MOLECULAR EVALUATION AND MICROPROPAGATION OF FIELD SELECTED ELITES OF *R. DAMASCENA*

N. Kaur[1]*, R. K. Sharma[1], M. Sharma[2], V. Singh[2], P. S. Ahuja[1]

[1]40 Bee St. Apt. 318, Charlesto, South Carolina-29403-58, Division of Biotechnology,
[2] Natural Plant Product, Institute of Himalayan Bioresource Technology (CSIR) Palampur-176061 (H.P.), India

Received 8 May 2007

Summary. Molecular characterization of field selected elites of *R. damascena* is important in order to establish the genetic differences of the selections to protect the IPRs as well as to ascertain the genetic nature of the clones. Fifty eight informative primers were chosen for evaluation of the genetic diversity among six selected elites of *R. damascena*. In total 368 fragments were generated of which 43 were polymorphic representing 11.68 % polymorphism with selected primers. One decamer primer OPV4 (CCCCTCACGA) was identified that could distinguish 6 oil-rich varieties of *R. damascena* screened through field selections of existing germplasm of rose. This is because the primer OPV-4 might be amplifying a highly repeated genomic region, probably the microsatellite region of the rose genome. The protocol for micropropagation developed by Pati et al. (2001) was successfully adopted for multiplying the elite selections of *R. damascena* Mill. from cultivars Jwala and Himroz: IHBT 1, IHBT 2, IHBT 3 and IHBT 4 needed for continuous supply of the selected elites to the farmers.

*Corresponding author, e-mail: navtej2209@yahoo.co.in

***Key words**:* field selections, micropropagation, RAPD, scented rose, variety

Abbreviations: MS - Murashige and Skoog, PGRs - plant growth regulators, BA-6 -benzyl adenine, IBA - indole butyric acid, PCR - polymorphic chain reaction, RAPD -random amplified polymorphic DNA.

INTRODUCTION

Rose is one of the most important ornamental and aromatic crops and represents a major commodity in the commercial market of the floriculture as well as the essential oil industry. The flavour and fragrance of rose is unmatched and most sought after in the food and cosmetic industry. Rose oil, rose water, rose concrete and rose absolute are some of the most important products that are in great demand. Among the 200 species of roses distributed throughout the temperate regions, only a few are scented (Gudin, 2000). These include *Rosa damascena* Mill., *R. gallica* Linn., *R. centifolia* Linn., *R. bourboniana* Desportes., *R. chinensis* Jacq., *R. moschata* Herrm. and *R. alba* Linn. There are mainly four species of roses for oil production. These are *Rosa damascena* Mill., *R. gallica* Linn., *R. centifolia* Linn. and *R. moschata* Herrm. (Tucker and Maciarello, 1988). Amongst them, *R. damascena* is generally preferred for its highly prized rose oil, which is commonly used in perfumery, cosmetics, beverages, soft drinks, ice-creams, and as a fragrance component in ointments and lotions etc. (Douglas, 1993).

Accurate identification of clones, cultivars and varieties, and knowledge of their genetic interrelationships are essential for breeding, variety control and registration, stock handling and the protection of plant breeders' rights. Therefore, the molecular markers have been applied in various studies to evaluate the genetic relationships in roses (Hubbard et al., 1992; Torres et al., 1993; Debener et al., 1996, Agaoglu et al., 2000 and Baydar et al., 2004).

The present study was aimed to investigate the genetic relationships among six field selected elites of *R. damascene*: IHBT 1, IHBT 2, Jwala

and three of *R. damascena* var. Himroz: IHBT 3, IHBT 4, Himroz using RAPD markers. The micropropagation of these varieties was carried out for continuous supply of the stock to the farmers.

MATERIALS AND METHODS

Plant Material

A population of approximately 50000 plants raised from mixed stem cuttings collected from perennial rose plantations at the University of Agriculture, Udaipur, Rajasthan and maintained in the field of the Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh were utilized as an original gene pool of *R. damascena*. Two varieties, Jwala and Himroz were diversified through selections of desirable traits (morphological/oil content) across 25000 plants. The six elites, three of *R. damascena* var. Jwala (IHBT 1, IHBT 2, Jwala) and three of *R. damascena* var. Himroz (IHBT 3, IHBT 4, Himroz) were developed through field selections and maintained at the Natural Plant Product Experimental Farm of the Institute.

RAPD analysis

Isolation of genomic DNA and quantification

Genomic DNA was isolated from young leaves collected and transported in an ice box from the field to the laboratory and subsequently ground in liquid nitrogen using a mortar and pestle. Total DNA was isolated according to the protocol described by CTAB method (Doyle et al, 1990). Plant material (1g) was homogenized in liquid nitrogen and transferred to 50 ml polypropylene centrifuge tube containing 15 ml of pre-warmed (65 ^{o}C) DNA extraction buffer. The suspension was incubated for 1 h at 65 ^{o}C. The mixture was emulsified with an equal volume of chloroform:iso-amyl alcohol (24:1) for 5-10 min by inversion followed by centrifugation at 15,000 rpm for 15 min. The aqueous phase was transferred to a fresh centrifuge tube with a wide bore pipette and 0.6 volume of iso-propanol

was added to it and mixed by gentle inversion. The precipitated DNA was washed twice with 70 % alcohol. The pellet was dried under vacuum and dissolved in 1 ml of TE buffer. Total DNA was purified and its quality and quantity was varified spectrophotometrically as well as using 0.8 % agarose gel with uncut λ DNA as a standard.

PCR conditions for RAPD analysis

Decamer oligonucleotides (Operon Technologies Inc.USA), Taq DNA polymerase and its corresponding buffer (Bangalore Genei, India) (10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01 % gelatin) and 200 µM each of dNTPs (MBI Fermentas) were used for the PCR reaction in a final volume of 25 µl. All DNA amplifications were carried out in a thermal cycler (Gene Amp 9700; Perkin Elmer, USA), which was programmed for 1 cycle of 4 min at 94 °C followed by 45 cycles of 1 min at 94 °C, 1 min at 37 °C and 2 min at 72 °C. The last extension cycle was programmed at 72 °C for 7 min. The PCR products were resolved in 1.5 % agarose gel after staining in ethidium bromide and photographed using a gel documentation system.

Data analysis

The amplified products were scored as present (1) or absent (0) for each hybrid. The data entry was done into a binary data matrix as discrete variables. Jaccard's coefficient of similarity was calculated and a dendrogram based on the similarity coefficient was generated using an unweighed pair group method based on arithmetic means (UPGMA) through the computer package NTSYS-PC 2.0e (Rohlf,1998).

Initiation of aseptic cultures from nodal explants

For initiating aseptic cultures, nodal segments (2.5-3.0 cm) were excised from six field selected elites of *R. damascena*. After removal of leaves and thorns, explants were thoroughly washed with a liquid detergent (Labolene, Ranbaxy, India) using a sable-hair brush and then treated with

an aqueous solution of 0.1 % Bavistin and 0.1 % streptomycin sulphate for about 30 min on a horizontal shaker (100 rpm) and rinsed with running tap water. Thereafter explants were surface sterilized with 70 % alcohol for 2 min before repetitive washings in sterile distilled water. The explants were finally exposed to 0.04 % (w/v) HgCl solution treatment with a drop of liquid detergent for 10-15 min and again washed thoroughly with sterile distilled water in a laminar flow cabinet. On both ends, a few mm portions exposed to the sterilant were removed and explants were placed upright in test tubes containing half strength MS medium supplemented with sucrose (3 %, w/v) and agar (0.8 %, w/v). After 4 weeks the sprouted shoots were transferred to a BA (2.0 mg/l) supplemented MS medium (liquid and solid) for multiple shoot proliferation. The cultures were regularly sub-cultured at a 4-week interval.

Rooting and hardening of micro shoots

To achieve *in vitro* rooting of microshoots a half-strength MS liquid medium supplemented with IBA (2.0 mg/l) and sucrose (3.0 %) was used as reported earlier (Pati et al., 2000). Cultures were initially kept in dark for one week. Later on, the microshoots were transferred to liquid MS medium without PGRs and with the same concentration of sucrose for 3-4 weeks.

The rooted microshoots were taken out of the culture vessels and then washed thoroughly in lukewarm water before they were transferred to Hikko trays/pots containing sand in the hardening chamber for 3 weeks and later on transferred to a greenhouse for further growth.

RESULTS

Variey identification by DNA fingerprinting

Six new varieties of Damask rose were evaluated for productivity and quality profile of the oil (Table 1). IHBT 4 recorded a maximum number (228.36) of flowers per plant but the average flower weight was maximum for IHBT 1. The highest flower yield per plant as well as flower yield per hectare were recorded from IHBT 4 with an oil content of 0.052 %. The

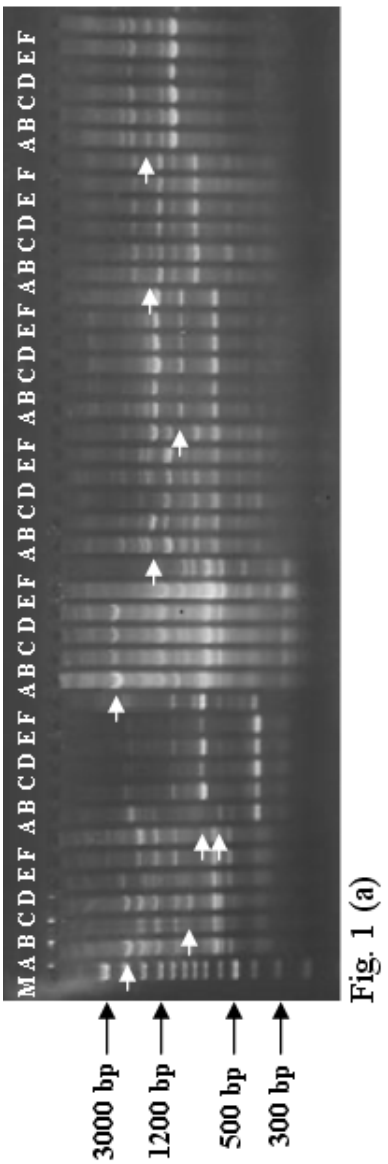

Fig. 1 (a)

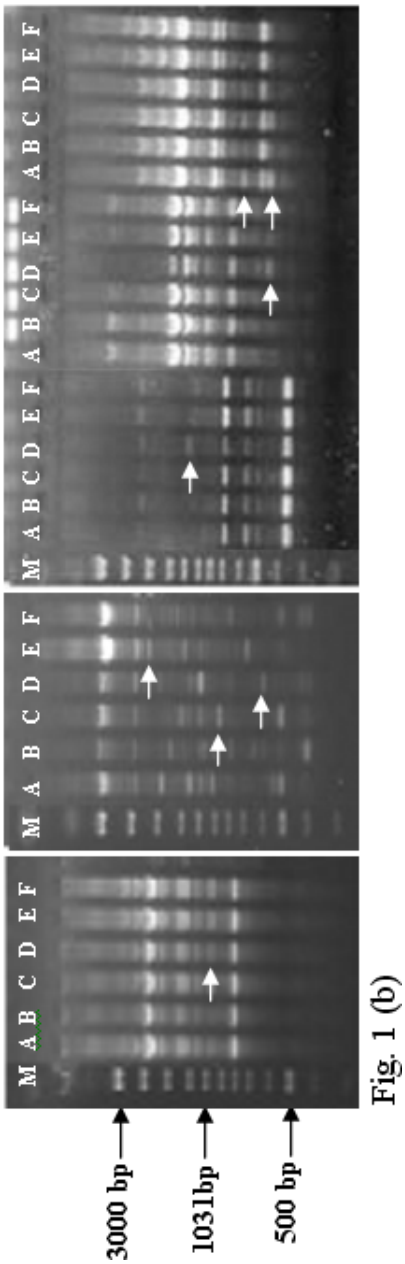

Fig. 1 (b)

Fig. 1. (a-b): RAPD profile for 6 oil-rich rose varieties using primers (a) OPV-7, OPV-9, OPV-10, OPV-12, OPS-1, OPS-5, OPS-6 (b) OPS-15, OPV-4, OPM-4, OPM-8, OPM-14 where M represents 100 bp DNA ladder, A-IHBT 1, B-IHBT 2, C-Himroz 3, D-IHBT 3, E-IHBT 4, F-Jwala.

maximum content was recorded in IHBT 3.

RAPD analysis

A total of 180 primers of ten different operon series (OPA1-20; OPC1-20; OPE1-20; OPF1-20, OPG1-20; OPM1-15; OPO1-20; OPS1-20; OPV1-20; OPT1-5) were screened across three genomic lines belonging to two different species: *R. damascena* and *R. bourboniana*. Fifty eight RAPD primers were found to be evincing polymorphism among the two species and were utilized for DNA fingerprinting and evaluation of genetic diversity across the 6 oil rich varieties of *R. damascena*. Among 368 loci, which were scored, 43 (11.68 %) were polymorphic. The size of bands varied between 300 and 2800 bp. One decamer primer CCCCTCACGA of OPV4 series was identified as the best RAPD marker that could distinguish between the varieties studied (Fig. 1b). The number of products generated by each primer varied from a minimum of 1 with OPS-8 to a maximum of 17 with OPV-4 with an average of 6 bands per primer. The distribution of the amplified fragments in six varieties of *R. damascena* is presented in Table 2. At least 43 markers were found to be informative to develop a fingerprint for the varieties studied. A representative profile of RAPD primer distinguishing rose varieties is shown in Fig. 1. Based on the RAPD data, we could develop fingerprints which could give details of a minimum number of primers to be used for the identification of these varieties. Fingerprints based on 43 fragments across the field selected elites of *R. damascena* are shown in Fig. 3.

IHBT 1	IHBT 2	Himroz	IHBT 3	IHBT 4	Jwala
1.0000000					
0.9488636	1.0000000				
0.9291785	0.9230769	1.0000000			
0.8954802	0.9000000	0.9186047	1.0000000		
0.9070423	0.9171429	0.9360465	0.9294118	1.0000000	
0.8972222	0.9178470	0.9255014	0.9077810	0.9475219	1.0000000

Fig. 2. Jaccard's similarity values in 6 oil-rich varieties based on RAPD data.

Cluster analysis

RAPD marker data were used to construct a dendrogram on the basis of shared fragments. It is evident that 6 oil rich varieties of rose developed through field selections fall in 2 major clusters (Fig. 4). Cluster I includes IHBT 1, IHBT 2 and Himroz. IHBT 1 showed 92 % and 94 % similarity with Himroz and IHBT 2, respectively. Cluster II involves IHBT 3, IHBT 4 and Jwala. The similarity matrix ranged from 90 % between Jwala and IHBT 3 to 94 % between Jwala and IHBT 4. The reported genetic similarity across IHBT 3 and group I was 0.90 and 0.92 with IHBT 4. Jwala shared 0.91 similarity with group I. IHBT 1 and IHBT 3 developed through the field selections of Himroz and Jwala showed 89 % similarity, suggesting that the level of diversity across them was very low (11%) (Fig. 2). In conclusion, the primers (OPV-4, OPV-7, OPV-9, OPV-10, OPV-12, OPS-5, OPS-6, OPM-8, OPM-14) were suitable to generate polymorphism and the information generated was of importance in registration of these field selections as novel genotypes.

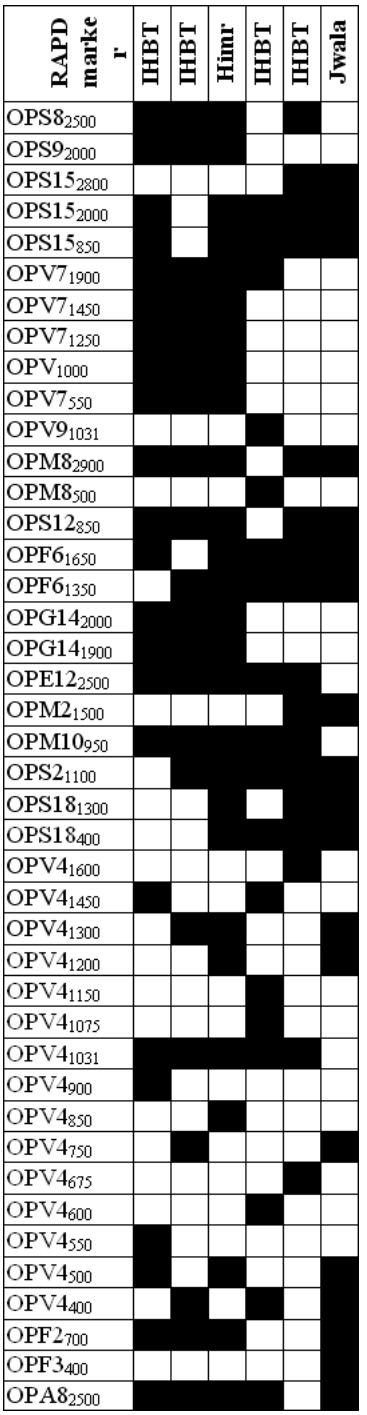

Fig. 3. Diagrammatic representation of fingerprint for 6 field selected elites of *R. damascena* based on 43 RAPD markers.

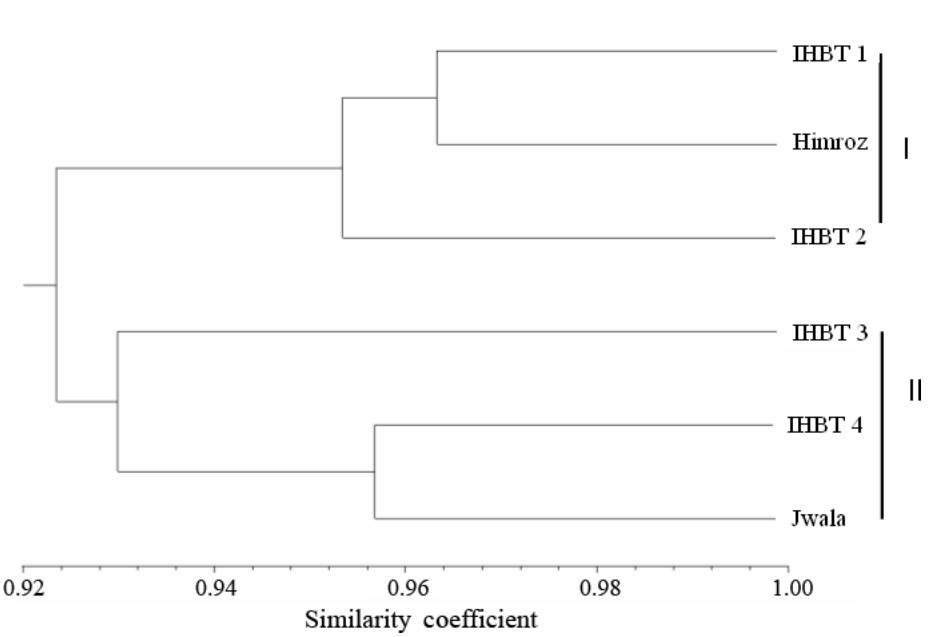

Fig. 4. Dendrogram for 6 oil-rich varieties of rose. The scale indicates Jaccard's similarity coefficient.

Micropropagation of selected elites

Nodal segments of field selected elites of *R. damascena cvs.* Jwala and Himroz namely IHBT 1, IHBT 2, Himroz, IHBT 3, IHBT 4, and Jwala could be successfully established in ½ MS medium supplemented with 3.0 % sucrose. The axillary bud proliferated after 2-3 weeks of inoculation. Such proliferated buds were transferred to MS medium supplemented with BA (2.0 mg/l). Shoot proliferation started after 2 weeks of culture. Proliferated shoots were regularly sub-cultured onto fresh medium at a 4-weeks interval. Proliferating shoots were transferred to a liquid MS medium supplemented with BA (2.0 mg/l). In such cases 20 ml medium per 250 ml flask was used. There was an increase (by 6.08 %) in the liquid static culture medium in case of IHBT 3 followed by IHBT 1 (5.84 %) and Jwala (5.52 %) (Table 3). Shoots turned brown in case of agar gelled medium while in liquid medium shoots were healthy and showed high multiplication rate after 4 weeks

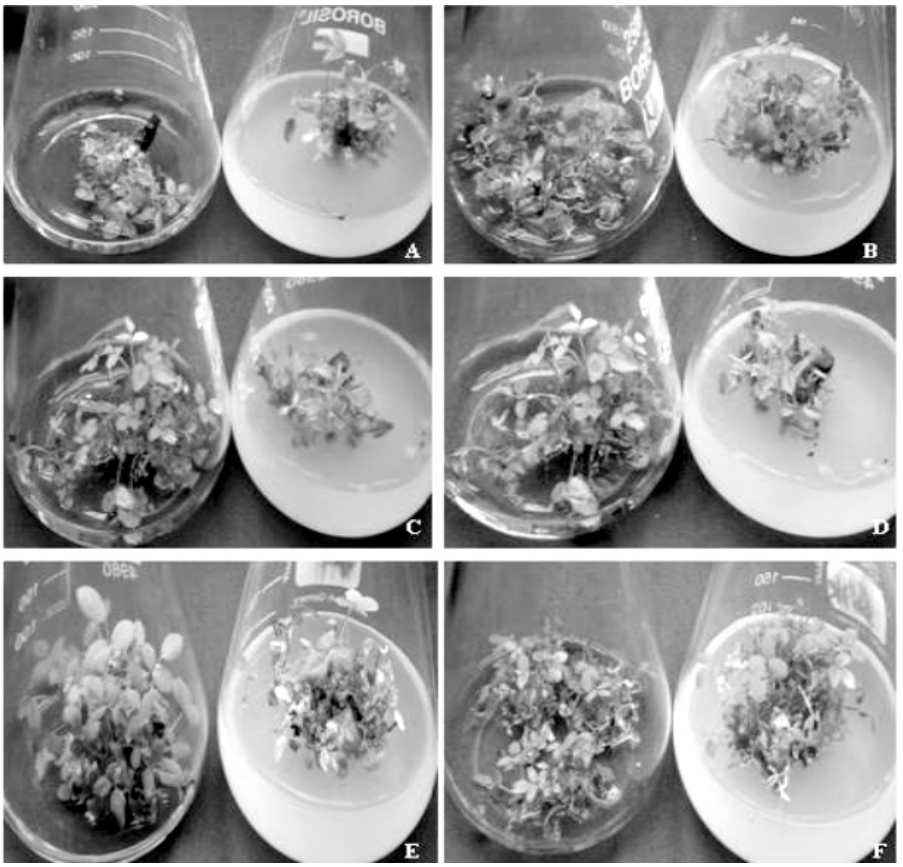

Fig. 5. Comparison of growth of six field selected elites of R. damascena var. Jwala and Himroz on liquid and agar gelled MS media supplemented with BA (2.0 mg/l) where A represents IHBT 1, B- IHBT 2, C-Himroz and D-IHBT 3, E- IHBT 4 and F- Jwala.

(Fig. 5). The results largely confirm the earlier findings of Pati et al. (2001), however, in the present study differences in micropropagation and potential were observed for different genotypes. For root induction microshoots were inoculated on a half-strength MS liquid medium supplemented with IBA (2.0 mg/l) and sucrose (3.0 %) as reported earlier (Pati et al., 2000). Cultures were initially kept in the dark for one week. Later on, the microshoots were transferred to a liquid MS medium without PGRs and with the same

Table 1. Comparative performance of improved strains of *R. damascene.*

Code	Cultivars	Average flowers (No./plant)	Average flower weight (g)	Flower yield (g/plant)	Oil content (%)	Flower yield (q/ha)
A	IHBT 1	133.83	2.66	355.62	0.040	41.43
B	IHBT 2	101.19	2.43	246.14	0.049	28.68
C	Himroz	183.24	2.38	435.20	0.052	50.70
D	IHBT 3	170.57	2.27	387.77	0.055	45.18
E	IHBT 4	228.36	2.51	573.64	0.052	66.83
F	Jwala	207.69	2.41	501.28	0.047	58.40

concentration of sucrose. Root initiation was observed after 7 days of culturing in IHBT 1 and IHBT 3 followed by 8 days in IHBT 2 and IHBT 4 and 9-10 days for Himroz and Jwala. The shoots with a well-developed root system were available after 4 weeks of maintenance on PGR-free medium at 25±2 °C. The rooted microshoots were taken out of the culture vessels and transferred to pots containing sand and kept in a green house covered with plastic jars for 3-4 weeks, and subsequently transferred to bigger pots filled with sand:soil (1:1) and maintained under green house conditions. In the greenhouse 80-85 % shoots with a stem diameter of 2.0-2.5 mm, shoot length of 5.0-5.5 cm and root length of 5.5-7.0 cm were established.

DISCUSSION

Variety identification by DNA fingerprinting within *R. damascene*

Genetic analysis reduces ambiguities that can arise when examining morphological properties. Although many techniques exist to study DNA, RAPD is a sensitive method that is widely used to differentiate between plant cultivars/varieties/clones (Hu and Quiros, 1991; Rani et al., 1995 and Wang et al., 1994; Iwata et al., 2000).

Among 368 loci, which had been scored, 43 (11.68 %) were found polymorphic across 6 oil rich varieties of *R. damascena* screened through field selections of existing germplasm of rose. The polymorphic fragments generated by OPV4 primer can be identified as RAPD markers that could distinguish all the 6 selected clones from each other. This is possibly due

Table 2. Distribution of amplified fragments in 6 oil-rich varieties of rose.

Parameters	Detail
Total number of primers screened	180
Number of primers producing polymorphism	58
Total number of loci scored	368
Total number of polymorphic loci	43
Total number of monomorphic loci	325
Size of amplified band	300-2800 bp
Average number of polymorphic fragments per primer	6
Percentage of total bands which are polymorphic	11.68%
*Data pooled from studies on 6 oil-rich varieties of *R. damascena*	

to the primer OPV-4 (CCCCTCACGA) amplifying a highly repeated genomic region, probably the microsatellite region, of the rose genome. Due to the hypervariability of this region, the primer could distinguish six closely related field selections of *R. damascena*. This is in contrast to an earlier investigation with RAPD markers where no variability could be detected among *R. damascena* plants (Ağaoğlu et al., 2000). These authors did not employ the primer OPV-4. Narrow genetic variation (>4 %) between the two vars. of *R. damascena* (Jwala and Himroz) may be due to the development of these varieties through field selections from the already existing clones of *R. damascena*. However, the plants can be distinguished by morphological characters, which may be due to point mutations not detected by the existing molecular markers (Baydar et al., 2004). Previous observations have shown that mutant sprouts with distinguishable mutated phenotypes show identical DNA marker patterns to the single mutations leading to the altered phenotypes (Debener et al., 2000).

Since Himroz as well as Jwala originated from the old accessions at the Udaipur Agriculture University, Rajasthan, there was a high degree of similarity within and between the groups except that there were minor

Table 3. Comparison of shoot multiplication rate of different genotypes on a MS medium with BA (2.0 mg/l).

Code	Cultivars	Multiplication Rate (%) after 4 weeks on	
		Agar gelled	Liquid
A	IHBT 1	3.58±1.48	5.84±1.64
B	IHBT 2	2.52±1.14	3.60±1.58
C	Himroz	2.72±1.81	4.36±1.92
D	IHBT 3	3.04±1.51	6.08±1.14
E	IHBT 4	2.40±1.73	3.60±1.87
F	Jwala	2.44±1.30	5.52±1.81

adaptation differences at the phenotypic levels. Furthermore, flowering behaviour and flower productivity varied within the groups as is evident from the morphological data. This phenotypic variation could be explained by differential expression of certain structural genes regulated by epigenetic changes. The existence of such a correlation between changes in the methylation state of certain gene sequences and the expression of a mutant phenotype has been clearly shown by Cubas et al. (1999). It was important for us to use molecular analysis to establish the genetic differences of the selections, so as to protect the IPRs around the selections as well as to ascertain the genetic nature of the clones.

Micropropagation of selected elites of *R. damascene*

The protocol for micropropagation developed by Pati et al. (2001) was successfully adopted to achieve the objective of multiplying the elite selections of *R. damascena* Mill. from cultivars Jwala and Himroz: IHBT 1, IHBT 2, IHBT 3 and IHBT 4. This protocol demonstrates the positive effect of BAP (2.0 mg/l) included in the static liquid MS medium on multiple shoot proliferation. This is comparable with earlier results (Hasegawa, 1980; Wulster and Sacalis, 1980), showing that inclusion of BAP (1.0-10.0 mg/l) in the agar solidified culture medium was essential for bud break and shoot multiplication of *R. hybrida*. Bressan et al. (1982) reported maximum promotive effect with BAP as compared to 2-isopentyladenine (2-iP). The inclusion of auxins at low levels neither enhanced nor repressed shoot

multiplication regardless of the BAP concentration. Vijaya et al. (1991) reported that BAP was the most effective growth regulator in stimulating shoot proliferation. This work facilitated the early stocking of elite selections for further distribution to farmers.

***Acknowledgements*:** Financial assistance from the University Grant Commission is fully acknowledged.

References

Ağaoğlu, Y.S., Ergül, A. and Baydar, N. 2000. Molecular analysis of genetic diversity oil rose (*Rosa damascena* Mill.) grown Isparta (Turkey) region. Biotechnol. Biotechnol. Eq., 14, 16-18.

Baydar, N.G., Baydar, H. and Debener, T. 2004. Analysis of genetic relationships among *Rosa damascena* plants grown in Turkey by using AFLP and microsatellite markers. J. Biotech., 111, 263-267.

Bressan, P.H., Kim, Y.J., Hyndman, S.E., Hasegawa, P.M. and Bressan, R.A. 1982. Factors affecting in vitro propagation of rose. J. Amer. Soc. Hortic. Sci., 107, 979-990.

Cubas, P., Vincent, C. and Coen, E. 1999. An epigenetic mutation responsible for natural variation in floral symmetry. Nature, 401, 157-161.

Debener, T., Bartels, C. and Mattiesch, L. 1996. RAPD analysis of genetic variation between a group of rose cultivars and selected wild rose species. Mol. Breed., 2 (4), 321-327.

Debener, T., Janakiram, T. and Mattiesch, L. 2000. Sports and seedlings of rose varieties analysed with moleculr markers. Plant Breed., 119, 71-74.

Douglas, M. 1993. Rose-*Rosa damascena* 'Trigintipetala'. Crop and Food Research., pp. 1-5.

Doyle, J. J., Doyle, J. L. 1990. Isolation of plant DNA from fresh tissue. Focus., 12, 13-15.

Gudin, S. 2000. Rose: Genetics and breeding. In: Janick, J. (ed.), Plant Breeding Reviews, vol. 17, John Wiley and Sons, Inc. pp.159-189.

Hasegawa, P.M. 1980. Factors affecting shoot and root initiation from cultured rose shoot tips. J. Amer. Soc. Hort. Sci., 105, (2), 216-20.

Hubbard, M., Kell, J., Rajapakse, S., Abbott, A. and Ballard, R. 1992. Restriction fragment length polymorphism in rose and their use for cultivar identification. Hort. Sci., 27, 172-173.

Hu, J. and Quiros, C.F., 1991. Identification of broccoli and cauli flower cultivars with RAPD markers. Plant Cell Rep., 10**,** 505-511.

Iwata, H., Kato, T. and Ohno, S., 2000. Triparental origin of Damask roses. Gene, 259, 53–59.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant.,15, 473–497.

Pati, P.K., Sharma, M. and Ahuja, P.S., 2001. Micropropagation, Protoplast Culture and its implications in the improvement of scented roes. In: Proceedings of the Third International Symposium on Rose Research and Cultivation. Acta Hort., 547, 147-158.

Rani, V., Parida, A. and Raina, S.N., 1995. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. Plant Cell Rep., 14**,** 459–462.

Rohlf, F. J., 1998. NTSYS-PC Numerical Taxonomy and Multivariate Analysis System, version 2.0. Exeter Software, Setauket, NewYork.

Torres, A.M., Millan, T. and Cubero, J.I., 1993. Identifying rose cultivars using random amplified polymorphic DNA markers. Hort. Sci., 28 (4), 333-334.

Tucker, A.O. and Maciarello, M., 1988. Nomenclature and chemistry of the Kazanlak Damask rose and some potential alternatives fron the horticultural trade of North America and Europe. In: Flavors and Fragrances: A world Perspective. Elsevier, Amsterdam. pp. 99-114.

Vijaya, N., Satyanarayana, G., Prakash, J. and Pierik, R.L.M., 1991. Effect of culture media and growth regulators on *in vitro* propagation of rose. Curr. Plant Sci. Biotech. Agric., 12, 209-214.

Wang, G., Castiglione, S., Zhang, J., Fu, R., Ma, J., Li, W., Sun, Y. and Sala, F., 1994. Hybrid rice (*Oryza sativa* L.): identification and parentage determination by RAPD fingerprinting. Plant Cell Rep., 14**,** 112–115.

Wulster, G. and Sacalis, J., 1980. Effects of auxins and cytokinins on ethylene evolution and growth of rose callus tissue in sealed vessels. Hort. Sci., 15, 736-737.