Rapid Protocol for in vitro Multiplication of Citrus limonia Osbeck Rootstock


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 (GA3) 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 explant source. 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 auranti-folia 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-

Keywords: BA, NAA, nodal segment, shoot induction, shoot tip
Abbreviations: BA, 6-benzyl adenine; NAA, α-naphthalene acetic acid; GA₃, 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 auranti-folia 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-

Therefore, this study was conducted to assess the best concentrations of α-naphthylene acetic acid (NAA), BA and gibberellic acid (GA3) 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. The explants were washed under running tap water for 20 min and dipped in 0.1% HgCl2 [(w/v)] (Qualigens, Mumbai, India)] for 5 and 10 min, respectively. After treatment both explants were again washed thrice 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-h 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 GA3 on in vitro shoot multiplication

The experiment consisted of 8 treatment combinations i.e. two explants derived each from nodal segment and shoot tip explants, two strengths of MS medium (full and half) and two GA3 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.

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 GA3 on full-strength MS medium; (I) Shoot elongation on half-strength MS medium containing 0.1 mg/L GA3.
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 \( \mu \text{M} \) NAA inhibited bud differentiation but this was largely overcome in the presence of 22 \( \mu \text{M} \) BA.

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

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

Interaction among factors

| A x B | * | NS | * | * | * |
| A x C | * | NS | NS | * | * |
| B x C | * | NS | NS | * | * |
| A x B x C | * | NS | NS | * | * |

* indicates significant difference
NS indicates none significant difference

* 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.

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 \( \times \) NAA concentration, NAA concentration \( \times \) BA concentration and explant \( \times \) NAA concentration \( \times \) 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 ‘Jaf’. Rastgoo (2008) reported more leaves in pummel nodal segment explants with 0.75 mg/l BA in MS medium.
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 GA3

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 GA3 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 GA3 (2 mg/L) resulted in excessive shoot regeneration in Citrus depressa.

### Impact of MS medium with or without 0.1 mg/L GA3 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 GA3 formed more leaves per shoot than MS medium without GA3. Symal et al. (2007) also reported a maximum number of leaves in Citrus aurantium folia Swingle shoot tip explants in MS medium containing 0.1 mg/L GA3 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 GA3 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 GA3 compared with MS medium without GA3 (Fig. 1I). No significant differences were observed among all interactions with respect to shoot length. This might be attributed to the function of GA3 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 trifoliatea stem internodes onto MS medium containing 1 μM of GA3. Paudyal and Haq (2000) reported that the addition of GA3 (5.8 μM) to shoot proliferation medium improved shoot elongation in pummelo.

### 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 GA3 in shoots derived from nodal segments.

### ACKNOWLEDGEMENTS

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### Table 2 Effect of MS medium with or without GA3 on shoot multiplication of Rangpur lime.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of regenerated shoots(a) (Mean)</th>
<th>No. of leaves / shoot(b) (Mean)</th>
<th>Shoot length(c) (Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microshoot (A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodal segment</td>
<td>2.25 a</td>
<td>10.00 a</td>
<td>1.79 a</td>
</tr>
<tr>
<td>Shoot tip</td>
<td>2.25 a</td>
<td>9.45 a</td>
<td>1.62 b</td>
</tr>
<tr>
<td>MS medium strength (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full strength</td>
<td>2.75 a</td>
<td>10.00 a</td>
<td>1.67 a</td>
</tr>
<tr>
<td>Half strength</td>
<td>1.75 b</td>
<td>9.35 a</td>
<td>1.75 a</td>
</tr>
<tr>
<td>GA3 concentration (mg/L) (C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>1.95 b</td>
<td>7.20 b</td>
<td>1.65 b</td>
</tr>
<tr>
<td>0.1</td>
<td>2.55 a</td>
<td>12.25 a</td>
<td>1.77 a</td>
</tr>
</tbody>
</table>

* indicates significant difference
NS indicates none significant difference

\(a\) Data were recorded at 5 weeks after culturing explants onto multiplication medium

\(b\) 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

\(c\) Interaction among factors

\(\text{Mean} \)
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