic embryos of *E. oleracea* can be accomplished by obtaining and analyzing images through a stereomicroscope and via Scanning Electron Microscopy (SEM) (Fig. 22.8).

For SEM, the collected samples are fixed in Karnovsky (Johansen 1940) for 24 h, rinsed in a sodium cacodylate buffer (0.05 M, pH 7.1) and post-fixed in 2% osmium tetroxide and a sodium cacodylate buffer for 3 h. They are then washed three times in the same fixation buffer, dehydrated in an increasing ethanolic series (25–100%), subjected to the critical point with CO_2 and metallized with gold-palladium. Observations and image capture are performed on a SEM.

The anatomical changes during the different stages of somatic embryogenesis in açaí palm can also be monitored. For such, samples of calluses and somatic embryos are cut and immersed in an FAA anti-fade solution (Formaldehyde, acetic acid, ethanol 70%, at a ratio of 1:1:18 v/v) (Johansen 1940), under vacuum for 24 h. The samples are then dehydrated in an increasing alcoholic series (70–100%) under vacuum for 1 h in each series, and steeped in historesin (Leica®). Crosswise and lengthwise serial cuttings (5 μm thick) are obtained in a manual rotating microtome, smoothed and placed on microscope slides on a plaque heated at 40 °C. Subsequently, the sections are stained with Toluidine blue for structural characterization (O'Brien et al. 1964) and the images obtained under a microscope with a digital still camera.

In general, histological analysis reveals that embryogenesis from fused zygotic embryos occurs via a multicellular route, demonstrated by the formation of meristematic cell masses that subsequently acquire protoderm and the presence of clusters of fused somatic embryos, and via a unicellular route, evidenced by the

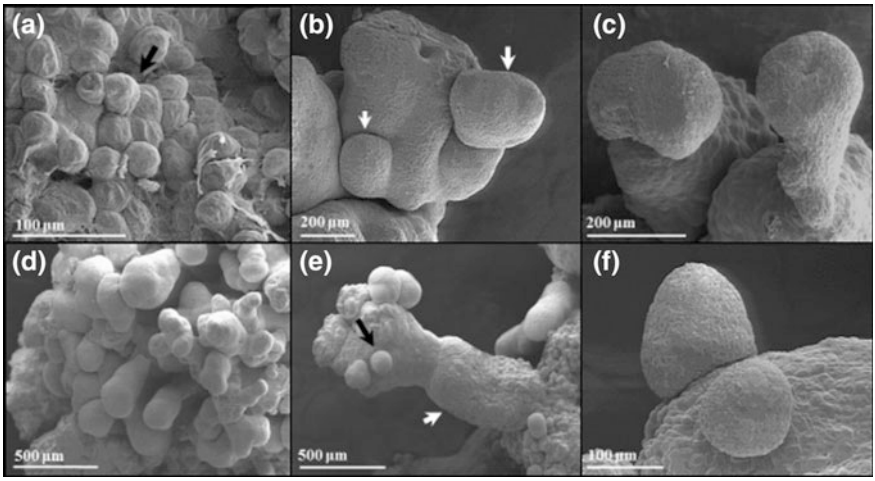
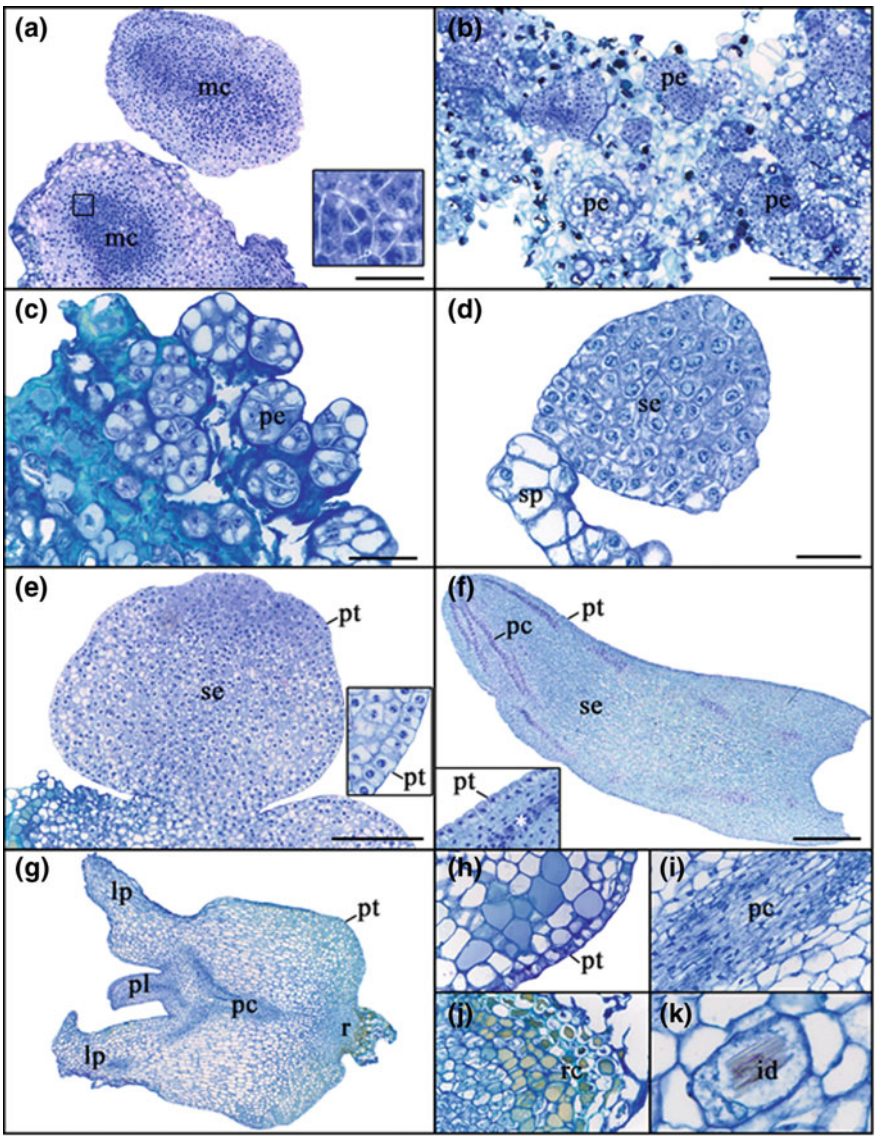


Fig. 22.8 Scanning electron micrograph during the induction of somatic embryos of *Euterpe oleracea* from zygotic embryos. **a** Callus with isodiametric cells. **b** Initial development of globular structure (arrow) and globular somatic embryos (larger arrow). **c** Somatic embryo with suspensor. **d** Cluster of somatic embryos in different stage of development. **e** Globular somatic embryo with suspensor (white arrow), exhibiting development of secondary somatic embryos (black arrow). **f** Figure **e** (black arrow) enlarged, secondary somatic embryos

presence of proembryos and suspensor-like structures. Additionally, the germination of the somatic embryos is confirmed by the presence of a plumule, root in protrusion, defined protoderm, and presence of procambium (Fig. 22.9). Furthermore, one can observe idioblasts containing raphides, common structures in zygotic embryos of germinating palms (Ribeiro et al. 2012; Magalhães et al. 2013).

22.7 Discussion

Initiation In several plant families, not unlike Arecaceae, analogues considered as sources of auxin have played a crucial role in the embryogenic process (Viñas and Jiménez 2011), especially 2,4-dichlorophenoxyacetic acid (2,4-D) (Fehér et al. 2003; Karami and Said 2010; Nic-Can and Loyola-Vargas 2016). However, the use of 2,4-D in high concentrations and for prolonged periods has been associated with the occurrence of abnormalities during the formation of somatic embryos (Rodriguez and Wetzstein 1998; Pescador et al. 2008; Konieczny et al. 2012). In this context, Picloram appears as an alternative of growth regulator inducing somatic embryogenesis, which has provided good results in palms such as *Bactris gasipaes* (Valverde et al. 1987; Steinmacher et al. 2007a, b, c), *Calamus merrillii and C. subinermis* (Goh et al. 1999, 2001), and *E. oleracea* (Scherwinski-Pereira et al. 2012). According to Karun et al. (2004), the superiority of Picloram can be



attributed to its effectiveness in absorption and mobilization thereof, as well as its rapid metabolization at specific sites.

Although for most plant species, mainly monocots, embryogenic competence is associated with the cells of certain tissues with embryonic or meristematic origin (Fehér 2006), already differentiated somatic cells can potentially recover this competence through previous callus formation (Mahdavi-Darvari et al. 2014), through a process of cell dedifferentiation, induced by various growth regulators and/or stressors. In this protocol, despite the juvenile nature of the explants used,

◂**Fig. 22.9** Histological characterization of somatic embryogenesis in *Euterpe oleracea* from zygotic embryos. **a** Nodular embryogenic callus consisting of regions with meristematic cells; note the detail of the meristematic region in intense cell division (square). **b**, **c** Development of proembryos. **d** Globular somatic embryo in formation, with suspensor showing. **e** Development of somatic embryo in the globular stage; note the defined protoderm (square). **f** Cross-section of somatic embryo in a post-globular developmental stage, exhibiting protoderm (square) and defined procambial strands (square—*). **g** Somatic embryo germinating; note the development of apical meristems. **h** Detail of protoderm. **i** Detail of procambial strands. **j** Detail of root cap. **k** Detail of raphide-containing idioblasts. Abbreviations: (cm) meristematic cell, (id) idioblast, (lp) leaf primordium, (pc) procambium, (pe) proembryo, (pl) plumule, (pt) protoderm, (r) root, (rc) root cap, (se) somatic embryo and (sp) suspensor. Bars = **a**, **b**, **e**, **f**: 200 μm, **c**, **d**, **h**, **i**, **j**, **k**: 50 μm and **g**: 500 μm

somatic embryogenesis does not occur directly, but rather by the formation of masses of cells or calli, characterizing the indirect type of somatic embryogenesis.

It is worth noting that the indirect route of somatic embryogenesis, specifically from zygotic embryos of the açaí palm, does not seem to interfere with the genetic stability of calli and plants generated, according to flow cytometry analyses (Freitas et al. 2016). Despite the results contrary to those previously mentioned by Ledo et al. (2002) in *E. oleracea*, which obtained somatic embryos directly from mature zygotic embryos and reported failure in the use of immature zygotic embryos, the protocol proposed here allows the use of immature zygotic embryos as sources of viable explants for the somatic embryogenesis of açaí palm, corroborating Scherwinski-Pereira et al. (2012).

The advantages of using of immature zygotic embryos compared to mature embryos is also noteworthy, due to the ease of transportation and storage, given the recalcitrance and consequent perishability of ripe fruits, which are easily injured. Moreover, unripe fruits have a hard epicarp but a soft endosperm that facilitates the extraction of zygotic embryos. Eventually, ripe fruits may also be used as sources of explants for somatic embryogenesis of açaí palm (Fig. 22.3). However, it still seems necessary to progress in the conditions of regeneration of the somatic embryos generated.

It is mentioned that zygotic embryos are generally more responsive in terms of embryogenic callus production than the other explants used, based on the protocols proposed. Whereas zygotic embryos—regardless of the maturity stage—can produce up to 80% of embryogenic calli, inflorescences and leaves produce rates that rarely exceed 20%.

Differentiaton/Maturation In this protocol, the removal and/or reduction of the inducer growth regulator (Picloram) and addition of the combination of regulators 2i-P and NAA at low concentrations provide high efficiency (100%) in the differentiation/maturation of somatic embryos starting from embryogenic calli originating from zygotic embryos and inflorescences. As for explants from immature leaves, they exhibit (on average) around 20% of embryogenic calli with somatic embryos, which demands future optimization. The success of the auxin–cytokinin combination (change of source of auxin and addition of cytokinin) in the expression of somatic embryogenesis has been reported by several authors in different palm

trees (Guerra and Handro 1988, 1991; Ledo et al. 2002; Eshraghi et al. 2005; Steinmacher et al. 2007c; Scherwinski-Pereira et al. 2012; Balzon et al. 2013; Silva et al. 2014) and according to Lakshmanan and Taji (2000), efficiency in the formation of somatic embryos promoted by the addition of cytokinins most likely depends on the type of auxin used in the induction process.

According to the aforementioned protocol, specifically for zygotic embryos, the degree of maturity of the explant does not bring about significant differences in the percentage of formation of somatic embryos.

Regeneration and acclimatization Generally, palm tree somatic embryo germination/conversion is a slow process, with low efficiency and with obvious abnormalities, constituting one of the main bottlenecks for the use of this technique. However, this protocol provides a relatively satisfactory frequency of regeneration of açaí palm plants (58.7%) starting from immature zygotic embryos, the vast majority with normal development (issuance of aerial part and roots), although this is not reached for the other explants used. Even though in most somatic embryogenesis protocols, the germination of the embryos obtained is usually carried out in culture media lacking growth regulators, in this protocol BAP and GA_3 are added. The results obtained corroborate those reported by Perera et al. (2009), who consider the addition of GA_3 a crucial step for the regeneration of *Cocos nucifera*. There are reports of the use of other growth regulators in addition to those mentioned above, such as 3-indoleacetic acid (IAA), indolebutyric acid (IBA), and 1-naphthaleneacetic acid (NAA), in different palm species during the regeneration phase. In *Elaeis guineensis*, for example, Jayanthi et al. (2015) and Gomes et al. (2015) reported the use of regulators for the purpose of improved rooting, a fundamental aspect of the conversion process, since it supplies water and nutritional needs of the plants after transfer to ex vitro conditions (Ree and Guerra 2015). On the other hand, growth regulators were not necessary in *Euterpe oleracea* (Scherwinski-Pereira et al. 2012) and *Acrocomia aculeata* (Luis and Scherwinski-Pereira 2014).

The maturity of the explants used was decisive for the regeneration of açaí palm plants from somatic embryos obtained through zygotic embryos, being restricted only to those somatic embryos from immature zygotic embryos. According to Ronchi and Giorgetti (1995), immature tissues, as well as organs close to the embryogenic stage, when submitted to stressful conditions, present higher embryogenic capacity and, consequently, regeneration of plants. Accordingly, these explants are considered ideal for monocot species (Fehér 2006). It should be noted, however, that in the protocols described here, the immaturity of the somatic explants used (inflorescences and leaves) was not sufficient to provide regeneration of the somatic embryos obtained, suggesting that this stage still needs improvements.

For the acclimatization of plants, proper care is important, under the risk of high losses of material. In our studies, the process of acclimatization begins with the planting of well-developed plants (6–8 cm high), provided they have a vigorous root system. The regenerated plants are placed in containers filled with commercial substrate and washed sand (3:1 v/v), which are covered with transparent plastic

bags with small openings in the upper portion, seeking to produce a greenhouse effect in the environment. The plants are also pre-acclimatized, being conditioned for two or three weeks in a germination chamber (BOD) at 25 °C and 12-h photoperiod. After the first week, the plastic bags are removed and, at the end of the second or third week of pre-acclimatization, the plants are then brought to a greenhouse to complete the growth.

Anatomy Histological studies are of significant importance for the detailing of events during somatic embryogenesis, which may elucidate the mechanisms for acquiring totipotency in somatic tissues of palm trees, as well as the distinction between responsive and non-responsive cells.

In palm trees, specifically, most studies mention the occurrence of indirect somatic embryogenesis (Viñas and Jiménez 2011). Somatic embryos via the indirect route show a high probability of multicellular origin (Gaj 2004), a fact confirmed by the aforementioned protocol starting from zygotic embryos. The origin of somatic embryos from a single-cell route has also been reported in palm trees (Luis and Scherwinski-Pereira 2014), as well as the two routes in the same regeneration system, as reported in *B. gasipaes* (Almeida et al. 2012) and previously in *E. oleracea* (Scherwinski-Pereira et al. 2012).

It is worth mentioning that both direct and indirect somatic embryogenesis can be derived from a single cell or from several cells (Quiroz-Figueroa et al. 2006), and that the first divisions necessary for somatic embryo formation have not often been observed in detail, since the cell or group of cells from which the embryo originates is not always easy to identify (Winkelmann 2016).

Our results clearly evidence the occurrence of somatic embryogenesis from the in vitro culture of immature zygotic embryos of *E. oleracea* under high concentrations of Picloram. Such embryogenic structures do not have a vascular connection with the matrix tissue, and the development of somatic embryos is evident at stages similar to those of the zygotic embryos and, subsequently, plant formation.

The results obtained characterize the protocol starting from zygotic embryos as a model of somatic high-frequency, repetitive and non-synchronized embryogenesis, via indirect route. Use of the growth regulator Picloram, combined with activated charcoal, NAA, 2i-P, BAP, and GA_3 significantly optimize the development of somatic embryogenesis in açaí palms starting from zygotic embryos. The other explants—in spite of the initial responsiveness and production capacity of somatic embryos—require optimization of the protocols, particularly of the final stages.

22.8 Identification of Steps Required for Protocol Modification

The regeneration of açaí plants through somatic embryogenesis is described in this protocol, starting from zygotic embryos. However, studies are still needed in order to: (1) reduce the time to obtain embryogenic calli; (2) increase the frequency of germination of somatic embryos; (3) involve high-efficiency systems, such as

temporary immersion bioreactors, in order to optimize the regeneration and production of large-scale somatic embryos. Additionally, a seemingly arduous path still seems to be necessary for somatic embryogenesis to be used for large-scale cloning of elite genotypes starting from somatic tissues (leaves and inflorescences). Notably, this fact should be considered with much research, although our research group has already obtained good results with these types of propagules, now requiring the protocols to be optimized.

Acknowledgements The authors thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for financial support and scholarships. We also wish to extend our thanks to Embrapa Eastern Amazon, Belém, PA, Brazil, for providing the biological material for experiments.

References

Abul-Soad AA (2011) Micropropagation of date palm using inflorescence explants. In: Jain SM, Al-Khayri JM, Johnson DV (eds) Date Palm Biotechnology. Springer, Dordrecht, pp 91–118

Almeida M, Almeida CV, Graner EM, Brondani GE, Abreu-Tarazi MF (2012) Pre-procambial cells are niches for pluripotent and totipotent stem-like cells for organogenesis and somatic embryogenesis in the peach palm: a histological study. Plant Cell Rep 31:1495–1515. https://doi.org/10.1007/s00299-012-1264-6

Balzon TA, Luis ZG, Scherwinski-Pereira JE (2013) New approaches to improve the efficiency of somatic embryogenesis in oil palm (*Elaeis guineensis* Jacq.) from mature zygotic embryos. Vitro Cell Dev Biol Plan 49(1):41–50. http://dx.doi.org/10.1007/s11627-012-9479-3

Barbosa PO, Pala D, Silva CT, Souza MO, Amaral JF, Vieira RAL, Folly GAF, Volp ACP, Freitas RNF (2016) Açai (*Euterpe oleracea* Mart.) pulp dietary intake improves cellular antioxidant enzymes and biomarkers of serum in healthy women. Nutrition 32:674–680. https://doi.org/10.1016/j.nut.2015.12.030

Bem GF, Costa CA, Oliveira PRB, Cordeiro VSC, Santos IB, Carvalho LCRM, Souza MAV, Ognibene DT, Daleprane JB, Sousa PJC, Resende AC, Moura RS (2014) Protective effect of *Euterpe oleracea* Mart (açaí) extract on programmed changes in the adult rat offspring caused by maternal protein restriction during pregnancy. J Pharm Pharmacol 66:1328–1338. https://doi.org/10.1111/jphp.12258

Carey AN, Miller MG, Fisher DR, Bielinski DF, Gilman CK, Poulose SM, Shukitt-Hale B (2015) Dietary supplementation with the polyphenolrich açaí pulps (*Euterpe oleracea* Mart. and *Euterpe precatoria* Mart.) improves cognition in aged rats and attenuates inflammatory signaling in BV-2 microglial cells. Nutr Neurosci 20(4):238–245. https://doi.org/10.1080/1028415X.2015.1115213

Carvalho D, Boletim: Produção Nacional de Açaí. SEBRAE (2015) hhttp://www.bibliotecas.sebrae.com.br/chronus/ARQUIVOS_CHRONUS/bds/bds.nsf/64153228c3c444bcdb587b6b501fa076/$File/5827.pdf. Accessed on Aug 7 2017

Companhia Nacional de Abastecimento (CONAB). Açaí (fruto) (2016) Conjuntura Nacional. http://www.conab.gov.br/OlalaCMS/uploads/arquivos/16_05_09_11_55_19_conjutura_de__acai_abr_-16-1.pdf. Accessed on Aug 7 2017

Costa MR, Oliveira MSP, Moura EF (2001) Variabilidade genética em açaizeiro Pesquisa (*Euterpe oleraceae* Mart.). Biotecnologia Cienc Desenvolv 21:46–50

Costa MGM, Ooki GN, Vieira ADS, Bedani RB, Saad SMI (2017) Synbiotic Amazonian palm berry (açai, *Euterpe oleracea* Mart.) ice cream improved *Lactobacillus rhamnosus* GG survival

to simulated gastrointestinal stress. Food Funct. 8(2):731–740. https://dx.doi.org/10.1039/c6fo00778c

Dudits D, Györgyey J, Bögre L, Bako L (1995) Molecular biology of somatic embryogenesis. In: Thorpe TA (ed) *In vitro* Embryogenesis in Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 267–308

Elhiti M, Stasolla C, Wang A (2013) Molecular regulation of plant somatic embryogenesis. Vitro Cell Dev Biol Plant 49:631–642. https://doi.org/10.1007/s11627-013-9547-3

Emons AMC (1994) Somatic embryogenesis: cell biological aspects. Acta Bot Neerl 43(1):1–14

Eshraghi P, Zarghami R, Mirabdulbaghi M (2005) Somatic embryogenesis in two Iranian date palm cultivars. Afr J Biotechnol 4:1309–1312

Fehér A (2015) Somatic embryogenesis—stress-induced remodeling of plant cell fate. Biochim Biophys Acta 1849(4):385–402. https://doi.org/10.1016/j.bbagrm.2014.07.005

Fehér A (2006) Why somatic plant cells start to form embryos? In: Mujib S, Samaj J (eds) Somatic embryogenesis plant cell monographs. Springer, Heidelberg, pp 85–101

Fehér A, Pasternak T, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult 74:201–228. https://doi.org/10.1023/A:1024033216561

Feio CA, Izar MC, Ihara SS, Kasmas SH, Martins CM, Feio MN, Maués LA, Borges NC, Moreno RA, Póvoa RM, Fonseca FA (2012) *Euterpe oleracea* (açai) modifies sterol metabolism and attenuates experimentally-induced atherosclerosis. J Atheroscler Thromb 19 (3):237–245. https://doi.org/10.5551/jat.11205

Ferreira DS, Gomes AL, Silva MG, Alves AB, Agnol WHD, Ferrari RA, Carvalho PRN, Pacheco MTB (2016) Antioxidant capacity and chemical characterization of açaí (*Euterpe oleracea* Mart.) fruit fractions. Food Sci Technol 4(5):95–102. https://doi.org/10.13189/fst.2016.040502

Fki L, Masmoudi R, Kriaâ W, Mahjoub A, Sghaier B, Mzid R, Mliki A, Rival A, Drira N (2011) Date palm micropropagation via somatic embryogenesis. In: Jain SM, Al-Khayri JM, Johnson DV (eds) Date Palm Biotechnology. Springer, Dordrecht, pp 47–68

Fragoso MF, Prado MG, Barbosa L, Rocha NS, Barbisan LF (2012) Inhibition of mouse urinary bladder carcinogenesis by açai fruit (*Euterpe oleracea* Martius) intake. Plant Foods Hum Nutr 67:235–241. https://doi.org/10.1007/s11130-012-0308-y

Freitas EO, Monteiro TR, Nogueira GF, Scherwinski-Pereira JE (2016) Somatic embryogenesis from immature and mature zygotic embryos of the açaí palm (*Euterpe oleracea*): Induction of embryogenic cultures, morphoanatomy and its morphological characteristics. Sci Hort 212:126–135. https://doi.org/10.1016/j.scienta.2016.09.044

Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regul 43:27–47. https://doi.org/10.1023/B:GROW.0000038275.29262.fb

Garzón GA, Narváez-Cuenca C-E, Vincken J-P, Gruppen H (2017) Polyphenolic composition and antioxidant activity of açai (*Euterpe oleracea* Mart.) from Colombia. Food Chem 217:364–372. https://doi.org/10.1016/j.foodchem.2016.08.107

Goh DKS, Bon M-C, Aliotti F, Escoute J, Ferrière N, Monteuuis O (2001) *In vitro* somatic embryogenesis in two major rattan species: *Calamus merrillii* and *Calamus subinermis*. Vitro Cell Dev Biol Plant 37:375–381. https://doi.org/10.1007/s11627-001-0066-2

Goh DKS, Michaux-Ferrière N, Monteuuis O, Bom M-C (1999) Evidence of somatic embryogenesis from root tip explants of the rattan *Calamus manan*. Vitro Cell Dev Biol Plant 35:424–427. https://doi.org/10.1007/s11627-999-0060-7

Gomes HT, Bartos PMC, Scherwinski-Pereira JE (2015) Optimizing rooting and survival of oil palm (*Elaeis guineensis*) plantlets derived from somatic embryos. Vitro Cell Dev Biol Plant 51:111–117. https://doi.org/10.1007/s11627-015-9669-x

Gordon A, Cruz APG, Cabral LMC, Freitas SC, TaxI CMAD, Donangelo CM, Mattietto RA, Friedrich M, Matta M, Marx F (2012) Chemicalcharacterization and evaluation of antioxidant

properties of açaí fruits (*Euterpe oleraceae* Mart) during ripening. Food Chem 133:256–263. https://doi.org/10.1016/j.foodchem.2011.11.150

Guerra MP, Handro W (1988) Somatic embryogenesis and plant regeneration in embryo cultures of *Euterpe edulis* mart. (palmae). Plant Cell Rep 7:550–552. https://doi.org/10.1007/BF00272754

Guerra MP, Handro W (1998) Somatic embryogenesis and plant regeneration in different organs of *Euterpe edulis* Mart. (Palmae): control and structural features. J Plant Res 111: 65-71. https://doi.org/10.1007/BF02507151

Guerra MP, Handro W (1991) Somatic embryogenesis in tissue cultures of *Euterpe edulis* Mart. (Palmae). In: Ahuja MR (ed), Woody plant biotechnology. Plenwn Press, New York, pp 189–196

Heinrich M, Dhanji T, Casselman I (2011) Açai (*Euterpe oleracea* Martius): a phytochemical and pharmacological assessment of the species' health claims. Phytochem Lett 4:10–21. https://doi.org/10.1016/j.phytol.20https://doi.org/10.11.005

Henderson A, Galeano G (1996) Euterpe, Prestoea, and Neonicholsonia (Palmae: Euterpeinae). New York Botanical Garden, New York

Hogan S, Chung H, Zhang L, Li J, Lee Y, Dai Y, Zhou K (2010) Antiproliferative and antioxidant properties of anthocyanin-rich extract from açai. Food Chem 118:208–214. https://doi.org/10.1016/j.foodchem.2009.04.099

Instituto Brasileiro de Geografia e Estatística (IBGE) (2015) Produção da Extração Vegetal e da Silvicultura 2015. https://sidra.ibge.gov.br/pesquisa/pevs/quadros/brasil/2015. Accessed on Aug 7 2017

Isah T (2016) Induction of somatic embryogenesis in woody plants. Acta Physiol Plant 38(118):1–22. https://doi.org/10.1007/s11738-016-2134-6

Jayanthi M, Susanthi B, Nandiganti MM, Mandal PK (20.15) *In vitro* somatic embryogenesis and plantlet regeneration from immature male inflorescence of adult dura and tenera palms of *Elaeis guineensis* (Jacq.). SpringerPlus. 4(256):1–7. https://doi.org/10.1186/s40064-015-1025-4

Jiménez VM (2005) Involvement of plant hormones and plant growth regulators *on in vitro* somatic embryogenesis. Plant Growth Regul 47:91–110. https://doi.org/10.1007/s10725-005-3478-x

Johansen DA (1940) Plant microtechnique. McGraw-Hill Book Co. Inc., New York, 523 p

Kang J, Li Z, Wu T, Jensen GS, Schauss AG, Wu X (2010) Anti-oxidant capacities of flavonoid compounds isolated from acai pulp (*Euterpe oleracea* Mart.). Food Chem 122:610–617. https://doi.org/10.1016/j.foodchem.2010.03.020

Kang J, Thakali KM, Xie C, Kondo M, Tong Y, Ou B, Jensen G, Medina MB, Schauss AG, Wu X (2012) Bioactivities of açaí (*Euterpe precatoria* Mart.) fruit pulp, superior antioxidant and anti-inflammatory properties to *Euterpe oleracea* Mart. Food Chem 133:671–677. https://doi.org/10.1016/j.foodchem.2012.01.048

Karami O, Said A (2010) The molecular basis for stress-induced acquisition of somatic embryogenesis. Mol Biol Rep. 37:2493–2507. https://doi.org/10.1007/s11033-009-9764-3

Karun A, Siril EA, Radha E, Parthasarathy VA (2004) Somatic embryogenesis and plantlet regeneration from leaf and inflorescence explants of arecanut (*Areca catechu* L.). Curr Sci 86:1623–1628

Konieczny R, Sliwinska E, Pilarska M, Tuleja M (2012) Morphohistological and flow cytometric analyses of somatic embryogenesis in *Trifolium nigrescens* Viv. Plant Cell Tissue Organ Cult 109:131–141. https://doi.org/10.1007/s11240-011-0081-x

Lakshmanan P, Taji A (2000) Somatic embryogenesis in leguminous plants. Plant Biology. 2:136–148. https://doi.org/10.1055/s-2000-9159

Ledo AS, Lameira OA, Benbadis AK, De Menezes IC, Oliveira MSP, Medeiros Filho S (2002) Somatic embryogenesis from zygotic embryos of *Euterpe oleracea* Mart. Rev Bras Frutic 24:601–603. https://doi.org/10.1590/S0100-29452002000300004

Lorenzi H, Souza HM, Costa JTM, Cerqueira LSC, Behr N (1996) Palmeiras do Brasil: nativas e exóticas. Instituto Plantarum, Nova Odessa, 303 p

Luis ZG, Scherwinski-Pereira JE (2014) An improved protocol for somatic embryogenesis and plant regeneration in macaw palm (*Acrocomia aculeata*) from mature zygotic embryos. Plant Cell Tissue Organ Cult 118:485–496. https://doi.org/10.1007/s11240-014-0500-x

Machado DE, Rodrigues-Baptista KC, Alessandra-Perini J, Moura R. S, Santos TA, Pereira KG, Silva YM, Souza PJC, Nasciutti LE, Perini JA (2016) *Euterpe oleracea* extract (açaí) is a promising novel pharmacological therapeutic treatment for experimental endometriosis. PlosONE 11(11). http://dx.doi.org/10.1371/journal.pone.0166059

Magalhães HM, Lopes PSN, Ribeiro LM (2013) Sant'anna-Santos, B. F.; Oliveira, D. M. T. Structure of the zygotic embryos and seedlings of *Butia capitata* (Arecaceae). Trees 27:273–283. https://doi.org/10.1007/s00468-012-0797-1

Mahdavi-Darvari F, Noor NM, Ismanizan I (2014) Epigenetic regulation and gene markers as signals of early somatic embryogenesis. Plant Cell Tissue Organ Cult 120(2):407–422. https://doi.org/10.1007/s11240-014-0615-0

Martins CC, Bovi MLA, Nakagawa J, Júnior GG (2004) Temporary storage of Jussara palm seeds: effests of time, temperature end pulp on germination and vigor. Hortic. Bras. 22:271–276. https://doi.org/10.1590/S0102-05362004000200021

Moura RS, Ferreira TS, Lopes AA, Pires KMP, Nesi RT, Resende AC, Souza PJC, Silva AJR, Borges RM, Porto LC, Valenca SS (2012) Effects of *Euterpe oleracea* Mart. (açaí) extract in acute lung inflammation induced by cigarette smoke in the mouse. Phytomedicine 19:262–269. https://doi.org/10.1016/j.phymed.2011.11.004

Mulabagal V, Keller WJ, Calderón AI (2012) Quantitative analysis of anthocyanins in *Euterpe oleracea* (açaí) dietary supplement raw materials and capsules by Q-TOF liquid chromatography/mass spectrometry. Pharm Biol. 50(10):1289–1296. https://doi.org/10.3109/13880209.2012.674141

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nascimento WMO (2008) Açaí *Euterpe oleracea* Mart., Informativo Técnico Rede de Sementes da Amazônia n.18, Embrapa Amazônia Oriental

Nascimento WMO, Cicero SM (2010) Novembre, A. D. L. C. Conservação de sementes de açaí (*Euterpe oleracea* Mart.). Rev Bras Sementes 32(1):024–033. http://dx.doi.org/10.1590/S0101-31222010000100003

Nic-Can GI, Loyola-Vargas VM (2016) The role of the auxins during somatic embryogenesis. In: Loyola-Vargas VM, Ochoa-Alejo (eds) Somatic embryogenesis: fundamental aspects and applications. Springer, Switzerland, pp 171–182

Oliveira MSP (2002) Biologia floral do açaizeiro em Belém do Pará. Embrapa Amazônia Oriental, Boletim de Pesquisa 08, Belém, 26 p

O'Brien TP, Feder N, Mccylly ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373. https://doi.org/10.1007/BF01248568

Perera PIP, Vidhanaarachchi VRM, Gunathilake TR, Yakandawala DMD, Hocher V, Verdeil JL, Weerakoon LK (2009) Effect of plant growth regulators on ovary culture of coconut (*Cocos nucifera* L.). Plant Cell Tissue Organ Cult 99:73–81. https://doi.org/10.1007/s11240-009-9577-z

Pescador R, Kerbauy GB, Viviani D, Kraus JE (2008) Anomalous somatic embryos in *Acca sellowiana* (O. Berg) Burret (Myrtaceae). Rev Bras Bot 31(1):155–164. http://dx.doi.org/10.1590/S0100-84042008000100014

Portal Brasil (2017) http://www.brasil.gov.br/economia-e-emprego/2017/02/assistencia-tecnica-e-investimentos-devem-impulsionar-producao-de-acai. Accessed on Aug 7 2017

Poulose SM, Fisher DR, Larson J, Bielinski DF, Rimando AM, Carey AN, Schauss AG, Shukitt-Hale B (2012) Anthocyanin-rich acai (*Euterpe oleracea* Mart.) fruit pulp fractions attenuate inflammatory stress signaling in mouse brain BV-2 microglial cells. J Agric Food Chem 60:1084–1093. https://doi.org/10.1021/jf203989k

Quiroz-Figueroa F, Rojas-Herrera R, Galaz-Avalos RM, Loyola- Vargas VM (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell Tissue Organ Cult 86:285–301. https://doi.org/10.1007/s11240-006-9139-6

Ree JF, Guerra MP (2015) Palm (Arecaceae) somatic embryogenesis. Vitro Cell Dev Biol Plant. 51(6):589–602. https://doi.org/10.1007/s11627-015-9722-9

Ribeiro JC, Antunes LM, Aissa AF, Darin JD, De Rosso VV, Mercadante AZ, Bianchi MLP (2010) Evaluation of the genotoxic and antigenotoxic effects after acute and subacute treatments with acai pulp (*Euterpe oleracea* Mart.) on mice using the erythrocytes micronucleus test and the comet assay. Mutat Res 695:22–28. https://doi.org/10.1016/j.mrgentox.2009.10.009

Ribeiro LM, Oliveira DMT, Garcia QS (2012) Structural evaluations of zygotic embryos and seedlings of the macaw palm (*Acrocomia aculeata*, Arecaceae) during *in vitro* germination. Trees. 26:851–863. https://doi.org/10.1007/s00468-011-0659-2

Rocha AP, Carvalho LC, Sousa MA, Madeira SV, Sousa PJ, Tano T, Schini-Kerth VB, Resende AC, Moura RS (2007) Endothelium-dependent vasodilator effect of *Euterpe oleracea* Mart. (acai) extracts in mesenteric vascular bed of the rat. Vascul Pharmacol 46:97–104. https://doi.org/10.1016/j.vph.2006.08.411

Rodriguez APM, Wetzstein HY (1998) A morphological and histological comparison of the initiation and development of pecan (*Carya illinoinensis*) somatic embryogenic cultures induced with naphthaleneacetic acid or 2,4-dichlorophenoxyacetic acid. Protoplasma. 204:71–83. https://doi.org/10.1007/BF01282295

Ronchi VN, Giorgeiti L (1995) The Cell's Commitment to Somatic Embryogenesis. In: Bajaj YPS (ed), Biotechnology in agriculture and forestry: somatic embryogenesis and synthetic Seed. Springer—Verlag, Berlin, pp 3–19

Sabbe S, Verbeke W, Deliza R, Matta VM, Damme PV (2009) Consumer liking of fruit juices with different açaí (*Euterpe oleracea* Mart.) concentrations. J Food Sci 74(5):171–176. https://doi.org/10.1111/j.1750-3841.2009.01146.x

Saldanha CW, Martins-Corder MP (2012) *In vitro* germination and embryogenic competence acquisition of *Euterpe edulis* Martius immature zygotic embryos. Crop Breed Appl Biotechnol 12:171–178. https://doi.org/10.1590/S1984-70332012000300002

Scherwinski-Pereira JE, Guedes RS, Da Silva RA, Fermino PCP Jr, Luis ZG, Freitas EO (2012) Somatic embryogenesis and plant regeneration in açaí palm (*Euterpe oleracea*). Plant Cell Tissue Organ Cult 109:501–508. https://doi.org/10.1007/s11240-012-0115-z

Schumann G, Ryschka U, Schulze J, Klocke E (1995) Anatomy of Somatic Embryogenesis. In: Bajaj YPS (ed), Biotechnology in agriculture and forestry: somatic embryogenesis and synthetic seed. Springer—Verlag, Berlin, pp 71–86

Silva AKN, Beckman JC, Rodrigues AMC, Silva LHM (2017) Avaliação da composição nutricional e capacidade antioxidante de compostos bioativos da polpa de açaí. R Bras Tecnol Agroindustr 11(1):2205–2216

Silva RC, Luis ZG, Scherwinski-Pereira JE (2014) The histodifferentiation events involved during the acquisition and development of somatic embryogenesis in oil palm (*Elaeis guineensis* Jacq.). Plant Growth Regul 72:67–80. https://doi.org/10.1007/s10725-013-9837-0

Silvestre WVD, Silva PA, Palheta LF, Neto CFO, Souza RORM, Festucci-Buselli RA, Pinheir HA (2017) Differential tolerance to water deficit in two açaí (*Euterpe oleracea* Mart.) plant materials. Acta Physiol Plant 39:4. https://doi.org/10.1007/s11738-016-2301-9

Staritsky G (1970) Tissue culture of the oil palm (*Elaeis guineensis* Jacq.) as a tool for vegetative propagation. Euphytica 19:288–292

Steinmacher DA, Cangahuala-Inocente GC, Clement CR, Guerra MP (2007a) Somatic embryogenesis from peach palm zygotic embryos. Vitro Cell Dev Biol Plant 43:124–132. https://doi.org/10.1007/s11627-007-9032-y

Steinmacher DA, Clement CR, Guerra MP (2007b) Somatic embryogenesis from immature peach palm inflorescence explants: towards development of an efficient protocol. Plant Cell Tissue Organ Cult 89:15–22. https://doi.org/10.1007/s11240-007-9207-6

Steinmacher DA, Krohn NG, Dantas ACM, Stefenon VM, Clement CR, Guerra MP (2007c) Somatic embryogenesis in peach palm using the thin cell layer technique: Induction, morphohistological aspects and AFLP analysis of somaclonal variation. Ann Bot 100:699–709. https://doi.org/10.1093/aob/mcm153

Sun X, Seeberger J, Alberico T, Wang C, Wheeler CT, Schauss AG, Zou S (2010) Açaí palm fruit (*Euterpe oleracea* Mart.) pulp improves survival of flies on high fat diet. Exp Gerontol 45:243–251. https://doi.org/10.1016/j.exger.2010.01.008

Taylor L (2017) Tropical plant database: Açaí. Carson City, NV. https://www.rain-tree.com/açaí.htm. Accessed on Aug 7, 2017

Teixeira JB, Söndahl MR, Kirby EG (1994) Somatic embryogenesis from immature inflorescences of oil palm. Plant Cell Rep 13:247–250. https://doi.org/10.1007/BF00233313

Tomlinson, P. B.; Huggett, B. A. Cell longevity and sustained primary growth in palm stems. Am J Bot 99(12):1891–1902. http://dx.doi.org/10.3732/ajb.1200089

Valverde R, Arias O, Thorpe TA (1987) Picloram-induced somatic embryogenesis in pejibaye palm (*Bactris gasipaes* HBK). Plant Cell Tissue Organ Cult 10:149–150. https://doi.org/10.1007/BF00035913

Viñas M, Jiménez VM (2011) Factores que influyen en la embriogénesis somática *in vitro* de palmas (Arecaceae). Rev Colomb Biotecnol 13:229–242. http://dx.doi.org/10.15446/rev.colomb.biote

Von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tissue Organ Cult 69:233–249. https://doi.org/10.1023/A:1015673200621

Williams ES, Maheswaran B (1985) Origin and development of somatic embryoids formed directly on immature embryos of *Trifolium repens in vitro*. Ann Bot 56:619–630. https://doi.org/10.1093/oxfordjournals.aob.a087052

Winkelmann T (2016) Somatic versus zygotic embryogenesis: learning from seeds. In: Germanà MA, Lambardi M (eds) *In vitro* embryogenesis in higher plants - Methods in molecular biology. Springer, New York, pp 25–46

Xie C, Kang J, Lic Z, Schauss AG, Badgera TM, Nagarajana S, Wu T, Wua X (2012) The açaí flavonoid velutin is a potent anti-inflammatory agent: blockade of LPS-mediated TNF-α and IL-6 production through inhibiting NF-κB activation and MAPK pathway. J Nutr Biochem 23:1184–1191. https://doi.org/10.1016/j.jnutbio.2011.06.013

Yamaguchi KKL, Pereira LFR, Lamarão CV, Lima ES, Veiga-Junior VF (2015) Review Amazon acai: chemistry and biological activities: A review. Food Chem 179:137–151. https://doi.org/10.1016/j.foodchem.2015.01.055

Zavattieri MA, Frederico AM, Lima M, Sabino R, Arnholdt-Schmitt B (2010) Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron J Biotechnol 13(1):1–9. https://doi.org/10.2225/vol13-issue1-fulltext-4

Chapter 23
Somatic Embryogenesis and Somatic Embryo Cryopreservation of the Tree-Fern *Cyathea Delgadii* Sternb.

Karolina Tomiczak, Małgorzata Grzyb, Jan J. Rybczyński and Anna Mikuła

23.1 Introduction

Cyathea delgadii Sternb. belongs to the *Cyathea* clade of the genus *Cyathea*, family Cyatheaceae, also known as Scaly Tree-Ferns. It is one of the many evergreen, non-seasonal tree-fern species from Central and South America, which grow in gallery, montane, cloud and rain forests, as well as in open locations and along paths in Costa Rica, Panama, Venezuela, Columbia, Peru, Ecuador, Bolivia, Argentina, Paraguay, and Brazil (Tryon and Tryon 1982; Large and Braggins 2004).

Cyathea delgadii is a fast growing, medium-sized species, with an erect, slender 'trunk', reaching as much as 10 m tall, and some 5–15 cm in diameter (Bittner and Breckle 1995; Large and Braggins 2004), and the latter provides a suitable habitat for a number of different epiphytes that include other ferns, mosses and liverworts (Bittner and Trejos-Zelaya 1997; Vital and Prado 2006). Its bi-pinnate fronds measure up to 3 m in length, and these form a large, delicately arching crown. The frond stipes are brown and bear spines, whereas rachises, which are also brown, are clothed with small scales. Sori occur on either side of the pinnule mid-vein and are protected by globose indusia (Large and Braggins 2004). Spores are produced throughout the year, and a single frond can produce nearly 300 million spores. These germinate in diffuse light, but germination of stored spores diminishes with time (Simabukuro et al. 1998a).

This species is frequently cultivated as an ornamental tree for gardens. To do well in cultivation, plants should be provided with a substratum of consistently

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_23

moist and well-drained humus. This species does best when sheltered from the wind and grown under warm conditions (Large and Braggins 2004).

Prior to 2010, very limited data relating to the in vitro propagation of *C. delgadii* was available. Until then, only experiments involving spore sterilization (Simabukuro et al. 1998b) and spore germination (Hiendlmeyer and Randi 2007) had been carried out. At about the same time, gametophyte development, the process of zygotic embryogenesis and the production of sporophytes were also described by Goller and Rybczyński (2007) and Rybczyński and Mikuła (2011).

The process of somatic embryogenesis has only very recently been reported for *C. delgadii* (Mikuła et al. 2015b). It is worth stating that this last publication represents not only the first documentation of somatic embryogenesis in ferns, but also for the entire clade Monilophyta. Since Monilophyta contains the closest living relatives of spermatophytes, the recognition and description of somatic embryogenesis for a representative of this group of plants not only has the potential to improve the large-scale propagation and conservation of this species, but may also help us understand the evolution and regulation of this remarkable process.

Somatic embryos of *C. delgadii* can be easily and efficiently induced on stipe explants, measuring only a few millimeters, excised from very young etiolated sporophytes. There is no requirement for any exogenous plant growth regulators or stress factors (Mikuła et al. 2015b). The best result for somatic embryogenesis was achieved using a simple medium containing half-strength MS macro- and micronutrients, a full complement of vitamins, and 1% sucrose (Mikuła et al. 2015a). It is, however, also possible to induce somatic embryos on *C. delgadii* stipe explants cultured on 1/4 MS or 1/8 MS medium, or even on 2% solution of sucrose solidified with agar (Mikuła et al. 2015a).

Initially, it is necessary to induce somatic embryos of *C. delgadii* on stipe explants collected from zygotic, embryo-derived sporophytes. These embryos soon develop into young sporophytes and become the source of new stipe explants for the second cycle of somatic embryogenesis. This cycle can be repeated over and over with no reduction in efficiency. Furthermore, the prolongation of stipe explant culture up to 8 months, without sub-culturing, can improve the efficiency of the somatic embryogenesis seven-fold (Mikuła et al. 2015b).

The key factor to successful induction of somatic embryogenesis in *C. delgadii* is the etiolation of the plant material that is the source of explants (Mikuła et al. 2015a). Explants excised from sporophytes cultured under 16/8 h photoperiod conditions were incapable of somatic embryogenesis, regardless of the light conditions used for their subsequent culture. In such cases, only the development of aposporic gametophytes was observed (Mikuła et al. 2015a). More detailed studies revealed that light conditions under which the donor plant material was kept, significantly modulated the level and balance of endogenous hormones (Grzyb et al. 2017). The concentration of abscisic acid (ABA) in etiolated explants was almost 12-fold lower than that in explants collected from sporophytes grown under photoperiodic conditions. Conversely, the concentration of IAA and total cytokinins was 2-fold and 9.4-fold greater, respectively. Following explant excision, the content of all phytohormones present dropped dramatically, but their ratios

remained almost unchanged, thus triggering embryogenic competence in epidermal cells of the stipe fragment (Grzyb et al. 2017). Cyto-morphological and proteomic analyses indicated that starch accumulation could be another important factor associated with the induction phase of somatic embryogenesis. Moreover, the emergence or increased expression of enzymes engaged in maintenance of cell wall plasticity and enrichment of the latter with lipid substances, together with flavonoid biosynthesis and production of plastid isoprenoids seemed to be closely associated with this phase (Domżalska et al. 2017).

It is speculated that the phase of somatic embryogenesis expression in *C. delgadii* commences 10 days following stipe explant excision and culture initiation, when the first cell divisions occur. A rapid increase in sucrose content 4 days previously can be considered the switch necessary for initiating this phase (Grzyb et al. 2017). At the proteomic level, an increase in the abundance of proteins was observed. These proteins were involved in the conversion of sugars and the production of metabolically usable energy, the regulation of ubiquitination and 26S proteasome activity, as well as being associated with amino acid metabolism. An abundance of enolase involved in the synthesis of storage compounds was detected during the expression phase of *C. delgadii* somatic embryogenesis (Domżalska et al. 2017).

The entire phenomenon of somatic embryo formation in *C. delgadii* continues to be investigated in great depth. Besides recording detailed cyto-morphological description and elucidating the physiological and molecular regulation of this process, experiments involving its practical application to the conservation of species are also currently being undertaken. Although cryopreservation procedures have been developed for gametophytes of *C. delgadii* (Mikuła et al. 2011), the use of somatic embryos that develop directly into sporophytes would shorten the time required to obtain mature plants from material stored in liquid nitrogen.

This chapter describes the well-established protocol for regenerating sporophytes of *C. delgadii* via somatic embryogenesis from stipe explants and the preliminary protocol for the cryopreservation of *C. delgadii* somatic embryos using the encapsulation-dehydration technique.

23.2 Materials and Culture Conditions

Plant material:

Fronds of *Cyathea delgadii* bearing mature sori on the underside of frond pinnules.

Apparatus and instruments:

1. Laminar-flow hood, dissecting stereo-microscope, tissue culture chamber, gyratory shaker, water bath that can be set at a temperature of 38 °C, cryogenic storage dewar, 1 ml automatic pipette;

2. Forceps, scalpel, dissecting needle, sterile filter paper;
3. Plastic Petri dishes (Ø9 cm), 1 ml tips with end cut off, 2 ml cryo-vials;
4. Large glass Petri dishes (about Ø15 cm) or ceramic plates, 250 ml glass jars, 250 ml Erlenmeyer flasks, 20 ml glass breakers; 150 ml glass crystallizing dish;

Media and solutions:

1. Commercial bleach containing 5% sodium hypochlorite (e.g. Domestos), 70% ethanol, sterile distilled water, liquid nitrogen;
2. Media and sucrose solutions (see Tables 23.1 and 23.2), 3% sodium alginate solution, 0.1 M calcium chloride ($CaCl_2$) solution.

Basic medium composition is listed in Table 23.1. Modifications required for different culture stages are listed in Table 23.2. The pH should be adjusted to 5.8 with 1 M NaOH or 1 M HCl prior to autoclaving at 121 °C for 18 min. Pour about 30 ml agar medium to Ø9 cm plastic Petri dishes and 50 ml sucrose solution to 250 ml Erlenmeyer flasks.

Light conditions:

1. Constant darkness;
2. 16/8 h photoperiod at a light intensity of 50 μM m^{-2} s^{-1}.

Table 23.1 Composition of basal 1/2 MS medium

Chemicals	Concentration in mg/l
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
KH_2PO_4	170
FeNaEDTA	36.7
HBO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
KI	0.83
NaMoO4.$2H_2O$	0.25
$CuSO_4.7H_2O$	0. 0125
$CoCl_2$.6H2O	0.0125
Nicotinic acid	0.5
Thiamine HCl	0.1
Pyridoxin HCl	0.5
Glycine	2.0
Myo-inositol	100

Table 23.2 Formulations of media and sucrose solutions used for induction of somatic embryogenesis and cryopreservation of somatic embryos of *C. delgadii*

Component	Growth Medium (GM)	Induction Medium (IM)	Preculture Medium (PM)	0.3 M sucrose solution	0.5 M sucrose solution	0.75 M sucrose solution	1.0 M sucrose solution
Basal 1/2 MS medium	1×	1×	1×	1×	1×	1×	1×
Sucrose (M)	0.06	0.03	0.06	0.3	0.5	0.75	1.0
ABA (μM)	–	–	10	–	–	–	–
Agar (g/l)	7.5	7.5	7.5	–	–	–	–

The pH of all media are adjusted to 5.8

23.3 Methods

The protocol for the somatic embryogenesis of *Cyathea delgadii* includes the following steps: (1) spore disinfection, sowing and gametophyte culture initiation, (2) sporophyte initiation and induction of the first cycle of somatic embryogenesis, (3) induction of the second cycle of somatic embryogenesis, (4) sporophyte culture and acclimatization. The protocol of cryopreservation of somatic embryos by encapsulation-dehydration includes: (1) encapsulation of somatic embryos, (2) preculture, (3) osmotic dehydration, (4) air desiccation, (5) freezing, (6) thawing and post-freezing culture.

23.3.1 Somatic Embryogenesis of Cyathea delgadii

23.3.1.1 Spore Disinfection, Sowing and Gametophyte Culture Initiation

Collected mature fronds of *C. delgadii* should be placed in paper envelopes and left to dry naturally in a cool, airy place for a few days. Released spores are visible at the bottom of the envelope as a fine powder. They can be separated from the sporangia by gentle shaking or blowing the contents of the opened envelope. Sporangial material, together with infertile spores are slightly lighter than fertile spores, and so it will tend to move first, whereas heavier spores should remain (Large and Braggins 2004).

1. Wrap released and dried spores in a piece of filter paper folded to form a small packet (Fig. 23.1a).
2. Immerse the spore packet in a 150-ml crystallizing dish containing about 60 ml 70% (v/v) ethanol for 30 s (Fig. 23.1b).

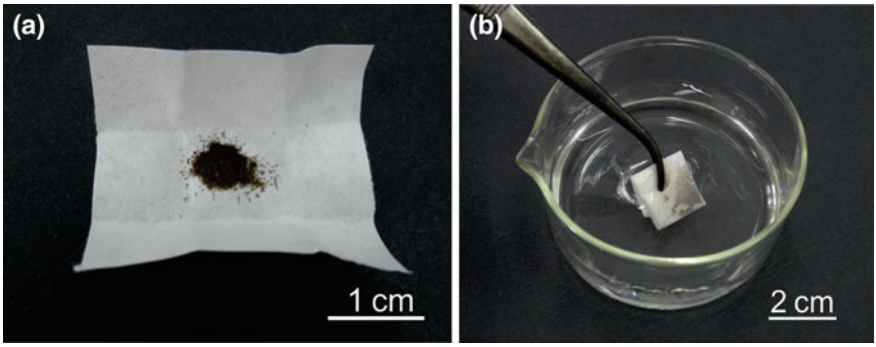

Fig. 23.1 Spore disinfection. **a** Preparation of a small packet with spores; **b** disinfection of the wrapped spores

3. Remove ethanol and treat the packet with 5% (v/v) commercial bleach (Domestos) for 20 min.
4. Wash the packet 3 times with sterile distilled water, 5 min each time.
5. Blot the spores from the packet onto GM medium (Table 23.1) in Ø9 cm plastic Petri dishes.
6. Leave the spores to germinate in a tissue culture chamber at 22 ± 1 °C, under a 16/8 h photoperiod and at a light intensity of 3.5 µM m^{-2} s^{-1}.
7. When young gametophytes (Fig. 23.2a) develop, transfer them separately onto fresh GM medium.

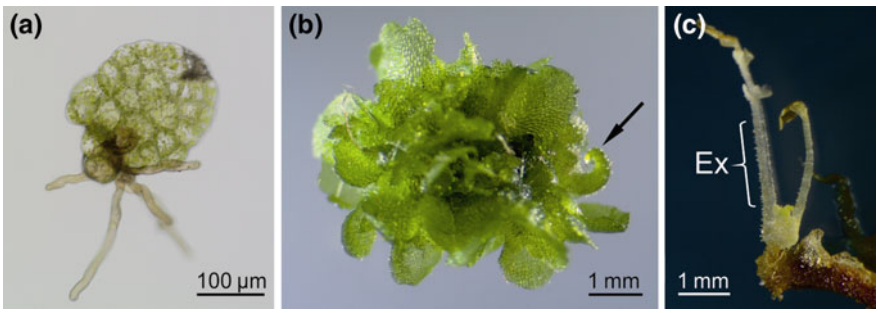

Fig. 23.2 Derivation of donor plant material for induction of the first cycle of somatic embryogenesis. **a** A young gametophyte of *C. delgadii* after 3 weeks from spore sowing; **b** intense gametophyte proliferation on 1/2 MS agar medium, *arrow* indicates the appearance of a crosier of a young sporophyte; **c** a young sporophyte obtained via syngamy, *Ex*—stipe fragment used as an initial explant for the first cyle of somatic embryogenesis

23.3.1.2 Sporophyte Initiation and Induction of the First Cycle of Somatic Embryogenesis

Carefully check the gametophyte culture for the appearance of sporophytes (Fig. 23.2b). Syngamy occurs spontaneously following development of archegonia and antheridia. In order to accelerate the process of gametophyte maturation, sex organ formation and fertilization, transfer gametophytes onto 1/2 MS medium either with concentration of NH_4NO_3 reduced to 412.5 mg/l or completely lacking NH_4NO_3. For the initiation of the first cycle of somatic embryogenesis, use young sporophytes at the first crozier stage (Fig. 23.2c).

1. Using sterile forceps and scalpel excise, under a dissecting stereo-microscope, a 2.5-mm-long stipe fragment from the base of the first frond and place this on IM medium (Table 23.2). Keep the cultures in a tissue culture chamber at 22 ± 2 °C in constant darkness and at a relative humidity of 35–55%.
2. After 2 weeks of culture, the first divisions of epidermal cells of stipe explants should be visible under a dissecting stero-microscope. The next few divisions, also perpendicular to the polar axis of the explant, result, within about 3 weeks following culture initiation, in the formation of linear somatic embryos. Subsequent anticlinal, periclinal and inclined cell divisions lead to the formation of four-segmented pro-embryos. Further development of somatic embryos results in differentiation of the embryonic leaf, then, emergence of the lamina primordium of the first frond, and finally, development of the shoot apex primordium.
3. During transition from embryo to juvenile sporophyte occurring in darkness, the lamina of the embryonic leaf becomes pale green, and the whole leaf elongates. The next steps of sporophyte development are the formation of the second frond and the elongation of the root.
4. Following elongation of the second leaf and the development of the third, the young etiolated sporophyte can be used as a source of explants for the second cycle of somatic embryogenesis.

23.3.1.3 Induction of the Second Cycle of Somatic Embryogenesis

For the initiation of the second cycle of somatic embryogenesis, use young etiolated, embryo-derived sporophytes which have developed 2-4 leaves (Fig. 23.3a).

1. Using sterile forceps and scalpel, excise 2.5-mm-long stipe fragments from the two youngest fronds under a dissecting stereo-microscope and place on IM medium. Keep the cultures in a tissue culture chamber at 22 ± 2 °C in constant darkness and at a relative humidity of 35–55%.
2. The initiation of the second cycle of somatic embryogenesis and the development of somatic embryos proceeds in similar manner to the first cycle, but is much more efficient since the percentage of responding explants in the second

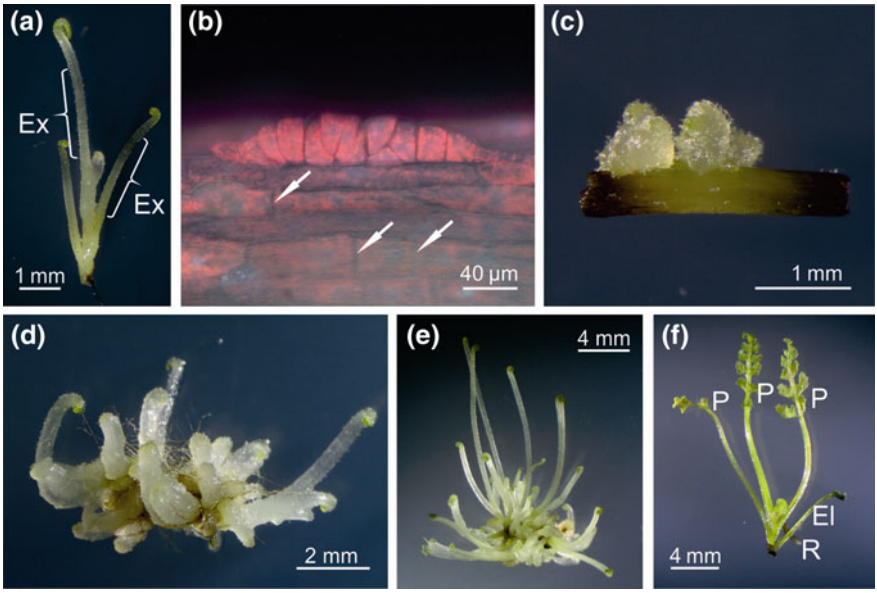

Fig. 23.3 The second cycle of somatic embryogenesis of *C. delgadii*. **a** Young, etiolated, somatic embryo-derived sporophyte, *Ex*—stipe fragments constituting initial explants for somatic embryogenesis; **b** formation of a linear pro-embryo from epidermal cells of stipe explants of *C. delgadii* after 2 weeks, *arrows* indicate cell divisions of epidermal cells perpendicular to the polar axis of the stipe; **c** somatic embryos at the late embryonic leaf stage after 4 weeks of culture; **d** very young sporophytes obtained after 2 months of culture; **e** young etiolated sporophytes with elongated leaves obtained after 4 months of culture in darkness; **f** single young sporophyte with embryonic leaf (*El*), three pinnate fronds (*P*) and a root (*R*) obtained after 2-month-long culture in darkness followed by another 2 months under photoperiod conditions

cycle of somatic embryogenesis is four-fold greater than the percentage of responding explants in the first cycle of somatic embryogenesis (Mikuła et al. 2015b).

3. First divisions of epidermal cells can already be observed after 10 days of culture; linear pro-embryos are evident even after 2 weeks (Fig. 23.3b). Somatic embryos reach the early and late embryonic leaf stage (Fig. 23.3c) after about 21 and 28 days of culture, respectively. A dozen or so young sporophytes can be obtained from one responding explant after 2 months (Fig. 23.3d).
4. When kept constantly in darkness, the somatic embryo-derived stipe explants are able to produce new somatic embryos that develop into etiolated sporophytes (Fig. 23.3e) within 10 months without any sub-culturing. The efficiency of the process increases gradually, eventually resulting in 80% of the explants producing, on average, 80 somatic embryos each between months 9 and 10 of culture.

5. After 7 months of culture the first sings of sporophyte senescence, such as browning of the fronds, become visible. However, these aging fronds can spontaneously produce new somatic embryos, directly on their laminae and stipes, or indirectly from embryogenic callus tissue, which can form on the surface of laminae and stipes.

23.3.1.4 Sporophyte Culture and Acclimatization

Sporophytes obtained from somatic embryos are able to turn green and grow normally following exposure to a 16/8 h photoperiod (Fig. 23.3f). They can develop into plants without sub-culturing but owning to their quick growth, they should be transferred to larger containers, e.g. glass jars (Fig. 23.4a). For acclimatization, sporophytes that were cultured under 16/8 h photoperiod condition should be used.

1. Transfer the sporophytes to pots containing an autoclaved mixture of peat and perlite (3:1) and maintain in mini greenhouses. Alternatively, sporophytes can be transferred firstly to jars with perlite and a small volume of liquid basal 1/2 MS medium, so as to promote root elongation (Fig. 23.4b), but this step is not obligatory.
2. Ventilate the mini greenhouses daily and mist the sporophytes periodically over a period of 4–6 weeks. By now, the plantlets should be ready to be transferred to a greenhouse (Fig. 23.4c).
3. During the next 6 months, the sporophytes grow to maturity and produce spores capable of germination.

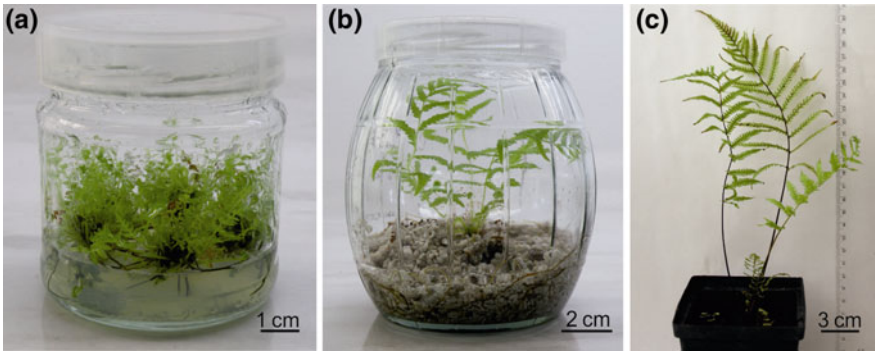

Fig. 23.4 Acclimatization of somatic embryo-derived sporophytes of *C. delgadii*. **a** Young, green young sporophytes after 2 months of culture under 16/8 h photoperiod conditions; **b** seven-month-old sporophytes in perlite, before acclimatization; **c** acclimatized sporophyte in a greenhouse

23.3.2 *Cryopreservation of* C. delgadii *Somatic Embryos by Encapsulation-Dehydration*

23.3.2.1 Encapsulation of Somatic Embryos

For encapsulation, somatic embryos of *C. delgadii* at the embryonic leaf stage should be used. Experiments conducted so far indicate that embryos at the late embryonic leaf stage survive both dehydration and the whole procedure of cryopreservation better than do embryos at the early embryonic leaf stage.

1. Gently detach the somatic embryos from stipe explants using forceps or dissecting needle.
2. Place the embryos in a 20 ml beaker, together with 3–5 ml of sodium alginate solution for 10 min.
3. Mix embryos with the solution and, using an automatic pipette whose tip has been cut off, make alginate beads by adding drops of the alginate solution (each containing 1–3 somatic embryos) to the $CaCl_2$ solution. Leave for 30 min.
4. Pour off the $CaCl_2$ solution and rinse the beads with a small volume of distilled water. Drain beads on sterile filter paper.

23.3.2.2 Preculture

As with gametophytes (Mikuła et al. 2011), somatic embryos of *C. delgadii* show a higher survival rate following cryopreservation if the pre-culture step is conducted on agar rather than in liquid medium.

1. Place alginate beads on PM medium (Table 23.2; 15 beads per Petri dish) and culture in darkness for 2 weeks at 22 °C.

23.3.2.3 Osmotic Dehydration

1. Place 50 alginate beads into a 250 ml Erlenmeyer flask containing 0.3 M sucrose solution (Table 23.2) and culture in darkness for 48 h on gyratory shaker (100 rpm) at 22 °C.
2. Replace the 0.3 M sucrose solution with 0.5 M sucrose solution and culture for 48 h.
3. Replace the 0.5 M sucrose solution with 0.75 M sucrose solution and culture for 48 h.
4. Replace the 0.75 M sucrose solution with 1.0 M sucrose solution and culture for 48 h.

23.3.2.4 Air Desiccation

The length of air-desiccation considerably affects the survival of somatic embryos following cryopreservation. A drastic reduction in embryos in the survival of embryos was observed following desiccation for 5 h. Conversely, desiccation for too short a period was equally destructive.

1. Pour off the 1.0 M sucrose solution and place the beads separately on inverted glass dishes or on ceramic plates.
2. Air-dry the beads in a laminar flow cabinet at room temperature for 4.5 h.

23.3.2.5 Freezing

1. Place the beads in 2-ml cryo-vials (12 beads per cryo-vial).
2. Freeze the cryo-vials by direct immersion in liquid nitrogen for at least 24 h.

23.3.2.6 Thawing and Post-freezing Culture

1. Thaw the cryo-vials in a water bath at 38 °C for 3 min.
2. Place the beads on GM medium supplemented with 2% sucrose and culture in darkness.
3. After 1 week of post-freezing culture, carefully examine all beads under the dissecting stereo-microscope in order to identify any pale green surviving fragments of somatic embryos. Regeneration of whole somatic embryos from surviving cells is evident after 2 weeks of culture (Fig. 23.5a). The young sporophytes grow shortly thereafter (Fig. 23.5b).
4. Extended post-freezing culture in darkness results in subsequent, abundant regeneration of further somatic embryos that develop into etiolated sporophytes (Fig. 23.5c, d).
5. When post-freezing culture is carried out under photoperiod conditions, only gametophyte regeneration occurs (Fig. 23.5e, f).

23.4 Conclusion and Future Prospects

To date, the use of somatic embryogenesis for plant production and genetic improvement has been restricted to spermatophytes. Here we provide a complete, simple, fast, and effective protocol for the propagation of a species of tree-fern by exploiting this same phenomenon. Currently, regeneration of *C. delgadii* sporophytes via somatic embryogenesis from epidermal cells of stipe explants is the most

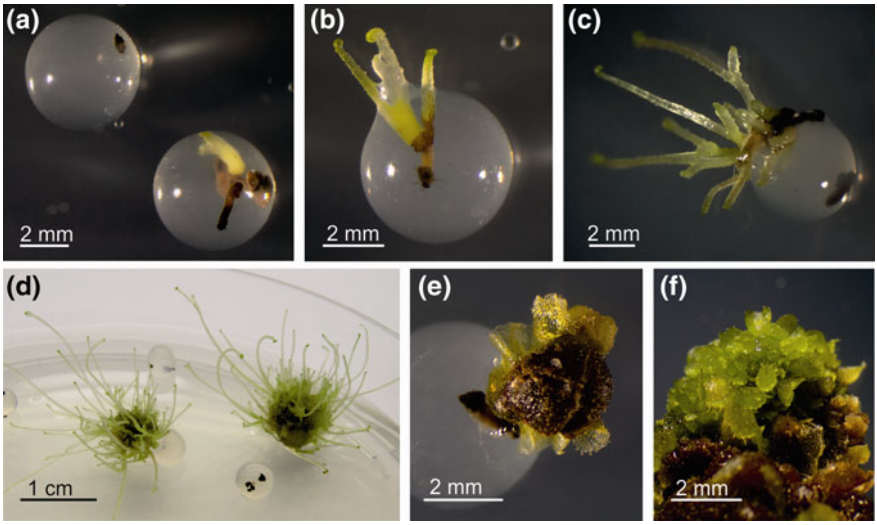

Fig. 23.5 Cryopreservation of *C. delgadii* somatic embryos. **a** Regeneration of somatic embryo in alginate bead after 3 weeks of post-freezing culture in darkness; **b** growth of a young sporophyte after 4 weeks of post-freezing culture in darkness; **c** young etiolated sporophytes after 4 and **d** 6 months of post-freezing culture in darkness; **e** regeneration of gametophytes after 3 and **f** 4 weeks of post-freezing culture under photoperiod conditions

investigated and best understood methodology. However, development of similar protocol for the efficient regeneration of *C. delgadii* somatic embryos from other types of explant is already well advanced and optimization of the cryopreservation of somatic embryos is also currently the subject of detailed study. Both techniques can be used to advantage for rapidly and reliably propagating and conserving tree-ferns and should now be extended to the propagation of other endangered and commercially valuable species.

References

Bittner J, Breckle S-W (1995) The growth rate and age of tree fern trunks in relation to habitats. Am Fern J 85:37–42. https://doi.org/10.2307/1547463

Bittner J, Trejos-Zelaya J (1997) Analysis of the vascular epiphytes of tree ferns in a montane rain forest in Costa Rica. Rev Matemática Teoría y Apl 4:63–73

Domżalska L, Kędracka-Krok S, Jankowska U et al (2017) Proteomic analysis of stipe explants reveals differentially expressed proteins involved in early direct somatic embryogenesis of the tree fern *Cyathea delgadii* Sternb. Plant Sci 258:61–76. https://doi.org/10.1016/j.plantsci.2017.01.017

Goller K, Rybczyński JJ (2007) Gametophyte and sporophyte of tree ferns in vitro culture. Acta Soc Bot Pol 76:193–199. https://doi.org/10.5586/asbp.2007.022

Grzyb M, Kalandyk A, Waligórski P, Mikuła A (2017) The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb. Plant Cell Tiss Org Cult 129:387–397. https://doi.org/10.1007/s11240-017-1185-8

Hiendlmeyer R, Randi AM (2007) Response of spores and young gametophytes of *Cyathea delgadii* Sternb. (Cyatheaceae) and *Blechnum brasiliense* Desv. (Blechnaceae) to different light levels. Acta Bot Bras 21:909–915. https://doi.org/10.1590/S0102-33062007000400015

Large MF, Braggins JE (2004) Tree Ferns. Timber Press

Mikuła A, Makowski D, Walters C, Rybczyński JJ (2011) Exploration of cryo-methods to preserve tree and herbaceous fern gametophytes. In: Fernández H, Kumar A, Revilla A (eds) Working with ferns: issues and applications. Springer, New York, pp 173–192. https://doi.org/10.1007/978-1-4419-7162-3_13

Mikuła A, Pożoga M, Grzyb M, Rybczyński JJ (2015a) An unique system of somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb.: the importance of explant type, and physical and chemical factors. Plant Cell Tiss Org Cult 123:467–478. https://doi.org/10.1007/s11240-015-0850-z

Mikuła A, Pożoga M, Tomiczak K, Rybczyński JJ (2015b) Somatic embryogenesis in ferns: a new experimental system. Plant Cell Rep 34:783–794. https://doi.org/10.1007/s00299-015-1741-9

Rybczyński JJ, Mikuła A (2011) Tree ferns biotechnology: from spores to sporophytes. In: Fernández H, Kumar A, Revilla A (eds) Working with ferns: issues and applications. Springer, New York, pp 135–147. https://doi.org/10.1007/978-1-4419-7162-3_10

Simabukuro EA, De Carvalho MA, Felippe GM (1998a) Reserve substances and storage of *Cyathea delgadii* Sternb. spores. Braz J Bot 21:149–152. https://doi.org/10.1590/S0100-84041998000200006

Simabukuro EA, Dyer AF, Felippe GM (1998b) The effect of sterilization and storage conditions on the viability of the spores of *Cyathea delgadii*. Am Fern J 88:72–80

Tryon RM, Tryon AF (1982) Cyatheaceae. Ferns and allied plants: with special reference to tropical America. Springer, New York, pp 166–212

Vital DM, Prado J (2006) Moss and liverwort epiphytes on trunks of *Cyathea delgadii* in a fragment of tropical rain forest, São Paulo State, Brazil. Trop Bryol 27:51–54

Chapter 24
Avocado (*Persea americana* Mill.)

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24.1 Introduction

Avocado (*Persea americana* Mill.) is an ancient tree species first domesticated by humans over 8000 years ago (Galindo-Tovar et al. 2008). Today, huge demand for avocado, particularly in the developed world, has made it one of the fastest growing horticultural industries globally. However, despite a 30% increase in production, to over 5 million tonnes/year, in the last decade, global producers are unable to keep pace with the booming demand (Fulgoni et al. 2013). Much of this results from the popularity of avocado as a luxurious yet healthy 'super food'. It is unique in containing all the essential macroelements (carbohydrates, proteins and fats) plus a wide spectrum of vitamins (A, B, C, D, E and K) and minerals (Pierce 1959). A high content of unsaturated fatty acids makes avocado even more valuable for human nutrition, as it facilitates the bioavailability of fat soluble vitamins and phytochemicals (Dreher and Davenport 2013). In addition, avocado has been shown to reduce cholesterol and blood pressure in animal studies (Salazar et al. 2005; Imafidon and Amaechina 2010).

The vast majority (70%) of avocado is produced in the Americas, predominantly by Mexico (FAOSTAT 2014), where it has become a high value cash crop. Mexico is also thought to be the center of origin of the species (Storey et al. 1986), although it is found naturally in almost all tropical and subtropical regions of the world (Scora and Bergh 1992; Koppa 1996). The cultivated *Persea* species, *Persea americana*, is one of over 81 species in the family Lauraceae, which boasts a rich genetic and physiological diversity (Ben-Ya'acov et al. 1992; Davis et al. 1998). Within *P. americana*, there are at least three races (Furnier et al. 1990); Mexican (var. americana), Guatemalan (var. guatamalensis) and West Indian (var. drymifolia);

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_24

although at least 3 additional races are suggested to exist (Furnier et al. 1990; Ben-Ya'acov and Michelson 1995; Silva and Ledesma 2014).

The diversity within *Persea* represents a huge resource for crop improvement. Preservation of this genetic diversity through global repositories or germplasm banks is recognized as key for safeguarding food and nutritional security in the context of climate change. Avocado seeds however, like for many tropical and subtropical crops, are recalcitrant to storage. Therefore, the world's avocado germplasm is maintained as living field repositories. These are under constant threat of natural disasters, pest and diseases (Villalobos et al. 1991; Ben-Ya'acov et al. 1992; Encina et al. 2014). In addition to the problems of preserving avocado diversity, utilizing this diversity in breeding programs for commercial uptake of new cultivars is difficult. This results from the long life-cycle of avocado plus its open pollination syndrome (protogynous dichogamy), which means new cultivars cannot be clonally propagated through seed (Zirari and Lionakis 1994; Silva and Ledesma 2014). Therefore, while a number of breeding programs have been initiated to improve disease and stress resistances, fruit yield, flavour, pulp composition, and post-harvest quality (Platt 1976; Escobedo and Escobedo 2010; Silva and Ledesma 2014), relatively few elite cultivars are in commercial production (Schaffer et al. 2013; Witney et al. 2017). The single fruiting cultivar, Hass, comprises 80% of the global avocado fruit trade, and this presents a significant risk to the global industry inherent to monocultures.

In order to best preserve global avocado diversity and, in future, utilize it for intensive selection and breeding programs, it is essential to develop improved technologies for avocado conservation, breeding/improvement and propagation (Furnier et al. 1990; Hiti-Bandaralage et al. 2017; Mhameed et al. 1997). In vitro somatic embryogenesis has direct importance to these objectives (Encina et al. 2014; Guan et al. 2016). Somatic embryogenesis is the process by which somatic cells give rise to totipotent embryogenic cells capable of becoming complete plants (Kulkarni et al. 2006), with each step in the process characterized by distinct biochemical and molecular events. Somatic embryogenesis can be a robust tool to regenerate genetically clonal plants from single cells chosen from selected plant material, or genetically engineered cells (Márquez-Martín et al. 2012). The application of somatic embryos to cryopreservation holds huge potential for safely and cost-effectively conserving the large number of avocado accessions relevant to breeding programs worldwide. This technique requires regenerative cells of an organism, which are stored at ultra-low temperatures (-196 ± 1 °C) using liquid nitrogen. Much interest has also been placed on applying somatic embryos to develop genetic transformation protocols for avocado improvement, directly targeting resistance to diseases including *Phytophthora* root rot, *Rosellinia* root rot, *Verticillium* wilt, anthracnose, *Cercospora* spot and sun blotch viroid (Sánchez-Romero et al. 2006). In addition, mutagenesis of somatic embryos under in vitro conditions (somaclonal variation) can be advantageous to create variability for selection. Recovery of plantlets from somatic embryos and clonal multiplication in vitro is an essential step for commercial application of this technology to crop improvement.

The first successful attempt at somatic embryogenesis in avocado was achieved by Pliego-Alfaro and Murashige in 1981 using immature zygotic embryos of cv. 'Hass' (Pliego-Alfaro and Murashige 1988). Since then, it has become clear that the embryogenic capacity of avocado is highly genotype dependent (Witjaksono and Litz 1999; Litz and Litz 2000). Moreover, optimization of nutrient media components, hormone type and concentration, type and concentration of gelling agent, and light intensity is also vital for successful embryo development (Mujib and Šamaj 2006). The type of embryonic cells (somatic embryo or proembryonic masses) as well as the inoculum density and suspension duration also affect the maturation of somatic embryos (Márquez-Martín et al. 2012). To date embryogenic culture development has predominantly used immature zygotic embryos, although nucellus tissues and protoplasts have also been used successfully (Jain et al. 1999). Somatic embryos have also been recovered even from genetically transformed embryogenic avocado callus (explants of immature fruit) (Cruz-Hernandez et al. 1998). As yet, however, neither leaf nor root tissue from any developmental stage of avocado have successfully produced embryogenic callus or induced direct embryogenesis.

Once somatic embryos are induced, regeneration from embryogenic cultures is still a major problem in most woody species (Jain et al. 1999; Jain and Gupta 2005; Márquez-Martín et al. 2012). The embryogenic regeneration pathway of avocado has been studied in some detail, with many reports that avocado somatic embryos can lose their morphogenic competence in as little as 3–4 months after induction depending on the genotype (Mooney and Staden 1987; Pliego-Alfaro and Murashige 1988; Witjaksono et al. 1998; Witjaksono and Litz 1999; Litz 2004; Kulkarni et al. 2006; Suarez et al. 2006). In addition to the loss of viability over time, the main factor limiting conversion of somatic embryos into plantlets is incomplete maturation (Ammirato 1986). During maturation somatic embryos accumulate or store products and change colour from translucent to white opaque (Cailloux et al. 1996). This morphological feature has been used as an indicator of the efficiency of maturation and regeneration (Witjaksono et al. 1998).

Two types of regeneration occur after maturation; unipolar (only shoot apex or root) and bipolar (both shoot apex and root). Shoots regenerated from unipolar embryos can either be rooted or rescued using in vitro micrografting (Raharjo and Litz 2005). The percentage of high quality bipolar embryos from avocado somatic embryos is extremely low at 2–3% or less and, again, is genotype dependent (Pliego-Alfaro and Murashige 1988; Witjaksono et al. 1998; Raharjo and Litz 2005). This low rate of somatic embryo conversion is currently the main bottleneck in avocado regeneration via somatic embryogenesis (Witjaksono and Litz 1999; Litz 2005).

This chapter presents improved protocols for somatic embryogenesis from zygotic embryos of immature fruit of four avocado cultivars, based on our original published work (Encina et al. 2014). The main stages; (1) induction, (2) maintenance and proliferation, (3) maturation and germination, (4) in vitro culture of recovered plants and (5) acclimatization, are described. Furthermore, we present a protocol for successful cryopreservation of these somatic embryos and post-freezing plant recovery, with direct application for future cryobank conservation of avocado germplasm.

24.2 Materials

24.2.1 Somatic Embryogenesis

1. Immature fruitlets (5–10 mm) of *Persea americana* cvs. 'Reed', 'Hass', 'Duke 7' and 'A10'.
2. Ethanol (70%), Sodium hypochlorite (0.5%), Tween-20 (0.1%), sterile distilled water, 125 mL Erlenmeyer flasks, 100 mL measuring cylinders.
3. Petri dishes, forceps, scalpel blades, 1–20 mL serological pipettes, and 10–1000 μL air-displacement piston pipettes and tips.
4. Dissecting microscope.
5. Picloram, 2,4-dicholrophenoxyacetic acid, 6-benzyladenine, Gibberellic acid, Indole-3-butyric acid, tissue culture agar (Sigma A-1296), Gellan gum (Gelrite® Merk), activated charcoal and sucrose.
6. Myo-inositol, Thiamine HCl, Nicotinic acid, Pyridoxine HCl, Glycine, Glutamine, Proline.
7. Laminar-flow hood with ultraviolet light.
8. Culture incubation chambers with F40 Gro-lux tubes (Sylvania Lighting Australia) and shaking incubators.
9. Culture media (see Tables 24.1 and 24.2).
10. Peat, perlite and vermiculite as potting media and 100 mL plastic pots.

24.2.2 Cryopreservation

1. Somatic embryos at proliferation stage of cvs. 'A10', 'Reed', 'Velvick' and 'Duke-7'.
2. Glycerol, ethylene glycol, dimethyl sulfoxide (DMSO), acetone and sucrose.

Table 24.1 Somatic embryo induction medium

Chemicals	Quantity in mg/L	Chemicals	Quantity in mg/L
$NaH_2PO_4.H_2O$	138	$Na_2MoO_4.2H_2O$	0.25
KNO_3	2527.50	$CoCl_2.6H2O$	0.025
$CaCl_2.2H_2O$	147	$CuSO_4.5H_2O$	0.025
$(NH_4)_2SO_4$	132.2	Myo-inositol	100
$MgSO_4.7H_2O$	246.50	Thiamine HCl	4
KI	0.83	Picloram	0.1
$ZnSO_4.4H_2O$	8.6	Sucrose	30,000
$MnSO_4.4H_2O$	22.3	Agar	8000
H_3BO_3	6.2	pH	5.7

This media is a modification of B5 major salts (Gamborg et al. 1968) and MS minor salts (Murashige and Skoog 1962); pH was adjusted to 5.7 with 1 M NaOH or 1 M HCl prior to autoclaving at 121 °C for 20 min. 15 mL aliquots poured under sterile conditions into 60 mm × 15 mm Petri plates

Table 24.2 Maintenance and proliferation media

MSP Witjaksono and Litz (1999)		MMSE Pateña et al. (2002)	
Chemicals	Quantity in mg/L	Chemicals	Quantity in mg/L
KNO_3	1900	$NaH_2PO_4.H_2O$	138
$CaCl_2.2H_2O$	440	KNO_3	2527.50
$MgSO_4.7H_2O$	370	$CaCl_2.2H_2O$	147
KH_2PO_4	170	$(NH_4)_2SO_4$	132.2
Na_2.EDTA	37.3	$MgSO_4.7H_2O$	246.50
KI	0.83	KI	0.83
$ZnSO_4.4H_2O$	8.6	$ZnSO_4.4H_2O$	8.6
$MnSO_4.4H_2O$	22.3	$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2	H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25	$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025	$CoCl_2.6H2O$	0.025
$CuSO_4.5H_2O$	0.025	$CuSO_4.5H_2O$	0.025
Myo-inositol	100	Na_2.EDTA	37.3
Thiamine HCl	4	Myo-inositol	100
Picloram	0.1	Thiamine HCl	1
Sucrose	30,000	Nicotinic acid	0.5
Agar	8000	Pyridoxine HCl	0.5
pH	5.7	Glycine	2
		Glutamine	400
		2,4-dichlorophenoxy acetic acid	0.5
		Sucrose	60,000
		Coconut water*	100 (mL/L)
		Gellan gum	2500
		pH	5.7

*Coconut water is given in mL/L. Mango shoot proliferation (MSP) is based on Murashige and Skoog (1962) and Mango medium for somatic embryogenesis (MMSE) is based on Pateña et al. (2002). The pH was adjusted to 5.7 with 1 M NaOH or 1 M HCl prior to autoclaving at 121 °C for 20 min. Aliquots of 30 mL poured under sterile conditions into 90 mm × 15 mm Petri plates

3. Liquid nitrogen (LN) and 1 L canister.
4. Fluorescein diacetate (FDA) stain (Sigma-Aldrich).
5. MMSE media excluding gelling agents and growth regulators (Table 24.2).
6. Sterile Disposable Petri dishes, sterile cryo tubes (2 mL).
7. Aluminum foil strips (8 mm × 25 mm) dry sterilized.
8. Watch glasses, forceps, scalpel blades, 1–20 mL serological pipettes, and 10–1000 μL air-displacement piston pipettes and tips and Parafilm®.
9. Myo-inositol, Thiamine HCl, Nicotinic acid, Pyridoxine HCl, Glycine, Glutamine, Proline.
10. Laminar-flow hood with ultraviolet light.
11. Culture incubation chambers with F40 Gro-lux tubes (Sylvania Lighting Australia) and shaking incubators.
12. UV illumination and yellow filter for visualization.

The loading solution (LS: 2 M glycerol, 0.4 M sucrose and MMSE basal salts), PVS2 (30% v/v glycerol, 15% v/v ethylene glycol, 15% v/v DMSO, 0.4 M sucrose and MMSE basal salts) and unloading solution (MMSE basal + 1.2 M sucrose) are prepared as liquid media. The pH is adjusted to 5.7 with 1 M NaOH or 1 M HCl prior to filter sterilization (Millipore 0.22 μM filter top unit) in a laminar flow cabinet. Solid MMSE media excluding plant growth regulators are prepared, pH is adjusted to 5.7 with 1 M NaOH or 1 M HCl prior to autoclaving at 121 °C for 20 min. Aliquots of 30 mL are poured under sterile conditions into 90 mm × 15 mm Petri plates.

24.3 Method

Zygotic embryos were extracted from immature fruits (5–10 mm long) of avocado (*Persea americana* Mill.) cvs. 'Reed', 'Hass', 'Duke 7', 'Velvick' and 'A10' following the protocols of (Witjaksono and Litz 1999) (Fig. 24.1a–c).

Induction of somatic embryogenesis from zygotic embryos of immature fruits includes: (1) surface disinfestation of ex-plants and embryogenic culture induction, (2) maintenance and proliferation of embryogenic cultures, (3) embryo maturation and germination (4) in vitro culture of regenerated plants and (5) acclimatization and field transfer.

24.3.1 Surface Disinfestation of Immature Fruits

1. Harvest fruits 5–10 mm in size from the field at the start of the growing season; fruits should be left at room temperature before dissection.
2. Remove pendula from the end of the fruit.
3. Wash the immature fruits with a detergent such as Microshield® (2% Chlorhexdine) and rinse in running tap water for 20 min.
4. In a laminar-flow hood, immerse immature fruits in a solution of 0.5% (v/v) Sodium hypochlorite and 0.1% (v/v) Tween-20 for 10 min.
5. Rinse three times with autoclaved distilled water, still in laminar flow hood.

24.3.2 Extraction of Zygotic Embryos and Embryogenic Culture Initiation

1. In the laminar-flow hood, place fruit in a sterile Petri dish base.
2. Remove the chalazal end of the ovule.
3. Make an incision on the pericarp to pare away the layers and reveal the embryo sac.
4. Under the dissecting microscope, using a scalpel blade, carefully lift out the embryo (0.6–0.8 mm) embedded in gelatinous endosperm of the ovules' micropylar end.

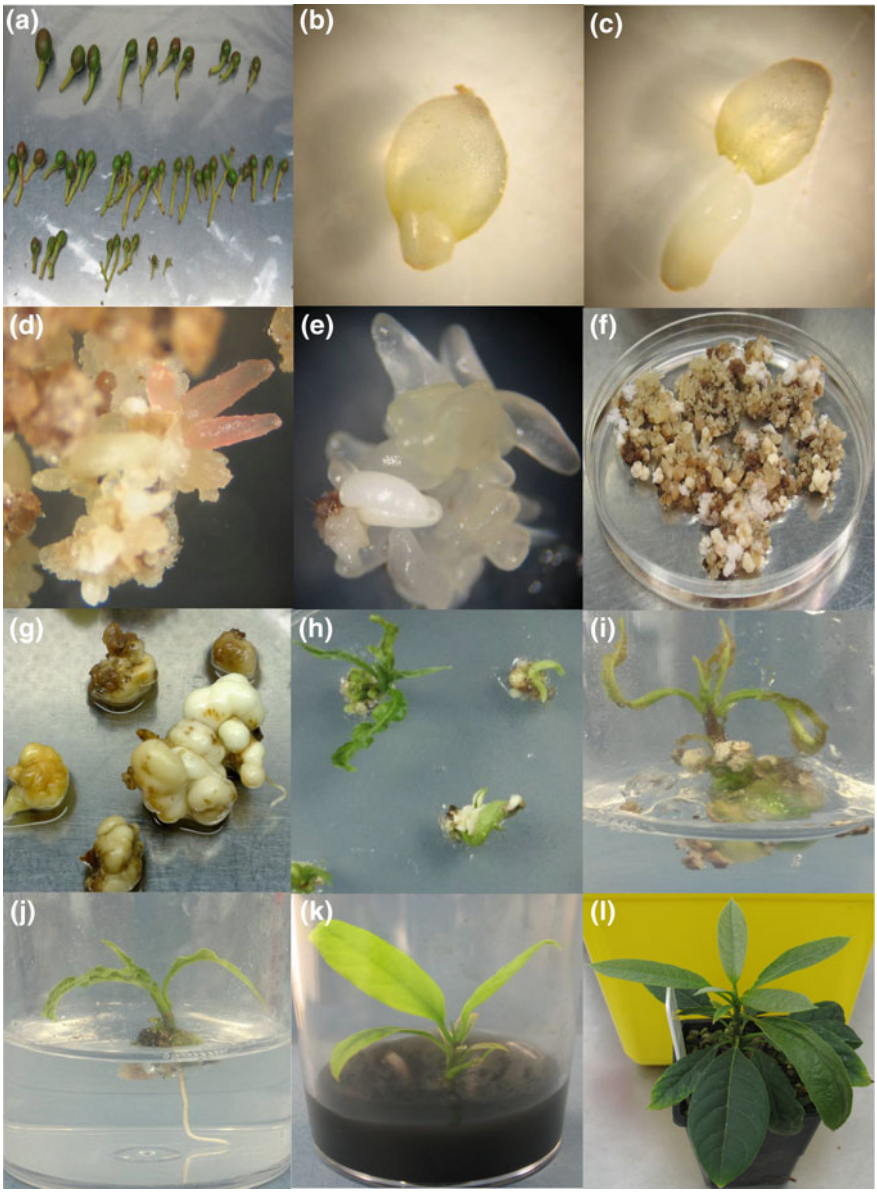

Fig. 24.1 Induction, multiplication, maintenance and regeneration of 'Reed' avocado somatic embryos. **a** Immature avocado fruitlets of 5–10 mm in size; **b**, **c** immature avocado embryos removed from embryo sac; **d** primary somatic embryo; **e** secondary somatic embryo; **f** somatic embryos on proliferation MMSE; **g** white-opaque somatic embryos on embryo development media; **h** germinating embryos; **i** unipolar shoot; **j** bipolar shoot; **k** complete plantlet on medium supplemented with activated charcoal; **l** acclimatized plant

5. Immediately place the isolated zygotic embryo horizontally on the surface of a 60 × 15 mm Petri plate containing somatic embryo induction media and seal the plates with Parafilm®.
6. Incubate the cultures in darkness at 25 ± 1 °C for 3 months.

24.3.3 Maintenance and Proliferation of Embryogenic Cultures

Tissues from immature zygotic embryos develop into either somatic embryos and/or secondary embryos (pro-embryonic masses) (Guzmán-García et al. 2013). Depending on the cultivar, proliferation media composition (MSP or MMSE) can be used to maintain and proliferate proembryonic masses from primary somatic embryos that were established on embryo induction media.

1. Transfer white-opaque somatic embryos and pro-embryogenic masses (Approximately 50–100 mg) to proliferation media (30 mL in 90 mm × 15 mm Petri plates). Discard necrotic cells with black to dark brown appearance. When transferring, distribute somatic embryos evenly on the surface of the media. Either of two different proliferation media compositions (MSP and MMSE) can be used (Table 24.2).
2. Seal the plates containing the embryos with Parafilm® and incubate in darkness at 25 ± 1 °C (Table 24.3).
3. The culture vigour is maintained by sub-culturing every four weeks on fresh MSP or MMSE.

NOTES: As shown by Witjaksono and Litz (1999) and Encina et al. (2014) prolonged subculture on MSP media will result in a gradual decrease in multiplication rates and fail to recover viable somatic embryos. According to Encina et al. (2014) somatic embryos die out after 11 months in vitro for some cultivars. Meanwhile, proliferation on MMSE media sustained a higher rate of multiplication during the 11 months compared to MSP media for 4 different avocado cultivars (Fig. 24.2). 'Reed' showed a lower multiplication rate on MSP media throughout, but increased proliferation rates during 5–11 months on MMSE media. 'A10' showed good proliferation rates from 3–10 months on MMSE while it declined on MSP. 'Hass' and 'Duke 7' showed a lower multiplication rate on MSP as compared to MMSE during the 11 months. To maintain viability on MMSE somatic embryos were routinely sub-cultured every 4 weeks. The main differences between MSP and MMSE medium are listed in Table 24.4.

Somatic embryos of all four cultivars differed in their size, as shown in Fig. 24.3. 'Duke-7' and 'A10' somatic embryos were noticeably the smallest and largest in size, respectively. The size of 'Reed' somatic embryos ranged from medium to large and 'Velvick' somatic embryos showed maximum variation from small to large.

Table 24.3 Embryo development media

Chemicals	Quantity in mg/L	Chemicals	Quantity in mg/L
KNO_3	1900	Myo-inositol	100
$CaCl_2.2H_2O$	440	Thiamine HCl	4
NH_4NO_3	1650	6-benzyleadenine	0.5
$MgSO_4.7H_2O$	370	Gibberellic acid	1.0
KH_2PO_4	170	Coconut water*	100 mL/L
Na_2.EDTA	36.7	#Proline	400
KI	0.83	#Glutamine	1000
$ZnSO_4.4H_2O$	8.6	Sucrose	45,000
$MnSO_4.4H_2O$	22.3	Gellan gum	6000
H_3BO_3	6.2	pH	5.7
$Na_2MoO_4.2H_2O$	0.25		
$CoCl_2.6H_2O$	0.025		
$CuSO_4.5H_2O$	0.025		

*Coconut water is given in mL/L

#Either Glutamine at 1000 mg/L or Proline at 400 mg/L is added

The pH was adjusted to 5.7 with 1 M NaOH or 1 M HCl prior to autoclaving at 121 °C for 20 min. Aliquots of 40 mL poured under sterile conditions it to 100 mm × 20 mm Petri plates

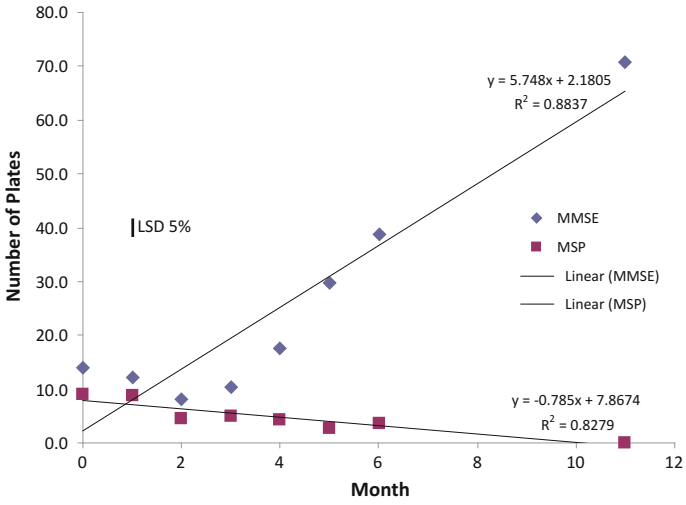

Fig. 24.2 Linear regression trend lines for the effect of media on the multiplication rates of avocado somatic embryos of the four cultivars over a period of 11 months. The plot shows that the difference between the two media was significant after 3 months with a negative slope on MSP and a positive slope on MMSE

Table 24.4 Comparison of MSP and MMSE medium

Medium composition	MSP	MMSE
Basal medium	Murashige and Skoog Macros	Gamborg B5 Macros
Gelling agent	Agar	Phytagel
Coconut water	No	Yes
Auxin used	Picloram	2-4D

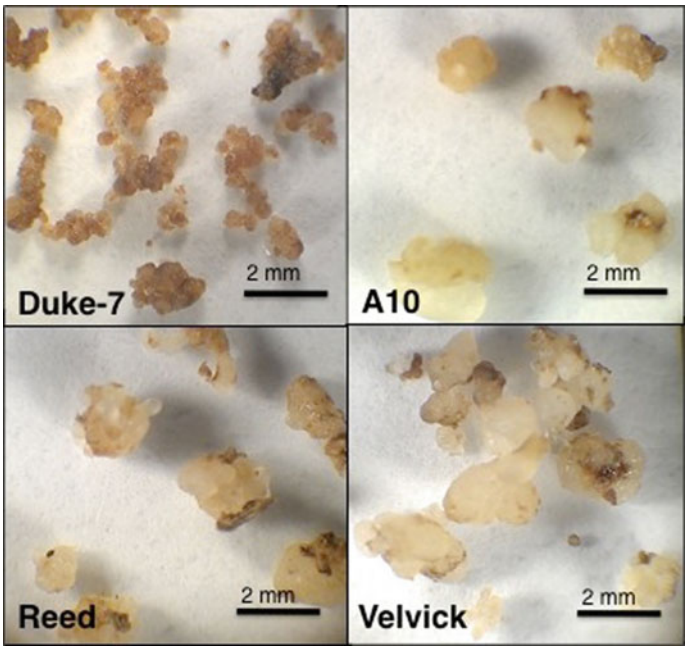

Fig. 24.3 Comparisons of somatic embryo size of cvs. 'Duke-7', 'A10', 'Reed' and 'Velvick' on MMSE medium after 2 weeks of subculture

24.3.4 Maturation and Germination (One-Step) of White-Opaque Somatic Embryos

1. Select white-opaque somatic embryos of approximately 5–8 mm diameter from actively growing cultures on MMSE and transfer to solid embryo development media (Table 24.3) supplemented with either glutamine 1000 mg/L or proline 400 mg/L, 40 mL in 100 mm × 20 mm Petri plates. Seal the plates with Parafilm®.
2. Incubate plates in culture room at 25 ± 1 °C, under standard light conditions of 40 molm/s using cool white fluorescent tubes (F40 tubes Gro-lux, Sylvania Lighting Australia) for 6 weeks.

3. Subculture to fresh embryo development media at six weeks.
4. Results should be recorded after 12 weeks growth in terms of somatic embryo survival, greening average (green embryos vs. white embryos), unipolar (corresponding to shoot only regeneration) and bipolar regeneration (shoot + root regeneration).

NOTES: White-opaque somatic embryos of cultivar 'Reed' were used for germination protocols. Embryo development media devoid of amino acids (proline and glutamine) supplements can result in 95% survival of somatic embryos. However, they do not develop into unipolar or bipolar individuals. Unipolar and bipolar development can be achieved by either adding proline or glutamine individually to the media. In our hands, incorporating 400 mg/L proline resulted in a maximum of 12.5% unipolar shoot regeneration and 5% bipolar shoot regeneration. The survival rates can be over 85%, with 65–93% becoming green during the incubation period. Glutamine alone in 1000 or 2000 mg/L resulted in 10% unipolar shoot regeneration and no bipolar shoots (Encina et al. 2014). Glutamine concentrations beyond 4000 mg/L did not produce any regenerated shoots. Often avocado shoots regenerated from somatic embryos display hyperhydricity symptoms that will disappear during sub-culture. Also, other abnormalities such as scythe leaves (Fig. 24.4d), apical necrosis (Fig. 24.4e) and callus overgrowth (Fig. 24.4c) prevail as a result of high concentration of GA_3 in embryo development media.

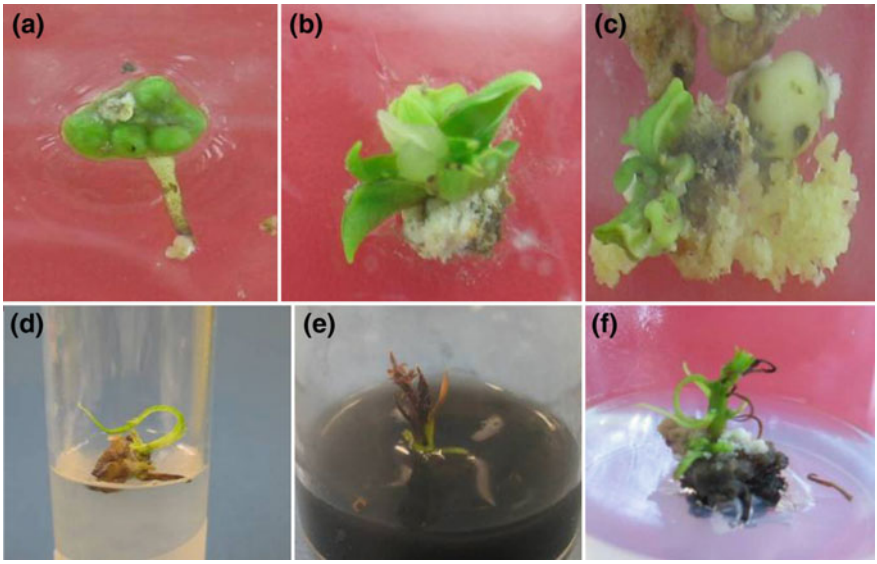

Fig. 24.4 Abnormalities shown by avocado somatic embryos regenerates. **a** Root without shoot **b** callus overgrowth **c** secondary somatic embryo growth without shoot production **d** scythe leaves **e** apical necrosis and **f** abnormal shoot

24.3.5 Germination in Two-Step Regeneration System

1. Select white-opaque somatic embryos with approximately 5–8 mm diameter from actively growing cultures on MMSE and transfer to 25 mL of liquid embryo development media of same composition supplemented with either 1000 mg/L glutamine or 400 mg/L proline in an Erlenmeyer flask.
2. Maintain flasks in a rotary shaker at 80–85 rpm for 12–15 days.
3. Transfer the white-opaque somatic embryos to solid embryo development medium and maintain in culture room at 25 ± 1 °C, under standard light conditions of 40 molm/s using cool white fluorescent tubes (F40 tubes Gro-lux, Sylvania Lighting Australia) for 6 weeks. Subculture to fresh embryo development media at six weeks.
4. Results were recorded after 12 weeks in terms of somatic embryo survival, average number of green embryos, unipolar (shoot-only) regeneration, or bipolar regeneration (shoot + root).

NOTES: White-opaque somatic embryos are germinated in a two-step procedure; using liquid phase for 12–15 days followed by culture in solid phase for 12 weeks with a subculture onto the same media at the 6 week period. Adding either proline or glutamine in media produced different outcomes in regeneration and germination. Introduction of liquid phase with no proline or glutamine resulted in 29% plant regeneration. Incorporation of 400 mg/L proline or 1000 mg/L glutamine during both phases (liquid and solid) improved germination. Proline at 400 mg/L with the two-step procedure resulted in 35% plantlet regeneration while glutamine at 1000 mg/L could regenerate 58.3% of white somatic embryos into plantlets for cultivar 'Reed', of which 43.3% were bipolar (Fig. 24.1i–h).

24.3.6 Culturing Regenerated Plantlets

Regeneration from white-opaque somatic embryos will give rise to both bipolar and unipolar plantlets. Root regeneration of unipolar shoots is needed in vitro through root induction methods. Unipolar shoots can also be rescued through micro-grafting to an in vitro germinated rootstock (cultivars must be graft compatible).

1. Subculture bipolar plants recovered from two-step germination procedure in 60 mL of media (embryo development medium excluding growth hormones and including 1 g/L charcoal) for 15 days in 120 mL plastic tissue culture containers.
2. Culture unipolar plants in MSP media, minus picloram, but supplemented with 25 mg/L indole-3-butyric acid, for 3 days in plastic tissue culture jars. Then transfer to the same media composition excluding indole-3-butyric acid but supplemented with 1 g/L activated charcoal for root induction.

24.3.7 Acclimatization of Regenerated Plants

In vitro generated plants from somatic embryos exhibit structural and functional differences to plants grown in the open environment. Plants under in vitro conditions have less available CO_2 due to enclosed sterile containment and are supplied with a saccharide based carbon source forcing them to be semi autotrophic (Lesar et al. 2012). Many anatomical features required to survive in the harsh natural environment, such as a fully developed cuticle and adequate amount of stomata, as well as basic plant functional units for photosynthesis such as a well-defined palisade and amount of spongy parenchyma, are altered under in vitro conditions (Wetzstein and Sommer 1982; Fabbri et al. 1986). Therefore, gradual acclimatization of shoot and root to dynamic external environmental factors is essential.

1. De-flask plantlets (about 3 cm height) and remove all nutrient media by washing root system with running tap water.
2. Submerge plantlets a 1 g/L solution of Fongarid® (Systemic fungicide) for 10 min.
3. Prepare potting media by mixing fine potting mix, vermiculite and perlite (1:1:1) and fill into 50 mL pots (mix should be sterilized prior to use).
4. Plant the fungicide treated plants and water the pot to its field capacity.
5. Place individual pots in a sealable plastic bag and maintain sealed for 2 weeks inside a growth cabinet at 25 ± 1 °C, 16 h light under 78% relative humidity (RH).
6. After 2 weeks remove the plastic bag and maintain plants in the growth cabinet for 4 weeks before transferring to glasshouse conditions.

24.3.8 Glasshouse Acclimatization and Field Transfer

1. Transfer plants to square plastic pots (e.g. Fisher Scientific) containing a perlite: peatmoss: vermiculite (1:1:1 v/v) mixture and place in a greenhouse.
2. Allow plants to grow to ~100 cm in height before planting in the field (throughout the process transfer plants through a decreasing % of shade cloth until full sun is achieved).
3. Plant in the field by digging an appropriate size hole. Remove the pot and pre soak roots in a solution of Agri-phos 600 (600 g/L Phosphorus Acid) for a few minutes. Place plant in the hole and backfill the hole carefully with soil.

24.3.9 Cryopreservation of Somatic Embryos

Cryopreservation, coupled with advancement in tissue culture and marker technologies to tag the germplasm, offers an attractive approach to conserve germplasm. To date somatic embryos of almost 40 different species of crop, fruit and forest trees have been successfully cryopreserved (Bertrand-Desbrunais et al. 1989; Mycock et al. 1995;

Pérez et al. 1998; Dulloo et al. 2009; Engelmann 2011). Cryopreservation is associated with advantages such as prolonged storage life, less storage space requirements, reduced cost of maintenance and reduced duplication in collection (Bouman and de Clerk 1990; Grout and Crisp 1995; Engelmann 2004; Panis and Lambardi 2005; Benson 2008; Kaity et al. 2008; Kaczmarczyk et al. 2012). Moreover, this overcomes the problem of seed storage recalcitrance for many tropical tree species including avocado, and offers a sustainable alternative to field repositories. Cryopreservation of avocado somatic embryos offers an attractive pathway to conserve avocado germplasm. Though heterozygous in nature when regenerated using zygotic embryos as explants, avocado somatic embryos can be a useful material in the absence of methods for cryopreservation of true somatic tissues such as shoot tips.

According to literature, cryopreservation of avocado somatic embryos has had limited success. Efendi (2003) described the effect of cryogenic storage on five avocado cultivars ('Booth 7, 'Hass', 'Suardia', 'Fuerte' and 'T362) using two cryopreservation protocols (controlled-rate freezing and classical-vitrification). In terms of controlled-rate freezing, three out of five embryogenic cultivars were successfully cryopreserved with a recovery of 53–80%. Using classical vitrification, cultivar 'Suardia' had 62% recovery whereas 'Fuerte' had only a 5% recovery. Guzmán-García et al. (2013) employed a droplet-vitrification method on two 'Duke-7' embryogenic cell lines that gave promising results ranging from 78 to 100% recovery for both lines. Protocols employed in both studies cannot be applied in general to multiple cultivars pointing to the need for more intensive research in this field.

This chapter describes the protocol developed by O'Brien et al. (2016) and includes two cryopreservation protocols; cryovial and droplet-vitrification, for the conservation of avocado somatic embryos applicable to multiple cultivars. Optimizations included; loading sucrose concentration, treatment temperature and duration of plant vitrification solution 2 (PVS2). Viability achieved ranged from 59 to 100% with cryovial and droplet-vitrification methods with somatic embryos of cultivars 'A10', 'Reed', 'Velvick' and 'Duke-7' after short and long-term LN exposure.

24.3.10 Loading of Somatic Embryos and Vitrification Optimization with PVS2

1. Dispense 30 mL of freshly prepared LS in a sterile petri dish.
2. Place somatic embryos in a petri dish containing LS.
3. Incubate somatic embryos in LS for a pre-optimised time (Time of exposure cultivar dependant, e.g. refer to Fig. 24.6 for relevant cultivar times).
4. Pre-chill freshly prepared PVS2 solution to 0 ± 1 °C.
5. Remove LS using a pipette from somatic embryo petri dishes.

6. Dispense 30 mL of pre-chilled PVS2 into the petri dishes containing loaded somatic embryos.
7. Incubate somatic embryos in PVS2 solution for the pre-optimised time for different cultivars (e.g. refer to Fig. 24.5).
8. Treat somatic embryos in unloading solution for 20 min.
9. Carefully inoculate the somatic embryos in clumps onto petri dishes containing gelled MMSE.
10. Seal the plates with Parafilm® and incubate in darkness at 25 ± 1 °C.
11. Assessment for viability can be carried out for new growth/proliferation after 20 days (Fig. 24.5).

NOTES: Different cultivars showed varied viability results for PVS2 treatments. Cultivar 'A10' recorded viability of 73% at 0 ± 1 °C treated up to 60 min and declined drastically thereafter. At 25 ± 1 °C PVS2 exposure for just 30 min caused significant decrease in viability of 'A10' (47%). Cultivar 'Reed' had 80% viability for PVS2 at 0 ± 1 °C up to 60 min and declined thereafter. When incubated at 25 ± 1 °C, for 60 min viability was 67% but further exposure caused significant reduction in viability. Cultivar 'Velvick' at 0 ± 1 °C for an incubation period of 60 min resulted 86% viability. At 25 ± 1 °C and 30 min incubation reduced the viability to 47%. Overall the best temperature for all cultivars tested was 0 ± 1 °C.

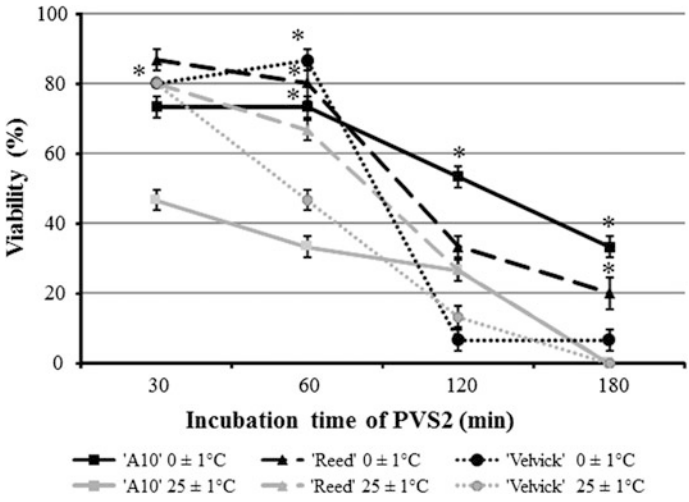

Fig. 24.5 Viability (%) of 'A10,' 'Reed,' and 'Velvick' avocado somatic embryos after exposure to PVS2 for different time durations, at 0 ± 1 °C and 25 ± 1 °C. Data were recorded 20 days after treatment. Values are means of 15 biological replicas. Vertical bars represent SEM

24.3.11 Cryovial-Vitrification of Somatic Embryos Using PVS2

Once the loading and vitrification of somatic embryos has been optimized with PVS2 for your cultivar of interest, follow the next steps for cryovial-vitrification.

1. Place somatic embryos prepared using Steps 1–7 above into 2 mL of pre-chilled fresh PVS2 in cryo tubes.
2. Seal cryo tubes and plunge into LN. Leave in LN for desired time of cryopreservation (minimum 60 min when testing protocols).
3. For recovery from cryopreservation, remove cryo tubes from LN. Treat somatic embryos in unloading solution for 20 min. Replace unloading solution three times within the 20 min period and gently swirl intermittently.
4. Inoculate somatic embryos in clumps onto Petri dishes containing gelled MMSE. Remove excess unloading solution with a sterile pipette.
5. Seal the plates containing the embryos with Parafilm® and incubate in darkness at 25 ± 1 °C.
6. Assessment for viability can be carried out for new growth/proliferation after 14 days (Fig. 24.8b), however results were taken after 5 weeks of growth (Fig. 24.6).

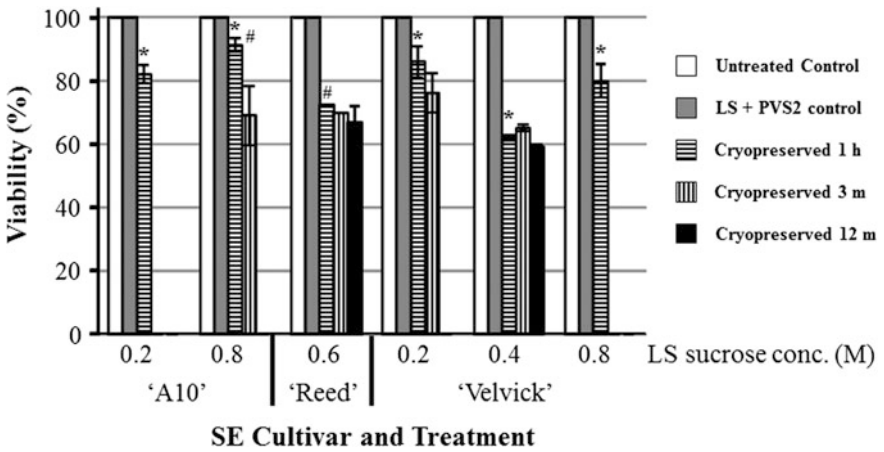

Fig. 24.6 Viability (%) of 'A10', 'Reed' and 'Velvick' SE after application of the cryovial-vitrification method. SE were in LS with different concentrations of sucrose and different incubation times of PVS2 (60 min for 'A10' and 'Velvick'; 30 min for 'Reed') with (+LN) or without (−LN) immersion in LN for 60 min, 3 or 12 months. Data was recorded 5 weeks after treatment. Values are means of 15 biological replicas ± SEM (Standard error of mean). (*) significance level of $p \leq 0.05$; a Tukey's multiple comparison test was used except for samples less than 4 in which case an unpaired t test was performed

24.3.12 Droplet-Vitrification of Somatic Embryos Using PVS2

Once the loading and vitrification of somatic embryos has been optimized with PVS2 for your cultivar of interest, follow the next steps for droplet-vitrification.

1. Pre-chill Aluminium foil strips in the freezer just before starting the experiment.
2. Dispense 30 mL of freshly prepared LS into a sterile petri dish.
3. Place somatic embryos in the petri dish containing LS.
4. Incubate somatic embryos in the LS for a pre-optimised time (Time of exposure cultivar dependant, see Fig. 24.9).
5. Pre-chill freshly PVS2 and a few petri dishes to 0 ± 1 °C.
6. Remove LS using a pipette from somatic embryo petri dishes.
7. Dispense 30 mL of pre-chilled PVS2 into the petri dishes containing loaded somatic embryos.
8. Incubate somatic embryos in PVS2 for the pre-optimised time for different cultivars (see Fig. 24.9).
9. Place Aluminium foil on an ice block and pipette out fresh PVS2 droplets of 15–25 μL without letting the cooled PVS2 warm up (Fig. 24.7).
10. Place somatic embryos in the droplets of PVS2 and top up droplets with pre-chilled PVS2 making sure entire somatic embryo is encapsulated.
11. Place the foil strip with encapsulated somatic embryos in a LN-chilled cryo tube by carefully inserting the foil strips while holding the cryo tube in a horizontal position.
12. Seal the cryo tube and plunge into LN, still horizontally. Leave in LN for desired time of cryopreservation (minimum of 60 min when testing protocols).

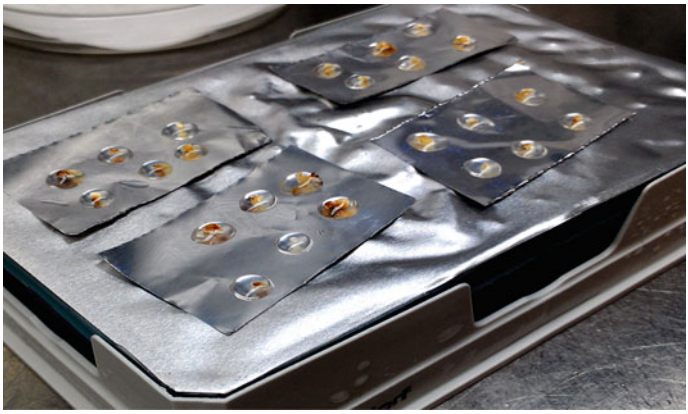

Fig. 24.7 Somatic embryos suspended in droplets of PVS2 on aluminum foil strips

13. To regenerate plants post cryopreservation, take cryo tubes out of LN. Treat somatic embryos in unloading solution for 20 min. Replace unloading solution three times within the 20 min period and gently swirl intermittently.
14. Inoculate clumps of somatic embryos on petri dishes containing MMSE. Remove excess unloading solution with a sterile pipette.
15. Seal the plates containing the embryos with Parafilm® and incubate in darkness at 25 ± 1 °C.
16. Viability of somatic embryos are assessed for new growth/proliferation after 14 days (Fig. 24.8b), however results were taken after 5 weeks growth (Fig. 24.9).

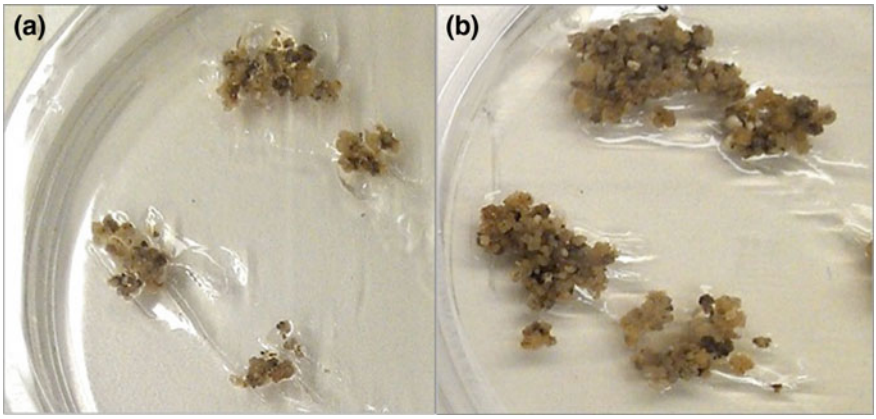

Fig. 24.8 Somatic embryo clumps inoculated **a** immediately after LN treatment and **b** 14 days after LN treatment

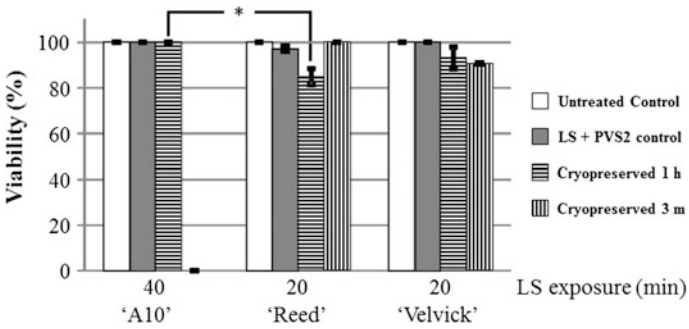

Fig. 24.9 Viability (%) of 'A10', 'Reed,' and 'Velvick' SE after application of the droplet-vitrification method, in LS with 0.2 M sucrose for 20 or 40 min, followed by incubation in PVS2 at 0 ± 1 °C for 60 min, with (+LN) or without (−LN) immersion in LN for 60 min or 3 months. Data was recorded 5 weeks after treatment. Values are means of 15 biological replicas ± SEM. (*) indicate a significance level of $p \leq 0.05$; a Tukey's multiple comparison test was performed

24.3.13 FDA Staining for Viability Post Cryopreservation

1. Prepare a 1% (w/v) stock of Fluorescein diacetate (FDA) stain (Sigma-Aldrich) in acetone.
2. Dilute 1% FDA stock solution to a 2% (v/v) working solution in water.
3. In a watch glass place the somatic embryos (at any stage in which viability to be tested) and add the stain directly and incubate at room temperature for 5 min in the dark.
4. Visualize through a yellow filter under a UV illumination. Somatic embryos with green fluorescent illumination are viable while non fluorescent embryos are non-viable.

NOTES: We have shown that somatic embryos of all cultivars tested were successfully stained with FDA after 4 weeks of cryopreservation treatments using cryovial and droplet vitrification methods (O'Brien et al. 2016) (Figs. 24.9, 24.10). Only viable living cells are able to be stained. The somatic embryos from negative controls (not treated for cryopreservation but LN treated) were mostly unstained with only a slight colouring expected from dying tissue.

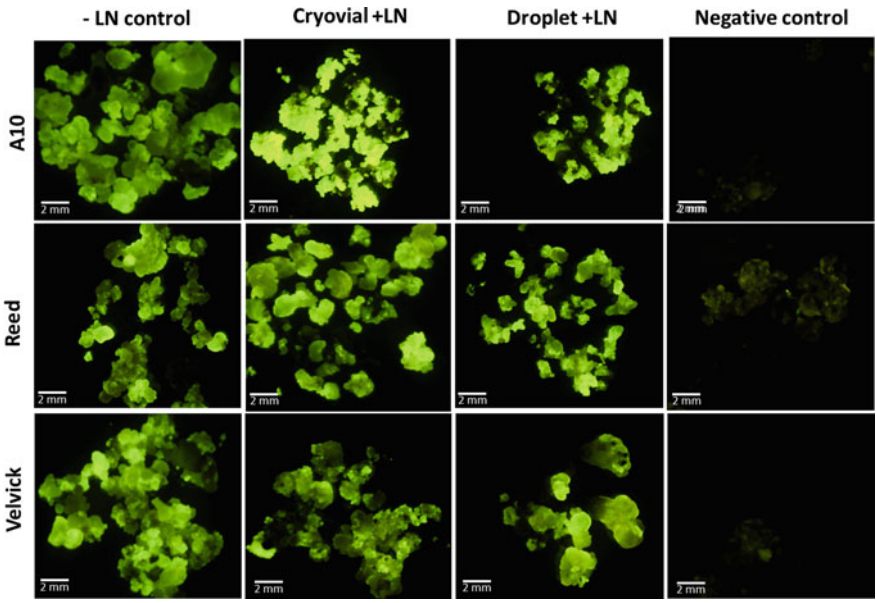

Fig. 24.10 FDA vital staining of avocado somatic embryos 4 weeks post LN treatment. 'A10' somatic embryos: Cryovial—0.8 M loading solution 20 min, PVS2 at 0 ± 1 °C for 60 min, Droplet vitrification—0.2 M loading solution 40 min, PVS2 at 0 ± 1 °C for 60 min. 'Reed' somatic embryos: Cyrovial—0.6 M loading solution 20 min, PVS2 at 0 ± 1 °C for 60 min, Droplet vitrification of 0.2 M loading solution 20 min, PVS2 at 0 ± 1 °C for 60 min. 'Velvick' somatic embryos: Cyrovial—0.8 M loading solution 20 min, PVS2 at 0 ± 1 °C for 60 min, Droplet vitrification—0.8 M loading solution 20 min, PVS2 at 0 ± 1 °C for 60 min

24.3.14 Conclusion and Future Prospects

Long-term maintenance and regeneration of plantlets from avocado somatic embryos has been a major barrier in the development of new avocado cultivars. The development of efficient somatic embryogenesis protocols for avocado, as well as large-scale in vitro regeneration and propagation technologies are critical towards the development and commercialization of new superior cultivars of this valuable horticultural crop.

We have demonstrated an improved multiplication system for multiple cultivars of somatic embryos by using MMSE medium, as well as an improved regeneration protocol for one cultivar. The regeneration of avocado somatic embryos was significantly improved by using a two-step regeneration system which involved the transfer of white opaque somatic embryos to liquid medium as an intermediate step for inducing germination between subcultures in solid medium. This liquid incubation step significantly enhanced shoot/plant development from somatic embryo tissue. In addition to liquid incubation the supplementation of 1 g/L glutamine in the medium for 12–15 days followed by the subculture of the regenerants in the same solidified media increased the shoot regeneration and regenerants survival from 10 and 5% in solid medium to 58.3 and 36.7%, respectively.

At present field collections are constantly exposed to abiotic and biotic stresses. Moreover, the size of gene pool, number of replications and quality of maintenance are also largely restrained by the local environmental conditions, space and funding. The safest and cheaper option for long-term conservation of plant genetic resources is cryopreservation (Abdelnour-Esquivel and Engelmann 2002). Cryopreservation of somatic embryos is valuable as it is readily retrievable for further biotechnology manipulations as well as storage of biotechnology products such as genetically transformed lines (Engelmann, 2011; Sánchez-Romero et al. 2006). It is vitally important that somatic embryos can be grown into healthy and viable plants after exposure to LN if protocols are to be effectively applied for conservation programs (Ashmore et al. 2001). We have shown that somatic embryos of avocado cultivars 'A10', 'Reed', 'Velvick' and 'Duke-7' can be cryopreserved through the application of the cryovial and droplet-vitrification methods.

A new era of avocado advancement and improvement will depend on continued innovations in avocado such as improved tissue culture techniques and marker-assisted technology. Further work is required on shoot tip cryopreservation for avocado, to ensure clones of mother-stock trees in fields are preserved instead of using somatic embryos which are zygotic and highly heterozygous. Although undesirable somatic embryos, still they are useful in conserving *Persea* germplasm. Continued work is also required to increase the efficiency of the regeneration process of avocado plants via somatic embryogenesis as well as to increase the quality of the regenerants using different genotypes of avocado. If successful, this can add considerable value globally to the developmental and genetic transformation studies on the generation of new and improved avocado varieties as well as cryopreservation of somatic embryos for germplasm conservation including the

maintenance of diversity within the *Persea* genera and wild related species. Somatic embryogenesis and cryopreservation of somatic embryos/shoot tips have the ability to be adapted to lead to establishment of a global Cryo-Bank conserving biodiversity and offering source of disease-free genetic material. The technologies developed could be translated to germplasm/accessions available across the globe for a wide range of horticultural crops e.g. tropical and sub-tropical crops such as macadamia and mango, as well as other difficult-to-conserve tree species.

References

Abdelnour-Esquivel A, Engelmann F (2002) Cryopreservation of c hayote (Sechium edule JACQ: SW.) zygotic embryos and shoot-tips from in vitroplantlets. Cryo Lett 23, 299–308 (Alcaraz, M.L., Hormaza, J.I., 2009)

Ammirato PV (1986) Organizational events during somatic embryogenesis

Ashmore S, Azimi M, Drew R (2001) Cryopreservation trials in *Carica papaya*. Acta Hortic 560:117–120

Benson EE (2008) Cryopreservation of phytodiversity: a critical appraisal of theory & practice. Crit Rev Plant Sci 27:141–219. https://doi.org/10.1080/07352680802202034

Ben-Ya'acov A, Bufler G, Barrientos-Priego AF et al (1992) A study of avocado germplasm resources, 1988–1990. I. General description of the international project and its findings. In: Proceedings of the second World Avocado congress, pp 535–541

Ben-Ya'acov A, Michelson E (1995) Avocado Rootstocks. In: Horticultural reviews, pp 381–429

Bertrand-Desbrunais A (Institut F de RS pour le D en C), Fabre J, Engelmann F et al (1989) Adventive embryogenesis recovery from coffee (*Coffea arabica* L.) somatic embryos after freezing in liquid nitrogen. In: Bulletin de la Societe Botanique de France Actualites Botaniques (France)

Bouman H, de Clerk GJ (1990) Cryopreservation of Lilly meristems. In: Flower bulbs. Acta Horticulturae

Cailloux F, Julien-Guerrier J, Linossier L, Coudret A (1996) Long-term somatic embryogenesis and maturation of somatic embryos in *Hevea brasiliensis*. Plant Sci 120:185–196. https://doi.org/10.1016/S0168-9452(96)04491-3

Cruz-Hernandez A, Litz RE, Lim MG (1998) *Agrobacterium tumefaciens*–mediated transformation of embryogenic avocado cultures and regeneration of somatic embryos. Plant Cell Rep 17:497–503

Davis J, Henderson D, Kobayashi M et al (1998) Genealogical relationships among cultivated avocado as revealed through RFLP analyses. J Hered 89:319–323. https://doi.org/10.1093/jhered/89.4.319

Dreher ML, Davenport AJ (2013) Hass avocado composition and potential health effects. Crit Rev Food Sci Nutr 53:738–750. https://doi.org/10.1080/10408398.2011.556759

Dulloo ME, Ebert AW, Dussert S et al (2009) Cost efficiency of cryopreservation as a long-term conservation method for coffee genetic resources. Crop Sci 49:2123–2138. https://doi.org/10.2135/cropsci2008.12.0736

Efendi D (2003) Transformation and cryopreservation of embryogenic avocado (*Persea americana* Mill.) cultures. University of Florida

Encina CL, Parisi A, O'Brien C, Mitter N (2014) Enhancing somatic embryogenesis in avocado (*Persea americana* Mill.) using a two-step culture system and including glutamine in the culture medium. Sci Hortic 165:44–50. https://doi.org/10.1016/j.scienta.2013.10.019

Engelmann F (2004) Plant cryopreservation: progress and prospects. Vitro Cell Dev Biol Plant 40:427–433. https://doi.org/10.1079/IVP2004541

Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. Vitro Cell Dev Biol Plant 47:5–16. https://doi.org/10.1007/s11627-010-9327-2

Escobedo V, Escobedo JA (2010) Adventitious root formation without rooting medium in etiolated shoots of "Duke" avocado (*Persea americana*) growing on nurse plants. In: XXVIII International horticultural congress on science and horticulture for people (IHC2010): International symposium on Micro 923, pp 227–232

Fabbri A, Sutter E, Dunston SK (1986) Anatomical changes in persistent leaves of tissue cultured strawberry plants after removal from culture. Sci Hortic 28:331–337. https://doi.org/10.1016/0304-4238(86)90107-X

FAOSTAT (2014) FAOSTAT. In: FAOSTAT. http://www.fao.org/faostat/en/#data/QC/visualize. Accessed 29 Sept 2017

Fulgoni VL, Dreher M, Davenport AJ (2013) Avocado consumption is associated with better diet quality and nutrient intake, and lower metabolic syndrome risk in US adults: results from the National Health and Nutrition Examination Survey (NHANES) 2001–2008. Nutr J 12:1. https://doi.org/10.1186/1475-2891-12-1

Furnier GR, Cummings MP, Clegg MT (1990) Evolution of the avocados as revealed by DNA restriction fragment variation. J Hered 81:183–188. https://doi.org/10.1093/oxfordjournals.jhered.a110963

Galindo-Tovar ME, Ogata-Aguilar N, Arzate-Fernández AM (2008) Some aspects of avocado (*Persea americana* Mill.) diversity and domestication in Mesoamerica. Genet Resour Crop Evol 55:441–450. https://doi.org/10.1007/s10722-007-9250-5

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158. https://doi.org/10.1016/0014-4827(68)90403-5

Grout BWW, Crisp PC (1995) Cryopreservation of germplasm of tomato. SpringerLink, 371–380. https://doi.org/10.1007/978-3-662-03096-7_26

Guan Y, Li S-G, Fan X-F, Su Z-H (2016) Application of somatic embryogenesis in woody plants. Front Plant Sci. https://doi.org/10.3389/fpls.2016.00938

Guzmán-García E, Sánchez-Romero C, Panis B, Carpentier SC (2013) The use of 2D-DIGE to understand the regeneration of somatic embryos in avocado. Proteomics 13:3498–3507. https://doi.org/10.1002/pmic.201300148

Hiti-Bandaralage JCA, Hayward A, Mitter N (2017) Micropropagation of avocado (*Persea americana* Mill.). Am J Plant Sci 8:2898–2921

Imafidon KE, Amaechina FC (2010) Effects of aqueous seed extract of *Persea americana* Mill. (avocado) on blood pressure and lipid profile in hypertensive rats. Adv Biol Res 4:116–121

Jain SM, Gupta PK (eds) (2005) Protocol for somatic embryogenesis in woody plants. Springer, Dordrecht/New York

Jain SM, Gupta PK, Newton RJ (eds) (1999) Somatic embryogenesis in woody plants. Springer, Dordrecht

Kaczmarczyk A, Funnekotter B, Menon A et al (2012) Current tissues in plant cryopreservation. Curr Front Cryobiol, 417–438

Kaity A, Ashmore SE, Drew RA, Dulloo ME (2008) Assessment of genetic and epigenetic changes following cryopreservation in papaya. Plant Cell Rep 27:1529–1539. https://doi.org/10.1007/s00299-008-0558-1

Koppa LE (1996) A taxonomic revision of the genus Persea in the Western hemisphere (Persea-Lauraceae). Mem NY Bot Grad 14:1–117

Kulkarni V, Suprasanna P, Bapat V (2006) Plant regeneration through multiple shoot formation and somatic embryogenesis in a commercially important and endangered Indian banana cv. Rajeli. Curr Sci 90:842–850

Lesar H, Hlebec B, Čeranič N et al (2012) Acclimatization of terrestrial orchid *Bletilla striata* Rchb. f. (Orchidaceae) propagated under in vitro conditions. Acta Agric Slov 99:69–75

Litz RE (2005) Biotechnology of fruit and nut crops. CABI

Litz RE (2004) Effect of gamma irradiation on embryogenic avocado cultures and somatic embryo development. Plant Cell, Tissue Organ Cult 77:139–147

Litz RE, Litz W (2000) Somatic embryogenesis of avocado (*Persea americana*) and its application for plant improvement. In: International symposium on tropical and subtropical fruits. Acta Horticulturae, pp 133–138

Márquez-Martín B, Barceló-Muñoz A, Pliego-Alfaro F, Sánchez-Romero C (2012) Somatic embryogenesis and plant regeneration in avocado (*Persea americana* Mill.): influence of embryogenic culture type. J Plant Biochem Biotechnol 21:180–188. https://doi.org/10.1007/s13562-011-0091-0

Mhameed S, Sharon D, Kaufman D et al (1997) Genetic relationships within avocado (*Persea americana* Mill) cultivars and between *Persea* species. Theor Appl Genet 94:279–286. https://doi.org/10.1007/s001220050411

Mooney PA, Staden JV (1987) Induction of embryogenesis in callus from immature embryos of *Persea americana*. Can J Bot 65:622–626

Mujib A, Šamaj J (2006) Somatic embryogenesis. Springer Science & Business Media

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Mycock DJ, Wesley-Smith J, Berjak P (1995) Cryopreservation of somatic embryos of four species with and without cryoprotectant pre-treatment. Ann Bot 75:331–336. https://doi.org/10.1006/anbo.1995.1030

O'Brien C, Constantin M, Walia A et al (2016) Cryopreservation of somatic embryos for avocado germplasm conservation. Sci Hortic 211:328–335. https://doi.org/10.1016/j.scienta.2016.09.008

Panis B, Lambardi M (2005) Status of cryopreservation technologies in plants (crops and forest trees). In: Food and Agriculture Organization of the United Nations (ed) The role of biotechnology in exploring and protecting agricultural genetic resources. Food & Agriculture Org., pp 43–54

Pateña LF, Carlos-Refuerzo LR, Barba RC (2002) Somatic embryogenesis and plantlet regeneration in mango (*Mangifera indica* L.). Vitro Cell Dev Biol Plant 38:173–177. https://doi.org/10.1079/IVP2001275

Pérez RM, Mas O, Navarro L, Duran-Vila N (1998) Production and cryoconservation of embryogenic cultures of mandarin and mandarin hybrids. Plant Cell, Tissue Organ Cult 55:71–74. https://doi.org/10.1023/A:1026433405701

Pierce HF (1959) The nutritional value of the avocado. California avocado society Year Book

Platt RG (1976) Current techniques of avocado propagation. In: Proceedings of the first international tropical fruit short course: the avocado. Florida Cooperative Extension Service and Institute of Food and Agricultural Services, University of Florida, Gainesville, pp 92–95

Pliego-Alfaro F, Murashige T (1988) Somatic embryogenesis in avocado (*Persea americana* Mill.) in vitro. Plant Cell, Tissue Organ Cult 12:61–66

Raharjo SH, Litz RE (2005) Micrografting and ex vitro grafting for somatic embryo rescue and plant recovery in avocado (*Persea americana*). Plant Cell, Tissue Organ Cult 82:1–9

Salazar MJ, El Hafidi M, Pastelin G et al (2005) Effect of an avocado oil-rich diet over an angiotensin II-induced blood pressure response. J Ethnopharmacol 98:335–338. https://doi.org/10.1016/j.jep.2005.01.044

Sánchez-Romero C, Márquez-Martín B, Pliego-Alfaro F (2006) Somatic and zygotic embryogenesis in avocado. Somat Embryogenesis, 271–284

Schaffer BA, Wolstenholme BN, Whiley AW (2013) The avocado: botany, production and uses. CABI

Scora RW, Bergh B (1992) Origin of and taxonomic relationships within the Genus *Persea*. University of California, Riverside, pp 505–574

Silva TA, Ledesma N (2014) Avocado history, biodiversity and production. In: Sustainable horticultural systems. Springer International Publishing, Cham, pp 157–205

Storey WB, Bergh B, Zentmyer GA (1986) The origin, indigenous range and dissemination of the avocado. Calif Avocado Soc Yearb 70:127–143

Suarez IE, Schnell RA, Kuhn DN, Litz RE (2006) Recovery and indexing of avocado plants (*Persea americana*) from embryogenic nucellar cultures of an avocado sunblotch viroid-infected tree. Plant Cell, Tissue Organ Cult 84:27–37. https://doi.org/10.1007/s11240-005-7532-1

Villalobos VM, Ferreira P, Mora A (1991) The use of biotechnology in the conservation of tropical germplasm. Biotechnol Adv 9:197–215

Wetzstein HY, Sommer HE (1982) Leaf anatomy of tissue-cultured *Liquidambar styraciflua* (Hamamelidaceae) during acclimatization. Am J Bot 69:1579–1586. https://doi.org/10.2307/2442913

Witjaksono, Litz RE (1999) Induction and growth characteristics of embryogenic avocado cultures. Plant Cell, Tissue Organ Cult 58:19–29

Witjaksono, Litz RE, Grosser JW (1998) Isolation, culture and regeneration of avocado (*Persea americana* Mill.) protoplasts. Plant Cell Rep 18:235–242

Witney WG, Arpaia M, Clegg MT, Douhan G (2017) Avocado germplasm preservation and breeding program in California. In: New germplasm and new breeding programmes. Tauranga, New Zealand

Zirari A, Lionakis SM (1994) Effect of cultivar, explant type, etiolation pretreatment and the age of plant material on the *in vitro* regeneration ability of avocado (*Persea americana*). Acta Hortic 365:69–76

Chapter 25
Date Palm Somatic Embryogenesis from Inflorescence Explant

Adel A. Abul-Soad and Jameel M. Al-Khayri

25.1 Introduction

Date palm (*Phoenix dactylifera* L.), is a dioecious tropical and subtropical monocot fruit species cultivated mainly in the Middle East. Nowadays, date palm, as a salt and drought tolerant plant, attracts the attention of growers in various countries in Africa, Asia, Europe, and the Americas (Al-Khayri et al. 2015a, b). Sugary date fruit is a good source of minerals and vitamins in addition to a variety of uses of all parts of the adult tree (Zaid and de Wet 2002). World dates production in 2014 has reached 7.6 million mt harvested from 1.127 million ha. The top date producers are Egypt, Iran, Algeria, Saudi Arabia, Iraq, Pakistan, Sudan, Oman, United Arab Emirates, Tunisia, Libya, Kuwait and Morocco (FAOSTAT 2014).

An efficient propagation is a key element for the developmental process of the date palm sector. Micropropagation provides a safe way to obtain disease-free plant materials for establishing new plantations and the replacement of trees. This approach prevents the spread of the fatal pests that may be associated with offshoots separated from mother trees grown in the field. Researchers have tested various explant types from the date palm tissue; however, only explants isolated from the shoot tips of young offshoots (Tisserat 1984; Veramendi and Navarro 1997; Abul-Soad et al. 2002; Al-Khayri and Naik 2017) and immature inflorescence (Bhaskaran and Smith 1992; Fki et al. 2003; Abul-Soad 2011; Abul-Soad et al. 2005) have proved commercially viable. The use of the shoot tip sacrifices the

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_25

mother tree; whereas, the use of immature inflorescence is nondestructive to the source tree. Moreover, the latter is the only option when offshoots are scarce. A case in point, the inflorescence explant was successfully used to conserve the distinction-threatened Egyptian date palm cultivar Gazal found in the Siwa oasis (Abul-Soad et al. 2017a, b, c). Moreover, a single inflorescence gives plenty of explants sufficient to produce a large number of in vitro plantlets. For example, a single inflorescence of the Pakistani cultivar Gulistan produced 10,000 plantlets (Abul-Soad and Mahdi 2010).

Date palm propagation through tissue culture technique is a viable method as compared to the offshoots propagation (Abul-Soad 2011). However, in some cases, several genetic and epigenetic variations may appear after transplanting the ex vitro plants in the open field. This may be attributed to variability in the quality of plant materials used and some stress conditions that may occur during the in vitro phase. The most prominent somaclonal variation reported in shoot tip tissue culture plants are fruit of multiple carpels, albinism or variegated leaves, change in fruit quality, terminal bud bending and twisted leaf and inflorescence (Hassanpour-Estahbanati and Hamidian 2007; Alkhateeb 2008). Utilizing immature inflorescence avoided these abnormalities although occasional dwarfism was reported (Mirbahar et al. 2014). Nonetheless, the assurance of genetic conformity can only be achieved by molecular verification. Genetic fidelity of micropropagated plants is a major concern especially in commercial production.

This chapter describes an in vitro regeneration protocol for date palm based on somatic embryogenesis from the immature inflorescence explants. It provides detailed steps for inflorescence excision, surface sterilization and explant preparation, culture medium preparation for various culture stages, culture conditions and procedures to obtain direct embryogenic callus and subsequent differentiation into viable somatic embryos, followed by germination, rooting and complete plantlets formation, and finally greenhouse acclimatization and open field transplanting.

25.2 Protocol of Somatic Embryogenesis from Inflorescence

25.2.1 Culture Medium

1. The basal medium used for date palm micropropagation in the current protocol is based on MS salts (Murashige and Skoog 1962) with some modifications (Table 25.1). Sometimes, the minor elements of MS basal salts has been used in combination with the major elements of B5 medium (Gamborg et al. 1968), particularly during the first stages of cultures (initiation, maturation and differentiation) to avoid the high concentration of the major elements present in the MS medium in order to reduce the osmotic pressure of the medium to provide more suitable conditions for the growth of the young explant and the undifferentiated callus tissues.

Table 25.1 The composition of the basal B5 medium (Gamborg et al. 1968) and basal MS medium (Murashige and Skoog 1962) used for in vitro culture for date palm inflorescence

Constituents	B5 medium (mg l^{-1})	MS Medium (mg l^{-1})
Major nutrients		
Ammonium nitrate	–	1650
Ammonium sulfate	134	–
Potassium nitrate	2500	1900
Calcium chloride-2H_2O	113.23	440
Magnesium sulfate-7H_2O	122.090	370
Sodium phosphate	130.42	170
Minor nutrients		
Potassium iodide	0.75	0.83
Boric acid	3	6.2
Manganese sulfate-4H_2O	10	22.3
Zinc sulfate-7H_2O	2	8.6
Sodium molybdate-2H_2O	0.13	0.25
Cupric sulfate-5H_2O	0.025	0.025
Cobalt chloride-6H_2O	0.025	0.025
Iron–EDTA		
Iron sulfate-7H_2O	27.8	27.8
Ethylenediamine tetraacetic acid disodium salt dihydrate	37.3	37.3
Vitamins		
Myo-Inositol	100	100
Nicotinic acid	1	1
Pyridoxine hydrochloride	1	1
Thiamine hydrochloride	1	1
Glycine	2	2
Biotin	1	1
Sugar and gelling substances		
Sucrose	30,000	30,000
Agar	2400	2400
Gerlite	1400	1400
Other additives	See Table 25.2	See Table 25.2
pH	5.7	5.7

2. This protocol consists of five culture stages differing in plant growth regulators (PGR) and some other additives as described in Table 25.2. Add these additives accordingly and adjust the medium to the desired final volume.
3. Adjust medium to pH 5.7 with 1 *N* KOH and HCl, and dispense into 150 × 25-mm culture tubes (15 ml medium per tube) in initiation stage or in 350-ml culture jars. Autoclave for 15 min at 121 °C and 1 × 10^5 Pa (1.1 kg cm^{-2}).

Table 25.2 Plant growth regulators and other additives supplemented to the basal medium for various culture stages, the duration and incubation of each stage of the date palm regeneration from inflorescence explant

Culture stage	Basal medium	Medium additives (mg l^{-1})	Plant growth regulators (mg l^{-1})	Culture stage duration (No. of subcultures)	Subculture interval (Weeks)	Light and temperature conditions
Initiation	B5	170 Potassium orthophosphate 100 Glutamine 40 Adenine sulfate	0.1 2,4-D + 0.1 IAA + 5 NAA + 1 2iP	2–8	3–4	Darkness, 28 ± 2 °C
Maturation	B5	170 Potassium orthophosphate 100 Glutamine 40 Adenine sulfate 1500 Activated charcoal	0.1 2,4-D + 1 2iP	1–3	4–6	Darkness, 28 ± 2 °C
Differentiation	B5	170 Potassium orthophosphate 100 Glutamine 40 Adenine sulfate 1500 Activated charcoal	0.1 NAA + 0.1 Kinetin	1–2	6–8	16 h, 20 µmol m^{-2} s^{-1}, 27 ± 2 °C
Proliferation	MS		0.1 NAA + 0.05 BA	5–25	6–8	16 h, 40 µmol m^{-2} s^{-1}, 27 ± 2 °C
Rooting	MS	3000 Activated charcoal	0.1 NAA	3–5	8–16	16 h, 40 µmol m^{-2} s^{-1}, 27 ± 2 °C

4. It is well known that using about 3–4 g l^{-1} gelrite (gellan gum) as gelling agent in the tissue culture medium is better than 7–8 g l^{-1} agar due to the chemical purity of the former which resulted in faster response of the explants. Moreover, using Gelrite results in a clear medium which is advantageous for checking cultures for bacterial contamination. However, continuous re-culturing on Gerlite-solidified medium for more than 1–2 subcultures may cause more hyperhydricity compared to the agar. Consequently, a formula was developed, consisting of 1.4 g l^{-1} Gerlite and 2.4 g l^{-1} agar, for the use in all stages of this protocol (Table 25.1). In addition being cost-effective, this formula combines both benefits of the gelling substances and achieve better in vitro responses compared to using them individually.
5. Note that any types of culture vessels can be used, but it was observed that the growth of the explants in the small size culture vessel (150 × 25-mm culture tubes) was 1–2 weeks earlier than in the larger jars (350-ml). For rooting medium, start with small culture tubes for 1–2 subcultures only then plantlets must be transferred into larger culture tubes (250 × 25 mm) in order to accommodate the erect growth of date palm simple leaves (fronds later). In case of growing the leaves more than 25 cm, it can be trimmed or twisted to be accommodated within the available space of the culture tubes.
6. Plastic closures are usually used to cap the culture vessels, but sometimes they can be replaced with pieces of aluminum foil sheet or piece of cotton. Cotton plugs limit gases exchange, particularly useful during initiation stage. Whereas, aluminum foil are preferred during the rooting stage to allow more aeration and reduces the humidity buildup inside the culture vessel.

25.2.2 Inflorescence Excision and Explant Preparation

1. Select a healthy field-grown parent tree of a commercial cultivar, an endangered cultivar or a land race of elite fruit quality from which you intend to excise the immature inflorescence. No difference between female and male inflorescences concerning the excision procedures. Start cutting off the outer fronds from only one side of the parent tree till the first immature inflorescence appears. A second way is to guess the place of the immature inflorescence based on the usual spathes' emergence places above the last year remaining sheaths (The outer cover of the inflorescence) and also the fact of presence an inflorescence in each alternative frond. Then cut the fronds from that point to expose the inflorescence (Abul-Soad 2011).
2. Excise immature inflorescence by cutting at the spathe base without exposing the tender tissue within. The excised inflorescence (Fig. 25.1a) is carefully cleaned with a piece of cotton cloth to remove dust particles then placed in an ice box (about 4 °C) to transfer to the laboratory. It must be kept in dry conditions during the transfer process by covering the inflorescence with paper tissues within a plastic-transparent bag in the ice box. Note that excessive

moisture around the inflorescence during this transfer trip leads to splitting the outer soft protective spathe near the base of the immature inflorescence.
3. Once the inflorescence arrived to the laboratory, start the surface sterilization procedure by first washing the intact inflorescence under running tap water and then immerse in a detergent solution for 2–3 min (Fig. 25.1b).
4. Avoid making any damage to the intact spathe either during detachment process from the parent tree, during transportation and during surface sterilization procedure (Abul-Soad et al. 2017b). Longer use of detergent solution may cause splitting of the outer soft immature spathe (Fig. 25.1c).
5. Immerse the inflorescence in 5.25% w/v sodium hypochlorite solution (50% v/v commercial bleach) containing 10–15 drops of Tween 20 per 100 ml solution. Gently shake for 10 min by hand or by placing on a rotary shaker 100 rpm/min. Rinse the inflorescence with sterile distilled water three times and keep dry throughout the explant excision process. Note that young inflorescence of 20–30 cm length appeared most responsive to callus formation compared to younger and older inflorescences (Abul-Soad et al. 2017b).

25.2.3 Culture Initiation and Callus Induction

1. For explant excision and culture initiation, the outer cover is carefully opened using scalpel and forceps. First make a transverse cut just after the base of the intact inflorescence and another perpendicular cut to the first cut to give T shape. Then two to three other perpendicular cuts near to the second cut to open a small window to extract the spikelets one by one (Fig. 25.1d).
2. Immature spikelets are white to creamy color and differ in their lengths (Fig. 25.1e). Spikelet 2–3-cm long are cultured whole whereas the longer spikelets are cut into 2–3 pieces, each piece contained a few florets. No noticeable differences in the response of intact spikelet compared to the spikelet pieces. Culture the explants individually in culture tubes containing the initiation medium as specified in Table 25.2. Position the spikelet explant on the initiation medium submerged into the nutrient medium (Fig. 25.1f). Explants cultured in test tubes are positioned vertically submerged into the medium; whereas, explants cultured in jars are positioned horizontally to achieve submersion. Partially submerged explants showed severe browning particularly in the parts not in direct contact with the nutrient medium. Note that callus induction from inflorescence explants is relatively rapid; it occurs within 2–8 months compared to the long duration associated with callus induction from shoot tip explants which requires 8–18 months (Abul-Soad et al. 2002).
3. Bacterial or fungal contaminations are rarely found in the cultured explants following the described disinfection and inoculation procedures. This is another advantage of using inflorescence compared to the frequently contaminated cultures initiated from shoot tip explants. Note that bacteria-contaminated floral

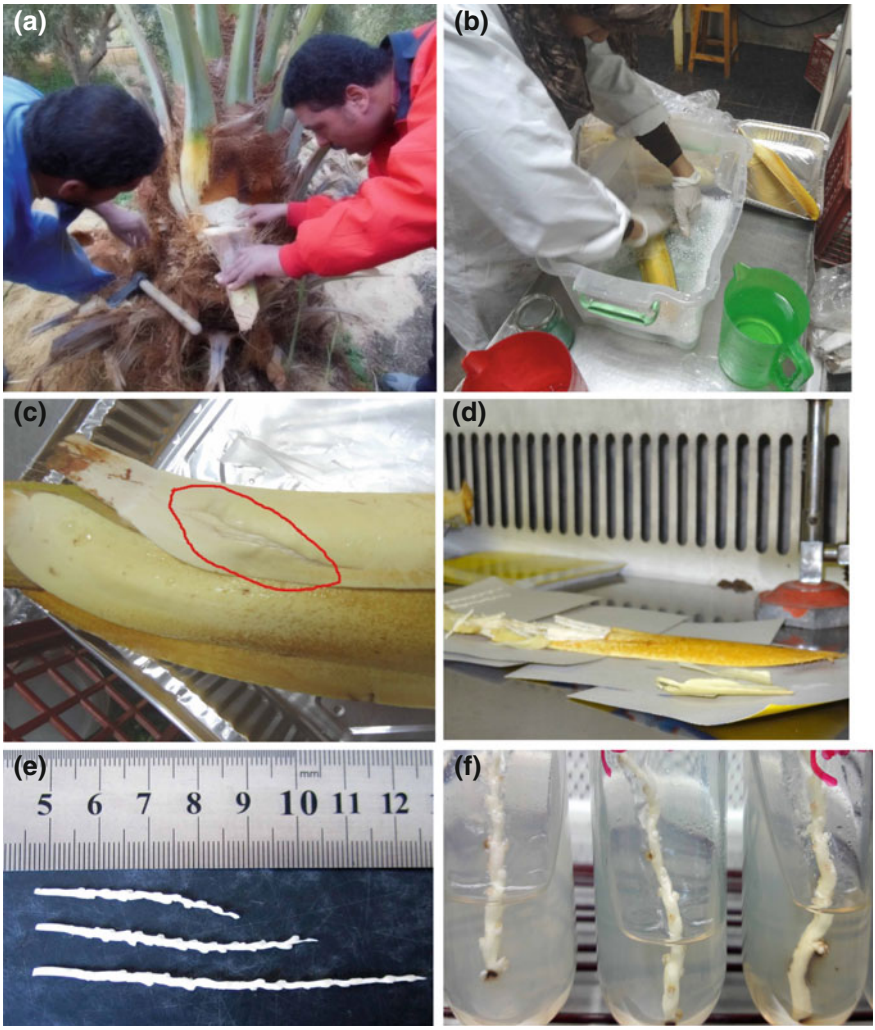

Fig. 25.1 Inflorescence excision from date palm tree and explant preparation for culture initiation. **a** Excising the immature inflorescence from a parent date palm tree, **b** Surface sterilization with detergent solution to remove dust particles, **c** Damage occurred to the soft outer sheath of the spathe because of long exposure to sterilization solution and hard shaking, **d** Opening the inflorescence under aseptic conditions and getting the spikelets explants, **e** Excised spikelets at different lengths from a single inflorescence each consisting of about 10–15 florets, **f** The spikelet explant submerged in the nutrient medium in upright position

explants are identified by the red color appearance on the whole explant (Abul-Soad et al. 2017b). Contaminated explants must be discarded; hence, it is advisable to culture explants in individual vessels to reduce contamination losses.

4. Incubate cultures under full darkness (Fig. 25.2a) at 28 ± 2 °C. Maintaining incubation temperature at this level is crucial especially during the callus induction phase.
5. In contrast to shoot tip explants, it is not advisable to add activated charcoal to the initiation medium as the floral explants show minimal browning, especially after a few subcultures, but normally does not affect explants response. Transferring the explants to a fresh medium at 3–4-week intervals during the initiation stage is recommended.
6. Transfer the explants regularly to a fresh initiation medium until embryogenic callus form. Callus formation may be apparent after the first subculture, but sometimes callus formation is visible in the initial culture. Subsequently, the embryogenic callus becomes clearly visible after 3–7 subcultures. Note that swelling of the florets on the spikelet explants is an excellent indication of survivability and potential development. Spikelet explants with collapsed florets or without any change do not induce callus. Callus formation from the florets is exhibited in four forms. The 1st form takes place where some cultivars show direct formation of clear cut embryogenic callus on the initiation medium and rapidly proliferate on the same medium (Fig. 25.2b). The 2nd form of the induced callus which commonly emerges from among the floral parts of a floret (Fig. 25.2c). The 3rd form of callus which can be induced from the spikelet explants is unfriable callus which appears watery somewhat and is not embryogenic (Fig. 25.2d). This type needs more time to develop into embryogenic callus. The 4th form bursting a bulk of embryogenic callus within the swelled and mostly brownish florets due to long period of in vitro sub-culturing (Fig. 25.2e, f). In order to obtain the latter callus type, such floret explants have to be transferred onto nutrient medium supplemented with 3 mg l^{-1} 2-4-D after 8 subcultures with no apparent callus formation (Abul-Soad 2012). The 1st form is the best one in terms of the potential to differentiate into intact somatic embryos after maturation. This form could be obtained after only 2 subcultures when all factors control somatic embryogenesis were optimized. These factors include the cultivar, optimal time to excise the immature inflorescence from a parent date palm tree, quality of excised inflorescence from excision time to the in vitro culture inside the laboratory, composition of the nutrient medium, manipulation of the explants and ambient incubation conditions (Abul-Soad et al. 2017b). Nonetheless, for unknown reasons, in vitro cultured explants may fail to response for a long time. However, sometimes, callus of the 4th form develops after 8 subcultures after floret swelling occurs (Fig. 25.2e) as it may contain slow-growing embryogenic callus within the floret (Fig. 25.2f).
7. Keep in mind that callus induction from the inflorescence explants is related mainly to the genetic background, i.e. cultivar and the group of date palm. Date palm cultivars belonging to the dry date group is very difficult to give direct

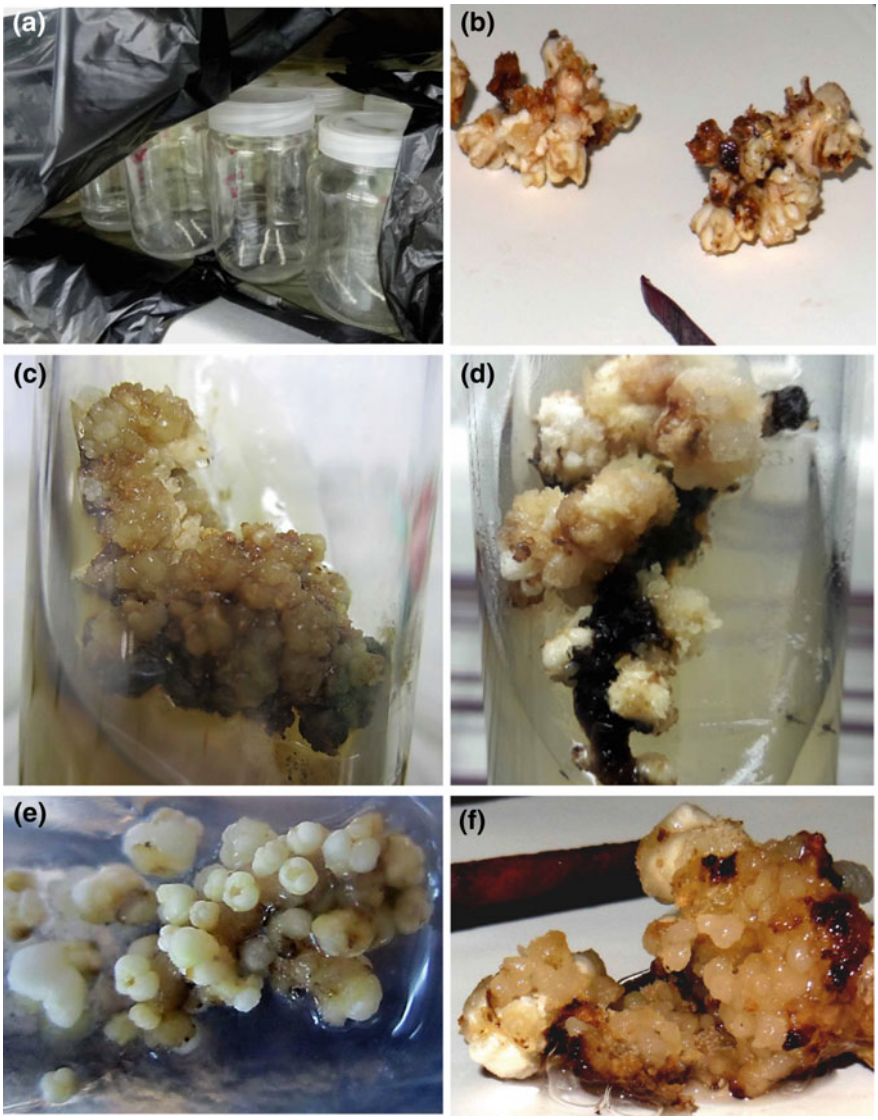

Fig. 25.2 Callus formation in the date palm inflorescence explants. **a** Initial spikelets explants cultured onto the initiation medium and incubated under full darkness, **b** direct formation of embryogenic callus within the florets after 1st subculture (6–8 weeks) on the spikelets explants, **c** the targeted form of embryogenic callus develops directly on the spikelet explants after 6-8 weeks of in vitro culture, **d** unfriable callus induced mainly on 2,4-D containing medium (Photo by Najam ul-deen Solangi), **e** spikelet explant consisting of swelled white florets after 1st subculture, **f** embryogenic callus formation on a 2,4-D medium within the swelled florets after 10–15 subcultures ready to differentiate into embryos

callus formation as compared to those of soft or semi-dry date groups. It could be because of the low moisture content of explants from the dry date cultivars. Moreover, this may be attributed to the impact of climate conditions, particularly the extreme high temperature, on the growing inflorescences before excision from the date palm tree. Date palm trees planted in one location may vary in the onset of flowering, subsequently the inflorescences vary in age. Thus, it is importance of note the differentiation degree of the excised inflorescence to identify the most suitable time for collecting the inflorescences for successful in vitro culturing (Drira and Benbadis 1985; Abul-Soad 2012; Kriaa et al. 2012).

8. The embryogenic callus from inflorescence explants is transferred directly to the maturation medium (Table 25.2), unlike to the callus from shoot tip explants where a proliferation step is required (Abul-Soad et al. 2004). Consequently, obtaining regenerated plantlets from inflorescence protocols require shorter duration than the shoot tip explants protocols. This is considered an important advantage especially for commercial production because it contributes to reduction of operation expenses.

25.2.4 Somatic Embryogenesis

1. Subculture the embryogenic callus on the maturation medium (Table 25.2). Notice more callus growth occurs in addition to the development of some white and small somatic embryos (Fig. 25.3a). After a few weeks, globular embryos begin to appear which subsequently elongate forming bipolar-shaped embryos (Fig. 25.3b). These embryos have no connection with the original explants and can be easily separated from the original explants and other embryogenic callus. Portions of explants tend to turn brown to black during this stage and have connection with some other immature embryos and embryogenic callus. These portions should not be cut at this stage but left to be separated later on after further growing of the attached organs and somatic embryos.
2. After 1–3 subsequent subcultures maintained under full dark conditions, transfer the matured embryogenic callus to the differentiation medium (Table 25.2). Incubate the cultures starting the differentiation stage in 16-h photoperiods (20 μmol m^{-2} s^{-1}) provided by white cool-fluorescent lamps at 27 ± 2 °C for 1–2 subcultures only (Fig. 25.3c). After each subculture harvest the developed somatic embryos and transfer them to the proliferation medium or directly to rooting medium based on the type of differentiated structures whether they are individual somatic embryos or cluster of shoots (Table 25.2).
3. After harvesting the differentiated cultures, the remaining embryogenic callus gradually losses potential to differentiate, i.e. form shoots or somatic embryos. Also, the differentiated structures after two subcultures become small in size and abnormal in shape (Abul-Soad 2011). Nonetheless, new colonies of embryogenic callus develop, but without the ability to differentiate into

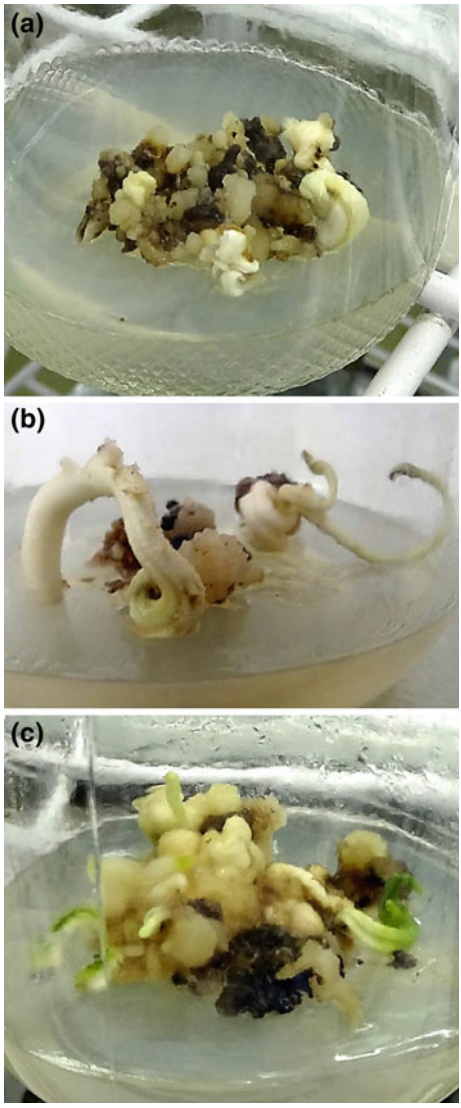

Fig. 25.3 Date palm somatic embryogenesis from inflorescence cultures. **a** Matured embryogenic callus, **b** and development of somatic embryogenesis under darkness. Note that, embryogenic callus and the somatic embryos can be easily separated, **c** Differentiated somatic embryos from a male inflorescence explants under low light conditions

somatic embryos. This could be because of increasing the concentration of some endogenous hormones such as abscisic acid (ABA) in the new colonies of embryogenic callus that may inhibit the development of additional somatic embryos. It was reported that the concentration of endogenous ABA in the embryogenic callus was about 50 µg/100 g fresh weight whereas it was only 17 µg/100 g FW in the matured somatic embryos (Zayed and Abd Elbar 2015).

25.2.5 Proliferation and Rooting

1. Classify the differentiated somatic embryos from the differentiation stage into two types, multiple embryo or shoot clusters and single embryos (Abul-Soad et al. 2004; Abul-Soad 2011). The multiple embryos cluster consists of several somatic embryos and can not be detached from each other without making cut which is unadvisable at this stage. Cuts will damage the organs causing excretion of phenolic compounds and the tissue turns pale green and may die. In addition, this will allow time for the growing somatic embryos and shoots to grow further and constitute complete independent embryos or shoots easily to be separated from each other. On the other hand, making cuts in these juvenile tissues encourage the production of secondary somatic embryos within a couple of months only which may increase the chance of producing somaclonal variations. The multiple embryos form green shoot growth but often root development is delayed and can be stimulated with auxin supplement later on after shoots elongate (Fig. 25.4a).
2. Three types of cultures can be detected at the beginning of the proliferation stage: the first type, cluster of somatic embryos only some of them are entirely individual and can be easily separated from the cluster; the second, a cluster of only shoots without almost root outgrowths which is a type of organogenesis; the third type, a mixture of shoots and somatic embryos.
3. It is preferred to use a plant growth regulators-free medium for proliferation or rooting (Table 25.2) for a single subculture every 2–3 subcultures to rebalance the endogenous plant growth regulators. Furthermore, the presence of PGR encourages the development of embryogenic callus and secondary somatic embryos on the bases of rooting plantlets. Using a hormone-free medium and sometimes activated charcoal enhance the growth of the growing plants. Open field observation of ex vitro plants based on phenotype showed a minimal somaclonal variation of plants produced after 25 subcultures during the proliferation stage. Nonetheless, the optimal number of subcultures is 10–11 subcultures after then the proliferating shoots may turn pale green and leaves become very week and easily drop from the plants which mostly show infection with latent bacterial contamination. It is worthy to mention that the response after prolonged culturing depends mainly of the quality of first cluster used in the proliferation stage, i.e. vigorous, normal phenotype and latent bacteria-free.
4. The singular somatic embryos which differenced individually from the embryogenic callus without any attached embryogenic callus or secondary somatic embryos produce a single shoot and a primary white root. Once these embryos produce green leaves, transfer them to the rooting medium (Table 25.2) (Fig. 25.4b).
5. Although, shoots produced from inflorescence culture are rarely found contaminated with latent bacteria; occasionally, week proliferated shoots showed bacterial latent contamination. Treatment with 10 mg L^{-1} Cefotax, a wide spectrum antibiotic that was added to the nutrient medium after autoclaving, failed to eliminate this contamination (Abul-Soad et al. 2017b).

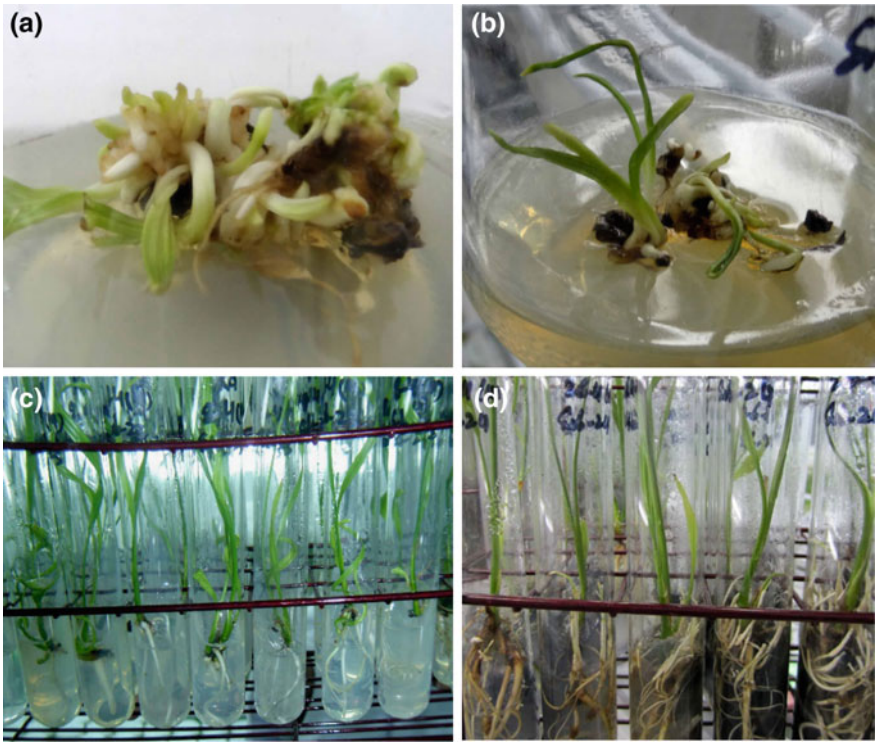

Fig. 25.4 Proliferation and rooting of date palm plantlets. **a** Multiple somatic embryos and cluster of shoots produced on the differentiation medium can be used as the first cluster in shoot multiplication, **b** the differentiated singular somatic embryos that go directly into the in vitro rooting medium for more growth, **c** separated shoots after trimming the primary root which encouraging the adventitious root formation on the rooting medium, **d** note secondary root growth and the thickness of the trunk of in vitro rooted plantlet ready for transfer to the greenhouse for acclimatization

6. To encourage adventitious root formation, it is advised to trim the primary root with the scalpel before placing on the rooting medium (Fig. 25.4c). Moreover, it is preferred to subculturing at 2–4 months during the rooting stage instead of the 4–6 week intervals during the other stages of this protocol. This measure increases the thickness of the plantlets' trunk, allows more time for proper root differentiation, and enhances the hardening of the plantlets before transfer to the acclimatization stage. These improvements are attributed to the stress incurred by the gradual depletion of nutrient in the rooting medium during this extended culture period. As the culture progress, the pH of the medium decreases because of the excreted acidic metabolites causing the solid medium becomes liquid. Decreased pH to 6.4 increases the absorption of the nutrients which may explain the improved growth of subculturing at 2–4 months.

7. Continue re-culturing the directly differentiated somatic embryos of 3–7 cm long and easily separated shoots from a proliferated cluster of shoots to the rooting medium (Table 25.2). After 3–5 subcultures, first couple of subcultures on the rooting medium but without activated charcoal to encourage the production of adventitious roots, then add the activated charcoal in the last 2–3 subcultures to increase the greenish of the leaves and the branching of secondary roots (Fig. 25.4d) as explained by Abul-Soad and Jatoi (2014).

25.2.6 Acclimatization

1. Gradual transfer of the plants from autotrophic conditions to heterotrophic conditions is necessary a few weeks before transfer to the greenhouse. This can be done through covering the tube with aluminum foil instead of the plastic closures and making holes gradually to reduce the moisture inside the tube. Also, liquid medium may be used to reduce the concentration of basal salts and sugar to the half the normal concentrations (15 g L^{-1} instead of 30 g L^{-1}), (Abul-Soad 2011).
2. Select the rooted plantlets with 2–3 leaves of 10–25 cm length and a thick trunk and branched 3–5 adventitious roots with secondary roots.
3. Transfer the in vitro plantlets to the greenhouse and leave them for an hour then gently remove the plantlets from culture tubes and immerse them in a water bath and rinse off the roots from adhering agar.
4. Note although the date palm is full sun plant, but shade must be provided during the growth of the ex vitro plants in the greenhouse. To reduce light intensity net cloth could be used. In addition, plastic low tunnels installed inside the greenhouses (Fig. 25.5a, b) are recommended to control relative humidity.
5. Avoid transferring the in vitro plants to the acclimatization stage in mid of winter as this encourages fungal infection of the transplanted plants. In addition, avoid shaking off the low tunnel sheet as the condensed droplets of evaporated water from the soil and the plants spread the fungi infection inside the tunnel.
6. Treat the plantlets with fungicide, 3 g L^{-1} Topsin M, and plant them in 5-cm × 25-cm plastic pots containing potting mixture consisting of 1:1 washed sand and peat moss and mixed with 10–20% perlite (Jatoi et al. 2015). Place some stones at the bottom of the pot for enhanced aeration. Mist the plantlets with water during the process of soil transfer to prevent desiccation.
7. Place the pots inside the tunnel for a month and never water or fertilize the transplants during this period. They rely on the moist soil bed to provide them with their limited requirements at this stage.
8. Remove the low tunnel gradually after first month of transplanting. Water the plants 1–2 times during second and third months after removal of the low tunnel (Fig. 25.5a). After three months of transplanting and complete removal of the tunnel, fertilize the plants with 100 mg L^{-1} N-P-K fertilizer (20-20-20) subsequently as needed.

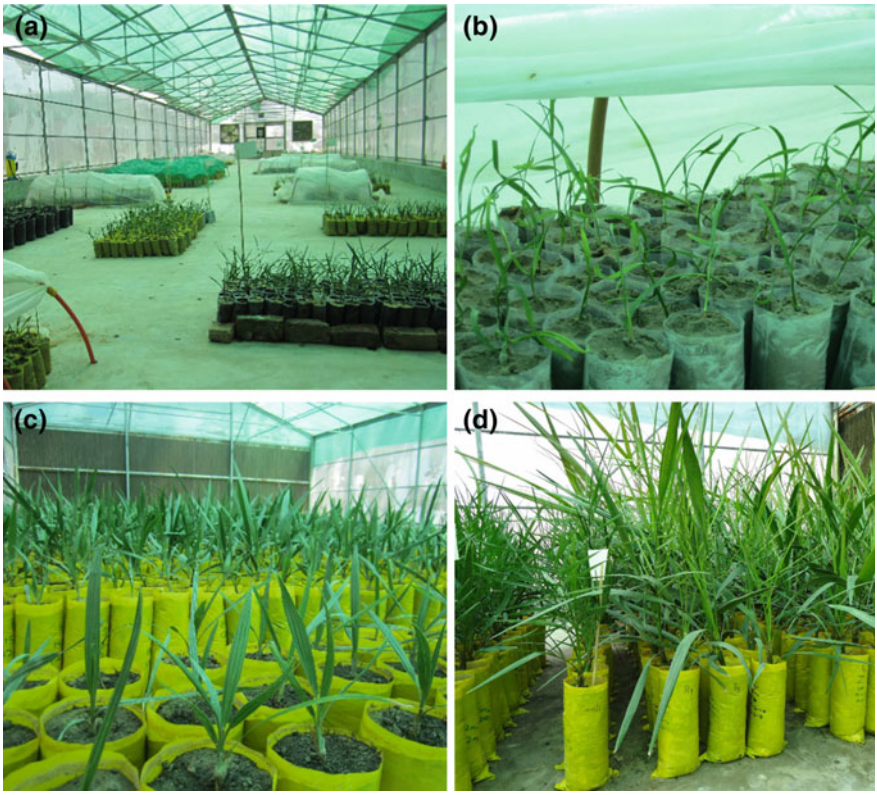

Fig. 25.5 Acclimatization and ex vitro date palm plant establishment. **a** Various stages of ex vitro plantlets acclimatization in the greenhouse which contains plastic low tunnels, **b** plantlets under plastic low tunnel throughout the first 3 weeks after soil transplanting, **c** seven to twelve-month-old ex vitro plants growing in the greenhouse after removing from the plastic low tunnels, **d** ex vitro 2-year-old plants with feathered leaves in of 30-cm-height bags ready to be planted in the permanent field

9. After 6–7 months transfer the ex vitro plants to larger plastic pots of 25 cm height × 20 cm wide or polyethylene bags of 30 cm height × 15 cm wide, and maintain them in the greenhouse (Fig. 25.5c). Note that date palm growth vigor varies among cultivars.
10. After producing feathered leaves (Fig. 25.5d), the plants can be transferred to the open field (Fig. 25.6a). Normally, fruits are produced in the third or fourth year of field growth (Fig. 25.6b, c).
11. Note that it is possible to shift ex vitro date palm plants of 7–12 months old to the open field but protection must be provided to eliminate attacks by farm animals and reduce the impact of strong winds. However, avoid leaving the ex vitro plants more than 2.5 years inside the greenhouse in order to avoid root twisting densely inside the bag or penetrating the bottom of the bag. This root distortion was found to slow plant growth in the field and delay flowering for a year.

Fig. 25.6 Date palm plants produced via immature inflorescence technique. **a** Plant after 1 year in the open field, **b**, **c** normal fruiting of ex vitro plants after 3 years in the open field

25.2.7 Performance of Micropropagated Plants in Open Field

Although phenotypic characteristics can be used to recognize abnormalities, molecular markers are most reliable to detect genetic aberrations (Mirbahar et al. 2014). Surveys conducted on the tissue culture derived plants produced by this protocol at Date Palm Research Institute, Khairpur, Sindh, Pakistan, showed about 2% of phenotypic deformations in vegetative or reproductive characters (Abul-Soad et al. 2015). Six types of abnormalities were recorded during the first couple of years from releasing the plants to open fields. These were dwarf plants, ceased flowering, twisted inflorescence, fertilization failure, multiple carpels and bastard offshoots. However, the majority of these malformations developed into normal growth starting the fourth year. A second phase of the survey is currently in progress based on the use of molecular markers to confirm genetic and epigenetic variations. On the other side, producing productive date palm plants from

inflorescence in thousands of which 98% showed normal growth and fruiting proved that the origin of the induced embryogenic callus from the inflorescence explants was somatic not haploid cells. Also, the histological observations of initial explants showed that the young florets are consisted of meristematic cells and the callus originates from the surface of these meristematic cells at the fourth week of culture (Zayed and Abd Elbar 2015). In fact, this reconfirms the high efficiency of this protocol and potential applicability for commercial tissue culture laboratories.

25.3 Research Prospects

It is evident that inflorescence provide an excellent source of explants for date palm micropropagation. On the average, the lab manipulation process require only 1–1.5 year. One disadvantage is that explants availability is limited to the flowering season. Storing the inflorescences while maintaining their viability will provide extended time for culturing and widen the window of explants availability during the year. In this respect, research is needed to determine the appropriate storage conditions of inflorescences for subsequent culture initiation. Like the case in other date palm explants, response to the culture medium is cultivar dependent (Al-Khayri and Al-Bahrany 2004). At the present, this technique is yet to be demonstrated in most commercial cultivars. Optimization of the culture medium and the inclusion of proper additives are important considerations for achieving regeneration of other date palm cultivars (Al-Khayri 2001). The use of organic additives to attain enhanced plant regeneration from inflorescence explants is another area meriting investigation (Al-Khayri 2010, 2011). As more understanding of the factors controlling the differentiation and dedifferentiation processes of the inflorescence tissue is gained, the use of inflorescence as explant source is expected to expand due to the convenience it provides and the low or absent contamination associated with this type of protected explants.

Acknowledgements Some of the results presented in this chapter were obtained through a research project (Grant No. 5011) funded by the Science and Technology Development Fund (STDF), Egypt.

References

Abul-Soad AA (2011) Micropropagation of date palm using inflorescence explants. In: Jain SM, Al-Khayri JM, Johnson DV (eds) Date palm biotechnology. Springer, Dordrecht, pp 91–118

Abul-Soad AA (2012) Influence of inflorescence explant age and 2,4-D incubation period on somatic embryogenesis of date palm. Emirates J Food Agric 24(5):434–443

Abul-Soad AA, Jatoi MA (2014) Factors affecting in vitro rooting of date palm (*Phoenix dactylifera* L.). Pak J Agri Sci 51(2):477–484

Abul-Soad AA, Mahdi SM (2010) Commercial production of tissue culture date palm (*Phoenix dactylifera* L.) by inflorescence technique. J Genet Eng Biotech 8(2):39–44

Abul-Soad AA, Ibrahim AI, El-Sherbeny NR, Baker SI (2002) In vitro optimization for plant regeneration of date palm (*Phoenix dactylifera* L.). Minia J Agric Res Develop 22(2D): 2265–2282

Abul-Soad AA, Ibrahim AI, El-Sherbeny NR, Baker SI (2004) Improvement and characterization of somatic embryogenesis in date palm (*Phoenix dactylifera* L.). In: Proceedings of the international conference of genetic engineering and its applications. Egyptian Society of Genetics and Suez Canal University, Sharm El Sheikh City, South Sinai, Egypt, 8–11 Apr, pp 359–373

Abul-Soad AA, El-Sherbeny N, Baker S (2005) Date palm (*Phoenix dactylifera* L. cv. Zaghloul) propagation using somatic embryogenesis of female inflorescence. In: 3rd conference on recent technologies in agriculture. Cairo University, Giza, Egypt, pp 423–441

Abul-Soad AA, Mahdi SM, Markhand GS (2015) Date palm status and perspective in Pakistan. In: Jain SM, Al-Khayri JM, Johnson DV (eds) Date palm genetic resources and utilization, vol 2. Asia and Europe. Springer, Dordrecht, pp 153–205

Abul-Soad AA, Abdallah AS, Khalil OA, Mahdi SM (2017a) Conservation of superior Egyptian date palm genotypes through in vitro culture of inflorescence explants. Egypt J Hortic (in press)

Abul-Soad AA, Emara KS, Abdallah AS, Mahdi SM (2017b) Somatic embryogenesis in *Phoenix dactylifera* L. using floral bud explants. In: IX International Symposium on In Vitro Culture and Horticultural Breeding, ISHS 2016. Acta Hortic 1187:13–28

Abul-Soad AA, Jain SM, Jatoi MA (2017c) Biodiversity and conservation of date palm. In: Ahuja MR, Jain SM (eds) Biodiversity and conservation of woody plants, sustainable development and biodiversity. Springer International Publishing AG, pp 313–353. https://doi.org/10.1007/978-3-319-66426-2_12

Alkhateeb AA (2008) A review the problems facing the use of tissue culture technique in date palm (*Phoenix dactylifera* L.). Sci J King Faisal Univ Basic Appl Sci 9:85–104

Al-Khayri JM (2001) Optimization of biotin and thiamine requirements for somatic embryogenesis of date palm (*Phoenix dactylifera* L.). In Vitro Cell Dev Biol Plant 37:453–456

Al-Khayri JM (2010) Somatic embryogenesis of date palm (*Phoenix dactylifera* L.) improved by coconut water. Biotechnology 9:477–484

Al-Khayri JM (2011) Influence of yeast extract and casein hydrolysate on callus multiplication and somatic embryogenesis of date palm (*Phoenix dactylifera* L.). Sci Hortic 130:531–535

Al-Khayri JM, Al-Bahrany AM (2004) Genotype-dependent *in vitro* response of date palm (*Phoenix dactylifera* L.) cultivars to silver nitrate. Sci Hortic 99:153–162

Al-Khayri JM, Naik PM (2017) Date palm micropropagation: advances and applications. Ciênc Agrotec 41(4):347–358

Al-Khayri JM, Jain SM, Johnson DV (eds) (2015a) Date palm genetic resources and utilization, vol 1, Africa and the Americas, 546 p. Springer, Dordrecht

Al-Khayri JM, Jain SM, Johnson DV (eds) (2015b) Date palm genetic resources and utilization, vol 2, Asia and Europe, 566 p. Springer, Dordrecht

Bhaskaran SH, Smith RH (1992) Somatic embryogenesis from shoot tip and immature inflorescence of *Phoenix dactylifera* L. cv. Barhee. Plant Cell Rep 12:22–25

Drira N, Benbadis A (1985) Vegetative multiplication of date palm (*Phoenix dactylifera* L.) by reversion of in vitro cultured female flower buds. J Plant Physiol 119(3):227–235

FAOSTAT (2014) Food and Agricultural Organization of the United Nations. Available from: www.fao.org/faostat/en/. Accessed 10 Nov 2017

Fki L, Masmoudi R, Drira N, Rival A (2003) An optimized protocol for plant regeneration from embryogenic suspension cultures of date palm, *Phoenix dactylifera* L., cv. Deglet Nour. Plant Cell Rep 21:517–524

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Hassanpour-Estahbanati A, Hamidian S (2007) Morphological abnormalities in tissue culture derived date palm plants (*Phoenix dactylifera* L.) in Southern province of Iran. In: 4th international symposium on date palm in Saudi Arabia, King Faisal Univ., Al-Hassa, 5–8 May

Jatoi MA, Abul-Soad AA, Markhand GS, Solangi N (2015) Establishment of an efficient protocol for micropropagation of some Pakistani cultivars of date palm (*Phoenix Dactylifera* L.) using novel inflorescence explants. Pak J Bot 47(5):1921–1927

Kriaa W, Sghaier B, Masmoudi F, Benjemaa R, Drira N (2012) The date palm (*Phoenix dactylifera* L.) micropropagation using completely mature female flowers. CR Biol 335: 194–204

Mirbahar AA, Markhand GS, Khan S, Abul-Soad AA (2014) Molecular characterization of some Pakistani date palm (*Phoenix dactylifera* L.) cultivars by RAPD markers. Pak J Bot 46: 619–625

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Tisserat B (1984) Propagation of date palms by shoot tip cultures. HortScience 19:230–231

Veramendi J, Navarro L (1997) Influence of explant sources of adult date palm (*Phoenix dactylifera* L.) on embryogenic callus formation. J Hort Sci 72:665–671

Zaid A, de Wet PF (2002) Origin, geographical distribution and nutritional values of date palm. In: Zaid A (ed) Date palm cultivation. FAO Plant Production and Protection, vol 156. Food and Agricultural Organization of the United Nations, Rome

Zayed EMM, Abd Elbar OH (2015) Morphogenesis of immature female inflorescences of date palm in vitro. Ann Agri Sci 60(1):113–120

Chapter 26
Haploid Embryogenesis in Tea

R. Bajpai and R. Chaturvedi

26.1 Introduction

Plants have been a boon to mankind, from serving as a source of food and energy for ever increasing world's population to generating employment to millions of people and being hub of potentially beneficial medicinal compounds (Namita et al. 2012). Thus, considerable efforts have been made to improvise the existing farming practices and to obtain new varieties with high quality traits that could meet the demands of the ever increasing population (Olmedilla 2010). Genetic improvement in commercially important woody perennials, such as Tea, Neem, Citrus, Populous etc., including grain crops, such as wheat, rice, rye, barley, is nearly impossible following traditional breeding approach, owing to their long reproductive cycle, self-incompatibility, high inbreeding depression and extreme heterozygous nature (Germana 2006, ; Srivastava and Chaturvedi 2008). Producing genetically modified (GM) crops using transgenic technique has been another option to achieve value added crop improvement but regulatory measures involved in propagation of GM crops restricts their usage (Comai 2014). Thus, in vitro haploid plant production serves as a legitimate solution to overcome the shortcomings of conventional breeding and, thereby, to develop genetically stable elite clones with high quality traits (Islam and Tuteja 2012; Mishra et al. 2017).

Haploids may be defined as sporophytic plants with gametic chromosomal constitution (Palmer and Keller 2005; Germana 2011; Shen et al. 2015). Doubling of haploid chromosomes, using chemical mutagens, results in 100% homozygosity in a single generation (Hazarika et al. 2013; Dwivedi et al. 2015). It proved to be the shortest possible route of attaining pure breeding lines, which otherwise requires

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_26

several generations of selfing (Seran 2007; Srivastava and Chaturvedi 2008; Shen et al. 2015; Mishra et al. 2017) using conventional approaches.

Nature bestows plants with a life cycle that alternates between two sequential phases, the dominant sporophytic (2n) phase involving formation of numerous haploid spores (n) as a result of meiosis. These haploid spores undergo mitotic divisions forming multicellular haploid gametophyte where each cell is haploid. The gametophytic phase on the other hand, involves formation of gametes by the process called mitosis, whereby this multicellular haploid gametophyte produces male gametes (sperms) and female gametes (eggs). The gametes (male and female) unite together and give rise to diploid zygote i.e. a sporophyte (2n), repeating the sporophytic phase (Germana 2011; Hazarika et al. 2013) (Fig. 26.1). Plants with

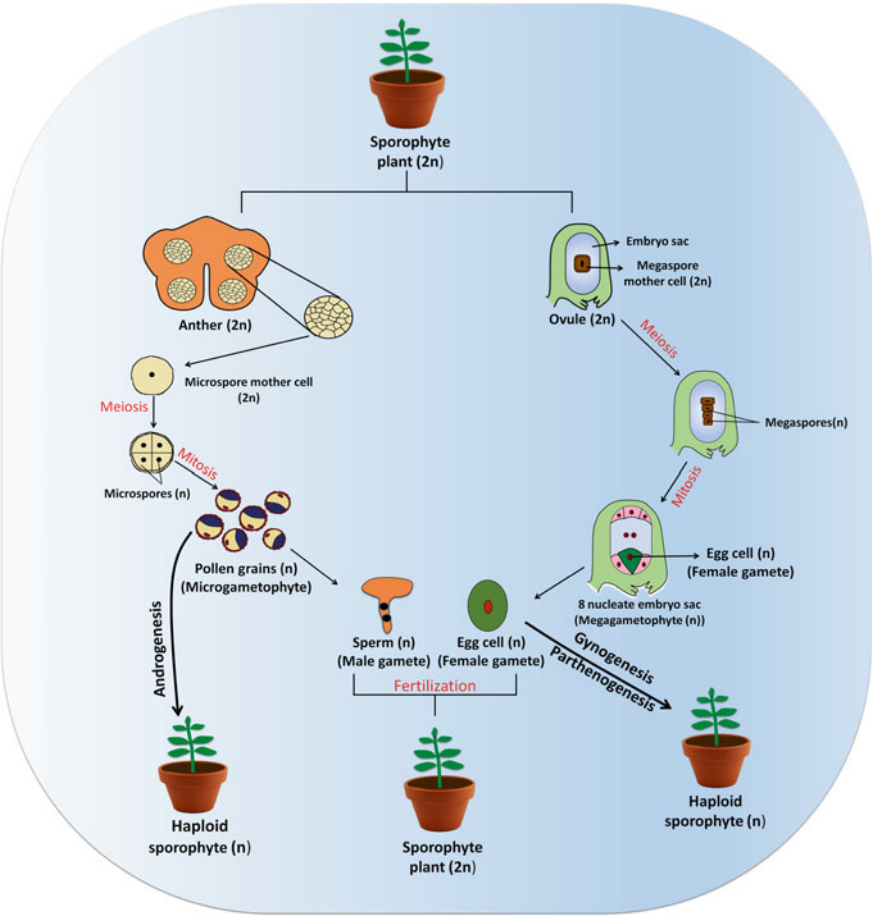


Fig. 26.1 Development of male and female gametes and their involvement in haploid plant development

gametophytic constitution (n) are sterile in nature and are considered to have reduced size compared to their diploid counterparts (Bhojwani and Razdan 1996). The breakthrough discovery of naturally occurring haploid plants in *Datura stramonium* L. (Blackslee et al. 1922), followed by remarkable journey of obtaining haploids through anther cultures in *Datura annoxia* (Guha and Maheswari 1964, 1966), induction of haploid embryos via interspecific crosses in barley (*Hordeum vulgare* L.) (Kasha and Kao 1970) and from unfertilized cells of female gametophyte (embryo sac) in barley (San Noeum 1976), revolutionised the definition of haploid plants and proved their utility in plant breeding.

Spontaneous occurrence of haploids is a rare phenomenon (0.001–0.1%) in nature, which limits their utility in breeding programs (Bhojwani and Razdan 1996; Hazarika et al. 2013; Karasawa et al. 2015). Therefore, adopting artificial methods of haploid induction using in vivo techniques (parthenogenesis, polyembryony, distant hybridization using chromosome elimination technique) and in vitro methods, by inoculating immature explants, like anthers bearing haploid microspores (n, microgametophyte, process referred as androgenesis) and ovary/ovules bearing unfertilized female gamete (n, egg cell; process referred as gynogenesis) into culture medium, becomes a necessity to uplift the agricultural productivity. In vitro methods of haploid production are the most preferred of all existing approaches and have been reported in more than 250 plant species till date, where most of them are legumes (Segui 2010; Dunwell 2010). However, it has gained limited success in woody plants, such as tea, owing to its recalcitrant nature (Mondal et al. 2004; Hazarika et al. 2013; Mishra and Goswami 2014; Mishra et al. 2017).

Tea is an economically important medicinal plant and a source of beverages consumed throughout world next to water (Katiyar and Mukhtar 1996). High demand and low rate of production necessitates development of high yielding elite varieties in tea (Akula et al. 2000). More than a decade of strenuous efforts, varietal improvement within the existing varieties of tea were attained following conventional breeding approaches, but, have been narrowed down due to the long reproductive cycle, extremely heterozygous nature and intense inbreeding depression within cultivated taxa (Mondal et al. 2004). In such a scenario, developing haploid plants followed by their diploidization will not only shorten the long gestation period but, will also aid in development of genetically stable clones. Current chapter represents a successful protocol of obtaining complete haploid plantlets in tea.

26.2 Methods for Haploid Inception

Many research groups attempted generation of haploids using in vivo and in vitro methods and the choice vary from crop to crop (Magoon and Khanna 1963; Kasha 1974; Maluszynski et al. 2003; Xu et al. 2007; Touraev et al. 2009; Dunwell 2010). In majority of the crops microspore mediated (androgenic) haploid development was obtained, while in case of barley distant hybridization was a method of choice.

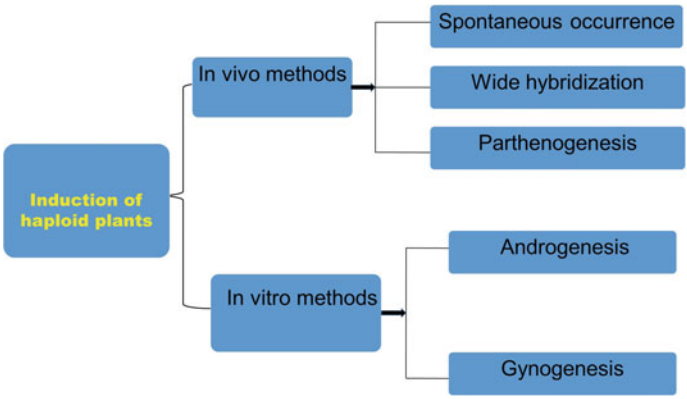


Fig. 26.2 Methodologies of haploid induction

In a few other plants, gynogenesis or parthenogenesis in unfertilized female gametophyte resulted in haploids. Occasionally, spontaneous occurrence of haploids was also reported (Fig. 26.2).

26.2.1 In Vivo Haploid Induction

This technique involves the spontaneously occurring haploids, haploids induced by wide hybridization and by parthenogenesis.

26.2.1.1 Spontaneous Occurrence of Haploids

The rare occurrences of haploids are noticed in nature but at low frequency of 0.001–1% (Bhojwani and Razdan 1996). After the discovery of first spontaneously occurring haploid plants in *Datura* with the cytological evidences of their existence by Dorothy Bergner in 1921 (Blackslee 1922), several other reports followed. Although spontaneously occurring haploids have been used for producing DH lines, but the frequency of homozygous development was low as compared to other methods (Palmer and Keller 2005).

26.2.1.2 Wide Hybridization

It is an effective method of achieving haploidy in plants via interspecific or intergeneric crosses between the cultivated species and their wild relatives. In addition to it, hybridization facilitates creation of new varieties through transfer of agronomically important characters (Zenkteler and Nitzsche 1984). This process is

prevalent mostly in cereal crops, such as wheat, rye and barley (Froster et al. 2007). In most of the cases, haploid formation precedes fertilization of female gametophyte via pollinators from distantly related species or an intergeneric plant, resulting in viable embryo and functional endosperm but in certain cases the endosperm may be absent or abnormal (Laurie and Bennet 1988). The other crossing over method, known as *Hordeum bulbosum* method, was described by (Kasha and Kao 1970) after haploid plants in *H. vulgare* were achieved during its cross with *H. bulbosum* following selective elimination of chromosome of the latter. Chromosomal elimination could be referred as an extension of wide hybridization process where the nuclei of the zygote formed after cross inherits the genome from both parents but subsequently gets eliminated; the elimination is a selective elimination (Dunwell 2010; Comai 2014). Generation of haploids using hybridization process has certain advantages over in vitro methods, which are sometimes encountered with albinism in microspore/egg cell derived haploid embryos and may also cause mortality. On the other hand, the wide hybridization method has limitations due to its dependency on simultaneous flowering in both the parents involved in crosses (Froster et al. 2007).

26.2.1.3 Parthenogenesis

Development of an embryo from an egg cell without involving the fusion of egg cell with the male/sperm nuclei is termed as parthenogenesis. This method is rarely observed in nature (Dunwell 2010), but could be induced via pollination through irradiated or inactive pollen or by using chemicals (Khush and Virmani 1996).

26.2.2 In Vitro Methods of Haploid Production

In vitro development of haploid plants is achieved by triggering immature male or female gametophyte with certain stimulus, generally a temporary stress treatment that diverts their mode of development from gametophytic to sporophytic giving rise to haploids (Srivastava and Chaturvedi 2008; Germana 2011). The process of gametogenesis could be termed as androgenesis or gynogenesis on the basis of gametic explants (male/female) chosen for culture initiation. These haploid explants undergo the gametic embryos development, directly or preceded by callusing. The embryos germinate into complete haploid sporophytic plants. Both the process have been reported to develop haploids efficiently, but, androgenesis is preferred over gynogenesis due to the presence of several anthers with indefinite number of haploid pollen grains within a flower in contrast to the presence of single ovary with limited number of ovules (Srivastava and Chaturvedi 2008; Srivastava et al. 2011; Soriano et al. 2013) and a single haploid egg cell inside each ovule. A brief description of both the methods is described in the subsequent sub-sections followed by a detailed protocol on tea haploid embryogenesis as case study.

This will provide a vision on the implementation of in vitro haploid technology and its intervention to generate homozygous diploid plants (pure breed lines) even in strictly cross pollinating tree species where conventional methods are difficult to implement.

26.2.2.1 Androgenesis

In androgenesis the male gametophyte (pollen grain/microspore) undergoes transition from its normal gametophytic mode of development to sporophytic mode under the influence of a stress pre-treatment that could be either a physical or chemical treatment (Srivastava and Chaturvedi 2008). The mechanism of androgenic haploid production (Fig. 26.3) involves either detachment of intact entire immature anthers from stamens or isolation of microspores from anthers by methods, like centrifugation, simple stirring, squeezing, and inoculating them into

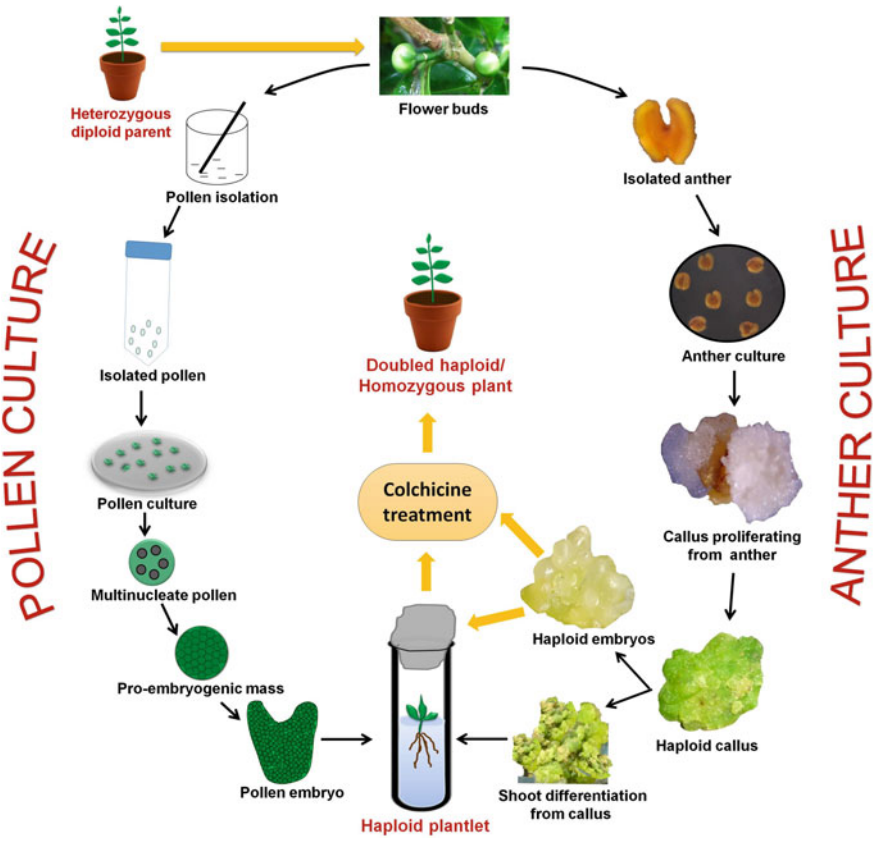


Fig. 26.3 Androgenesis mediated production of doubled haploid plants

the culture medium (Ferrie and Caswell 2011). Anther culture has been reported to be a simple, one step protocol for achieving pure breeding lines (Chaturvedi et al. 2003; Hazarika et al. 2013). Anthers in medium provide better growth conditions to the microspores and simultaneously prevent them from being directly exposed to the stress (Soriano et al. 2013). The culture of isolated microspores on the other hand is complex and requires more skill and highly enriched medium for androgenic induction (Germana 2011; Mishra and Goswami 2014).

Anther culture has been reported as an easier and more efficient method of producing haploid plants (Hofer 2004; Germana 2011), however it has certain drawbacks, such as delayed response time due to the presence of anther wall, which need to be burst open to release microspores (Foster et al. 2007; Soriano et al. 2013) and the presence of mixed stages of microspores development. The other limitation with anther culture is simultaneous proliferation of the somatic cells of the anther wall along with the microspore proliferation, which results into chimera formation. Thus crucial observation of the cultures has to be made to abstain any callusing from anther wall (Bhojwani and Razdan 1996). The problem of chimera formation during anther culture could be avoided, if the haploids would be induced from isolated microspores (Germana 2005; Ferrie and Caswell 2011; Germana 2011). Androgenic haploids have been obtained in more than 200 genera. In tea, however no report on complete plant development via androgenesis was available until before the report by Mishra et al. (2017).

26.2.2.2 Gynogenesis

Is an alternative strategy of attaining in vitro haploids in plants when anther culture is not successful and encountered with recalcitrance or white plant production (Bohanec 2009). Immature ovaries or ovules or unopened flower-buds bearing unfertilised egg cell are generally used to obtain gynogenic haploids. Families such as *Chenopodiacea*, *Liliaceae* and *Cucurbitaceae,* where androgenesis seems difficult, or in certain plants with male sterility or those with dioecious nature, gynogenic cultures serves as best possible route for achieving haploid plants (Thomas et al. 2000; Bhatt and Murthy 2007; Chen et al. 2010). The technique has equally been used in crops, like maize, rice, barley, where haploids have successfully been attained through anther or microspore culture (Sita 1996). However, this technique is considered to be very cumbersome and less efficient method for induction of haploids (Palmer and Keller 2005; Froster et al. 2007) though it favours genetic stability of doubled haploids and produces green plant production as compared to androgenesis (Palmer and Keller 2005). No literature on gynogenesis in tea was available until before Hazarika and Chaturvedi (2013). The transverse sections of the unpollinated ovaries were used in the author's laboratory to induce gynogenic haploids in tea (*Camellia sinensis* (L.) O. Kuntze) (Hazarika et al. 2013).

26.3 In Vitro Haploid Induction in Tea—*A Case Study*

The methodology on haploid plant development via androgenic embryos in tea has been adopted from the author's laboratory. Certain crucial parameters involved in successful regeneration of a haploid plantlet are elaborated in the below subsections.

26.3.1 Explant Selection

Fresh flower-buds, measuring 4 mm in diameter (Fig. 26.5a), from tea (*Camellia assamica* ssp. *assamica* (Masters) plantations were plucked in the early morning hours, between 6 am and 7 am, during the months from October to December. Buds were dissected and anthers were taken to determine the correct stage of microspores, at early-to-late uninucleate stages, using acetocarmine. Later, 4 mm sized buds were routinely used to initiate anther cultures.

26.3.2 Inception of Aseptic Cultures

Immature flower buds of appropriate size, as mentioned in above section, were collected from field. The selected buds were taken inside the laminar-air-flow cabinet and surface sterilized with 0.8% (v/v) sodium hypochlorite for 7 min, after which they were washed at least three times with sterile distilled water. Following sterilization, the buds were dissected under the stereo-microscope with the help of fine needles and forceps. Any of the anthers, if damaged were discarded. Twenty anthers from a single bud were cultured into pre-sterilised Petri plates (60 × 15 mm) containing 10 ml of callus induction medium mentioned in section below. After inoculation the plates were sealed with Parafilm and subjected to different conditions of light and temperature treatment mentioned in ensuing sections.

26.3.3 Preparation of Culture Medium

Murashige and Skoog (1962, MS) medium in combination of different growth regulators and certain additives was used to initiate androgenic cultures in tea. The concentration of the stock solutions used for preparation of MS medium is described below:

- Macronutrient stock solution (10X); Micronutrients (20X); Iron stock (20X) and Vitamins (20X).
- All the growth regulators used during the experiment were at the concentration of 1×10^{-3} M. GA_3 (Gibbrellic acid) used at certain stages in medium was filter sterilised with 0.2 μM filter and added to medium after autoclaving.
- Additives like L-glutamine and L-serine were prepared freshly every time, filter sterilised and added to the medium following autoclaving.

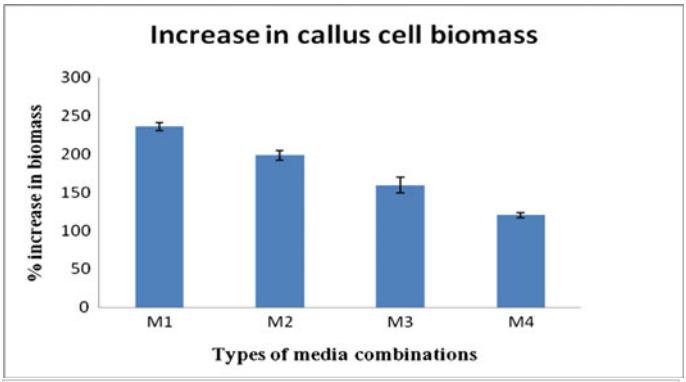
The stock solutions, growth regulators and the additives were refrigerated at 4 °C until use. Sucrose and myoinositol were added afresh while the medium was prepared. The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1 N HCl as per requirement. Agar at a concentration of (0.8%) was used to solidify the media. The medium was autoclaved for 15 min at 121 °C at 15 psi and finally poured into the vessels (Petriplates or test-tubes used for experiment).

26.3.4 Culture Condition

Stress conditions, such as high temperatures, cold shock and sucrose starvation, have been reported to promote callusing in anthers (Chaturvedi et al 2003; Srivastava and Chaturvedi 2008). Therefore, following published reports on androgenesis in author's laboratory, the tea anthers in culture medium were exposed to temperature pre-treatment, either cold (5 °C) or heat (33 °C shock) in completely dark conditions. The incubation of cultures at 25 °C in light and dark served as control experiments. After the initial exposure to dark conditions for 5 days, all cultures were maintained at 25 ± 2 °C, humidity of 50–60% under (16/8 h) photoperiod. Cultures were observed regularly to monitor any changes in their morphology.

26.3.5 Callus Induction and Multiplication

The isolated anthers (Fig. 26.5b) were inoculated on MS (Murashige and Skoog 1962) medium supplemented with auxins, like 2,4 dichlorophenoxyacetic acid (2, 4-D), α-naphthalene acetic acid (NAA) and cytokinins, such as 6-furfurylaminopurine (Kinetin) and 6-benzylaminopurine (BAP), at different concentrations together with L-glutamine and L-serine that acted as additional sources of nitrogen in the medium. Two different carbon sources (glucose and sucrose) at varying concentration between 3%, 6% and 12% were tested to identify which of them induces callusing faster. Different media combinations were tested for haploid callus induction. Maximum callusing (96%) from inside anther locules was achieved on callus induction medium consisting of MS with glucose (6%), 2, 4-D (5 μM), Kinetin (5 μM), L-glutamine (800 mg/L) and L-serine (200 mg/L).



M1: MS + sucrose (3%) + 2,4-D (5 μM) + Kinetin (5 μM) + L-glutamine (800 mg/L) + L- serine (200 mg/L)

M2: MS + glucose (3%) + 2,4-D (5 μM) + Kinetin (5 μM) + L-glutamine (800 mg/L) + L- serine (200 mg/L)

M3: MS + sucrose (3%) + 2,4-D (3 μM) + TDZ (18 μM)

M4: MS + sucrose (3%) + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM)

Fig. 26.4 Increase in callus cell biomass

Profusely growing light green callus (Fig. 26.5c) was attained on this medium. But, a significant increase in cell biomass proliferation was observed (Fig. 26.4) when glucose in the induction medium was substituted with sucrose (3%).

26.3.6 Callus Differentiation and Embryo Maturation

Highly proliferating callus on maintenance medium MS + Sucrose (3%) + 2,4-D (5 μM) + Kinetin (5 μM) + L-glutamine (800 mg/L) and L-serine (200 mg/L) showed variations in its morphology and developed as nodulated callus after 8 weeks (2 subcultures of 4 week each on same medium). The callus with nodules showing signs of regeneration was transferred to MS + BAP (10 μM) + GA_3 (3 μM) + L-glutamine (800 mg/L) and L-serine (200 mg/L) medium where it developed into embryos. Maturation of asynchronously growing embryos showing various stages of embryo development (Fig. 26.5d) was attained when the

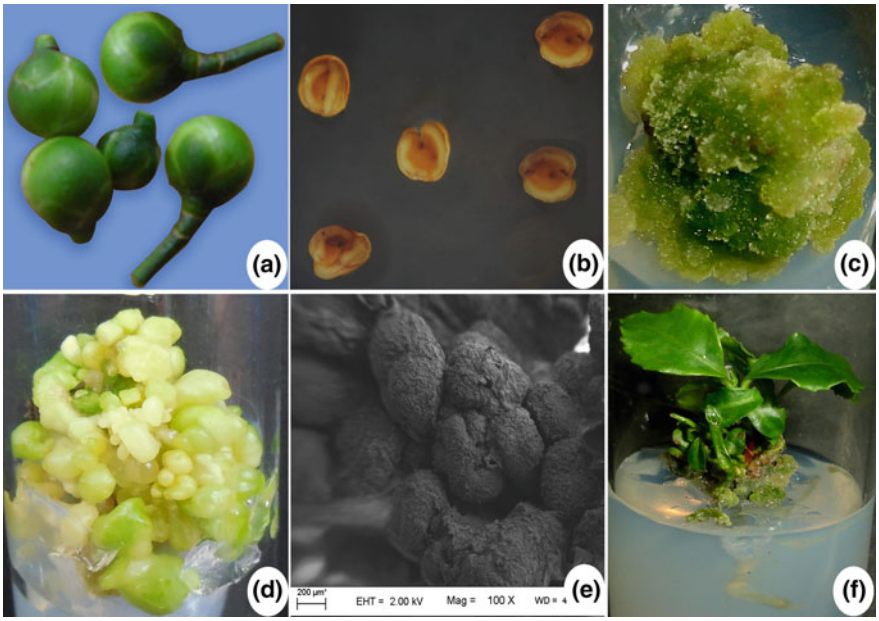


Fig. 26.5 Anther culture in tea. **a** Flower buds bearing anthers at correct stage of microspores **b** Isolated anthers inoculated on callus induction medium **c** Highly proliferating callus induced from anthers in culture on induction medium. **d** Asynchronously growing cluster of haploid embryos at different stages of development. **e** Same as **d**, scanning electron micrograph image of embryos in cluster. **f** Complete developed haploid plantlet

concentration of growth regulators and supplements in the embryogenesis medium were reduced by 10 folds. The structure of embryos obtained was determined through scanning electron micrographic technique (Fig. 26.5e).

26.3.7 Embryo Germination and Development of Haploid Plantlet

The germination of embryos into complete haploid plantlets (Fig. 26.5f) was attained when the embryos were transferred to MS + BAP (10 µM) + IBA (1 µM) + GA_3 (0.5 µM) + L-glutamine (80 mg/L) and L-serine (20 mg/L) medium. Further growth of the plantlets was attained when the concentration of the major salts in the above mentioned germination medium was reduced to half (1/2). Cytological squash preparation from root-tips of the regenerated plants and the flow cytometric analysis of their leaves, discussed in section below, confirmed the haploid status of the regenerated plants.

26.4 Factors Affecting Haploid Embryogenesis

The in vitro haploid induction is governed by certain crucial factors, such as the physiological condition of the donor plant, genotype, stage of the explant, stress pre-treatments to explants, composition of the medium and culture conditions used to initiate cultures.

26.4.1 Physical Condition of the Donor Plant

Successful induction of gametic embryogenesis greatly relies on availability of flower-buds from diseases free plants. The quality of light and temperature conditions provided to the donor plants affect the responses from the explants, anthers/ovaries (Ferrie and Caswell 2011). Growing donor plants in green house would reduce the contamination that is often high in experimental material collected from field (Ferrie and Caswell 2011). Age of the donor plant plays a vital role in promoting gametogenesis (Maheswari et al. 1982; Jacquard et al. 2006; Mishra and Goswami 2014).

26.4.2 Genotype of Anther/Ovary Donor

Numerous studies revealed that genotype of the donor plant is a key player in deciding the efficiency of embryogenesis (Olmedilla 2010). The response of different cultivars within same species varies in terms of embryo induction ability; for example in *Brassica napus*, Topas, DH 4079 cultivars are highly embryogenic while the other members of the same family i.e. *Brassica olerecea* ssp *italica*, Shogun is the variety with high embryogenic potential (da Silva Dias 2001). The exact mechanism as to how the genotype influences the rate of embryogenesis is not yet clear. However, several studies conducted on micropspore embryogenesis reveal that the frequency of normal green plant formation via anther culture technique varies with genotype and is genetically controlled by additive effect of nuclear genes whose expression is largely influenced by environmental factors and also by certain cytoplasmic factors (Larsen et al.1991; Datta 2005; Olmedilla 2010).

26.4.3 Stage of the Explant

Stage of the explant is one of the most crucial factors to induce haploid embryogenesis (Bhojwani and Razdan 1996; Olmedilla 2010). Touraev et al. (1997) indicated that microspores are liable to vary their developmental pattern within

wide developmental window. The immature anthers with early-to-late uninucleate stages of microspores have been suggested to be most responsive stage for attaining androgenic haploids for example in Barley (Clapham 1971); *Azadirachta indica* (Chaturvedi et al. 2003; Srivastava and Chaturvedi 2008; Srivastava et al. 2011); *Eribotrya japonica* (Germana 2006); *Camellia assamica* (Mishra et al. 2017). In a few others, like *Arabidopsis thaliana* (Gress hoff and Doy 1972), *Albizzia lebbek* L (Gharyal et al. 1982), *Camellia assamica ssp assamica* (Gharyal et al. 1983), Maize (Gaillard et al. 1991), only the late uninucleate stage of microspore was responsive for haploid induction. Similarly, determining the correct stage of embryo sac appears to be a crucial parameter while initiating haploid generation through gynogenesis. It is quite difficult to identify the stage of embryo sac, but, this could be done through histological sections of the ovule (Hazarika and Chaturvedi 2013).

26.4.4 Composition of the Medium

The culture medium not only acts as the source of nutrient but also routes the development of embryos (Datta 2005). There is no subscribed medium for induction of haploids via androgenesis or gynogenesis since the nutritional requirement of each plant species varies (Mishra and Goswami 2014). Thus, supplementing the medium with proper concentrations of mineral salts, carbon source, suitable growth regulators and certain additives regulates the fate of haploid explants in culture (Olmedilla 2010). Usually a combination of cytokinin and auxin has been found to successfully induce haploids in woody plants (Nair et al. 1983), using BAP in combination with 2,4-D at varying concentrations resulted in haploid callus induction in Neem (Chaturvedi et al. 2003), *Allium* spp. (Alan et al. 2003), *Vitis labruscana* (Nakajima et al. 2000), *Camellia sinensis* (L.) O Kuntze (Hazarika and Chaturvedi 2013); *Camellia assamica* ssp *assamica* (Masters) (Mishra et al. 2017). Optimization of type and concentration of carbon source is another vital factor that regulates androgenic response (Bhojwani and Razdan 1996). Sucrose is mostly the preferred carbon source in nutrient media for in vitro culture (Mishra and Goswami 2014), but the concentration at which it is used varies in different plant species (Olmedilla 2010). Sucrose at higher concentrations up to 12% was used for callus induction from anthers in Neem, but for further regeneration of callus into embryos a lower concentration of sucrose 3% was suitable (Chaturvedi et al. 2003; Srivastava and Chaturvedi, 2008). Similar observation was made in *Camellia assamica* ssp *assamica* (Masters) (Mishra et al. 2017); both carbon sources, glucose and sucrose, were used in the range of 3–12% but, best response in terms of callus induction from anther locules was achieved when (6%) glucose was used while higher concentrations of carbohydrate showed inhibition of callus proliferation. Following this, embryogenesis from calli and germination from these haploid embryos was attained on MS medium with 3% sucrose content (Mishra et al. 2017).

26.4.5 Stress Pre-treatment for Culture Initiation

Application of stress treatments is a pre-requisite for inducing haploids as it facilitates reprogramming of gametic explants and deviates their mode of development from gametophytic to sporophytic (Chaturvedi et al. 2003; Olmedilla 2010; Germana 2011; Karawasa et al. 2016; Mishra et al. 2017). The kind of stress pre-treatment varies from plant to plant; it could be temperature, osmotic stress, gamma irradiation, addition of ethanol, heavy metal and hypertonic environment (Islam and Tuteja 2012) or a combination of them. Cold temperature pre-treatment provided to the anthers promoted androgenesis in Dhatura (Sapory and Maheswari 1976), Wheat (Hu and Kasha 1997), Neem (Chaturvedi et al. 2003; Srivastava and Chaturvedi 2008); Tea (Mishra et al. 2017). In a few other plants, such as *Brassica campestries* (Keller and Armstrong 1979), *Brassica napus* ssp *oleifera* (Dunwell 1985), *Brassica napus* (Custers et al. 1994), Wheat (Touraev et al. 1996), high temperature pre-treatment was used to induce haploids. Starvation conditions maintained in the medium by depriving the cultures with carbon and/or nitrogen source have also proven beneficial for inducing haploids in *Brassica campestries* (Keller and Armstrong 1979), Wheat (Touraev et al. 1996), *Quercus suber* (Bueno et al. 1997). The starvation conditions have often been applied in combination of high temperature to induce haploid plants in *Brassica compestries* (Keller and Armstrong 1979), *Quercus suber* (Bueno et al. 1997). In addition to the temperature stresses, certain reports reveal the application of osmolytes, such as maltose in enhancing haploid production in Barley (Scott and Lyne 1994, 1995), Tobacco (Touraev et al. 1996), Wheat (Touraev et al. 1996) and Fabaceae (Ochatt et al. 2009).

26.5 Screening Haploids and Doubled Haploid Plants

Spontaneous diploidization that may occur in in vitro haploid cultures and somatic cell proliferation along with pollen callusing in anther cultures may cause mixing of haploid and diploid calli. Therefore, it is very essential to screen the plants regenerated from these calli (Germana 2011). The ploidy status of the regenerated plants can be determined by methods, such as cytological squash preparation of cells, flow cytometric analysis, isoenzyme analysis and utilization of molecular markers, such as RAPD (Randomly amplified polymorphic DNA), SCAR (sequence characterized amplified region) and SSR (simple sequence repeats) (Chen et al. 1998; Bartosova et al. 2005; Srivastava and Chaturvedi 2008; Mishra et al. 2017). Ploidy of the androgenic haploid plants was determined using flow cytometry and chromosomal counting via cytological squash preparation.

26.5.1 Flow Cytometry

Fresh, young leaves from in vitro regenerated haploid plantlets and the field grown plant (control) were utilized to estimate the ploidy through flow cytometer. The leaves were chopped in nuclear isolating buffer consisting of 0.2 M Tris HCl, 4 mM $MgCl_2.6H_2O$, 2.5 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM Sodium Metabisulfite, 1.5% Triton-X-100 and 2% PVP-10. The pH of the buffer was adjusted to 7.5. The nuclear suspension thus obtained was filtered using 30.0 μm nylon net filter membrane. Rnase A and propidium iodide, both at a concentration of 50 μg/ml were added to the obtained filtrate. The prepared samples were analysed using photomultiplier voltage of the instrument. The peak position of the reference (control) was determined following which the ploidy of the unknown sample was analysed keeping the instrument settings constant. The flowcytometric analysis from leaves of in vitro regenerated haploid plant shown in (Fig. 26.6a) exhibit major G1/G0 peak at channel position 100 and a smaller G2 peak at channel position 200 while (Fig. 26.6b), is the histogram obtained from the leaves of parent plant showing major G0/G1peak at channel position 200 and a smaller G2 peak at channel 400. The flowcytometry analysis confirms haploid nature of in vitro regenerated plants.

26.5.2 Cytological Squash Preparation

Chromosome counting is one of the most commonly used, reliable method for determining the ploidy of regenerated plants (Mishra and Goswami 2014). It has been reported as a best method representing exact origin of plants (Maluszynska 2003). Cytological analysis of haploid plantlets obtained via anther culture in tea and the field grown *Camellia assamica* ssp *assamica* plant (Control) was performed. The root tips from in vitro developed haploid plantlets and the shoot tips from the field grown plant were washed with sterile distil water. The roots were then treated with 8-hydroxyquinoline and refrigerated at 4 °C for 4 h. Following this the treated roots were fixed in modified Carnoy's solution (7:3:3:1 v/v/v/v absolute ethanol: chloroform: methanol: glacial acetic acid) and kept at 4 °C for 48 h. The material was stored in 70% ethanol until use (Chaturvedi et al 2003). The material to be analysed was stained with 1% (w/v) aceto-orcein dye and 1 N HCL mixture and heated gently. It was transferred to a glass slide, covered with coverslip and squashed. The results were analysed under the Nikon 80i microscope. The cytological analysis of the root-tips from in vitro regenerated haploid plants (Fig. 26.6c) revealed the haploid chromosomal constitution as (2n = 2X = 15) which was exactly half of the chromosomal count from shoot-tip of (control) parent plant (2n = 2X = 30) (Fig. 26.6d). The study further confirms the haploid status of regenerated plants.

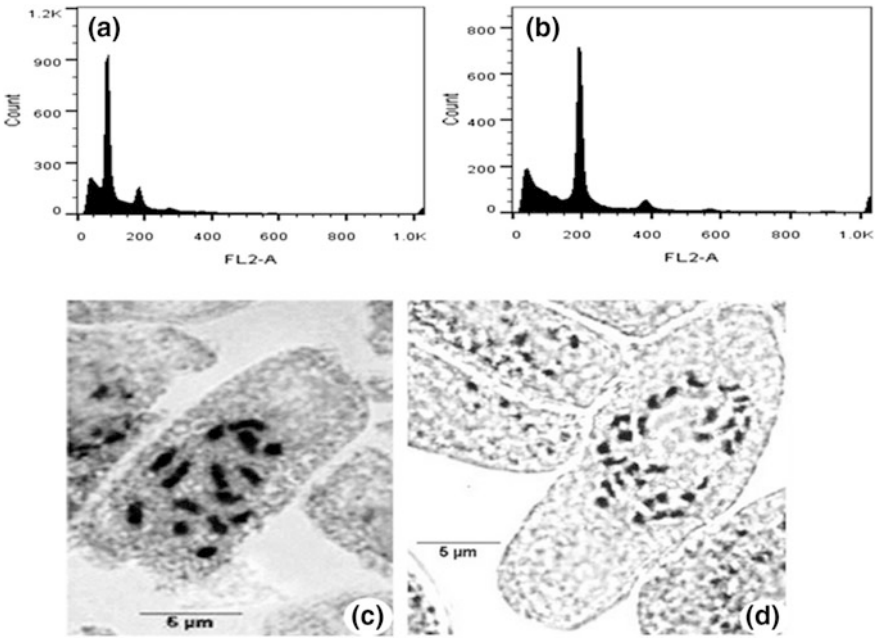


Fig. 26.6 The main objective is to determine the ploidy of the haploids and of control plants. The two methods for the ploidy analysis are (i) Flow cytometry that determines the total DNA count, and (ii) Cytological squash preparation that determines the total number of chromosomes in a given ploidy. Since the two techniques are followed for the same objective of ploidy analysis, hence, all the four figures, which belongs to this objectives are placed together and have been given the same figure number. This is as per the norms

26.6 Uses of Haploids and Doubled Haploid Plants

Haploid embryogenesis becomes a pre-requisite of the present day plant breeders providing speedy generation of pure breeding lines in heterozygous plants with long reproductive cycle (Srivastava and Chaturvedi 2008). Regeneration of doubled haploid plants would facilitate easier detection of recessive mutations at plant level which otherwise cannot be noticed in the presence of dominant alleles (Germana 2011; Mishra et al. 2017) in heterozygous diploid plants. The totipotent nature of microspores has been recognised as a readily available single origin target that could be utilised for transformation (Touraev et al. 1997). The genetic stability within the doubled haploid population makes it easy to mark QTLs within them and, thus, to study phenotype of plants in different environmental locations (Datta 2005). Although QTLs are of great importance in trait related studies, but gathering information about the genes for those traits is a tricky process. Use of recombinant chromosome substitution lines in such instances provide accurate mapping for both

QTLs and doubled haploids towards specific target (Thomas et al. 2000). Doubled haploid production is of great benefit as the seeds produced by them could be grown again and harvested for faster analysis using marker system (Maluszynski 2003). Generation of aneuploids during anther culture in certain species, such as rice, neem, help in studying extra chromosome or the chromosomal behaviour and thereby aid in genome construction (Datta 2005). The developed doubled haploids may overcome the limitations of conventional breeding methods and lead to the generation of elite homozygous breeding lines in strictly cross-pollinating, heterozygous plants with high inbreeding depression and, thereby, to mediate their overall genetic improvement (Mishra and Goswami 2014; Mishra et al. 2017).

26.7 Conclusion

Potential application of gamete biology in the field of plant breeding has revolutionised its status and has led to the development of genetically stable populations in variety of plant species, including vegetables, cereal crops and other difficult to cultivate woody perennials. Haploid embryogenesis is a cost effective, rapid method of attaining pure breeding lines with increased crop productivity in a short span of time. This book chapter summarises widely available literature on haploid induction and provides details on crucial parameters that needs to be addressed while implementing haploidy in plants. Although molecular aspects related to gametogenesis and analysis of regenerated doubled haploid populations has covered long journey. However, exact mechanism behind reprogramming of microspores to switch over from gametogenesis to embryogenesis has yet to be deciphered.

References

Akula A, Becker D, Bateson M (2000) High-yielding repetitive somatic embryogenesis and plant recovery in a selected tea clone, 'TRI-2025', by temporary immersion. Plant Cell Rep 19:1140–1145

Alan AR, Mutschler MA, Brants A, Cobb E, Earle ED (2003) Production of gynogenic plants from hybrids of *Allium cepa L.* and *A. roylei* Stearn. Plant Sci 165:1201–1211

Bartošová Z, Obert B, Takacomá Š, Kormutak A, Pretová A (2005) Using enzyme polymorphism to identify the gametic origin of flax regenerants. Acta Biol Cracov Bot 47:173–178

Bhatt JG, Murthy HN (2007) Factors affecting in vitro gynogenic haploid production in niger (*Guizotia abyssinica* (L. f.) Cass.). Plant Growth Regul 52:241–248

Bhojwani SS, Razdan MK (1996) Haploid Production. Plant tissue culture: theory and practice. Elsevier Scientific Publishers, Amsterdam, The Netherlands, pp 113–142

Blakeslee AF, Belling J, Farnham ME, Berner AD (1922) A haploid mutant in Jimson weed, *Dhatura stramonium*. Science 55:646–647

Bohanec B (2009) Doubled haploids via gynogenesis. In: Touraev A, Forster BP, Jain SM (eds) Advances in haploid production in higher plants. Springer, Dordrecht, Netherlands, pp 35–46

Bueno MA, Gómez A, Boscaiu M, Manzanera JA, Vicente O (1997) Stress-induced formation of haploid plants through anther culture in cork oak (*Quercus suber*). Physiol Plant 99:335–341
Chaturvedi R, Razdan MK, Bhojwani SS (2003) Production of haploids of neem (*Azadirachta indica* A. Juss.) by anther culture. Plant Cell Rep 21:531–537
Chen JF, Cui L, Malik AA, Mbira KG (2010) In vitro haploid and dihaploid production via unfertilized ovule culture. Plant Cell Tissue Organ Cult 104:311–319
Chen Y, Kenaschuk E, Dribnenki P (1998) High frequency of plant regeneration from anther culture in flax, *Linum usitatissimum* L. Plant Breed 117:463–467
Clapham D (1971) In vitro development of callus from the pollen of *Lolium* and *Hordeum*. Z Pflanzeniichtg. 65:285–292
Comai L (2014) Genome elimination: translating basic research into a future tool for plant breeding. PLoS Biol 12:e1001876
Custers JB, Cordewener JH, Nöllen Y, Dons HJ, Campagne MM (1994) Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep 13:267–271
Datta SK (2005) Androgenic haploids: factors controlling development and its application in crop improvement Curr Sci 1870–1878
Da Silva DJC (2001) Effect of incubation temperature regimes and culture medium on broccoli microspore culture embryogenesis. Euphytica 119:389–394
Dunwell JM, Cornish M, De Courcel AGL (1985) Influence of genotype, plant growth temperature and anther incubation temperature on microspore embryo production in *Brassica napus* ssp. oleifera. J Exp Bot 36:679–689
Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol J 8:377–424
Dwivedi SL, Britt AB, Tripathi L, Sharma S, Upadhyaya HD, Ortiz R (2015) Haploids: constraints and opportunities in plant breeding. Biotechnol Adv 33:812–829
Ferrie AMR, Caswell KL (2011) Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tissue Organ Cult 104:301–309
Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12:368–375
Gaillard A, Vergne P, Beckert M (1991) Optimization of maize microspore isolation and culture conditions for reliable plant regeneration. Plant Cell Rep 10:55–58
Germanà MA (2005) Protocol of somatic embryogenesis from Citrus spp. anther culture. In: Jain SM, Gupta PK (eds) Protocol for somatic embryogenesis in woody plants. Springer, Netherlands, pp 191–207
Germana MA (2006) Doubled haploid production in fruit crops. Plant Cell Tissue Organ Cult 86:131–146
Germanà MA (2011) Anther culture for haploid and doubled haploid production. Plant Cell Tissue Organ Cult 104:283–300
Gharyal PK, Maheshwari SC (1982) In vitro differentiation of plantlets from tissue cultures of Albizzia lebbeck L. Plant Cell Tissue Organ Cult 2(1):49–53
Gharyal PK, Rashid A, Maheshwari SC (1983) Androgenic response from cultured anthers of a leguminous tree *Cassia siamea Lam*. Protoplasma 118:91–93
Gresshoff PM, Doy CH (1972) Haploid Arabidopsis thaliana callus and plants from anther culture. Aust J Biol Sci 25:259–264
Guha S, Maheswari SC (1964) In vitro production of embryos from anthers of Datura. Nature 204:497
Guha S, Maheswari SC (1966) Cell division and differentiation of embryos in the pollen grains of Datura in vitro. Natur 212:97–98
Hazarika RR, Chaturvedi R (2013) Establishment of dedifferentiated callus of haploid origin from unfertilized ovaries of tea (*Camellia sinensis* (L.) O. Kuntze) as a potential source of total phenolics and antioxidant activity. In Vitro Cell Dev Biol Plant 49:60–69

Hazarika R, Mishra V K, Chaturvedi R (2013) In Vitro Haploid Production—A Fast and Reliable Approach for Crop Improvement. In: Tuteja N, Gill S (eds) Crop improvement under adverse conditions. Springer, New York, pp 171–212

Höfer M (2004) In vitro androgenesis in apple—improvement of the induction phase. Plant Cell Rep 22:365–370

Hu T, Kasha KJ (1997) Performance of isolated microspore-derived doubled haploids of wheat (*Triticum aestivum* L.). Can J Plant Sci 77:549–554

Islam SS, Tuteja N (2012) Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. Plant Sci 182:134–144

Jacquard C, Asakaviciute R, Hamalian AM, Sangwan RS, Devaux P, Clement C (2006) Barley anther culture: effects of annual cycle and spike position on microspore embryogenesis and albinism. Plant Cell Rep 25:375–381

Karasawa MMG, Chiancone B, Gianguzzi V, Abdelgalel AM, Botta R, Sartor C, Germanà MA (2016) Gametic embryogenesis through isolated microspore culture in *Corylus avellana* L. Plant Cell Tissue Organ Cult 124:635–647

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*Hordeum vulgare* L.). Natur 225:874–876

Kasha KJ (1974) Haploids in higher plants: advances and potential. In: Proceedings of the 1st international symposium, University of Guelph, Guelph

Katiyar S, Mukhtar HA (1996) Tea in chemoprevention of cancer. Int J Oncol 8:221–238

Keller WA, Armstrong KC (1979) Stimulation of embryogenesis and haploid production in *Brassica campestris* anther cultures by elevated temperature treatments. Theor Appl Genet 55:65–67

Khush GS, Virmani SS (1996) Haploids in plant breeding. In: Jain SM, Sopory SK, Veilleux R (eds) In Vitro haploid production in higher plants. Springer, Netherlands, pp 11–33

Larsen ET, Tuvesson IK, Andersen SB (1991) Nuclear genes affecting percentage of green plants in barley (*Hordeum vulgare* L.) anther culture. Theor Appl Genet 82:417–420

Laurie DA, Bennett MD (1988) The production of haploid wheat plants from wheat x maize crosses. Theor Appl Genet 76:393–397

Maluszynska J (2003) Cytogenetic tests for ploidy level analyses—chromosome counting. In: Maluszynski M, Kasha K.J, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Springer, Dordrecht, pp 391–395

Magoon ML, Khanna KR (1963) Haploids Caryologia 16:191–235

Maheshwari SC, Rashid A, Tyagi AK (1982) Haploids from pollen grains–retrospect and prospect. Am J Bot 69:865–879

Maluszynski M, Kasha KJ, Szarejko I (2003) Published doubled haploid protocols in plant species. Doubled haploid production in crop plants. Springer, Netherlands, pp 309–335

Mishra VK, Bajpai R, Chaturvedi R (2017) An efficient and reproducible method for development of androgenic haploid plants from in vitro anther cultures of *Camellia assamica* ssp. *assamica* (Masters). In Vitro Cell Dev Biol Plant 53:239–248

Mishra VK, Goswami R (2014) Haploid production in higher plant. Int J Chem Biol Sci 1:26–45

Mondal BA, Laxmikumaran M, Ahuja PS (2004) Recent advances of tea (*Camellia sinensis*) biotechnology. Plant Cell Tissue Organ Cult 76:195–254

Murashiage T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473–497

Nair S, Gupta PK, Mascarenhas AF (1983) Haploid plants from in vitro anther culture of *Annona squamosa* Linn. Plant Cell Rep 2:198–200

Nakajima I, Kobayashi S, Nakamura Y (2000) Embryogenic callus induction and plant regeneration from unfertilized ovule of 'Kyoho'grape. J Jpn Soc Hortic Sci 69:186–188

Namita P, Mukesh R, Vijay KJ (2012) *Camellia sinensis* (green tea): a review. Global J Pharmacol 6:52–59

Ochatt S, Pech C, Grewal R, Conreux C, Lulsdorf M, Jacas L (2009) Abiotic stress enhances androgenesis from isolated microspores of some legume species (*Fabaceae*). Plant Physiol 166:1314–1328

Olmedilla A (2010) Microspore embryogenesis In: Plant developmental biology-biotechnological perspectives. Springer, Berlin Heidelberg, pp 27–44
Palmer CD, Keller WA (2005) Overview of haploidy. In: Haploids in crop improvement II. Springer, Berlin Heidelberg, pp 3–9
San Noeum LH (1976) Haploïdes d' Hordeum vulgare L. par culture in vitro d'ovaires non fécondés. Ann Amelior Plant 26:751–754
Scott P, Lyne RL (1994) The effect of different carbohydrate sources upon the initiation of embryogenesis from barley microspores. Plant Cell Tissue Organ Cult 36:129–133
Scott P, Lyne RL, Rees T (1995) Metabolism of maltose and sucrose by microspores isolated from barley (*Hordeum vulgare* L). Planta 197:435–441
Seguí-Simarro JM (2010) Androgenesis revisited. Bot Rev 76:377–404
Seran TH (2007) Anther culture in tea improvement. Med Aromat Plant Sci Biotechnol 1:234–239
Shen Y, Pan G, Lübberstedt T (2015) Haploid strategies for functional validation of plant genes. Trends Biotechnol 33:611–620
Sita GL (1996) Gynogenic haploids in vitro. In: In vitro haploid production in higher plants. Springer, Netherlands, pp 175–193
Sopory SK, Maheswari SC (1976) Development of Pollen Embryoids in Anther Cultures of Datura innoxia: I. General observations and effects of physical factors. J Exp Bot 27:49–57
Soriano M, Li H, Boutilier K (2013) Microspore embryogenesis: establishment of embryo identity and pattern in culture. Plant Reprod 206:181–196
Srivastava P, Chaturvedi R (2008) In vitro androgenesis in tree species: an update and prospect for further research. Biotechnol Adv 26:482–491
Srivastava P, Chaturvedi R (2011) Increased production of azadirachtin from an improved method of androgenic cultures of a medicinal tree *Azadirachta indica* A. Juss Plant Signal Behav 6:974–981
Thomas WTB, Newton AC, Wilson A, Booth A, Macaulay M, Keith R (2000) Development of recombinant chromosome substitution lines-a barley resource. SCRI annual report, pp 99–100
Touraev A, Ilham A, Vicente O, Heberle-Bors E (1996) Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Rep 15:561–565
Touraev A, Vicente O, Heberle-Bors E (1997) Initiation of microspore embryogenesis by stress. Trends Plant Sci 2:297–302
Touraev A, Forster BP, Jain SM (2009) (eds) Advances in haploid production in higher plants. Berlin, Springer, pp 1–208
Xu L, Najeeb U, Tang GX, Gu HH, Zhang GQ, He Y, Zhou WJ (2007) Haploid and doubled haploid technology. Adv Bot Res 45:181–216
Zenkteler M, Nitzsche W (1984) Wide hybridization experiments in cereals. Theor Appl Genet 68:311–315

Chapter 27
Somatic Embryogenesis in Neem

Vartika Srivastava and Rakhi Chaturvedi

27.1 Introduction

Neem, *Azadirachta indica* A. Juss, beholded as a potential and versatile medicinal tree, possessing exclusive therapeutic properties and is deep rooted in the field of medicine, forestry, agriculture and industry. The word '*Neem*' originates from Sanskrit word '*Nimba*' means to bestow health. It possesses most promising healing properties against cancer, diabetes, HIV, malaria, cardiovascular diseases, bacterial infections, skin diseases, allergies, and other ailments. It is well-known in Ayurvedic, Unani and Homeopathic forms of medicine. It affects the physiology and behavior of the insects and acts as the most effective insect-repellent. Its wood is termite resistant and is a valuable resource of timber. The auspicious attributes of the plant corresponds to its secondary metabolites. The most active constituent of neem is Azadirachtin (Koul 2004). Hence, the commercial importance of neem is always on a hike.

On the contrary, neem is accompanied with certain limitations. It constitutes high genetic variability due to cross-pollinating nature. The seeds are highly heterozygous and their germination capacity is lost soon after maturity. Moreover, neem is considered as recalcitrant species with long reproductive cycle (Puri 2003). Additionally, heterozygosity in neem causes variations in the production of secondary metabolites, consequently, it affects the commercial market in the field of medicine and agriculture. These attributes have been challenging and have attracted the biotechnologist for scientific research worldwide. Therefore, genetic diversity studies have also been performed via Amplified Foreign Length Polymorphism (AFLP; Singh et al. 1999) and Rapid Amplified Polymorphic DNA (RAPD) marker analysis (Deshwal et al. 2005).

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9_27

Biotechnology perspectives, especially plant tissue culture, offers substantial methodologies for genetic improvement, production of homozygous lines and large-scale propagation of neem. Somatic embryogenesis is one of the way to achieve large scale plant propagation with an added advantage of increasing the number of somatic embryos through secondary embryogenesis, their enhanced automated production in bioreactor and serving as material for synthetic seed production, providing material for physiological, genetic and biochemical research (Singh and Chaturvedi 2013).

Somatic embryogenesis in neem is governed by several factors, such as effect of basal medium constituents, plant growth regulators (PGRs) and culture conditions. Their requirements may vary from explant to explant on the initiation and expression of somatic embryos, their multiplication and complete plant development. The chapter describes the methodology and morphogenic development of somatic embryos in Neem (*Azadirachta india*). Ontogenic analysis is rewarding at this level for proper differentiation of the observed structures and their conversion into complete plantlets. Successful generation of somatic embryos, consecutively via cyclic embryogenesis, have been added-on perspective of our study.

27.2 Somatic Embryogenesis: The General Aspect

The era of somatic embryogenesis began after 60 years of theory of totipotency by Haberlandt, is now one of the progressive areas of research in plant science (Loyola-Vargas 2016). The first successful generation of somatic embryos was reported in *Oenanthe aquatica* in 1958 (Miettinen and Waris 1958; Waris 1959). Fostering somatic cells to develop totipotency and walk through the pathway of embryogenesis is a complex coordinated process and requires skilled and specific approach.

Somatic embryogenesis is defined in two stages: Induction and Expression. The key ingredient in this process is endogenous auxin content, which is affected by the changes in genetic regulation and external application of exogenous PGRs. Developmental changes related to morphology and ultrastructural modifications are essential part of the process. Reviewing the molecular behavior of embryo development, SERK (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE) gene is mostly upregulated in the regions where there is enhanced auxin content but the later do not induce the former (Hecht et al. 2001). Embryo development could be associated with or without callus development depending upon the plant species, therefore, expression of WUS (WUSCHEL) gene is increased in pro-embryogenic mass of callus (PEMs) (Zuo et al. 2002). Interestingly, callus cells being diverse in nature, are not the composite of embryogenic origin alike zygote or differentiated parts of the plant. They undergo induction process and reprogramming for attaining embryo development.

27.3 Somatic Embryogenesis in Neem: Key Factors

27.3.1 Explant Type

Explant is congregation of diversity of cells and tissues and only a few of them have the potential for embryogenic induction. These tissues are more competent and specific for embryogenic potential and responds well to the induction treatment than the non-competent ones. Numerous changes occur at the molecular and physiological level of the explant at various stages of somatic embryogenesis. Upon PGR treatment, the differentiated competent cells of the explant undergo reprogramming. The entire process channelizes the cells to halt and re-activate the cell cycle. The target somatic cells are now destined for embryogenesis directly from the explants or indirectly via an intervening callus development. The type of explant is a critical factor for obtaining maximum embryogenic response. The immature zygotic embryos are served to be the best explant and the advanced stages of it showed considerable somatic embryogenesis as compared to the younger embryos; globular stage embryos exhibited browning after a few days of culture initiation. Hypocotyl region have also proved to be an alternative explant for better embryogenic response. Additionally, leaf, stem and root segments were also utilized for induction of somatic embryos but response was not satisfactory.

In neem, the most appropriate explant utilized for the study is immature zygotic embryos, which showed maximum embryogenic response in least number of days (Table 27.1) followed by immature cotyledons. Immature zygotic embryos at early dicotyledonous stage were effective in inducing somatic embryos on the explant directly (Chaturvedi et al. 2004) or indirectly via callus formation (Rout 2005). Immature zygotic embryos were efficiently used in other plants to obtain somatic embryogenesis, such as *Castanea sativa* Mill. (Sezgin and Dumanoğlu 2014), *Fraxinus mandshurica* Rupr. (Kong et al. 2012), *Acacia senegal* (L.) Willd. (Rathore et al. 2012), *Cassia angustifolia* (Parveen and Shahzad 2014). These explants are more proficient for somatic embryo induction, metabolically and biochemically, due to presence of milky white content in immature cotyledons of neem (Gairi and Rashid 2005). Chaturvedi et al. (2004) reported the differentiation of neomorphs (embryo-like organized shiny structures) from torpedo stage of zygotic embryos (Fig. 27.1).

27.3.2 Nutrient Requirements

An optimum nutritional requirement in association with suitable environment is crucial for the growth and development of the explant that considerably mimics the natural growth of the plant. Nutrient medium is supplied with both inorganic and organic form of nitrogen, carbon source and growth regulators, and retained at an optimum pH of 5.8.

Table 27.1 Type of explant and the percent response for embryo induction from various explants

S. No.	Explant	Media	% Response	Days required for embryo induction	Remarks	References
1.	Immature zygotic embryo (Early dicotyledonous)	MS + BAP (5 μM)	57	28	–	Chaturvedi et al. (2004)
2.	Immature zygotic embryo (Early dicotyledonous)	MS + TDZ (0.1 μM) + ABA (4 μM)	76.16	28	–	Singh and Chaturvedi (2009)
		MS + BAP (1 μM) + IAA (0.5 μM)	100	56	Secondary embryos preceded by callusing	
3.	Mature seeds	MS + TDZ (10 μM)	75	35	–	Murthy and Saxena (1998)
4.	Immature zygotic embryo	MS + BAP (1.11 μM) + 2,4-D (0.45 μM)	82.8	56	Intervening callus formation	Rout (2005)
5.	Immature Cotyledons	MS + TDZ (0.5 μM)	90–100	7	–	Gairi and Rashid (2005)
6.	Leaflets (Juvenile)	MS + IAA (1.5 mg/L) + Kinetin (1.5 mg/L)	–	28	Initially formation of embryogenic calli occurred	Shekhawat et al. (2009)
7.	Nodal segments	MS + CH (1000 mg/L)	66.2	56–70	–	Akula et al. (2003)
	Root segments	MS + CH (1000 mg/L)	72	56–70	–	
	Leaf	MS + TDZ (4.5 μM)	44	84	Via callusing	
8.	Stem	MS + BAP (1 mg/L)	–	20–25	–	Phukan et al. (2017)
9.	Cotyledons and Hypocotyl	MS + NAA (0.5 mg/L + BAP (1 mg/L) + CH (1 g/L) + Sucrose (5%)	–	28–35	Formation of embryogenic callus	Su et al. (1997)
		MS + CH (1 g/L) + Zeatin (0.2 mg/L) + Sucrose (5%)	67	14	Somatic embryos	

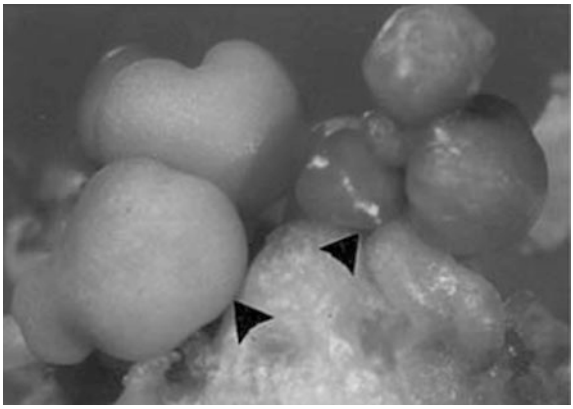


Fig. 27.1 Development of neomorphs directly on explant on MS + 2,4-D (5 μM)

27.3.2.1 Effect of Inorganic Nitrogen Source

Generally, ammonium and nitrate are the major sources of inorganic form of nitrogen, of which, ammonium being readily utilized by the plant. However, it causes decrease in pH of the medium leading to poor growth and development of the plant. Somatic embryogenesis in neem is achieved mostly in full strength Murashige and Skoog (1962, MS) medium. Differentiation of somatic embryos, neomorphs and shoots were tested in both MS and Gamborg et al. (1968, B5) medium but the MS medium provided a commendable response than B5 medium. This could be due to the presence of high amount of nitrogen in MS medium (in the form of ammonium and nitrate) as compared to B5 medium. Therefore, MS basal medium was chosen for all the experiments. Collateral effect could be observed in reports of Shekhawat et al. (2009), where additional supplementation of ammonium sulphate (50 mg/L) and potassium nitrate (100 mg/L) produced maximum number of somatic embryos. During the process of germination of somatic embryos, half-strength MS medium was proclaimed to have an alike effect as full strength MS (Shekhawat et al. 2009; Su et al. 1997). Effect of ammonium and nitrate have been reported in several plant systems portraying a varied response.

27.3.2.2 Effect of Carbon Source

Apart from the nitrogen source, emphasis of carbon source is also crucial for playing a dual role of carbon source and osmoticum (Biahoua and Bonneau 1999). In general 3% sucrose is used for embryonic development, plant regeneration and cyclic embryogenesis (Chaturvedi et al. 2004; Singh and Chaturvedi 2009). However, low sucrose level (2%) also supported the growth of SE (Gairi and Rashid 2004, 2005) and even 1% sucrose was sufficient for plantlet regeneration.

Su et al. (1997) observed somatic embryogenesis in Neem at higher concentration of sucrose (5%). Maltose at 3% concentration also proved an efficient alternative for conversion of somatic embryos into plantlets (Akula et al. 2003).

27.3.3 *Effect of Plant Growth Regulators on Initiation of Somatic Embryos*

During embryogenesis, exogenous auxin treatments are provided to the explants. These inducers affect the regulatory processes of somatic embryos by causing changes in the endogenous indole acetic acid (IAA) content. Cytokinins also favors in somatic embryo initiation processes but majorly contributes to the cell division and reactivation of cell cycle in competent cells. During the stages of embryo development, embryos attain a bilateral symmetry. To achieve this state, endogenous auxin content plays a major role.

Chaturvedi et al. (2004) investigated on effects of different plant growth regulators (PGRs) on induction of somatic embryogenesis in Neem. Among all, role of cytokinins was found to be more influential where benzylaminopurine (BAP) at 5 μM concentration gave maximum embryogenic induction when early dicotyledonous and torpedo stages of embryos were used as explants. The sole presence of auxin (2,4-dichlorophenoxyacetic acid; 2,4-D) in the medium was observed ineffective in inducing embryogenesis. However, combined presence of auxins (2,4-D, IAA) or 1-naphthaleneacetic acid (NAA) and indole butyric acid (IBA) along with BAP had cumulative effect on embryogenesis but was preceded by callus.

In addition to the frequently used PGRs, the effect of Thidiazuron (TDZ), a substituted urea compound, was also explored on induction of somatic embryogenesis. TDZ alone was favorable for embryogenic induction but its combined presence with GA_3 promoted enhanced embryogenic responses (100%) but the number of somatic embryos per culture were low ($\sim$9 embryos/culture). However, the combined presence of TDZ and ABA in the medium promoted maximum number of somatic embryos ($\sim$32 embryos/culture) though percentage cultures showing embryogenesis was low (37%). The somatic embryos were induced on hypocotyl and plumular regions when immature zygotic embryos at early dicotyledonous and torpedo stages were used as explants (Fig. 27.2a).

A few other reports have emphasized the use of TDZ to induce somatic embryogenesis in Neem (Murthy and Saxena 1998, Gairi and Rashid 2004, 2005). Studies revealed that the effects of TDZ has two-way response: major cytokinin and minor auxin-like effect. It promotes induction of embryos at low concentration in lesser time compared to BAP as is seen in peanuts (Victor et al. 1999). The growth regulators, ABA and GA_3, promotes the regulatory process of embryogenesis, hence, augments the rate of embryo induction (Jiménez 2005) as observed in the author's laboratory. Apart from induction of somatic embryos, direct shoot organogenesis was also observed from the explants (Fig. 27.2b) along with appearance of somatic embryos at respective stages of development (Fig. 27.2c, d).

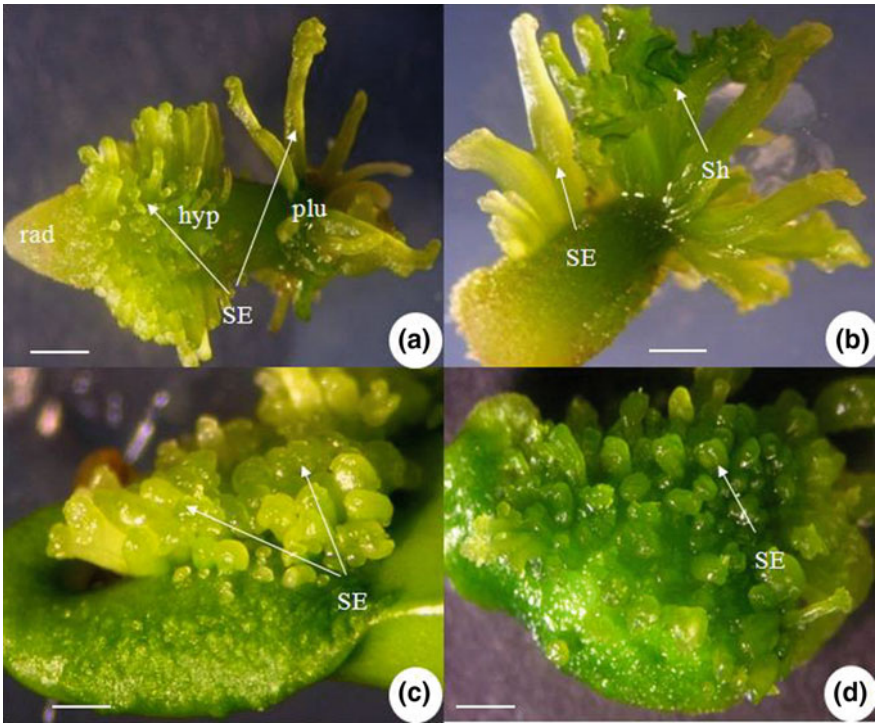


Fig. 27.2 Induction of somatic embryos (SEs) on immature zygotic embryo explants, **a** 4-week-old culture, showing SEs from hypocotyl and plumular regions of explants, **b** Shoot induction along with induction of somatic embryos, **c** and **d** Appearance of different stages of SEs on explants

27.3.4 Effect of PGRs on Expression of Somatic Embryos

The inductive stage of somatic embryogenesis involves the activation of embryogenic competence of the explant by providing the appropriate blend of growth hormones, medium constituents and culture conditions. Subsequently, expression of somatic embryos takes place and is marked by the reduced level of endogenous auxin content in several plant species. The transition from induction to the maturation period is usually moderate as the cells adapt themselves for further development and undergo the stages of zygotic embryo development, viz, globular, heart, torpedo and cotyledonary stages (Fig. 27.3). Studies on the removal of auxin or exogenous addition of cytokinins, ABA and GA_3, for the maturation and germination of somatic embryos are unclear which vary with the plant species. Certainly, these somatic embryos must pass through the maturation phase or else they failed to germinate to form complete plantlets.

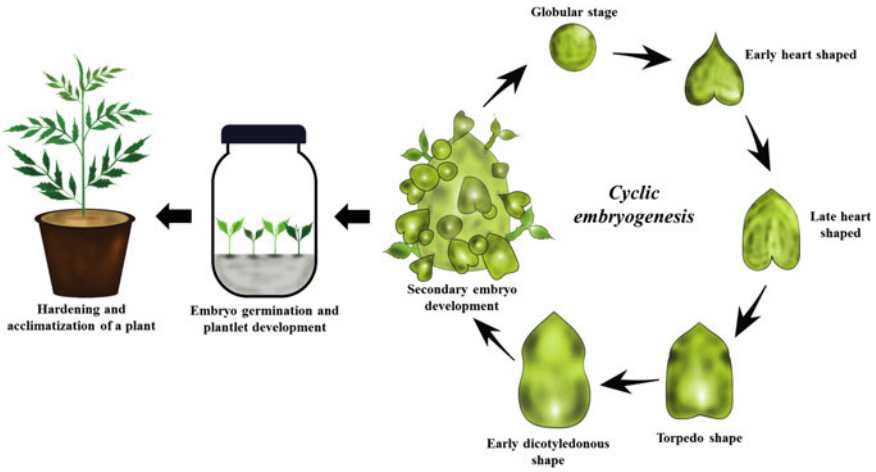


Fig. 27.3 A progressive view of different stages of embryo development and cyclic embryogenesis

27.3.5 Cyclic Embryogenesis

The process begins with the onset of generation of primary somatic embryos whose superficial tissues, such as cotyledon or hypocotyl regions serve as a bedrock for the generation of secondary and tertiary embryos (Fig. 27.3). Formulation of the medium composition for cyclic embryogenesis should focus on cyclic production of embryos rather than progressing them for further germination. In the author's laboratory, initially, primary somatic embryogenesis was obtained on MS + TDZ + ABA/GA_3. Thereafter, secondary embryos developed on the surface of hypocotyl regions of the primary embryos. Cyclic embryogenesis was successfully achieved on MS + IAA + GA_3 either directly or preceded by callus formation. Embryogenic potential of the cultures was observed to remain stable with consecutive passages (Singh and Chaturvedi 2009). Repetitive embryogenesis did not occur on the basal medium rather it caused germination in maximum cultures. Akula et al. (2003) reported repetitive embryogenesis exhibiting 4–7 fold multiplication within two to three cycles. Cyclic embryogenesis demonstrates a reproducible methodology for constant production of somatic embryos at large scale, thus, providing fundamental material for scale-up, metabolic and downstream processing studies.

27.4 Ontogenic Analysis of Somatic Embryos

The entire developmental process of somatic embryogenesis is accompanied with morphological, cellular and ultrastructural changes and accumulation of proteins and storage lipids serve as energy source. Histological analysis of the embryos at

different stages of embryonic development provided an insight into the structural and cellular details of the embryo, pro-embryogenic masses (PEMs) and callus. The staining techniques solely depends upon the cell content, such as Astra blue stains the cellulosic wall and the cytoplasm while safranin stains the cutinized cell wall and lignified membrane of vascular bundles (Cutler et al. 2007). Scanning electron microscopy provided ultrastructural studies of different stages of the developed embryos (Fig. 27.4a, b). Certain morphological deformity in somatic embryos, including presence of cotyledons or no cotyledons or fused cotyledons or presence of more than two cotyledons, were also observed. In a few cases, the radicular end of the somatic embryos were poorly differentiated and the hypocotyl was elongated consisting of cotyledons and provascular strands (Chaturvedi et al. 2004; Singh and Chaturvedi 2013). Histological studies differentiated the normal and abnormal development of somatic embryos successfully.

Apart from embryos, in the author's laboratory, occasionally, neomorphs were also developed on the explants (as shown in Sect. 27.3.1). These neomorphs, which appeared to be suppressed embryos with epidermal origin and closed provascular strands, were germinated in a monopolar fashion and gave rise to only shoots. The morphological differences with respect to the neomorphs and embryos were clearly understood by histological analysis. These structures differentiated mostly from torpedo stage embryo explants on MS + 2,4-D (5 μM) (Table 27.2). They are green, with smooth shiny surface and solid interior. Some exhibited spherical structure with visible appendages while others showed notches like heart-shaped embryos, some population developed foliar protuberances at the tip.

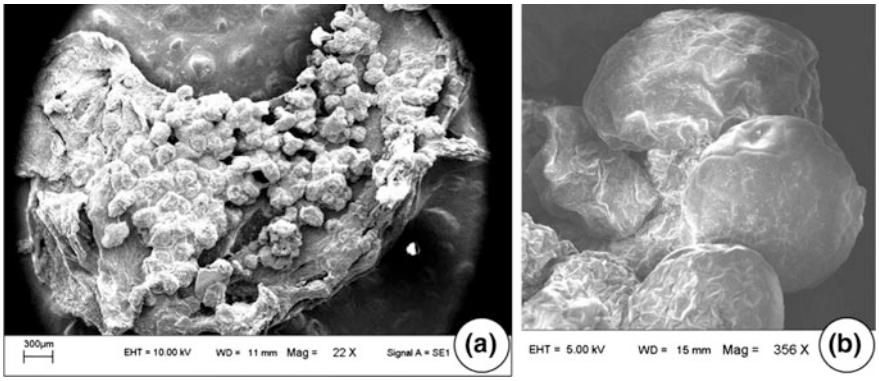


Fig. 27.4 **a** Scanning electron microscopic analysis showing various stages of somatic embryos, **b** Same as **a**, depicting enlarged view of globular-shaped embryos

Table 27.2 Stage of zygotic embryzygotic embryo at culture

Regenerants ► Treatment (μM)	Globular embryo			Heart shape embryo			Torpedo shape embryo			Early dicot embryo		
	Shoots	SE	NEO	Shoots	SE	NEO	Shoots	SE	NEO	Shoots	SE	NEO
MS	–	–	–	–	–	–	–	–	–	–	–	–
CH (1000 mgl−1)	–	–	–	–	–	–	–	–	–	–	–	–
2,4-D (1)	–	–	20 ± 1.0 (3)	–	–	20 ± 1.0 (2)	–	–	20 ± 1.0 (4)	–	–	25 ± 3.0 (4)
2,4-D (5)	–	–	20 ± 1.0 (3)	–	–	40 ± 1.0 (4)	–	–	66 ± 1.0 (6)	–	–	25 ± 1.0 (2)
BAP (5)	–	–	–	–	20 ± 1.0 (20)	–	25 ± 1.0 (6)	40 ± 1.0 (10)	40 ± 1.0 (5)	57 ± 1 (18)	57 ± 3.0 (25)	–
BAP (10)	–	–	–	–	–	–	–	8 ± 1.0 (6)	40 ± 1.0 (3)	24 ± 1.0 (6)	22 ± 1.0 (9)	40 ± 1.0 (5)
TDZ (0.1)	–	–	–	–	25 ± 2.0 (10)	–	25 ± 1.0 (10)	25 ± 1.0 (20)	–	–	22 ± 1.0 (10)	–
BAP (5) + 2,4-D (1)	–	–	–	20 ± 1.0 (5)**	–	–	25 ± 3.0 (5)**	–	–	30 ± 1.0(5)**	50 ± 1.0 (6)**	50 ± 1.0 (2)**
BAP (5) + IAA (2)	–	–	–	–	–	33 ± 1.0 (5)	35 ± 1.0 (5)	–	30 ± 1.0 (4)	–	16 ± 1. (8)	–
BAP (5) + NAANaphthalenacetic acid (NAA) (2)	–	–	–	–	–	–	33 ± 1.0 (7)	–	33 ± 1.0 (5)	–	–	20 ± 1.0 (5)
BAP (5) +IAA (2) +NAANaphthalenacetic acid (NAA)(2)	–	–	–	–	–	–	25 ± 0.0 (6)	–	25 ± 1.0 (4)	33 ± 1.0 (7)	–	–
BAP (5) +Kn (5) +IBA (0.5)	–	–	–	20 ± 1.0 (3)	–	–	16 ± 1.0 (5)**	–	–	–	50 ± 4.0 (8)**	50 ± 4.0 (2)**
BAP (5) + CH (1000 mgl−1)	–	–	–	–	–	–	–	–	–	–	–	–
BAP (10) + CH (1000 mgl −1)	–	–	–	–	–	–	33 ± 1.0 (5)	–	–	21 ± 0.5 (5)	42 ± 3.0 (20)	–
2,4-D (5) + CH (1000 mgl −1)	–	–	–	–	–	–	–	–	55 ± 2.0 (5)	–	–	–
TDZ (0.1) + CH (1000 mgl −1)	–	–	–	–	–	–	–	–	–	–	–	–
IAA (3) + BAP (3)												
+CH (1000mgl-1)	–	–	–	–	–	–	–	28 ± 3.0 (8)**	–	33 ± 1.0 (6)	33 ± 1.0 (4)	–

± = Significant error

Note Control MS; *Growth Period* 4 weeks

SE Somatic embryos; *NEO* Neomorphs

*The figures in parenthesis represent the number of regenerants per explant

**Indirect differentiation, from callused explant

27.5 Methodology

27.5.1 Establishment of Cultures

Immature fruits from experimental neem plant were cleaned with 1% savlon (v/v) followed by washing with sterile distilled water (SDW). Under aseptic conditions in the laminar airflow, they were quick rinsed with 90% ethanol for 30 s followed by washing with SDW thrice. The fruits were finally surface sterilized with 0.1% $HgCl_2$ for 10 min and then washed thrice with SDW. The sterilized immature fruits were dissected under stereo-microscope for isolation of the embryos at its different stages of development. They were inoculated on basal media, MS and B5, fortified with growth regulators at various concentrations and combinations, 3% sucrose and 0.8% agar, to obtain the best responsive stage of zygotic embryo. The cultures were kept in diffused light (1000–2000 lx) at optimum temperature of 25 °C and 50–60% humidity. They were observed periodically, however, the data was recorded after four weeks.

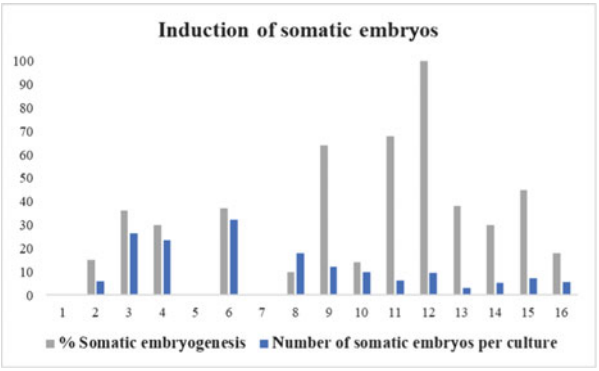
27.5.2 Induction Process

Various forms of structures, like shoots, somatic embryos and neomorphs, were observed directly from the inoculated immature zygotic embryo explants or indirectly via callusing of explants (Table 27.2). The frequency of differentiation of these structures varied with the stage of the zygotic embryo and the culture medium. With BAP (5 μM), the heart shape, torpedo shape and early dicotyledonous embryos differentiated SEs but shoot differentiation was exhibited only by torpedo shape and early dicot embryos. The latter showed differentiation of shoots and SEs with higher frequencies (57%) than the former. Maximum population of neomorphs (66%) were observed on MS with 2,4-D (5 μM), when torpedo shape zygotic embryos were used as an explant.

Another set of experiment displayed somatic embryogenesis on plumular and hypocotyl regions of zygotic embryos on MS supplemented with ABA and GA_3 at various concentrations (Fig. 27.5).

27.5.3 Cyclic Embryogenesis

It refers to a swift repetitive process of somatic embryogenesis on a large scale. During the process, secondary embryos were developed on the surface of primary embryos. The repetitive embryogenesis was obtained on MS + BAP (1 μM) + IAA (0.5 μM) where 100% cultures showed embryogenesis with an average of 14 embryos per explant, however, it is preceded by callusing of



Media Type	TDZ (μM)	ABA (μM)	GA_3 (μM)
1	-	-	-
2	0.5	-	-
3	1.0	-	-
4	2.0	-	-
5	0.5	1.0	-
6	1.0	1.0	-
7	2.0	1.0	-
8	0.5	4.0	-
9	1.0	4.0	-
10	2.0	4.0	-
11	0.5	-	1.0
12	1.0	-	1.0
13	2.0	-	1.0
14	0.5	-	4.0
15	1.0	-	4.0
16	2.0	-	4.0

Fig. 27.5 Graphical representation of embryonic induction with respect to TDZ, ABA and GA_3

explants. The number of secondary somatic embryos increased until the end of 8 weeks of culture initiation. The somatic embryos was also utilized for artificial seed production as mentioned in Sect. 27.6.

27.5.4 Shoot Elongation, Multiplication, Rooting and Acclimatization

Individual regenerated shoots (~2 cm) were detached from the explant and transferred on MS medium supplemented with BAP (0.5 μM), which resulted into better elongation of shoot in 4 weeks (Fig. 27.6a). Shoot multiplication via axillary bud proliferation in nodal segment culture was obtained on MS medium fortified with BAP (1 μM) and casein hydrolysate (250 mg/L) (Fig. 27.6b). Around 4 cm long shoot was transferred for rooting on ¼ MS (major) supplemented with IBA (0.5 μM) (Fig. 27.6c). Transplantation of rooted plants was performed in soilrite followed by their transfer to soil with 0.1% urea and Bavistin (1:1) for hardening (Fig. 27.6d). The plant was completely acclimatized within 8 months.

27.5.5 Ontogenic Analysis

Somatic embryos at different stages were fixed in glutaraldehyde and dehydrated with ethyl-alcohol series gradually, followed by complete drying of the material. The dried material were finally gold-coated for SEM analysis as shown in Fig. 27.4a, b. Histological studies were also performed with these regenerants. They were fixed in FAA (Formalin: acetic acid: 70% ethanol) followed by sequential dehydration through tertiary-butyl-alcohol series. The section was

Fig. 27.6 **a** A 4-week-old elongated shoot on MS + BAP (0.5 μM), **b** A 4-week old shoot on MS + BAP (1 μM) + CH (250 mg/L), **c** A 4-cm long shoot from **b**, exhibiting rooting on ¼ MS + IBA (0.5 μM), **d** An elongated mature plantlet transferred for hardening

embedded in paraffin wax for microtome sectioning. The sections were double stained with Astra blue (1%) and Safranin (1%), and visualized under stereomicroscope.

27.6 Applications and Future Endeavors

The worldwide usage of neem for healthcare, industry or research have always uplifted the economical value of the plant. Despite of the fact that it undergo limitations for natural propagation, scientists have flourished neem plantations via plant tissue culture technology successfully. Generation of somatic embryos aids in providing a common platform for other tissue culture methodologies, hence, it is the most influential tool for research and industry. An overview of the applications of somatic embryogenesis is presented in Fig. 27.7.

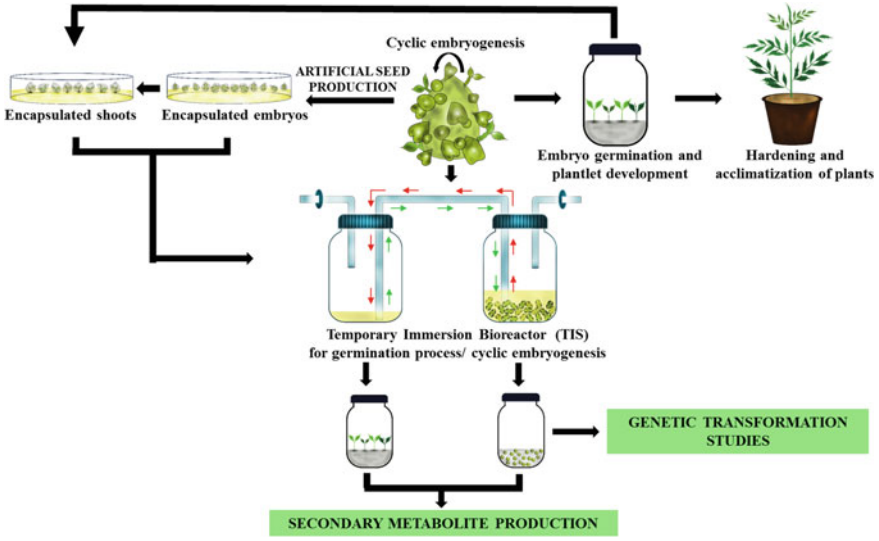


Fig. 27.7 Overview of applications of somatic embryogenesis

27.6.1 Synthetic Seed Production

Synthetic seeds are considered as an efficient way of plant propagation especially in case of recalcitrant species, and is regarded to be better than vegetative and micropropagation techniques because it requires low input area, can be directly transplanted to soil, hence, reducing the total cost. Moreover, somatic embryos are one of the prerequisite for the production of artificial seeds but latter is again a better choice as they remain viable for long duration and can be easily stored or transported (Saiprasad 2001). In neem, cyclic somatic embryogenesis have been successfully established in the author's laboratory along with production of synthetic seeds (Fig. 27.8), which could serve as a platform for efficient scale-up studies. Germplasm conservation via synthesis of artificial seeds technology is also advantageous.

27.6.2 Scale-up Via Bioreactor

Bioreactor studies assist in large-scale production of secondary metabolites from in vitro cell suspension cultures. According to Prakash and Srivastava (2007), scale-up studies in stirred tank bioreactor have been performed in *Azadirachta indica* and evaluated scale-up parameters for maximum production of biomass and azadirachtin content. The recent advances, such as temporary immersion system opens up more ways for mass propagation of plant and natural metabolites. Apart

Fig. 27.8 Synthetic seeds of somatic embryos of neem

from in vitro cell suspension cultures, somatic embryos (Etienne et al. 1999; Steinmacher et al. 2011), artificial seeds as well as in vitro shoots (Pérez-Alonso et al. 2012) could be propagated on a wide scale via temporary immersion system.

27.6.3 Secondary Metabolite Production

Neem offers several advantages and is a reservoir of secondary products of which azadirachtin is the most potent metabolite among all. Neem seed oil have also been utilized as biodiesel production (Sekhar et al. 2009) as well as biopesticide (Weathersbee et al. 2005). Neem, being recalcitrant and cross-pollinating in nature, is diversified in case of its natural way of propagation and secondary metabolite production. Plant tissue culture technologies, such as somatic embryogenesis offers advantages for constant and enhanced production of bioactive compounds. Statistical media optimization studies (Singh and Chaturvedi 2012), haploid production (Srivastava and Chaturvedi 2011) and hairy root culture (Allan et al. 2002) provide enhanced benefits for increased production of the compounds.

27.6.4 Genetic Transformation Studies

Somatic embryogenesis aids in proficient usage for genetic transformation studies. The plant species, which are recalcitrant, possess long reproductive cycle and breeding limitations are the important targets for transformation studies. Regeneration of transformed plants is often a setback (Giri et al. 2004), which can be overcome by somatic embryogenesis and other plant tissue culture techniques. Somatic embryogenesis have now become a common platform for transferring

genetic material and enhances the target trait. Tuominen et al. (1995) overexpressed iaaM and iaaH by *Agrobacterium* mediated transformation in *Populus* and demonstrated changes in wood formation properties and overall development of the plant. Allan et al. (2002) developed hairy root cultures through *Agrobacterium* mediated transformation via *Agrobacterium rhizogenes* in *Azadirachta indica* and found improvement in the developmental pattern of the plant. According to the recent advances in research, the molecular depth of somatic embryogenesis of neem and its genetic manipulation remains a topic of research.

27.7 Conclusion

Somatic embryogenesis is adapted as a promising plant tissue culture approach for high yield of elite clones of plant species. It is multi-target methodology for substantial propagation of plant, genetic transformation studies, and germplasm conservation. Somatic embryos are rich source of bioactive metabolites, paving a smooth path for pharmaceutical industries. Somatic embryogenesis in neem have been investigated thoroughly, however, proper germination and plantlet development from somatic embryos remains the area of interest along with scale-up studies. Effect of nutritional requirements on the initiation and expression of somatic embryos was critically analyzed. Methodology for cyclic embryogenesis have been speculated that can harbor large-scale production of plant species and constant production of target metabolites.

References

Allan E, Eeswara J, Jarvis A, Mordue A, Morgan E, Stuchbury T (2002) Induction of hairy root cultures of *Azadirachta indica* A. Juss. and their production of azadirachtin and other important insect bioactive metabolites. Plant Cell Rep 21:374–379

Akula C, Akula A, Drew R (2003) Somatic embryogenesis in clonal neem, *Azadirachta indica* A. Juss. and analysis for in vitro azadirachtin production. In Vitro Cell Dev Biol-Plant 39:304–310

Biahoua A, Bonneau L (1999) Control of in vitro somatic embryogenesis of the spindle tree (*Euonymus europaeus* L.) by the sugar type and the osmotic potential of the culture medium. Plant Cell Rep 19:185–190

Chaturvedi R, Razdan MK, Bhojwani SS (2004) In vitro morphogenesis in zygotic embryo cultures of neem (*Azadirachta indica* A. Juss.). Plant Cell Rep 22:801–809

Cutler DF, Botha CEJ, Stevenson DW (2007) Plant Anatomy, an applied approach. Practical Microtechnique. Blackwell Publishing Ltd, USA, pp 170–194

Deshwal RS, Singh R, Malik K, Randhawa GJ (2005) Assessment of genetic diversity and genetic relationships among 29 populations of *Azadirachta indica* A. Juss. using RAPD markers. Genet Resour Crop Evol 52:285–292

Etienne-Barry D, Bertrand B, Vasquez N, Etienne H (1999) Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and regeneration of plants. Plant Cell Rep 19:111–117

Gairi A, Rashid A (2004) TDZ-induced somatic embryogenesis in non-responsive caryopses of rice using a short treatment with 2, 4-D. Plant Cell Tiss Organ Cult 76:29–33
Gairi A, Rashid A (2005) Direct differentiation of somatic embryos on cotyledons of *Azadirachta indica*. Biol Plantarum 49:169–173
Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158
Giri CC, Shyamkumar B, Anjaneyulu C (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. Trees 18:115–135
Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt ED, Boutilier K, Grossniklaus U, de Vries SC (2001) The Arabidopsis Somatic Embryogenesis Receptor Kinase 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol 127:803–816
Jiménez VM (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regul 47:91–110
Kong DM, Preece JE, Shen HL (2012) Somatic embryogenesis in immature cotyledons of Manchurian ash (*Fraxinus mandshurica* Rupr.). Plant Cell Tiss Organ Cult 108:485–492
Koul O, Wahab S (2004) (eds) *Neem: today and in the new millennium*. Kluwer Academic Publishers, Dordrecht, USA
Loyola-Vargas VM (2016) The history of somatic embryogenesis. In: Victor M, Loyola-Vargas, Neftali Ochoa-Aljo (eds) Somatic embryogenesis: fundamental aspects and applications. Springer International Publishing AG, Switzerland, pp 11–22
Miettinen JK, Waris H (1958) A chemical study of the neomorphosis induced by glycine in *Oenanthe aqnatica*. Physiol Plant 11:193–199
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497
Murthy BN, Saxena PK (1998) Somatic embryogenesis and plant regeneration of neem (*Azadirachta indica* A. Juss.). Plant Cell Rep 17:469–475
Parveen S, Shahzad A (2014) Somatic embryogenesis and plantlet regeneration of *Cassia angustifolia* from immature cotyledon-derived callus. Biol Plant 58:411–418
Pérez-Alonso N, Capote A, Gerth A, Jiménez E (2012) Increased cardenolides production by elicitation of *Digitalis lanata* shoots cultured in temporary immersion systems. Plant Cell Tiss Organ Cult 110:153–162
Phukan H, Kumar R, Mitra PK (2017) Plant Regeneration by Somatic Embryogenesis in *Azadirachta indica* A. Juss. (Neem). Int Res J Eng Technol 4:3212–3217
Prakash G, Srivastava AK (2007) Azadirachtin production in stirred tank reactors by *Azadirachta indica* suspension culture. Process Biochem 42:93–97
Puri HS (2003) Neem: the divine tree *Azadirachta indica*. Harwood Academic Publishers, The Netherlands
Rathore JS, Rai MK, Shekhawat NS (2012) Induction of somatic embryogenesis in gum arabic tree [*Acacia senegal* (L.) Willd.]. Physiol Molecular Biology Plants 18:387–392
Rout GR (2005) In vitro somatic embryogenesis in callus cultures of *Azadirachta indica* A. Juss—a multipurpose tree. J For Res 10:263–267
Saiprasad GV (2001) Artificial seeds and their applications. Resonance 6:39–47
Sekhar MC, Mamilla VR, Mallikarjun MV, Reddy KV (2009) Production of biodiesel from neem oil. Int J Eng Stud 1:295–302
Sezgin M, Dumanoğlu H (2014) Somatic embryogenesis and plant regeneration from immature cotyledons of European chestnut (*Castanea sativa* Mill.). In Vitro Cell Dev Biol-Plant 50:58–68
Shekhawat GS, Mathur S, Batra A (2009) Role of phytohormones and nitrogen in somatic embryogenesis induction in cell culture derived from leaflets of *Azadirachta indica*. Biol Plant 53:707
Singh A, Negi MS, Rajagopal J, Bhatia S, Tomar UK, Srivastava PS, Lakshmikumaran M (1999) Assessment of genetic diversity in *Azadirachta indica* using AFLP markers. Theor Appl Genet 99:272–279

Singh M, Chaturvedi R (2009) An efficient protocol for cyclic somatic embryogenesis in neem (*Azadirachta indica* A Juss.). Int J Environ Sci Eng 1:49–51

Singh M, Chaturvedi R (2012) Statistical optimization of media for enhanced azadirachtin production from redifferentiated zygotic embryo cultures of neem (*Azadirachta indica* A. Juss.). In Vitro Cell Dev Biol-Plant 48:92–98

Singh M, Chaturvedi R (2013) Somatic embryogenesis in Neem (*Azadirachta indica* A. Juss.): current status and biotechnological perspectives, In: Junaid Aslam P, Srivastava S, Sharma MP (eds) Somatic embryogenesis and Gene expression. Narosa Publishing House, New Delhi, pp 35–55

Srivastava P, Chaturvedi R (2011) Increased production of azadirachtin from an improved method of androgenic cultures of a medicinal tree *Azadirachta indica* A. Juss Plant Signaling Behav 6:974–981

Steinmacher DA, Guerra MP, Saare-Surminski K, Lieberei R (2011) A temporary immersion system improves in vitro regeneration of peach palm through secondary somatic embryogenesis. Ann Bot 108:1463–1475

Su WW, Hwang WI, Kim SY, Sagawa Y (1997) Induction of somatic embryogenesis in *Azadirachta indica*. Plant Cell Tiss Organ Cult 50:91–95

Tuominen H, Sitbon F, Jacobsson C, Sandberg G, Olsson O, Sundberg B (1995) Altered growth and wood characteristics in transgenic hybrid aspen expressing *Agrobacterium tumefaciens* T-DNA indoleacetic acid-biosynthetic genes. Physiol Plant 109:1179–1189

Victor JM, Murch SJ, KrishnaRaj S, Saxena PK (1999) Somatic embryogenesis and organogenesis in peanut: the role of thidiazuron and N6-benzylaminopurine in the induction of plant morphogenesis. Plant Growth Regul 28:9–15

Waris H (1959) Neomorphosis in seed plants induced by amino acids I: *oenanthe aquatica*. Physiol Plant 12:753–766

Weathersbee AA III, McKenzie CL (2005) Effect of a neem biopesticide on repellency, mortality, oviposition, and development of *Diaphorina citri* (Homoptera: Psyllidae). Fla Entomol 88:401–407

Zuo J, Niu QW, Frugis G, Chua NH (2002) The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. Plant J 30:349–359

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S. M. Jain and P. Gupta (eds.), *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants*, Forestry Sciences 85,
https://doi.org/10.1007/978-3-319-79087-9