In vitro micropropagation of scented geranium (*Pelargonium graveolens* L. Her. ex Ait: syn *P. roseum* willd)

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The objective of this study was to develop a rapid and efficient system for regenerating shoots from nodal explants of scented geranium (*Pelargonium graveolens* L. Her. ex Ait: syn. *P. roseum* willd). Single node stem explants were inoculated in MS media containing different combinations of 6-benzylaminopurine (BAP) with indole-3-acetic acid (IAA) or naphthalene acetic acid (NAA) (0, 0.5, 1.0, 2.0 mg/l) in a 4 × 4 factorial experiment. Multiple shoots were induced in media supplemented with BAP and IAA. Maximum number of shoots (56 per explant) were observed in the medium containing BAP and IAA at 1 mg/l each, 30 days after inoculation. Micro shoots were subcultured once in every four weeks. Adventitious shoots were induced from *in vitro* grown leaves and petioles. Several regenerated shoots were rooted on MS half-strength medium supplemented with 0.5 mg/l indole-3-butyric acid (IBA) and the plantlets were hardened in the growth chamber. This micropropagation system could be used for rapid and large-scale production of scented geranium.

PLANTS are a valuable source of a vast array of chemical compounds. Many species of higher plants synthesize and accumulate extractable organic substances in quantities sufficient to be economically useful raw materials for various commercial applications. Economically important plants serve as an irreplaceable source of flavour and fragrances, pharmaceuticals, etc., and many more products[1-3].

Pelargonium graveolens, commonly known as rose-scented geranium, belongs to the family Geraniaceae, yields an oil on distillation which is commercially known as 'oil of geranium'. It is one of the important essential oils widely used in soaps, perfumery and cosmetic industries. Even though many species of Pelargonium grow in India, only *P. graveolens* is cultivated mainly for the production of oil. The total annual production of geranium oil in India stands at about 20 tonnes. As the production is inadequate to meet the growing demand of the Indian perfumery industry, an additional 20 tonnes of oil worth ten million rupees is being imported annually. The imports during the last ten years have shown a sharp increase[4]. Hence, there is large scope for cultivation of this important plant in India.

Geranium is propagated by stem cuttings. One of the constraints is the non-availability of sufficient planting material for large-scale cultivation. Considerable amount of work has been done on ornamental geraniums[5-11], but work on *in vitro* multiplication of oil-yielding crop is limited. Tissue culture offers an effective alternative method for rapid multiplication of desirable clones containing high oil content. Relatively few studies have been published on the mass *in vitro* clonal propagation of oil-yielding species of Pelargonium. Pillai and Hildebrandt[12], Stefainiak and Zenkteler[13] and Cassels and Carney[14] described the use of adventitious regeneration. Micropropagation by axillary bud proliferation has proved to be the most reliable method for large-scale production of many crop plants. The present investigation was undertaken to serve the need to standardize *in vitro* techniques for mass multiplication of high oil-yielding varieties.

Stem cuttings of *P. graveolens* were collected from the field-grown plants from Central Institute of Medicinal and Aromatic Plants (CIMAP), Bangalore. The nodal explants were thoroughly washed in running water after removing the leaves and stipules for an hour. Explants were surface-sterilized first with a surfactant Tween-20 (two drops per 100 ml solution), followed by rinsing in 0.1% mercuric chloride for 10 min and washed thrice with sterile distilled water. The explants of 1 cm length were inoculated aseptically in Murashige and Skoog[15] (MS) medium supplemented with various concentrations of auxins, IAA, NAA and cytokinins, kinetin (KIN) and BAP. The pH was adjusted to 5.7–5.8 and 0.3% w/v phytagel (Sigma) was added before autoclaving at 1.06 kg/cm^2 pressure for 20 min. Leaf lamina and petioles used for the induction of adventitious shoots were cut into approximately 5 × 7 mm and 5 mm pieces, respectively. The number of shoots initiated from the cut ends of leaves and petioles was recorded every four weeks after inoculation.

MS media supplemented with BAP and IAA or NAA (0, 0.5, 1.0, 2.0 mg/l) were tested in 4 × 4 factorial combinations for the induction of multiple shoots from nodal explants of *Pelargonium*. For further proliferation of these multiple shoots, MS media supplemented with different combinations of IAA and KIN (0, 0.2, 0.5, 1.0 mg/l), 2% sucrose and high myoinositol (1 g/l) were used. After inoculation, cultures were maintained at 25 + 2°C under 16 h daily illumination with fluorescent light (15 μE m^{-2} s^{-1}). In all experiments 20 replicates were used and each experiment was repeated at least three times. Root induction was attempted with MS half-strength medium supplemented with NAA, IAA or IBA at 0.5 mg/l each, along with 2% sucrose. All observations were recorded every seventh day. The results exclude the cultures that got contaminated or died after inoculation (up to 2%). Among the media combinations tested for different stages, the following combinations were found to be superior (concentrations in mg/l).

MS1: MS full-strength medium supplemented with IAA (1.0) + KIN (1.0) + myoinositol (1000.0) along with 2% sucrose.

MS2: MS full-strength medium supplemented with BAP (1.0) + IAA (1.0) along with 2% sucrose.

MS3: MS half-strength major elements with IBA (0.5) along with 2% sucrose.

Among the different explants tested (leaves, nodal and internodal explants), only nodal explants responded positively within 12–15 days after inoculation. Induction

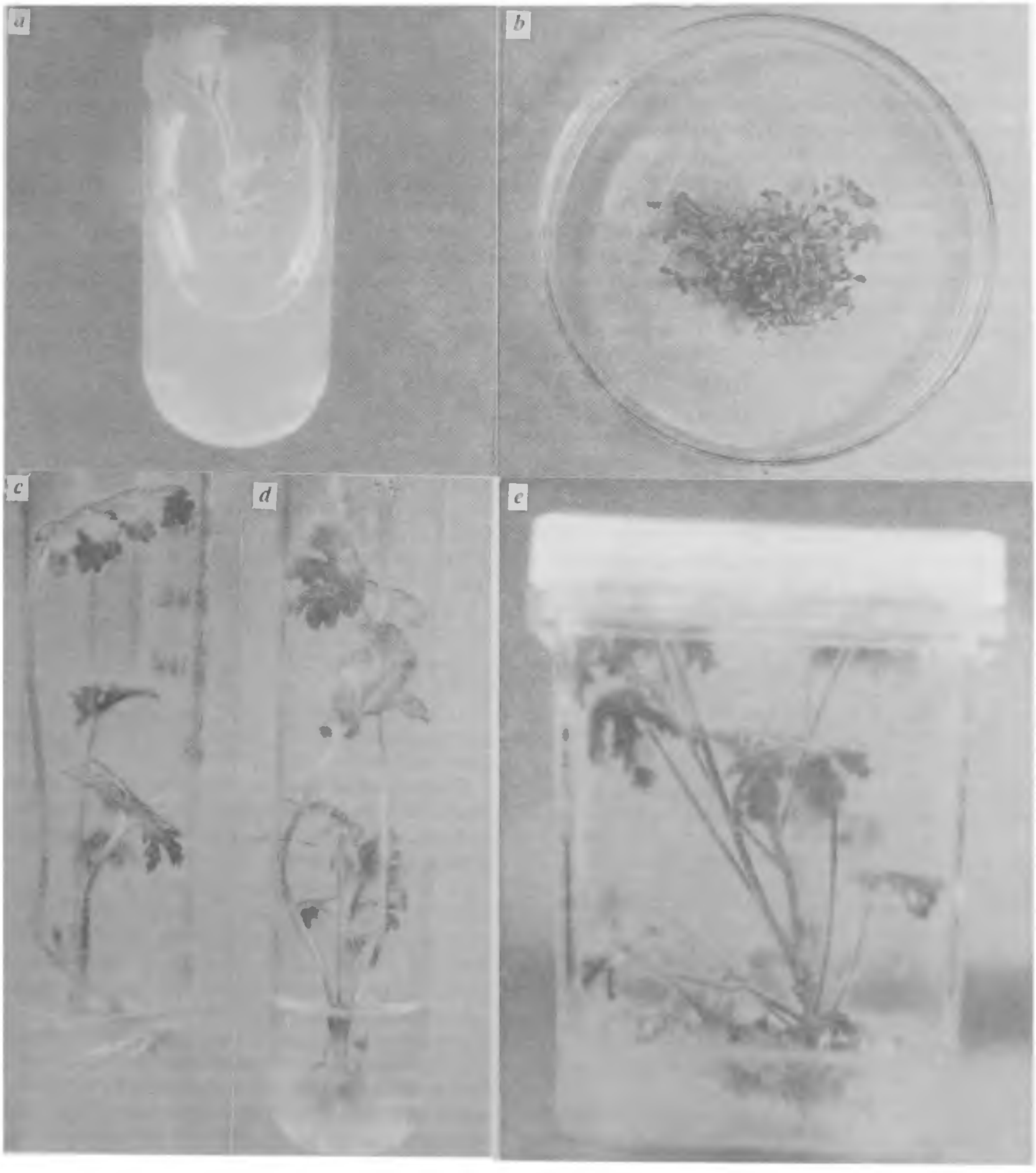

Figure 1. Micropropagation of *P. graveolens* from nodal explants. *a*, Single node cutting with the initial axillary bud sprouting. *b*, Multiple shoots induced from nodal explants in MS2 media 30 days after inoculation. *c*, Root development 11 days after transfer to MS4 solid media. *d*, Hardening in liquid media. *e*, Vigorous proliferation of shoots.

of axillary bud into single shoot (Figure 1 *a*) was observed from the nodal explant inoculated on MS1 medium. Even though nodal explants inoculated in the same medium with the normal inositol concentration (100 mg/l) responded similarly, there was a marked reduction in the petiole length and also leaf size. Axillary buds were first visible as short, swollen protrusions similar to those described as dwarf shoot primordia by Romberger[16]. Axillary shoots proliferated rapidly while attached to the original explant. Shoots were then removed and subcultured on the same medium for further proliferation. It was observed that proliferation of shoots was more vigorous in magenta jars compared to test tubes. All developmental stages up to plantlet development are shown in Figure 1.

Nodal explants on BAP with IAA combinations responded better than those on BAP with NAA. Induction of multiple shoot was achieved in IAA and BAP media 3–4 weeks after inoculation of nodal explants, especially from bulged axillary regions and cut ends. Among all the 16 concentrations tested (Table 1) the best response was noticed in MS2, with an average of 56 shoots per explant (Figure 1 *b*). As the concentration of BAP was increased up to 1 mg/l, there was a marked increase in the mean number of multiple shoots per explant, which started decreasing at higher levels. Explants inoculated in BAP alone and in BAP with NAA combinations turned brown. The presence of NAA in the regeneration medium inhibited multiple shoot initiation regardless of BAP concentration. This is consistent with the results of Valobra and James[17]. Induction of adventitious shoots with different concentrations of BAP alone was observed in ornamental geranium[10].

Table 1. Effect of BAP and IAA on multiple shoot induction from nodal explants of *P. graveolens*. Data represent mean of independent values ± standard deviation taken 30 days after inoculation

Growth regulators (mg/l)		Number of shoots/explant
BAP	IAA	
0 0	0.0	—
0.5	0 0	—
1 0	0 0	—
2.0	0.0	—
0.0	0.5	—
0 5	0 5	25 8 ± 0.84
1.0	0.5	38.5 ± 1.12
2.0	0 5	33 6 ± 0 89
0 0	1.0	—
0.5	1.0	—
1.0	1.0	56.0 ± 1.22
2.0	1 0	29.2 ± 0 84
0.0	2.0	—
0 5	2 0	—
1.0	2.0	—
2 0	2 0	28 8 ± 1 30

Large clumps of multiple shoots were separated and subcultured as small clusters for further proliferation in nutrient media supplemented with different concentrations and combinations of cytokinins (BAP, KIN), auxins (IAA, NAA) and GA. Maximum proliferation rate was obtained in MS1 with 0.5 mg/l GA. Addition of GA along with MS1 was found to help in elongation as well as proliferation (Figure 1 *c*). To obtain large number of regenerants in regal *Pelargonium*, Cassells and Carney[14] found that exposure to a pulse of auxin was critical. Subsequent transfer to cytokinin-containing auxin-free medium resulted in adventitious regeneration. However, some other authors[12] have used a conventional auxin–cytokinin factorial approach. *In vitro* leaf lamina and petioles were inoculated in different combinations of BAP and IAA. Adventitious shoots were obtained from the cut ends of both petioles and leaf lamina (Figure 2 *a*), whereas no response was seen in the field-grown explants. The number of shoots initiated from lamina and petioles were less compared to nodal explants.

The shoots developed *in vitro* (3–5 cm in length) were transferred for rooting to MS media containing half-strength major salts supplemented with IBA or NAA or IAA (0.5 mg/l). MS3 media showed the maximum rooting (100%) within 10–12 days after the transfer. The roots developed directly from the shoots without callus formation (Figure 1 *d*). Rooted plantlets were transferred to half MS-strength mineral solution (Figure 1 *e*) for about a week, and then planted in autoclaved 'Soilrite' and kept in a growth chamber maintained at 80% RH and 25°C. After 25 days, the hardened plants were gradually transferred to pots (Figure 2 *b*). Rooting of the individual shoots *in vitro* is one of the important steps in micropropagation[18]. Hamdorf[19] reported that micropropagation stock may yield 40% more cuttings than conventional stock.

The addition of vitamins (folic acid, biotin and pantothenic acid) or complex organic substances like casein hydrolysate, yeast extract, malt extract and coconut water did not enhance shoot multiplication any further. However, increased myoinositol (1.0 g/l) was found to enhance the proliferation along with KIN and IAA (1.0 mg/l each). In less than six months, complete plantlets could be obtained. Rate of shoot multiplication was improved further by careful selection.

In ornamental geraniums, induction of adventitious shoots from hybrid seeds and direct somatic embryogenesis from hypocotyl explants has been reported[9, 10]. Ornamental geraniums have attracted the attention of tissue culturists from quite some time. Growth and regeneration potential of meristem and shoot tips *in vitro* has been demonstrated in many species. Organogenesis was the only route available until recently for *in vitro* regeneration of geranium plants[6]. Recently,

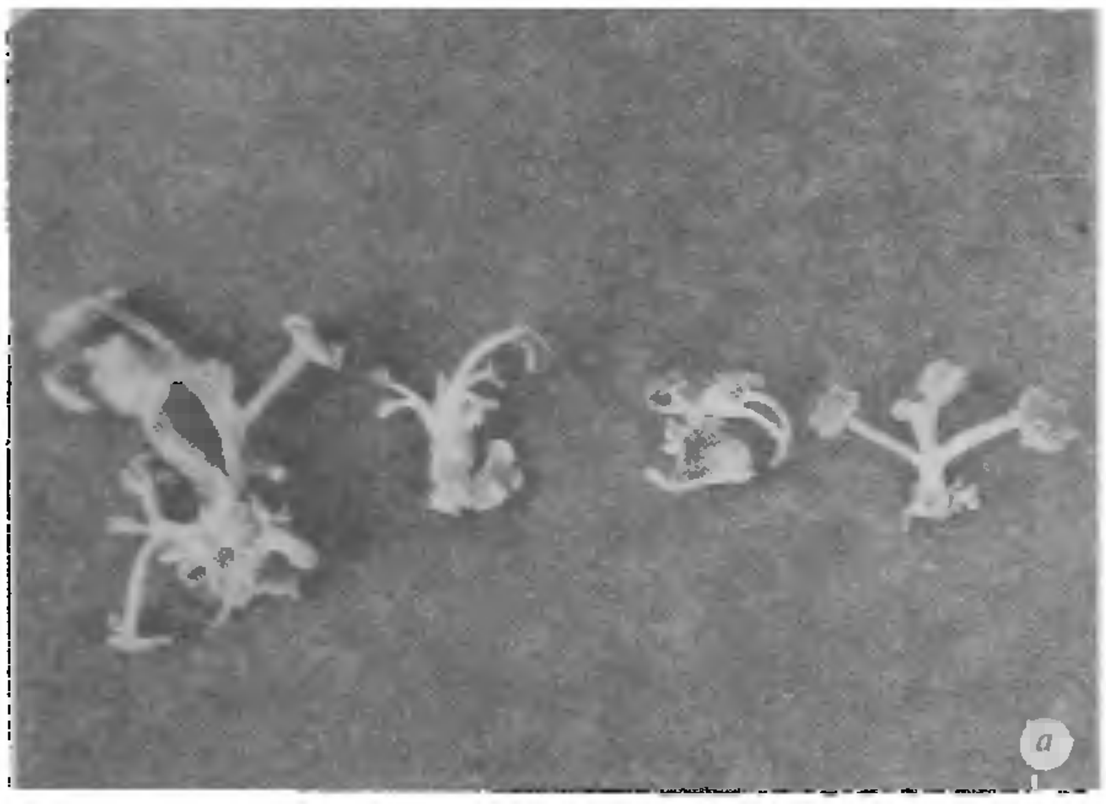

Figure 2. *a*, Adventitious shoots developed from the cut ends of lamina, *b*, Plantlets transferred to soil after hardening.

somatic embryogenesis has been reported from some ornamental species by Qureshi and Saxena[10]. However, organogenesis has not been completely satisfactory, as calli lose the regeneration capacity early.

On the other hand, in scented geranium the study of biotechnological approaches is limited to the establishment of callus cultures and investigations on accumulation of essential oil. Although this is of academic interest, it is not yet practical for the large-scale extraction of oil. Charlwood and Charlwood[20] showed that monoterpenes are not accumulated in recognizable concentrations in unorganized callus. Differentiated callus did show some increase in the accumulation of the oil. However, organogenesis was not as easy as expected and the desired increase in the essential oil content was not achieved. ,Commercialization of *in vitro* techniques is yet to be realized in oil-yielding species. Our results by enhanced axillary shoot multiplication show enormous potential for mass multiplication, with a high rate of up to 50–60 shoots per subculture. Rooting is uniform and establishment in pots is as high as 95%. Field trials are being conducted to compare the biomass and oil yield of these *in vitro* multiplied plants with that of the mother plant. Plant regeneration from tissue culture system is a critical step in the crop improvement programme. It has been emphasized that induction of direct multiple shoots is a superior method for getting true-to-type plants compared to regeneration from callus cultures, where chances of variation are high. Mass multiplication of a high oil-yielding genotype by enhanced axillary bud proliferation as reported here seems to be an ideal method for increasing the oil yield per unit land area.

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