An efficient plant regeneration protocol from callus cultures of *Citrus jambhiri* Lush

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Abstract *Citrus jambhiri* Lush. (family Rutaceae), commonly known as ‘rough lemon’, is one of the favourite rootstocks for lemons, oranges, mandarins, grape fruits and kinnows in Punjab. The present investigation deals with development of an efficient micropropagation protocol for *Citrus jambhiri* Lush. using cotyledons as explant. Maximum callus induction (91.66 %) was observed on MS medium supplemented with 2,4-D (2 mg/L) in combination with ME (500 mg/L). Green healthy calli were cut into small pieces and cultured on MS medium for regeneration. Maximum shoot regeneration (87.50 %) was observed with BA (3 mg/L). Effect of increasing age of callus was also studied which showed that callus retained regeneration capacity (58.33 %) even after 420 days of culture. Regenerated shoots were separated out and cultured on rooting medium. Maximum rooting response (91.67 %) was observed on half strength MS medium supplemented with NAA (0.5 mg/L). After hardening and acclimatization the plantlet were transferred to the field and showed 67 % survival.

Keywords Tissue culture · Rough lemon ·
2,4-dichlorophenoxyacetic acid · 6-benzylaminopurine ·
Malt extract · Callus induction

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
BA	6-benzylaminopurine
ME	Malt extract
KN	Kinetin
NAA	Naphthalene acetic acid
IBA	Indole butyric acid
IAA	Indole acetic acid

Introduction

Citrus jambhiri Lush. (family Rutaceae), commonly known as ‘rough lemon’, is the most favourite rootstock for lemons, oranges, mandarins, grape fruits and kinnows in Punjab, because of its high vigour and well adaptation to warm humid areas with sandy soils. Locally in Punjab, it is known as “Jatti Khatti” and scions grafted on it produce large trees with high yield of fruits. However, its main drawback is that it is very sensitive to cold and susceptible to *Phytophthora*, which requires development of new cultivars. Improvement of *Citrus spp.* by conventional breeding methods is hampered by various aspects of citrus biology like nucellar polyembryony, heterozygosity, sexual incompatibility, juvenility etc. (Button et al. 1977; Koltunow 1993). Under such circumstances, *in vitro* culture techniques hold potential and could offer solution to these problems. Besides improving the existing cultivars, micropropagation techniques have the potential for mass scale generation of novel plants in a comparatively short time span when compared to conventional breeding. Plant tissue culture has emerged as a powerful tool for propagation and improvement of many woody plant species including *Citrus*. *In vitro* culture has the potential to eliminate diseases and provides

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scope for development of new cultivars through somaclonal variations (Hammschlag et al. 1995). Production of callus and its subsequent regeneration are the prime steps in crop plants to be manipulated by biotechnological means and to exploit somaclonal variations (Islam et al. 2005). The composition of culture medium and culture conditions have been shown to be crucial for the growth of *in vitro* cultures (Duran-Vila et al. 1992). Tissue culture and micropropagation protocols have been described for a number of *Citrus spp.* using a wide range of explant sources (Grinblat 1972; Barlass and Skene 1982; Duran-Vila et al. 1989; Beloualy 1991; Carimi et al. 1995; Altaf and Ahmad 1997; Normah et al. 1997; Al-Khayri and Al-Bahrany 2001; Khawale and Singh 2005; Ali and Mirza 2006; Altaf et al. 2008; Altaf et al. 2009a, b; Khan et al. 2009; Laskar et al. 2009; Sharma et al. 2009; Pe'rez-Tornero et al. 2010; Singh and Rajam 2009; 2010). However, a little work has been carried out on the tissue culture of *C. jambhiri* (Raman et al. 1992; Altaf and Ahmad 1997; Khawale and Singh 2005; Ali and Mirza 2006; Altaf et al. 2008; Sharma et al. 2009; Savita et al. 2010). The development of tissue culture protocol is essential to be used routinely as a research tool for improvement of this plant. Keeping this in mind, the present study was designed to develop an efficient and reproducible protocol for callus induction from cotyledons of *C. jambhiri* and regeneration of calli. The effect of age of callus on its regeneration capacity was also determined.

Materials and methods

Surface sterilization

Fresh seeds from the fruits of rough lemon plants growing in the Botanical Garden of Guru Nanak Dev University, Amritsar were collected and soaked in water overnight, washed with teepol for 5 minutes followed by running tap water for 30 minutes. These seeds were surface sterilized with 0.1 % mercuric chloride for 15 min and rinsed with autoclaved double distilled water 3–4 times in laminar flow hood. After peeling off both the seed coats, seeds were again sterilized by washing with 0.1 % mercuric chloride solution for 5 minutes and rinsed with distilled water as described above.

Callus induction

In order to induce callus, cotyledons were separated out from surface sterilized seeds and injured with the help of scalpel and inoculated on freshly prepared medium. Callus was initiated in 25x150 mm culture tubes containing 40 ml of MS medium (containing 0.8 % agar and 3 % sucrose) supplemented with different concentrations and combinations of plant growth regulators like 2,4-dichlorophenoxy

acetic acid (2,4-D), 3-benzyl amino purine (BA), kinetin (KN), malt extract (ME), indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA). For each treatment, 24 culture tubes were inoculated for individual treatment and the experiment was repeated 3 times. Cultures were maintained at 26 ± 2 °C with 16 h-day-length provided by fluorescent tubes.

Shoot regeneration

For regeneration, green healthy friable calli were divided into small pieces and cultured on MS medium (containing 0.8 % agar and 3 % sucrose) supplemented with different concentrations and combinations of plant growth regulators like BA, KN, ME, NAA and Indole-3-acetic acid (IAA). To determine the regeneration capacity of long term callus cultures, calli of different age groups were transferred to optimized shoot regeneration medium (MS medium supplemented with 3 mg/L of BA). For each treatment, 24 culture tubes were inoculated for individual treatment and the experiment was repeated 3 times. Cultures were maintained at 26 ± 2 °C with 16 h-day-length provided by fluorescent tubes.

Rooting of regenerated shoots

For rooting, the regenerated shoots (induced on best shoot regeneration medium) were separated out and cultured on half strength MS (containing 0.8 % agar and 3 % sucrose) medium supplemented with different concentrations of NAA, IBA and IAA.

For each treatment, 24 culture tubes were inoculated for individual treatment and the experiment was repeated 3 times. Cultures were maintained at 26 ± 2 °C with 16 h-day-length provided by fluorescent tubes. Visual observations were taken every three days and the effect of different treatments on percentage of explants showing response for callus induction, shoot regeneration and root induction was recorded after 30 days of culture.

Comparative anatomical study

For comparative anatomical study shoots regenerated from nodal segments and through callus were used. Thin sections were cut with the help of fine razor, dehydrated with ethanol series and stained with safranin and fast green and mounted with DPX. Photographs were clicked with camera (Olympus Imaging Corp.) attached to the microscope (Olympus MLXi).

Hardening and acclimatization

Regenerated plantlets were washed with water in order to remove any adhering medium and transferred to autoclaved plastic pots containing a mixture of garden soil, sand and vermiculite in the ratio of 3:1:1. Hardening of potted

plantlets was accomplished in culture room set at 26 ± 2 °C, 16 hr-day-length ($40 \mu\text{mole m}^{-2} \text{s}^{-1}$) by covering them with polyethylene bags to maintain high humidity. After 12–15 days, polyethylene bags were removed initially for a short duration (15–30 min) daily for about one week. Gradually, the daily exposure time was increased by 30 min for each day. Polyethylene bags were completely removed after 20 days. Subsequently, the plantlets were transferred to earthen pots containing only garden soil and kept in the polyhouse for one month for acclimatization, and then transferred to screen house (Singh et al. 2006).

Statistical analysis

Statistical computations were performed using computer software. The data pertaining to callus induction, shoot

regeneration and rooting were subjected to one-way analysis of variance (ANOVA) and the differences among means were compared by high-range statistical domain (HSD) using Tukey's test.

Results

Callus induction

Effect of different concentrations and combinations of 2,4-D, BA, KN, NAA, ME and IBA on percent callus induction from cotyledons of *Citrus jambhiri* is given in Table 1. Among different concentrations of 2,4-D tested, maximum callus induction response (52.66 %) was observed with 2,4-D at 2 mg/L. This response increased to 83.33 % when 2,4-D

Table 1 Effect of different concentrations and combinations of 2,4-D, BA, KN, NAA, ME and IBA on per cent callus induction from cotyledonary explants of *Citrus jambhiri* Lush

Supplement	Concentration (mg/L)	Percent callus induction* (Mean $\pm$ SE)
Control	0.00	0.00
2,4-D	1.00	25.00 $\pm$ 2.30 ^b
	2.00	52.66 $\pm$ 3.52 ^a
	4.00	40.66 $\pm$ 1.33 ^b
	6.00	33.33 $\pm$ 2.60 ^b
		F(df 3,8)=20.90; HSD=11.62
2,4-D (2 mg/L)+BA	0.25	47.22 $\pm$ 3.67 ^b
	0.50	52.77 $\pm$ 1.39 ^b
	0.75	83.33 $\pm$ 2.49 ^a
	1.00	44.44 $\pm$ 1.39 ^b
		F(df 3,8)=55.549; HSD=10.8985
2,4-D (2 mg/L)+ME	100	33.33 $\pm$ 2.60 ^c
	200	46.00 $\pm$ 2.30 ^b
	300	79.00 $\pm$ 2.30 ^a
	400	81.66 $\pm$ 2.60 ^a
	500	91.66 $\pm$ 2.30 ^a
	600	79.00 $\pm$ 2.30 ^a
		F(df 5,12)=106.61;HSD=10.065
2,4-D (2 mg/L)+IBA	0.25	83.33 $\pm$ 2.60 ^a
	0.50	79.00 $\pm$ 2.30 ^a
	0.75	71.00 $\pm$ 2.30 ^a
	1.00	61.33 $\pm$ 3.80 ^a
		F(df 3,8)=11.632; HSD=12.857
2,4-D (2 mg/L)+NAA	0.25	44.44 $\pm$ 1.39 ^b
	0.50	51.39 $\pm$ 1.39 ^b
	0.75	58.35 $\pm$ 2.42 ^b
	1.00	79.17 $\pm$ 2.40 ^a
		F(df 3,8)=58.026; HSD=8.922
2,4-D (2 mg/L)+KN	0.25	36.11 $\pm$ 1.39 ^a
	0.50	47.22 $\pm$ 3.67 ^a
	0.75	58.33 $\pm$ 4.81 ^a
	1.00	44.44 $\pm$ 1.39 ^a
		F(df 3,8)=8.316; HSD=14.4185

*Out of 24 cultures inoculated for each concentration and each experiment repeated 3 times.

Same alphabets indicate that values are not significantly different at $p \leq 0.05$ level.

Observations recorded after 30 days

(2 mg/L) was used in combination with BA at 0.75 mg/L or with IBA at 0.25 mg/L. Maximum callus induction (91.66 %) was observed when 2,4-D (2 mg/L) was used in combination with ME (500 mg/L). The callus induced from cotyledons was green and fragile, and suitable for regeneration (Fig. 1a).

Shoot regeneration

For regeneration, 60 day old green healthy friable calli were divided into small pieces which were cultured on MS medium supplemented with different concentrations and combinations of BA, KN, ME, NAA and IAA (Table 2).

Fig. 1 (a) Callus induction from cotyledonary explants of *C. jambhiri* on MS medium supplemented with 2,4-D (2 mg/L) in combination with ME (500 mg/L). (b) Callus maintenance on MS medium supplemented with 2,4-D (2 mg/L) in combination with ME (500 mg/L). (c) Shoot regeneration on MS medium supplemented

with BA (3 mg/L). (d) Shoot multiplication on same medium. (e) Rooting of regenerated shoot with NAA (0.5 mg/L). (f) Transplanted plantlets in plastic pots. (g) Hardened plantlets transferred to screen house. (h) Healthy plant of *Citrus jambhiri* successfully transferred to the field

Table 2 Effect of different concentrations and combinations of BA, KN, ME, NAA and IAA on regeneration of callus induced from cotyledonary explants of *Citrus jambhiri* Lush

Supplement	Concentration (mg/L)	Per cent callus regeneration* (Mean±SE)
Control	0.00	0.00
BA	1.00	33.33±2.40 ^d
	2.00	73.61±1.39 ^b
	3.00	87.50±2.40 ^a
	4.00	62.50±2.40 ^c
		F(df 3,8)=109.6020; HSD=9.9523
KN	1.00	25.00±2.30 ^b
	2.00	50.00±2.30 ^a
	4.00	33.33±2.60 ^b
	6.00	12.66±2.60 ^b
		F(df 3,8)=19.00; HSD=13.880
ME	100	8.33±2.60 ^d
	200	25.00±2.30 ^c
	300	37.66±2.60 ^b
	400	47.33±3.52 ^b
	500	46.00±2.30 ^b
	600	64.00±3.00 ^a
		F(df 12,5)=49.96; HSD=12.135
BA (3 mg/L)+NAA	0.25	63.28±1.76 ^a
	0.50	54.94±1.39 ^b
	0.75	45.83±2.40 ^b
	1.00	33.33±2.40 ^c
		F(df 3,8)=41.6363; HSD=9.2393
BA (3 mg/L)+IAA	0.25	33.33±1.39 ^a
	0.50	45.83±2.40 ^a
	0.75	57.06±6.07 ^a
	1.00	44.44±2.40 ^a
		F(df 3,8)=7.4969; HSD=15.4419

*Out of 24 cultures inoculated for each concentration and each experiment repeated 3 times.

Same alphabets indicate that values are not significantly different at $p \leq 0.05$ levels.

Observations recorded after 30 days

Maximum callus regeneration response (87.5 %) was observed with BA (3 mg/L) (Fig. 1c). However, when BA (3 mg/L) was used in combination with increasing dose of NAA (0.25–1.00 mg/L), a dose dependent decrease in regeneration response was observed. Similarly, addition of different doses of IAA to MS medium along with BA (3 mg/L) was also shown to decrease the regeneration response. Among different doses of KN studied, maximum regeneration response (50 %) was

observed at 2 mg/L where as for ME, maximum response (64 %) was observed at 600 mg/L. Table 3 shows the regeneration capacity of calli of different ages cultured on MS medium supplemented with BA (3 mg/L). Per cent callus regeneration, number of shoots per culture and height of shoots decreased with increase in age of callus. The callus induced from cotyledons of *C. jambhiri* was found to regenerate even after 420 days and showed 58.33 % callus regeneration.

Table 3 Effect of increasing age on regeneration capacity of callus induced from cotyledonary explants of *C. jambhiri* Lush

Age of callus	%Callus regeneration (Mean ± SE)	No. of shoots/culture (Mean ± SE)	Height of shoot (Mean ± SE)
60 days old	87.50±2.40 ^a	5.28±0.09 ^a	4.03±0.08 ^a
180 days old	71.00±2.30 ^b	4.92±0.32 ^b	3.84±0.01 ^b
300 days old	66.67±2.60 ^c	4.19±0.08 ^c	3.79±0.01 ^c
420 days old	58.33±2.33 ^d	4.06±0.03 ^c	3.51±0.02 ^d
	F(df 3,8)=106.601; HSD=5.9523	F(df 3,8)=29.502; HSD=4.310	F(df 3,8)=11.302; HSD=0.3240

*Out of 24 cultures inoculated for each concentration and each experiment repeated 3 times.

Same alphabets indicate that values are not significantly different at $p \leq 0.05$ levels.

Table 4 Effect of different concentrations of NAA, IBA and IAA on rooting response of shoots regenerated from callus induced from cotyledonary explants of *Citrus jambhiri* Lush. on half strength MS medium

Supplement	Concentration (mg/L)	Percent rooting* (Mean $\pm$ SE)	Number of roots/shoot* (Mean $\pm$ SE)	Root length (cm)* (Mean $\pm$ SE)
Control	0.00	0.00	0.00	0.00
NAA	0.5	91.67 $\pm$ 4.17 ^a	5.76 $\pm$ 0.07 ^a	6.45 $\pm$ 0.04 ^a
	1	58.33 $\pm$ 4.17 ^b	4.13 $\pm$ 0.07 ^b	5.80 $\pm$ 0.04 ^b
	2	100 (Callus+Roots) ^a	4.21 $\pm$ 0.05 ^b	4.77 $\pm$ 0.05 ^c
	3	100 (Callus+Roots) ^a	4.23 $\pm$ 0.05 ^b	3.50 $\pm$ 0.01 ^d
		F(df 3,8)=45.33; HSD=13.347	F(df 3,8)=193.467; HSD=0.254	F(df 3,8)=1154.75; HSD=0.172
IBA	0.5	79.17 $\pm$ 4.17 ^b	1.60 $\pm$ 0.01 ^d	3.59 $\pm$ 0.05 ^c
	1	70.83 $\pm$ 4.17 ^b	2.00 $\pm$ 0.00 ^c	3.82 $\pm$ 0.04 ^c
	2	91.67 $\pm$ 4.17 ^a	2.47 $\pm$ 0.02 ^b	4.51 $\pm$ 0.04 ^b
	3	95.83 $\pm$ 4.17 ^a	2.90 $\pm$ 0.06 ^a	5.76 $\pm$ 0.07 ^a
		F(df 3,8)=7.583; HSD=18.875	F(df 3,8)=331.36; HSD=0.140	F(df 3,8)=340.69; HSD=0.2427
IAA	0.5	41.67 $\pm$ 4.17 ^b	1.67 $\pm$ 0.09 ^d	2.84 $\pm$ 0.02 ^c
	1	58.33 $\pm$ 4.17 ^b	2.00 $\pm$ 0.00 ^b	2.84 $\pm$ 0.01 ^c
	2	79.17 $\pm$ 4.17 ^a	2.47 $\pm$ 0.03 ^a	3.91 $\pm$ 0.02 ^b
	3	70.83 $\pm$ 4.17 ^a	2.68 $\pm$ 0.02 ^a	4.91 $\pm$ 0.02 ^a
		F(df 3,8)=15.333; HSD=18.875	F(df 3,8)=88.605; HSD=0.220	F(df 3,8)=3133.94; HSD=0.08

*Out of 24 cultures inoculated for each concentration and each experiment repeated 3 times. Same alphabets indicate that values are not significantly different at $p \leq 0.05$ levels. Observations recorded after 30 days.

Rooting of regenerated shoots

For rooting, the regenerated shoots were separated out and cultured on half strength MS medium supplemented with different concentrations of NAA, IBA and IAA (0.5–3 mg/L). Maximum rooting response (91.67 %) was observed on MS medium supplemented with NAA (0.5 mg/L). Similar results in terms of percent rooting were also observed with IBA (2 mg/L) but number of roots per shoot (5.76 ± 0.07) as well as length of roots was higher in case of NAA (0.5 mg/L) than with IBA (2 mg/L) (Table 4; Fig 1e).

Hardening and acclimatization

The plantlets derived from callus were removed from the test tubes, freed from agar and transferred to the field by the procedure mentioned in ‘Materials and Methods’. The plantlets rooted on NAA (0.5 mg/L) were used for hardening and acclimatization. Out of 110 plants transferred to the field 73 plants survived after one year indicating 67 % survival rate.

Comparative anatomical study

For comparison of anatomy, the transverse sections of shoots regenerated from nodal segments and callus induced from cotyledonary explants were studied. No variability was observed in the anatomy of shoots obtained from nodal segments and callus. Structure of cells of different parts was compared eg. cortex, pericycle, xylem, phloem, xylem vessels etc. (Fig. 2a,b,c,d).

Discussion

The micropropagation protocol is required to produce disease free plants of *C. jambhiri*. To produce fungal resistant plants, seed could be a good source of explant because pathogens don't reach to the seed level even after severe infection to the plant. Thus, cotyledons were used as explant in the present investigation. Since, Citrus seeds have very short period of viability which decreases with increasing time of storage (Johnston 1968), therefore, during present investigation freshly isolated seeds from

Fig. 2 (a) Transverse section (T.S.) of stem of *Citrus jambhiri* Lush. produced from nodal segment taken from mother plant. (b) Transverse section (T.S.) of stem of *Citrus jambhiri* Lush. regenerated from callus induced from cotyledonary explant. (c) Enlarged transverse section of (a). (d) Enlarged transverse section of (b). **Abbreviations:** C-cortex; Ph- phloem; X-xylem; R-xylem rays; Per-pericycle; V-xylem vessels

fruits were used. The effect of different plant growth regulators on callus induction from cotyledons was studied. Creamish, friable, non-embryogenic calli were produced from injured portion of cotyledons within 15–20 days of culture on MS medium supplemented with 2,4-D alone and in combination with other plant growth regulators or supplements like BA, KN, NAA, IBA and ME. Best response of callus induction was observed with 2,4-D (2 mg/L)+ME (500 mg/L) where as 2,4-D (2 mg/L)+BA (0.75 mg/L) produced slightly lesser percentage of callus induction. Our results are in conformity with some of the earlier studies on different *Citrus spp.* which showed good callus induction response under the influence of ME in combination with 2,4-D or other plant growth regulators (Grosser et al. 1988; Carimi et al. 1999; Madhav et al. 2002; Miah et al. 2002; Hao and Deng 2003; Singh et al. 2006). Many reports are available on callus induction from different explants in various species of *Citrus* using 2,4-D (Einset et al. 1980; Gmitter et al. 1990; Beloualy 1991; Das et al. 2000; Tao et al. 2002; Singh et al. 2006). Chakraborty and Goswami (1999) reported best callus induction response on MS medium supplemented with 2,4-D (1 mg/L)+BA (0.5 mg/L). Ill et al. (1995) reported callus induction with NAA and KN in *C. reticulata*.

Maximum shoot regeneration response was observed with BA (3 mg/L). Earlier also culture of callus of different spp. of *Citrus* on MS medium supplemented with different concentrations of BA has shown good regeneration response (Pena et al. 1995a, b; Normah et al. 1997; Cervera et al. 1998; Chakraborty and Goswami 1999; Pena and Navarro 1999; Costa et al. 2002). Similarly, Sharma et al. (2009) reported best shoot regeneration response from shoot tip callus cultured on MS medium supplemented with BA (1 mg/L). Absence of 2,4-D and presence of BA and KN in medium initiates shoot induction from the callus as reported earlier in other species too (Raman et al. 1992).

Kaneyoshi et al. (1994) used MS medium containing BA (5 mg/L) in combination with NAA (0.1 mg/L) for regeneration of *Poncirus trifoliata* Rad. Similar reports are also available on *Citrus* for indicating best shoot regeneration from callus on medium supplemented with BA and NAA (Chaturvedi and Mitra 1974; Beloualy 1991). However, in our study when BA (3 mg/L) was used in combination with increasing dose of NAA (0.25–1.00 mg/L), a dose dependent decrease in regeneration response was observed. The callus induced from cotyledons of *C. jambhiri* could be maintained in culture for more than a year and was found to regenerate (in 58 % of cultures) even after 420 days in culture. However, the regeneration capacity of the callus decreased with increasing age of the callus. These results are in conformity with those of Chakraborty and Goswami (1999).

Among different plant growth regulators tested for root induction from regenerated shoots, NAA at a concentration

of 0.5 mg/L was found to give the best rooting response. This is in conformity with some of the earlier studies (Pena et al. 1995a; Kaneyoshi et al. 1994; Chakraborty and Goswami 1999; Normah et al. 1997; Usman et al. 2005; Rani et al. 2004).

In the present investigation, we have established an efficient and simple protocol for the plant regeneration of *C. jambhiri* using callus cultures induced from cotyledonary explants. This protocol may be applied for genetic transformation in *C. jambhiri*.

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