IDENTIFICATION OF MOLECULAR MARKERS FOR DEVELOPING BREEDING STRATEGIES IN ROSE

By

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THESIS

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DECLARATION

I, Cinu Sebastian (2004-11-32) hereby declare that this thesis entitled **'Identification of Molecular Markers for Developing Breeding Strategies In Rose'** is a bonafide record of research work done by me during the course of research and this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Certified that this thesis, entitled **'Identification of Molecular Markers for Developing Breeding Strategies In Rose'** is a record of research work done independently by **Ms. Cinu Sebastian** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Cinu Sebastian

Affectionately dedicated to my beloved parents and my only brother

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ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µM	Micromole
BAP	Benzyl Amino Purine
cm	Centimeters
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DAP	Days after pollination
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxy Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
IAA	Indole Acetic Acid
M	Mole
mg/l	Milligram per litre
ml	Millilitre
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
nm	Nanometer
NTSYS	Numerical Taxonomy System of Multivariate Statistical Programme
°C	Degree celcius
OD	Optical Density
p mols	Picomoles
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
ppm	Parts per million
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
rpm	Revolutions per minute
TAE	Tris Acetate EDTA
Taq	Thermus aquaticus
TE	Tris EDTA
U	Unit
UPGMA	Unweighted Pair Group Method of Arithmetic Averages
UV	Ultra violet
v/v	Volume by volume
w/v	Weight by weight
kb	Kilo base

INTRODUCTION

1. INTRODUCTION

Rose has enthralled mankind, captured his imagination and taken roots in his thoughts ever since man first beheld it and inhaled it's scent. Known as the 'Queen of flowers' the rose is a pure enchantment suggesting beauty, pleasure and delight. Added to beauty, rose also offers a delightful fragrance, which has made it important throughout the world.

Rose is an important member of the Rosaceae family. Ancestors of modern roses are wild roses that grow over wide areas in the world. Roses are being cultivated from ancient times in France, Cyprus, Greece, India, Iran, Italy, Morocco, USA and Bulgaria for the production of perfume. Modern garden roses, developed from the wild species, came from France. Later England, Germany, Belgium, Holland and other countries also became important producers. The other rose growing countries are China, Japan, Burma, USA, etc. (Pal, 1991).

In India, several species are found growing wild mostly in the Himalayan ranges. Among these are *Rosa webbiana*, characteristic of the dry Kashmir and Western Himalayan belt and the moisture loving *R. gigantea* which is found in the extreme eastern portion of the range, especially in Sikkim, Meghalaya, Manipur and areas of Burma and South West China. Other interesting wild roses of the Himalayas are two members of the Musk rose complex *R. brunonii* found in Nepal and Garhwal areas of Himalayas and *R. longicuspis* found in the Khasia and Mishmi Hills of Eastern India. Both have a characteristic fragrance. The third member of the Musk rose is the southern *R. leschinaulliana* which is found in Nilgiri and Palani Hills of South India. Other wild roses found in the Himalayas include the pink *R. webbiana*, as well as the four petalled white flower *R. serica*. *R. foetida*, the yellow rose of Iran, is found in similar climatic belt of Kashmir. *R. ecae*, also with bright yellow flowers, is grown as hedge around Kargil. *R. clinophylla* can grow throughout the Indian plains, especially in the plains of Bengal, near streams and marshy places.

As far as the cultivated garden roses are concerned, only eight species have played an important role. They are *R. chinensis*, *R. damascena*, *R. foetida*, *R. gallica*, *R. gigantea*, *R. moschata*, *R. multiflora* and *R. wichuraiana*. Perpetually flowering forms derived from *R. chinensis* and *R. gigantea* were crossed with new introductions which gave rise to important groups of Noisettes and Bourbons. Further complex crossing gave rise to the Tea roses and Hybrid Perpetuals. The Tea roses are prized for their continuous flowering habit and Hybrid Perpetuals for their big size, rich perfume and hardy nature. A distinct group of roses known as Polyanthas Pompons or Dwarf Polyanthas developed parallel to Hybrid Teas from the species *R.polyantha* (now called *R. multiflora*). Continous bloom and hardiness are it's advantages. The Floribunda group which emerged later on by hybridization between the Dwarf Polyantha roses and Hybrid Teas, became highly popular for producing large quantities of blooms with better shape. The Persian rose and Edouard (*R. borboniana*) are extensively cultivated in Uttar Pradesh, Rajasthan, Punjab and Tamil Nadu.

Although rose breeding in India began in 1935 and the first variety was developed by Roy Choudhary from a cross between 'Hedley' and 'Fascination' known as 'Dr. S.D. Mukherjee', rose has received little attention at the hands of geneticists. Among the named varieties, many otherwise desirable roses are female sterile and hence pose a real limitation to breeding. The range of variability, which is of potential value in the development of new varieties, is enormous in roses, but most of it remains unexploited. This wealth of untouched material has to be used meaningfully by planning crosses in a way that will avoid unproductive hybrids and thus save time, effort and resources. Hence developing molecular markers that can readily identify a fertile female parent will be a boon to rose breeders.

Roses in nature are usually cross pollinated by insects, especially bees, which are attracted by the colour and scent. Many are not aware that roses bear fruits which contain seeds. If flowers are not cut off, the lower rounded portion below the calyx lobes will swell and develop into a fruit called the 'hip' or 'hep' which turns orange or red when ripe. Not all varieties develop them. Hip formation is particularly profuse in some of the

wild roses. Seeds from naturally formed rose fruits may give a variable progeny so that even without resorting to artificial crossing or hybridization, new forms may be obtained.

Lammerts (1946) reported that the breeding of roses is occasionally hampered by premature abortion of the developing embryo resulting in few or no viable seeds. Lack of germination is due to mechanical restriction of embryo expansion by the presence of a thick pericarp or dormancy regulated by the growth inhibitors within the achene. Therefore embryo rescue is very useful in the production of rose hybrids.

Commercial cultivation of roses for cut flower has increased during the last three decades. The major rose producing regions in India are Karnataka, Maharashtra, Punjab, Uttar Pradesh, Delhi, Gujarath, Haryana, Himachal Pradesh, Madhya Pradesh, Rajasthan, Tamil Nadu and West Bengal (Yadav *et al*.,1989).

In this context, present study has been undertaken with the following objectives

- To develop RAPD based molecular markers for identification of elite parents.
- To standardize embryo rescue techniques for regeneration of rose hybrids.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 INTRODUCTION

The present study aims at the identification of molecular markers for developing breeding strategies in rose and attempting embryo rescue for regeneration. A comprehensive literature relevant to the present study is reviewed in this chapter.

2.2 BOTANY

Rose belongs to the family *Rosaceae* and genus *Rosa*. It is an ornamental shrub with upright or climbing stems, usually prickly. The leaves are alternate, compound, oddly pinnate with stipules adherent to leaf stalks. Flowers are solitary or in corymbs. Calyx is five lobed, lobes either simple or compound and inserted at the top of a roundish or pear shaped fleshy tube. Petals and sepals are generally five but many more in cultivars due to transformation of stamens. It has numerous carpels, inserted at the base of calyx tube, free and with simple projecting style and stigma. The ripe fruit (hip or hep) consists of many one-seeded carpels in a fleshy tube and the seeds are hard (Yadav *et al.*, 1989).

2.3 DISTRIBUTION

Ancestors of modern roses are wild roses that grow over wide areas in the world. Roses are being cultivated from ancient times in France, Cyprus, Greece, India, Iran, Italy, Morocco, USA and Bulgaria for the production of perfume. Modern garden roses, developed from the wild species, came from France. Later England, Germany, Belgium, Holland and other countries also became important producers. The other rose growing countries are China, Japan, Burma, USA, etc. (Pal, 1991).

In India, several species are found growing wild mostly in the Himalayan ranges. The Persian rose and Edouard (*R. borboniana*) are extensively cultivated in Uttar Pradesh, Rajasthan, Punjab and Tamil Nadu. Commercial cultivation of roses for cut flower has increased during the last three decades. The major rose producing regions are Karnataka, Maharashtra, Punjab, Uttar Pradesh, Delhi, Gujarath, Haryana, Himachal Pradesh, Madhya Pradesh, Rajasthan, Tamil Nadu and West Bengal (Yadav *et al*.,1989).

2.4 *IN VITRO* TECHNIQUES IN *ROSA*

There are several routes of *in vitro* propagation in rose. They include embryo culture, somatic organogenesis, suspension cultures and enhanced release of axillary buds.

2.4.1 Embryo culture

The first tissue culture of rose established by Lammerts in 1946 was embryo culture. Asen and Larson (1951) have detailed their procedure for rose embryo culture. Rose has two sets of seed coat and both are involved in dormancy. They safely removed the outer seed coat by soaking them for 9-17 hours in Cross and Bevans solution (1:2 Zinc chloride: Hydrochloric acid w/w). The inner seed coat which is water impermeable must be broken to permit germination.

The potential use of embryo culture to rescue a particular hybrid embryo, which otherwise abort is one of the goals of a rose breeder. Another practical use is the rapid seed germination, which can take place *in vitro*. Von and Hand (1956) found that when seeds of a fertile cross were planted in soil, 66.9 per cent of seeds germinated after 180 days. However if seeds were planted on embryo germination medium, 98 per cent germination occurred in 14 days. Graifenberg (1973) has reported that when the achene of *R. canina* is broken and the naked embryo is excised and placed on Knudson C medium, embryo grows quite well.

2.4.2 Anther culture

Tabaeezadeh and Khui (1981) studied the response of anther of two *Rosa* species to various levels of auxins and cytokinins at different stages and light conditions. MS with 2.0 mg/l IAA and 0.4 mg/l kinetin was generally best for anther culture of *Rosa damascena* while medium with 7.5 mg/l IAA and 0.8 mg/l kinetin was optimum for *Rosa hybrida*. Both species produced more callus in the dark than in light, at the stage of bud development.

2.4.3 Protoplast culture

Strauss and Potrykus (1980) isolated protoplasts from cell suspension cultures and achieved sustained deviation of protoplast by plating them on agar solidified medium. The first change in plated protoplast was deviation from spherical shape due to cell wall synthesis on the third day at 27^0C. Later, micro and macro colonies developed and were plated to form calli. Efficient protoplast culture procedures have been worked out from cell suspensions by Pati *et al.* (2001).

2.4.4 Callus culture

Rout *et al.* (1991) reported successful *in vitro* regeneration of shoots from callus cultures of *Rosa hybrida* L. cv. Landora. Adventitious shoot buds differentiated within 5-6 weeks, by subculturing calli on modified induction medium consisting of ½ MS with 0.2 mg/l BA, 0.01 mg/l NAA, 5, 10 or 20 GA_3 and 600 mg/l of either L proline or L glutamine. Regenerated shoots were successfully rooted in ten days on liquid medium with half MS basal salts and 0.1 mg/l NAA.

2.4.5 Somatic embryogenesis

Rout *et al.* (1989) developed the protocol for the induction of somatic embryogenesis in callus cultures of *Rosa hybrida* cv.Landora. Friable callus was

obtained from immature leaf and stem internode segment on MS medium supplemented with 0.5 mg/l BAP, 1.0 mg/l NAA, 0.5 –2.0 mg/l 2,4-D and 30 mg/l sucrose. Somatic embryos were initiated on half strength MS supplemented with 0.5 mg/l BAP, 0.01 mg/l NAA, 0.1 mg/l GA_3 and various concentrations of l-proline (200-800 mg/l). But the embryos showed abnormalities in shape, structure and number of cotyledons.

Rout *et al.* (1991) observed that some of the somatic embryos were morphologically normal showing distinct cotyledons and radicals. The embryos were loosely attached to the mother callus with short suspensor like structures at the basal end. Somatic embryos also arose from the basal region of other embryos in clusters indicating a clear case of secondary somatic embryogenesis. On sub culturing the somatic embryos on a regeneration medium 12 per cent of the embryos elongated and formed leaves but failed to develop into a plantlet. Somatic embryogenic callus was initiated from *in vitro* derived explants on MS medium with 2,4-D or NAA and several further subcultures on MS media with TDZ (Dohm *et al.*, 2001).

The effects of different concentrations of TDZ, BA and abscisic acid alone or in combinations, on proliferation and germination of secondary embryos were evaluated by Li *et al.* (2002). Highest frequency of somatic embryogenesis was observed on medium containing either 2.3μ mol/l TDZ, 2.9 μmol/l GA_3 or a combination of 2.9 μmol/l GA_3 with either 2.3 μ mol/l TDZ or 2.2 μmol/l BA.

2.4.6 Micropropagation

Propagation *in vitro* is a rapid and cheaper method producing flowering rose. Success has been made using explants such as shoot apices (Hasegawa, 1979), axillary buds (Bressan *et al.*, 1982; Rout *et al.*, 1989). Dormant axillary buds were used as explants for *in vitro* propagation of *Rosa hybrida* cv. Landora (Rout *et al.*, 1989). Early bud break was noticed in BA supplemented media. Incorporation of GA_3 at low concentrations (0.25- 0.50 mg/l) in this medium showed 90-95 per cent improved

response. Axillary buds nearest to or furthest from the apex either failed to or showed longest time to develop compared to those in the middle portion of the shoot (Bressan *et al.*, 1982). Shoot proliferation of the cv. Forever Yours was achieved using a modified MS medium supplemented with BA at 2.0 and NAA at 0.1 mg/l. Shoots rooted easily on quarter strength MS media and later transferred to soil (Skirvin and Chu, 1979). Shoot tips and lateral buds of cv. Improved blaze proliferated multiple shoots with 3.0 mg/l BA and 0.3 mg/l IAA (Hasegawa, 1979). Micropropagation protocols using nodal segments were established in R. damascene and R. bourboniana (Pati *et al.*, 2001).

2.5 ROSE BREEDING

Rose, which is one of the most important commercial cut flower crops, is usually propagated by budding or grafting. Breeding programs in rose are focused on the improvement of various characteristics to enhance the ornamental value, including the colour, size, form and keeping quality of the bloom and reactions of plants to the environment. Although, desirable traits were introduced by classical breeding, there were limitations to this technique; firstly because of limited gene pool; secondly, distant crosses were limited by incompatibility or difference in ploidy level between putative parents and thirdly, characters such as uniform growth and synchronous flowering were polygenic (Rout *et al.*, 1999).

2.5.1 Incompatibility, cross compatibility and sterility studies

Banerjee (1969) reported the possibility of physiological factors being responsible for sterility in case of polyantha rose. Shahare and Shastry (1963) found structural hybridity in roses to be the common factor responsible for high pollen sterility in rose. Pal (1991) was of the opinion that hybrid sterility is an important factor conditioning the development of new types. Meenakshi (1977) found cross incompatible cultivars like 'Super Star' where sterility is considered to be a genetically controlled character. Highly female sterile and self-compatible varieties were also found.

Pal (1991) stated that amateur rose breeder's problem is whether the crossed seed obtained by them is likely to be viable or not. Khalatkar (1992) has done scanning of rose pericarp by electron microscope and has shown that achene pericarp and the endocarp thickness can determine germination. The thickness is controlled by environmental factors like temperature during maturation of achenes and genetic factors influencing rate of embryo development since they are of hybrid origin.

2.5.2 Polyploidy and cytological abnormalities

The hybrid produced on crossing two parents in interspecific mating may not always be fertile. Cytological studies (Hurst 1941) indicated cause of male and female sterility. The basic chromosome number n = 7 and many of the spp. like *R. moschata*, *R. gigantea, R. multifora, R. wichuriana* and *R. chinensis* are diploid 2n = 14 while species like *R. gallica, R. foetida, R. damascena* and *R. centifolia* are tetraploids with 2n = 28. They were fertile and gave rise to hybrid perpetuals with crosses between *R. damascena.* In the study at I.A.R.I., a cluster flowered hyper triploid (2n = 22 or 21 + 1) or trisomic 'Mohini' has been obtained by crossing a diploid and tetraploid while its sister seedling 'Prema' is a normal triploid. Many species are diploid (2n = 14), tetraploid (2n = 28), hexaploid (2n = 42) and octaploid (2n = 56). Crossing between tetraploids of European origin and diploids of Asian origin has resulted in triploids and aneuploids.

2.5.3 Pollination and seed set

Banerjee (1969) observed that in diploid cv. 'Chattilon' pollination, fertilization and embryo development followed normal pattern until 22 to 24 days after pollination. Thereafter flowers dried up and development ceased. Lata (1971) made crosses among six strongly fragrant hybrid tea varieties of garden roses and reported that all these tetraploid varieties are mainly self-incompatible. Seed setting and female sterile varieties were seen in intervarietal crosses.

2.6 EMBRYO RESCUE TECHNOLOGY

Embryo culture is the sterile isolation and growth of an immature or mature embryo *in vitro,* with the goal of obtaining a viable plant. Mature embryo culture utilizes ripe seeds whereas immature embryo culture or embryo rescue is the culture of immature embryos. Embryo rescue is mainly used to avoid embryo abortion caused by pre and post fertilization barriers (Chawla, 2002). Embryo abortion is normally encountered in the seeds of unsuccessful crosses. Although fertilization occurs normally in such crosses and embryos begin to develop in a relatively normal way, a number of irregularities subsequently set in, resulting in the eventual death of embryos and collapse of seeds.

Artificial culture methods are applied to breeding work to make it possible to bring to maturity anomalous individuals, which might otherwise fail to develop into mature plants and subsequently to perpetuate them by horticultural methods of vegetative propagation. Works have been conducted in culturing the embryos of apple, pear, peach, plum and rose.

2.6.1 Recovery of hybrid embryos in rose and other crops

Embryo rescue techniques are most often used in order to obtain inter specific progenies (Bajaj *et al.*, 1982). Sometimes, when embryo abortion and or fruit abscission is very early, ovule culture and even ovary culture have to be performed.

The pioneer work in embryo culture was of Tukey (1932; 1933) who isolated and cultured immature embryos of early ripening varieties of sweet cherry and peach. In roses, although mature embryo culture has been described (Lammerts, 1946), immature embryos were not used. Where no progeny was obtained by conventional means, because of poor hip set (2 %) and no mature seed germination occurred, Gudin (1994) reported that plantlets were obtained by embryo culture. Burger *et al.* (1990) used immature embryos as explant source for *in vitro* organogenesis and plant regeneration.

Artificial embryo culture has been successfully done by earlier workers like Hanning in *Cruciferae* and Dietrich in *Cruciferae* and *Graminae* (Chawla, 2002). *In ovulo* embryo culture was found to be the most promising method for the rescue of immature tulip embryos. Improved seedling and bulblet formation were also observed (Custers *et al.*, 1992). Interspecific hybridization has been reported in the genus *Arachis* through embryo culture (Bajaj *et al.*, 1982). Interspecific hybridization between perennial *Medicago* species using ovule culture followed by embryo culture has been successful in recovering hybrid embryos which would not mature *in vivo* (Bauchan, 1987). Rajashekhar *et al.* (2003) has reported *in vitro* culture of interspecific hybrid ovules and embryos of cotton. Interspecific hybrids of genus Helianthus were obtained using immature embryos as explants (Tiwari and Tripathy, 2004).

2.6.2 Factors affecting embryo rescue.

2.6.2.1 Dormancy

A major problem in rose breeding programmes is that of seeds (achenes) failing to germinate or exhibiting a low percentage germination. Lack of germination is usually attributed to a mechanical restriction for embryo expansion by the presence of a thick pericarp. Several workers Asen and Larson, (1951); Lammerts, (1946); von Abrams and Hand, (1956) have reported that the dormancy of rose seed is conditioned by inhibiting effects of the seed coat. von Abrams and Hand, (1956) have conducted a detailed five year study indicating that embryo is generally complete and that its germination potential was not influenced by climatic variation encountered. Semeniuk *et al.* (1963) confirmed the role of seed coat in imposition of dormancy. Jackson and Blundell, (1963) showed that extracts from achenes inhibited germination of *Rosa arvensis*. Marchant *et al.* (1994) confirmed that low germination percentage of achenes is not solely limited to the presence of pericarp or testa as a physical barrier. They suggested the complete removal of pericarp and testa which produced a diffusible substance that suppressed embryo germination.

2.6.2.2 Stage of embryo

Gudin (1994) noticed that further development was obtained only when the rose embryos had reached the heart shaped stage before they were isolated. Mohapathra and Rout (2005) used 0.25 mm long immature embryos for their experiments.

Tukey (1932) observed that cherry embryos 5 to 6 mm in length showed best results. Maheshwaran and Williams (1986) reported that somatic embryoids were induced from superficial cells of the hypocotyl region of torpedo stage of sexual embryos of *Trifolium repens*.

2.6.2.3 Surface sterilization

Aseptic procedures are to be followed in the excision of embryos and their transfer to nutrient medium. Since the embryos are lodged in sterile environment of the ovule, surface sterilization of the embryos, as such, is not necessary. Instead entire ovules, seeds or capsules containing ovules are sterilized and embryos aseptically freed from the surrounding tissues. Splitting open the seeds and transferring embryos to the nutrient medium is the simplest technique that can be used with seeds. Procedures for the isolation of comparatively smaller embryos require that they can be removed intact from ovules without damage. This can best be achieved by carrying out the operations under a dissection microscope (Raghavan, 1976).

According to Marchant *et al.* (1994) intact rose hips were washed in running water for at least an hour after which the hips were surface sterilized by immersion in 0.1 per cent mercuric chloride solution for fifteen min followed by thorough rinsing with sterile distilled water. The hips were opened under aseptic conditions using forceps and scalpel and the achenes removed. Achenes, awaiting dissection were placed in sterile distilled water to prevent dessication. The pericarp was removed by making lateral cuts and the thin papery testa was also removed. Mohapathra and Rout (2005) has established a protocol for sterilization in floribunda roses. Rose hips were washed in 1 per cent v/v detergent Teepol solution and subsequently washed with tap

water for 30 min. This was followed by surface sterilization with 0.1 per cent w/v mercuric chloride for 30 min and rinsing with autoclaved water.

2.6.2.4 Culture requirements

The most important aspect of embryo culture work is the selection of the medium necessary to sustain continued growth of embryos. Gudin (1994) tried six media containing Murashige and Skoog (1962) macro and micro elements (MS) and sucrose at different concentrations and found that the medium characterized by the highest mineral salt and lowest sugar concentration of all media tested in the experiment (MS, 2 % sucrose) gave the best results.

Marchant *et al.* (1994) found that no significant differences were observed between the percentage germination values of embryos cultured on media containing MS salts and vitamins (Murashige and Skoog, 1962) compared to those cultured on media containing the mineral salts described by Asen and Larson (1951).There was no significant difference between germination on media containing 3.0 mg/l BAP compared to those lacking this growth regulator. Germination of embryos on media containing monosaccharides, glucose and fructose was significant compared to their counterparts sucrose and maltose. Mohapathra and Rout (2005) recommends a combination of BA (2.5 mg/l) with GA_3 (0.5 mg/l) than BA alone. There was no sign of development of immature embryos in MS media without growth regulators. However, Kathryn *et al.* (2005) reported that maximum maturation and conversion of embryos occurred when callus was cultured on MS medium without growth regulators. Omission or lowering the concentration of 2,4-D from culture medium helped embryo development and germination as reported in many cultures of *Rosa* species (Mathews *et al*., 1991).

Burger *et al* .(1990) reported induction of adventitious shoot buds in the callus derived from immature embryos of *Rosa hybrida* cv. Bridal pink (female parent) and several *Rosa hybrida* cultivars (pollen parent) on a modified half strength MS media supplemented with 1.0 mg/l BA and 0.5 mg/l NAA .

2.6.2.5 Media oxidation

In rose, problems of browning could be overcome by iniating the cultures in dark for a day or two after inoculation, because the polyphenol oxidase activity was found to be induced by light (Rout *et al.*, 1999). Studies of Mohapathra and Rout (2005) also revealed that the cultures initially incubated in dark for two weeks and subsequently transferred to light at 16 hour photoperiod had high germination percentage.

2.7 MOLECULAR MARKERS

The genus *Rosa* contains more than 120 species and numerous interspecific hybrids. Many morphological and cytological traits were described for the majority of species and their hybrids. The genus has been divided into four subgenera (Hulthemia, Platyrhodon, Hesperrodos and Eurosa) and several botanical sections. A better knowledge of the genetical resources available in the genus *Rosa* is necessary before undertaking any genetic improvement in order to use plant material in breeding programs.

Breeding within taxonomic groups of garden roses will not produce resistant varieties since many traits are found singly in distantly related species and cultivars (Svejda, 1975). New types of resistant varieties can be developed only through hybridization of distantly related species and cultivars, which are in diploid and tetraploid groups. Hence knowledge about the fertility status of parent cultivars is also essential. Direct crosses between diploid resistant rose species and tetraploid roses result in sterile triploids. Thus characterization of ploidy level is important, as no exhaustive identification key exists. Chromosome counting in cells in division is a reference method to determine ploidy level of plants but it is slow and expensive when numerous plants are to be studied. Estimation of ploidy levels on a cheaper, quicker and large scale is possible with flow cytometry technique. Due to variability between genotypes, flow cytometry has not always permitted to determine the ploidy level precisely (Jacob *et al.*, 1996). When two distantly related diploid resistant species are

crossed they also result in sterile diploids although chromosome numbers are the same (Ballard *et al.*, 1996). Fertile amphiploids can be produced from sterile diploid hybrids by doubling chromosome number using colchicines. Nevertheless, these artificial amphidiploids only have low to moderate fertility and progeny of amphidiploids segregated for a wide range of fertility (Byrne *et al.*, 1996). Identification of elite parents and their characterization can be done using molecular markers.

2.7.1 The Polymerase Chain Reaction (PCR) and the Random Amplified Polymorphic DNA (RAPD)

Mullis and Faloona (1987) demonstrated that oligonucleotide primers could be used to rapidly amplify specific segments of DNA. This technology was later known as PCR technique. Also known as molecular photocopying, PCR exploits the remarkable property of natural polymerase enzymes to copy the genetic material, which can be either DNA or RNA. The technique is useful in detecting polymorphism but the PCR based polymorphism assay known as Amplified Sequence Polymorphism (ASP) requires target DNA sequence information for the design of amplification primers (Skolnick and Wallace, 1988). The time and cost of obtaining this sequence information is prohibitive for many large scale genetic mapping applications.

Williams *et al.* (1990) described a new DNA polymorphism assay based on the amplification of random DNA sequence with single primers of arbitrary nucleotide sequence. The primers detected polymorphism in the absence of specific nucleotide sequence information and the polymorphism, which functioned as genetic marker was called RAPD marker after Random Amplified Polymorphic DNA. Their results suggested that the minimum useful primer length was an oligonucleotide of nine bases and a GC content of 40 per cent or greater was required to generate detectable levels of amplification products. They also reported that single base changes in the arbitrary primer could cause a complete change in the set of amplified DNA segments.

Welsh and Mc Clelland (1990) reported that simple and reproducible fingerprints of complete genomes could be generated using single arbitrarily chosen primers and the polymerase chain reaction. The method was called arbitrarily primed

PCR (AP-PCR) and involved two cycles of low stringency amplification followed by a PCR at higher stringency.

The quality of genomic DNA is a major factor that affects the reproducibility of RAPD patterns by affecting the primer annealing. Different workers have adopted different protocols for extraction of genomic DNA from rose. Debener and Mattiesch (1996) reported the extraction of DNA according to the protocol of Doyle and Doyle (1987). Ballard *et al.* (1996) followed the protocol using CTAB (Roger and Bendich, 1994) for the extraction of DNA for RAPD analysis. Aloisi and Bollereau (1996) extracted DNA by the procedure derived from Maroof *et al.* (1984). Micheli *et al.* (1994) reported that ethanol precipitable contaminants like low molecular weight DNA and RNA in the genomic DNA preparation alter the formation of productive template-primer complexes and hence influence the reproducibility of RAPD patterns.

Stift *et al.* (2003) did the comparative study of RAPD fragment separation in agarose and polyacrylamide gels. They found that better resolution of the bands and more number of polymorphic bands are obtained in polyacrylamide gels.

Cubero *et al.* (1996) suggested that RAPD was advantageous over RFLP as it required lower cost, equipment and supplies, had higher speed of analysis, required minimal quantities of DNA and had high degree of polymorphism.

2.7.2 Identification of cultivars using molecular markers

Rosa cultivars are normally identified by visual descriptions of numerous morphological traits. This methodology is both labor intensive and time consuming. It also depends on environment fluctuations and human judgement. Quick and unambiguous method independent of environmental conditions was provided by molecular analysis.

Kuhnz and Fretz (1978) studied the isozyme banding patterns from six enzyme systems for eight rose cultivars. Differences in growth habit among the cultivars and

different optimum locations for enzymes posed difficulties in setting a standard location. Results indicated that all cultivars could be distinguished by difference in their enzyme banding patterns if results from several systems were employed.

Molecular level markers have been used for cultivar identification purposes with RAPD's by Torres *et al.* (1992), with RFLP's by Hubbard *et al.* (1992), Rajapakse *et al.* (1992), and by DNA fingerprinting by Tzuri *et al.* (1992) and Vanstein and Meir (1994).

Tzuri *et al.* (1991) reported DNA fingerprinting analysis of ornamental plants. Minisatellite and microsatellite DNA probes were hybridized to many loci in rose, gerbera and carnation DNA which enabled cultivar identification in these ornamentals.

Restriction fragment length polymorphisms (RFLP) were investigated as a means of identifying rose cultivars for patent protection by Rajapakse *et al.* (1992). They identified 15 probes that displayed RFLP useful in cultivar identification and a total of 16 cultivars have been distinguished.

Five rose cultivars were analysed by Torres *et al.* (1993) using random amplified polymorphic DNA (RAPD) markers. Using eight primers, all cultivars were distinguished by comparing the differences in DNA banding patterns.

Vainstein and Meir (1994) reported the use of mini and micro satellite probes to hybridize DNA of 24 rose genotypes. The resultant DNA fingerprints were shown to be genotype specific, thereby enabling cultivars identification at DNA level. Full-sib family analysis of DNA fingerprints revealed 32 parental specific bands out of the 128 observed in parents.

Cubero *et al.* (1996) reported varietal identification in *Rosa* by using isozyme and RAPD markers. The results indicated that RAPD technique was more reliable than isozyme studies. Five cultivars were analysed using ten enzyme systems and distinguishable banding patterns were observed only in one variety. The remaining

four cultivars had the same banding patterns. However, with only eight primers, all the cultivars could be distinguished by comparing differences in DNA banding patterns using RAPD technique.

RFLP and RAPD profiles were used for identification of rose cultivars and genome mapping by Ballard *et al.* (1996). They characterized 20 of 22 cultivars examined using RAPD markers. A genetic mapping programme to identify molecular markers associated with black spot resistance in roses was also initiated. RAPD analysis revealed a high degree of genetic polymorphism existed between all the resistant amphidiploids and susceptible tetraploid parent rose cultivars.

Matsumoto and Fukui (1996) reported that the identification of rose cultivars and clonal plants by RAPD technique. Analyses were carried out in nine rose cultivars and three clonal plants and all the cultivars were identified using only three primers.

Genetic variability based on RAPD markers was analysed among ten cultivated rose varieties and nine wild species from three series of the genus *Rosa* (Debener *et al.*, 1996). Thirteen RAPD primers produced 104 polymorphic DNA fragments with a high potential to differentiate rose genotypes. A dendrogram displaying the relative genetic similarities among the genotypes showed the existence of large genetic diversity among the cultivated roses as compared to wild species.

The utility of RAPD markers for the identification of interspecific rose hybrids otherwise indistinguishable from their seed parent was investigated in three crosses by Debener *et al.* (1997). Eighty nine different RAPD primers and primer combinations were used to identify markers specific for the parental genome. Reproducible and specific markers were subsequently analysed in the progeny.

Variety identification and genetic diversity of rose was studied using RAPD techniques by Hai *et al.* (2002). A total of 30 cultivars were analysed where three specific RAPD markers were observed from 65 RAPD bands amplified with 12 primers.

2.7.3 Detection of genetic variation using molecular markers

Detection of genetic differences and determination of genetic relationships between genotypes are for proprietary rights protection and utilization of plant genetic resources. Phenotypic differences are not necessarily concordant with the underlying gene mutations and differences in genetic events.

Debener and Mattiesch (1996) have developed a series of crosses between diploid genotypes and studied the segregation of genes for morphological characters, disease resistance and other agronomically important traits in the rose genome with RAPD markers. They have also proceeded to construct a chromosome linkage map with this molecular marker technique.

Genetic variability in the genus *Rosa* sub genus *Eurosa* consisting of rose trees was analyzed through RAPD markers by Aloisi and Bollerau (1996). Ten genotypes belonging to different sections of the species were analysed and 77 RAPD's were obtained. Results indicated that identical individuals exhibit same patterns in RAPD studies and a good section grouping was observed. The study helped to solve identification problems commonly encountered in the botanical species.

Moreno *et al.* (1996) analyzed 22 wild species of rose from Royal Botanical Garden, Madrid and Cordoba using isoenzymes and RAPD markers. Each ten based arbitrary primer produced a specific DNA banding pattern that grouped together plants belonging to same species and botanical sections as predicted from their genetic background.

Meir *et al.* (1997) assessed the applicability of DNA fingerprinting using mini and micro satellite sequences for the identification of genotypes and to the establishment of genetic distances between rose (*Rosa hybrida*) plants. 119 accessions and 213 markers of 36 rose species that include eight sections of the subgenus *Eurosa* and one species each from the subgenera *Hesperhodus* and *Platyrhodon* were used by

Jan *et al.* (1998) to calculate a similarity matrix, which was clustered with the unweighted pair group method using arithmetic means (UPGMA). The RAPD markers distinguished between all the rose accessions and species grouped into their respective sections.

Nineteen species of rose were analysed using RAPD markers by Milan *et al.* (1996). Hundred and seventy five amplification products were examined by cluster analysis to assess the genetic relationship among species and their genetic distances. With ten primers and seventy five PCR amplification products a particular species could be distinguished from the rest. Twelve were common to all, 31 were unique to a species and 32 were phenetically informative. Multivariate analysis of 16 primers amplifying a total of 100 reproducible fragments enabled the grouping of plants belonging to same species and botanical sections as predicted from their genetic background. Interspecific analysis revealed a high degree of genetic diversity. 31 were specific to each section and ten of them were specific to a species.

Debener and Spethmann (1997) studied the utility of RAPD markers for the identification rose hybrids otherwise undistinguishable from their seed parents. Eighty nine different RAPD primers and primer combinations were used to mark specific parental genomes. Reproducible and specific markers were subsequently analyzed in the progeny.

Phylogenetic analyses of the genus *Rosa* were made by Matsumoto *et al.* (1998) on 17 species from 7 sections within subgenus *Rosa*. Six out of the 7 species that contributed to modern roses were found to belong to one clade.

An effective pairwise combination of long primers for RAPD analysis in roses was done by Debener and Mattiesch (1998). Twenty four primers of different lengths (eight each of 10, 15 and 20 bp) were tested each in RAPD reactions with DNA of *Rosa multiflora* and *Rosa canina*. All primer classes produced fragment patterns of comparable complexity.

Genetic relatedness in *Chaenomeles* (Rosaceae) was studied by RAPD analysis in 42 plants representing accessions of three wild species and one hybrid taxon (Bartish *et al.*, 1999). Amplification with 17 primers yielded a total of 156 polymorphic RAPD bands.

Molecular markers and their utilization in *Rubus* (Rosaceae) research and breeding are briefly reviewed by Klemola (1999). Markers have been employed in cultivar and hybrid identification, estimation of genetic similarities, and taxonomy and population genetic studies of various *Rubus* species. RAPD studies for cultivar identification in raspberry produced cultivar specific profiles with a minimum of three primers. Comparisons between the amount of genetic similarity detected in the RAPD pattern and calculated from pedigree information of ten raspberry cultivars showed that RAPD analysis provided a more accurate estimate of the real amount of genetic relatedness. Parentage testing and identification of hybrids between two Rubus genotypes were also done using molecular markers. RAPD patterns have proved useful in taxonomic classification within Rubus.13 species, which represented 13 subgenera, were correctly examined using ten RAPD primers. Pamfil *et al.* (1996) reported an informative RAPD analysis of 44 Rubus species representing seven subgenera. RAPD technique was also employed in determining genotypic distributions and amount of genetic variation in wild Rubus populations.

Debener *et al.* (2001) mapped 60 additional markers to the existing core map for diploid roses which comprised 305 molecular markers. This proved to be a useful tool for genetic studies, map based cloning and in projects on chromosome evolution within the Rosaceae family.

Wen *et al.* (2004) have characterized genetic relationships of *Rosa roxburghii* (Tratt) and it's relatives using morphological traits, RAPD and AFLP markers. This ornamental plant, which has cancer preventing effects, shows immunity to black spot disease and is of great potential for its exploitation in ornamental breeding. Thirty six morphological traits, 251 RAPD markers amplified with 29 arbitrary primers and 1685

AFLP markers generated from 54 primer combinations were employed to discriminate between 15 accessions and to evaluate the relatedness with a sample set of its relatives. Genotype specific RAPD or AFLP markers, yielded from the four most polymorphic primers or the six most polymorphic primer combinations respectively, could efficiently identify the genotypes.

Sports and seedlings of rose variety were analyzed with molecular markers by Debener *et al.* (2000). Between 695 and 752 RAPD and AFLP fragments were used to infer genetic difference between the sports, original variety and seedlings of these varieties.

Ahmad *et al.* (2004) reported the identification and characterization of plum and pluot cultivars by microsatellite markers. 28 markers were used to investigate the genetic diversity among 14 plums, 6 pluots and one plumcot using 7 apricot cultivars as reference.

An overview of the molecular tools currently applied to rose research and breeding has been presented by Debener *et al.* (2004). Analysis of morphological characters and resistance to two of the most important pathogens, those causing blackspot and powdery mildew, both genetically and molecularly were done. Several morphological traits including single genes and quantitative trait loci were mapped relative to molecular markers. Strategies for the marker assisted selection against the genetic background of wild donor species in introgression programmes were also developed.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled 'Identification of molecular markers for developing breeding strategies in rose' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara and RARS, Ambalavayal during the period from 2004 to 2006. The materials used and the methodologies adopted in this study are described below.

3.1 MATERIAL

The fifty varieties of rose, twenty five of which were fertile (Plate 1: a, b and c) and twenty five sterile (Plate 2: a, b and c), maintained at Regional Agricultural Research Station, Ambalavayal were used for the present study. These plants were subjected to morphological and molecular characterisation using RAPD markers.

3.2 METHODS

3.2.1 Morphological markers

The selected plants were morphologically analysed for the following characters.

Seed setting ability : seed setting / non seed setting.

Floral characters

1. Number of flowers
2. Size : Big / medium / miniature.
3. Fragrance : High/ moderate/ poor.
4. Flower colour
5. Petal number

Akebono

Alliance

Appreciation

Babylon

Bridal Blush

Casanova

Chardony

Dagen Hams Show

Devigayathri

Plate 1a: Selected seed setting varieties of rose maintained at RARS, Ambalavayal.

Dream cloud

Fontain Bleau

Gavina

Golden Choice

Golden gate

Happiness

Jogan

Lavender Lassie

Plate 1b: Selected seed setting varieties of rose maintained at RARS, Ambalavayal.

Prosperity

Regal Red

Scala

Senshei

Soraya

Sweet Heart

Sweet N Pink

Viamala

Plate 1c: Selected seed setting varieties of rose maintained at RARS, Ambalavayal.

Atoll

Belange

Cabaret

Chitrangini

Festival Funfare

Foster

Golden Giant

Love Story

Plate 2a: Selected non-seed setting varieties of rose maintained at RARS, Ambalavayal.

Lovers Meeting

My Choice

Orange Flame

Peach Melba

Pilgrim

Pink Panther

Pristine

Proud Land

Plate 2b: Selected non-seed setting varieties of rose maintained at RARS, Ambalavayal.

Red Fountain

Red Letter Day

Rosa Guajard

Rosa Rouletti

Rosa Royalty

Simon Bolivor

Softeen

Softy Softy

Twinkeless

Plate 2c: Selected non-seed setting varieties of rose maintained at RARS, Ambalavayal.

3.2.2 Molecular characterization

3.2.2.1 Isolation of genomic DNA

Fresh tender leaves were collected from RARS, Ambalavayal and stored at -20°C until use. Genomic DNA was isolated from the selected fifty varieties. Different protocols were attempted to determine the best method from those available in literature.

Procedure 1: Doyle and Doyle (1987) method

Reagents

1. Extraction buffer (4X)
2. Lysis buffer
3. Sarcosine (5 %)
4. TE buffer
5. Ice-cold Isopropanol
6. Chloroform-Isoamyl alcohol (24:1 v/v)
7. Ethanol (70% v/v)

(Chemical compositions of reagents are given in Appendix I)

Procedure 1.1

Leaf samples weighing 1 g were freeze powdered in liquid nitrogen and ground with 6 ml of 1X extraction buffer, 50 µl β mercaptoethanol and a pinch of sodium metabisulphate using chilled autoclaved mortar and pestle. The homogenate was transferred to a 50 ml oakridge tube containing 6 ml pre warmed lysis buffer and 2 ml sarcosine. The tubes were incubated in a water bath at 65°C for 10 min, with intermittent shaking. The tubes were removed from the water bath and equal volume of chloroform:isoamyl alcohol mixture (24:1 v/v) was added and mixed by gentle inversion. Centrifugation was carried out at 10,000 rpm for 15 min at 4°C. The clear aqueous phase was transferred to a fresh tube. To this, 0.6 volume ice-cold isopropanol was added and after gentle mixing, kept in -20°C deep freezer for 20 min

for complete precipitation of DNA. DNA was pelleted by centrifuging at 10,000 rpm for 15 min at 4°C. The isopropanol was poured out and the pellet was washed with 70 per cent (v/v) ethanol. The supernatant was discarded and the pellet air-dried. Dried pellet was dissolved in 100 µl of TE and stored at – 20°C until further use.

Procedure 1.2

A modification of the above protocol was tried with 6 ml 1X extraction buffer, 12 ml lysis buffer and 2 ml sarcosine.

Procedure 2: Rogers and Bendich (1994) method

Reagents

1. 2X CTAB extraction buffer
2. 10X CTAB extraction buffer
3. TE buffer
4. Ice-cold Isopropanol
5. Chloroform-Isoamyl alcohol (24:1 v/v)
6. Ethanol 70 per cent (v/v)

(Chemical compositions of reagents are given in Appendix I)

Procedure 2.1

Leaf sample weighing 1 g was ground using a pre-chilled mortar and pestle in the presence of liquid nitrogen. The ground tissue was transferred into a 50 ml oak ridge tube containing 5 ml pre-warmed 2X CTAB extraction buffer. The contents were mixed well and incubated at 65°C for 15 min. Then equal volume of chloroform: isoamyl alcohol mixture was added, mixed gently by inversion and centrifuged at 10,000 rpm for 15 min at 4°C. The mixture separated into three distinct phases from which the upper aqueous phase containing DNA was pipetted out into a fresh 50 ml oakridge tube. To this, 1/10th volume 10 per cent CTAB was added and mixed gently by inversion. Equal volume of chloroform: isoamyl alcohol mixture was added, mixed

gently to form an emulsion and centrifuged at 10,000 rpm for 15 min at 4°C. The aqueous phase was collected in a fresh oakridge tube and 0.6 volume of chilled isopropanol was added and mixed gently to precipitate the DNA. It was incubated at -20°C for 20 min. The contents were then centrifuged at 10,000 rpm for 5 min at 4°C to pellet the DNA. The isopropanol was poured off retaining the DNA pellet. It was washed first with 70 per cent alcohol and then with absolute alcohol. The DNA pellet was air dried to remove the alcohol and then dissolved in 100 µl of TE buffer.

Procedure 2.2

A modified protocol by increasing the quantity of extraction buffer to 7 ml 2X CTAB extraction buffer was tried.

Procedure 2.3

A modification of the above protocol was tried in which β-mercaptoethanol also was added during extraction. 25µl of β-mercaptoethanol was added while grinding the leaf tissue.

3.2.2.2 Purification of DNA

The DNA isolated contains RNA and protein as contaminants. The DNA sample was hence treated with RNase and Proteinase K.

Preparation of RNase and Proteinase K

Ribonuclease A (Genei, Bangalore) was dissolved at a concentration of 10 mg/ml in 0.01 M sodium acetate (pH 5.2). The solution was heated at 100°C for 15 min and then cooled to room temperature. The pH was finally adjusted by adding 100µl Tris base (pH 7.4) and stored at -20°C.

Proteinase K (Genei, Bangalore) was prepared at a concentration of 20 mg/ml in distilled water and stored at -20°C

RNase and Proteinase treatment

100 µl of DNA suspended in TE buffer was treated with 2 µl of RNase solution and incubated at 37°C for 1 hour. After that 2 µl of Proteinase K solution was added and again incubated at 45°C for 1 hour.

The total volume was made upto 500 µl with distilled water and equal volume of phenol: chloroform - isoamyl alcohol mixture (1:1) was added. It was centrifuged at 10,000 rpm for 10 min at 4°C. The top layer was transferred to a fresh eppendorf tube and equal volume of chloroform - isoamyl alcohol mixture was added. The upper layer was saved and this step was repeated twice. The final aqueous phase was collected into a fresh eppendorf tube and 0.6 volume of chilled isopropanol was added, mixed gently and incubated at -20°C for 30 min to precipitate the DNA. It was centrifuged at 10,000 rpm for 10 min at 4°C. The DNA pellet was retained and washed first with 70 per cent alcohol and then with absolute alcohol. It was then air dried and dissolved in 25 µl TE buffer.

3.2.2.3 Quality estimation of DNA samples

The quality of isolated DNA samples were evaluated through agarose gel electrophoresis.

Materials Required

1. Agarose
2. 50X TAE buffer (pH 8)
3. Gel casting tray, comb, electrophoresis unit and power pack.
4. Ethidium bromide solution (stock 10 mg/ml; working concentration, 0.5 µg/ml)
5. 6X loading dye
6. Double digest marker
7. UV transilluminator
8. Gel documentation and Analysis system

(Chemical compositions of the buffer and dyes are given in Appendix II)

Procedure for casting the gel and electrophoresis

1X TAE buffer was prepared from 50 X TAE stock buffer. Agarose (0.8 per cent (w/v) for genomic DNA and 1.2 per cent (w/v) for RAPD) was weighed and added to the volume of buffer required in the casting tray. It was then dissolved by boiling. The open ends of the gel-casting tray were sealed properly with cello tape and arranged on a horizontal level platform. The comb was placed at an open end of the tray such that it is 0.5-1.0 mm from the base. After the agarose dissolved completely it was cooled to lukewarm temperature and ethidium bromide was added to a final concentration of 0.5 µg/ml. It was poured into the gel mould and allowed to solidify for about 20-30 min at room temperature. The comb and cellotape were removed carefully and the gel was placed in the electrophoresis unit with the well side directed towards the cathode. 1X TAE buffer was added to the buffer tank so as to cover the gel to a depth of 1 mm. 5 µl DNA sample was mixed with $1/6^{th}$ volume tracking dye and carefully loaded into the wells using a micropipette. The λDNA/EcoRI HindIII Double Digest (Bangalore Genei) was used as molecular weight marker. The cathode and the anode of the electrophoresis unit were connected to the power pack (Biotech) and the gel was run at constant voltage. The power was turned off when three- fourth running was completed which could be identified from the position of the tracking dye.

Gel Documentation

The gel was taken from the electrophoresis unit and the separated DNA bands were viewed under UV light in a transilluminator. The DNA fluoresces under UV light on account of intercalating ethidium bromide dye. The image was stored in the gel documentation system (Alpha Imager).

3.2.2.4 Quantification of DNA using spectrophotometer

The quantity of extracted DNA was further evaluated by spectrophotometry. The absorbance values at 260 nm and 280 nm, purity and quantity in ng/ml were automatically read using Nanodrop spectrophotometer ND-1000. 1µl volume distilled

water was loaded at the loading point for initializing the instrument. This was loaded as the blank also. 1 µl volume of DNA was loaded and reading was taken. The purity of DNA was assessed from the ratio of Optical Density (OD) at 260 nm and 280 nm. A ratio of 1.8 indicates good quality DNA.

3.2.2.5 RAPD markers

Random amplified polymorphic DNA (RAPD) analysis was carried out using good quality DNA from all the selected varieties. This technique allows DNA amplification using random primers. The differences in the patterns of bands amplified from genetically distinct individuals behave as genetic markers. Random decamer primers were used to generate the amplification patterns and many loci were analysed as each primer anneals to a different region of the DNA which was viewed on an agarose gel.

Composition of RAPD reaction mixture

A total volume of 25 µl mixture was prepared for each reaction. The RAPD reaction mixture consists of 10X assay buffer for Taq DNA polymerase, $MgCl_2$, dNTPs, template DNA, random primer and Taq polymerase enzyme which was subjected to cycling among three temperatures for denaturing, annealing and extension. The composition of reaction mixture is as follows

10X Assay buffer for Taq DNA polymerase (15 mM $MgCl_2$)	. 2.5 µl
$MgCl_2$	. 1.0 µl
dNTP mix (100µM each of dATP, dCTP, dGTP, dTTP)	- 1.0 µl
DNA template (20 – 50 ng)	- 2.0 µl
Random decamer primer (2.5pM)	- 2.0 µl
Taq DNA polymerase (0.6 U)	- 2.0 µl
Milli Q water	- 14.5µl
Total volume	**- 25 µl**

Primer screening

Different primers were screened to identify those which gave maximum amplification products. Fifty random primers belonging to OPE, OPF, OPAA (Operon Technologies, USA) and C series (Integrated Technologies) were used for screening. Details of the primers are given in Annexure III.

From the fifty one primers used for initial screening 10 primers were selected and a secondary screening was tried with five varieties each of the seed setting and sterile varieties. Finally four primers that produced distinct and reproducible bands were selected and RAPD analysis of all the fifty varieties were done.

RAPD analysis

Reaction mixture (25.0 µl) containing 2 µl genomic DNA (20-50 ng), 2.5 µl 10X assay buffer (Bangalore Genei), 1 µl Mg Cl_2 (Bangalore Genei), 1 µl dNTPs (100 µM each of dATP, dGTP, dCTP, dTTP), 2 µl of primer (2.5 pM), 2 µl of Taq polymerase enzyme (Bangalore Genei) and 14.5 µl Milli Q water was prepared in 0.2 ml PCR tubes. Genomic DNA was replaced by equal volume of Milli Q water keeping all other constituents same in one PCR tube which was taken as control. Polymerase Chain Reaction was carried out in the thermal cycler of model PTC200 of MJ Research, USA programmed for an initial denaturing period of 94°C for 3 min followed by 40 cycles of 1 min denaturation at 92°C, 1 min primer annealing at 37°C and 2 min polymerisation at 72°C. After completion of amplification, the reaction was held at 4°C for 10 min.

The amplified products were resolved on a 1.2 per cent w/v agarose gel with ethidium bromide as intercalating agent for visualisation. The agarose gel was prepared in 1X TAE buffer and run in a horizontal electrophoresis (Bangalore Genei) at 100V supplied from a power pack (Biotech). Double digest marker λDNA/EcoRI HindIII (Bangalore Genei) was used as molecular weight marker and visualised under

UV light in a transilluminator (Herolab) and documented with the help of an alpha imager (Alpha Innotech, USA).

Analysis of RAPD data

The amplification products for the selected primers were scored across the lanes as 1 or 0 by the presence or absence of bands of identical molecular weights. Statistical analysis with Unweighted Pair Group Method of Arithmetic Averages (UPGMA) was done and dendrogram was generated using NTSYS software. Similarity indices were computed as JACCARD's coefficient through 'Simqual' routine and clustering was done using Sequential Agglomerative Heirarchial Nested Clustering (SAHN) routine of the NTSYS package.

3.2.3 *In vitro* embryo culture

3.2.3.1 Collection of explant

Green hips about four to five weeks after pollination were collected from RARS, Ambalavayal. They were stored at 4°C until use.

3.2.3.2 Sterilization of glassware

Borosilicate glass wares from Borosil and Corning were used for the experiments. They were initially soaked in hot water at 100^0C for half an hour. On cooling, they were washed with detergent solution, rinsed with potassium dichromate solution in sulphuric acid, washed free of detergent using tap water and finally rinsed with distilled water. Washed glass wares were dried in hot air oven at 60^0C and were stored away from dust and contaminants. Standard procedures were adopted (Gamborge and Shyluk, 1981) for the preparation of media. The pH of the medium was adjusted to 5.7. Semi solid medium was prepared by adding good quality agar (0.70%). Sterilization of media was done by subjecting them to temperature of 121^0C at a pressure of 15psi for 20 min. After sterilization, the media were allowed to cool to room temperature and stored in cool, dry place.

3.2.3.3 Preparation of culture medium

Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium, the most extensively used medium for plant tissue culture was used as the nutrient medium in the present study. Composition of MS medium is given in Annexure III. Half strength MS was also tried for embryo rescue along with growth regulators *viz.*, Indole acetic acid (IAA), Naphthalene acetic acid (NAA) and Benzyl amino purine (BAP). Separate stocks were prepared for macronutrients, micronutrients, Fe-EDTA, vitamins and aminoacids. For making 1 litre full strength MS, 20:20:10:10:10 ml was taken from five stocks respectively. Separate stocks were prepared for each of the growth regulators used. Sucrose (Sisco Research Laboratories and Sigma, USA) was used as the carbon source at the concentration of 30g/l in the experiments. Myo-inositol at the concentration 0.1 g/l was used as an osmoticum. The pH of the medium was adjusted 5.7 - 5.8 before adding agar. For solidifying the culture medium, agar (Sisco Research Laboratories and Sigma, USA) was used at the concentration of 7 g/l. Distilled water from Aquaguard was used for the preparation of stocks and media. Sterilization of media was done by subjecting them to temperature of 121^{0}C at a pressure of 15 psi for 20 min. After sterilization, the media were allowed to cool to room temperature and stored in cool, dry place.

3.2.3.4 In vitro pollination

In vitro pollination was attempted to obtain seed set and embryo in various crosses that do not normally set seed.

Estimation of pollen fertility

Pollen grains scooped out from flower buds at the time of anthesis were stained in acetocarmine (1%) and viewed at 40X magnification. All the pollen grains that were well stained were counted as fertile and unstained as sterile. The observations were recorded from ten microscopic fields. The fertility percentage was calculated using the formula.

$$\frac{\text{Number of well stained pollen grains in a field} \times 100}{\text{Total number of pollen grains in a field}}$$

Pollination technique

Flower buds ready to dehisce were collected on the day of anthesis prior to flower opening. The epicalyx and calyx surrounding the base of the corolla tube of the flower bud was removed. Ovaries were scooped out without injury, style excised and the ovaries were placed on a clean slide. Pollen grains were suspended in a drop of water and pollination was carried out. After pollination it was transferred to inoculation medium and the cultures were incubated in dark.

3.2.3.5 Embryo culture technique

Surface sterilization

Method of surface sterilization followed by Marchant *et al.* (1994) was modified and used. Intact rose hips were washed in 1 per cent (v/v) detergent solution (Teepol) and subsequently washed with tap water and sterile distilled water. The hips were then washed with 0.1 per cent mercuric chloride solution. After sterilisation the hips were opened under aseptic conditions using forceps and scalpel and the achenes were removed. Achenes awaiting dissection were placed in sterile distilled water to prevent desiccation (Plate 3).

Embryo culture

The pericarp was removed by making lateral cuts, with a scalpel, on each side of the achene. After removal of seed coat, the immature embryo was placed on MS medium supplemented with different concentrations (Plate 4: a, b and c).

Embryo culture studies were done with achenes and embryo excised from hips and inoculated in different media combinations involving auxins, cytokinins and GA_3 as given in Table 1.

Table 1. Media combinations tried for culture establishment.

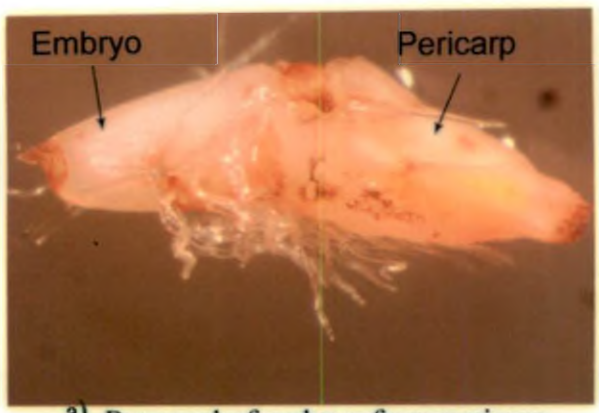
Basal media	Growth regulators (mg l^{-1})
MS	1.5 BA + 0.5 GA_3
MS	2.0 BA + 0.5 GA_3
MS	2.5 BA + 0.5 GA_3
MS	0.5 BA
MS	1.0 BA
MS	1.5 BA
MS	2.0 BA
MS	2.5 BA
MS	0.5 NAA
MS	1.0 NAA
MS	1.5NAA
MS	1.0 IBA+ 1.0 2iP
MS	1.0 IBA + 2.0 2iP
MS	1.0 IBA + 2.5 2iP
MS	1.0 BA + 1.0 IAA
MS	1.0 BA + 1.5 IAA
MS	1.0 BA + 2.0 IAA
½ MS	1.0 BA + 1.0 IAA
MS	0.5 2,4-D
MS	1.0 2,4-D
MS	0.5 IAA
MS	1.0 IAA
MS	0.5 IBA
MS	1.0 IBA
MS	1.5 IBA
MS	1.5 IAA
MS	1.5 2,4-D
MS	0.5 kinetin
MS	1.0 kinetin
MS	1.5 kinetin

After removal of seed coat, embryos excised from hips were cultured in various media combinations to get direct or indirect organogenesis (Table 2). Trials were done varying the culture conditions and the time for surface sterilisation.

Table 2. Media tried for regeneration

Basal Media	Growth regulators (mg l^{-1})
½ MS	1.0 BA + 0.5 NAA
½ MS	1.5 BA + 0.5 NAA
½ MS	1.0 BA + 0.5 NAA + 0.5 GA_3
½ MS	1.0 BA + 0.5 NAA + 0.1 GA_3
½ MS	1.5 BA
½ MS	1.0 BA

Plate 3: Rose achene taken as explant (10X).



a) Removal of embryo from pericarp.

b) Embryo before removal of testa.

c) Embryo after removal of testa.

Plate 4: Preparation of explant when immature embryo is taken (10X)

RESULTS

4. RESULTS

The study on "Identification of molecular markers for developing breeding strategies in rose" was carried out at Centre for Plant Biotechnology and Molecular Biology, COH, Vellanikkara during the period 2004-2006. The results obtained are described here.

4.1 ASSESSMENT OF VARIABILITY

From the germplasm collection at Regional Agricultural Research Station, Ambalavayal, fifty varieties were selected based on morphological characters. The characters included seed setting ability, number of flowers, flower size, fragrance, flower colour and petal number.

Seed setting ability: The ability to set seeds was taken as the main criterion for selection of varieties. Twenty five each of seed setting varieties and non seed setting varieties were selected.

Floral characters

Number of flowers: Number of flowers in a stalk were observed and recorded.
Flower size: Size of the flower was characterized as big, medium and small from its appearance and recorded.
Fragrance: Fragrance was rated as strong, medium and poor and observations were noted.
Flower colour: Colour of the flower was noted and recorded.
The observations are presented in the Tables 3 and 4.

4.2 MOLECULAR CHARACTERIZATION

4.2.1 Isolation of genomic DNA

Tender fresh leaves collected from RARS, Ambalavayal, were used for the isolation of genomic DNA.

Table 3. Floral morphology of seed setting varieties of rose

Sl No.	Variety code	Variety name	No. of flowers	*Size	Fragrance	Flower colour	Petal number
1.	V1	Fontain bleau	3	Big	Strong	Pink to violet	20
2.	V2	Dream cloud	4	Medium	Medium	Pink	14
3.	V3	Dagen Hams Show	2	Big	Medium	Peach	21
4.	V4	Scala	7	Big	Medium	Pink to red	26
5.	V5	Bridal blush	3	Big	Medium	White	28
6.	V6	Alliance	1	Big	Strong	Creamish pink	20
7.	V7	Akebono	1	Medium	Strong	Red	17
8.	V8	Sweet Heart	1	Big	Poor	Cream	20
9.	V9	Golden Gate	2	Big	Poor	Red	18
10.	V10	Sweet N Pink	1	Big	Poor	Pink	38
11.	V11	Jogan	3	Big	Strong	Cream	35
12.	V12	Senshei	2	Big	Medium	Reddish orange	35
13.	V13	Prosperity	5	Medium	Poor	White	18
14.	V14	Chardony	2	Big	Strong	Creamish yellow	20
15.	V15	Golden Choice	1	Big	Medium	Yellowish	28
16.	V16	Casanova	1	Medium	Medium	Creamish sandal	20
17.	V17	Devigayathri	3	Medium	Medium	White	17
18.	V18	Happiness	1	Medium	Medium	Red	20
19.	V19	Babylon	2	Big	Medium	Reddish pink	25
20.	V20	Appreciation	3	Big	Medium	Reddish pink	23
21.	V21	Lavender Lasssie	16	Miniature	Medium	Pinkish violet	30
22.	V22	Viamala	2	Big	Strong	Yellowish white	22
23.	V23	Soraya	3	Medium	Medium	Red to pink	23
24.	V24	Regal Red	2	Medium	Medium	Red	10
25.	V25	Gavina	1	Big	Medium	Violet	38

*Size : < 4" = miniature
4-6" = medium
> 6" = big

Table 4. Floral morphology of non seed setting varieties

Sl No.	Variety code	Variety name	No. of flowers	Size	Fragrance	Flower colour	Petal number
1.	V26	Atoll	3	Big	Medium	Orange red	40
2.	V27	Chitrangini	2	Medium	Poor	Orangish with a tinge of pink	20
3.	V28	Rosa royalty	23	Miniature	Poor	Rose to white	7
4.	V29	Belange	2	Big	Medium	Pink	21
5.	V30	Foster	3	Medium	Medium	Reddish orange	18
6.	V31	Pristine	2	Big	Medium	Creamish with pink at tips	32
7.	V32	Lovers meeting	2	Medium	Strong	Orange at base pinkish at tip	18
8.	V33	Red letter day	1	Medium	Medium	Red to pink	10
9.	V34	Twinkeless	7	Miniature	Medium	Pinkish white	18
10.	V35	Rosa guajard	2	Miniature	Poor	Reddish pink	10
11.	V36	Softeen	4	Medium	Poor	White	25
12.	V37	Simon bolivor	3	Medium	Medium	Creamish yellow	18
13.	V38	Pilgrim	1	Big	Medium	Pink red	20
14.	V39	My choice	1	Medium	Medium	Pinkish	18
15.	V40	Golden giant	2	Big	Strong	Light yellow	30
16.	V41	Pink panther	2	Big	Strong	Pink	28
17.	V42	Festival funfare	4	Medium	Poor	Creamish with red streaks	9
18.	V43	Rosa rouletti	14	Miniature	Poor	Pinkish red	8
19.	V44	Love story	2	Medium	Strong	Orange pink	15
20.	V45	Orange flame	1	Medium	Medium	Orange	12
21.	V46	Cabaret	2	Big	Medium	Pink with dark pink streaks	25
22.	V47	Softy softy	2	Medium	Strong	Pinkish	20
23.	V48	Peach melba	4	Big	Strong	Peach	10
24.	V49	Proud land	5	Medium	Poor	Peach	18
25.	V50	Red fountain	1	Medium	Poor	Red	20

*Size : < 4" = miniature
4-6" = medium
> 6" = big

Among the different protocols tried for extraction of genomic DNA, the quality of DNA isolated using Rogers and Bendich CTAB method using 7 ml extraction buffer and 25 ml β-mercaptoethanol was found to be the best. Considerable amount of RNA was present in the isolated samples which were completely removed by treatment with RNAse and Proteinase K.

The quantity and quality of DNA isolated were analyzed by spectrophotometry (Tables 5 and 6). Good quality DNA was obtained which was assessed from the ratio of absorbance at 260 nm and 280 nm. A factor of 1.8 indicated best quality.

4.2.2 RAPD Assay

Fifty varieties selected based on morphological characters were subjected to RAPD assay.

4.2.2.1 Screening of random primers

Fifty one random primers from four different series OPE, OPF, OPAA (Operon Technologies) and C (Integrated Technologies) were screened using the genomic DNA isolated. The reaction mixture and thermal cycle followed were found to be ideal and gave good amplification.

On secondary screening of these fifty one primers with five varieties each of the fertile and sterile varieties, ten primers were selected for further studies.

OPAA series

The results of screening thirteen primers of this series are summarized in Table 7. A good amplification was obtained with the number of bands ranging upto five. Out of the primers showing good amplification, OPAA 2, 4 and 10 were selected for further screening.

Table 5. Estimation of DNA quality and quantity of fertile varieties

Variety code	Variety name	Ratio of 260/280	Concentration (ng/µl)
V1	Fountain blue	1.81	1685.51
V2	Dream cloud	1.78	1292.23
V3	Dagen Hams Show	1.81	1641.88
V4	Scala	1.81	1685.35
V5	Bridal blush	1.93	3245.27
V6	Alliance	1.78	2810.26
V7	Akebono	1.83	2773.00
V8	Sweet Heart	1.85	1438.16
V9	Golden Gate	1.78	1377.78
V10	Sweet N Pink	1.85	809.47
V11	Jogan	1.91	1178.06
V12	Shenshei	1.78	985.22
V13	Prosperity	1.85	1499.96
V14	Chardony	1.81	1032.75
V15	Golden Choice	1.78	1215.90
V16	Casanova	1.97	1162.11
V17	Devigayathri	1.81	1292.23
V18	Happiness	1.81	807.18
V19	Babylon	1.97	1050.25
V20	Appreciation	1.78	1773.25
V21	Lavender Lasssie	1.86	866.07
V22	Viamala	1.97	1662.11
V23	Soraya	1.73	1292.23
V24	Regal Red	1.84	1215.90
V25	Gavina	1.78	887.67

Table 6. Estimation of DNA quantity and quality of sterile varieties

Variety code	Variety name	Ratio of 260/280	Concentration (ng/µl)
V26	Atoll	1.81	1215.90
V27	Chitrangini	1.78	927.98
V28	Rosa royalty	1.92	1685.35
V29	Belange	1.88	1641.88
V30	Foster	1.83	985.22
V31	Pristine	1.78	2810.26
V32	Lovers meeting	1.83	2040.29
V33	Red letter day	1.77	882.18
V34	Twinkeless	1.81	1449.96
V35	Rosa guajard	1.79	1509.69
V36	Softeen	2.00	1509.69
V37	Simon bolivor	1.82	809.47
V38	Pilgrim	1.97	1050.50
V39	My choice	1.85	877.36
V40	Golden giant	1.81	1641.88
V41	Pink panther	1.78	1141.78
V42	Festival funfare	1.93	2245.27
V43	Rosa rouletti	1.79	1172.45
V44	Love story	1.93	1245.27
V45	Orange flame	1.85	1226.95
V46	Cabaret	1.89	807.18
V47	Softy softy	2.06	753.36
V48	Peach melba	1.93	2245.27
V49	Proud land	1.85	1773.00
V50	Red fountain	1.87	1108.75

Table 7. Amplification pattern produced by random primers of OPAA series

Sl. No.	Primer	Sequence	Number of bands		Amplification pattern
			Fertile	Sterile	
1.	OPAA -1	AGA CGG CTC C	3	3	Good
2.	OPAA-2	GAG ACC AGA C	4	3	Good
3.	OPAA -3	TTA GCG CCC C	3	2	Average
4.	OPAA -4	AGG ACT GCT C	3	5	Good
5.	OPAA -5	GGC TTT AGC C	0	0	No amplification
6.	OPAA -6	GTG GGT GCC A	3	0	Poor
7.	OPAA -7	CTA CGC TCA C	2	2	Average
8.	OPAA -8	TCC GCA GTA G	1	2	Poor
9.	OPAA -9	AGA TGG GCA G	3	3	Good
10.	OPAA -10	TGG TCG GGT G	4	3	Good
11.	OPAA- 11	ACC CGA CCT G	3	3	Good
12.	OPAA -12	GGA CCT CTT G	2	2	Average
13.	OPAA -13	GAG CGT CGC T	0	0	No amplification

Good : 3-3 and above
Average : 2 and above 2.
Poor : 2 and below 2.
No amplification : 0 and 0.

Table 8. Amplification pattern produced by random primers of OPF series

Sl. No.	Primer	Sequence	Number of bands		Amplification pattern
			Fertile	Sterile	
1.	OPF - 1	ACG GAT CCT G	0	0	No amplification
2.	OPF- 2	GAG GAT CCC T	0	0	No amplification
3.	OPF –3	CCT GAT CAC C	0	0	No amplification
4.	OPF –4	GGT GAT CAG G	0	0	No amplification
5.	OPF –5	CCG AAT TCC C	0	0	No amplification
6.	OPF –6	GGG AAT TCG G	0	0	No amplification

Good : 3-3 and above
Average : 2 and above 2.
Poor : 2 and below 2.
No amplification : 0 and 0.

Table 9. Amplification pattern produced by random primers of OPE series

Sl. No.	Primer	Sequence	Number of bands		Amplification pattern
			Fertile	Sterile	
1.	OPE - 3	CCA GAT GCA C	0	1	Poor
2.	OPE – 5	TCA GGG AGG T	0	0	No amplification
3.	OPE – 6	AAG ACC CCT C	0	0	No amplification
4.	OPE - 8	TCA CCA CGG T	0	2	Poor
5.	OPE - 9	CTT CAC CCG A	0	0	No amplification
6.	OPE – 10	CAC CAG GTG A	1	0	Poor
7.	OPE - 13	CCC GAT TCG G	2	1	Poor
8.	OPE – 16	GGT GAC TGT G	0	0	No amplification
9.	OPE – 17	CTA CTG CCG T	1	2	Poor
10.	OPE – 18	GGA CTG CAG A	3	3	Good
11.	OPE – 19	ACG GCG TAT G	0	2	Poor
12.	OPE – 20	AAC GGT GAC C	2	3	Average

Good : 3-3 and above
Average : 2 and above 2.
Poor : 2 and below 2.
No amplification : 0 and 0.

Table 10. Amplification pattern produced by random primers of C series

Sl. No.	Primer	Sequence	Number of bands Fertile	Sterile	Amplification pattern
1.	C - 1	TTC GAG CCA G	5	4	Good
2.	C - 2	GTG AGG CGT C	2	2	Average
3.	C - 3	GGG GGT CTT T	2	4	Average
4.	C - 4	CCG CAT CTA C	4	5	Good
5.	C - 5	GAT GAC CGC C	6	2	Average
6.	C - 6	GAA CGG ACT C	2	2	Average
7.	C - 7	GTC CCG ACG A	4	3	Good
8.	C - 8	TGG ACC GGT G	3	3	Good
9.	C - 9	CTC ACC GTC C	4	1	Poor
10.	C - 10	TGT CTG GGT G	2	0	Poor
11.	C - 11	AAA GCT GCG G	2	2	Average
12.	C - 12	TGT CAT CCC C	2	1	Poor
13.	C - 13	AAG CCT CGT C	4	3	Good
14.	C - 14	TGC GTG CTT G	4	4	Good
15.	C - 15	GAC GGA TCA G	4	4	Good
16.	C - 16	CAC ACT CCA G	4	3	Good
17.	C - 17	TTC CCC CCA G	3	2	Average
18.	C - 18	TGA GTG GGT G	2	1	Poor
19.	C - 19	GTT GCC AGC C	5	5	Good
20.	C – 20	ACT TCG CCA C	2	2	Average

Good : 3-3 and above
Average : 2 and above 2.
Poor : 2 and below 2.
No amplification : 0 and 0.

OPF series

Six primers from the OPF series, OPF 1, 2, 3, 4, 5 and 6 were used for screening. Good amplification was not observed and none were selected from this series (Table 8).

OPE series

The amplification patterns produced for twelve primers in the OPE series are presented in the Table 9. The number of bands varied from zero to three.

C series

Twenty random primers from 1 to 20 were used for screening (Table 10). The number of amplification products varied from zero to six. Seven primers of this series C4, C7, C8, C13, C14, C15 and C19, which gave good amplification, were selected for further studies.

4.2.2.2 Screening of rose varieties with selected primers

A secondary screening was done with the ten selected primers C4, C7, C8, C13, C14, C15, C19, OPAA 2, 4 and 10. Five rose varieties belonging to the fertile series and five from the sterile varieties were used for screening. Out of the primers showing good amplification C 4, C 15, C 19 and OPAA 2 were selected for further screening of all the fifty varieties.

Fifty rose varieties selected were screened using the four primers OPAA 2, C4, C15 and C19 belonging to OPAA series and C series.

4.2.3 Genetic analysis

The amplification products for the selected primers were scored across the lanes as 1or 0 by the presence or absence of bands of identical molecular weights. A total of

331 amplification products were generated .C4 generated two prominent bands for the ten rose varieties (Plate 5). The first band was found to be present in most of the fertile varieties and absent in sterile varieties except V27. The 2nd band was monomorphically represented both in sterile and fertile varieties. C15 generated five polymorphic bands of which the last two bands were absent in sterile varieties except V47, V46, V48 and V49 (Plate 6). The first band was monomorphic to thirteen sterile varieties. It was completely absent in the fertile varieties except for four varieties. Seven bands were generated by C19 (Plate 7) of which the 6th band was feebly represented in V4, V5, V12, V22 and V23 and prominently represented in the sterile varieties. Four polymorphic bands were generated by OPAA2 as shown in Plate 8. The 4th band though not prominent, was absent in all sterile varieties. The remaining three bands were represented in both fertile and sterile varieties.

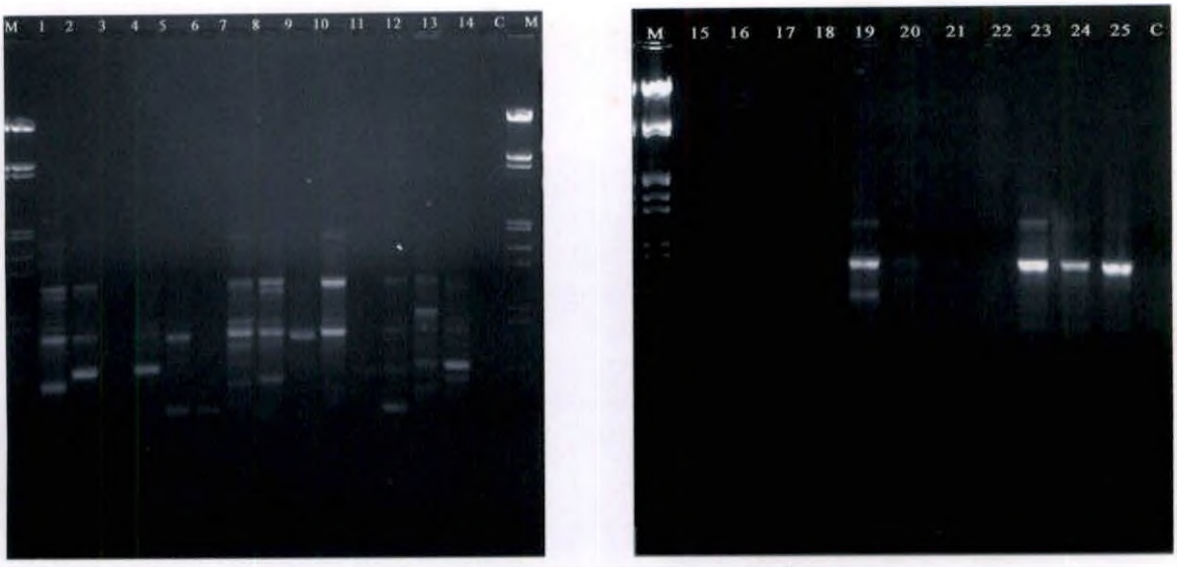
The RAPD data was used to generate a similarity matrix using the SIMQUAL programme. Based on estimated Genetic Similarity Matrix the highest (100 %) similarity was observed between V3, V15, V16 and V17 and between V29 and V31.

The dendrogram derived from data based on RAPD analysis presented two major clusters. The first and second major clusters consisted of twenty two varieties and twenty eight varieties respectively. The first cluster was dominated by fertile varieties with seven varieties belonging to the other group. Maximum similarity of about 94 per cent was observed between V7 and V10, V4 and V12 and V9 and V11. The second cluster predominated by sterile varieties had ten varieties which were sterile. Maximum similarity was observed between the varieties V3, V15, V16 and V17 and between V29 and V31 (100%).

4.3 *IN VITRO* EMBRYO CULTURE

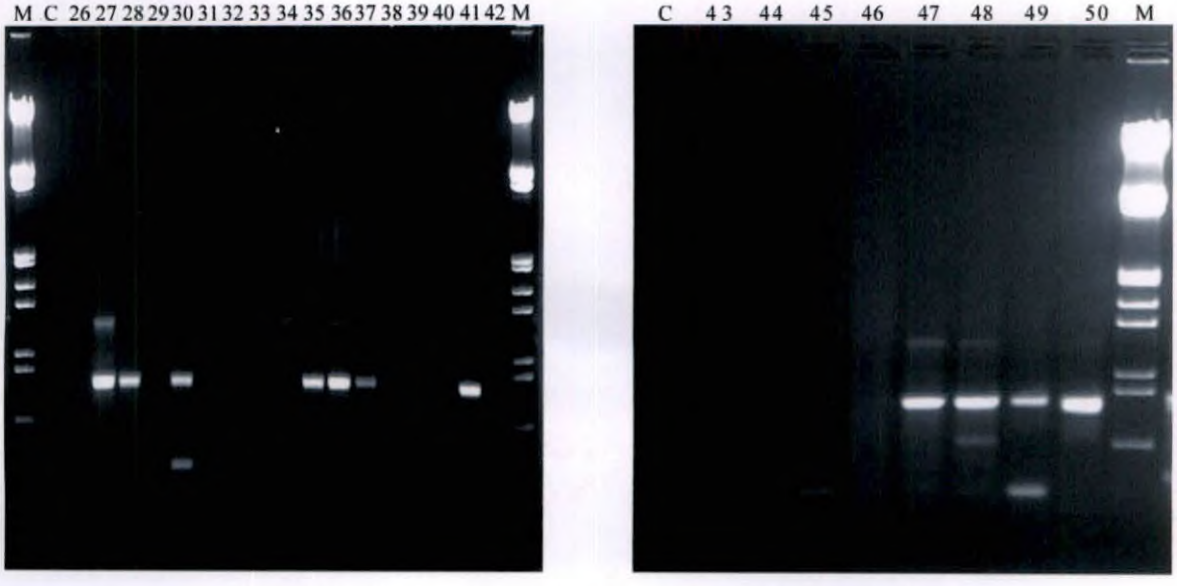
4.3.1 Estimation of pollen fertility

Pollen grains from two male parents Lovers meeting and My choice were scooped out from flower buds just before anthesis. Pollen fertility was assessed by



Amplification pattern of seed setting varieties

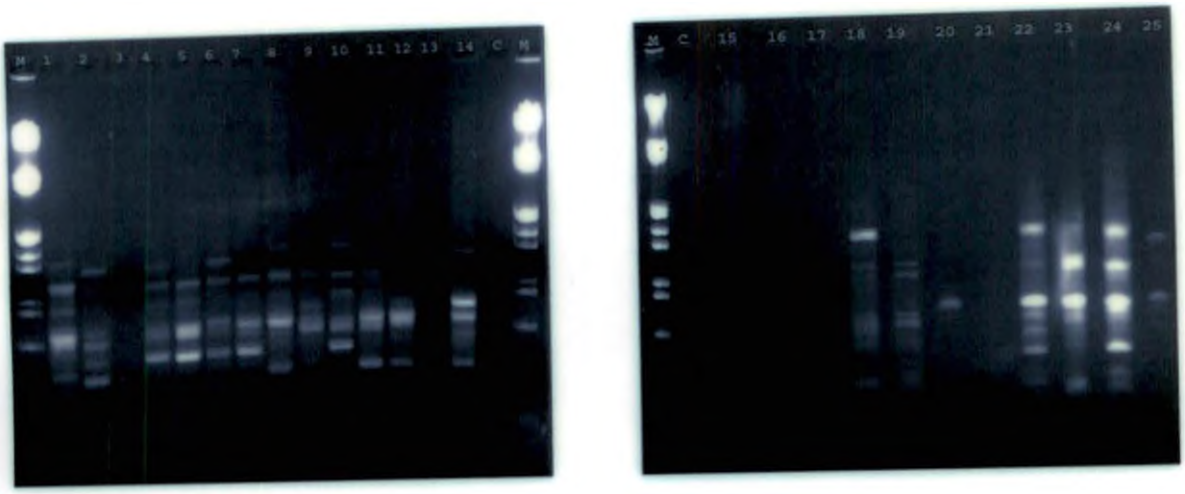
M = marker, C = control, Lane 1- 25: 1 =Fontain Blue, 2 = Dream Cloud, 3 = Dagen Hams Show, 4 = Scala, 5 = Bridal Blush, 6 = Alliance, 7 = Akebono, 8 = Sweet Heart, 9 = Golden Gate, 10 = Sweet N Pink, 11 = Jogan , 12 = Shensei, 13 = Prosperity, 14 = Chardony, 15 = Golden Choice, 16 = Casanova, 17 = Devigayathri, 18 = Happiness, 19 = Babylon, 20 = Appreciation, 21 = Lavender Lassie, 22= Viamala, 23 = Soraya, 24 = Regal Red, 25= Gavina.



Amplification pattern of non - seed setting varieties

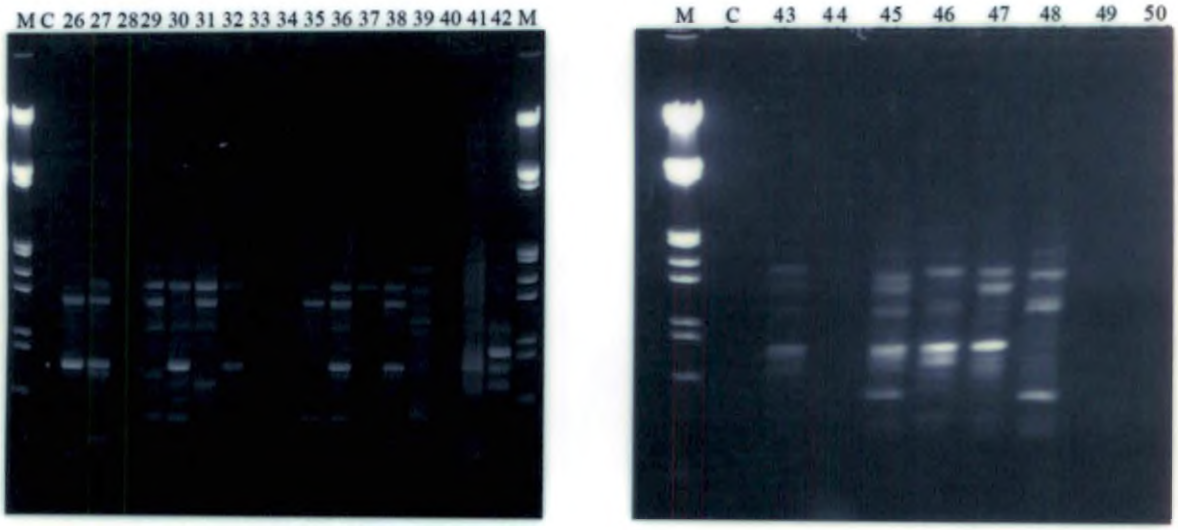
M = marker, C = control, Lane 26 - 50: 26 = Atoll, 27 = Chitrangini, 28 = Rosa Royalty, 29 = Belange, 30 = Foster, 31 = Pristine, 32 = Lover's Meeting, 33 = Red Letter day, 34 = Twinkeless, 35 = Rosa Guajard, 36 = Softeen, 37 = Simon Bolivor, 38 = Pilgrim, 39 = My Choice, 40 = Golden Giant, 41 = Pink Panther, 42 = Festival Funfare, 43 = Rosa Rouletti, 44 = Love Story, 45 = Orange Flame, 46 = Cabaret, 47 = Softy Softy, 48 = Peach Melba, 49 = Proud Land, 50 = Red Fountain.

Plate 5: Amplification pattern of selected 50 rose varieties with the primer C 4



Amplification pattern of seed setting varieties

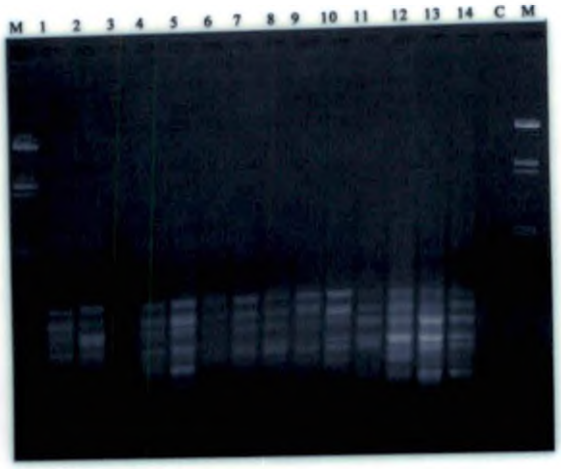
M = marker, C = control, Lane 1- 25: 1 =Fontain Blue, 2 = Dream Cloud, 3 = Dagen Hams Show, 4 = Scala, 5 = Bridal Blush, 6 = Alliance, 7 = Akebono, 8 = Sweet Heart, 9 = Golden Gate, 10 = Sweet N Pink, 11 = Jogan , 12 = Shensei, 13 = Prosperity, 14 = Chardony, 15 = Golden Choice, 16 = Casanova, 17 = Devigayathri, 18 = Happiness, 19 = Babylon, 20 = Appreciation, 21 = Lavender Lassie, 22= Viamala, 23 = Soraya, 24 = Regal Red, 25= Gavina.

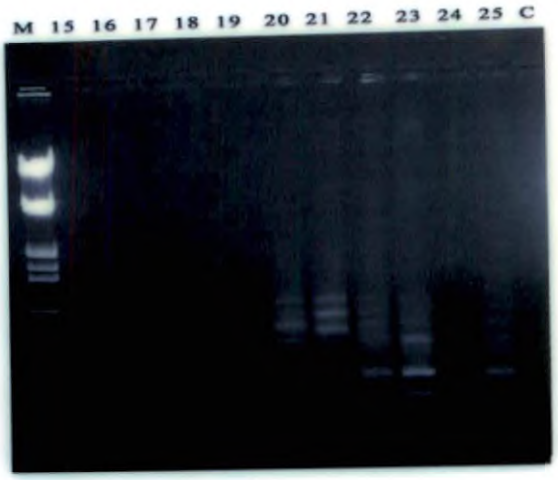


Amplification pattern of non - seed setting varieties

M = marker, C = control, Lane 26 - 50: 26 = Atoll, 27 = Chitrangini, 28 = Rosa Royalty, 29 = Belange, 30 = Foster, 31 = Pristine, 32 = Lover's Meeting, 33 = Red Letter day, 34 = Twinkeless, 35 = Rosa Guajard, 36 = Softeen, 37 = Simon Bolivor, 38 = Pilgrim, 39 = My Choice, 40 = Golden Giant, 41 = Pink Panther, 42 = Festival Funfare, 43 = Rosa Rouletti, 44 = Love Story, 45 = Orange Flame, 46 = Cabaret, 47 = Softy Softy, 48 = Peach Melba, 49 = Proud Land, 50 = Red Fountain.

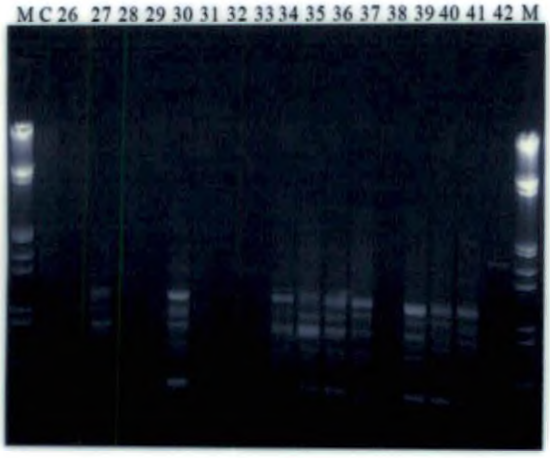
Plate 6: Amplification pattern of selected 50 rose varieties with the primer C 15

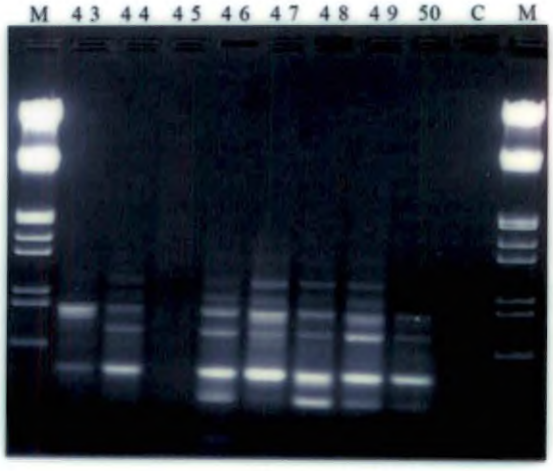




Amplification pattern of seed setting varieties

M = marker, C = control, Lane 1- 25: 1 =Fontain Blue, 2 = Dream Cloud, 3 = Dagen Hams Show, 4 = Scala, 5 = Bridal Blush, 6 = Alliance, 7 = Akebono, 8 = Sweet Heart, 9 = Golden Gate, 10 = Sweet N Pink, 11 = Jogan , 12 = Shensei, 13 = Prosperity, 14 = Chardony, 15 = Golden Choice, 16 = Casanova, 17 = Devigayathri, 18 = Happiness, 19 = Babylon, 20 = Appreciation, 21 = Lavender Lassie, 22= Viamala, 23 = Soraya, 24 = Regal Red, 25= Gavina.

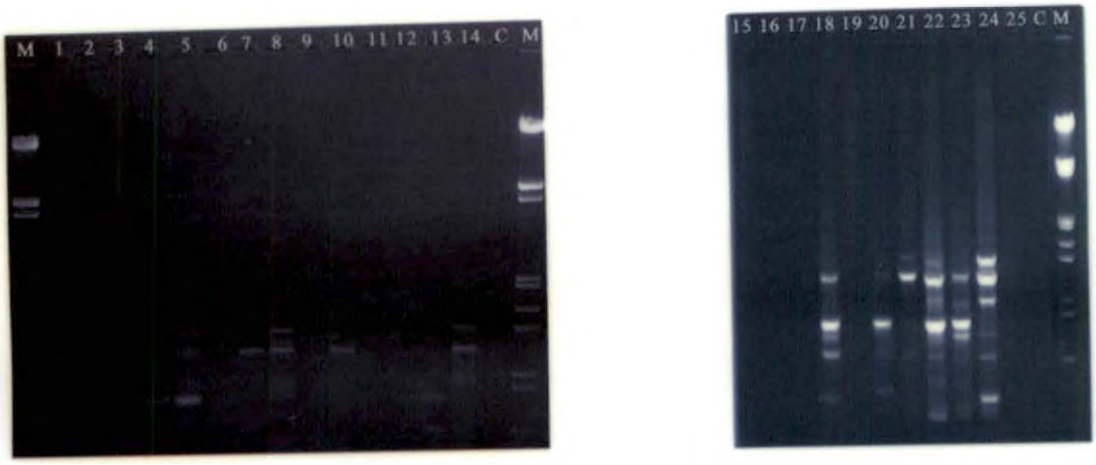




Amplification pattern of non - seed setting varieties

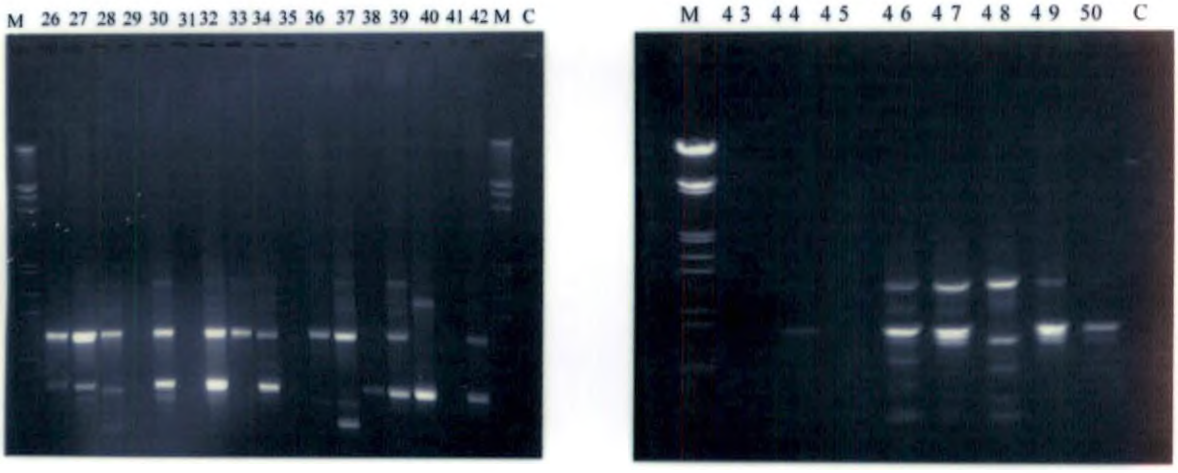
M = marker, C = control, Lane 26 - 50: 26 = Atoll, 27 = Chitrangini, 28 = Rosa Royalty, 29 = Belange, 30 = Foster, 31 = Pristine, 32 = Lover's Meeting, 33 = Red Letter day, 34 = Twinkeless, 35 = Rosa Guajard, 36 = Softeen, 37 = Simon Bolivor, 38 = Pilgrim, 39 = My Choice, 40 = Golden Giant, 41 = Pink Panther, 42 = Festival Funfare, 43 = Rosa Rouletti, 44 = Love Story, 45 = Orange Flame, 46 = Cabaret, 47 = Softy Softy, 48 = Peach Melba, 49 = Proud Land, 50 = Red Fountain.

Plate 7: Amplification pattern of selected 50 rose varieties with the primer C 19



Amplification pattern of seed setting varieties

M = marker, C = control, Lane 1- 25: 1 =Fontain Blue, 2 = Dream Cloud, 3 = Dagen Hams Show, 4 = Scala, 5 = Bridal Blush, 6 = Alliance, 7 = Akebono, 8 = Sweet Heart, 9 = Golden Gate, 10 = Sweet N Pink, 11 = Jogan , 12 = Shensei, 13 = Prosperity, 14 = Chardony, 15 = Golden Choice, 16 = Casanova, 17 = Devigayathri, 18 = Happiness, 19 = Babylon, 20 = Appreciation, 21 = Lavender Lassie, 22= Viamala, 23 = Soraya, 24 = Regal Red, 25= Gavina.



Amplification pattern of non - seed setting varieties

M = marker, C = control, Lane 26 - 50: 26 = Atoll, 27 = Chitrangini, 28 = Rosa Royalty, 29 = Belange, 30 = Foster, 31 = Pristine, 32 = Lover's Meeting, 33 = Red Letter day, 34 = Twinkeless, 35 = Rosa Guajard, 36 = Softeen, 37 = Simon Bolivor, 38 = Pilgrim, 39 = My Choice, 40 = Golden Giant, 41 = Pink Panther, 42 = Festival Funfare, 43 = Rosa Rouletti, 44 = Love Story, 45 = Orange Flame, 46 = Cabaret, 47 = Softy Softy, 48 = Peach Melba, 49 = Proud Land, 50 = Red Fountain.

Plate 8: Amplification pattern of selected 50 rose varieties with the primer OPAA2

staining with 1 per cent acetocarmine solution. Observations were taken after 5 min from five microscopic fields. The fertile pollen retain the red colour of the acetocarmine stain as shown in Plate 9. Mean fertility was observed to be 74.6 per cent (Table 11).

4.3.2 *In vitro* pollination

The ovaries were pollinated with the pollen grains from flowers ready for anthesis. Observations after a week showed seed set and swelling. A good per cent of successful seed set was observed in the cross (82 per cent).

4.3.3 Embryo culture

4.3.3.1 Standardization of surface sterilization

Achenes and immature embryos were taken as explants. Surface sterilization with 0.1 per cent $HgCl_2$ was tried at varying time intervals as represented in the Table 12. Surface sterilization at 30 sec was found to give the best results.

4.3.3.2 Incubation conditions

The cultures that were incubated in dark for a period of 14 days showed embryo development and proliferation in contrast to those continuously kept in light (Table 13).

4.3.3.3 Refinement of media for culture establishment and selection of explant

Influence of basal media

Attempts were made by varying the salt concentration of the medium. MS basal medium alone did not elicit any response. Half MS media with growth regulators was found to produce embryoids in 92 per cent cases when compared to 68 per cent produced by full MS (Table 14).

Plate 9: Viable pollen grain of rose stained with 1 % acetocarmine (100X)

Table 11. Assessment of pollen fertility of varieties: Lovers meeting and My choice (male parents)

Microscopic field	No. of fertile pollen grains	Total no. of pollen grains	Pollen fertility (%)
Variety: Lovers Meeting			
1	2	3	66.6
2	6	8	75.0
3	4	5	80.0
4	2	3	66.6
5	3	4	75.0
Variety: My choice			
6	2	3	66.6
7	2	4	50.0
8	2	2	100.0
9	2	3	66.6
10	2	2	100.0
Mean fertility			74.6

Table 12. Effect of time of surface sterilization on percentage survival

Explant taken	Time for surface sterilization	Percentage survival after a week
Achene	15min	0.0
	1min	0.0
	30sec	23.07
Hips	15min	0.0
	1min	44.1
	30sec	66.6

Table 13. Effect of incubation conditions on percentage survival

Incubation condition	Percentage of cultures showing response after three weeks
Continuous light	15.3
Incubation in dark for two weeks	66.6

Media : MS + 1.0 mg/l BA + 1.0 mg/l IAA

Table 14. Influence of basal media on percentage survival

Basal media	Growth regulators (mg/l)	Percentage of cultures showing positive response
MS	1.0 BA + 1.0 IAA	68.0
½ MS	1.0 BA + 1.0 IAA	92.0

Experiments with achene as explant

Effect of auxins on achene explant

The auxins NAA, IAA, IBA and 2,4-D were used to study the effect when achenes were used as explant. Darkening of the achenes was observed after a week in all experiments.

Effect of cytokinins on achene explant

BA and kinetin were used at varying concentrations to study their effect on achenes. Kinetin (0.5, 1 and 1.5 mg/l) and BA (0.5, 1, 1.5 and 2 mg/l) gave negative response when observations were taken after a week.

Effect of combinations of growth regulators with achene explant

Combinations of auxins and cytokinins were used in the media with achene as explant. Combinations of BA (1.5, 2, 2.5 mg/l) with GA_3 (0.5 mg/l), IBA (1 mg/l) with 2 iP (1, 2 and 2.5 mg/l) and BA (1 mg/l) with IAA (1,1.5 and 2 mg/l) were tried which gave no response.

The observations are shown in the Table 15.

Experiments with embryo as explant

Achenes were split open by making lateral cuts and the seed coat was removed. Immature embryo (4-5 week old) was inoculated in different media combinations.

Effect of auxins on embryo as explant

Embryos showed no response to the varying concentrations of NAA, IAA, IBA and 2,4-D (0.5, 1 and 1.5 mg/l). Observations taken after a week showed darkened embryos in all cultures.

Table 15. Response to growth regulators (mg/l) with achene as explant.

Growth regulators (mg l^{-1})	Response
MS +1.5 BA + 0.5 GA_3	Nil
MS +2.0 BA + 0.5 GA_3	Nil
MS +2.5 BA + 0.5 GA_3	Nil
MS +0.5 BA	Nil
MS +1.0 BA	Nil
MS +1.5 BA	Nil
MS +2.0 BA	Nil
MS +2.5 BA	Nil
MS +0.5 NAA	Nil
MS +1.0 NAA	Nil
MS +1.5NAA	Nil
MS +1.0 IBA+ 1.0 2iP	Nil
MS +1.0 IBA + 2.0 2iP	Nil
MS +1.0 IBA + 2.5 2iP	Nil
MS +1.0 BA + 1.0 IAA	Nil
MS +1.0 BA + 1.5 IAA	Nil
MS +1.0 BA + 2.0 IAA	Nil
½ MS +1.0 BA + 1.0 IAA	Nil
MS +0.5 2,4-D	Nil
MS +1.0 2,4-D	Nil
MS +0.5 IAA	Nil
MS +1.0 IAA	Nil
MS +0.5 IBA	Nil
MS +1.0 IBA	Nil
MS +1.5 2,4-D	Nil
MS +1.5 IAA	Nil
MS +1.5 IBA	Nil
MS +0.5 kinetin	Nil
MS +1.0 kinetin	Nil
MS +1.5 kinetin	Nil
MS basal	Nil

Observations taken after 7 days and subcultured after 21 days.

Effect of cytokinins on embryo explant

BA (0.5, 1, 1.5 and 2 mg/l) and kinetin (0.5, 1, 1.5 mg/l) were used to study the effect of cytokinin on embryo explant. Negative response was noticed in all cultures.

Effect of combinations of growth regulators with immature embryo as explant

The combinations of different growth regulators tried are given in the Table 16. Combination of BA and IAA gave positive response when embryo of Akebono variety was used as explant. Basal media of half MS gave a better response of 92 per cent when compared to 68 per cent produced by full MS. Fresh embryoids were observed as shown (Plate 10).

Half MS media with 1 BA+ 1 IAA gave best results for embryoid formation.

Proliferation and Regeneration Media

Effect of BA on proliferation and regeneration

BA alone in the basal media of half and full MS showed no effect on developing embryo. Embryoids formed dried after 60 days of inoculation.

Effect of BA and NAA

Subculturing from media containing BA and IAA into a combination of BA and NAA gave positive results as shown in the Table 17 and Plate 11.

Subculturing into ½ MS + 1.5 mg/l BA + 0.5 mg/l NAA from inoculation media ½ MS + 1 mg/l BA+ 1 mg/l IAA was found to produce maximum proliferation.

Table 16. Response to growth regulators (mg/l) with embryo as explant

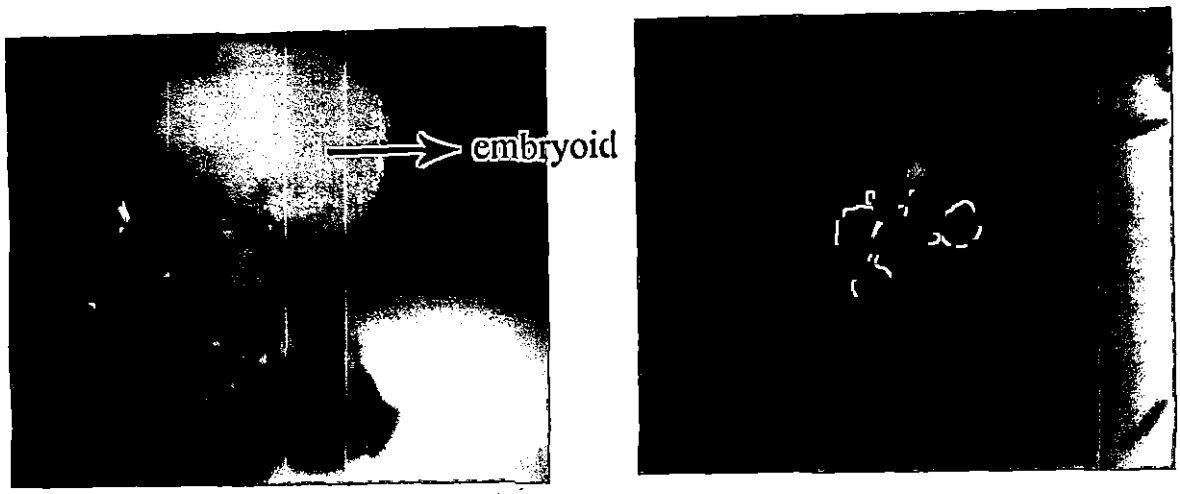
Growth regulators (mg l^{-1})	Response
MS +1.5 BA + 0.5 GA_3	Nil
MS +2.0 BA + 0.5 GA_3	Nil
MS +2.5 BA + 0.5 GA_3	Nil
MS +0.5 BA	Nil
MS +1.0 BA	Nil
MS +1.5 BA	Nil
MS +2.0 BA	Nil
MS +2.5 BA	Nil
MS +0.5 NAA	Nil
MS +1.0 NAA	Nil
MS +1.5NAA	Nil
MS +1.0 IBA+ 1.0 2iP	Nil
MS +1.0 IBA + 2.0 2iP	Nil
MS +1.0 IBA + 2.5 2iP	Nil
MS +1.0 BA + 1.0 IAA	Positive response (68%)
MS +1.0 BA + 1.5 IAA	Positive response (35%)
MS +1.0 BA + 2.0 IAA	Positive response (27%)
½ MS +1.0 BA + 1.0 IAA	Positive response (92%)
MS +0.5 2,4-D	Nil
MS +1.0 2,4-D	Nil
MS +0.5 IAA	Nil
MS +1.0 IAA	Nil
MS +0.5 IBA	Nil
MS +1.0 IBA	Nil
MS +1.5 2,4-D	Nil
MS +1.5 IAA	Nil
MS +1.5 IBA	Nil
MS +0.5 kinetin	Nil
MS +1.0 kinetin	Nil
MS +1.5 kinetin	Nil
MS basal	Nil

Observations taken after 7 days and subcultured after 21 days.

Table 17. Response to media tried for regeneration

Basal Media	Growth regulators (mg l^{-1})	Media of inoculation	Observation	Percentage proliferation
½ MS	1.0 BA + 0.5 NAA	MS + 1.0 BA+ 1.0 IAA	Fresh and green with slight proliferation	25
½ MS	BA + 0.5 NAA	½ MS + 1.0 BA+ 1.0 IAA	Fresh and green with slight proliferation	40
½ MS	1.5 BA + 0.5 NAA	MS + 1.0 BA + 1.0 IAA	Fresh and green with slight proliferation	33
½ MS	1.5 BA + 0.5 NAA	½ MS + 1.0 BA + 1.0 IAA	Fresh green with good proliferation	80
½ MS	1.5 BA	MS+ 1.0 BA + 1.0 IAA	Darkening and slight drying	0
½ MS	1.5 BA	½ MS + 1.0 BA + 1.0 IAA	Fresh upto five days and then dark	0
½ MS	1.0 BA	MS+ 1.0 BA + 1.0 IAA	Darkening and slight drying	0
½ MS	1.0 BA	½ MS + 1.0 BA+ 1.0 IAA	Fresh upto five days and then dark	0
½ MS	1.0 BA + 1.0 IAA+ 0.5 GA_3	MS+ 1.0 BA + 1.0 IAA	Darkening	0
½ MS	1.0 BA + 1.0 IAA+ 0.5 GA_3	½ MS + 1.0 BA + 1.0 IAA	Darkening	0
½ MS	1.0 BA + 1.0 IAA+ 1.0GA_3	MS + 1.0 BA + 1.0 IAA	Darkening	0
½ MS	1.0 BA + 1.0 IAA+ 1.0.0GA_3	½ MS + 1.0 BA + 1.0 IAA	Darkening	0
½ MS	1.0 BA + 0.5 NAA + 0.5 GA_3	MS + 1.0 BA + 1.0 IAA	Darkening	0
½ MS	1.0 BA + 0.5 NAA + 0.5 GA_3	½ MS + 1.0 BA + 1.0 IAA	Darkening	0
½ MS	1.0 BA + 0.5 NAA + 0.1 GA_3	MS+ 1.0 BA + 1.0 IAA	Darkening	0
½ MS	1.0 BA + 0.5 NAA + 0.1 GA_3	½ MS + 1.0 BA + 1.0 IAA	Darkening	0

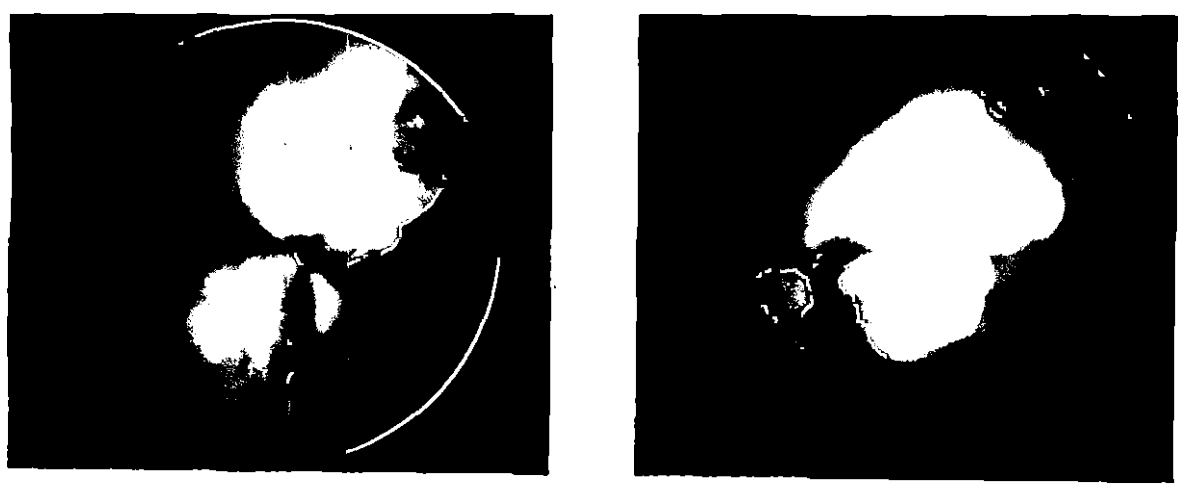
Observation after 14 days of sub culturing.



Full MS+1mg/l BA+1mg/l IAA media

Half MS+1mg/l BA+1mg/l IAA media

Plate 10: Development of embryoids in the rose variety Akebono (10X)



Half MS+1mg/l BA+0.5mg/l NAA

Half MS+1.5mg/l BA+0.5mg/l NAA

Plate 11: Proliferation of *in vitro* developed embryo (10X)

DISCUSSION

5. DISCUSSION

Rose, known as the 'Queen of flowers' is one of the most important member of the *Rosaceae* family. It represents a major commercial market within the horticultural industry. Now days it is a favourite ornamental for landscapes as well. Roses are cultivated world wide in glasshouses and in the open field. Breeders are always looking for new and novel varieties to meet the ever-increasing demand of the consumers for new colour, disease resistant flowers and enhanced fragrance and shelf life of flowers.

During the 1930s and 1940s, a number of researchers reported on the possibility of isolating and artificially culturing, embryos removed from seeds of several trees like peach, cherry, pear and apple (Tukey, 1932). Later, attention was focused on the possibility of rescuing embryos from rose achenes (Asen and Larson, 1951). There are few reports on the culture of embryos *in vitro* (Lammerts, 1946; Asen and Larson, 1951) but attempts were poorly defined. Burger *et al.*(1990) used immature embryo as explant source for *in vitro* organogenesis and plant regeneration. Subsequently, Gudin (1994), Marchant *et al.*(1994) and Mohapathra and Rout (2005) have reported embryo rescue of *Rosa hybrida*, English roses and floribundas. As many desirable roses are female sterile, unproductive hybrids may result on crossing thereby wasting resources, time and energy. Developing a molecular marker that can readily identify the female parent and attempts to rescue the aborting hybrid under our conditions were the main aims to be satisfied under the present study.

5.1 MORPHOLOGICAL MARKERS

Morphological characterization of the rose varieties was done based on seed setting characters. Out of the fifty varieties selected, it was found that the number of flowers ranged according to the group to which the plants belonged. The Hybrid tea varieties were found to have one to two flowers per plant, each branch terminating in a single flower. The Floribundas had three to six flowers while the miniature varieties bore 15-20 flowers. The fragrance ranged from strong to poor and the petal number

varied from 7 to 40. The flower colours were observed and recorded. Red, pink and white were the common colours. Cream, yellow and orange were also recorded. Streaks of pink and red were characteristic to the varieties Cabaret and Festival funfare.

5.2 MOLECULAR MARKERS

Different workers have adopted different protocols for extraction of genomic DNA from rose. Debener and Mattiesch (1996) reported the extraction of DNA according to the protocol of Doyle and Doyle (1987). Ballard *et al.* (1996) followed the protocol using CTAB (Roger and Bendich, 1994) for the extraction of DNA for RAPD analysis. Aloisi and Bollereau (1996) extracted DNA by the procedure derived from Maroof *et al.*(1984).Good quality genomic DNA, which is a prerequisite for RAPD assay, was isolated from the selected rose varieties using CTAB method. CTAB method was found to be more effective (Ballard *et al.*, 1996) and a modification using 7ml extraction buffer and 25 µl β-mercaptoethanol was found to be the best.

The detergent CTAB present in the extraction buffer helps in the release of nucleic acids after disruption of the cell membrane. EDTA, the chelating agent, present in the buffer prevents the action of DNAse by blocking Mg^{2+} which is the major co-factor of DNAse enzyme.

Tender leaves were chosen for DNA extraction. Cubero *et al.* (1996) reported the use of young leaf tissue for DNA extraction as this tissue produced the greatest yield of DNA. Aloisi and Bollereau (1996) also obtained good quality DNA from young leaves. Young leaves usually contain actively dividing cells with lesser concentration of extra nuclear materials like protein, oil, carbohydrates and other metabolites that interfere with nucleic acid extraction (Babu, 1997). Micheli *et al.* (1994) reported that RNA in genomic DNA often influences the reproducibility of RAPD patterns. The contaminants were removed by RNAse and Proteinase K

treatment and the DNA was purified by phenol: chloroform-isoamyl alcohol (1:1) treatment.

5.2.1 RAPD Assay

RAPD analysis was carried out in the thermal cycler (PTC 200, MJ Research) programmed for an initial denaturing period of 94°C for 3 min followed by 40 cycles of 1 min at 92°C, 1 min at 37°C and 2 min at 72°C. Amplification products were separated by electrophoresis in 1.2 per cent agarose gels and visualized by ethidium bromide staining. Optimum amplification has been obtained under similar thermal cycle conditions by Ballard *et al.* (1996).

5.2.2 Primer Screening

Fifty one primers were screened for the RAPD analysis of the fifty rose varieties. Twelve primers gave no amplification at all and twelve gave poor amplification. The remaining twenty seven primers produced reasonably good amplification. Williams *et al.* (1990) has reported that the DNA segments amplified could be completely changed by even a single base change in the primer sequence. They also found that the amplification was influenced by the GC content in the decamer primer and that a GC content of 40 per cent or more was needed in the primer sequemce to generate detectable levels of amplified products. Welsh and Mc Clelland (1990) observed that primers of similar length but different sequence gave a different set of pattern since the template-primer interactions were different. A secondary screening was done with ten selected primers for five rose varieties belonging to the sterile and fertile group. Four primers that produced maximum number of bands and differences in the banding patterns among the fertile and sterile groups were selected for the final analysis of the fifty rose varieties.

5.2.3 RAPD profiling and genetic analysis

The amplification products for the selected primers were scored across the lanes as 1 or 0 by the presence or absence of bands of identical molecular weights

(Fig.1). The scored data for all the primers were used for the similarity-based analysis using NTSYS pc. (ver 21) using SIMQUAL programme is depicted in Fig. 2a & b. Three hundred and thirty one amplification products were generated of which all were polymorphic. This could be due to high genetic variability of cultivars. Primer C 4 generated a 1.1 kb band that was present only in fertile varieties. The number of conspicuous bands generated by primer C 15 was more in fertile varieties and one band of 1.5 kb was unique to sterile varieties. C19 produced seven bands. The 0.4 kb size band was found to be prominent in sterile varieties. OPAA 2 failed to generate any specific bands but produced three bands common to both sterile and seed setting varieties. Amplification pattern of the DNA of the varieties showed that certain bands were specific when amplified with the aforesaid primers. These results could be utilized for early detection of fertility status involving the above as female parent.

The dendrogram derived from data based on RAPD analysis presented two major clusters (Fig. 3). The first cluster was dominated by fertile varieties and the other cluster by non-seed setting varieties. In the first cluster maximum similarity of about 94 per cent was observed between V7 and V10, V4 and V12 and V9 and V11. The second cluster showed maximum similarity between V3, V15, V16 and V17 and between V29 and V31. The clustering of varieties, with the seed setting and non-seed setting varieties almost completely represented in different clusters, reflect a considerable similarity at the DNA level. A variation of upto 50 per cent is observed among the varieties while the clusters show a variation of 38 per cent to 49 per cent .The clusters show a difference of 11 per cent among themselves. Moreno *et al.* (1996) have also reported from their dendrogram results that RAPD technology is a rapid sensitive method to estimate relationships between closely related and more distantly related species and cultivars of *Rosa*. Characterization of genetic diversity in Genus *Rosa* by RAPD has also been reported by Aloisi and Bollereau (1996).

The polymorphic bands generated by the primer that were predominantly present only in the seed setting or sterile varieties suggest that they are specific for those varieties. However, in order to assess and confirm this specificity, a much higher number of primers should be used. RAPD markers are dominant and so it is not

Primers \ Varieties	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25	V26	V27	V28	V29	V30	V31	V32	V33	V34	V35	V36	V37	V38	V39	V40	V41	V42	V43	V44	V45	V46	V47	V48	V49	V50
C 15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	0	1	0	1	1	1	1	0	0	0	1	1	1	1	0	0	0	1	0	1	1	1	1	0	0
	1	0	0	1	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	1	1	0	1	1	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0
	1	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	1	0	1	0	0	0	1	0	1	0	0	1	1	1	0	1	1	1	0	0	0
	1	1	0	1	1	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
	0	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
C 4	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	0	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	1	1	0	1	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	1	1	1	1
C 19	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	1	0	0	0	1	1	1	1	0	1	1	1	0	1	1	0	1	1	1	1	0
	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1	1	0	1	1	1	0	0	1	0	1	1	1	1	0
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OPAA 2	1	0	0	0	1	1	1	1	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	1	0	1	1	1	0	1	0	1	1	1	0	1	1	0	1	0	0	1	0	0	0	1	1	1	1	1
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	0	0	1	1	1	0	1	0	1	0	1	0	0	0	1	1	1	0	1	0	0	0	1	1	0	1	1
	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 1: Scoring for the amplification products derived by RAPD for the 50 varieties

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20	V21	V22	V23	V24	V25
V1	1.00E+00																								
V2	8.33E-01	1.00E+00																							
V3	4.44E-01	5.00E-01	1.00E+00																						
V4	6.67E-01	7.22E-01	4.44E-01	1.00E+00																					
V5	7.78E-01	7.22E-01	3.33E-01	7.78E-01	1.00E+00																				
V6	7.22E-01	5.56E-01	7.22E-01	6.11E-01	6.11E-01	1.00E+00																			
V7	8.33E-01	7.78E-01	3.89E-01	7.22E-01	7.22E-01	6.67E-01	1.00E+00																		
V8	8.33E-01	7.78E-01	3.89E-01	7.22E-01	7.22E-01	5.56E-01	6.89E-01	1.00E+00																	
V9	7.22E-01	7.78E-01	5.00E-01	8.33E-01	7.22E-01	5.56E-01	7.78E-01	8.89E-01	1.00E+00																
V10	8.89E-01	8.33E-01	3.33E-01	7.78E-01	7.78E-01	6.11E-01	9.44E-01	9.44E-01	8.33E-01	1.00E+00															
V11	6.67E-01	7.22E-01	5.56E-01	8.89E-01	6.67E-01	6.11E-01	7.22E-01	8.33E-01	9.44E-01	7.78E-01	1.00E+00														
V12	6.11E-01	6.67E-01	5.00E-01	9.44E-01	7.22E-01	5.56E-01	6.67E-01	7.78E-01	8.89E-01	7.22E-01	9.44E-01	1.00E+00													
V13	3.89E-01	5.56E-01	6.11E-01	6.11E-01	5.00E-01	4.44E-01	5.56E-01	5.56E-01	6.67E-01	5.00E-01	7.22E-01	6.67E-01	1.00E+00												
V14	6.11E-01	5.56E-01	5.00E-01	7.22E-01	6.11E-01	6.67E-01	7.78E-01	7.78E-01	7.78E-01	7.22E-01	8.33E-01	7.78E-01	7.78E-01	1.00E+00											
V15	4.44E-01	5.00E-01	1.00E+00	4.44E-01	3.33E-01	7.22E-01	3.89E-01	3.89E-01	5.00E-01	3.33E-01	5.56E-01	5.00E-01	6.11E-01	5.00E-01	1.00E+00										
V16	4.44E-01	5.00E-01	1.00E+00	4.44E-01	3.33E-01	7.22E-01	3.89E-01	3.89E-01	5.00E-01	3.33E-01	5.56E-01	5.00E-01	6.11E-01	5.00E-01	1.00E+00	1.00E+00									
V17	4.44E-01	5.00E-01	1.00E+00	4.44E-01	3.33E-01	7.22E-01	3.89E-01	3.89E-01	5.00E-01	3.33E-01	5.56E-01	5.00E-01	6.11E-01	5.00E-01	1.00E+00	1.00E+00	1.00E+00								
V18	3.89E-01	2.22E-01	7.22E-01	2.78E-01	3.89E-01	6.67E-01	3.33E-01	3.33E-01	3.33E-01	2.78E-01	3.89E-01	3.33E-01	4.44E-01	4.44E-01	7.22E-01	7.22E-01	7.22E-01	1.00E+00							
V19	6.11E-01	5.56E-01	8.33E-01	3.89E-01	3.89E-01	6.67E-01	5.56E-01	5.56E-01	5.56E-01	5.00E-01	5.00E-01	4.44E-01	4.44E-01	4.44E-01	8.33E-01	8.33E-01	8.33E-01	6.67E-01	1.00E+00						
V20	3.89E-01	4.44E-01	8.33E-01	3.89E-01	2.78E-01	6.67E-01	3.33E-01	3.33E-01	4.44E-01	2.78E-01	5.00E-01	4.44E-01	5.56E-01	4.44E-01	8.33E-01	8.33E-01	8.33E-01	6.67E-01	6.67E-01	1.00E+00					
V21	5.00E-01	5.56E-01	7.22E-01	5.00E-01	5.00E-01	6.67E-01	5.56E-01	5.56E-01	5.56E-01	5.00E-01	6.11E-01	5.56E-01	6.67E-01	6.67E-01	7.22E-01	7.22E-01	7.22E-01	6.67E-01	5.56E-01	7.78E-01	1.00E+00				
V22	5.56E-01	3.89E-01	3.33E-01	5.56E-01	6.67E-01	6.11E-01	3.89E-01	3.89E-01	3.89E-01	4.44E-01	4.44E-01	5.00E-01	2.78E-01	3.89E-01	3.33E-01	3.33E-01	3.33E-01	6.11E-01	2.78E-01	5.00E-01	3.89E-01	1.00E+00			
V23	5.00E-01	3.33E-01	3.89E-01	2.78E-01	5.00E-01	4.44E-01	4.44E-01	4.44E-01	3.33E-01	3.89E-01	2.78E-01	3.33E-01	4.44E-01	4.44E-01	3.89E-01	3.89E-01	3.89E-01	5.56E-01	5.56E-01	4.44E-01	3.33E-01	6.11E-01	1.00E+00		
V24	5.56E-01	3.89E-01	5.56E-01	3.33E-01	5.56E-01	7.22E-01	5.00E-01	3.89E-01	3.89E-01	4.44E-01	3.33E-01	2.78E-01	2.78E-01	3.89E-01	5.56E-01	5.56E-01	5.56E-01	6.33E-01	6.11E-01	5.00E-01	5.00E-01	6.67E-01	5.00E-01	1.00E+00	
V25	5.00E-01	5.56E-01	9.44E-01	3.89E-01	3.89E-01	6.67E-01	4.44E-01	4.44E-01	5.56E-01	3.89E-01	5.00E-01	4.44E-01	5.56E-01	4.44E-01	9.44E-01	9.44E-01	9.44E-01	6.67E-01	6.89E-01	7.78E-01	6.67E-01	2.78E-01	4.44E-01	6.11E-01	1.00E+00
V26	5.56E-01	3.89E-01	7.78E-01	4.44E-01	4.44E-01	8.33E-01	5.00E-01	3.89E-01	3.89E-01	4.44E-01	4.44E-01	3.89E-01	3.89E-01	5.00E-01	7.78E-01	7.78E-01	7.78E-01	7.22E-01	7.22E-01	7.22E-01	6.11E-01	5.56E-01	5.00E-01	6.67E-01	7.22E-01
V27	6.67E-01	6.11E-01	4.44E-01	4.44E-01	5.56E-01	6.11E-01	7.22E-01	6.11E-01	5.00E-01	6.67E-01	4.44E-01	3.89E-01	3.89E-01	5.00E-01	4.44E-01	4.44E-01	4.44E-01	5.00E-01	6.11E-01	5.00E-01	6.11E-01	4.44E-01	6.11E-01	5.56E-01	5.00E-01
V28	5.00E-01	4.44E-01	8.33E-01	2.78E-01	3.89E-01	6.67E-01	4.44E-01	4.44E-01	4.44E-01	3.89E-01	3.89E-01	3.33E-01	4.44E-01	4.44E-01	8.33E-01	8.33E-01	8.33E-01	6.67E-01	7.78E-01	7.78E-01	6.67E-01	3.89E-01	5.56E-01	6.11E-01	8.89E-01
V29	4.44E-01	3.89E-01	6.89E-01	4.44E-01	3.33E-01	7.22E-01	3.89E-01	3.89E-01	5.00E-01	3.33E-01	5.56E-01	5.00E-01	5.00E-01	5.00E-01	8.89E-01	8.89E-01	8.89E-01	8.33E-01	8.33E-01	7.22E-01	6.11E-01	4.44E-01	5.00E-01	6.67E-01	8.33E-01
V30	5.00E-01	5.56E-01	3.89E-01	5.00E-01	7.22E-01	4.44E-01	5.56E-01	4.44E-01	4.44E-01	5.00E-01	3.89E-01	4.44E-01	5.56E-01	4.44E-01	3.89E-01	3.89E-01	3.89E-01	4.44E-01	3.33E-01	4.44E-01	5.56E-01	6.11E-01	6.67E-01	5.00E-01	4.44E-01
V31	4.44E-01	3.89E-01	6.89E-01	4.44E-01	3.33E-01	7.22E-01	3.89E-01	3.89E-01	5.00E-01	3.33E-01	6.56E-01	5.00E-01	5.00E-01	5.00E-01	8.89E-01	8.89E-01	8.89E-01	8.33E-01	8.33E-01	7.22E-01	6.11E-01	4.44E-01	5.00E-01	6.67E-01	8.33E-01
V32	4.44E-01	3.89E-01	7.78E-01	3.33E-01	3.33E-01	7.22E-01	3.89E-01	2.78E-01	2.78E-01	3.33E-01	3.33E-01	2.78E-01	3.89E-01	3.89E-01	7.78E-01	7.78E-01	7.78E-01	7.22E-01	6.11E-01	7.22E-01	6.11E-01	5.56E-01	5.00E-01	6.67E-01	7.22E-01
V33	5.00E-01	4.44E-01	9.44E-01	3.89E-01	3.89E-01	7.78E-01	4.44E-01	4.44E-01	4.44E-01	3.89E-01	5.00E-01	4.44E-01	5.56E-01	5.56E-01	9.44E-01	9.44E-01	9.44E-01	7.78E-01	7.78E-01	7.78E-01	7.78E-01	3.89E-01	4.44E-01	6.11E-01	8.89E-01
V34	4.44E-01	5.00E-01	8.67E-01	5.56E-01	5.56E-01	6.11E-01	5.00E-01	5.00E-01	5.00E-01	4.44E-01	5.56E-01	6.11E-01	6.11E-01	6.11E-01	6.67E-01	6.67E-01	6.67E-01	5.00E-01	5.00E-01	7.22E-01	8.33E-01	4.44E-01	5.00E-01	3.33E-01	6.11E-01
V35	5.56E-01	6.11E-01	6.67E-01	6.67E-01	6.67E-01	6.11E-01	6.11E-01	6.11E-01	7.22E-01	5.56E-01	6.67E-01	7.22E-01	6.11E-01	6.11E-01	6.67E-01	6.67E-01	6.67E-01	5.00E-01	7.22E-01	6.11E-01	7.22E-01	3.33E-01	5.00E-01	4.44E-01	7.22E-01
V36	5.56E-01	5.00E-01	4.44E-01	5.56E-01	7.78E-01	6.11E-01	6.11E-01	5.00E-01	5.00E-01	5.56E-01	4.44E-01	5.00E-01	5.00E-01	5.00E-01	4.44E-01	4.44E-01	4.44E-01	6.11E-01	5.00E-01	3.89E-01	6.11E-01	5.56E-01	6.11E-01	8.67E-01	5.00E-01
V37	4.44E-01	5.00E-01	5.56E-01	4.44E-01	8.67E-01	5.00E-01	5.00E-01	5.00E-01	5.00E-01	4.44E-01	4.44E-01	5.00E-01	6.11E-01	5.00E-01	5.56E-01	5.56E-01	5.56E-01	6.11E-01	5.00E-01	5.00E-01	7.22E-01	4.44E-01	6.11E-01	5.56E-01	6.11E-01
V38	4.44E-01	3.89E-01	7.78E-01	4.44E-01	3.33E-01	7.22E-01	3.89E-01	2.78E-01	3.89E-01	3.33E-01	4.44E-01	3.89E-01	3.89E-01	3.89E-01	7.78E-01	7.78E-01	7.78E-01	7.22E-01	7.22E-01	7.22E-01	5.00E-01	5.56E-01	5.00E-01	6.67E-01	7.22E-01
V39	3.89E-01	4.44E-01	6.11E-01	5.00E-01	6.00E-01	5.56E-01	4.44E-01	4.44E-01	4.44E-01	3.89E-01	5.00E-01	5.56E-01	5.56E-01	5.56E-01	6.11E-01	6.11E-01	6.11E-01	5.56E-01	4.44E-01	6.67E-01	7.78E-01	5.00E-01	5.56E-01	3.89E-01	5.56E-01
V40	4.44E-01	5.00E-01	7.78E-01	5.56E-01	4.44E-01	6.11E-01	3.89E-01	3.89E-01	5.00E-01	3.33E-01	5.56E-01	6.11E-01	6.11E-01	5.00E-01	7.78E-01	7.78E-01	7.78E-01	5.00E-01	6.11E-01	8.33E-01	7.22E-01	4.44E-01	5.00E-01	3.33E-01	7.22E-01
V41	5.56E-01	7.22E-01	6.67E-01	6.67E-01	6.67E-01	6.11E-01	6.11E-01	5.00E-01	6.11E-01	5.56E-01	5.56E-01	6.11E-01	6.11E-01	5.00E-01	6.67E-01	6.67E-01	6.67E-01	3.89E-01	6.11E-01	6.11E-01	7.22E-01	3.33E-01	3.89E-01	4.44E-01	7.22E-01
V42	5.00E-01	4.44E-01	8.33E-01	3.89E-01	3.89E-01	7.78E-01	4.44E-01	3.33E-01	3.33E-01	3.89E-01	3.89E-01	3.33E-01	4.44E-01	4.44E-01	8.33E-01	8.33E-01	8.33E-01	6.67E-01	6.67E-01	7.78E-01	6.67E-01	5.00E-01	4.44E-01	6.11E-01	7.78E-01
V43	4.44E-01	5.00E-01	5.56E-01	7.78E-01	5.56E-01	6.11E-01	6.11E-01	5.00E-01	6.11E-01	5.56E-01	6.67E-01	7.22E-01	6.11E-01	6.11E-01	5.56E-01	5.56E-01	5.56E-01	5.00E-01	5.00E-01	5.00E-01	5.00E-01	5.56E-01	5.00E-01	4.44E-01	5.00E-01
V44	4.44E-01	6.11E-01	6.67E-01	7.78E-01	5.56E-01	5.00E-01	6.11E-01	6.11E-01	7.22E-01	5.56E-01	7.78E-01	8.33E-01	8.33E-01	7.22E-01	6.67E-01	6.67E-01	6.67E-01	3.89E-01	5.00E-01	6.11E-01	7.22E-01	3.33E-01	3.89E-01	2.22E-01	6.11E-01
V45	5.56E-01	5.00E-01	7.78E-01	5.56E-01	4.44E-01	8.33E-01	5.00E-01	3.89E-01	5.00E-01	4.44E-01	5.56E-01	5.00E-01	3.89E-01	5.00E-01	7.78E-01	7.78E-01	7.78E-01	7.22E-01	7.22E-01	6.11E-01	5.00E-01	5.56E-01	3.89E-01	7.78E-01	7.22E-01
V46	5.00E-01	5.56E-01	3.89E-01	6.11E-01	6.11E-01	4.44E-01	4.44E-01	4.44E-01	4.44E-01	5.00E-01	5.00E-01	5.56E-01	5.56E-01	5.56E-01	3.89E-01	3.89E-01	3.89E-01	3.33E-01	2.22E-01	4.44E-01	5.56E-01	6.11E-01	5.56E-01	2.78E-01	3.33E-01
V47	5.56E-01	5.00E-01	2.22E-01	5.56E-01	7.78E-01	3.89E-01	5.00E-01	5.00E-01	5.00E-01	5.56E-01	4.44E-01	5.00E-01	5.00E-01	5.00E-01	2.22E-01	2.22E-01	2.22E-01	3.89E-01	2.78E-01	2.78E-01	3.89E-01	6.67E-01	7.22E-01	4.44E-01	2.78E-01
V48	5.00E-01	5.56E-01	3.89E-01	5.00E-01	7.22E-01	4.44E-01	5.56E-01	5.56E-01	5.56E-01	5.00E-01	5.00E-01	5.56E-01	6.67E-01	6.67E-01	3.89E-01	3.89E-01	3.89E-01	4.44E-01	3.33E-01	3.33E-01	5.56E-01	5.00E-01	6.67E-01	5.00E-01	4.44E-01
V49	4.44E-01	5.00E-01	4.44E-01	4.44E-01	6.67E-01	3.89E-01	5.00E-01	5.00E-01	5.00E-01	4.44E-01	4.44E-01	5.00E-01	7.22E-01	6.11E-01	4.44E-01	4.44E-01	4.44E-01	3.89E-01	3.89E-01	5.00E-01	6.11E-01	4.44E-01	7.22E-01	3.33E-01	5.00E-01
V50	3.89E-01	4.44E-01	6.11E-01	3.89E-01	5.00E-01	4.44E-01	4.44E-01	4.44E-01	4.44E-01	3.89E-01	3.89E-01	4.44E-01	5.56E-01	5.56E-01	6.11E-01	6.11E-01	6.11E-01	4.44E-01	5.56E-01	5.56E-01	5.56E-01	3.89E-01	6.67E-01	3.89E-01	6.67E-01

Figure 2a: Similarity matrix of the 50 rose varieties generated from RAPD data

	V26	V27	V28	V29	V30	V31	V32	V33	V34	V35	V36	V37	V38	V39	V40	V41	V42	V43	V44	V45	V46	V47	V48	V49	V50
V1																									
V2																									
V3																									
V4																									
V5																									
V6																									
V7																									
V8																									
V9																									
V10																									
V11																									
V12																									
V13																									
V14																									
V15																									
V16																									
V17																									
V18																									
V19																									
V20																									
V21																									
V22																									
V23																									
V24																									
V25																									
V26	1.00E+00																								
V27	6.67E-01	1.00E+00																							
V28	6.33E-01	6.11E-01	1.00E+00																						
V29	7.78E-01	5.56E-01	7.22E-01	1.00E+00																					
V30	5.00E-01	7.22E-01	5.56E-01	3.89E-01	1.00E+00																				
V31	7.78E-01	5.56E-01	7.22E-01	1.00E+00	3.89E-01	1.00E+00																			
V32	8.89E-01	6.67E-01	6.33E-01	7.78E-01	6.11E-01	7.78E-01	1.00E+00																		
V33	8.33E-01	5.00E-01	6.89E-01	6.33E-01	4.44E-01	8.33E-01	8.33E-01	1.00E+00																	
V34	6.67E-01	6.67E-01	7.22E-01	5.56E-01	7.22E-01	5.56E-01	6.67E-01	7.22E-01	1.00E+00																
V35	5.56E-01	6.67E-01	6.11E-01	6.67E-01	6.11E-01	6.67E-01	4.44E-01	6.11E-01	7.78E-01	1.00E+00															
V36	5.56E-01	7.78E-01	5.00E-01	5.56E-01	6.33E-01	5.56E-01	5.56E-01	5.00E-01	6.67E-01	7.78E-01	1.00E+00														
V37	4.44E-01	6.67E-01	6.11E-01	5.56E-01	6.33E-01	5.56E-01	5.56E-01	6.11E-01	7.78E-01	7.78E-01	6.89E-01	1.00E+00													
V38	8.89E-01	6.67E-01	7.22E-01	6.89E-01	5.00E-01	6.89E-01	6.89E-01	7.22E-01	5.56E-01	5.56E-01	5.56E-01	4.44E-01	1.00E+00												
V39	6.11E-01	7.22E-01	6.67E-01	6.11E-01	7.78E-01	6.11E-01	7.22E-01	6.67E-01	9.44E-01	7.22E-01	7.22E-01	6.33E-01	6.11E-01	1.00E+00											
V40	6.67E-01	5.56E-01	7.22E-01	6.67E-01	6.11E-01	6.67E-01	6.67E-01	7.22E-01	6.89E-01	7.78E-01	5.56E-01	6.67E-01	6.67E-01	6.33E-01	1.00E+00										
V41	5.56E-01	6.67E-01	6.11E-01	5.56E-01	7.22E-01	5.56E-01	5.56E-01	6.11E-01	7.78E-01	6.89E-01	7.78E-01	7.78E-01	5.56E-01	7.22E-01	7.78E-01	1.00E+00									
V42	9.44E-01	6.11E-01	6.89E-01	7.22E-01	5.56E-01	7.22E-01	9.44E-01	8.89E-01	7.22E-01	5.00E-01	5.00E-01	5.00E-01	6.33E-01	6.67E-01	7.22E-01	6.11E-01	1.00E+00								
V43	5.56E-01	5.56E-01	3.89E-01	6.67E-01	6.11E-01	6.67E-01	5.56E-01	5.00E-01	5.56E-01	6.67E-01	6.67E-01	5.56E-01	6.67E-01	6.11E-01	5.56E-01	6.67E-01	5.00E-01	1.00E+00							
V44	4.44E-01	4.44E-01	5.00E-01	5.56E-01	6.11E-01	5.56E-01	4.44E-01	6.11E-01	7.78E-01	7.78E-01	5.56E-01	6.67E-01	4.44E-01	7.22E-01	7.78E-01	7.78E-01	5.00E-01	7.78E-01	1.00E+00						
V45	7.78E-01	5.56E-01	6.11E-01	8.89E-01	3.89E-01	8.89E-01	7.78E-01	7.22E-01	4.44E-01	5.56E-01	5.56E-01	4.44E-01	8.89E-01	5.00E-01	5.56E-01	5.56E-01	7.22E-01	6.67E-01	4.44E-01	1.00E+00					
V46	5.00E-01	6.11E-01	4.44E-01	3.89E-01	7.78E-01	3.89E-01	6.11E-01	4.44E-01	7.22E-01	5.00E-01	6.11E-01	6.11E-01	5.00E-01	7.78E-01	6.11E-01	6.11E-01	5.56E-01	6.11E-01	6.11E-01	3.89E-01	1.00E+00				
V47	4.44E-01	6.67E-01	3.89E-01	3.33E-01	8.33E-01	3.33E-01	4.44E-01	2.78E-01	5.56E-01	5.56E-01	7.78E-01	6.67E-01	4.44E-01	6.11E-01	4.44E-01	5.56E-01	3.89E-01	5.56E-01	4.44E-01	3.33E-01	8.33E-01	1.00E+00			
V48	2.78E-01	5.00E-01	4.44E-01	3.89E-01	7.78E-01	3.89E-01	3.89E-01	4.44E-01	6.11E-01	6.11E-01	7.22E-01	8.33E-01	2.78E-01	6.67E-01	5.00E-01	6.11E-01	3.33E-01	5.00E-01	6.11E-01	3.89E-01	6.67E-01	7.22E-01	1.00E+00		
V49	4.44E-01	5.56E-01	6.11E-01	3.33E-01	8.33E-01	3.33E-01	4.44E-01	5.00E-01	7.78E-01	6.67E-01	6.67E-01	7.78E-01	3.33E-01	7.22E-01	6.67E-01	6.67E-01	5.00E-01	4.44E-01	6.67E-01	2.22E-01	7.22E-01	7.78E-01	8.33E-01	1.00E+00	
V50	6.11E-01	5.00E-01	7.78E-01	5.00E-01	6.67E-01	5.00E-01	6.11E-01	6.67E-01	7.22E-01	6.11E-01	5.00E-01	6.11E-01	5.00E-01	6.67E-01	6.11E-01	6.11E-01	6.67E-01	5.00E-01	6.11E-01	3.89E-01	6.67E-01	6.11E-01	6.67E-01	8.33E-01	1.00E+00

Figure 2b: Similarity matrix of the 50 rose varieties generated from RAPD data

Figure 3

Fertile varieties

V1 = Fontain bleau, V2 = Dream cloud , V3 = Dagen Hams Show, V4 = Scala, V5 = Bridal blush, V6 = Alliance , V7 = Akebono, V8 = Sweet Heart , V9 = Golden Gate, V10 = Sweet N Pink , V11 = Jogan, V12 = Senshei , V13 = Prosperity, V14 = Chardony , V15 = Golden Choice, V16 = Casanova , V17 = Devigayathri, V18 = Happiness , V19 = Babylon, V20 = Appreciation , V21 = Lavender Lasssie, V22 = Lavender Lasssie, V23 = Soraya , V24 = Regal Red , V25 = Gavina

Sterile varieties

V26 = Atoll , V27 = Chitrangini, V28 = Rosa royalty , V29 = Belange, V30 = Foster , V31 = Pristine, V32 = Lovers meeting , V33 = Red letter day, V34 = Twinkeless, V35 = Rosa guajard, V36 = Softeen , V37 = Simon bolivor, V38 = Pilgrim , V39 = My choice, V40 = Golden giant , V41 = Pink panther, V42 = Festival funfare , V43 = Rosa rouletti, V44 = Love story , V45 = Orange flame, V46 = Cabaret , V47 = Softy softy, V48 = Peach melba , V49 = Proud land, V50 = Red fountain

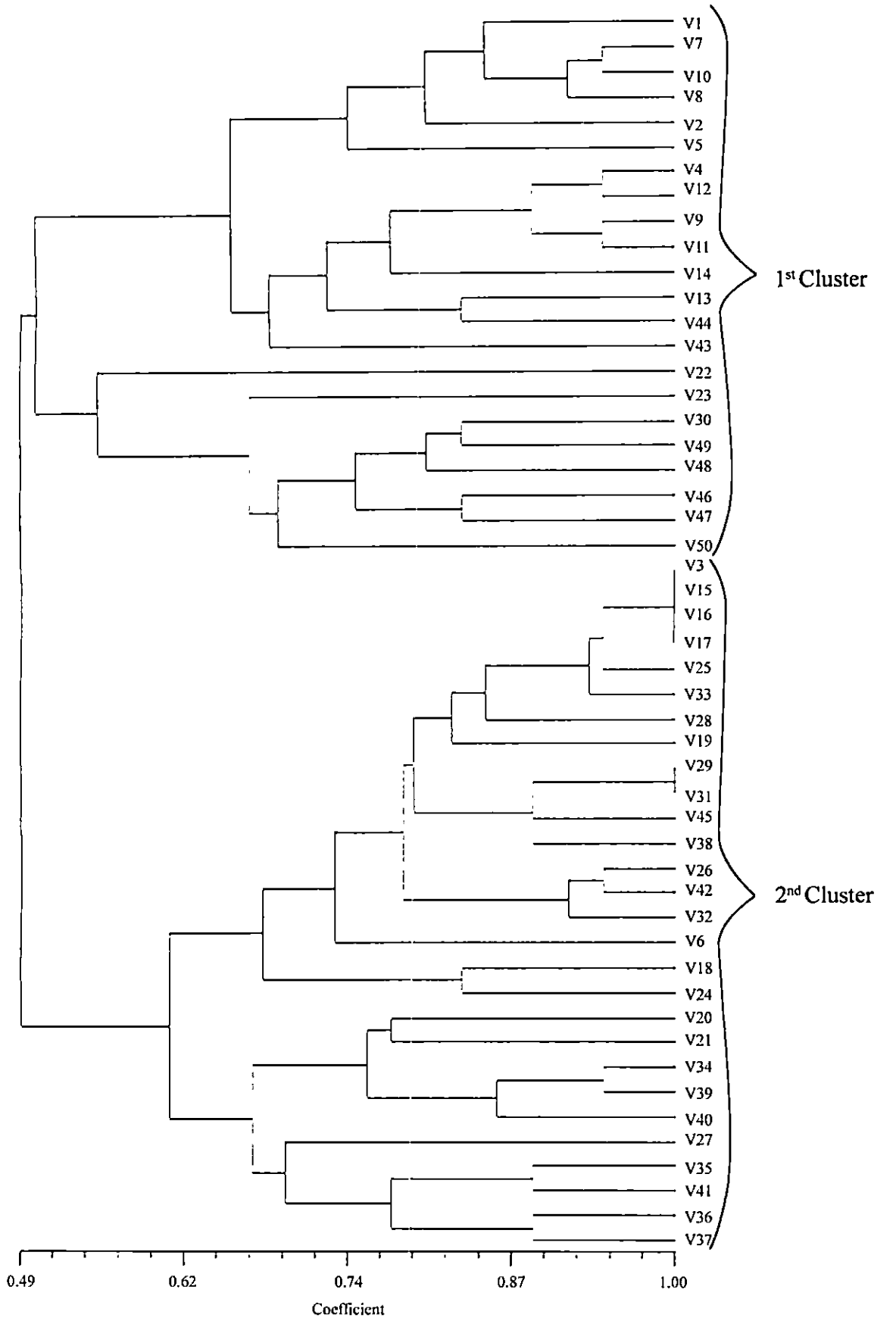

Figure 3 : Dendrogram derived from the RAPD analysis of 50 rose varieties using for random primers.

possible to distinguish whether a DNA segment is amplified from a locus that is heterogeneous or homozygous. Sequencing the amplified product can further increase the utility of RAPD characterization.

5.3 EMBRYO CULTURE STUDIES

Surface sterilization was done with 0.1 per cent mercuric chloride at varying time intervals and surface sterilization of hips at 30 seconds was found to give the best results. This was a modification of the method reported by Marchant *et al.* (1994) where 15 min surface sterilization was given. The present study indicates that growth response of immature embryos on culture medium incubated in the dark for the initial two weeks and subsequently in continuous light was significantly more than that incubated in continuous light. This is in accordance with the reports of Mohapathra and Rout (2005) and Marchant *et al.* (1994). This might be due to low production of polyphenolic substances from the surrounding tissues of the excised embryos that help in better response (Rout *et al.*, 1999). The finding in the present study that MS basal salt alone without growth regulators did not elicit any response was also in agreement with the report of Mohapathra and Rout (2005).

Experiments with intact achenes as explants generated poor response when compared to the immature embryos obtained by completely removing the pericarp and testa . Similar results were obtained in the experiments of Marchant *et al.* (1994). The reduction in germination rate when rose embryos were cultured in the presence of pericarp or testa suggested that it contained a diffusible substance that suppressed embryo germination. In intact achenes this could be transported to the embryo directly thereby inhibiting germination. These results are in general agreement with those of Jackson and Blundell (1963) that showed that extracts from achenes inhibited the germination of *Rosa arvensis.* The presence of stable inhibitors of embryo growth in endosperm and seed coat has been reported in other crops like Iris (Raghavan, 1976).

Experiments were done to determine the effect of auxins and cytokinins on the explant independently and in combination. The growth regulators alone did not show

any response while combinations of auxins and cytokinins showed positive response. The role of auxins in supporting ovary development into fruit is well documented. The developing seeds are a rich source of auxin (Nitsch, 1952), which is utilized for normal fruit growth in apple, pear and strawberry. Synthetic auxins and 2,4-D can replace stimulus provided by pollination. Euewens and Schwala (1975) have reported that in general, high auxins IAA and GA_3 levels have been associated with active seed growth and fruit growth by cell expansion. The aforesaid reports explain the need for combination of growth regulators especially auxins and cytokinins for the development of seed after fertilization.

The present study showed that a basal inoculation media of half MS with 1 mg/l BA and 1 mg/l IAA gave a better response using immature embryo as explant and subculturing into half MS with 1.5 mg/l BA and 0.5 mg/l NAA gave maximum proliferation. Induction of adventitious shoot buds in the callus derived from immature embryos of *Rosa hybrida* cv. Bridal pink (female parent) and several *Rosa hybrida* cultivars (pollen parent) on a modified half MS media supplemented with 1.0 mg/l BA and 0.5 mg/l NAA was reported by Burger *et al.*,(1990). Vijaya *et al.* (1991) has investigated the use of three auxins in combination with BAP and found that NAA was more effective than IAA or IBA in the production of multiple shoots. However, in the present study a definite protocol for regeneration has yet to be identified. Arunachalam and Kaicker have found that freshly harvested achenes showed higher germination percentage that can be included in further studies. Juvenlity of embryo and endosperm tissues will be a deciding factor in regeneration.

SUMMARY

6. SUMMARY

The present investigation "Identification of molecular markers for developing breeding strategies in rose" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara with the objective of developing RAPD based molecular markers for identification of elite parents for use in rose breeding and to standardize embryo rescue techniques for regeneration of rose hybrids. The salient findings of the study are stated below.

1) The study was conducted with fifty rose varieties maintained at RARS, Ambalavayal. The morphological characters like seed setting ability, number of flowers, flower size, fragrance and colour were observed and recorded.

2) The genomic DNA isolated from tender leaves of rose plants using CTAB method of Roger and Bendich (1994) with slight modifications gave good results.

3) RNAse and Proteinase K treatment enabled purification of isolated DNA and was used for RAPD analysis.

4) Out of the fifty one primers screened, belonging to different series, OPAA 2, C - 4, C-15 and C-19 were identified as the best. These primers were found to produce maximum number of bands and differences in the banding patterns among seed setting and non-seed setting varieties.

5) Molecular characterization by RAPD assay showed some bands specific to or prominent in seed setting varieties or sterile varieties.

6) The clustering of fertile and non seed setting varieties, mostly into two separate clusters indicated their similarity at the genetic level.

7) Pollen fertility was assessed by acetocarmine staining and mean fertility was observed to be 74.6 per cent.

8) Surface sterilization of hips with 0.1 per cent mercuric chloride for 30 seconds was found to be the best.

9) The present study indicates that an initial dark period of two weeks generated better growth response from the immature embryos than incubation in continuous light.

10) Intact achenes as explants generated poor response but removal of pericarp and testa elicited further development of embryo.

11) The reduction in development of rose embryos cultured in the presence of pericarp or testa suggests that it contained a diffusible substance that suppresses embryo germination.

12) The present study indicates that MS basal medium without growth regulators is not sufficient for producing response.

13) Half MS with 1 mg/l BA and 1 mg/l IAA as inoculation media and subculturing into half MS with 1.5 mg/l BA and 0.5 mg/l NAA gave maximum response. However, a definite protocol for regeneration has yet to be identified.

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* Originals not seen

APPENDICES

APPENDIX I

The reagents used for DNA isolation

A) Doyle and Doyle's Method of DNA extraction.

1) Extraction buffer (4X)

Sorbitol	- 2.5 g
Tris- HCl	- 4.8 g
EDTA	- 0.74g

The chemicals were dissolved in 60 ml sterile distilled water. The pH was adjusted to 7.5 and final volume was made up to 100 ml with distilled water and then autoclaved.

2) Lysis buffer

1M Tris-HCl (pH-8.0)	- 20 ml
0.25 M EDTA	- 20 ml
5 M NaCl	- 40 ml
CTAB	- 2 g
Distilled water	- 20 ml

Cetyl Trimethyl Ammonium Bromide (CTAB) was dissolved in 20 ml sterile distilled water. To this solution the required volumes of other stock solutions are added.

3) Tris-HCl 1M (pH-8.0)

Tris-HCl 15.76g was dissolved in 60 ml sterile distilled water. The pH was adjusted to 8.0 and final volume was made up to 100 ml with distilled water and then autoclaved.

4) EDTA 0.25 M

Ethylene Diamine Tetra Acetic acid (EDTA) 9.305 g was dissolved in 100 ml sterile distilled water and autoclaved.

5) NaCl 5M

Sodium chloride 29.22 g was dissolved in 100 ml sterile distilled water and autoclaved.

6) Sarcosine 5 %

Sarcosine 5 g was dissolved in 100 ml sterile distilled water and autoclaved.

7) TE buffer

(Tris HCl -10.0 mM; EDTA -1.0 mM)

Tris-HCl 1.0 M (pH 8.0) - 1.0 ml

EDTA 0.25 M (pH 8.0) - 0.4 ml

Distilled water - 98.6 ml

Autoclaved and stored at room temperature.

8) Ice-cold Isopropanol

9) Chloroform-Isoamyl alcohol (24:1 v/v)

To 24 parts of chloroform, 1 part of isoamyl alcohol was added and mixed properly. The mixture was stored in refrigerator before use.

10) Ethanol 70 per cent.

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

B) Reagents for DNA isolation as per Rogers and Bendich (1994)

1) 2X CTAB extraction buffer

2% CTAB (w/v)

100 mM Tris (pH 8)

20 mM EDTA (pH 8)

1.4 M NaCl

1% PVP

2) 10% CTAB solution

10% CTAB (w/v)

0.7M NaCl

3) TE Buffer

10 mM Tris pH 8

1 mM EDTA pH 8

4) Iso-propanol

5) Chloroform: Isoamyl alcohol mixture (24:1, v/v)

6) Ethanol 100% and 70%

APPENDIX II

Buffer and dyes used in gel electrophoresis

1) 6x Loading/Tracking dye

Bromophenol blue	- 0.25 %
Xylene cyanol	- 0.25 %
Glycerol	- 30 %

The dye was prepared and kept in fridge at 4°C

2) Ethidium Bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg /ml in water and was stored at room temperature in a dark bottle.

3) 50x TAE buffer (pH 8.0)

Tris base	- 242.0 g
Glacial acetic acid	- 57.1 ml
0.5 M EDTA (pH 8.0)	- 100 ml
Distilled water	- 1000 ml

The solution was prepared and stored at room temperature.

APPENDIX III

Composition of Murashige and Skoog basal medium

Stock	Composition	Concentration (mg/l)	Stock strength	Quantity/L medium
I.	**Macronutrients**		50X	20 ml
	NH_4NO_3	1650.00		
	KNO_3	1900.00		
	$CaCl_2.2H_2O$	440.00		
	KH_2PO_4	170.00		
	$MgSO_4.7H_2O$	370.00		
II.	**Micronutrients**		50X	20 ml
	H_3BO_3	6.20		
	$MnSO_4.4H_2O$	22.30		
	KI	0.83		
	$ZnSO_4.7H_2O$	8.60		
	$Na_2MoO_4.2H_20$	0.25		
	$CuSO_4.5H_2O$	0.025		
	$CoCl_2.6H_2O$	0.025		
III.	**Fe stock**		100X	10 ml
	Na_2EDTA	37.30		
	$FeSO_4.7H_2O$	27.80		
IV.	**Vitamins**		100X	10 ml
	Thiamine HCl	0.10		
	Nicotinic acid	0.50		
	Pyridoxine HCl	0.50		
V.	**Amino acid**		100X	10 ml
	Glycine	2.00		

IDENTIFICATION OF MOLECULAR MARKERS FOR DEVELOPING BREEDING STRATEGIES IN ROSE

By

CINU SEBASTIAN

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the
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ABSTRACT

Rose, one of the most important flowering ornamentals is a favourite for landscaping and an important commercial cut flower. Breeders are always looking for new and novel varieties to meet the ever increasing demand of consumers. However, many years are required to develop a new variety through conventional methods.

Many desirable roses are female sterile and hence pose a real limitation to breeding. Developing a molecular marker that can readily identify a female parent can go a long way to avoid unproductive hybrids. Premature abortion of developing embryos resulting in few or no viable seeds is another major set back.

The present investigation entitled 'Identification of molecular markers for developing breeding strategies in rose' was held out at this context at the Centre of Plant Biotechnology and Molecular Biology (CPBMB) with the aim of determining a molecular marker and attempting embryo rescue of rose.

Fifty rose varieties were selected based on morphological characters *viz.*, seed setting ability. Variations in foliar characters of plants were recorded. Genomic DNA extraction from tender leaves of rose plants using Roger and Bendich's method (1994) with slight modification was found to be the best.

Out of fifty-one primers screened four primers belonging to OPAA 2, C 4, C 15 and C 19 were identified as the best. Molecular characterization by RAPD assay generated a total of 331 amplification products. Some bands were specific or prominent to the group. The clustering of sterile and fertile varieties mostly into two separate clusters indicated their similarity at the genetic level. Further studies have to be conducted by increasing the number of primers used, for identification of fertility status of more varieties.

In view of the problems faced by breeders regarding unproductive hybrids, an attempt was made for embryo rescue. Surface sterilization of hips with 0.1 per cent mercuric chloride was standardized. The pollen fertility was assessed by acetocarmine

staining and mean fertility was observed to be 74.6 per cent. The poor response of germination observed for achenes was due to both physical and chemical restriction on the embryo. Effect of media type and combination of growth regulators were assessed. A high germination rate was observed in cultures incubated for two weeks in dark and subsequently transferred to light. Inoculation media with BA and IAA and subculturing media with BA and NAA combined with low salt concentration (half MS) was found to give maximum response. Further trials can lead to the identification of a definite protocol for regeneration.