Chapter 17
Rough Lemon (*Citrus Jambhiri* Lush.)

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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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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after the appearance of the new citrus threat by *Citrus tristeza virus* (CTV), the dominant sour orange rootstock has been replaced by rough lemon rootstock which was tolerant to CTV.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)

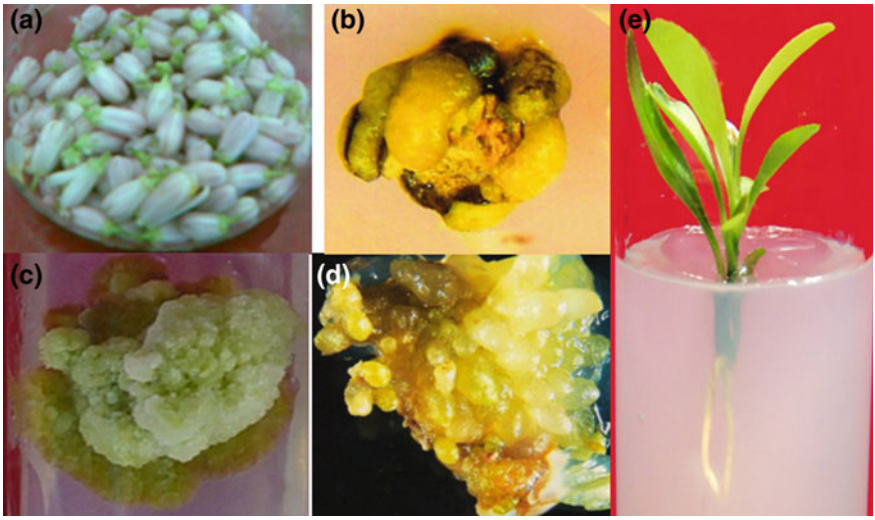

Fig. 17.1 Plantlet regeneration via somatic embryogenesis from ovaries; **a** Unopened flower buds; **b** Ovary showing swelling after 60–90 days of inoculation; **c** callus induction from the wall of ovary; **d** somatic embryo induction and **e** somatic embryo germination

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.

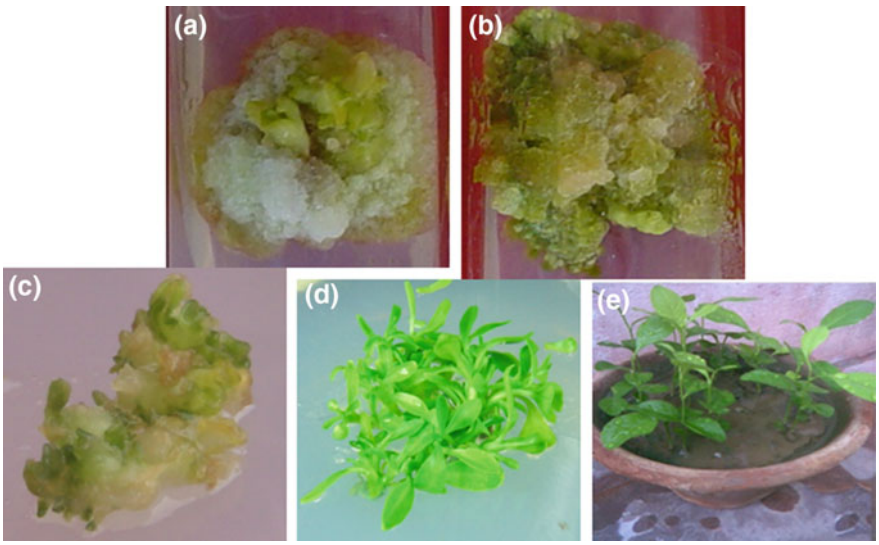

Fig. 17.2 Plantlet regeneration via somatic embryogenesis from nucellar tissues; **a** callus induction from nucellar tissue; **b** callus multiplication; **c** somatic embryo induction; **d** somatic embryo germination; **e** plantlets transferred to earthen pot for hardening in polyhouse

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.

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