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Chemical and Molecular Fingerprinting of Different Cultivars of *Pelargonium graveolens* (L' Herit.) viz., Reunion, Bourbon and Egyptian

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Abstract: The present study was carried out to compare three elite cultivars of *Pelargonium graveolens* viz., Reunion, Bourbon and Egyptian on the basis of chemical and molecular fingerprinting. The germplasm collection of Kelkar Education Trust's Scientific Research Centre has identified a Geraniol rich variety called Reunion which is suitable for commercial cultivation in the plateau region of Maharashtra. Attempts were made to differentiate Reunion cultivar from the other varieties like Bourbon and Egyptian. For this study 6 plants of each cultivar viz., Reunion, Bourbon and Egyptian were randomly selected. Essential oil was extracted using Hydrodistillation method. Chemical fingerprinting was done using GC analysis. Chemically the essential oil of each cultivar showed different composition, among which essential oil of Reunion cultivar was found to have most desirable rosy aroma. For molecular fingerprinting RAPD method was used. Out of 20 primers from Kit OPB, 6 primers were more informative for molecular screening of the three cultivars. Molecular studies revealed the difference among all the three cultivars at genetic level. Thus the three cultivars of *P. graveolens* viz., Reunion, Bourbon and Egyptian were effectively differentiated using chemical and molecular fingerprinting.

Key words: *Pelargonium graveolens*, Hydrodistillation, GC analysis, Chemical fingerprinting, RAPD, PCR, Molecular fingerprinting

INTRODUCTION

Pelargonium graveolens also known as rose scented Geranium (Family-Geraniaceae) is highly valued aromatic plant native to Cape Province of South Africa. It is mainly grown for its essential oil called Geranium oil which has pronounced rose like odour (Bijalwan and Kediyal, 2006). The rosy odour of the oil is primarily due to chief constituents Geraniol and Citronellol often known as rose alcohols that occur in varying proportions depending upon the origin of oil (Mahindru, 1992). There are several other secondary constituents, which also contribute to the olfactory value of commercial Geranium oil, amongst which 6,9-guaidiene and 10-epi-γ-eudesmol are ranked the highest (Saxena *et al*., 2000).

Geranium oil is one of the top 20 essential oils in the world which has wide application in perfumery, cosmetics and flavour industry. It forms a part of many high grade perfumes (Ravindra *et al*., 2004). It also possess insect repellant (Jaenson-Thomas *et al*., 2006), antifungal and antibacterial (Aggarwal *et al*., 2000) properties, making it useful in medicinal and agrochemical field. It has wide application in aromatherapy as well as food product industry (Lawless, 1995).

However, against its own requirement of approximately 200 tonnes, India produces less than 20 tonnes of Geranium oil annually and meets its requirement by imports (Navale and Mungse, 2002). Mild climate with low humidity is ideal for growth of Geranium. On the other hand high humidity, heavy rainfall with mist, fog and water logging are detrimental for its growth (Rajeswara-Rao, 2000).

In India different cultivars of *Pelargonium graveolens* are cultivated in varied agroclimatic zones. Three cultivars of *P. graveolens* namely Algerian (Hemanti), Bourbon (Bipuli) and Egyptian (Kunti) are cultivated commercially (Rajeswara-Rao and Bhattacharya, 1992). Among these cultivars Algerian variety is grown in Northern Plains of Uttar Pradesh while Bourbon variety is recommended for plantation in Southern India especially Nilgiris (Rajeswara-Rao, 2000). These varieties are very difficult to differentiate morphologically. But they could be differentiated by Gas chromatograhy on the basis of relative proportion of Geraniol and Citronellol. Always Geraniol rich varieties are preferred which impart rosy smell to the essential oil.

In Maharashtra the plateau region with low humidity is suitable for Geranium cultivation. Germplasm collection of Kelkar Education Trust's Scientific Research Center,

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Mulund (West), Mumbai, has identified a Geraniol rich variety of *P. graveolens* called Reunion which could be made available to farmers for its commercial cultivation in Maharashtra.

Though the different cultivars are difficult to differentiate on morphological basis, they can be easily differentiated on proportion of Geraniol to Citronellol. Molecular markers have already been successfully used to differentiate the cultivars viz., Algerian, Bourbon and Egyptian (Shasany *et al.*, 2002). In present communication attempts have been made to differentiate Reunion cultivar from rest of the cultivars viz., Bourbon and Egyptian on the basis of chemical and molecular fingerprinting.

MATERIALS AND METHODS

Chemical fingerprinting

Extraction of essential oil: For oil extraction one year old 6 plants of each cultivar viz., Reunion, Bourbon and Egyptian were randomly selected from Medicinal and Aromatic Plant Garden, Kelkar Education Trust's Scientific Research Center, Mulund (West), Mumbai (March 2006-April 2009). Fifty gram of leaf material was used for oil extraction. Oil extraction was done by Hydrodistillation method using Clevenger's apparatus for 6 hrs continuously (Misra *et al.*, 2000). The oil layer was separated in Dichloromethane and was analyzed by Gas Chromatography (GC).

GC analysis of oil: GC was carried out on Varian 3800 Gas Chromatographic apparatus with two FIDs, a Data Handling System and a Vaporizing Injector Port along with Varian Chromatographic Star Workstation Software. Injector Port was equipped with a HP-1 Column cross linked with Methyl Silicon (Length -25 m, Inner Diameter -0.33 μm, Film Thickness -1.05 μm). Port temperature was 250°C. The Gas Oven temperature was programmed as follows: Initial temperature 60°C (1 min) with rise at the rate of 5°C min^{-1} to 250°C. Hydrogen was used as a Carrier Gas at the rate of 1.5 mL min^{-1} with constant volume. The samples were injected using split sampling method in a ratio of 1:50. The composition in relative percentage was computed by the normalization method from the GC peak area. The peaks obtained in graphs of experimental samples were identified by comparison with authentic graphs of Geranium oil of Reunion, Bourbon and Egyptian cultivars (Provided by Library, S.H. Kelkar and Company, Mulund-West, Mumbai).

Molecular fingerprinting

DNA isolation

Plant material: For DNA isolation one year old 6 plants of each cultivar viz., Reunion, Bourbon and Egyptian were randomly selected from Medicinal and Aromatic Plant Garden, Kelkar Education Trust's Scientific Research Center, Mulund (West), Mumbai (March 2006-April 2009). Three gram of fresh leaf material from each plant was used as sample for extracting DNA.

Reagents and chemicals

Extraction buffer: Tris buffer (100 mM, pH 8.0), EDTA-disodium (25 mM, pH 8.0), NaCl (1.5 M), CTAB (Cetyltrimethylammoniumbromide) (2.5% w/v), PVP (Polyvinyl pyrrolidone) (1% w/v) (add just before the use), β-mercaptoethanol (0.2% v/v) (add just before the use).

Other reagents: Chloroform:Isoamylalcohol (24:1), 5 M NaCl, High Salt TE Buffer, 80% Ethanol, RNAse, 1X TAE Buffer.

Extraction and quantification of genomic DNA: DNA was extracted by the method as described (Khanuja *et al.*, 1999). The quality of DNA was checked by running it on 0.8% Agarose gel in 1X TAE buffer containing ethidium bromide (0.5 μg mL^{-1}) at 50V for 4 h. Similarly the quantity of DNA was assessed by loading the samples in a range of 1, 2, 3, 4 and 5 μL. The purity and concentration of the extracted DNA was further measured by UV spectrophotometer (Varian Cary 50) at 260 and 280 nm. Purity was analyzed by absorbance ratios at 260/280 nm.

Amplification conditions

RAPD-PCR conditions: PCR amplification was carried out using 10 oligonucleotide (decamer) primers (Kit OPB) obtained from Operon Technologies Inc., CA, USA. Amplification was performed in a 25 μL reaction volume containing 40 ng genomic DNA, 2.5 μL 1X assay buffer, 1 μL dNTPs mixture, 2 μL primer and 1.2 units Taq DNA polymerase containing 20 mM Tris HCl (pH 8.0), 100 mM KCl, 0.1 M EDTA, 1 M DTT, 0.5% Tween-20 and 50% Glycerol. Reaction mixture was overlaid with 15 μL of Mineral oil to prevent evaporation. All the chemicals required for PCR reactions were purchased from Bangalore Genei, India. The PCR conditions were set as described (Shasany *et al.*, 2002). The PCR reactions were carried out in a thermal cycler programmed for initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min (denaturation), 35° C for 1 min (annealing) and 72°C for 2 min (extention) with final extension at 72°C for 5 min. The hold temperature was maintained at 8°C. After amplification, PCR products were stored at 4°C till electrophoresis.

Visualization of RAPD-PCR products: PCR products were mixed with 5 μL of 10X loading dye [0.25% (w/v) bromophenol blue and 40% (v/v) Glycerol]. The electrophoresis was carried out on 2% Agarose gel in 1X TAE buffer containing ethidium bromide (0.5 μg mL^{-1}) at 120 V for 2 h. A 100 bp DNA ladder was used as a standard molecular weight marker. The RAPD patterns were photographed and stored as digital pictures in gel documentation system.

RESULTS

Morphological differentiation: Morphologically plants of Reunion cultivar showed variation from the rest of the two cultivars viz., Bourbon and Egyptian, which appeared similar to each other. The habit of Reunion cultivar was robust because of the less internodal growth. The leaves of Reunion were thick and less dentate as compared to the thin and heavily dentate leaves of Bourbon and Egyptian (Fig. 1a-c). The leaf lamina of Reunion cultivar was highly pubescent because of heavy growth of glandular hairs.

Chemical fingerprinting: The oil yield was 0.2, 0.4 and 0.6% for cultivar Reunion, Bourbon and Egyptian respectively. It was observed that the yellowish green color of the oil was seen in the increasing order viz., Reunion < Bourbon < Egyptian.

The essential oil extracted was subjected to GC analysis. Gas Chromatogram of essential oil showed almost all major components viz., Geraniol, Citronellol, Rose oxides, Isomenthone, Linalool, 6-9-guiadiene and 10-epi-γ-eudesmol (Fig. 2a-c) as observed in standard oil profile of Geranium oil (Provided by Library, S.H. Kelkar and Company, Mulund-West, Mumbai). The minor components like Geranyl formate, Citronellyl formate, Geranyl tiglate, Citronellyl tiglate, Geranyl propionate and Citronellyl propionate were also present. However, Menthone which was reported in other reports was absent in all the samples.

As seen in Table 1 the Geraniol to Citronellol (G/C) ratio was 1:1, 1:2 and 1:4 in Reunion, Bourbon and Egyptian respectively. The other components that were affecting the rosy note were 6-9-guiadiene and 10-epi-γ-eudesmol. In Reunion 10-epi-γ-eudesmol was totally absent and 6-9-guiadiene was as high as 8.8%. In Bourbon 6-9-guiadiene was comparatively low (4-5%) and 10-epi-γ-eudesmol was also present though in small quantity (0.7-1.7%). In Egyptian amount of 10-epi-γ-eudesmol was as high as 16.7%. Rose oxides were highest in Reunion followed by Bourbon and then Egyptian. Isomenthone content was highest in Bourbon and Egyptian while Linalool was more in Reunion. Minor components like Geranyl tiglate, Citronellyl tiglate, Geranyl propionate and Citronellyl propionate were present in low concentration in all the cultivars. Geranyl formate and Citronellyl formate were present in Reunion and Bourbon whereas in Egyptian they were totally absent.

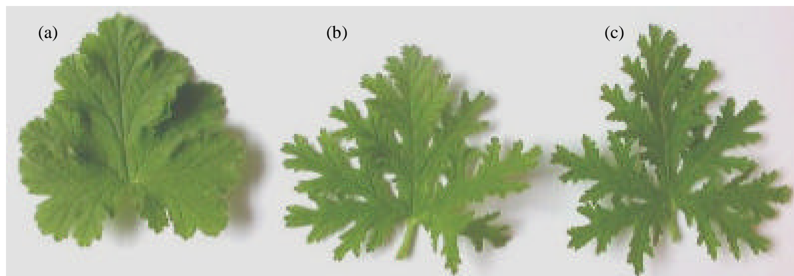

Fig. 1: Difference in leaf morphology of different cultivars of *Pelargonium graveolens* (L' Herit.). (a) Cultivar: Reunion, (b) Cultivar: Bourbon and (c) Cultivar: Egyptian

Table 1: Peak area percentage of different constituents of essential oil extracted from three cultivars of *Pelargonium graveolens* (L' Herit) viz., Reunion, Bourbon and Egyptian

	Reunion						Bourbon						Egyptian					
Components	R1	R2	R3	R4	R5	R6	B1	B2	B3	B4	B5	B6	E1	E2	E3	E4	E5	E6
Geraniol	18.1	18.5	18.6	19.0	19.1	18.0	18.1	19.7	18.6	19.7	19.8	18.8	12.1	12.8	11.7	10.0	11.6	11.9
Citronellol	21.0	21.2	19.4	21.0	20.7	21.7	30.7	34.8	31.1	30.1	30.7	32.1	45.7	39.3	45.1	38.1	42.6	42.8
6,9-guaidiene	8.21	8.8	7.1	7.0	8.1	7.8	4.66	5.1	5.8	5.0	4.1	4.3	8.99	7.5	6.5	8.9	7.6	7.4
10-epi-γ-eudesmol	-	-	-	-	-	-	0.79	0.6	1.1	1.7	0.8	0.9	16.7	12.5	15.1	16.1	15.6	14.6
Rose oxides	2.52	2.1	3.0	2.6	2.9	2.4	1.52	1.1	1.0	1.6	1.1	1.4	-	-	-	-	-	-
Isomenthone	4.12	4.4	3.9	4.1	4.9	4.4	6.65	6.4	6.5	5.9	5.7	5.6	5.73	5.0	5.6	6.7	6.7	5.4
Linalool	5.95	5.18	4.19	5.52	5.21	4.27	3.67	3.95	4.35	4.59	4.39	5.77	2.45	2.71	2.16	2.33	2.87	3.15
Menthone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

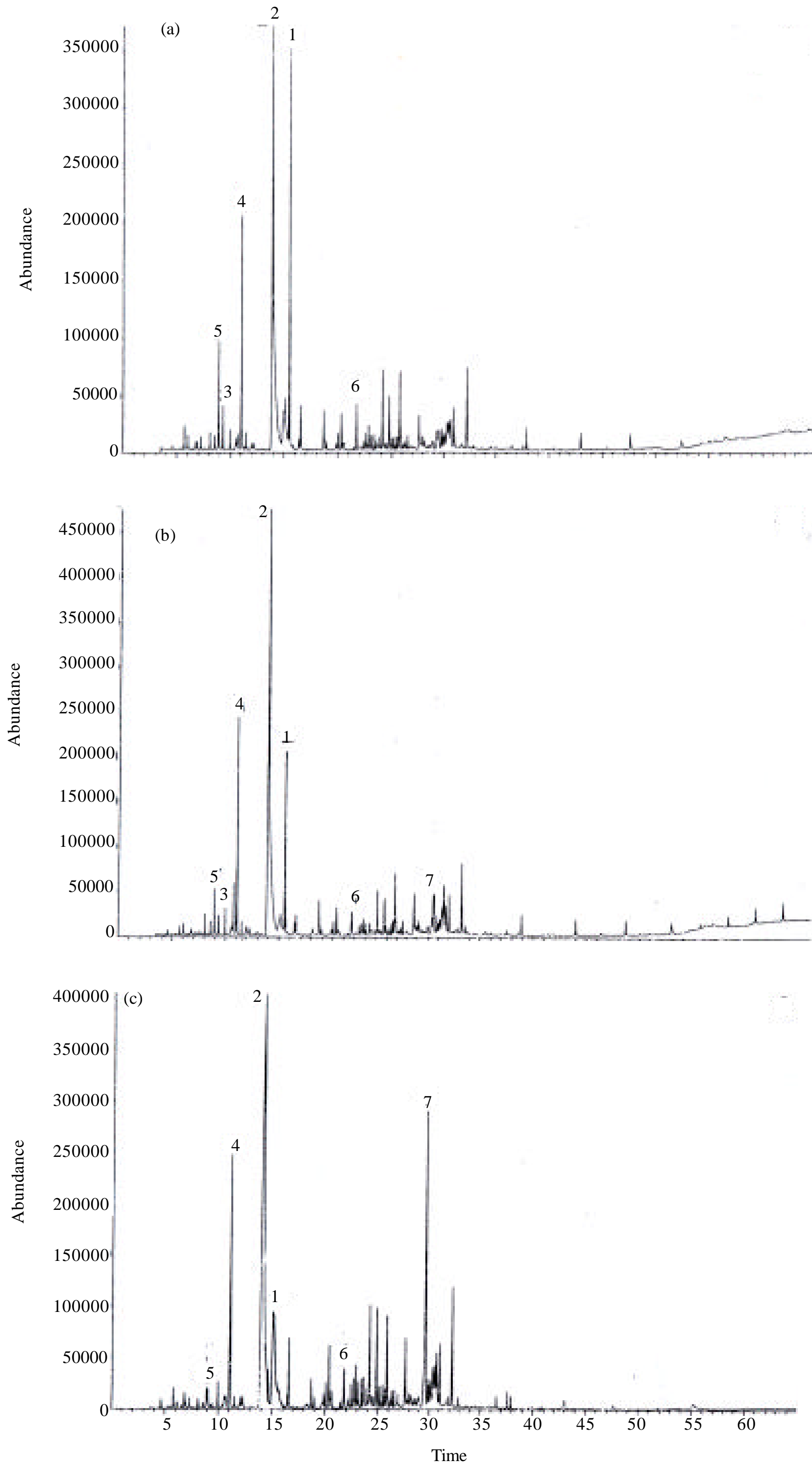

Fig. 2: GC chromatograms of essential oil extracted from leaves of *Pelargonium graveolen* (L' Herit.). Peak Identification: 1. Geraniol, 2. Citronellol, 3. Rose Oxides, 4. Isomenthone, 5. Linalool, 6. 6,9-guaidiene and 7. 10-epi-γ-eudesmol. (a) Cultivar: Reunion, (b) Cultivar: Bourbon and (c) Cultivar: Egyptian

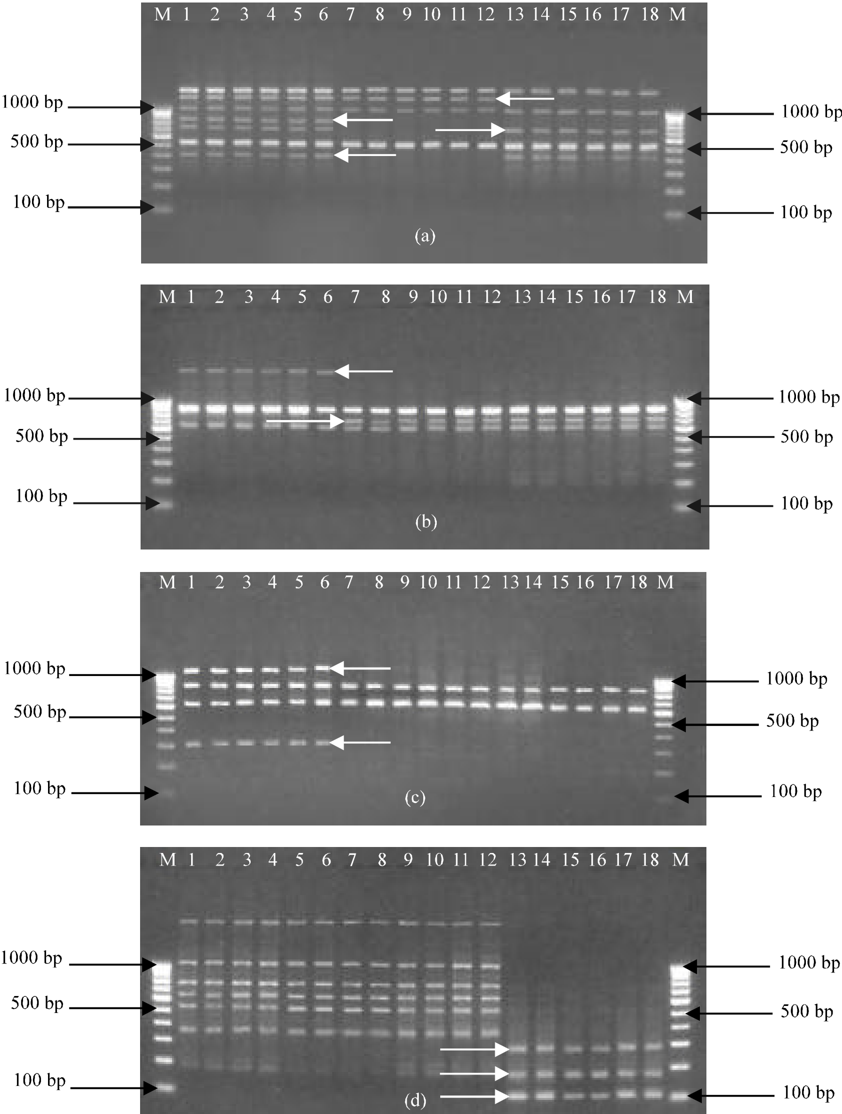

Fig. 3: RAPD pattern of three cultivars of *Pelargonium graveolens* (L' Herit.) viz., Reunion, Bourbon and Egyptian. Lane M: represents 100 bp DNA ladder, Lane 1-6: Cultivar: Reunion, Lane 7-12: Cultivar: Bourbon and Lane 13-18: Cultivar: Egyptian. (a) OPB 19, (b) OPB 13, (c) OPB 04 and (d) OPB 05

Molecular fingerprinting

Extraction of genomic DNA: The purity and concentration of DNA was assessed visually as well as by absorbance ratios. The absorbance ratio at 260/280 nm for all the DNA samples was between 1.8-2.2 that proved the purity of extracted DNA sample. Similarly the visual analysis showed that 2 μL of each DNA sample corresponds to 80 ng of DNA.

RAPD-PCR analysis: Initially 30 plants of each cultivar were grown in pots out of which 6 plants were selected randomly for molecular fingerprinting. For this study 20 primers from Kit OPB (Operon Technologies Inc., CA, USA) were used. Out of these 20 primers, 15 primers could successfully produce amplification for all the three cultivars. Out of 15 primers, 9 primers showed monomorphic banding patterns for all the three cultivars while 6 primers namely OPB 04, OPB 05, OPB 12, OPB 13, OPB 16 and OPB 19 were found to be more informative in differentiating the cultivars. OPB 12, OPB 16 and OPB 19 produced unique fragments for each cultivar.

Figure 3a-d represent the RAPD amplification patterns generated by Primers viz., OPB 19, OPB 13, OPB 04 and OPB 05, respectively.

- Lane M represents 100 bp DNA ladder
- Lane 1-6 represents Reunion Cultivar
- Lane 7-12 represents Bourbon Cultivar
- Lane 13-18 represents Egyptian Cultivar

Three primers namely OPB 12, OPB 16 and OPB 19 could differentiate between the three cultivars. As seen in Fig. 3a with OPB 19 bands at 550, 1000 and 1200 bp were seen in all the cultivars. In Reunion a specific band at 900 bp was seen which represents as a marker for Reunion (Lane 1-6), it was absent in other two cultivars (Lane 7-12 and 13-18). A band of 1100 bp was present in both Reunion (Lane 1-6) as well as Bourbon (Lane 7-12) and was absent in Egyptian (Lane 13-18). Similarly bands at 400 bp and 700 bp were present in Reunion (Lane 1-6) as well as Egyptian (Lane 13-18) and were absent in Bourbon (Lane 7-12).

Two primers namely OPB 04 and OPB 13 were useful in differentiating Reunion from other two cultivars. As seen in Fig. 3b with OPB 13 two bands at 600 and 900 bp were seen in all the three cultivars. A specific band at 700 bp was seen in Bourbon (Lane 7-12) and Egyptian (Lane 13-18) cultivars but was totally absent in Reunion (lane 1-6). Similarly a band at tentatively 1400 bp was seen only in Reunion (lane 1-6) whereas it was totally absent in rest of the 2 cultivars. With OPB 04 specific bands at 300 and 1000 bp were seen in Reunion (Lane 1-6) but they were totally absent in Bourbon (Lane 7-12) and Egyptian (Lane 13-18) cultivars (Fig. 3c).

One primer OPB 05 differentiated Egyptian from rest of the two cultivars. As seen in Fig 3d with OPB 05 Egyptian (Lane 13-18) showed three unique bands at 100, 200 and 300 bp which were totally absent in Reunion (Lane 1-6) and Bourbon (Lane 7-12) cultivars.

DISCUSSION

Morphological differentiation: Morphologically it was difficult to differentiate between Bourbon and Egyptian cultivars. Cultivar Reunion could be differentiated on the basis of pubescent, thick and less dentate leaves.

Chemical fingerprinting: The three cultivars could be differentiated on the basis of their oil profile. In Egyptian the ratio of Geraniol to Citronellol (G/C) was almost 1:4 which imparted citrusy note to the cultivar. In Bourbon the proportion of Geraniol increased and that of Citronellol decreased with G/C ratio of 1:2. In Reunion the proportion of Citonellol still decreased to about 20% and G/C ratio appeared to be almost 1:1 that imparted characteristic rosy note to the cultivar which is indicative of better odour quality desired in the market. The other component that were affecting the rosy note were 6,9-guaidiene and 10-epi-γ-eudesmol in a desirable range of 0.2-4.5 and 0-2.4%, respectively (Saxena *et al.*, 2008). In Reunion, 10-epi-γ-eudesmol was totally absent and 6,9-guaidiene was as high as 8.8%. This contributed to desired rosy note of the oil. In Bourbon, 6,9-guaidiene was comparatively low (4-5%) and 10-epi-γ-eudesmol was also present though in small quantity (0.7-1.7%). In Egyptian, amount of 10-epi-γ-eudesmol was as high as 16.7% which imparted undesirable citrusy note to the oil. Rose oxides were highest in Reunion followed by Bourbon and then Egyptian.

Molecular fingerprinting: Morphologically Reunion cultivar appears to be different from Bourbon and Egyptian which appear similar to each other. This was supported by the banding pattern produced by OPB 04 and OPB 13, which produced unique profile for Reunion cultivar. When the essential oil composition is considered, Reunion is closer to Bourbon, possessing sweet rosy note. At molecular level, out of 20 primers used from Operon Kit OPB, only one primer OPB 05 could show the similarity between Reunion and Bourbon. On the whole all the three cultivars show certain degree of variation from each other at morphological and chemical level which is strongly supported by molecular screening. Primers OPB 12, OPB 16 and OPB 19 produced unique profiles that could differentiate all the three cultivars at genetic level. A similar work was carried out where Algerian or Tunisian (Hemanti), Bourbon or Reunion (Bipuli) and Kelkar or Egyptian (Kunti) cultivars of *P. graveolens* were successfully differentiated by RAPD analysis (Shasany *et al.*, 2002). A total 120 primers were used from Kit MAP, OPA, OPB, OPJ, OPO and OPQ. Among these primers used 16 primers were more informative in differentiating three cultivars. Out of these 16 primers, 12 could generate unique banding patterns for at least one cultivar and 2 could successfully differentiate all the three cultivars. However, they treated Reunion and Bourbon as the same cultivar (Shasany *et al.*, 2002). Our studies revealed that Reunion and Bourbon are two different cultivars which showed variation at morphological, chemical and molecular level.

Thus chemical and molecular fingerprinting could be used as an effective tool to differentiate the three cultivars of *P. graveolens* viz., Reunion, Bourbon and Egyptian which will help in supply of genetically pure germplasm.

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