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

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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 callilones 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 callilones 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-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 requirements 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 Palini hills of southern India (Rajeswara Rao et al., 1992). 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 paraxenous 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; Veileux 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; Hakkart 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

Expant 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) salveon solution (a germicide containing chlorhexidine gluconate 0.3% v/v and cetrimide 0.6% w/v), followed by 0.1% (v/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 photo-period at 35 μmol·m⁻²·s⁻¹ flux density. Observations were recorded at 1-week intervals. After 4 weeks of culture, the percentage of...
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 ten 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 fresh weight, leaf dry weight, number of flowers, number of seeds, and disease resistance (Table 3).

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., 1996). GC-MS analyses were also performed as described earlier (Dunbar and Stephens, 1989) in other species (Dunbar and Stephens, 1989) and that on other media, as revealed by t-test with 95% level of confidence.

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 calli within 12–15 d of culture initiation. The resultant callus was highly friable and yellow in color. Lower amounts of compact and light 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.
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% ± 2.5% shoot regeneration was recorded on medium containing 2.5 mg L⁻¹ BA and 0.1 mg L⁻¹ NAA. The callus cultures on hormone-free solid MS medium and attained a size of 1.5–2.5 cm in 10 d. For studying the differentiation of shoot and root system from the regenerated shoots, the following hormones were used: 1.5 mg L⁻¹ 2,4-D and 0.1 mg L⁻¹ BA; and 0.1 mg L⁻¹ NAA. It is observed that in hormone-free solid MS medium, the root system was formed and root length was significantly lower (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 same medium supplemented with 5.0 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 2,4-D supplementation of MS to 25 mg L⁻¹ BA and 0.1 mg L⁻¹ NAA were healthy, those produced on the same medium supplemented with 5.0 mg L⁻¹ NAA were abnormal and vitrified. Stimulatory effect of 2,4-D in multiple-shoot induction from callus culture of the Egyptian oil–type Pelargonium graveolens cv. Algerian has been reported earlier (Lakshmana Rao, 1994).

Different cytokinins at different levels proved to be effective 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. A maximum of 61.2% ± 2.5% shoot regeneration was recorded on medium containing 2.5 mg L⁻¹ BA and 0.1 mg L⁻¹ NAA. The callus cultures on hormone-free solid MS medium and attained a size of 1.5–2.5 cm in 10 d. For studying the differentiation of shoot and root system from the regenerated shoots, the following hormones were used: 1.5 mg L⁻¹ 2,4-D and 0.1 mg L⁻¹ BA; and 0.1 mg L⁻¹ NAA. It is observed that in hormone-free solid MS medium, the root system was formed and root length was significantly lower (Table 3). Moreover, the plantlet growth on the same medium supplemented with 5.0 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 2,4-D supplementation of MS to 25 mg L⁻¹ BA and 0.1 mg L⁻¹ NAA were healthy, those produced on the same medium supplemented with 5.0 mg L⁻¹ NAA were abnormal and vitrified. Stimulatory effect of 2,4-D in multiple-shoot induction from callus culture of the Egyptian oil–type Pelargonium graveolens cv. Algerian has been reported earlier (Lakshmana Rao, 1994).
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, and 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 homologues 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 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 (Raj 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-cedesmol contents in their essential oils as compared with essential oil of the wild type. The quality of the
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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, somacline B22 is the most productive genotype among the available cultivars in rose-scented geranium for producing essential oil.

![Fig. 3. DNA profiles of selected somaclonal variants and the parent 'Bipul' of rose-scented geranium Pelargonium graveolens: [left to right] lane 1 has molecular weight markers; lane 2 is 'Bipul' control; and lanes 3–7 are 'Bipul' calliclines B3, B6, B13, B22, and B17.](image-url)