



## ***In vitro* plant regeneration in rough lemon (*Citrus jambhiri* Lush.) through epicotyl segments by direct shoot organogenesis**

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**Abstract:** The effect of Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of growth hormones on direct regeneration from one month old epicotyl segments of *in vitro* grown rough lemon (*Citrus jambhiri* Lush.) seedlings was studied. The earliest bud induction in 7.5 days, highest bud induction frequency (98.50%), percent regeneration (90.53) were obtained on MS medium supplemented with 6-Benzylaminopurine (BAP) ( $1\text{mg lit}^{-1}$ ) with an average number of 12.50 buds per explants. The epicotyl segments with proliferated buds were transferred to elongation media in order to improve the recovery of normal shoots. Maximum number of elongated shoots (8.50) was obtained on MS medium having BAP ( $0.5\text{mg lit}^{-1}$ ) + Gibberellic Acid ( $\text{GA}_3$ ) ( $1.0\text{mg lit}^{-1}$ ). These elongated shoots were then rooted on MS medium containing Indole-3-butyric acid (IBA) ( $0.1\text{mg lit}^{-1}$ ) + Indole-3-acetic acid (IAA) ( $0.5\text{mg lit}^{-1}$ ) with highest rooting percentage (96%) and root number (5.0). Early (10.10 days) rooting was observed in MS medium supplemented with NAA  $1.0\text{mg lit}^{-1}$  + IBA  $0.5\text{mg lit}^{-1}$ . The plantlet survival was 98.52%, when plantlets were transferred to plastic pots containing a mixture of garden soil and vermiculite (1:1). The hardened plants were successfully established in the soil. The present study developed protocol which can be reliably used for *in vitro* regeneration of rough lemon and for gene transfer studies in rough lemon, especially to induce salinity and *Phytophthora* tolerance.

**Keywords:** *Citrus jambhiri* Lush., Direct regeneration, Epicotyl segments, *In vitro*, Rooting, Rough lemon

### **INTRODUCTION**

Citrus fruits are one of the most important fruit crop of the world planted in tropical and subtropical regions. 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 (Chaturvedi *et al.*, 2001) that can be used for the production of virus free rootstock plants through micropropagation (Roistacher *et al.*, 1976). Advances in biotechnology have generated new opportunities for citrus genetic improvement. Improvement of citrus by conventional method is hampered by polyembryony, sexual incompatibility and male or female sterility (Guo and Deng, 2001; Grosser and Gmitter, 2005). However, the occurrence of polyembryony in citrus species hinders the production of uniform rootstock population. There is also an urgent need to propagate disease-free planting material for the sustenance of citrus industry, particularly in the northern regions of the country. *In vitro* multiplication is a techno-economically viable and eco-friendly approach to produce disease free planting material on a large scale, utilizing relatively small space and time (Thorpe and Harry, 1997). Although several citrus rootstocks have been propagated through tissue culture (Hiregoudar *et*

*al.*, 2005), very few reports are available on *in vitro* multiplication of *Citrus jambhiri* (Chaturvedi *et al.*, 2001). The success rate of callus regeneration in *Citrus jambhiri* is very low (Raman *et al.*, 1992). Moreover, regeneration through callus is not a reliable approach to produce planting material. On the other hand, higher success rate with more number of shoots/explants can be achieved via direct shoot regeneration and this process possesses less probability of somaclonal variation among regenerants in comparison to callus mediated regeneration system is also a necessary pre-requisite for genetic improvement and genetic resource conservation. Genetic transformation is an alternative to overcome these difficulties. For successful transformation, regeneration of whole plants from the transformed cells is a prerequisite. For hormone, the effect of auxin on shoot regeneration was rarely concerned, though the main hormone effect on bud formation was due to the addition of Benzyl adenine (Garcia-Luis *et al.*, 1999). *In vitro* propagation has therefore been a great potential tool to overcome problems related with the field culture for such species (Hidaka and Omara, 1989). Genotype affects the morphogenic response of *in vitro* grown epicotyl segments of citrus rootstocks. The qualitative and quantitative differences in the organogenic response indicated that the conditions for regeneration must be optimized for each genotype

(Bordon *et al.*, 2000). Tissue culture of rough lemon has also been studied by Raman *et al.* (1992); Altaf and Ahmad (1997); Ali and Mirza (2006); Saini *et al.* (2010); Rattanpal *et al.* (2011). Furthermore, an efficient system of regeneration through organogenesis is the step for genetic transformation of these species (Gmitter *et al.*, 1992). This study was conducted with aim to explore regenerative ability via direct regeneration, appropriate media and epicotyl segments as explants of rough lemon (*Citrus jambhiri* Lush). The significance of the present study to develop the protocol which can be used successfully for the plant micropropagation and genetic transformation of rough lemon (*Citrus jambhiri* Lush).

## MATERIALS AND METHODS

**Plant material and explant preparation:** The experimental material consisted of seeds extracted from ripened fruits of citrus rootstock rough lemon (*Citrus jambhiri* Lush.) collected from the Citrus germplasm collection block of New Orchard of Department of Fruit Science, Punjab Agricultural University, Ludhiana during 2009-10 and 2010-11. Fresh fruit taken from field grown trees were washed with water containing 1-2 drops of teepol and then washed thoroughly with running tap water. The seeds extracted from fruits were removed of testa (outer covering) and then surface sterilized under aseptic conditions with mercuric chloride 0.1% (w/v) for 4-5 minutes. The seeds were thoroughly washed with sterilized distilled water thrice before inoculation to remove the toxic effects of mercuric chloride. After sterilization, seeds were sown in Murashige and Skoog (1962) basal medium in culture jars. The cultured seeds were incubated at  $25 \pm 2^\circ\text{C}$  temperature in dark for two weeks for etiolation. After one week of sowing, seed germination started. After 4-5 weeks, these culture were shifted to light for 16 hours continuous fluorescent white light (2000lux) followed by a dark period of 8 hours.

**In vitro direct shoot regeneration/proliferation:** Epicotyl segments (1-2cm) from one month old seedlings were cultured on different direct shoot regeneration medias were incubated at  $25 \pm 2^\circ\text{C}$  temperature for 16 hours continuous florescent white light (2000 lux) followed by a dark period of 8 hours to produce maximum number of adventitious shoot buds and to know the efficiency of regeneration with different media in petri dishes. Explants with proliferated shoot buds, formed under optimal condition, were transferred to elongation medium. The number of elongated shoots (>1cm and >2cm) was counted after four weeks of culture.

**In vitro root induction and acclimatization:** Elongated shoots were placed vertically in 25x150mm culture tubes containing 25ml of different root induction medium. The percentage of shoots rooted, days to root, an average number of roots/shoot and root length were

recorded after four weeks of culture. The *in vitro* rooted plantlets were removed from the culture tubes, thoroughly washed under running tap water and placed into plastic pots containing different potting mixture of garden soil, sand and vermiculite (1:1). Initial watering was done using 0.1% Bavistin solution to reduce the mortality of plants due to fungal pathogens. The percent survival of plantlets in different potting mixtures was recorded.

**Statistical analysis:** The experimental data was analysed with Completely Randomized Block Design as described by Singh *et al.* (1998).

## RESULTS AND DISCUSSION

### Direct shoot regeneration from epicotyl segments:

The response of epicotyl segments for direct regeneration was assessed on different media containing MS fortified with variable concentrations of BAP and 1-Nephtalene acetic acid (NAA) (Table 1). The significant differences among the culture media were recorded. The number of days taken to regeneration (7.50), percent regeneration (90.53), bud induction frequency (98.50%) and average number of buds (12.50) were significantly higher on MS medium supplemented with BAP  $1\text{mg lit}^{-1}$  followed by MS medium fortified with BAP  $0.5\text{mg lit}^{-1}$ . The lowest bud induction frequency (50.00%), average number of buds (2.15), shoot regeneration response (25.50%) was observed on MS medium supplemented with BAP  $1\text{mg lit}^{-1}$  + NAA  $0.1\text{mg lit}^{-1}$  with more (14.82) number of days taken for regeneration (Table 1). These results find support from the previous workers like Costa *et al.* (2002) reported shoot regeneration at Benzyl adenine (BA) from 0.5-4.0  $\text{mg lit}^{-1}$  with the best at 2  $\text{mg lit}^{-1}$  for *Citrus paradisi* Macf. epicotyl explants. Te-Chato and Nudong (1998) reported that BA  $0.5\text{mg lit}^{-1}$  gave the best results (75%) of shooting response in *Citrus reticulata* Blanco cultivar Shogun from different explants of *invitro* raised seedlings. The results of the present study are in agreement with the previous reports as in most citrus species epicotyls and stem segments have been used as explants for direct regeneration with MS medium supplemented with BA 3  $\text{mg lit}^{-1}$  or BA 1  $\text{mg lit}^{-1}$  (Ghorbel *et al.*, 1999; Pena and Navarro, 1999; Dominguez *et al.*, 2000; Cervera *et al.*, 2000). However, Kaneyoshi *et al.* (1994) reported adventitious bud formation using MS medium supplemented with BA 5  $\text{mg lit}^{-1}$  and NAA  $0.1\text{mg lit}^{-1}$  in *Poncirus trifoliata* Rad. But, Ali and Mirza (2006) reported the maximum shoot regeneration with MS medium supplemented with BA 3  $\text{mg lit}^{-1}$  from stem and epicotyl segments of rough lemon. Dejam *et al.* (2006) also reported that the highest percentage of explants with the adventitious buds was obtained in concentration of MS + 2  $\text{mg lit}^{-1}$  of BA. Nwe *et al.* (2014) reported that cotyledonary node explants cultured on Murashige and Skoog (MS) medium supplemented with 8.88  $\mu\text{M}$  N6-benzyladenine (BA) and 0.54  $\mu\text{M}$   $\alpha$ -naphthaleneacetic

**Table 1.** Effect of different media on the bud induction and percent regeneration of epicotyl segments of rough lemon (*C. jambhiri* Lush.) .

Media	Days to bud induction	Bud induction frequency (%)	Percent re-generation	Average number of adventitious buds
MS+BAP (1mg lit <sup>-1</sup> )	7.50	98.50	90.53	12.50
MS+BAP (0.5mg lit <sup>-1</sup> )	9.00	88.05	80.50	9.76
MS+BAP (3mg lit <sup>-1</sup> ) + NAA(0.1mg lit <sup>-1</sup> )	9.50	85.12	75.23	8.00
MS+BAP (3mg lit <sup>-1</sup> )+NAA(0.2mg lit <sup>-1</sup> )	10.80	82.00	70.51	7.50
MS+BAP (2mg lit <sup>-1</sup> )+NAA(0.1mg lit <sup>-1</sup> )	11.51	80.25	65.33	5.00
MS+BAP (2mg lit <sup>-1</sup> )+NAA(0.2mg lit <sup>-1</sup> )	12.03	75.00	60.25	4.00
MS+BAP (1mg lit <sup>-1</sup> )+NAA(0.2mg lit <sup>-1</sup> )	13.82	60.50	50.00	3.25
MS+BAP (1mg lit <sup>-1</sup> )+NAA(0.1mg lit <sup>-1</sup> )	14.82	50.00	25.50	2.15
CD (5%)	1.65	2.86	2.83	1.42

**Table 2.** Effect of different media on bud elongation in rough lemon (*C. jambhiri* Lush.) .

Media	Percent Bud elongation		Average number of shoots
	(>1cm)	(>2cm)	
MS+GA <sub>3</sub> (1.0mg lit <sup>-1</sup> )	22.25	6.50	3.24
MS+BAP(0.5 mg lit <sup>-1</sup> ) +GA <sub>3</sub> (1.0 mg lit <sup>-1</sup> )	40.20	18.45	8.50
MS+ BAP(2.0 mg lit <sup>-1</sup> ) +GA <sub>3</sub> (1.0 mg lit <sup>-1</sup> )	31.50	10.35	6.50
MS+ BAP(1.0 mg lit <sup>-1</sup> )( Control)	11.35	1.00	2.00
CD (5%)	3.67	2.21	1.18

acid (NAA) developed more than five shoots per explant. The best results were obtained with 1 mg lit<sup>-1</sup> BAP for bud induction (Almeida *et al.*, 2002). It has been possible to obtain the adventitious shoot formation (caulogenesis) by the cultivation of epicotyl and internodal segments in culture mediums containing BAP in combination with NAA (Bordon *et al.*, 2000; Moreira-Dias *et al.*, 2000, 2001) or BAP alone (Moura *et al.*, 2001; Almeida *et al.*, 2002; Silva *et al.*, 2005). Rezadost *et al.* (2013) reported that the highest percentage of responsive explants (90%) obtained by using 2.5 mg lit<sup>-1</sup> BAP in combination with the 0.05 mg lit<sup>-1</sup> NAA which had 2 days pre-culture period of epicotyls for allowing to grow in the absolute darkness for 4 weeks, followed by 10 days in 16 hours photoperiod. The highest number of well-developed shoots was 4.2 shoots per explant and obtained with medium containing 0.5 mg lit<sup>-1</sup> GA<sub>3</sub>. Silva *et al.* (2010) by culturing *Citrus aurantium* epicotyl segments in MT (Murashige and Tucker, 1969) medium containing 1mg lit<sup>-1</sup> BAP and 0.3mg lit<sup>-1</sup> NAA were able to achieve 80.9% regeneration efficiency from which 3.1 buds per explants attained. The constituents of culture medium play an important role in the process of tissue differentiation. The beneficial effects of cytokinins has been reported in the induction of shoot bud primordia in different crop plants (Lesham, 1973), which may be due to more synthesis of nucleic acids and proteins required for organogenesis (Torres, 1988). The cytokinins are generally added to induce shoot formation and to inhibit root formation.

**Shoot elongation:** The adventitious buds induced on MS medium supplemented with BAP (1mg lit<sup>-1</sup>) were further sub-cultured on different elongation media. Because of the short size of buds induced, an elongation phase was necessary. It was noted that MS+BAP (0.5mg lit<sup>-1</sup>) + GA<sub>3</sub>(1.0mg lit<sup>-1</sup>) medium gave maximum (40.20) shoot elongation (>1cm) followed by

MS+BAP(2.0mg lit<sup>-1</sup>)+GA<sub>3</sub>(1.0mg lit<sup>-1</sup>)(31.50%) (Table 2). Similarly maximum shoot elongation (18.45%) of >2cm in length was observed in MS + BAP(0.5mg lit<sup>-1</sup>) + GA<sub>3</sub>(1.0mg lit<sup>-1</sup>) medium followed by MS+ BAP (2.0mg lit<sup>-1</sup>) + GA<sub>3</sub>(1.0mg lit<sup>-1</sup>)(10.35%). Minimum shoot elongation was observed in control (MS+ BAP(1.0mg lit<sup>-1</sup>)(Table 2). Similar results were noted by Saini *et al.* (2010) in that the shoot elongation percentage was maximum in MS medium fortified with BAP 0.5 mg lit<sup>-1</sup> and GA<sub>3</sub> 1.0 mg lit<sup>-1</sup>. The number of elongated shoots was improved with the addition of BA alone or with GA<sub>3</sub> to the elongation medium. In the present study, possibly lower concentration of BA continued to stimulate shoot initiation, while reducing the dominance of newly formed buds, whereas GA<sub>3</sub> promoted the elongation of shoot buds initiated on the induction medium. Omura and Hidaka (1992) also observed that the addition of cytokinin and GA<sub>3</sub> was found necessary for successful shoot tip culture of citrus.

**Rooting of regenerated shoots:** The root induction from *in vitro* produced shoots of rough lemon started with in 10-20 days on various modification of MS medium. About 2-3 cm lengthy *in vitro* regenerated shoots were separated out from epicotyls segments and transferred to the different rooting media. Among the seven media evaluated for rooting response, the percent rooting was maximum (96.00) on MS fortified with IBA 0.1 mg lit<sup>-1</sup> and IAA 0.5 mg lit<sup>-1</sup>, where as it was minimum (65.50) on MS fortified with NAA 1.0 mg lit<sup>-1</sup> and IBA 0.5 mg lit<sup>-1</sup> (Table 3). Rooting was significantly earlier (10 days) on MS fortified with NAA 1.0 mg lit<sup>-1</sup> and IBA 0.5 mg lit<sup>-1</sup>, where as it was delayed on rest of media with maximum duration (20.52 days) on MS supplemented with IBA 1.0 mg lit<sup>-1</sup>. Number of roots were significantly higher in MS medium fortified with IBA 0.1mg lit<sup>-1</sup> and IAA 0.5

**Table 3.** Effect of different media on rooting response of direct regenerated shoots from epicotyl segments of rough lemon (*C. jambhiri* Lush.).

Media	Rooting percentage	Days to rooting	Number of roots	Root length(cm)
MS+IBA(0.1mg $lit^{-1}$ )+IAA(0.5 mg $lit^{-1}$ )	96.00	14.11	5.00	9.96
MS+IBA(0.5mg $lit^{-1}$ )+IAA(0.5 mg $lit^{-1}$ )	90.00	17.00	4.50	10.00
MS+IBA(1.0 mg $lit^{-1}$ )	84.50	20.52	2.31	9.91
MS+NAA(0.5mg $lit^{-1}$ )	80.52	15.50	3.28	10.00
MS+IBA(0.5mg $lit^{-1}$ )+IAA(0.1 mg $lit^{-1}$ )	78.21	13.00	2.00	9.92
MS+NAA(1.0mg $lit^{-1}$ )+IBA(1.0mg $lit^{-1}$ )	70.58	12.51	1.82	9.90
MS+NAA(1.0mg $lit^{-1}$ )+IBA(0.5mg $lit^{-1}$ )	65.50	10.10	1.52	10.00
CD (5%)	3.03	2.03	1.19	NS

**Table 4.** Effect of different potting mixtures on the percent survival of the plantlets of rough lemon (*C. jambhiri* Lush.).

Potting Mixtures	Percent survival
Garden soil	60.36
Garden soil + sand(1:1)	75.5
Garden soil + sand(1:2)	70.20
Garden soil + sand(2:1)	65.75
Garden soil + vermiculite(1:1)	98.52
Garden soil + vermiculite (1:2)	88.25
Garden soil + vermiculite (2:1)	92.45
CD (5%)	3.14

mg $lit^{-1}$ , where as root length was found to be non-significant irrespective of media (Table3)

Similarly, Raman *et al.* (1992) also reported the rooting of the excised shoots derived from stem derived callus of rough lemon with the addition of NAA 1 mg $lit^{-1}$  and sucrose (2%) to the 1/2 MS medium. In this study, rooting of high frequency was achieved even in 1/2 MS medium devoid of growth regulators. This can be explained on the basis of growth regulators composition of shoot regeneration medium. In this study, the shoot differentiation was achieved in medium containing NAA 0.5 mg $lit^{-1}$ , BA 3.0 mg $lit^{-1}$  and kinetin 0.5 mg $lit^{-1}$ . Thus, the shoots were rich in endogenous auxin content and when transferred to the 1/2 MS medium, rooting was induced.

Previous workers also reported the supplementation of MS media with IBA alone (Oh *et al.*, 1991) or in combination (Chakravarty and Gosami, 1999) and NAA alone (Das *et al.*, 2000) has induced roots in the shoots of different citrus species. Similarly, Parkash (2003) observed the earliest and maximum rooting in *Citrus jambhiri* Lush. on MS supplemented with NAA 1.0 mg  $lit^{-1}$  and IBA 1.0 mg  $lit^{-1}$ . Auxins play an important role in rhizogenesis, which include division of meristematic cells, their elongation and differentiation into root primordial (Omura and Hidaka, 1992). The number of roots produced per shoot, root length and thickness of roots varied with the concentration and combination of auxins used in the medium. Many workers also reported the maximum rooting response on MS+NAA(1.5-2mg $lit^{-1}$ ) in *Pectinifera* rootstock (Gill and Gosal, 2002) and *Citrus sinensis* cv. Mosambi (Rashad *et al.*, 2005).

**Acclimatization:** The rooted plantlets were first hardened *in vitro* for 5-6 days in the incubation room and kept over moist cotton soaked in half MS salts. The plantlets in MS solution exhibited fresh root growth. This process of

hardening acclimatized the plantlets for their subsequent transfer to soil. After hardening, the plantlets were transferred to different potting mixtures in plastic pots, in which they exhibited varied survival rates (Table 4). The maximum plantlet survival (98.52 %) was observed in garden soil + vermiculite (1:1) potting mixture. Similar results were noted by Saini *et al.* (2010) that the maximum plantlet survival was noted in garden soil + vermiculite (1:1) potting mixture. In this experiment, the rooted plantlets grown in all the potting mixtures exhibited low mortality of the plantlets because vermiculite had high water holding capacity.

## Conclusion

The present study concluded that the maximum (90.53%) and earliest (7.5 days) shoot regeneration response was in epicotyls segments cultured on MS medium supplemented with BAP 1 mg $lit^{-1}$ . Early (10.10 days) rooting was observed in MS medium supplemented with NAA 1.0 mg $lit^{-1}$  + IBA 0.5 mg $lit^{-1}$  and the highest (96%) rooting percentage was noted in MS medium supplemented with IBA 0.1 mg  $lit^{-1}$  + IAA 0.5 mg $lit^{-1}$ . The present work defined organogenesis and plant regeneration protocols for rough lemon (*Citrus jambhiri* Lush) which can be used in plant transformation experiments.

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