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Rapid Protocol for *in Vitro* Multiplication of *Citrus limonia* Osbeck Rootstock

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ABSTRACT

Rangpur lime (*Citrus limonia* Osbeck) is the most used rootstock in the world and in India, particularly in the state of Andhra Pradesh on account of its superior performance over other rootstocks. However, plants produced from seedlings are not used in orchards due to variations caused by polyembryony. To overcome such variation, *in vitro* propagation of Rangpur lime was performed using nodal segments and shoot tips as explants. After surface sterilization, the explants were inoculated on Murashige and Skoog (MS) medium supplemented with 3% sucrose, 100 mg/L benomyl and 250 mg/L streptomycin. The most effective concentrations of auxin (α-naphthalene acetic acid; NAA), cytokinin (6-benzyl adenine; BA) and gibberellic acid (GA_3) for *in vitro* shoot induction and multiplication in Rangpur lime rootstock were determined. Almost all NAA and BA treatments resulted in almost 100% shoot induction except for at 0.0 and 0.1 mg/L NAA and at 1.5 and 2 mg/L BA. Nodal segments induced a higher percentage of explant response with longer shoots in a shorter period of time than shoot tips, which produced more shoots and leaves than nodal segments. The effect of different BA and NAA concentrations on various parameters of proliferation were studied. Full-strength MS medium produced more regenerated shoots and leaves per shoot than half-strength MS medium. In addition, longer shoots formed with 0.1 mg/L GA_3 than culture medium without this plant growth regulator.

Keywords: BA, NAA, nodal segment, shoot induction, shoot tip
Abbreviations: BA, 6-benzyl adenine; **NAA**, α-naphthalene acetic acid; **GA_3**, gibberellic acid; **LAF**, laminar air flow; **MS**, Murashige and Skoog

INTRODUCTION

Rangpur lime is native to the north eastern parts of the Indian subcontinent and became prominent in recent years as an important rootstock for sweet orange and mandarins. It is also a promising fruit for commercial utilization in the processing industry (Raju 1978; Singh and Singh 2001). It is a high-yielding natural hybrid exhibiting the characteristics of lemon and mandarins. Rangpur lime also grows well in loam and clay loam soils and Rangpur lime trees have performed well in areas where rough lemon is short-lived; Rangpur lime is highly salt- and lime-tolerant (Sonkar 2001). The trees are large and vigorous, tolerant to *tristeza*, high salt concentration and foot-rot, and adapt well to a wide range of soils (Randhawa and Srivastava 1986; Sonkar 2001; Davies and Albrigo 2003). Rangpur lime is thus considered a promising rootstock for oranges, grapefruits and mandarins (Batchelor and Webber 1948; Davies and Albrigo 2003). In addition, it is the most cultivated rootstock in southern Brazil, accounting for 80% of citrus produced, owing to its adequate performance under moderate water conditions, induction of early-bearing, adequate yield and compatibility with most scion cultivars (Filho *et al.* 2007). Citrus trees are declining on a large-scale due to diseases like *tristeza*, viruses, canker and various other factors; for example, more than 823 different insect species attached *Citrus* spp. which resulted in the spread of diseases (Shivankar and Rao 2001) causing a 69.67, 57.59 and 50.25% decline in mandarin cvs 'Honey' and 'Dancy' and sweet orange cv 'Jafa', respectively (Singh and Huchche 2001). For a viable and productive citrus industry, a disease-free foundation stock is necessary. At present, Rangpur lime rootstock propagated by seed shows wide variability in the performance of scion varieties. Furthermore, the seeds are recalcitrant and rapidly lose viability and germination ability, which are dependent on many factors such as moisture content, storage temperature, etc. (Barman *et al.* 2006). The seeds cannot be stored for a long time as the viability decreases rapidly upon storage i.e. 13.3 and 0% after 30 and 60 days of seeds extraction, respectively (Barman *et al.* 2006). These problems can be overcome by propagating Rangpur lime rootstock vegetatively. In addition, there is an urgent need to produce disease-free planting material of Rangpur lime rootstock for the budding of cv 'Sathgudi' sweet orange in India, particularly in the state of Andhra Pradesh.

In vitro regeneration of citrus plants was successfully achieved by several groups using tissue culture, specifically direct organogenesis in which the plants produced were true-to-type, a desired characteristic in the commercial production of citrus or other fruit crops. Al-Khayri and Al-Bahrany (2001) reported maximum multiple shoot formation when nodal explants of a mature tree (*Citrus aurantifolia* cv 'Binzahir') were cultured in Murashige and Skoog (MS) (1962) medium containing 1 mg/L 6-benzyl adenine (BA) and 0.5 mg/L kinetin. Cervera *et al.* (2008) reported that *in vitro* organogenesis of 'Clementine' mandarin (*Citrus clementina* hort. ex Tanaka) was possible only when shoots of the first flush were used as the explant source. Other achievements were also reported for citrus regenera-

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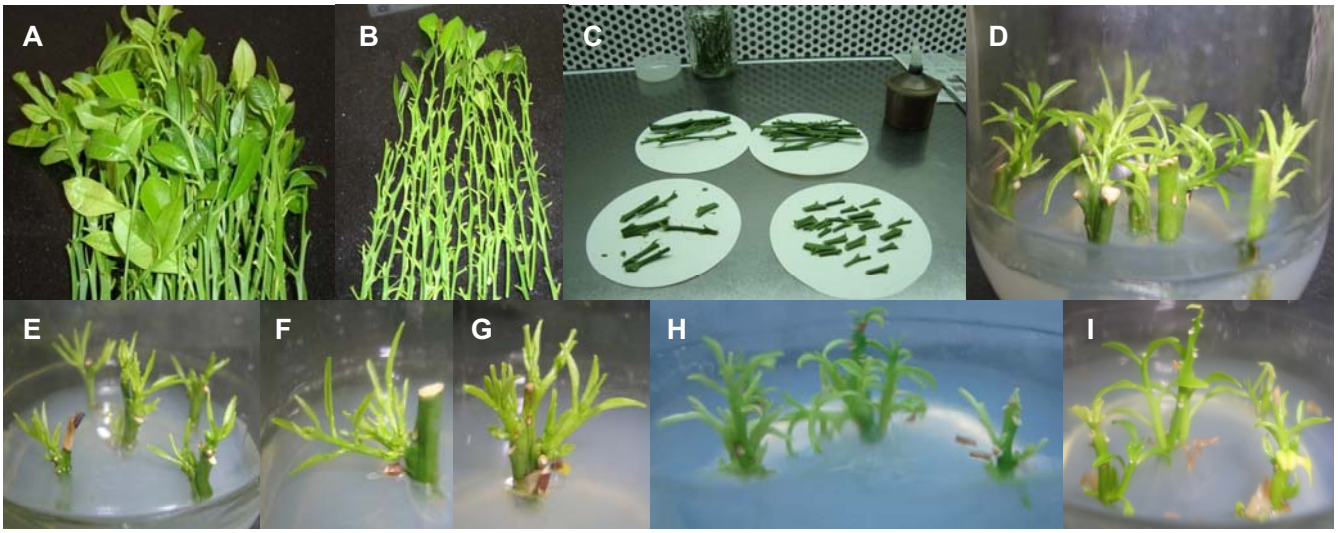

Fig. 1 ***In vitro*** **proliferation and multiplication in** ***Citrus limonia*** **Osbeck rootstock.** (**A**) 40-60 days-old shoots with shoot tips and nodal segments that served as explants; (**B**) Removal of thorns and leaves in the laboratory; (**C**) Preparation of nodal segments under LAF; (**D**) Rapid induction of shoots from nodal segments with 0.5 mg/L BA, 4 weeks after culture on full-strength MS medium; (**E**) Rapid induction of shoots from shoot tips with 0.5 mg/L BA, 4 weeks after culture on full-strength MS medium; (**F**) Multiple shoot induction from nodal segments with 0.5 or 1.0 mg/L BA; (**G**) Multiple shoot induction from shoot tips with 0.5 or 1.0 mg/L BA; (**H**) Shoot multiplication from nodal segments with 0.1 mg/L GA_3 on full-strength MS medium; (**I**) Shoot elongation on half-strength MS medium containing 0.1 mg/L GA_3.

tion on MS medium for *Citrus* spp. using either nodal segments (Desai *et al*. 1996; El-Wasel 2001; Kamble *et al.* 2002; Begum *et al.* 2004; Mukhtar *et al.* 2005; Usman *et al.* 2005; Kiran *et al.* 2008; Murkute *et al.* 2008, 2009; Rastgoo *et al.* 2009; Sen and Dhawan 2010) or shoot tips as explants (Kitto and Young 1981; Singh *et al.* 1994; Parthasarathy and Nagaraju 1996; Matsumoto *et al.* 1998; Mohanty *et al.* 1998; Meszaros *et al.* 1999; Mishra *et al.* 1999; Singh *et al.* 1999; Paudyal and Haq 2000; Parthasarathy *et al.* 2001; Kamble *et al.* 2002; Rana and Singh 2002; Mukhtar *et al.* 2005; Rathore *et al.* 2007; Symal *et al.* 2007; Murkute *et al.* 2008, 2009).

Therefore, this study was conducted to assess the best concentrations of α-naphthalene acetic acid (NAA), BA and gibberellic acid (GA_3) for *in vitro* shoot induction and multiplication of Rangpur lime rootstock.

MATERIALS AND METHODS

Collection of explants

Four-year-old field Rangpur lime trees (*Citrus limonia* Osbeck) were used as the source of nodal segments and shoot tip explants for the present investigation. Forty-days-old new shoots measuring 20-30 cm in length with 5-7 nodes were collected in polythene bags and brought to the laboratory for further treatments (**Figs. 1A, 1B**).

Preparation and sterilization of explants

Shoot tips and nodal segments 1-1.5 cm in length were excised under laminar air flow (LAF) and surface sterilized as standardized by Eed *et al.* (2010) (**Fig. 1C**). Explants were washed under running tap water for 20 min and dipped in 0.1% Tween 20 (Rolex-Laboratory-Reagent, Mumbai, India) for 15 min followed by three rinses in distilled water. They were treated with 70% ethanol (Changshu Yangyuan Chemical, analytical reagent, China) for 30 and 60 sec for shoot tips and nodal segments, respectively followed by three washes for 1-2 min with distilled water. Under LAF, the shoots and nodal segments were immersed in 0.1% $HgCl_2$ [(w/v) (Qualigens, Mumbai, India)] for 5 and 10 min, respectively. After treatment both explants were again washed three times for 1-2 min with sterile distilled water and inoculated onto culture medium defined below. Benomyl (Coromandel Fertilizers Ltd., 50% WP, Tamilnadu, India) at 100 mg/L and streptomycin (Himedia, Mumbai, India) at 250 mg/L were added to the culture medium before autoclaving. Earlier, the abaxial and adaxial surfaces of leaves of Rangpur lime plants grown in the field were sprayed with 1 g/L bavistin (BASF, Carbendazime 50% WP, Mumbai, India) mixed with 200 mg/L K-cyclin (Insecticide (India) Ltd., Jammu & Kashmir, India) (an antibiotic to control bacterial contamination) 3-4 days before explant collection.

Culture media and incubation conditions

The nutrients in culture media consisted of MS salts and the medium was solidified with 7% agar agar (Qualigens), supplemented with 3% sucrose (Qualigens) and NAA (Himedia) at 0, 0.1 and 0.2 mg/L alone or in combination with BA (Molychem, Mumbai, India) at 0.5, 1.0, 1.5 and 2.0 mg/L. The pH of the medium was adjusted to 5.8 before gelling with agar agar with 1N NaOH or 1N HCl. The MS medium was then dispensed into 250-ml culture bottles and autoclaved for 20 min at 121°C. The cultures were incubated at 25 ± 2°C under a 16-hr photoperiod under cool white fluorescent light (37.5 µmol m^{-2} s^{-1}).

Effect of NAA and BA on shoot proliferation

The experiment consisted of 24 treatment combinations i.e. two explants (nodal segments and shoot tips), three NAA concentrations (0.0 and 0.1 and 0.2 mg/L) and four BA concentrations (0.5, 1.0, 1.5 and 2.0 mg/L). The explants were inoculated onto culture bottles with 40 ml full-strength MS medium following surface sterilization; bottles were sealed with Parafilm® (Chicago, IL, USA). The data of different shoot proliferation parameters viz., percentage of explant response, number of days to shoot induction, number of shoots and leaves per explant and shoot length were recorded 4 weeks after culture.

Effect of GA_3 on *in vitro* shoot multiplication

The experiment consisted of 8 treatment combinations i.e. two shoots derived each from nodal segment and shoot tip explants, two strengths of MS medium (full and half) and two GA_3 concentrations (0.0 and 0.1 mg/L). The *in vitro* shoots derived from both explants obtained from any treatment in the BA+NAA experiment were subcultured on growth media supplemented with 0.5 mg/L BA, which was found to be the best concentration for inducing shoots. The data of different parameters of shoot multiplication *viz.*, number of regenerated shoots, number of leaves per shoot and shoot length were recorded 5 weeks after subculture.

Experimental design and data analysis

Experiments were conducted in a factorial completely randomized design (FCRD) with three replicates, each with 10 explants per replicate. Percentage and counting data obtained for various parameters were angular or square root transformed before analyzing data according to Gomez and Gomez (1983) and Sastry (2007). ANOVA values were obtained with Opstat1 software (O.P Sheron, Programmer, Computer Section, CCS HAU, Hisar, India) and means were separated with least significant difference (LSD) at $P = 0.05$.

RESULTS AND DISCUSSION

Effect of NAA and BA on explant response to shoot induction

The shoot induction response was significantly affected by the choice of explant (**Table 1**). Nodal segments showed higher shoot induction (99.44%) than shoot tips (97.50%). NAA at 0.2 mg/L or BA at 0.5 and 1.0 mg/L resulted in the highest shoot induction (100%) (**Table 1; Figs. 1D, 1E**). The high shoots induction response of both explant types is attributed to cytokinins, which break bud dormancy by activating meristems and causing shoots to proliferate (Murashige 1974). The outgrowth of axillary buds is in general related with the cytokinin level in the buds. Cytokinin may independently regulate the growth of axillary buds (Shimizu-Sato and Mori 2001). Chandan *et al.* (2009) also reported that <1 mg/L BA + 0.2 mg/L NAA resulted in most shoots in acid lime (*Citrus aurantifolia*) when axenic nodal segments were used. Kumar *et al.* (2001a, 2001b) reported higher shoot proliferation from epicotyl explants of sweet orange cv 'Mosambi' and 'Kinnow' mandarin with 1.0 mg/L BA than with higher (2.0 or 3.0 mg/L) concentrations of BA. This result was also in line with the findings of other groups: pectinifera (*Citrus depressa* Hyata) epicotyl segment explants with 1 mg/L BA (Gill and Gosal 2002); sweet orange 'Valencia' epicotyl explants with 1 mg/L BA (Almeida *et al.* 2006); pummel (*Citrus grandis* L. Osbeck) nodal segment explants with < 1 mg/L BA (Rastgoo 2008), specifically 0.75 mg/L BA (Rastgoo *et al.* 2009).

Effect of NAA and BA on number of days taken for shoot initiation

The number of days to shoot initiation was significantly dependent on explant type (**Table 1**). Nodal segments needed significantly fewer days to initiate shoots than shoot tips. MS medium without NAA needed less time (8.9 days) to initiate shoots than MS media with NAA (9.1-9.8 days). BA at 0.5 mg/L needed fewer days (8.7) to initiate shoots than other concentrations. All factorial interactions were significant (**Table 1**). Moreira-Dias *et al.* (2000) also reported that 5.4 µM NAA inhibited bud differentiation but this was largely overcome in the presence of 22 µM BA.

Effect of NAA and BA on number of shoots per explant

A significantly higher number of shoots was obtained with shoot tips than with nodal segments (**Figs. 1F, 1G**). However, no significant difference in the number of shoots was observed among the different concentrations of NAA. However, among the different concentrations of BA, 0.5 and 1.0 mg/L produced significantly more shoots than 1.5 and 2.0 mg/L. No significant differences were noticed among the various interactions (**Table 1**). Saini and Gill (2010) also reported maximum number of shoots with 0.5 mg/L BA in *Citrus jambhiri* cv. 'Rough lemon' explants. This was in agreement with other findings (sweet orange cv 'Mosambi' and 'Jaffa' with epicotyl segment explants using ≈ 1 mg/L BA and Kinnow mandarin with epicotyl explants using 1 mg/L BA (Kumar 2001a, 2001b); Troyer citrange (*Citrus sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf) with shoot explants using < 1 mg/l BA (Parthasarathy *et al.* 2001; Rastgoo 2008; Rastgoo *et al.* 2009). Shoot tips produced more shoots than nodal segments. Kitto and Young (1981) also reported that shoot tips formed more shoots than nodal sections perhaps because these contained more undeveloped buds.

Effect of NAA and BA on number of leaves per explant

The trend for this parameter was similar to that observed for the number of shoots per explant (**Table 1**). More leaves were obtained with 0.5 mg/L BA than at other concentrations. The explant × NAA concentration, NAA concentration × BA concentration and explant × NAA concentration × BA concentration interactions were all significant. Rastgoo *et al.* (2009) also reported more leaves in pummel nodal segment explants with < 0.1 mg/L BA in MS medium. Kumar *et al.* (2001a) reported a reduction in the number of leaves per explant with an increase in BA concentration in sweet orange (*Citrus sinensis* L. Osbeck) cv 'Mosambi' and 'Jafa'. Rastgoo (2008) reported more leaves in pummel nodal segment explants with 0.75 mg/l BA in MS medium.

Table 1 Effect of NAA and BA on shoot proliferation of Rangpur lime in MS medium.

Treatment	Response explant (%)[y] (Mean)[z]	No. of days to shoot induction[y] (Mean)[z]	No. of shoots/explant[y] (Mean)[z]	No. of leaves/explant[y] (Mean)[z]	Shoot length (cm)[y] (Mean)*
Explants (A)					
Nodal segment	99.44 a	9.15 b	2.40 b	12.75 b	1.27 a
Shoot tip	97.50 b	9.45 a	2.93 a	14.07 a	1.05 b
NAA concentration (mg/L) (B)					
0.0	98.33 b	8.99 b	2.68 a	13.78 a	1.13 b
0.1	97.08 b	9.15 b	2.58 a	13.25 a	1.26 a
0.2	100.0 a	9.76 a	2.58 a	13.20 a	1.08 b
BA concentration (mg/L) (C)					
0.5	100.0 a	8.70 c	3.17 a	16.40 a	1.28 a
1.0	100.0 a	9.19 b	3.13 a	14.87 b	1.24 a
1.5	98.89 a	9.57 a	2.53 b	11.77 c	1.05 b
2.0	95.00 b	9.75 a	2.47 b	10.60 d	1.03 b
Interaction among factors					
A x B	*	*	NS	*	*
A x C	*	NS	NS	NS	*
B x C	*	*	NS	*	*
A x B x C	*	NS	NS	*	*

[z] Similar letters indicate means which are not significantly different (LSD, $P = 0.05$), comparisons are made in each column within A, B and C, values represent as means .
[y]Data were recorded at 4 weeks after culturing explants onto shooting medium
* indicates significant difference
NS indicates none significant difference

Therefore, in general, more shoots are obtained with < 0.1 mg/L BA in *Citrus* spp., leading thus to the formation of more leaves.

Effect of NAA and BA on shoot length

Longer shoots formed from nodal segments than from shoot tips (**Table 1**). This may be attributed to active growth of almost all characters tested in nodal segments, particularly early shoot initiation. NAA at 0.1 mg/L and BA at 0.5 mg/L formed longer shoots than at other concentrations. All interactions among the different factors were significant (**Table 1**). Kumar *et al.* (2001a) also reported longer shoots in epicotyl segments explants of sweet orange with < 1.0 mg/L BA. El-Wasel (2001) reported a maximum shoot elongation of *Poncirus trifoliata* in MS medium containing 1.0 mg/l BA alone or with 0.1 mg/l NAA.

Effect of MS medium with or without 0.1 mg/L GA_3 on *in vitro* regenerated shoots

No significant difference was observed between shoots derived from nodal segments or shoot tips with respect to the number of regenerating shoots (**Table 2**). Full-strength MS medium regenerated significantly more shoots than half-strength MS medium whereas MS medium with 0.1 mg/L GA_3 regenerated more shoots than media without it (**Fig. 1H**). The explant × BA concentration interaction was significant (**Table 2**). Gill and Gosal (2002) also reported that MS medium supplemented with BA (1.0 mg/L) and GA_3 (2 mg/L) resulted in excessive shoot regeneration in *Citrus depressa*.

Impact of MS media with or without 0.1 mg/L GA_3 on number of leaves

No significant difference was observed between shoots derived from nodal segments or shoot tips explants between full- and half-strength MS medium with respect to the number of leaves (**Table 2**). However, MS media containing 0.1 mg/L GA_3 formed more leaves per shoot than MS medium without GA_3. Symal *et al.* (2007) also reported a maximum number of leaves in *Citrus aurantifolia* Swingle shoot tip explants in MS medium containing 0.1 mg/L GA_3 and 0.1 mg/L NAA. The explant × BA concentration and NAA concentration × BA concentration interactions were significant in terms of number of leaves per shoot.

Influence of MS medium with or without GA_3 on shoot length

Shoots derived from nodal segments and shoot tips significantly affected the resulting shoot length (**Table 2**). Shoots were longer when derived from nodal segments than from shoot tips. The strength of MS medium did not significantly affect shoot length. However, MS medium, irrespective of its strength, produced significantly longer shoots when it included 0.1 mg/L GA_3 compared with MS media without GA_3 (**Fig. 1I**). No significant differences were observed among all interactions with respect to shoot length. This might be attributed to the function of GA_3 in the plant which promotes internode elongation (Chawla 2004). Van Le *et al.* (1999) also reported shoot elongation after transferring thin cell layer explants excised from *Poncirus trifoliata* stem internodes onto MS medium containing 1 µM of GA_3. Paudyal and Haq (2000) reported that the addition of GA_3 (5.8 µM) to shoot proliferation medium improved shoot elongation in pummelo.

Table 2 Effect of MS medium with or without GA_3 on shoot multiplication of Rangpur lime.

Treatment	No. of regenerated shoots[y] (Mean)[Z]	No. of leaves / shoot[y] (Mean)[Z]	Shoot length[y] (cm) (Mean) [Z]
Microshoot (A)			
Nodal segment	2.25 a	10.00 a	1.79 a
Shoot tip	2.25 a	9.45 a	1.62 b
MS medium strength (B)			
Full strength	2.75 a	10.0 a	1.67 a
Half strength	1.75 b	9.35 a	1.75 a
GA_3 concentration (mg/L) (C)			
0.0	1.95 b	7.20 b	1.65 b
0.1	2.55 a	12.25 a	1.77 a
Interaction among factors			
A x B	NS	NS	NS
A x C	*	*	NS
B x C	NS	*	NS
A x B x C	NS	NS	NS

[Z]Similar letters indicate means which are not significantly different (LSD, $P = 0.05$), comparisons are made in each column within A, B and C, values represent as means
[y]Data were recorded at 5 weeks after culturing explants onto multiplication medium
* indicates significant difference
NS indicates none significant difference

CONCLUDING REMARKS

In this study, among the different concentrations of BA alone or in combination with NAA, BA at 0.5 and 1.0 mg/L were most suitable for shoot induction and longer shoots. Highest multiple shoot induction and longest shoots formed when shoot tips were used as explants although nodal segments were best for reducing the number of days to shoot initiation and increasing the percentage shoot induction. A higher number of regenerated shoots, leaves and longer shoots were observed with the treatment combination containing full-strength MS medium supplemented with 0.1 mg/L GA_3 in shoots derived from nodal segments.

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REFERENCES

Al-Khayri JM, Al-Bahrany AM (2001) *In vitro* micropropagation of *Citrus aurantifolia* (lime). *Current Science* **81 (9-10)**, 1242-1246

Almeida WABD, Filho FDAAM, Mendes BMJ, Rodriguez APM (2006) Histological characterization of *in vitro* adventitious organogenesis in *Citrus sinensis*. *Biologia Plantarum* **50 (3)**, 321-325

Barman P, Swami GSK, Patil CP, Thammaiah N (2006) Response of Rangpur lime seeds to various chemicals and bio-control agents for germination and viability of seeds. *Journal of Asian Horticulture* **3 (1)**, 22-26

Batchelor LD, Webber HJ (1948) *The Citrus Industry* (Vol 1, 1st Ed), University of California Press, Berkeley and Los Angeles, USA, 3 pp

Begum F, Amin MN, Islam S, Azad MAK (2004) A comparative study of axillary shoot proliferation from the nodal explants of three varieties pummelo (*Citrus grandis* [L.] Osb). *Biotechnology* **3 (1)**, 56-62

Chandan PM, Patil RS, Nagre PK, Raut VU (2009) *In vitro* propagation in seedless acid lime. *Abstracts of International Conference on Horticulture*, November, 09-12, 2009, Banglore, India, 297 pp

Chawla HS (2004) *Introduction to Plant Biotechnology*, Oxford IBH Publishing Co., Pvt. Ltd, New Delhi, India, pp 39-41

Davies FS, Albrigo LG (2003) *Citrus* (1st Ed), Cambridge University Press, UK, 90 pp

Desai RA, Patel BM, Shah RR (1996) *In vitro* propagation of acid lime (*Citrus aurantifolia* Swingle) var. 'Kagzi lime'. *Journal of Applied Horticulture Navsari* **2 (1/2)**, 91-95

Eed AM, Amrender-Reddy S, Malla-Reddy K, Teixeira da Silva JA, Verra-Reddy P, Begum H, Venkatsubbaiah PY (2010) Effect of antibiotics and fungicides on the *in vitro* production of *Citrus linonia* Osbeck nodal segment and shoot tip explants. *The Asian and Australasian Journal of Plant Science and Biotechnology* **4 (1)**, 66-70

El-Wasel AS (2001) Micropropagation of trifoliate orange rootstock (*Poncirus trifoliata* (L.) Raf.). *Arab University Journal of Agricultural Sciences* **9 (1)**, 21-34

Filho FDAAM, Espinoza-Nunez E, Stuchi ES, Ortega EMM (2007) Plant growth, yield, and fruit quality on four rootstocks. *Scientia Horticulturae* **114**,

45-49

Gill MIS, Gosal SS (2002) Micropropagation of pectinifera (*Citrus depressa* Hyata) potential citrus rootstock for sweet orange. *Indian Journal of Citriculture* **1 (1)**, 32-37

Gomez KA, Gomez AA (1983) *Statistical Procedures for Agricultural Research*, John Wiley and Sons, New York, pp 298-308

Kamble AB, More TA, Patil RS (2002) *In vitro* micropropagation and callus induction of acid lime (*Citrus aurantifolia* S.) cv. Sai Sharbati. *South Indian Horticulture* **50 (4-6)**, 329-334

Kiran K, Parshant B, Chahal TS (2008) *In vitro* multiplication of rough lemon (*Citrus jambhiri* Lush). *Indian Journal of Citriculture* **3 (1)**, 1-5

Kitto SL, Young MJ (1981) *In vitro* propagation of carrize citrange. *HortScience* **16 (3)**, 305-306

Kumar K, Dhatt AS, Gill MIS (2001a) *In vitro* plant regeneration in sweet orange (*Citrus sinensis* L. Osbeck) cv. Mosambi and Jaffa. *Indian Journal of Horticulture* **53 (3)**, 208-211

Kumar K, Dhatt AS, Gill MIS (2001b) *In vitro* plant regeneration in Kinnow mandarin (*Citrus nobilis* Lour. X *C. deliciosa* Terna). *Indian Journal of Horticulture* **58 (4)**, 299-302

Matsumoto K, Okuno K, Kawamura H, Hisajima S (1998) Application of citrus *in vitro* plants to environmental preservation (Part 1). Micropropagation of suntara orange (*Citrus reticulata* Blanco) and refinement of production procedure by cost simulation. *Journal of the Society of High Technology in Agriculture* **10 (1)**, 34-40

Meszaros A, Bellon A, Pinter E, Horvath G (1999) Micropropagation of lemon balm. *Plant Cell, Tissue and Organ Culture* **57 (2)**, 149-152

Mohanty S, Deka PC, Bhattacharya S (1998) Micropropagation of *Citrus sinensis* cultivar Musambi. *Indian Journal of Agriculture Science* **68 (2)**, 114-116

Moreira-Dias JM, Molina RV, Bordon Y, Guardiola JL, Garcia LA (2000) Direct and indirect shoot organogenic pathways in epicotyl cuttings of Troyer citrange differ in hormone requirements and in their response to light. *Annals of Botany* **85 (1)**, 103-110

Mukhtar R, Khan MM, Rafiq R, Shahid A, Khan FA (2005) *In vitro* regeneration and somatic embryogenesis in *Citrus aurantiifolia* and *Citrus sinensis*. *International Journal of Agricultural Biology* **7 (3)**, 518-520

Murashige T (1974) Plant propagation through tissue culture. *Annual Review of Plant Physiology* **25**, 135-166

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**, 473-497

Murkute AA, Sharma S, Singh SK (2008) Rapid clonal *in vitro* multiplication of *Citrus jambhiri* and *Citrus karna*. *Indian Journal of Horticulture* **65 (2)**, 127-133

Murkute AA, Sharma S, Singh SK (2009) Micropropagation of trifoliate orange (*Poncirus trifoliata*). *Indian Journal of Plant Physiology* **14 (2)**, 190-193

Parthasarathy VA, Barua A, Nagaraju V, Parthasarathy U (2001) Quadratic response of *Citrus* species to cytokinins and comparative efficacy on morphogenetic characters of *in vitro* proliferated shoots. *Indian Journal of Horticulture* **58 (4)**, 336-341

Parthasarathy VA, Nagaraju V (1996) *In vitro* shoot proliferation in certain citrus species. *Indian Journal of Horticulture* **53 (3)**, 171-174

Paudyal K, Haq N (2000) *In vitro* propagation of pummelo (*Citrus grandis* L. Osbeck). *In Vitro Cellular and Developmental Biology – Plant* **36**, 511-516

Raju ANY (1978) Studies on fruit growth and development in Rangpur lime (*Citrus limonia* Osbeck). MSc thesis, Andhra Pradesh Agricultural University, Tirupati, India, pp 1-2

Rana JS, Singh R (2002) *In vitro* clonal propagation of Kagzi lime (*Citrus aurantifolia* Swingle) through shoot tips. *Progress in Horticulture* **34 (1)**, 27-34

Randhawa GS, Srivastava KC (1986) *Citriculture in India* (1st Edn), Hindustan Publishing Corp., New Delhi, India, pp 101-102

Rastgoo S (2008) Studies on *in vitro* regeneration in pummelo (*Citrus grandis* [L.] Osb). PhD thesis, University of Agricultural Sciences, Bangalore, India, pp 106-117

Rastgoo S, Raju B, Sathyanarayana BN (2009) Optimization of growth regulator and culture medium for *in vitro* regeneration of pummelo. *Indian Journal of Horticulture* **66 (4)**, 423-428

Rathore JS, Rathore MS, Singh M, Singh RP, Shekhawat NS (2007) Micropropagation of mature tree of *Citrus limon*. *Indian Journal of Biotechnology* **6**, 239-244

Sastry EVD (2007) *Essentials of Agricultural Statistics*, Pointer Publishers, Jaipur, India, pp 260-266

Sen S, Dhawan V (2010) Development of a highly efficient micropropagation method for the *citrus* rootstock 'Swingle' Citrumelo [*Poncirus trifoliata* (L.) Raf. × *C. paradisi* McFaden]. *International Journal of Fruit Science* **10 (1)**, 65-78

Shimizu-Sato S, Mori H (2001) Control of outgrowth and dormancy in axillary buds. *Plant Physiology* **127**, 1405-1413

Shivahkar VJ, Rao CN (2001) Biological control of citrus insect pests. In: Singh S, Nagvi SAMH (Eds) *Citrus* (1st Ed), International Book Distribution Co., Lucknow, India, pp 347-367

Singh IP, Singh A (2001) Citrus germplasm and its utility. In: Singh S, Nagvi SAMH (Eds) *Citrus* (1st Ed), International Book Distribuation Co., Lucknow, India, 48 pp

Singh S, Huchche AD (2001) Citrus decline: A critical appraisal. In: Singh S, Nagvi SAMH (Eds) *Citrus* (1st Edn), International Book Distribution Co., Lucknow, India, pp 421-436

Singh S, Ray BK, Bhattacharyya, Deka PC (1994) *In vitro* propagation of *Citrus reticulata* Blanco and *Citrus limon* Burm.f. *HortScience* **29 (3)**, 214-1994

Singh S, Ray BK, Deka PC (1999) Micropropagation of *Citrus jambhiri* cv. Rough lemon. *Journal of Interacademicia* **3 (2)**, 140-145

Sonkar RK (2001) Citrus rootstock. In: Singh S, Nagvi SAMH (Eds) *Citrus* (1st Ed), International Book Distribution Co., Lucknow, India, 126 pp

Symal MM, Upadhyay S, Biswas S (2007) *In vitro* clonal propagation of Kagzi lime (*Citrus aurantifoia* Swingle). *Indian Journal of Horticulture* **64 (1)**, 84-86

Usman M, Sana M, Fatima B (2005) *In vitro* multiple shoot induction from nodal explants of citrus cultivars. *Journal of Central European Agriculture* **6 (4)**, 435-442

Van Le B, Thanh Ha N, Anh Hong LT, Tran Thanh Van K (1999) High frequency shoot regeneration from trifoliate orange (*Poncirus trifoliata* L. Raf.) using thin cell layer method. *Comptes Rendus de 1'Academie des Sciences Serie III, Sciences de la Vie* **322 (12)**, 1105-1111