African Journal of Biotechnology Vol. 10(63), pp. 13724-13728, 17 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1264
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

In vitro plant regeneration in rough lemon (*Citrus jambhiri* Lush) by direct organogenesis

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Accepted 4 May, 2011

This study was aimed to optimize the organogenesis in rough lemon (*Citrus jambhiri* Lush), an important citrus rootstock of India. Organogenesis was induced in epicotyl segments of rough lemon seedlings. Three important factors influencing organogenesis *in vitro* viz. hormone combination, cut modes and photoperiod were evaluated. Etiolation of seedlings had a positive effect on bud formation and higher number of buds per explant was obtained from etiolated seedlings. Among the cut modes, transverse cut performed better for its effect on the number of regenerated buds and shoots per explant. The best response regarding the number of adventitious buds formed per explant was observed with BAP at the concentration of 2.0 mg/l along with malt extract (500 ppm) and additional dose of 25 g/l sucrose.

Key words: Citrus, epicotyl, hypocotyl, etiolation, transverse cut, longitudinal cut, photoperiod.

INTRODUCTION

Improvement of citrus by conventional method is hampered by polyembryony, sexual incompatibility and male or female sterility (Grosser and Gmitter, 2005). Plant improvement via genetic transformation is an alternative to release new cultivars. For developing a routine genetic transformation protocol, an efficient system for *in vitro* regeneration is required. Source of explant, photoperiodic factors, cut modes, hormonal concentrations and additives may affect *in vitro* citrus shoot regeneration. Epicotyl segments excised from seedlings germinated in the dark for 3 - 6 weeks (Luth and Moore, 1999; Yang et al., 2000) and then transferred to a 16 h photoperiod that varied from 1 to 3 weeks (Cervera et al., 1998; Pena et al., 1995) improved the transformation efficiency. For hormones, the effect of auxin on shoot regeneration was rarely concerned, though the main hormone effect on bud formation was due to the addition of BAP (Garcia et al., 1999). Almeida et al. (2003) recorded maximum number of shoots when epicotyl segments were cultured on regeneration EME medium supplemented with 25 g/l additional sucrose and 1 and 2 mg/l BAP for sweet orange and rangpur lime, respectively. Among cut modes, transversal cut, the most popular cut mode (Moore et al., 1992; Pena et al., 2004) is simple to manipulate but produces the fewest adventitious buds. Longitudinal cut, a newly developed but infrequently used cut mode produced the most adventitious buds (Kayim et al., 2004). The goal of this study was to find efficient explant source, cut mode and optimize hormone combination and photoperiod exposure for multiple shoot induction and regeneration of rough lemon.

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Abbreviations: **BAP,** Benzyl amino purine**; NAA**, Napthaline acetic acid; **IBA,** Indoline butyric acid.

MATERIALS AND METHODS

Plant material and explant preparation

Seeds were extracted from ripe fruits of rough lemon. Fruits were collected from a citrus germplasm collection of Punjab Agricultural University, Ludhiana. Seed integuments were removed and disinfestation was done with 1% Mercuric Chloride for 5 min. Three washes in distilled and sterilized water were done before the seeds

were introduced in culture jars containing 50 ml of MS medium (Murashige and Skoog, 1962), supplemented with 25 g l^{-1} sucrose. The seeds were maintained at 27 ± 2°C in the dark for three weeks, followed by one, two or three week under a 16 h photoperiod (40 µmol $m^{-2}s^{-1}$).

Induction of adventitious buds and shoots

The 1 cm long epicotyl explants were cut transversely and longitudinally, hypocotyls were cut only transversely and cotyledonary leaves were cultured as such. Explants were cultured on modified Murashige and Skoog medium along with BAP (1 - 10 mg/l) supplemented with or without 0.1 mg/l NAA, 500 mg/l malt extract and additional dose of 25 g/l sucrose. Cultures were maintained at 27 ± 2°C, under 16-h photoperiod (40 µmol $m^{-2}s^{-1}$) for 45 days and then the cultures were evaluated. The experiment was laid in completely randomized design, with each treatment replicated 10 times. Each replication consisted of 10 culture jars with 5 explants segments. Percentage of explants with adventitious shoots longer than 1.0 cm per explant were evaluated.

Elongation of shoots

Explants with shoot buds were transferred to elongation medium. For shoot elongation, explants were cultured on MS Basal media along with 1 ppm GA. Cultures were kept at 27 ± 1°C under a 16 h photoperiod for 4 weeks. The number of elongated shoots (≥1 cm) was counted after 4 weeks of culturing. Individual shoots were excised from the proliferating basal shoot mass and transferred to rooting medium. The basal mass with shoot primordial was left on the same medium for a second cycle of regeneration (another 4 weeks).

Rooting of shoots

At the final stage of the *in vitro* development, elongated shoots were transferred to different strength MS liquid media fortified with 1.0 mg/l NAA and IBA each for rooting in polypropylene-capped 150 x 25 mm test tubes containing 15 ml of medium. One elongated shoot was placed in each test tube for rooting and there were 10 - 12 tubes from the respective elongation treatment. The cultures were maintained at 27 ± 2°C, under 16-h photoperiod (40 µmol $m^{-2}s^{-1}$).

RESULTS AND DISCUSSION

Induction of adventitious buds/shoots

Effect of photoperiod

After a few days in induction medium, many explants showed swelling at the cutting edges, from which adventitious buds developed after about two weeks in culture. Only part of the buds continued to develop to form shoots. Epicotyl segments (1 cm) from etiolated seedlings transferred to 16 h photoperiod for 2 weeks produced maximum number of explants with buds (88.0%) followed by photoperiod of one week (70.8%) and three weeks (63.8%) on modified Murashige and Skoog medium fortified with 2 ppm BAP, 500 ppm malt extract and additional dose of 25 g/l sucrose (data not given). Epicotyl sections from plants cultivated in fulltime darkness resulted in lower number of explants with buds (40%). Lower bud development efficiency in explants from plants cultivated in fulltime darkness was also observed by Mendes et al. (2002). Regeneration rate in epicotyl segments of Ponkan mandarin was found to increase after dark treatment for 20 days and then transplanting to continuous light conditions by Huang (2005).

Effect of explant and cut mode

Epicotyl segments (Table 1) excised from etiolated seedlings, transferred to 16 h photoperiod for 2 weeks showed the high efficiency (9.55) in bud induction on Murashige and Skoog medium supplemented with 2 ppm BAP, 500 ppm malt extract and additional dose of 25 g/l sucrose as compared to hypocotyls (6.08) and cotyledonary leaves (zero). No significant effect of explant viz., basal, median or apical segments of epicotyl on organogenesis was observed in *Citrus reshni* by Silva et al. (2005).

Longitudinal cut on an average gave higher number of buds and shoots per explant as compared to transverse cut (Figures 1, 2 and 3). Longitudinal cut could increase the wood area of epicotyl explants, resulting in more shoot regeneration than transversal cut (Figures 1 and 3). Among cut modes, Duan et al. (2007) observed that oblique cut performed the best for its effect on the number and quality of regenerated shoots and its convenience to manipulate in sweet orange.

Effect of media

Epicotyl and hypocotyl segments of rough lemon produced adventitious buds in all the media (Table 1) with all levels of BAP (1 - 10 mg/l). Bud formation increased when BA concentration was enhanced up to 2 ppm. However, concentration above 2 ppm suppressed the number of buds per explant. A complex interaction was observed between BA, NAA, malt extract and additional dose of sucrose for adventitious bud induction in epicotyl segments (Table 1). Higher number of buds per epicotyl segment was produced in medium M_8 as compared to M_2, M_{10} and M_{12}, though all the media were supplemented with 2 ppm BA. The mean number of buds reached a maximum of 9.6 per explant, among which 6.6 buds could elongate to shoots on modified Murashige and Skoog medium (M_8) supplemented with 2 ppm BAP, 500 ppm malt extract and additional dose of 25 g/l sucrose. On this medium, adventitious buds were observed on all the epicotyl and hypocotyls segments (Figure 2). The minimum number of buds and shoots were induced on modified Murashige and Skoog medium

Table 1. Effect of different media on shoot regeneration on epicotyl cuttings of rough lemon.

Media		Epicotyl				Hypocotyl	
		Number of bud/ expalnt	Number of shoot/ explant	Explant with bud (%)	Explans with shoot (%)	Number of bud/expalnt	Explant with bud (%)
M_1	MT + 500 ppm ME + 0.1 ppm NAA + 1 ppm BA	5.8	2.3	100.0	53.3	5.2	100.0
M_2	MT + 500 ppm ME + 0.1 ppm NAA + 2 ppm BA	6.9	3.2	100.0	76.7	5.0	69.5
M_3	MT + 500 ppm ME + 0.1 ppm NAA + 3 ppm BA	1.6	0.8	68.3	34.5	1.8	35.3
M_4	MT + 500 ppm ME + 0.1 ppm NAA + 4 ppm BA	1.5	0.5	42.8	17.2	1.5	37.3
M_5	MT + 500 ppm ME + 0.1 ppm NAA + 5 ppm BA	1.9	0.7	41.7	16.1	1.8	37.5
M_6	MT + 500 ppm ME + 0.1 ppm NAA + 10 ppm BA	0.9	0	37.5	0	1.1	32.4
M_7	MT + 500 ppm ME + 1 mg/l BAP + 25 g/l Sucrose	3.3	1.8	56.1	30.8	10.9	100.0
M_8	MT + 500 ppm ME + 2 mg/l BAP + 25 g/l Sucrose	9.6	6.7	100.0	88.4	8.9	100.0
M_9	MT + 500 ppm ME + 0.1 ppmNAA + 1 ppm BA +25 g/l Sucrose	7.8	2.8	88.4	65.6	6.2	90.3
M_{10}	MT + 500 ppm ME + 0.1 ppmNAA + 2 ppm BA +25 g/l Sucrose	5.6	1.8	81.3	58.3	4.6	78.5
M_{11}	MT + 0.1 ppm NAA + 1 ppm BA + 25 g /l sucrose	2.2	0.9	65.2	17.8	7.5	94.6
M_{12}	MT + 0.1 ppm NAA + 2 ppm BA + 25 g /l sucrose	1.2	0	51.3	0	6.2	87.5
	CD at 5%	2.36	1.45	12.37	8.35	0.7	6.5

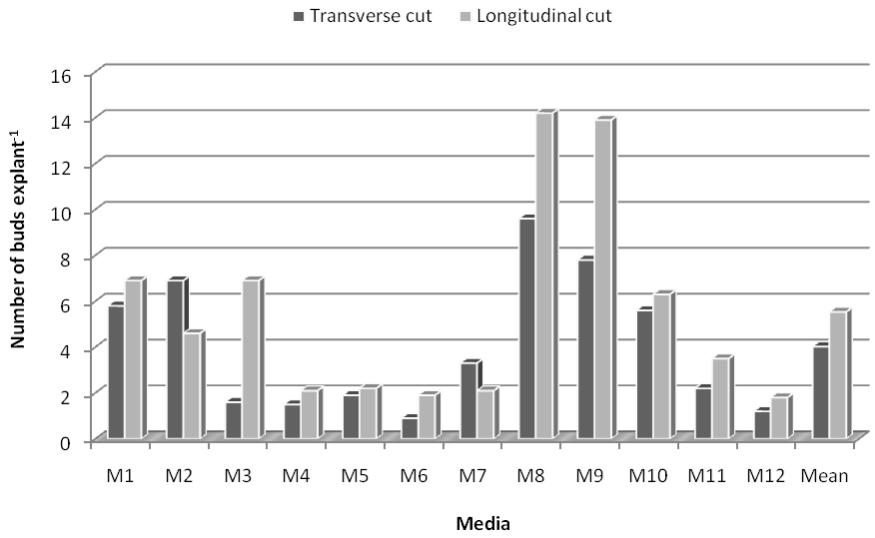

Figure 1. Effect of transverse and longitudinal cuts on number of bus explant^{-1} in epicotyl segments on different media.

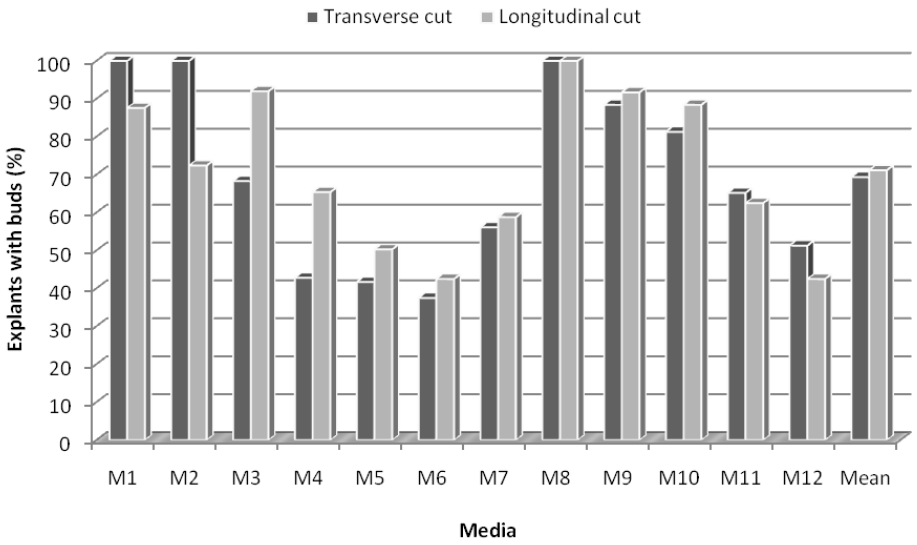

Figure 2. Effect of transverse and longitudinal cuts on percentage of explants producing buds in epicotyl segments on different media.

Figure 3. Adventitious bud formation and rooting of shoots obtained from epicotyl segments of rough lemon. (A) Multiple buds in longitudinally cut epicotyl segments, (B) shoot regeneration in epicotyl segments with longitudinal cut, (C) shoot regeneration in transversely cut epicotyl segments and (D) rooted plantlets.

(M_6) along with 10 ppm BAP, 0.1 ppm NAA and 500 ppm malt extract. *In vitro* organogenesis was found related to BAP concentrations in rangpur lime and sweet orange by Moura et al. (2001) and Almeida et al. (2002).

Shoot elongation

Because of the short size of buds induced, an elongation phase was necessary, except for the shoots regenerated from epicotyls with longitudinal cut on modified Murashige and Skoog medium along with 2 ppm BAP, 500 ppm malt extract and additional dose of 25 g/l sucrose, which reached to the height of ≥ 1 cm without transfer to any elongation media after 4 months of shoot induction and on average 5.1 elongated shoots per explant were obtained. For the rest of the media, 2.3 elongated shoots per explant were obtained on Murashige and Skoog medium supplemented with 1 ppm GA_3.

Root induction

At the final stage of the *in vitro* development, elongated shoots were transferred to different strength MS liquid media fortified with 1.0 mg/l NAA and IBA each for rooting. Rooting was initiated within twelve days after inoculation on the half strength MS and time taken for this medium was significantly lower as compared to other treatments. The maximum rooting (75.00%), number of roots per shoot (3.19 per shoot) and length of longest root (4.08 cm) was observed on half strength MS medium. The lowest rooting and rooting percentage was on the full strength MS medium. Improved rooting in lower strength media was attributed to reduction in nitrogen concentration by Hundman et al. (1982). They found that lowering of concentration of salts in the media to a certain limit lead to more favorable nitrogen concentration for root induction. Rooting of shoots of *Citrus junos* and *C. jambhiri* were also achieved easily on half strength MS medium by Rahman et al. (1996) and Oh et al. (1991), respectively.

Acknowledgment

This research was financially supported by the Department of Biotechnology, New Delhi.

REFERENCES

Almeida WAB, Mourao Filho FAA, Pino LE, Boscariol RL, Rodriguez APM, Mendes BMJ (2003). Genetic transformation and plant recovery from mature tissues of *Citrus sinensis* L. Osbeck. *Plant Sci.* 164: 203-211.

Almeida WAB, Mourao-Filho FAA, Mendes BMJ, Rodrigues APM (2002). *In vitro* organogenesis optimization and plantlet regeneration in *Citrus sinensis* and *C. limonia*. *Agricola*, 59: 35-40.

Cervera M, Pina JA, Juárez J, Navarro L, Peña L (1998). *Agrobacterium*-mediated transformation of citrange: factors affecting transformation and regeneration. Plant Cell Rep. 18: 271-278.

Duan Yn, Liu X, Fan J, Li DL, Wu R, Guo W (2007) Multiple shoot induction from seedling epicotyls and transgenic citrus plant regeneration containing the green fluorescent protein gene. Bot. Stud. 48(2): 165-171.

Garcia LA, Bordon Y, Moreira-Dias JM, Molina RV, Guardiola JL (1999). Explant orientation and polarity determine the morphogenic response of epicotyl segments of Troyer citrange. Ann. Bot. 84: 715-723.

Grosser JW, Gmitter FG Jr (2005). Applications of somatic hybridization and cybridization in crop improvement, with citrus as a model. *In Vitro* Cell Dev. Biol.-Plant 41: 220-225.

Huang J, Wu Z, Sun Z (2005). Several physiological factors influencing adventitious bud regeneration from the epicotyls of ponkan [*Citrus reticulata*]. Plant Physiol. Commun. 41(1): 37-40

Hundman SE, Hasegawa PM, Bressan RA (1982). Stimulation of root initiation from cultured rose shoots through the use of reduced concentrations of mineral salts. Hort. Sci. 17(1): 82-83.

Kayim M, Ceccardi TL, Berretta MJG, Barthe GA, Derrick KS (2004). Introduction of a citrus blightassociated gene into Carrizo citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.] by *Agrobacterium* mediated transformation. Plant Cell Rep. 23: 377-385.

Luth D, Moore GA (1999). Transgenic grapefruit plants obtained by Agrobacterium tumefaciens-mediated transformation. Plant Cell, Tissue Organ Culture, 57: 219-222.

Mendes BMJ, Boscariol RL, Filho FAAM, Almeida WAB (2002). Agrobacterium-mediated genetic transformation of 'Hamlin' sweet orange. *Pesq Agropec Brasileira* 37: 955-961.

Moore GA, Jacono CC, Neidigh JL, Lawrence SD, Kline K (1992). *Agrobacterium*-mediated transformation of citrus stem segments and regeneration of transgenic plants. *Plant Cell Rep.* 11: 238-242.

Moura TL de, Almeida WAB de, Mendes BMJ, Mourao AAFF de (2001). Citrus in vitro organogenesis related to BAP concentrations and explant section. Revista Brasileira de Fruticultura. 23(2): 240-245.

Oh SD, Song WS, Kim JS, Park EH (1991). *In vitro* microprapogation of Yooza (*Citrus junos* Tanaka) I. Plant regeneration from callus induced from shoot tips. J. Kor. Soc. Hort. Sci. 32: 87-96.

Pena L, Cervera M, Juarez J, Ortega C, Pina JA, Duran-Vila N, Navarro L (1995). High efficiency *Agrobacterium*-mediated transformation and regeneration of citrus. Plant Sci. 104: 183–191.

Pena L, Perez RM, Cervera M, Jurez JA, Navarro L (2004). Early events in *Agrobacterium*-mediated genetic transformation of citrus explants. Ann. Bot. 94: 67-74.

Rahman AAS, Nagaraju V, Parthasarthy VA (1996). Response of embryoids of certain citrus species to two cytokinins. Ann. Pl Physiol. 10: 45-49.

Silva RP da, Souza E dos S, Reboucas FS, Almeida WAB de (2005). Optimization of protocols for the in vitro regeneration of plants of mandarin (*Citrus reshni* Hort. ex Tan.) cv. Cleopatra. Revista Brasileira de Fruticultura, 27(3): 484-487.

Yang ZN, Ingelbrecht IL, Louzada ES, Skaria M, Mirkov TE (2000). *Agrobacterium*-mediated transformation of the commercially important grapefruit cultivar Rio Red (*Citrus paradise* Macf.). Plant Cell Reports 19(12): 1203-1211.