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SHORT COMMUNICATION

FACTORS AFFECTING SHOOT REGENERATION IN ROUGH LEMON (*CITRUS JAMBHIRI* LUSH) ROOTSTOCK

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SUMMARY

The aim of this work was to standardize tissue culture parameters for gene transfer in rough lemon rootstock. Epicotyl segments were cultured with different concentrations of 6-benzylaminopurine (BAP), cefotaxime and hygromycin. Epicotyl segments (1-2 cm) from etiolated seedlings transferred to 16 h photoperiod for 2 weeks produced maximum number of adventitious shoot buds on MS medium supplemented with BAP (0.5 mgl^{-1}). Longitudinal cut gave the highest number of buds per explant as compared to transverse cut. Maximum bud induction frequency was obtained on MS + BAP (0.5 mgl^{-1}) medium. The sensitivity of epicotyl segments cultured on regeneration medium MS + BAP (0.5 mgl^{-1}) to antibiotics, cefotaxime and hygromycin was observed at concentration of 400 ppm and 2 mgl^{-1}, respectively. The study can help in developing transgenics of rough lemon rootstock.

***Key words*: Citrus, cefotaxime, hygromycin, micropropagation, rough leaves.**

A prerequisite for successful gene transfer to plants is establishment of a suitable system of *in vitro* regeneration and recovery of whole plants from transformed cells. Regeneration of transgenic *citrus* plants has been achieved by organogenesis and somatic embryogenesis (Costa *et al.* 2002). 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. Several factors such as, the nature of explant, cut modes, hormonal concentrations and antibiotics may affect *in vitro* regeneration. Epicotyl segments excised from seedlings germinated in the dark for 3-6 weeks (Yang *et al.* 2000) and their transfer to 16 h photoperiod for 1-3 weeks improved the transformation efficiency in *Citrus* (Cervera *et al.* 1998). The role of BA in shoot bud formation is well established (Garcia- Luis *et al*. 1999). Among the cut modes, transverse cut, is simple to manipulate but produces very few adventitious buds (Pena *et al.* 2004). Longitudinal cut, a newly developed but infrequently used cut mode, produced the most adventitious buds (Yu *et al.* 2002, Kayim *et al.* 2004).

In any plant, genetic transformation system, availability of a selectable marker is essential to recover a high proportion of transgenic plants from the transformed cells. Genes conferring resistance to selective chemical agents, antibiotics or herbicides, are routinely used as selectable markers. However, in most transformation systems, the generation of a certain number of escapes is expected. The use of alternative selective agents- such as the antibiotic hygromycin (Costa *et al.* 2002) seems to be a promising strategy to circumvent the problem of escapes. The present study was carried out to establish tissue culture parameters as

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a pre-requisite for gene transfer in rough lemon rootstock.

Seeds of rough lemon (*Citrus jambhiri*. L) were peeled (removing the seed integument) and were surface disinfected with 0.1% mercuric chloride for 5 min followed by rinsing thrice with sterile distilled water. Seeds were cultured in jars containing 50 ml of basal medium consisting of Murashige and Skoog (MS) medium supplemented with 30 gl^{-1} sucrose and 7.5 gl^{-1} agar without plant growth regulators (pH 5.7). Cultures were maintained in dark for 4 weeks and then under a 16 h photoperiod (provided by cool white fluorescent light) for one, two or three weeks. The epicotyl explants were cut transversely and longitudinally and cultured on MS medium supplemented with different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mgl^{-1}) with or without, 500 mgl^{-1} malt extract.

In order to determine an appropriate concentration of cefotaxime and hygromycin for selection of transgenic shoots, epicotyl segments were cultured on regeneration medium [MS+ BAP (0.5 mgl^{-1})] containing different concentrations of cefotaxime (0,100, 200, 300 and 400 ppm) and hygromycin (0, 1, 2, 3 and 4 mgl^{-1}), respectively. The regeneration frequency of the segments, mean number of shoots per explant and shoot length was evaluated. Regeneration frequency was calculated as the number of segments producing buds/ shoots per total number of segments cultured.

Twenty cultures in each concentration formed one set. There were three similar replications per treatment. The data were analyzed as completely randomized block design (CRD) as described by Snedecor and Cochran (1967)**.**

Effect of *in vitro* raised seedlings in 16 h photoperiod or complete darkness on bud induction capacity of epicotyl segments was studied (Fig.1). The epicotyl segments (1-2 cm) from etiolated seedlings, transferred to 16 h photoperiod for 2 weeks produced maximum number of explants with adventitious buds (71/ 80), with bud induction frequency of 89 per cent on MS + BAP (0.5 mgl^{-1}) medium. Epicotyl sections from plants cultivated in complete darkness resulted in a slower bud development (32/80) and bud induction frequency (40%). The results are in accordance with Mendes *et al.* (2002) and Almeida *et al.* (2006) who also reported minimum bud development efficiency in explants from plants grown in complete darkness in *Citrus sinensis*.

Fig 1. Effect of seedling cultivation period in 16- h photoperiod or complete darkness on bud induction capacity of epicotyl segments in rough lemon

Epicotyl sections of rough lemon produced adventitious shoots in media (Table 1) with all concentrations of BAP. The increase in BAP concentration beyond 2 mgl^{-1} decreased the shoot regeneration capacity. Irrespective of cut mode, maximum bud induction frequency (95.09 %) was obtained in epicotyl segments of rough lemon on MS + BAP (0.5 mgl^{-1}) while minimum bud induction frequency (35.5 %) was obtained on MS + BAP (4.0 mgl^{-1}). Mendes *et al.* (2002) also reported higher percentage of transformation efficiency with BAP (0.5-1.0 mgl^{-1}).

Longitudinal cut gave the highest number of buds (14.15) and shoots (7.0) per explant as compared to transverse cut (Table 1). Longitudinal cut increased the wound area of epicotyl explants, resulting in more shoot regeneration as compared to the transverse cut (Fig 2a, 2b). In *Citrus*, longitudinal cut gave the highest number of buds per explant followed by oblique cut and transverse cut (Duan *et al.* 2007). Similar findings have been reported by Yu *et al.* (2002) and Kayim *et al*. (2004) in *Citrus*.

Table 1. Effect of transverse and longitudinal cuts on adventitious bud induction capacity of epicotyl segments on different media.

Media	Average no. of buds		Bud induction frequency (%)	
	Transverse cut	Longitudinal cut	Transverse cut	Longitudinal cut
MS+ BA 0.5 mgl^{-1}	9.55	14.15	95.09 (88.05)*	88.64 (70.58)
MS+ BA 1.0 mgl^{-1}	5.9	6.90	88.04 (70.33)	70 (56.97)
MS+ BA 1.5 mgl^{-1}	5.65	6.25	81.25 (65.08)	33.33 (35.40)
MS+ BA 2.0 mgl^{-1}	1.6	4.6	58.33 (50.00)	75 (60.29)
MS+ BA 2.5 mgl^{-1}	1.45	2.19	42.86 (40.81)	65.0 (53.74)
MS+ BA 3.0 mgl^{-1}	1.31	1.75	41.67 (40.29)	50.0 (44.98)
MS+ BA 3.5 mgl^{-1}	0.95	2.04	37.50 (37.44)	37.5 (37.73)
MS+ BA 4.0 mgl^{-1}	0.95	1.85	35.50 (36.74)	37.5 (37.73)
MS+ BA 0.5 mgl^{-1}+ME 500 mgl^{-1}	7.75	13.90	89.00 (72.05)	91.67 (73.27)
MS+ BA 1.0 mgl^{-1}+ME 500 mgl^{-1}	5.8	6.97	78.00 (62.05)	70 (56.97)
MS+ BA 2.0 mgl^{-1}+ME 500 mgl^{-1}	2.15	3.45	75.00 (64.97)	62.5 (52.58)
MS+ BA 3.0 mgl^{-1}+ME 500 mgl^{-1}	1.00	1.88	50.00 (44.98)	42.5 (40.74)
CD (5%)	A (Cut) - 0.54 B (Media) - 1.31 A x B - 1.86		A (Cut) - 3.46 B (Media) - 8.47 A x B - 11.97	

* Figures in parentheses are the transformed values.

The effectiveness of antibiotic cefotaxime on shoot regeneration was also investigated (Table 2). Maximum shoot regeneration was observed under control (98.26 %), followed by 100 ppm (90.04 %) and 200 ppm (59.56 %) cefotaxime. The LD_{50} value was observed at 400

Table 2. Effect of different concentrations of antibiotic cefotaxime on per cent shoot regeneration and shoot length.

Antibiotic cefotaxime conc (ppm)	Shoot regeneration (%)	Shoot length (cm)	
		Mean	Range
Control	98.26	1.62	1.0-2.6
100	90.04	2.84	1.8-4.6
200	59.56	3.47	2.0-5.0
300	56.89	2.60	1.0-4.0
400	50.00	1.53	0.5-2.0
CD (5%)	**12.62**	**0.18**	**-**

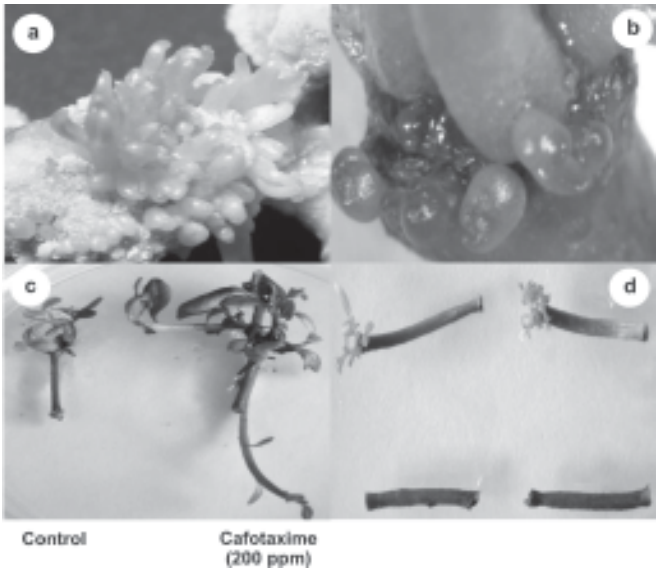

Fig 2. (a) Multiple buds in longitudinal cut epicotyl segments, (b) Bud induction at cut ends in transverse cut, (c) Increase in shoot length with cefotaxime (200 ppm) as compared to control and (d) LD_{50} value obtained with hygromycin 2 mgl^{-1}

ppm. The percent shoot regeneration at this value gets halved as compared to control. The percent shoot regeneration decreased with increase in concentration of cefotaxime. Maximum shoot length was recorded with 200 ppm cefotaxime (3.47 cm), followed by 300 ppm (Table 2, Fig 2c).

The shoot length increased with the increase in concentration of cefotaxime. Cefotaxime act indirectly, suppressing endogenous bacterial infection. This might have resulted in the reduction or removal of toxic microbial compounds from the medium or reduction of competition for nutrients (Valobra- Piagnani and James 1990). Mathias and Boyed (1986) suggested that cefotaxime acts as a plant growth regulator.

The percent shoot regeneration decreased with increase in the concentration of hygromycin as compared to control. In epicotyl explants of rough lemon, a concentration of 5 mgl^{-1} or higher completely inhibited shoot formation. The LD_{50} value was observed at 2 mgl^{-1} (Table 3, Fig 2d).

Table 3. Effect of different concentrations of antibiotic hygromycin on per cent shoot regeneration and number of shoots per culture.

Antibiotic hygromycin conc (mgl^{-1})	Shoot regeneration (%)	Number of shoots per culture
Control	94.66 (79.36)*	8.16
1	55.83 (48.35)	2.25
2	51.00 (45.55)	1.80
3	41.66 (39.98)	1.68
4	20.83 (26.89)	1.30
CD (5%)	**12.18**	**0.91**

* Figures in parentheses are the transformed values.

The number of shoots per culture also decreased with increase in concentration of hygromycin as compared to control. Maximum number of shoots were observed under control (8.16), followed by 1 mgl^{-1} (2.25) and 2 mgl^{-1} (1.80) of hygromycin (Table 3). Similar results were reported by Costa *et al.* (2002) and Mendes *et al.* (2008) using hygromycin in *Citrus*.

Rough lemon rootstock had slightly different requirements for optimal *in vitro* shoot regeneration. Complete bud and shoot inhibition occurred in presence of antibiotics**.**

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