Development of a Superior Somaclone of Rose-scented Geranium and a Protocol for Inducing Variants

Ritika Gupta, S. Banerjee, G.R. Mallavarapu, S. Sharma, S.P.S. Khanuja, A.K. Shasany, and Sushil Kumar¹

Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow-226015, India

Additional index words. *Pelargonium graveolens*, somaclonal variation, calliclones, essential oil yield and quality

Abstract. An efficient protocol has been established for generating somaclones in the Indian rose-scented geranium *Pelargonium graveolens* cv. Bipuli, which yields Reunion Island-type essential oil. Murashige and Skoog's (MS) medium supplemented with 4.5 mg·L⁻¹ BA and 1.0 mg·L⁻¹ NAA was found optimal for induction of callus from leaf explants. Callus regenerated shoots when transferred to MS medium with 2.5 mg·L⁻¹ BA and 0.1 mg·L⁻¹ NAA. The regeneration percentage as well as number of shoots per cm² of callus was greatly improved by addition of ADS at a concentration of 3.0 mg·L⁻¹. Regenerated shoots rooted within 20 days following transfer to half-strength MS medium with 0.1 mg·L⁻¹ NAA. Plantlets were acclimatized under glasshouse conditions with 80% to 85% survival. Randomly selected 30 individual calliclones were subjected to field trial with wild-type parent in randomized block design, replicated three times with 90% survival for two successive years. Characterization of these calliclones for essential oil yield and quality traits demonstrated induction of variability in all the characteristics examined in negative and positive directions in comparison with the wild-type parent. This screening led to the identification of somaclone B22, which out-yielded the wild-type parent as well as the rest of the somaclones. The quality of the essential oil of B22 was similar to that of the parent. Chemical names used: *N*⁶-benzyladenine (BA); naphthalene acetic acid (NAA); adenine di-sulphate (ADS).

Geranium oil is one of the most important essential oils, extensively used for imparting a pronounced and lasting roselike odor in high-grade perfumes and soaps. Many cultivars of rose-scented geranium *Pelargonium graveolens* are grown in various countries for distillation of geranium oil (Gulati, 1960; Lawrence, 1984). This crop is vegetatively propagated, and the annual production of oil is estimated to be in the range of ≈300 tonnes (Qinghua, 1993). The commercial value of geranium oil depends on its quality, which is mainly determined by the total rhodinol content and ratio of citronellol and geraniol. The highest quality geranium oil, which is obtained from the Bourbon cultivar of Reunion Island origin, contains almost equal amounts of geraniol and citronellol (Rajeswara Rao et al., 1995). The cultivars of Chinese and Egyptian origins produce relatively inferior quality oils. To meet the growing international re-

quirements for high-quality geranium oil, there is a need to produce more oil of the Reunion Island type.

Although several kinds of agroclimates in India appear suitable for geranium cultivation, only 5 tonnes of geranium oil are presently produced in India per year. Geranium cultivation is confined to the Nilgiri and Palni hills of southern India (Rajeswara Rao et al., 1969). The cultivation of geranium in this area dates to the early 18th century, when Portuguese priests introduced a small number of accessions of *P. graveolens*. Germplasm brought from Reunion Island and Algeria is now grown in India. The sensitivity of these genetic resources to several diseases and pests, especially white ant infestations and to water stagnation in the rainy season, does not permit economic cropping of geranium in the Indian plains. The development of suitable cultivars to meet these challenges is hindered by the narrow genetic base and sterility in the available accessions.

The parasexual genetic engineering and tissue culture-based somaclonal variation induction techniques offer possibilities for genetic improvement in the vegetatively propagated rose-scented geranium. Somaclonal variation generated through regeneration of plants from callus culture has already proved to be an effective and practical means to create increased variability relatively rapidly and without the deleterious genetic changes that get counterselected through the process of

whole-plant regeneration (Dunbar, 1997; Evans, 1989; Larkin and Scowcroft, 1981; Veilleux and Johnson, 1998).

Somaclonal variation techniques find their greatest applications for crop improvement only when a series of independently derived somaclones are judiciously evaluated under relatively stringent field conditions, and the desired variants are selected on the basis of improved economic characteristics. Somaclonal variation technique has been successfully employed for the improvement of ornamental geraniums *P. zonale* and *P. hortorum* (Abo-el-Nil and Hilderbrandt, 1973; Bottino and Hammerschlag, 1981; Chen and Galston, 1965, 1967; Dunbar and Stephens, 1989; Hakkaart and Hartel, 1979; Horst et al., 1976; Pillai and Hilderbrandt, 1968, 1969; Qureshi and Saxena, 1992), and scented geranium *Pelargonium graveolens* (Brown and Charlwood, 1986; Lakshmana Rao, 1994; Satyakala et al., 1995; Saxena et al., 2000; Skirvin and Janick, 1976a). One of these studies led to the development of a somaclonal variant, called 'Velvet rose' as a new cultivar (Skirvin and Janick, 1976b). The present work describes a method for somaclonal variation induction, and the spectrum of variation observed in the somaclones recovered from selection of a highly productive somaclone of the Bourbon-type rose-scented geranium cv. Bipuli.

Material and Methods

Explant source. The Bourbon-type rose-scented geranium cultivar Bipuli, which survives the northern Indian climatic conditions, was the source of explant for the present study. Healthy, potted 5- to 6-month-old glasshouse-grown plants obtained from 'Bipuli' stem cuttings served as the explant source. The explants, namely young leaves, were washed under running tapwater for 30 min and then surface-sterilized for 2–3 min in 2.5% (v/v) savlon solution (a germicide containing chlorhexidine gluconate 0.3% v/v and cetrimide 0.6% w/v), followed by 0.1% (w/v) HgCl₂ solution with constant agitation, and finally rinsed 3–5 times with sterile double-distilled water.

Culture medium and conditions. Murashige and Skoog's (Murashige and Skoog, 1962) medium, containing 3% sucrose and 100 mg·L⁻¹ myoinositol, was used as the basal medium. The MS medium was supplemented with NAA (0.1–1.0 mg·L⁻¹) in combination with BA (2.5–5.0 mg·L⁻¹). The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar (Hi-media Laboratories Private Ltd., Bombay, India) and autoclaved under 104 kPa at 121 °C for 15 min. Sterilized entire young leaves were placed horizontally with abaxial side down on the media surface in a 100-mL Erlenmeyer flask. Each treatment consisted of 10 explants; there were three replications. Cultures were maintained at 25 ± 1 °C, 60% relative humidity, and 16-h photoperiod at 35 μmol·m⁻²·s⁻¹ flux density. Observations were recorded at 1-week intervals. After 4 weeks of culture, the percentage of

Received for publication 13 Dec. 2000. Accepted for publication 13 June 2001. Grateful thanks are due to S. Ramesh, S.A. Hasan, and Shalini Rai for the GC analysis of oil and preparation of the manuscript, and to the Dept. of Biotechnology, Government of India, for partial financial support.

¹To whom reprint requests should be addressed: Prof. Sushil Kumar, National Center for Plant Genome Research (NCPGR), JNU Campus, Post Box 10531, New Delhi-110067, India. E-mail address: sushil2000_01@yahoo.co.in

callus induction from leaf segments, growth performance of the calli, percent callus explants forming shoots, and average number of shoots per cm² of callus were recorded and have been summarized in Tables 1 and 2, respectively.

After induction, the calli were subcultured on MS medium supplemented with reduced NAA (0.1–0.5 mg·L⁻¹), BA (2.5–3.5 mg·L⁻¹), and ADS (1.0–5.0 mg·L⁻¹) for inducing shoot regeneration.

Rhizogenesis. For rooting in vitro, regenerated shoots (2–3 cm in length) with four to five fully expanded leaves were excised and transferred to four media combinations differing in basic salts strength as well as in auxin concentration (Table 3).

Acclimatization and transfer to soil. Well-rooted plantlets with eight to 10 fully expanded leaves were removed from culture medium and the roots washed gently under running tapwater to remove the traces of medium. The plantlets were kept in a glasshouse at 26 ± 1 °C and 80% relative humidity for a week. The plantlets were transplanted into pots containing coarse sand for 2 weeks, and then moved to pots containing 1 sand : 1 soil : 1 farmyard manure. Fifty calliclones were established in a glasshouse, 30 of which were selected randomly for the field trial. These were multiplied via stem cuttings. The plants obtained were transferred to field trials during the third week of October. The calliclones were arranged in randomized blocks, replicated nine times. Cuttings of all 30 clones and a control parent were maintained under glasshouse conditions during the monsoon (July–September) and then were subjected to a second year of field trial starting the third week of October. Data were recorded for plant height, canopy size, herb yield, number of branches, number of leaves, leaf : stem ratio, leaf area, oil content, and oil yield for all the somaclones during both years. All data were subjected to the test of significance using the Critical Difference (CD) test at 5% and 1% levels of significance (Cochran and Cox, 1957).

Extraction of oil and gas chromatography. Six months after planting, all the plants were evaluated for oil content and oil composition. Oil content was determined by distilling herb samples in Clevenger's apparatus (Clevenger, 1928). Contents of 11 major constituents of oil were determined by gas chromatography (GC). GC and GC-MS analyses of the oils were performed as described earlier (Kulkarni et al., 1998). Data on oil content and oil composition were subjected to the test of significance using the CD test at 5% and 1% levels of significance.

Molecular analysis of hybrids. DNA was isolated from young leaves (1 g) of mature plants following the reported protocol (Khanuja et al., 1999) and was digested with EcoRI restriction endonucleases. A set of 20 decanucleotide primers (M/S Bangalore Genie, India) were used for PCR amplification. Polymerase chain reaction (PCR) was carried out in 25 µL reaction volume, containing 20–40 ng of plant genomic DNA, 125 µM of

Table 1. Effects of phytohormones on induction, establishment, and growth of callus cultures arising from leaf explants in rose-scented geranium (*Pelargonium graveolens* cv. Bipuli).

NAA/BA ^z (mg·L ⁻¹)	% Callus induction	Growth performance	Nature/color of callus
1.0/2.5	42	++	Friable to compact/creamish to light green
1.0/3.5	54	+	Compact/green
1.0/4.5	67	+++	Very friable/yellow

^zNAA= 1-naphthalene acetic acid; BA = 6- benzyl amino purine.

⁺25% increase over inoculum.

⁺⁺2-fold increase.

⁺⁺⁺3-fold increase.

Table 2. Effects of concentrations and combinations of growth regulators on shoot regeneration from calli in rose-scented geranium (*Pelargonium graveolens* cv. Bipuli).

Sl. no.	Phytohormone concn (mg·L ⁻¹)			% Callus segments that produced shoots	No. of shoots produced/cm ² of callus
	NAA ^z	BA ^y	ADS ^x		
1	0.5	2.5	0	39.0 ± 5.0	8.6 ± 0.5
2	0.5	3.5	0	34.6 ± 7.2	5.9 ± 0.9
3	0.1	2.5	0	61.2 ± 8.2	12.2 ± 1.2
4	0.1	3.5	0	56.2 ± 7.4	10.9 ± 3.6
5	0.1	2.5	1.0	82.1 ± 9.2	28.2 ± 2.8
6	0.1	2.5	3.0	94.6 ± 8.7 ^w	40.3 ± 5.2
7	0.1	2.5	5.0	92.8 ± 8.2	45.2 ± 6.4

^zNAA = 1-naphthalene acetic acid.

^yBA = 6-benzyl amino purine.

^xADS = adenine di-sulphate

^wRegeneration response on this medium was significantly superior to that on corresponding medium without ADS, as revealed by *t* test with 95% level of confidence.

Table 3. Effects of MS salts strength, auxin type, and concentration on the rooting of the in vitro–raised shoots of rose-scented geranium (*Pelargonium graveolens* cv. Bipuli).

Sl. no.	MS ^z strength	Auxin (mg·L ⁻¹)	% of shoots that rooted	No. of roots formed/shoot	Mean root length (cm) ^y
1	1/2	0.1 NAA ^x	96	9.7 ± 0.3 ^w	2.0 ± 0.4 ^w
2	1/2	0.5 NAA	60	3.2 ± 0.2	0.6 ± 0.2
3	1/2	0.5 IBA ^v	80	1.5 ± 0.2	0.3 ± 0.1
4	1/2	1.0 IBA	40	3.0 ± 0.2	0.6 ± 0.4

^zMS = Murashige and Skoog.

^yObservations were recorded at 20 d from transfer of shoots on to the rooting medium.

^xNAA= 1-naphthalene acetic acid.

^wRooting response on this medium was significantly superior to that on the other media, as revealed by *t* test, with 95% level of confidence.

^vIBA = indole butyric acid.

MgCl₂, 100 µM of each dNTP, 5p M of primer, and 0.2 units of Taq DNA polymerase. Amplification was carried out in DNA Engine PTC 200 (MJ Research Inc., Waltham, Mass.) thermal cycler programmed for 45 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C. The amplification cycle was concluded with final extension at 72 °C for 5 min. Amplification products were electrophoresed in 1.2% (w/v) agarose gel, visualized by ethidium bromide (0.5 µg·mL⁻¹) staining. The pictures of the gel were scanned for the presence of polymorphic fragments, which were scored for the presence (+) or absence (–) of bands. The data so generated were used for calculating the index of genetic similarity using Nei and Li's (1979) matching coefficient method. The similarity indices were calculated by the formula: 2 × (number of similar bands between the two accessions/total number of bands in the two accessions). These values were then used to generate a dendrogram with unweighted pair group method using arithmetic average (UPGMA) cluster analysis (Table 5; Fig. 3).

Results and Discussion

The present study demonstrated that the leaf explant of rose-scented geranium cv. Bipuli was highly responsive toward in vitro callusing, as has already been reported earlier in other species (Dunbar and Stephens, 1989) and a different cultivar of *P. graveolens* (Lakshmana Rao, 1994). All three combinations of BA and NAA tested for induction of callusing were found to be effective (Table 1); however, the media containing 4.5 mg·L⁻¹ BA and 1.0 mg·L⁻¹ NAA showed best callusing response where 67% of the leaf explants formed callus within 12–15 d of culture initiation. The resultant callus was highly friable and yellow in color. Lower amounts of compact and light green to green callus was formed on the other two media. The present findings are similar to the earlier report on *P. hortorum* (Pillai and Hilderbrandt, 1969) and that on *P. graveolens* cv. Hemanti (Saxena et al., 2000) in terms of the suitability of low auxin and high cytokinin concentrations for callus induction.

Table 4. Variation in the expression of essential oil yield and quality related traits among 27 somaclones and their wild-type parent in rose-scented geranium (*Pelargonium graveolens* cv. Bipuli).

Sl no.	Character	Measurement over 27 clones		Expression among 5 of the 27 somaclones selected for their superior performance					Control parent	Critical difference (CD at 5%)
		Range	Mean $\pm$ SE	B22 ^z	B13 ^z	B3 ^y	B17 ^y	B6 ^x		
a)										
				Oil yield related ^w						
1	Plant ht (cm)	61–88	75 $\pm$ 1	88 $\pm$ 2	75 $\pm$ 6 ⁸	71 $\pm$ 4	71 $\pm$ 6	76 $\pm$ 2	50 $\pm$ 2	7.0
2	Canopy (m ²)	0.6–1.6	1.2 $\pm$ 0.1	1.6 $\pm$ 0.2	1.0 $\pm$ 0.73	1.3 $\pm$ 0.04	1.4 $\pm$ 0.06	1.6 $\pm$ 0.03	0.81 $\pm$ 0.08	0.2
3	Herb yield (kg/pl ^v)	2.1–9.1	6.2 $\pm$ 0.4	9.1 $\pm$ 0.2	7.5 $\pm$ 0.1	5.7 $\pm$ 0.2	8.7 $\pm$ 0.1	8.9 $\pm$ 0.3	4.3 $\pm$ 0.06	0.7
4	No. of br/pl ^u	21–43	32 $\pm$ 1	43 $\pm$ 3	29 $\pm$ 4	36 $\pm$ 5	40 $\pm$ 6	40 $\pm$ 2	25 $\pm$ 2	1.0
5	No. of lvs/pl ^l	740–2933	824 $\pm$ 112	2690 $\pm$ 143	2366 $\pm$ 88	1800 $\pm$ 244	1966 $\pm$ 115	2366 $\pm$ 256	1437 $\pm$ 100	593
6	Leaf : stem ratio	0.58–0.97	0.78 $\pm$ 0.1	0.92 $\pm$ 0.1	0.91 $\pm$ 0.04	0.75 $\pm$ 0.07	0.75 $\pm$ 0.02	0.92 $\pm$ 0.06	0.67 $\pm$ 0.01	0.06
7	Leaf area (cm ²)	66–130	92 $\pm$ 3	112 $\pm$ 9	101 $\pm$ 6	80 $\pm$ 12	97 $\pm$ 3	91 $\pm$ 8	70 $\pm$ 7	22.0
8	Oil yield/pl (g/pl ^v)	4.5–36.1	1.9 $\pm$ 2.2	36.1 $\pm$ 1.6	29.2 $\pm$ 1.4	20.2 $\pm$ 1.0	35.2 $\pm$ 1.04	27.2 $\pm$ 0.22	10.5 $\pm$ 0.03	10.0
9	Oil content (%)	0.27–0.45	0.38 $\pm$ 0.1	0.45 $\pm$ 0.1	0.43 $\pm$ 0.02	0.44 $\pm$ 0.05	0.40 $\pm$ 0.05	0.34 $\pm$ 0.03	0.29 $\pm$ 0.01	0.11
b)										
				Oil quality related ^w (%)						
1	Geraniol	13.7–36.4	22.6 $\pm$ 0.6	21.0 $\pm$ 1.3	24.8 $\pm$ 0.4	26.3 $\pm$ 2.2	21.8 $\pm$ 0.9	23.4 $\pm$ 1.3	21.8 $\pm$ 3.3	6.5
2	Citronellol	21.6–39.3	27.2 $\pm$ 1.0	31.5 $\pm$ 0.6	29.7 $\pm$ 0.3	28.0 $\pm$ 2.5	33.7 $\pm$ 1.2	27.9 $\pm$ 2.3	34.5 $\pm$ 3.4	4.3
3	Linalool	2.4–8.5	5.8 $\pm$ 0.2	6.3 $\pm$ 2.6	5.1 $\pm$ 1.2	4.8 $\pm$ 0.6	6.4 $\pm$ 0.4	6.3 $\pm$ 1.9	4.3 $\pm$ 1.6	2.7
4	Cis-rose oxide	0.23–1.13	0.44 $\pm$ 0.1	0.40 $\pm$ 0.1	0.37 $\pm$ 0.1	0.37 $\pm$ 0.1	0.43 $\pm$ 0.1	0.30 $\pm$ 0.2	0.60 $\pm$ 0.3	0.3
5	Trans-rose oxide	0.08–0.50	0.29 $\pm$ 0.1	0.17 $\pm$ 0.1	0.13 $\pm$ 0.1	0.12 $\pm$ 0.1	0.20 $\pm$ 0.1	0.17 $\pm$ 0.1	0.30 $\pm$ 0.1	0.1
6	Isomenthone	6.0–8.7	7.3 $\pm$ 0.2	7.0 $\pm$ 0.3	6.9 $\pm$ 0.3	6.9 $\pm$ 0.4	6.6 $\pm$ 0.3	8.5 $\pm$ 0.8	7.7 $\pm$ 0.6	1.9
7	Menthone	0.10–0.20	0.15 $\pm$ 0.1	0.13 $\pm$ 0.1	0.13 $\pm$ 0.1	0.13 $\pm$ 0.1	0.10 $\pm$ 0.1	0.13 $\pm$ 0.1	0.13 $\pm$ 0.1	0.08
8	Citronellyl formate	5.8–10.2	7.7 $\pm$ 0.2	7.8 $\pm$ 0.1	7.3 $\pm$ 0.3	7.0 $\pm$ 0.1	7.3 $\pm$ 0.4	7.8 $\pm$ 0.7	7.8 $\pm$ 0.3	1.0
9	Geranyl formate	2.1–3.8	2.7 $\pm$ 0.1	2.5 $\pm$ 0.1	2.7 $\pm$ 0.2	3.3 $\pm$ 0.2	2.2 $\pm$ 0.1	2.9 $\pm$ 0.3	2.1 $\pm$ 0.2	0.5
10	10-epi-g-eudesmol	4.4–7.1	2.7 $\pm$ 0.1	5.8 $\pm$ 0.7	6.9 $\pm$ 0.2	6.1 $\pm$ 0.3	6.3 $\pm$ 0.1	5.9 $\pm$ 0.2	5.7 $\pm$ 0.3	0.5

^zClones selected for high herb and oil yields.^yClones selected for high oil yield.^xClones selected for high herb yield.^wObservations were recorded on the somaclones and their parent replicated grown in a replicated field trial during 1998–99, 1999–2000 cropping seasons.^vkg/plant.^uNumber of branches/plant.^tNumber of leaves/plant.^sGrams/plant.

In the course of the present study, shoot regeneration response could be recorded through repeated subculturing and prolonged growth of the calli on the MS medium containing 3.5 mg·L⁻¹ BA and 1.0 mg·L⁻¹ NAA. However, the resultant regenerants were malformed and vitrified in nature. Reduction in the level of auxin led to some degree of improvement in the morphology of the regenerants, as well as percent response and number of shoots per cm² of callus. A maximum of 61.2% $\pm$ 8.2% shoot regeneration was recorded on medium containing 2.5 mg·L⁻¹ BA and 0.1 mg·L⁻¹ NAA within 30 d of culture; each cm² of callus produced 12.2 $\pm$ 1.2 shoots (Table 2). The addition of ADS from 1.0 to 5.0 mg·L⁻¹ concentrations along with 2.5 mg·L⁻¹ BA and 0.1 mg·L⁻¹ NAA greatly improved the regeneration response up to 95%; and on average >40 shoots appeared on a cm² of callus. While the shoots formed with 1.0 to 3.0 mg·L⁻¹ supplementation of ADS to MS + 2.5 mg·L⁻¹ BAP + 0.1 mg·L⁻¹ NAA were healthy, those produced on the same medium supplemented with 5.0 mg·L⁻¹ ADS were abnormal and vitrified. Stimulatory effect of ADS in multiple-shoot induction from callus culture of the Egyptian oil-type *P. graveolens* cv. Algerian has been reported earlier (Lakshmana Rao, 1994).

Different cytokinins at different levels proved effective for in vitro regeneration of shoots from calli of different geranium species and cultivars. BAP in combination with some auxin has proved effective for optimum regeneration response in certain floricultural geranium species (Qureshi and Saxena, 1992) and scented geranium species (Brown and

Charlwood, 1986; Saxena et al., 2000) In certain other reports, either kinetin, zeatin, or 2iP was found effective for optimum level of regeneration response from callus cultures (Bottino and Hammerschlag, 1981; Dunbar and Stephens, 1989; Lakshmana Rao, 1994; Pillai and Hilderbrandt, 1969). The in vitro-regenerated shoots of the present study elongated further when subcultured on hormone-free solid MS medium and attained a size of 1.5–2.5 cm in 10 d.

Best rhizogenesis results, both in terms of percent response and number of roots per shoot, were obtained in one-half strength MS medium supplemented 0.1 mg·L⁻¹ NAA (Table 3). Comparable results were obtained with one-half strength MS medium supplemented with 0.5 mg·L⁻¹ IBA, but the number of roots formed and root length were significantly lower (Table 3). Moreover, the plantlet growth on the former medium was better with sturdier internodes suitable for in vivo acclimatization. The present finding differs from earlier reports (Lakshmana Rao, 1994; Satyakala et al., 1995; Saxena et al., 2000) where instead of NAA, IBA was found to be more effective for optimum rhizogenesis response.

Plantlets with expanded leaves and well-developed roots were transferred to a glasshouse for hardening. The plantlets were initially transferred to pots containing coarse sand and maintained under glasshouse conditions where 80% to 85% survival was noted. Subsequently, the healthy-looking plantlets were transferred to pots containing 1 garden soil : 1 coarse sand : 1 farmyard manure. Among the 50 individual calliclones estab-

lished in the glasshouse, 30 healthy-appearing, randomly selected individual clones along with their control parent were transferred to field conditions during Oct. 1998, where only 27 clones survived. All these clones were field-planted in randomized block design (replicated three times, with three plants per plot), were allowed to grow for 6 months, and then harvested for determining the yield and quality of oil. The field trial was again repeated in the winter–summer cropping season of 1999–2000. The averaged results of the two consecutive field trials are presented in Table 4.

It will be seen from Table 4 that in all the characteristics except height, somaclones harbored variation in both directions, i.e., more than as well as less than the value of the level of expression of characteristics observed in the control parent strain. Among the somaclones there was more than a 4-fold difference in herb yield and an 8-fold difference in oil yield. There were large differences (1.5–4 $\times$) among somaclones for characteristics such as canopy size, number of branches per plant, number of leaves per plant, leaf : stem ratio,

Table 5. Average similarity indices between 'Bipuli' parent cultivar accession and its selected five calliclones.

Accession	Bipuli	B3	B6	B13	B22	B20
Bipuli	1.00					
B3	0.72	1.00				
B6	0.55	0.75	1.00			
B13	0.73	0.73	0.67	1.00		
B22	0.64	0.89	0.79	0.75	1.00	
B20	0.80	0.98	0.78	0.75	0.92	1.00

Fig. 1. Regeneration of somaclonal variant(s) in the Indian cultivar Bipuli, rose-scented geranium *Pelargonium graveolens*: (A) induction of callus from leaf explant; (B) compact green callus undergoing regeneration; (C-E) regeneration of plantlets from callus; (F) in vitro rhizogenesis and complete plantlet formation in vitro.

leaf area, and oil content. The mean values for all the essential oil yield-related characteristics studied were higher for somaclones than for the corresponding values for the parental strain. Table 5 compares the expression of oil yield-related characteristics of the selected calliclones B3, B6, B22, B13, B17, and the 'Bipuli' parent cultivar. The herb yield of calliclones was more than 2-fold and the oil content 1.2- to 1.5-fold of 'Bipuli'.

The RAPD analysis of the DNAs of these clones and the 'Bipuli' parent using 20 decanucleotide primers was carried out to ascertain genetic differences between the somaclones and similarity between them and the parent (Fig. 3). In Table 5, which compares the similarity indices of the calliclone genotypes and control parent, calliclones of 'Bipuli' demonstrated homologies varying from 55%

to 80% with the parent cultivar and 67% to 98% homology among themselves. The presence of genetic differences between the calliclones and the parent proved that the calliclones were somaclonal mutants. Since the expression of characters in them was repeated in the two seasons of evaluation, it could be concluded that genotypic changes responsible for phenotypic differences between them and the parents were stably inherited.

Somaclone B22 was selected in which both herb and oil yields were significantly higher than those of other somaclones studied. The B22 plants were tall, highly branched, and carried a very large number of big leaves, such that a very large canopy was formed. The oil content in the shoot of B22 was 0.45%, highest among all the somaclones induced and the

Fig. 2. Morphologies of the field-grown plants of two accessions of the rose-scented geranium *Pelargonium graveolens*: (G) 24-week-old field-grown plant of 'Bipuli'; and (H) somaclone B22 developed from 'Bipuli'; typical leaves of (I) 'Bipuli' and (J) B22.

parent. Presumably, all these characteristics contributed towards highest herb and oil yields given by this clone in comparison with all the genotypes compared in the study. In our literature search, thus far no rose-scented geranium cultivars have been reported to contain essential oil in their herbage to the same extent as that present in clone B22 (Ram et al., 1995). Table 4 also presents the variation in profiles of essential oils of the wild type, and 27 somaclones developed in the study in terms of a set of 10 terpenoids that are important determinants of the quality of geranium oil. On average, the induced clones had somewhat lower citronellol, cis- and trans-rose oxides, citronellyl and geranyl formates, menthone, isomenthone, and 10 epi- γ -eudesmol contents in their essential oils as compared with essential oil of the wild type. The quality of the

Fig. 3. DNA profiles of selected somaclonal variants and the parent 'Bipuli' of rose-scented geranium *Pelargonium graveolens*: [left to right] lane 1 has molecular weight markers; lane 2 is 'Bipuli' control; and lanes 3-7 are 'Bipuli' calli clones B3, B6, B13, B22, and B17.

essential oil of B22 was, however, on par with that of the wild-type control parent.

We conclude that: 1) the somaclonal variation induction technique can be successfully used to widen the genetic variability in essential oil yield in the rose-scented, reproductively sterile, geranium genotype; 2) as far as the author knows, somaclone B22 is the most productive genotype among the available cultivars in rose-scented geranium for producing essential oil.

Literature Cited

- Abo-el-Nil, M.M. and A.C. Hilderbrandt. 1973. Origin of androgenetic callus and haploid geranium plants. *Can. J. Bot.* 51:2107-2109.
- Bottino, P. and F. Hammerschlag. 1981. Effect of plant age on callus growth, plant regeneration and anther culture of geranium. *J. Amer. Soc. Hort. Sci.* 106:114-116.
- Brown, J.T. and B.V. Charlwood. 1986b. The control of callus formation and differentiation in scented pelargoniums. *J. Plant Physiol.* 123:409-417.
- Chen, H.R. and A.W. Galston. 1965. Growth and development of *Pelargonium* pith cells *in vitro*. I. Induction of cell division. *Physiol. Plant.* 18:454-460.
- Chen, H.R. and A.W. Galston. 1967. Growth and development of *Pelargonium* pith cells *in vitro* II. Initiation and organized development. *Physiol. Plant.* 20:533-539.
- Clevenger J.F. 1928. Apparatus for the determination of volatile oil. *J. Amer. Pharm. Assoc.* 17:346.
- Cochran W.G. and G.M. Cox. 1957. Experimental designs. 2nd ed. Wiley, New York.
- Evans, David A. 1989. Somaclonal variation—Genetic basis and breeding applications. *Trends Genet.* 5:46-50.
- Dunbar, K.B. and C.T. Stephens. 1989. Shoot regeneration of hybrid seed geranium (*Pelargonium xhortorum*) and regal geranium (*Pelargonium xdomesticum*) from primary callus cultures. *Plant Cell Tiss. Org. Cult.* 19:13-21.
- Dunbar R.R. 1997. Tissue culture induced variations and crop improvement, p. 201-240. In: *Advances in agronomy*, vol. 58. Academic Press, New York.
- Gulati, B.C. 1960. Cultivation of geranium. *Ind. Oil Soap J.* 26(2):35-43.
- Hakkaert, F.A. and G. Hartel. 1979. Virus eradication from some *Pelargonium zonale* cultivars by meristem tip cultures. *Netherlands J. Plant Path.* 85:39-46.
- Horst, R.K., S.H. Smith, H.T. Horst, and W.A. Ogilvie. 1976. Virus, diseases of ornamentals. *Acta Hort.* 59:131-142.
- Kulkarni, R.N., G.R. Mallavarapu, K. Baskaran, S. Ramesh, and S. Kumar. 1998. Composition of essential oils of two isomenthone rich variants of geranium (*Pelargonium* spp.). *Flav. and Frag. J.* 13:389-392.
- Lakshmana Rao, P.V. 1994. *In vitro* regeneration of scented-leaved geranium *Pelargonium graveolens*. *Plant Sci.* 98:193-198.
- Larkin, P.J. and W.R. Scowcroft. 1981. Somaclonal variations—A novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 60:197-214.
- Lawrence, B.M. 1984. Progress in essential oils. *Perf. and Flav.* 9:987-995.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Nei, M. and W.H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* 74:5267-5273.
- Pillai, S.K. and A.C. Hilderbrandt. 1968. Geranium plants differentiated *in vitro* from stem tip and callus cultures. *Plant Dis. Rep.* 52:52-58.
- Pillai, S.K. and A.C. Hilderbrandt. 1969. Induced differentiation of geranium plants from undifferentiated callus *in vitro*. *Amer. J. Bot.* 56:52-58.
- Qinghua, Z. 1993. China's perfumery industry picks up. *Perf. and Flav.* 18: 47-48.
- Qureshi, J.A. and P.K. Saxena. 1992. Adventitious shoot induction and somatic embryogenesis with intact seedlings of several hybrid seed geranium varieties (*Pelargonium xhortorum* Bailey). *Plant Cell Rep.* 11:443-448.
- Rajeswara Rao, B.R., E.V.S.P. Rao, and M.R. Narayana. 1989. Rose geranium: An economical crop in the south Indian Plains. *Ind. Hort.* 36:14-17.
- Ram, M., M.M. Gupta, A.A. Naqvi, and S. Kumar. 1995. Commercially viable annual crop of geranium in northern Indian plains. *Current Res. on Medicinal and Aromatic Plants (now J. Medicinal and Aromatic Plant Sci.)* 17:17-20.
- Satyakala G., M. Muralidhar Rao, and G. Laxmi Sita. 1995. *In vitro* micropropagation of scented geranium (*Pelargonium graveolens* L. Her. Ex. Ait.: *Syn P. roseum* wild). *Curr. Sci.* 68(7):762-765.
- Saxena, G., S. Banerjee, L. Rahman, G.R. Mallavarapu, S. Sharma, and S. Kumar. 2000. An efficient *in vitro* procedure for micropropagation and generation of somaclones of rose-scented *Pelargonium*. *Plant Sci.* 155:133-140.
- Skirvin R.M. and J. Janick. 1976a. Tissue culture. Induced variation in scented *Pelargonium* spp. *J. Am. Soc. Hort. Sci.* 101:281-290.
- Skirvin R.M. and J. Janick. 1976b. "Velvet rose" *Pelargonium*: A scented geranium. *HortScience* 11:61-62.
- Veilleux, R.E. and A.A.T. Johnson. 1998. Somaclonal variations: Molecular analysis, transformation interaction and utilization, p. 229-268. In: J. Janick (ed.), *Plant Breeding Rev.* vol. 16. Wiley, New York.