High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (*Punica granatum* L.)

Soumendra K. Naik, Sitakanta Pattnaik, Pradeep K. Chand*

*Plant Tissue and Cell Culture Facility, Post-Graduate Department of Botany, Utkal University, Bhubaneswar 751004, Orissa, India*

Accepted 2 December 1999

**Abstract**

A complete protocol is presented for in vitro regeneration of pomegranate (*Punica granatum* L.), a tropical fruit tree, using cotyledonary nodes derived from axenic seedlings. Shoot development was induced from cotyledonary nodes on Murashige and Skoog (1962) (MS) medium supplemented with 2.3–23.0 μM benzyladenine (BA) or kinetin (Kn). Both type and concentration of cytokinin significantly influenced shoot proliferation. The maximum number of shoots (9.8 shoots/explant) was developed on a medium containing 9.0 μM BA. Shoot culture was established by repeatedly subculturing the original cotyledonary node on a fresh batch of the same medium after each harvest of the newly formed shoots. In vitro raised shoots were cut into nodal segments and cultured on a fresh medium for further multiplication. Thus, from a single cotyledonary node about 30–35 shoots were obtained in 60 days. Shoots formed in vitro were rooted on half-strength MS supplemented with 0.054–5.4 μM naphthaleneacetic acid (NAA). However, a medium containing 0.54 μM NAA resulted in the highest percentage rooting of shoots and significantly higher number of roots than other concentrations. Plantlets were successfully acclimated and established in soil. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Axillary proliferation; Cotyledonary node; In vitro regeneration; Fruit tree; *Punica granatum*

1. **Introduction**

The use of tissue culture techniques for fruit tree propagation has increased considerably over the past few decades. Reliable and efficient protocols for plant
regeneration in vitro through stimulation of axillary shoot proliferation from nodal stem segments and apical buds or through organogenesis or embryogenesis directly from various explants or callus have been developed for many important tropical and temperate fruit trees (Hutchinson and Zimmerman, 1987; Litz and Jaiswal, 1991; Grosser, 1994; Zimmerman and Swartz, 1994). Pomegranate (*Punica granatum* L.) is an important fruit tree of the tropics that is cultivated for its sweet edible fruits. In addition, the tree is also valued for its pharmaceutical properties and the stem and root bark, leaves and fruit rind are a good source of secondary products such as tannins, dyes and alkaloids (Anonymous, 1982).

An efficient in vitro regeneration protocol has not yet been developed for this fruit tree. Although somatic embryogenesis from seedling explants (Jaidka and Mehra, 1986) or petals (Nataraja and Neelambika, 1996) have been reported in a few cultivars of pomegranate, information on the conversion frequency of the somatic embryos into plantlets is absent. Organogenesis from callus derived from anther wall (Moriguchi et al., 1987) or leaf segments (Omura et al., 1987) has also been achieved in this fruit tree. However, in the former case the shoot forming potential of the anther wall — derived callus has been shown to be extremely poor. Only 10 out of 391 cultures showed shoot regeneration, each producing 1–2 shoots per culture (Moriguchi et al., 1987). On the other hand, in the latter case only 10–15% of the calli derived from the leaf segment of the fruit clone have been reported to exhibit shoot regeneration and the average number of shoots per explant was <1 (Omura et al., 1987).

In vitro propagation of an elite pomegranate cultivar through axillary proliferation from nodal explants of a mature tree has been reported earlier from our laboratory (Naik et al., 1999). However, the limitations of our previous protocol are browning of the culture medium followed by necrosis of the explants and the production of fewer shoots per explant. Cotyledonary nodes excised from axenic seedlings have been successfully used for in vitro regeneration of many tree species including *Anogeissus sericea* (Kaur et al., 1992), *Anogeissus acuminata* (Rathore et al., 1993), *Prosopis cineraria* (Nandwani and Ramawat, 1993), *Anacardium occidentale* (Das et al., 1996), *Sterculia urens* (Purohit and Dave, 1996), *Achras sapota* (Purohit and Singhvi, 1998) and *Dalbergia sissoo* (Pradhan et al., 1998) as they are more responsive than mature explants. However, a cotyledonary node-based regeneration system has not yet been reported for pomegranate. Therefore, our objective was to develop a regeneration protocol for pomegranate through high frequency axillary shoot proliferation from cotyledonary nodes excised from axenic seedlings.

2. Materials and methods

Seeds of “Ganesh” cultivar of pomegranate (*Punica granatum* L.) were collected from fully ripe fruits and washed free of the juicy testa. These seeds
were kept under running tap water for 30 min followed by a 2 min treatment with a 5% (v/v) aqueous solution of Laboline (Qualigens, India) and rinsed 5–6 times with distilled water. Seeds were surface sterilized for 2–3 min with a 0.01% (w/v) HgCl₂ aqueous solution (BDH, India) and finally rinsed 5–6 times with autoclaved distilled water. The surface-sterilized seeds were germinated in 30 ml screw-capped glass tubes containing half-strength MS medium (Murashige and Skoog, 1962) and finally rinsed 5–6 times with autoclaved distilled water. The surface-sterilized seeds were germinated in 30 ml screw-capped glass tubes containing half-strength MS medium (Murashige and Skoog, 1962) and 6 g/l agar (Bacteriological grade, Hi-media, India). The pH of the medium was adjusted to 5.8 prior to autoclaving at 120°C and 104 kPa for 15 min. The cultures were incubated at 25±1°C with 35 μmol m⁻² s⁻¹ photon flux density provided by cool white fluorescent tubes (Philips, India) and 60% relative humidity.

Twenty day-old axenic seedlings served as the source of explants. After removal of the radicle and the primary shoots the cotyledonary nodes were inserted into 300 ml screw-capped glass jars (2 explants/jar) containing MS medium supplemented with 2.3–23.0 μM benzyladenine (BA) or kinetin (Kn). The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar (BDH, India). The original cotyledonary nodes were repeatedly subcultured on shoot multiplication medium (MS+9.0 μM BA) after each harvest of the shoots. Shoots obtained from each harvest were cut into single node pieces (1.0–1.5 cm) and cultured on MS medium containing 4.5 μM BA or Kn. All cultures were maintained under similar conditions as described earlier for seed germination.

Shoots 2.5–3.0 cm in length were excised and transferred to half-strength MS medium containing 1.5 g/l phytagel (Sigma, USA) for rooting. The medium was further supplemented with 0.054–5.4 μM of NAA. After 5–7 days of root initiation the rooted shoots were transferred to half-strength MS medium for further elongation of roots and shoots.

In the shoot development experiment, each treatment consisted of nine replicates (culture vessels) and the experimental unit was two explants per vessel. In the rooting experiment, each treatment consisted of 12 replicates (culture tubes) and one explant per experimental unit. Data on shoot length, shoot number and root number were collected after 30 days. Each experiment was conducted twice. Data were analysed using analysis of variance (ANOVA) for a completely randomised design (CRD). Student–Newman–Keuls’ (SNK) multiple range test was used to separate the means for significant effects.

Well-rooted plantlets were transferred to plastic pots (7.5 cm diameter) containing autoclaved vermi-compost (Ranjan’s Agrotech, Bhubaneswar) and were covered with polyethylene bags to maintain high humidity. The potted plantlets were kept in the culture room at 25±1°C and a photon flux density of 50 μmol m⁻² s⁻¹. After 3 weeks, plantlets were transferred to larger clay pots (18 cm diameter) containing soil:compost (1:1) and kept under shade for another 3 weeks before transplanting in the field.
3. Results

Shoot development could not be induced from the cotyledonary nodes on MS medium free of growth regulators. Incorporation of a cytokinin to the medium was essential to induce axillary shoot proliferation from cotyledonary nodes. Of the two cytokinins tested, BA was more effective than Kn. Shoot development increased with the increase in the concentration of cytokinin (BA or Kn) up to 9.0 μM (Table 1). Ninety-four per cent of the explants exhibited shoot development within 7–8 days on a medium supplemented with 9.0 μM BA. On the other hand, the percentage of shoot development was reduced to 83% when Kn (9.0 μM) was substituted for BA (Table 1). Explants took 10–12 days for shoot development on a Kn supplemented medium. Analysis of variance revealed that shoot number and shoot length were significantly affected by the concentration

<table>
<thead>
<tr>
<th>Cytokinin concentration (μM)</th>
<th>Percentage shoot development</th>
<th>Shoot number</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>BA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>83</td>
<td>3.5bc</td>
<td>3.2d</td>
</tr>
<tr>
<td>4.5</td>
<td>89</td>
<td>4.1b</td>
<td>5.1ab</td>
</tr>
<tr>
<td>9.0</td>
<td>94</td>
<td>9.8a</td>
<td>5.5a</td>
</tr>
<tr>
<td>13.5</td>
<td>67</td>
<td>2.8bcd</td>
<td>2.9d</td>
</tr>
<tr>
<td>23.0</td>
<td>39</td>
<td>1.7de</td>
<td>–b</td>
</tr>
<tr>
<td>Kn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>50</td>
<td>1.0e</td>
<td>2.3e</td>
</tr>
<tr>
<td>4.5</td>
<td>78</td>
<td>1.5de</td>
<td>3.4c</td>
</tr>
<tr>
<td>9.0</td>
<td>83</td>
<td>2.4bcd</td>
<td>4.7b</td>
</tr>
<tr>
<td>13.5</td>
<td>72</td>
<td>1.0e</td>
<td>3.1cd</td>
</tr>
<tr>
<td>23.0</td>
<td>44</td>
<td>1.0e</td>
<td>–b</td>
</tr>
</tbody>
</table>

Source of variation: d.f. m.s. d.f. m.s.

<table>
<thead>
<tr>
<th>Analysis of variance summary table</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between the treatments (BA)</td>
</tr>
<tr>
<td>Between the treatments (Kn)</td>
</tr>
<tr>
<td>Between BA and Kn</td>
</tr>
<tr>
<td>Error</td>
</tr>
</tbody>
</table>

* Significant at P<0.05.
** Significant at P<0.01.

Means within a column having same letters are not statistically significant at P<0.05 according to Student–Newman–Keuls’ (SNK) multiple range test.

Shoot length could not be measured. Data were collected after 30 days of culture.
and type of cytokinin used (Table 1). Significantly higher number of shoots were developed on a medium containing 9.0 μM BA. Each cotyledonary node produced an average of 9.8 shoots in 30 days (Figs. 1 and 2) on this medium and they had an average length of 5.5 cm (Table 1). On the other hand, an average of 2.4 shoots were developed from a single cotyledonary node in 30 days on a medium containing 9.0 μM Kn (Fig. 3) and the average shoot length was 4.7 cm (Table 1). The frequency of shoot development was reduced markedly at higher concentration (23.0 μM) of BA or Kn. Moreover, shoots developed on medium containing a higher level of BA or Kn failed to elongate (Table 1).

Shoot culture was established by repeatedly subculturing the original cotyledonary nodes on fresh shoot multiplication medium (MS+9.0 μM BA) after each harvest of the newly formed shoots. Each explant could be subcultured for two consecutive times and produced an average of 3–4 shoots in 25 days. Shoots formed in vitro were also multiplied as nodal explants. About 70% of the nodes developed shoots (1–2 shoots/explant) in 25 days (data not shown). Therefore, from a single cotyledonary node about 30–35 shoots were obtained within 60 days.

Addition of an auxin to the medium was essential to induce rooting in the regenerated shoots. Root initiation occurred within 10–15 days on half-strength MS medium supplemented with 0.054–5.4 μM NAA. However, greatest percentage of shoots were rooted on a medium containing 0.54 μM NAA (Table 2). Analysis of variance showed that the root number was significantly influenced by the concentration of NAA used. Highest number of roots were developed (10.3 roots/shoot) on a medium containing 0.54 μM NAA (Table 2, Fig. 4). Rooted shoots were transferred to an auxin-free half-strength MS medium.

Fig. 1. Multiple shoot initiation from a cotyledonary node of P. granatum after 7–8 days of culture on MS+BA (9.0 μM).
Fig. 2. Prolific shoot development from a cotyledonary node of *P. granatum* after 30 days of culture on MS+BA (9.0 μM).

Table 2
Rooting of the in vitro formed shoots of *P. granatum*<sup>a</sup>

<table>
<thead>
<tr>
<th>Growth regulator concentration (μM)</th>
<th>Percentage rooting</th>
<th>Root number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>NAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.054</td>
<td>75</td>
<td>6.9b</td>
</tr>
<tr>
<td>0.54</td>
<td>92</td>
<td>10.3a</td>
</tr>
<tr>
<td>2.7</td>
<td>58</td>
<td>5.3c</td>
</tr>
<tr>
<td>5.4</td>
<td>33</td>
<td>3.6d</td>
</tr>
</tbody>
</table>

Source of variation
d.f.     m.s.
Between the treatments 3 96.3**
Error 44 0.43

** Significant at *P*<0.01.

<sup>a</sup> Means within a column having different letters are statistically significant at *P*<0.05 according to Student–Newman–Keuls' (SNK) multiple range test.
Fig. 3. Development of multiple shoots from a cotyledonary node of *P. granatum* after 30 days of culture on MS+Kn (9.0 μM).

Fig. 4. Rooting of the in vitro formed shoot of *P. granatum* after 20 days of culture on half-strength MS+NAA (0.54 μM).
on which they elongated further and attained an average length of 4.5 cm in 15 days.

Plantlets with well-developed roots were successfully acclimatized and eventually established in soil (Fig. 5). The percentage survival of the plantlets after transfer to vermi-compost was 60% and 75% of the plants transferred to soil survived.

4. Discussion

We have been successful in developing an efficient in vitro plant regeneration protocol for pomegranate using cotyledonary nodes from axenic seedlings. In our previous report (Naik et al., 1999), browning of the culture medium followed by necrosis of the explant was a common problem which influenced the establishment of shoot cultures. However, this problem was not encountered with the cotyledonary nodes used in the present study. The concentration and the type of cytokinin used had profound influence on shoot development from cotyledonary nodes of *P. granatum*. The highest per cent of shoot regeneration and maximum number of shoots per explant was recorded on a medium containing BA. BA is
reported to have favoured axillary shoot proliferation from cotyledonary nodes of several tree species including *Sterculia urens* (Purohit and Dave, 1996), *Dalbergia sissoo* (Pradhan et al., 1998) and *Achras sapota* (Purohit and Singhvi, 1998). The in vitro formed shoots of *P. granatum* were successfully rooted on a NAA-containing medium and a lower concentration of NAA resulted in the highest per cent of rooting. NAA-induced rooting was reported in many fruit trees including apple (Lane, 1978) and citrus (Barlass and Skene, 1982). However, unlike *P. granatum*, in these reports a higher concentration of NAA (5.4–10.8 μM) was shown to be more effective (Lane, 1978; Barlass and Skene, 1982). In the present case, we found that roots developed on a NAA-containing medium showed elongation following transfer to a medium free of growth regulators. This promotive effect of a growth regulator-free medium on root elongation has been recorded in several fruit trees such as *Malus* species (Lane, 1978), *Citrus* species (Barlass and Skene, 1982) and *Syzygium cuminii* (Yadav et al., 1990).

Keeping in view the high heterozygosity in seed-derived plants of fruit trees, in general, a cotyledonary node-based regeneration system could be an efficient strategy to obtain somaclonal variants of pomegranate possessing agronomically desirable traits.

References


