Micropropagation of rough lemon (*Citrus jambhiri* Lush.): Effect of explant type and hormone concentration

SHAWKAT ALI, BUSHRA MIRZA[*]

Department of Biological Sciences Quaid-i-Azam University Islamabad, Pakistan

The effect of various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BA) and α-naphthalene acetic acid (NAA) on regeneration of rough lemon (*Citrus jambhiri* Lush.) explants was studied. Optimal callus induction response was observed on Murashige and Skoog medium (MS), supplemented with 2,4-D 1.5 mg L^{-1} from all types of explants, with stem explants showing the highest response (92%). Maximum shoot regeneration response (70%) from callus was observed on MS medium supplemented with BA 3 mg L^{-1}. Direct shoot regeneration was highest in stem segment explants on MS medium with BA 3 mg L^{-1}. MS medium supplemented with NAA 0.5 mg L^{-1} provided 70 % rooting response. This is the first report on direct and indirect regeneration *in vitro* in rough lemon.

Key words: *Citrus*, microprogation, regeneration, callus, explant, hormone, rooting

Introduction

Citrus is the number one fruit of the world due to its high nutritional value, considerable production of fruit and fruit products and the citrus industry is considered to be a major fruit industry (CHATURVEDI et al. 2001). *Citrus* varieties are propagated by both sexual and asexual methods. Generally, rootstocks are propagated sexually through seeds, while most of the commercial varieties are propagated by various asexual methods (CHAUDHARY 1994). Micropropagation is an important asexual method that can be used for the production of virus-free rootstock plants (ROISTACHER et al. 1976). An efficient tissue culture protocol is a prerequisite for the *Agrobacterium*-mediated transformation of any plant. Tissue culture and micropropagation protocols have been described for a number of citrus species and explant sources (GRINBLAT 1972, CHATURVEDI and MITRA 1974, BARLASS and SKENE 1982, EDRISS and BURGER 1984, DURAN-VILA et al. 1989). The environmental conditions and composition of culture media are known to be crucial for the growth of tissue cultures (KOBAYASHI et al. 1985, DURAN-VILA et al. 1992, RANDALL 1994). Impact of MS and Murashige and Tucker (MT) media with various kinds and concentrations of cytokinins on the tissue culture of *Citrus reticulate* revealed that MT medium supplemented with 0.5 mg

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* Corresponding author, e-mail: bushra_dr@yahoo.com

L^{-1} BA gave the greatest percentage of shoots and roots numbers (TE-CHATO and NUDOUNG 1998). Most of the present work focuses on defining the optimal composition of culture media under constant environmental conditions.

Rough lemon is a commonly used root stock in south Asia; however, very little work has been carried out on the tissue culture of this plant. In order to determine the tissue culture protocol to be used routinely as a research tool for rough lemon (*Citrus jambhiri* Lush.), this study was conducted to identify the best type of explant from *in vitro* grown seedling and growth regulator concentration and /combination for callus induction, shoot regeneration from callus, direct shoot regeneration from explants and rooting of regenerated shoots.

Materials and methods

Plant material and preparation of explants

Seeds of rough lemon (*Citrus jambhiri* Lush.) were disinfected either for 10 minutes in a 0.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) tween-20 as a wetting agent after removing both seed coats or for 20 minutes in a 1% (v/v) sodium hypochlorite solution containing 0.1% (v/v) tween-20 before peeling and were rinsed three times with sterile distilled water under laminar flow hood. Seed sterilization after the peeling of the seed coat was also carried out in 0.1% (w/v) mercuric chloride (HgCl$_2$) solution for 5 minutes and rinsed by the same method as described above. The seeds were then placed individually in 25 x 150 mm culture tubes containing 25 ml of MS (MURASHIGE and SKOOG 1962) medium containing 5% sucrose and solidified with 0.8% agar. For germination the culture tubes were incubated in darkness at a constant 27 °C temperature for 2 weeks and then at 25 °C, in a growth chamber with 16 h of photoperiod, illumination of 45 µE m^{-2} s^{-1} and 60 % relative humidity for 3 weeks (PENA et al. 1995a).

Roots and stem segments were excised from 5-week-old *in vitro* grown seedlings and were cut into 0.5–1 cm pieces while leaf and cotyledon explants were prepared by cutting leaves and cotyledons perpendicularly to midrib with size of 0.5–1 cm^2 to use as explants for further manipulation.

Callus induction

Callus induction was initiated in a 100 mL flask containing 40 ml of MS medium containing 3% sucrose and solidified with 0.8% agar, having different concentrations of callus-inducing growth regulators individually and in combination. Thirty explants were used for each treatments and each experiment was conducted three times. Visual observations were taken every three days and the effect of different treatments was quantified on the basis of percentage of explants showing response for callus induction.

Shoot regeneration

Shoot regeneration was performed in 25 x 150 mm culture tubes containing 25 mL of MS medium containing 3% sucrose and solidified with 0.8% agar, having different concentrations of shoot-regenerating hormones individually or in combination (Tab. 3). Shoot re-

generation was carried out both from callus and from explants directly. In the case of shoot regeneration from callus, a healthy green portion of callus was taken and cut into pieces, and these pieces were then placed on a shoot regeneration medium. Twenty-five calli were raised for each treatment and each experiment was conducted three times. Visual observations were taken every three days and the effect of different treatments was quantified on the basis of percentage of calli showing response for shoot regeneration.

In the case of direct regeneration from explants, leaves, roots, stem segments, and cotyledons were excised from 5-week-old *in vitro* grown seedlings and were cut in to 0.5–1 cm pieces and placed on shoot regeneration medium having several different concentrations of hormones individually or in combination. Thirty explants of each type were used for each treatment in one experiment and each experiment was conducted three times.

Rooting of regenerated shoot

Rooting was performed in 25 x 150 mm culture tubes containing 25 mL of MS medium containing 3% sucrose and solidified with 0.8% agar, having 0.5 mg L^{-1} NAA or 1 mg L^{-1} 2,4-D. Twenty five regenerated shoots were cultured for rooting and each experiment was conducted three times. Visual observations were taken every three days and the effect on different shoots was quantified on the basis of percentage of shoots showing response for rooting.

Results

Seed surface sterilization

To identify the ideal surface sterilization conditions of seeds, either sodium hypochlorite or Mercuric chloride was used (Tab. 1). Seeds were exposed to sterilizing agents for varying durations, either before or after peeling. Maximum contamination (55%) was observed on sodium hypochlorite 1% (v/v) solution containing 0.1% (v/v) tween-20 for 20 minutes before peeling, followed by sodium hypochlorite 0.5% (v/v) solution containing 0.1% (v/v) tween-20 for 10 minutes, after peeling (40%). Mercuric chloride solution 0.1% (w/v) for 5 minutes showed the lowest contamination (10%) and hence was used to sterilize seeds for further studies.

Tab. 1. Percentage of contamination using different surface sterilizing agents. Data represent the mean value of three experiments. 100 seeds were used for each experiment.

Surface Sterilizing agent	Concentration (%)	Stage of application	Duration of exposure (min)	% of seeds showing contamination
Sodium hypochlorite	1.0 (v/v)	Before peeling	20	55
Sodium hypochlorite	0.5 (v/v)	After peeling	10	40
Mercuric chloride	0.1 (w/v)	After peeling	5	10

In vitro seed germination

Seeds were sown individually in 25 x 150 mm culture tubes after the peeling of both seed coats. Most of the seeds produced more than one seedling. The seeds used were either freshly isolated from fruits or stored at 4 °C. The freshly isolated seeds showed 75–80 % germination capacity, and started germination after 10 days,while the viability of stored seeds decreased with the passage of time,and after two months not even a single seed germinated (data not shown).

Callus induction

To determine the best callus induction response for explants of different types, various concentration and combination of hormones were tried (Tab. 2). The explants used include leaves, stem segments, cotyledons and roots excised from 5-week-old *in vitro* grown seedlings (Fig. 1A). The best callus induction response (83 %) of leaf explants was observed on MS medium supplemented with 1.5 mg L^{-1} 2,4-D (Fig. 1B) followed by MS medium supplemented with 1 mg L^{-1} 2,4-D (67 %) (Fig. 1C). The lowest callus induction response (33%) was observed on MS medium supplemented with 0.2 mg L^{-1} 2,4-D. Optimal callus induction response of stem explants was observed on MS medium supplemented with 1.5 mg L^{-1} 2,4-D (92 %) and (88 %) on both MS medium supplemented with 1 mg L^{-1} 2,4-D, and MS medium supplemented with 2,4-D 0.2 mg L^{-1} + BA 2 mg L^{-1} + NAA 0.2 mg L^{-1}. The lowest callus induction response (24%) of stem explants was observed on MS medium supplemented with 0.2 mg L^{-1} 2,4-D. The best callus induction response of cotyledons was observed on MS medium supplemented with 1 mg L^{-1} 2,4-D and MS medium supplemented with 1.5 mg L^{-1} 2,4-D (80 %). The lowest callus induction response (16%) of cotyledons was observed on MS medium supplemented with 0.2 mg L^{-1} 2,4-D. In root explants callus developed only on MS medium supplemented with 1.5 mg L^{-1} 2, 4-D (32%), which may be considered the best treatment.
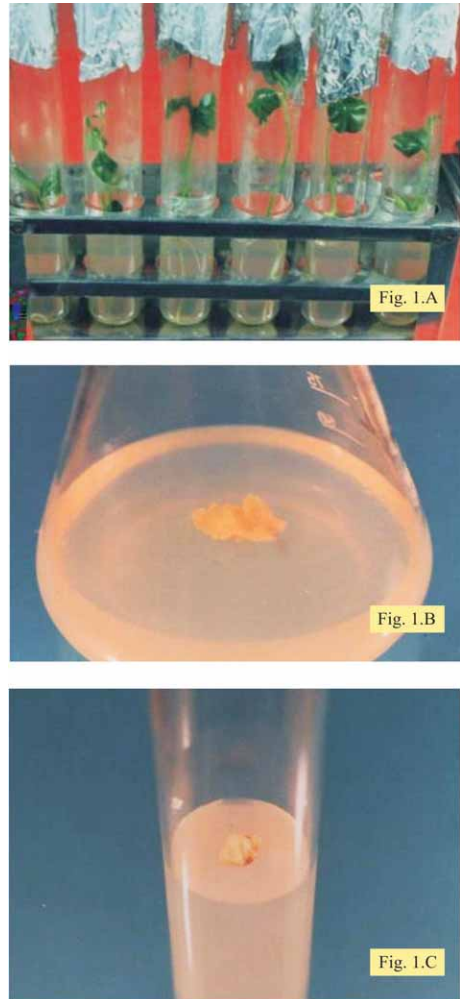

Fig. 1. Five-week *in vitro* grown seedling of rough lemon (A); callus response of explants on MS medium supplemented with 1.5 mg L^{-1} (B), and 1 mg L^{-1} 2, 4-D (*C*)

Tab. 2. Effect of hormone concentration on relative callus induction in different explants. All media contain MS salts and vitamins. Data represent the mean value of three independent experiments. Thirty explants were used for each treatment in each experiment.

*Medium	% of explants showing callus induction response			
	Leaf	Stem	Cotyledon	Root
2,4-D 0.2 mgL^{-1}	33	24	16	0
2,4-D 0.5 mgL^{-1}	50	72	40	0
2,4-D 1 mgL^{-1}	67	88	80	0
2,4-D 1.5 mgL^{-1}	83	92	80	32
2,4-D 2 mgL^{-1}	56	60	48	0
2,4-D0.2 + BA 2 mgL^{-1}	46	72	32	0
2,4-D 0.2 + BA 2 + NAA 0.2 mgL^{-1}	40	88	36	0

* All media contain MS salts and vitamins.

Shoot regeneration

For shoot regeneration the method of direct regeneration as well as regeneration from callus was performed. For indirect regeneration green healthy calli (obtained in the previous experiment from different type of explants) were cut into small pieces and these pieces were cultured on MS medium supplemented with BA 3 mg L^{-1} and MS medium supplemented with BA 2 mg L^{-1} + NAA 0.2 mg L^{-1}. Maximum shoot regeneration response (70%) was observed on MS medium supplemented with BA 3 mg L^{-1} (Fig. 2A) followed by 54% on MS medium supplemented with BA 2 mg L^{-1} + NAA 0.2 mg L^{-1} (Fig. 2B).

For direct shoot regeneration, the response of different explants on various growth regulator concentrations and combinations was observed (Tab. 3). Stem explants showed optimal shoot regeneration (83%) on MS medium supplemented with BA 3 mg L^{-1} (Fig. 2C) and 76% on MS medium supplemented with BA 0.5 mg L^{-1} (Fig. 2D). The lowest shoot re-

Tab. 3. Effect of hormone concentration on shoot regeneration in stem explants. All media contain MS salt and vitamins. Data represent the mean value of three independent experiments. Thirty explants were used for each treatment in each experiment.

*Medium	% of explants showing shoot regeneration response
BA 0.5 mgL^{-1}	76
BA 1 mgL^{-1}	30
BA 2 mgL^{-1}	33
BA 3 mgL^{-1}	83
BA 4 mgL^{-1}	26
BA 5 mgL^{-1}	40
BA 2 + NAA 0.1 mgL^{-1}	50
BA 5 + NAA 0.1 mgL^{-1}	20
BA 5 + NAA 0.2 mgL^{-1}	20
BA 3 + NAA 0.2mgL^{-1}	40
BA 3 + NAA 0.1 mgL^{-1}	16

* All media contain MS salts and vitamins.

generation response (16%) was observed on MS medium supplemented with BA 3 mg L^{-1} + NAA 0.1 mg L^{-1}. In cotyledon explants very little (13%) shoot regeneration response was observed on only one medium i.e MS supplemented with BA 3 mg L$^{-1,}$ while roots and leaf explants did not produce any shoots on any media tested (data not shown).

Rooting of regenerated shoots

For rooting, the developed shoots cut-off segments were cultured on rooting medium consisting of MS containing either 0.5 mg L^{-1} NAA or 1 mg L^{-1} 2,4-D. 70% rooting re-
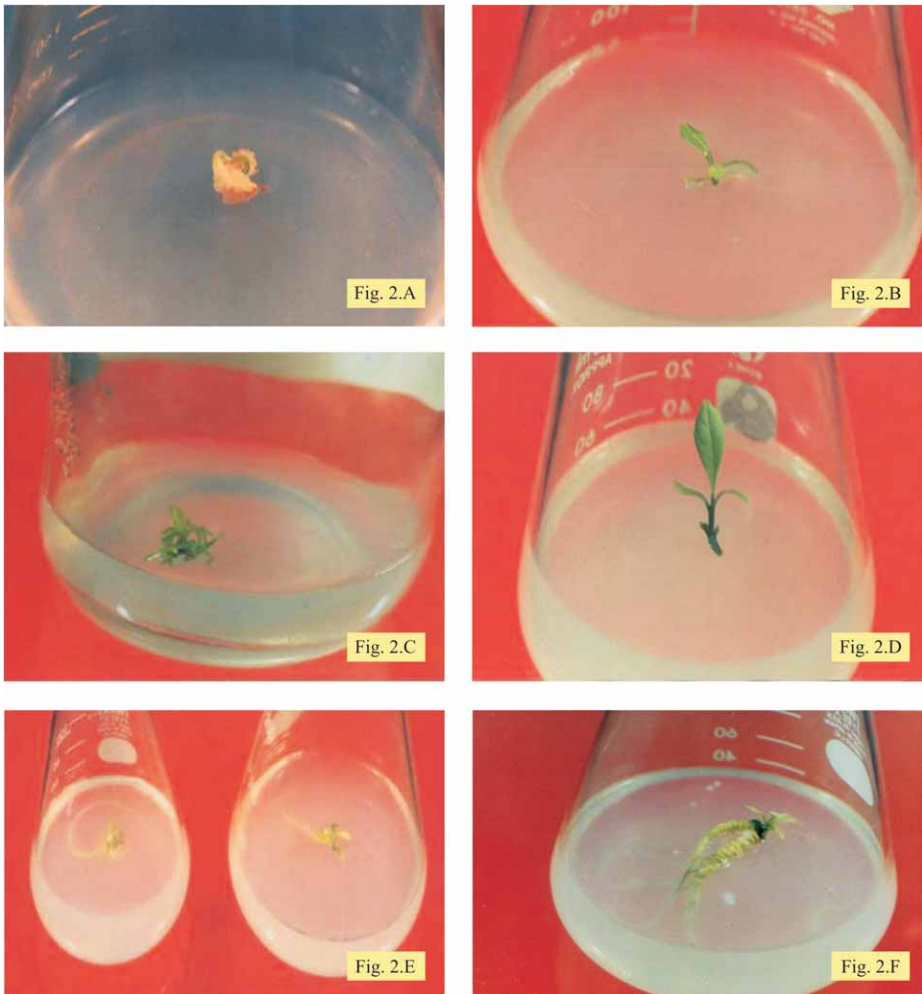

Fig. 2. Shoot regeneration and rooting response (*A*); shoot regeneration from callus on MS medium supplemented with BA 3 mg L^{-1} (*B*), and BA 2 mg L^{-1} + NAA 0.2 mg L^{-1} (*C*); explant showing shoot direct regeneration on MS medium supplemented with BA 3 mg L^{-1}; (*D*), and BA 0.5 mg L^{-1} (*E*); rooting of regenerated shoot on MS medium containing 0.5 mg L^{-1} NAA (*F*), and 1 mg L^{-1} 2,4-D.

sponse was observed on 0.5 mg L^{-1} NAA (Fig. 2E). Some rooting response (50%) was also observed on MS medium having 1 mg L^{-1} 2,4-D usually after induction of small callus (Fig. 2F).

Discussion

The present study was designed to identify the ideal conditions for micro propagation of rough lemon (*Citrus jambhiri* Lush.) because not much work has been done on the tissue culture and micro propagation of this plant. Citrus seeds have a very short life because they are injured by drying during storage and thus lose their viability (JOHNSTON 1968). Accordingly we mainly used freshly isolated seeds from fruits. These seeds showed 75–80% germination capacity, as reported earlier (CHAUDHARY 1994). In our experiments most seeds produced more than one seedling, because polyembryony is very common in citrus (CHAUDHARY 1994).

We tried various concentrations and combinations of hormones, and also different type of explants (leaves, stem segments, cotyledons and roots) for callus induction. The MS medium supplemented with 1.5 mg L^{-1} 2,4-D proved to be the best medium for callus induction for all types of explants, followed by MS medium supplemented with 1 mg L^{-1} 2,4-D. On MS medium with 1.5 mg L^{-1} 2,4-D up to 92% callus response was obtained in stem explants. Next to stem segments were leaf and cotyledons explants, while root explants did not prove to be suitable for callus induction showing only 5 % callus induction response. DAS et al. (2000) have also reported callus development in sweet orange (*Citrus sinensis*) on MS medium supplemented with 2,4-D 1 mg L^{-1} or NAA 1 mg L^{-1}.

Regeneration of different species of *Citrus* has been already investigated using MS medium supplemented with BA 3 mg L^{-1} or with BA 1 mg L^{-1} (PENA et al. 1995a, b, 1997; CERVERA et al. 1998a, b, 2000; GHORBEL et al. 1999: PENA and NAVARRO 1999; DOMINGUEZ et al. 2000;). KANEYOSHI et al. (1994) used MS medium supplemented with BA 5 mg L^{-1} and NAA 0.1 mg L^{-1} for regeneration of *Poncirus trifoliate* Rad. In order to establish the most suitable conditions for direct and indirect regeneration we tried some of the above reported and some other concentrations and combination of hormones. Maximum shoot regeneration response (70%) from callus was observed on MS medium supplemented with BA 3 mg L^{-1} followed by MS medium supplemented with BA 2 mg L^{-1} + NAA 0.2 mg L^{-1} (54%). In the case of direct shoot regeneration higher shoot regeneration response (83%) was observed on MS medium supplemented with BA 3 mg L^{-1} followed by MS medium supplemented with BA 0.5 mg L^{-1}. COSTA et al. (2002) reported shoot regeneration at BA from 0.5–4 mg L^{-1} with the best at 2 mg L^{-1} for *Citrus paradisi* (Macf) epicotyl explants. TE-CHATO and NUDOUNG, (1998) reported that BA 0.5 mg L^{-1} gave the best results (75%) of shooting response in *Citrus reticulata* Blanco cv Shogun from different explants of *in vitro* raised seedlings. We have obtained better regeneration response than those cited in these reports.

In our study direct shoot regeneration was observed only in stem explants, especially in young stem segments. Very little shoot regeneration was observed in cotyledons, while in leaves and root explants no shoot regeneration was observed at any concentration and combination of hormones. These results are in agreement with the previous reports as in most *Citrus* species epicotyls and stem segments have been used as explants for direct regenera-

tion (PENA et al. 1995a, b, 1997; CERVERA et al. 1998b, 2000; GHORBEL et al. 1999; PENA and NAVARRO 1999; DOMINGUEZ et al. 2000). However, KANEYOSHI et al. (1994) reported adventitious bud formation from epicotyls and root segments of trifoliate orange.

For rooting, the developed shoots cut off segments were cultured on MS medium supplemented with either 0.5 mg L^{-1} NAA or 1 mg L^{-1} 2,4-D. We obtained 70% and 50% rooting results in these two media respectively. Low rooting efficiency has been previously reported as major problem for *in vitro* production of *Citrus* plants (DURAN-VILA et al. 1989). Difficulties in inducing roots have been found in transformation procedures of tree species, like walnut (MC GRANAHAN et al. 1988), apple (JAMES et al. 1989), plum (MANTE et al. 1991) and *Carrizo citrange* (MOORE et al. 1992), and have resulted in relatively low production of regenerated plants. PENA et al. (1995a) used MS medium supplemented with 3 mg L^{-1} NAA for rooting of Sweet orange and got only 3.2%, rooting after 3 months of transfer of shoots to RM. Therefore in their further experiments they used an alternative method of shoot tip grafting for getting their transformed plants regenerated. However, KANEYOSHI et al. (1994), whose results were similar to ours, reported 81.1% rooting on MS medium supplemented with 0.5 mg L^{-1} NAA for trifoliate orange.

In summary, this work represents an ideal protocol for micropropagation of rough lemon. Our results show that up to 92% of stem segments can produce calli on MS with 1.5 mgL^{-1} 2,4-D, which can produce shoots on MS with BA 3 mg L$^{-1.}$ The same medium can be used to produce direct shooting of stem segments up to 83%. Rooting can be induced in up to 70% of these shoots by using MS supplemented with 0.5 mg L^{-1} NAA.

This is the first report on direct and indirect regeneration of rough lemon. Data generated in this study could be practically useful for efficient in vitro propagation of this plant.

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