Micropropagation of Elite Cultivars of Rose-scented Geranium (Pelargonium graveolens L’Herit.) for Industrial Production of Propagules

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Experiments were carried out to develop an efficient micropropagation procedure to produce rooted plantlets from stem and leaf explants of the elite cultivars of rose-scented geranium, Pelargonium graveolens. A set of 8 Murashige and Skoog (MS) salts based media were evaluated for eliciting adventitious shoot regeneration response from stem and leaf explants of three cultivars namely ‘Hemanti’, ‘Bipuli’ and ‘Kunti’. Stem and leaf explants without callus intervention produced 20 or more shoots in about 4 weeks. The directly regenerated shoots mass upon separation underwent good level of rooting on half strength MS medium. Plantlets thus obtained were planted in the field along with their vegetatively propagated control plants. In vitro raised plants were morphologically identical to control. Genetic fidelity of regenerants was further confirmed by gas chromatography of essential oil from aerial parts. Thus, an efficient procedure has become available for the multiplication of elite plant material of P. graveolens required for the intensive cropping in semi-tropical to tropical agro-climates of Indian hills and plains.

Keywords: adventitious shoot proliferation, plantlet multiplication, geranium essential oil, in vitro propagation, geranium planting material

Introduction

The aerial parts of Rose-scented Geranium, (Pelargonium graveolens L’Herit., Family-Geraniaceae) yield an industrially valuable essential oil, which has pronounced antibacterial (Lis-Balchin & Deans, 1996), antifungal (Chandravadana & Nidiry, 1994), pesticidal (Matsuda et al, 1996) and food preservative activities (Mallet et al, 1994). The oil is also used in high-grade perfumes and cosmetics. The uses of oil are getting diversified with the discovery of new activities of geranium whole oil and its components (Farooqui et al, 2000; Singh et al, 2001). In India, the commercial cultivation of geranium is restricted to semi-tropical to temperate agro-climates of Sheveroy, Nilgiri and Palni hills of southern India as a perennial crop (Rao et al, 1989), where it is propagated vegetatively through stem and leaf cuttings. Geranium can be grown in semi-tropical areas of Himalayan hills and sub-tropical areas of northern plains in winter-summer season. The adverse higher humidity in the monsoon season (June-September) leads to the death of field grown geranium plants (Ram et al, 1995; Kumar et al, 2000). Thus, renewed planting requires propagules in huge numbers for the geranium cropping in the semi-tropical agro-climates each year. The propagules for planting are made available either through maintaining the plants in glasshouses to serve as resource of cuttings or by transport of planting material from remote high altitude areas in southern or northern hills. Both are very inconvenient routes. A suitable in vitro micropropagation procedure is, therefore, required for the generation of scented-geranium propagules without risking the clonal fidelity. Of a number of reports on in vitro plant regeneration of ornamental geraniums, P. hortorum and P. regal available (Chen & Galston, 1965, 1967; Pillai & Hilderbrandt, 1969; Horst et al, 1976; Hakkart & Hartel, 1979); only a few describe such a procedure(s) for the rose-scented geranium (Charlwood & Charlwood, 1991; Satyakala et al, 1995; Saxena et al, 2000). The protocols reported earlier pertain to the development of some useful somaclones. However, the present study is aimed to standardize media and culture conditions for rapid adventitious clonal proliferation of elite cultivars of rose-scented geranium, to maintain clonal fidelity and field performance parameters.
Materials and Methods

Plant Material, Explant Type, Culture Medium and Conditions of Incubation

Three cultivars of *P. graveolens*, ‘Hemanti’, ‘Bipuli’ and ‘Kunti’ were obtained from CIMAP Farm, Lucknow. The genotypes were maintained as potted plants in a glasshouse. The node bearing (nodal) stem segments (1.5 cm) obtained from healthy potted 5-6 months old glasshouse grown plants of each cultivar were washed under running tap water for 30 min, surface sterilized for 2 min in 2.5% (v/v) Savlon solution, followed by 2.5 min sterilization by immersion in 0.1% (w/v) HgCl₂ solution with constant agitation, and rinsed five times with sterile distilled water.

These explants were inoculated on MS (Murashige & Skoog, 1962) medium containing 3% sucrose and 100 mg l⁻¹ myo-inositol for producing the aseptic shoot cultures, which were later used as explant material for further study. The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar and autoclaved under 104 KPa at 121°C for 15 min. Cultures were incubated at 25±1°C temp, 60% relative humidity and 16 hr photo-period at 35 μE m⁻² sec⁻¹ PAR.

The MS basal medium was supplemented with 2.5-5.0 μg ml⁻¹ 6-benzyl adenine (BA), 0.1-1.0 μg ml⁻¹ α-naphthalene acetic acid (NAA) and 1.0-3.0 μg ml⁻¹ adenine disulphate (ADS) either individually or in combinations (Table 1). Nodal explants were placed with the medium in culture tubes, while leaf explants, were placed on the medium in 100 ml flasks. Each treatment consisted of 10 explants. The experiment was carried out in triplicate. Cultures were maintained at parameters described above and observations were recorded every week. After 4 weeks of culture initiation, the explants with shoot buds were transferred to basal media for shoot elongation for another 4 weeks. The multiple shoots thus obtained were separated and counted before transferring to another medium for rooting.

Root Initiation

In vitro raised shoots (1-3 cm size) were transferred to four variants of the half strength basal medium, which varied in their auxin type and concentration (Table 2).

Acclimatization and Transfer to Soil

Well-rooted plantlets with 4-6 fully expanded leaves were removed from culture medium and the roots were washed gently under running tap water to remove the traces of medium. The plantlets, kept for a week in a glasshouse at 26±1°C and 80% relative humidity with roots sub-merged in Hoagland’s solution, were transplanted into pots containing coarse sand (gravel). After 2 weeks they were finally transferred either to the pots or to field, containing sand, soil and farmyard manure in 1:1:1 ratio.

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**Table 1—Adventitious shoot proliferation response from leaf and intercalary node bearing stem explants on agar solidified MS medium supplemented with varying phytohormonal compositions in the cv Hemanti of the rose-scented geranium *P. graveolens***

<table>
<thead>
<tr>
<th>No</th>
<th>Phytohormone concentration (μg ml⁻¹)</th>
<th>Adventitious shoot regeneration response in cv Hemanti</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BA</td>
<td>NAA</td>
</tr>
<tr>
<td>1</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>8</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>Calculated F-value</td>
<td>21.7**</td>
</tr>
<tr>
<td>10</td>
<td>LSD 5%</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>15</td>
</tr>
</tbody>
</table>

**significant at the level p > 95
Table 2—Adventitious shoot proliferation response from leaf and intercalary node bearing stem explants on agar solidified MS medium supplemented with 2.5 μg ml⁻¹ BA, 0.1 μg ml⁻¹ NAA and 1.0 μg ml⁻¹ of ADS, pH 5.8, in the cultivars Hemanti, Bipuli and Kunti of P. graveolens.

<table>
<thead>
<tr>
<th>No</th>
<th>Cultivar</th>
<th>Adventitious shoot regeneration response of</th>
<th>leaf explants</th>
<th></th>
<th>nodal explants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>explants that responded (%)</td>
<td>No of shoots per explant (%)</td>
<td>explants that responded (%)</td>
<td>number of shoots per explant</td>
</tr>
<tr>
<td>1</td>
<td>‘Hemanti’</td>
<td>92</td>
<td>36.6</td>
<td>85</td>
<td>30.0</td>
</tr>
<tr>
<td>2</td>
<td>‘Kunti’</td>
<td>85</td>
<td>28.4</td>
<td>86</td>
<td>21.8</td>
</tr>
<tr>
<td>3</td>
<td>‘Bipuli’</td>
<td>89</td>
<td>26.5</td>
<td>78</td>
<td>17.8</td>
</tr>
<tr>
<td>4</td>
<td>Calculated F-value</td>
<td>1.5</td>
<td>10.8**</td>
<td>1.6</td>
<td>15.5**</td>
</tr>
<tr>
<td>5</td>
<td>LSD 5%</td>
<td>9</td>
<td>5.4</td>
<td>8</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>14</td>
<td>7.8</td>
<td>12</td>
<td>7.5</td>
</tr>
</tbody>
</table>

**significant at p > 95

Field Evaluation of in vitro Raised Shoots

Two hundred in vitro raised plants of each cultivar were planted along with their control plants, vegetatively propagated in the field in randomized blocks replicated four times. Plant-wise data were recorded at the time of harvest for agronomical and oil quality traits, namely height, canopy size, number of branches, leaf area, leaf and stem ratio, per cent essential oil content and its composition.

Essential Oil Extraction, Quality Determination by Gas Chromatography (GC) and Statistical Analysis of Data

Essential oil from randomly selected five samples from each plot and glasshouse grown in vitro raised plants as well as control plants was extracted by hydro-distillation of shoots, separately for each sample. GC was performed as described by Kulkarni et al. (1998). The data on various parameters was statistically analyzed (Compton, 1994). Completely randomized design was applied for analysis of variance and means were compared by least significant differences (LSD).

Results and Discussion

In the first set of experiments, it was desired to find out media conditions in which the adventitious multiplication of shoots occurred efficiently in P. graveolens cv ‘Hemanti’. A total of 8 media in which various concentrations of BAP were combined with varied concentrations of NAA and growth promoting compound ADS, were used for supporting the growth from leaf and nodal explants. The behaviour of the explants on all the media was examined over 4 weeks of incubation. There was a considerable quantitative variation in the response of explants on the media of different phytohormonal compositions (Table 1). There was a close correspondence between the adventitious shoot regeneration response, measured in terms of % stem and leaf explants that regenerated shoots, and number of shoots formed per explant. Fifteen per cent more shoot formation was found from leaf explants as compared to the nodal explants, presumably because the epicenters for shoot regeneration in stem explants were the nodal meristems carried by them, whereas shoot regeneration occurred from several de novo originated meristematic points in leaf explants. A better response of the leaf explants with respect to the number of shoots formed per explant in scented-leaf geranium was also reported earlier (Saxena et al., 2000). Higher concentrations of NAA in the medium seemed to stimulate callus formation both from nodal as well as leaf explants with or without formation of adventitious shoots. The medium containing 2.5 μg ml⁻¹ BAP, 1 μg ml⁻¹ ADS and 0.1 μg ml⁻¹ NAA was found to be the most suitable for obtaining high level of direct adventitious shoot regeneration without intervention of callus from leaf and stem explants of P. graveolens cv Hemanti. Thus, it was used for evaluation of regeneration potential of the other two cultivars. Considering overall response in vitro, the three cultivars could be arranged in the following order in terms of their adventitious shoot regeneration response: Hemanti > Kunti > Bipuli. There were minor differences in the responses shown by stem and leaf explants of different varieties (Table 2).

Generally, the leaf explants initiated adventitious shoot regeneration earlier and more extensively than
the stem explants. Although MS medium containing NAA had failed earlier to support multiple shoot initiation in *P. graveolens* (Satyakala et al., 1995), it has successfully been utilized in the present study as the sole auxin source for all the three cultivars, as has also been reported earlier for both the scented leaf (Lakshamana Rao, 1994; Saxena et al., 2000) as well as ornamental geraniums (Dunbar & Stephens, 1989). Relatively lower doses of BA and NAA (2.5 µg ml⁻¹ BA and 0.1 µg ml⁻¹ NAA) gave better explant regeneration in all the cultivars tested contrasting to Saxena et al (2000), wherein higher doses of KN and NAA (8.0 µg ml⁻¹ KN, µg ml⁻¹ NAA) were required to achieve optimum regeneration in cv Hemanti. In the present study, the addition of ADS to the optimal hormonal combination for direct organogenesis has not only enhanced the explant response and number of shoots formed per explant but also promoted healthy growth without intervention of callus in all the cultivars. The genetic differences between the three varieties may be responsible for their different regeneration potentials under identical testing conditions.

The adventitious shoots produced on stem and leaf explants over MS medium supplemented with 2.5 µg ml⁻¹ BAP, 1 µg ml⁻¹ ADS and 0.1 µg ml⁻¹ NAA were used to determine the optimum conditions for rhizogenesis. The separated shoots were inoculated onto the half strength MS medium containing NAA or IBA; the concentration of NAA used was 0.1 µg ml⁻¹ or 0.5 µg ml⁻¹ and that of IBA was 0.5 µg ml⁻¹ or 1.0 µg ml⁻¹ (Table 3). All the cultivars gave good rooting response on media containing 0.1 µg ml⁻¹ NAA. Interestingly, the response was significantly lower if the concentration of NAA used was at higher level (0.5 µg ml⁻¹). The best rooting response on the adventitious shoots of cv Hemanti and Kunti was elicited on media containing 1.0 µg ml⁻¹ IBA. Comparable rooting response with shoots of cv Bipuli was observed when MS medium had been supplemented with lower amount of IBA at the rate of 0.5 µg ml⁻¹. There was a close correspondence between all the measures of rooting response: per cent shoots that got rooted, average number of roots formed and length of biggest root formed on the shoot. These experiments demonstrated that for the best rooting response on the shoots of Hemanti and Kunti varieties IBA at 1.0 µg ml⁻¹ concentration should be used to supplement the MS medium and in the case of Bipuli variety, NAA should be supplemented in MS medium at the rate of 0.1 µg ml⁻¹. Most of the rooted plantlets survived after transfer to coarse sand and then to sand, soil and farmyard manure, 1: 1: 1 mixture in pots. More than 95% of the rooted plants produced on the culture medium could be successfully established in the glasshouse as well as in the field.

Morphological characters evaluated at field level suggest that *in vitro* raised plants were slightly vigorous and so also the essential oil yields marginally higher in tissue culture raised plants as compared to their control plants. It may be because of better nutrition obtained by *in vitro* raised plants from the culture medium during the juvenile phase. The concentrations of quality determining components of essential oil were comparable as observed by GC for tissue culture raised versus the vegetatively propagated plants (Table 4). Citronellol and geraniol ratio, which is the distinguishing factor of essential oil quality (Lawrence, 1984) was 37:1, 2.7:1 and 1.3:1

<table>
<thead>
<tr>
<th>Phytohormone (auxin) concentration used (mg/l)</th>
<th>Rooting characteristics of <em>in vitro</em> regenerated adventitious shoots of the accession</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hemanti</td>
</tr>
<tr>
<td></td>
<td>shoots that got rooted %</td>
</tr>
<tr>
<td>0.1 NAA</td>
<td>85</td>
</tr>
<tr>
<td>0.5 NAA</td>
<td>45</td>
</tr>
<tr>
<td>0.5 IBA</td>
<td>84</td>
</tr>
<tr>
<td>1.0 IBA</td>
<td>92</td>
</tr>
<tr>
<td>Calculated F-value</td>
<td>53.3**</td>
</tr>
<tr>
<td>LSD 5%</td>
<td>9</td>
</tr>
<tr>
<td>1%</td>
<td>12</td>
</tr>
</tbody>
</table>

**Significant at p > 95**
Table 4—Expression of essential oil related characters in the tissue culture raised versus control plants of the P. graveolens, grown in the field in winter-summer season of 1999-2000 in subtropical agro-climate of Lucknow

<table>
<thead>
<tr>
<th>No</th>
<th>Character</th>
<th>Expression (mean) in 'Hemanti'</th>
<th>'Kunti'</th>
<th>'Bipuli'</th>
<th>LSD</th>
<th>5%</th>
<th>1%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Micro-propagules</td>
<td>Cuttings</td>
<td>Micro-propagules</td>
<td>Cuttings</td>
<td>Micro-propagules</td>
<td>Cuttings</td>
</tr>
<tr>
<td>1</td>
<td>Plant height (cm)</td>
<td>65</td>
<td>58</td>
<td>48</td>
<td>35</td>
<td>75</td>
<td>84</td>
</tr>
<tr>
<td>2</td>
<td>Canopy size (m²)</td>
<td>0.85</td>
<td>0.72</td>
<td>0.70</td>
<td>0.60</td>
<td>0.90</td>
<td>0.80</td>
</tr>
<tr>
<td>3</td>
<td>Herb yield (kg/plant)</td>
<td>5.1</td>
<td>3.6</td>
<td>1.9</td>
<td>2.0</td>
<td>5.9</td>
<td>5.3</td>
</tr>
<tr>
<td>4</td>
<td>Leaf area (cm²)</td>
<td>62</td>
<td>58</td>
<td>97</td>
<td>108</td>
<td>81</td>
<td>82</td>
</tr>
<tr>
<td>5</td>
<td>Leaf/Stem mass ratio</td>
<td>0.9</td>
<td>0.9</td>
<td>1.4</td>
<td>1.2</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>6</td>
<td>Trichomes per mm²</td>
<td>2.6</td>
<td>2.12</td>
<td>1.90</td>
<td>1.80</td>
<td>2.90</td>
<td>3.00</td>
</tr>
<tr>
<td>7</td>
<td>Petiole length (cm)</td>
<td>11.8</td>
<td>10.1</td>
<td>8.2</td>
<td>9.0</td>
<td>13.7</td>
<td>9.7</td>
</tr>
<tr>
<td>8</td>
<td>Number of branches/plant</td>
<td>22</td>
<td>21</td>
<td>24</td>
<td>22</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>Essential oil in shoot(%)</td>
<td>0.22</td>
<td>0.20</td>
<td>0.30</td>
<td>0.30</td>
<td>0.37</td>
<td>0.30</td>
</tr>
<tr>
<td>10</td>
<td>Essential oil yield (g/plant)</td>
<td>7.6</td>
<td>6.4</td>
<td>5.8</td>
<td>6.0</td>
<td>19.0</td>
<td>13.0</td>
</tr>
</tbody>
</table>

(ii). Quality determining components of essential oil (% )

<table>
<thead>
<tr>
<th>No</th>
<th>Component</th>
<th>Expression (mean) in 'Hemanti'</th>
<th>'Kunti'</th>
<th>'Bipuli'</th>
<th>LSD</th>
<th>5%</th>
<th>1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Geraniol</td>
<td>1.4</td>
<td>1.2</td>
<td>35.0</td>
<td>32.0</td>
<td>15.4</td>
<td>21.8</td>
</tr>
<tr>
<td>12</td>
<td>Citronellol</td>
<td>52.0</td>
<td>47.0</td>
<td>13.3</td>
<td>13.1</td>
<td>39.9</td>
<td>34.5</td>
</tr>
<tr>
<td>13</td>
<td>Linalool</td>
<td>0.8</td>
<td>1.2</td>
<td>6.4</td>
<td>5.1</td>
<td>6.4</td>
<td>4.4</td>
</tr>
<tr>
<td>14</td>
<td>Cit-Rose oxide</td>
<td>0.4</td>
<td>0.7</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>15</td>
<td>Trans-Rose oxide</td>
<td>0.1</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>16</td>
<td>Isomenthone</td>
<td>10.6</td>
<td>11.4</td>
<td>10.2</td>
<td>8.7</td>
<td>7.0</td>
<td>7.7</td>
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<tr>
<td>17</td>
<td>Menthone</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>18</td>
<td>Citronellyl formate</td>
<td>15.0</td>
<td>13.8</td>
<td>0.3</td>
<td>0.2</td>
<td>7.3</td>
<td>7.8</td>
</tr>
<tr>
<td>19</td>
<td>Geranyl formate</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>2.7</td>
<td>2.1</td>
</tr>
<tr>
<td>20</td>
<td>6,9-guaiadiene</td>
<td>0.4</td>
<td>0.3</td>
<td>1.3</td>
<td>1.1</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>21</td>
<td>10-epi-γ-endesmol</td>
<td>2.4</td>
<td>2.5</td>
<td>5.2</td>
<td>4.9</td>
<td>2.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>

for in vitro raised plants against 40 : 1 , 2.5 : 1 and 1.1 : 1 for vegetatively propagated plants of cv Hemanti, Kunti and Bipuli, respectively. While, 6, 9-guaiadiene and 10-epi-γ- endesmol the known quality detrimental components were 0.37, 2.44; 0.31, 5.2 and 0.1, 2.5% for in vitro raised plants versus 0.31, 2.5; 1.1, 4.9 and 0.5, 2.8% for the vegetatively propagated control plants of cv Hemanti, Kunti and Bipuli, respectively. These results showed that the in vitro handling of the explants under conditions described did not seem to introduce any new variation.

The present study has provided a procedure for the rapid micropropagation in elite rose-scented geranium cultivars (Fig. 1). Such a procedure is required for the production of propagules for their use in raising annual geranium crops in sub-tropical agro-climates of North Indo-Gangetic plains. This work also showed that leaf explants, because of their higher regeneration potential may be the preferable explants over hypocotyl explants used by Krishnaraj et al (1997) for genetic transformation experiments to create industrially valuable transgenic plants.

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Fig. 1 — Micropropagation procedure for the rose-scented geranium P. graveolens cultivars. 1 and 2. Adventitious shoot proliferation from leaf explants; 3. In vitro rooting of adventitious shoots; 4. Hardening of the in vitro raised plantlets; 5. In vitro produced plants seen growing in pots after 6 weeks of transfer to glasshouse.
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The authors thank Department of Biotechnology, Government of India for partial financial support, S Ramesh and A K Gupta for their help in the analysis of oil samples and for statistical analysis, respectively.

References