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Shri Mohan Jain
Pramod Gupta *Editors*

Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants

Volume II

Second Edition

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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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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

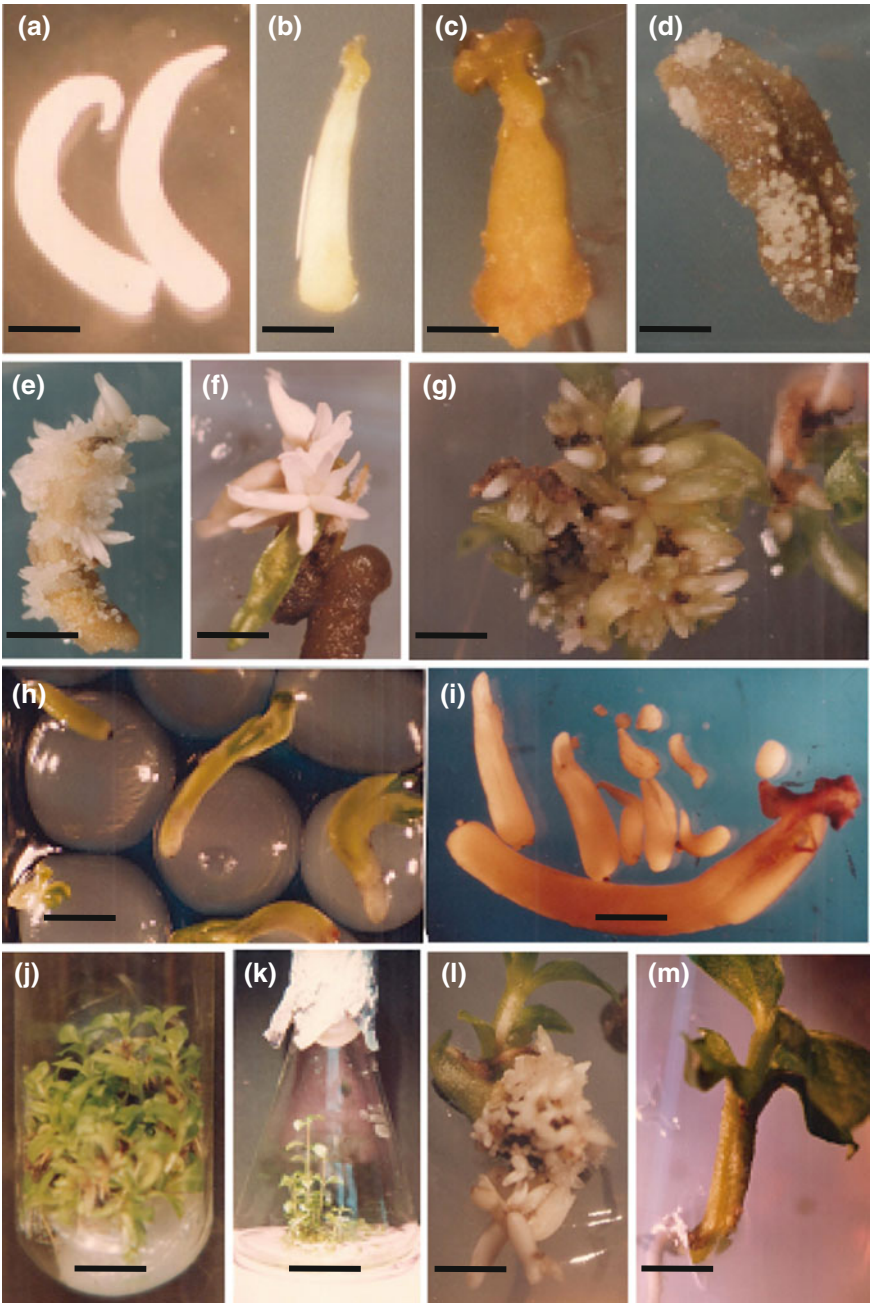
All these constituents are mixed appropriately to prepare a specific volume of normal strength medium. Other medium strength, need for agar, sucrose, growth regulators and other adjuncts are added as per the requirements of the experiments mentioned under different heads

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.

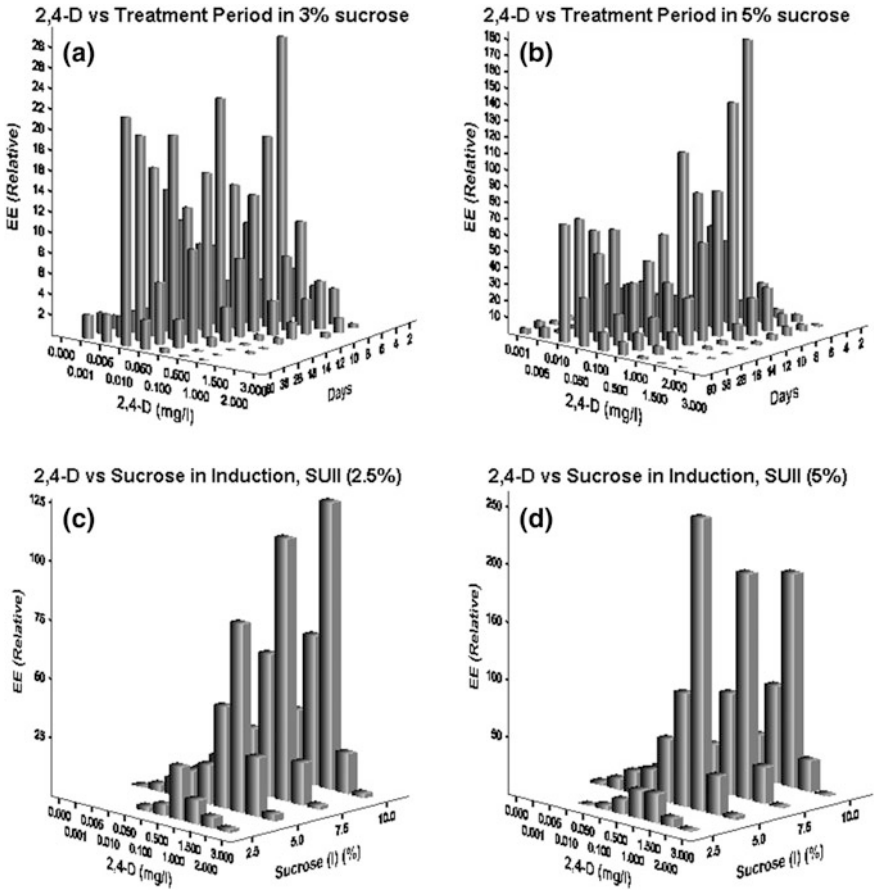

Fig. 1.2 Interactive effects of various factors on the induction and optimization of somatic embryogenesis in guava. **a** Interactions of 2,4-D concentrations and the treatment periods on the process of somatic embryogenesis from 10-weeks post anthesis zygotic embryo explants cultured in full strength 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

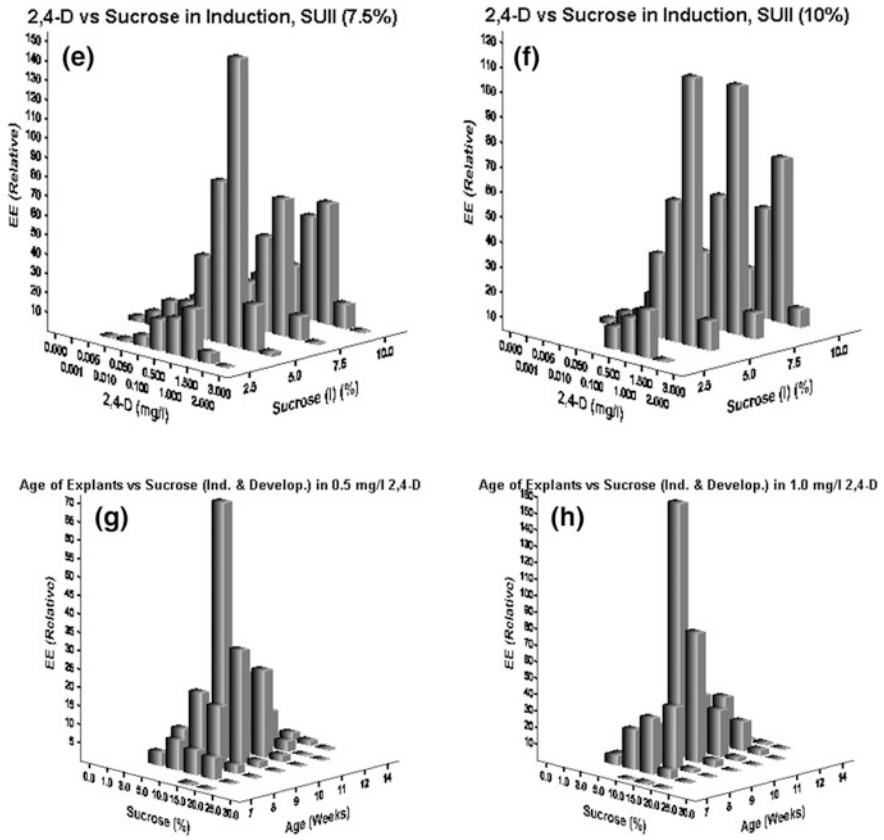

Fig. 1.2 (continued)

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.