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Effect of Explant Type and Different Plant Growth Regulators on Callus Induction and Plantlet Regeneration in *Citrus jambhiri* Lush

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Abstract

Citrus jambhiri (rough lemon) is an important rootstock for a number of *Citrus* fruit crops including lemons, oranges, grape fruits, kinnows and mandarins. The present study deals with establishment of protocol for micropropagation of *C. jambhiri* via callus induction and regeneration. Leaf segments, nodal segments and root segments excised from *in vitro* raised seedlings were used as explants. Maximum callus induction (98.66%) was observed from leaf segments on MS medium supplemented with 2, 4-D (4 mg/L). For nodal segments, maximum callus induction (96%) was observed with 2, 4-D (1 mg/L) and from root segments, it was 48.66% on MS medium supplemented with 2, 4-D (2 mg/L). Callus raised from leaf segments showed maximum regeneration (57%) with NAA (0.5 mg/L) + BA (1 mg/L) whereas nodal segments showed better regeneration (71.89%) with NAA (0.5 mg/L) + BA (3 mg/L). However, callus from root segments did not regenerate. Regenerated shoots were rooted on MS medium supplemented with different plant growth regulators and best response (71%) was observed with NAA (0.5 mg/L).

Keywords: Callus induction, *Citrus jambhiri*, Regeneration.

Introduction

In Punjab and nearby states, rough lemon (*Citrus jambhiri*) has been considered to be the most important rootstock for lemons, oranges, mandarins, grape fruits and kinnows, because of its high vigour, well adaptation to warm-humid areas with deep sandy soils and resistance to *Citrus tristeza virus*. Locally in Punjab, it is known as "Jatti-Khatti" and produces high yielding trees with large fruits. It grows easily in old citrus soil. However, it is very cold sensitive and susceptible to *Phytophthora.* The conventional breeding approaches have their own limitations to solve this problem of citrus industry because of perennial nature of the crop, long juvenility, nucellar polyembryony, heterozygocity etc. Recently, various

biotechnological approaches have been developed for improvement by creating the variations and selecting the variants for potential needs and it has been established that the plants multiplied through *in vitro* cultures can serve as sound plantation stock. Furthermore, micropropagation offers several advantages which are not possible with conventional propagation techniques. It provides reliable and economical method for maintaining pathogen free plants that can allow rapid multiplication and also allow international exchange of germplasm.

Tissue culture and micropropagation protocols have been described for a number of *Citrus* species and explant sources (Barlass and Skene, 1982; Duran-Vila *et al.,* 1989; Raman *et al.,* 1992; Normah *et al.,* 1997; Chakravarty and Goswami, 1999; Al-Khayri and Al-Bahrany, 2001; Filho *et al.,* 2001; Usman *et al.,* 2005; Altaf *et al.,* 2009a,b; Khan *et al.,* 2009; Laskar *et al.,* 2009; Sharma *et al.,* 2009; Pe´rez-Tornero *et al*., 2010). However, little work has been carried out on the tissue culture aspect of *C. jambhiri* (Altaf and Ahmad, 1997; Ali and Mirza, 2006; Altaf *et al.,* 2008). The present study deals with callus induction and plant regeneration using different explants (leaf, nodal and root segments) of *C. jambhiri.*

Materials and Methods

Collection of Explant

Seeds were harvested from healthy fruits of *Citrus jambhiri* tree growing in the Botanical Garden of Guru Nanak Dev University, Amritsar (India).

Surface sterilization

Fresh seeds from the fruits of healthy plant of rough lemon 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 seed coats, they were again sterilized by washing with 0.1% mercuric chloride solution for 5 minutes and rinsed with distilled water as described above.

In vitro seed germination

Surface sterilized seeds were placed individually in 25x150 mm culture tubes containing 25 ml of solidified (0.8% agar) MS medium supplemented with different concentrations of KN, IAA and IBA. For germination, the culture tubes were incubated in culture room at 26±2ºC with 16h photoperiod for two weeks.

Callus induction

Different explants like leaf segments, nodal segments and root segments (0.5-1.0 cm) were excised from 5 weeks old *in vitro* raised nucellar seedlings for callus induction. Callus was initiated in 25x150 mm culture tubes containing 40 ml of MS medium supplemented with different concentrations and combinations of plant growth regulators. For each explant, 24 culture tubes were inoculated for individual treatment and the experiment was repeated 3 times. Visual observations were taken

every three days and the effect of different treatments was quantified on the basis of the percentage of explants showing callus induction.

Callus regeneration

The green healthy friable calli induced from various explants were divided into small pieces and cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators i.e. BA, KN, ME, NAA and IAA for regeneration.

Rooting of regenerated shoots

The regenerated shoots were separated out and cultured on MS medium supplemented with different concentrations of NAA and IBA for rooting.

Hardening and acclimatization

Regenerated plantlets were washed with water in order to remove any adhering medium and transferred to plastic pots containing autoclaved 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 μmole m^{-2} s^{-1}) by covering them with polyethylene bags to maintain 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 green 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

***In vitro* seed germination**

The addition of plant growth regulators to the medium resulted in an increase or decrease in per cent germination from that of control value. Maximum seed germination (90.66%) was observed on MS medium supplemented with IBA (4 mg/L), followed by 86.33% with KN (2 mg/L) and 59.66% with IAA (2 mg/L) (Table 1) whereas 50% germination was observed without growth regulators on MS medium.

Callus induction from leaf segments

Leaf segments (0.5-1.0 cm) were excised from 5-weeks-old *in vitro* raised seedlings for callus induction. Among different plant growth regulators tested,

maximum callus induction (98.66%) was observed on MS medium supplemented with 2,4-D (4 mg/L) alone (Table 2). A decrease in callus induction was observed when 2,4-D (4 mg/L) was used in combination with different concentrations of BA alone and with NAA (Figure 1b).

Table 1 Effect of different concentrations of KN, IAA and IBA on seed germination of *Citrus jambhiri* Lush. inoculated on MS medium (Observations recorded after 30 days).

Plant growth regulator	Concentration (mg/l)	Per cent seed germination*
		Mean±SE
Control	-	50.00±1.33
KN	1	59.66±1.66[b]
	2	86.33±1.66[a]
	4	50.00±2.30[c]
	6	33.33±2.66[d]
		F(df 3,8)=111.413 HSD=9.520
IAA	1	33.66±2.66[c]
	2	59.66±3.84[a]
	4	58.33±2.66[a]
	6	37.66±2.60[b]
		F(df 3,8)=21.361 HSD=13.421
IBA	1	65.66±2.66[b]
	2	50.00±2.30[c]
	4	**90.66±1.33[a]**
	6	46.00±2.30[d]
		F(df 3,8)=83.899 HSD=10.016

***** Out of 24 cultures inoculated for each concentration, each experiment was repeated 3 times
Abbreviations: KN- Kinetin; IBA- Indole-3- butyric acid; IAA- Indole-3-acetic acid
Note: Different alphabets indicate that values are significantly different from each other at p≤0.05 levels.

Callus induction from nodal segments

Nodal segments (1-1.5 cm) were excised from 5-week-old seedlings. Transverse cuts were made on nodal segments and inoculated on callus induction medium. Maximum callus induction (96%) was observed on MS medium supplemented with 2, 4-D (1 mg/L) (Table 2). Callus induced from nodal segments was green, compact and capable of regeneration into shoots. A decrease in callus induction was observed when 2,4-D (1 mg/L) was used in combination with different concentrations and combinations of BA and NAA (Figure 1c).

Callus induction from root segments

The callus was developed (48.66%) only on MS medium supplemented with 2, 4-D (2 mg/L) from root explants (Table 2; Figure.1d).

Table 2. Effect of different concentrations and combinations of 2,4-D, NAA and BA on per cent callus induction from leaf, nodal and root segments excised from 5 weeks old seedlings of *Citrus jambhiri* Lush. (Observations recorded after 30 days)

Supplement	Concentration (mg/l)	Per cent callus induction*		
		Leaf segments	Nodal segments	Root segments
2,4-D	1	73.66±1.33[b]	**96.00±2.30[a]**	0.0
	2	79.00±2.30[b]	79.00±2.30[b]	**48.66±3.52**
	4	**98.66±1.33[a]**	61.33±3.84[c]	0.0
	6	77.66±1.33[b]	54.00±2.30[d]	0.0
		F(df3,8)=46.8432 HSD=7.397	**F(df 3,8)=45.9818 HSD=12.56**	**F(df3,8)=190.3216 HSD=7.99**
NAA	1	14.57±0.60[d]	27.45±2.28[c]	0.0
	2	31.99±1.00[c]	58.08±2.71[b]	0.0
	4	60.28±0.55[a]	74.33±1.45[a]	0.0
	6	45.50±0.32[b]	65.89±0.55[a]	0.0
		F(df3,8)=473.708 HSD=5.7066	**F(df 3,8)=149.929 HSD=12.241**	
2,4-D(4)+BA	0.25	34.72±1.39[d]	-	-
	0.50	41.66±2.40[c]	-	-
	0.75	57.66±6.07[a]	-	-
	1.00	44.44±1.39[b]	-	-
		F(df 3,8)=7.4969 HSD=15.4419		
2,4-D(4)+BA(0.75)+NAA	0.25	63.33±1.76[a]	-	-
	0.50	56.94±1.39[a]	-	-
	0.75	45.83±2.40[b]	-	-
	1.00	33.33±2.40[c]	-	-
		F(df3,8)=41.6363 HSD=9.2393		
2,4-D(1)+BA	0.25	-	68.06±1.39[b]	-
	0.50	-	77.78±1.39[a]	-
	0.75	-	62.5±2.41[b]	-
	1.00	-	45.83±2.41[c]	-
			F(df 3,8)=46.4599 HSD=8.8982	
2,4-D(1)+BA(0.5)+NAA	0.25	-	90.28±1.39[a]	-
	0.50	-	83.33±2.40[a]	-
	0.75	-	68.06±1.39[b]	-
	1.00	-	54.17±2.40[c]	-
			F(df 3,8)=41.6363 HSD=9.2393	

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

Abbreviations: 2,4-D- 2,4- dichlorophenoxyacetic acid; NAA- Naphthalene acetic acid; BA- 6-benzylaminopurine

Note: Same alphabets indicate that values are not significantly different at p≤0.05 levels.

Callus regeneration

The regeneration response (71.89%) was better from nodal segment derived callus cultures on MS medium supplemented with BA (3 mg/L) and NAA (0.5 mg/L); (Figure 1f), as compared with callus induced from leaf segments (57.55%) (Table 3; Figure 1e).

Table 3. Effect of different concentrations and combinations of NAA, BA and KN on shoot regeneration from callus (raised from leaf segments and nodal segments excised from nucellar seedlings)

Supplement (mg/L)			Regeneration from leaf callus		Regeneration from nodal callus	
NAA	**BA**	**KN**	**Percent* regeneration**	**Number of shoots* per culture**	**Percent* regeneration**	**Numberof shoots* per culture**
0.50	0.25	-	19.56±0.87^{f}	1.87±0.13^{f}	19.45±0.49^{f}	1.67±0.17^{g}
0.50	0.50	-	24.89±0.11^{e}	1.92±0.08^{f}	25.53±0.63^{e}	1.71±0.10^{f}
0.50	0.75	-	32.33±0.55^{d}	2.13±0.09^{e}	40.56±0.47^{d}	2.23±0.13^{e}
0.50	1.00	-	57.55±0.29^{a}	4.05±0.05^{a}	47.68±0.73^{c}	3.68±0.02^{d}
0.50	2.00	-	44.67±0.33^{b}	3.20±0.20^{b}	59.65±0.56^{b}	4.33±0.13^{c}
0.50	3.00		38.33±0.87^{c}	2.91±0.19^{c}	**71.89±0.89^{a}**	**5.83±0.03^{a}**
0.50	4.00		31.76±0.33^{d}	2.53±0.11^{d}	58.93±0.39^{b}	4.75±0.17^{b}
			F(df 4,10)=1217.54 HSD=2.0268	**F(df 4,10)=841.6363 HSD=0.2393**	**F(df 4,10)=139.573 HSD=5.2593**	**F(df 4,10)=679.8793 HSD=0.1896**
2.00	-	0.25	0.0	-	16.28±0.26^{d}	1.56±0.10^{f}
2.00	-	0.50	0.0	-	22.22±0.13^{d}	1.28±0.17^{e}
2.00	-	0.75	0.0	-	37.87±0.13^{c}	2.07±0.12^{d}
2.00	-	1.00	0.0	-	43.75±0.13^{b}	4.83±0.03^{a}
2.00	-	2.00	0.0	-	46.25±0.26^{b}	3.15±0.23^{c}
2.00	-	3.00	0.0	-	**63.95±0.48^{a}**	4.83±0.03^{a}
2.00	-	4.00	0.0	-	54.97±0.11^{b}	4.65±0.15^{b}
					F(df 4,10)=41.6363 HSD=9.2393	**F(df 4,10)=41.6363 HSD=0.1393**

***** Out of 24 cultures inoculated for each concentration and each experiment was repeated 3 times.
Abbreviations: NAA- Naphthalene acetic acid; BA- 6-benzylaminopurine and KN-Kinetin.
Note: Same alphabets indicate that values are not significantly different at p≤0.05 levels.

Rooting of regenerated shoots

Regenerated shoots were transferred to MS medium supplemented with NAA and IBA. Maximum rooting response (71%) was observed on MS medium supplemented with NAA at 0.5 mg/L (Table 4; Figure 1g). However, among different concentrations of IBA tested, maximum response was observed with 2 mg/L of IBA.

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 as mentioned above. The plants were transferred to the field and showed 55% survival (Figure 1h).

Table 4. Effect of different concentrations of NAA and IBA on per cent rooting response from shoots of *Citrus jambhiri* cultured on MS medium (Observations recorded after 30 days of inoculations).

Supplement (mg/L)		Per cent Rooting*
NAA	**IBA**	**Mean±SE**
0.25	-	52.63±0.14[c]
0.50	-	**71.00±0.29[a]**
0.75	-	61.83±0.44[b]
1.00	-	35.13±0.13[d]
2.00	-	27.26±0.16e
4.00	-	21.26±0.16[f]
		F(df 4,10)=3231.504; HSD=1.4980
-	0.25	22.67±0.17[f]
-	0.50	34.87±0.78[d]
-	0.75	40.00±0.58[c]
-	1.00	48.68±0.20[b]
-	2.00	51.58±0.29[a]
-	4.00	30.30±1.14[e]
		F(df 4,10)=342.4118 HSD=2.559

* Out of 24 cultures inoculated for each concentration and each experiment was repeated 3 times.
Abbreviations: NAA- Naphthalene acetic acid and IBA-Indole-3-butyric acid.
Note: Same alphabets indicate that values are not significantly different at p≤0.05 levels.

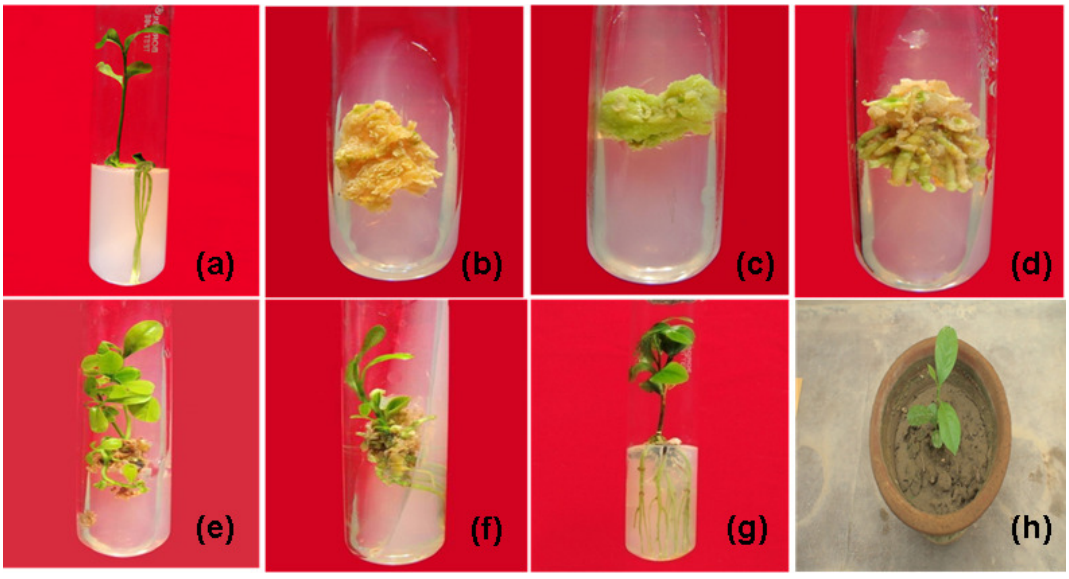

Figure 1 (a) *In vitro* raised nucellar seedling, (b) Callus induction from leaf segment, (c) Callus induction from nodal segment, (d) Callus induction from root segment, (e) Shoot regeneration from calli derived from leaf segment, (f) Shoot regeneration from calli derived from nodal segment, (g) Rooting of regenerated shoots and (h) Hardened plantlet transferred to pot.

Discussion

Since, *Citrus* seeds have very short period of viability (Johnston 1968), therefore, we used freshly isolated seeds for germination studies. Maximum germination response (90%) was observed on MS medium supplemented with IBA (4 mg/L). According to Chaudhary (1994), *Citrus* seeds showed 75-80% germination. Most seeds have shown polyembryony as reported earlier (Choudhary 1994). In the present study, we used various explants like leaf, root and nodal segments excised from 5 weeks old seedlings for callus induction, which showed different response. Among three explants used, nodal segments were best for callus induction as compared to leaf and root segments. The lower concentration of 2,4-D (1 mg/L) was sufficient to induce callus in 96% of cultures from nodal segments whereas for leaf segments higher concentration of 2,4-D (4 mg/L) was to be used to achieve 98% callus induction. Nodal segment derived calli were green and friable whereas those raised from leaf segments were brown and became necrotic after 2-3 subcultures. There was no callus induction from root segments except with the use of 2,4-D (2 mg/L). However, this callus could not be regenerated. This study showed that response to callus induction depended on explant type as well as concentration and type of plant growth regulator used. Similar results showing variations among explant types with respect to callus induction have also been reported in other plants like *Albizzia lebbek* (Lakshmana Rao and De, 1987); *Lonicera japonica* (Georges *et a.l,* 1993) and *Holarrhena antidysenterica* (Raha *et al.,* 2003).

Best callus regeneration response (71%) was observed with nodal segment derived callus cultured on MS medium supplemented with NAA (0.5 mg/L) and BA (3 mg/L). Earlier also combination of BA and NAA have been shown to be favourable for shoot regeneration from calli of different *Citrus* spp. (Chaturvedi and Mitra, 1974; Beloualy, 1991). Some studies have shown use of BA alone to be better treatment for shoot regeneration in different *Citrus* spp. (Raman *et al.,* 1992; Costa *et al.,* 2002). In our study, combination of KN with NAA was less effective as compared to BA with NAA. On the contrary, there are some reports indicating KN to be a better plant growth regulator treatment for shoot regeneration (Rahman *et al.*, 1996). Among two plant growth regulators tried for rooting, NAA was found to give better response (71%) as compared to IBA. Similar results have earlier been reported in *Citrus acida* (Chakravarty and Goswami, 1999). Other reports which showed NAA to be better rooting hormone for *Citrus* spp. included Pena *et al*. (1995a); Chakravarty *et al*. (1999); Normah *et al.* (1997); Usman *et al.* (2005); Rani *et al.* (2004).

Authors' contributions: ***Savita*** *(Senior Research Fellow), performed some of the experiments, worte the manuscript and calculation;* ***Vijay*** *(Lecturer) performed some of the experiment;* ***Dr. G.S. Virk*** *(Professor), Project leader, contributed in experiment design and final editing of the manuscript and* ***Dr. (Mrs.) Avinash Nagpal*** *(Professor), co-investigator for the project, contributed in experiment design, preparation and also corresponding author of manuscript.*

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