In vitro propagation and biochemistry of *Rosa hybrida* L. cv. 'First Red' and clonal fidelity of microplants

A Dissertation

Submitted in partial fulfillment of the requirement

for the award of degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

Under the guidance of

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July 2013

CERTIFICATE

This is to certify that the dissertation entitled **"*In vitro* propagation and biochemistry of *Rosa hybrida* L. cv. 'First Red' and clonal fidelity of microplants"** submitted by Miss. Amanpreet Kaur (R. No. 301101002) in the partial fulfillment of the requirement for the award of Master of Science in Biotechnology to Department of Biotechnology and Environment Sciences, Thapar University, Patiala. She has fulfilled all the requirements in completing this work under my supervision and guidance.

The work is an original contribution of the candidate and has not been submitted elsewhere for any other degree or diploma.

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DECLARATION

I hereby declare that the work which is being presented in this dissertation entitled **"*In vitro* propagation and biochemistry of *Rosa hybrida* L. cv. 'First Red' and clonal fidelity of microplants"** by the undersigned in the partial fulfillment of the requirement for the award of Degree of Master of Science in Biotechnology, Thapar University, Patiala is true and original record of my own independent and original research work carried out under the supervision of Dr. Anil Kumar, Assistant Professor, Department of Biotechnology and Environment Sciences, Thapar University, Patiala, India. This matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any other degree.

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ACKNOWLEDGMENT

I thank the almighty whose blessings have enabled me to accomplish my project work successfully. It is my privilege to express my sincere thanks and a sense of deep gratitude to Dr. Anil Kumar, Assistant Professor, Department of Biotechnology & Environmental Science, Thapar University for his valuable advice, splendid supervision, and immense patience due to which this work was able to take the shape in which it has been presented. It was his valuable discussions and constant encouragement through which I have gained a lot. No words can express my sincere and deep sense of reverence for him. I owe thanks to him for all the help, pain taking efforts and deep insight into the problem and thus improving the quality of work at all stages.

I owe my sincere thanks to Dr. M. S. Reddy, Head, Department of Biotechnology & Environmental Sciences, Thapar University and Coordinator of TIFAC CORE for his immense concern throughout my project work.

I am always thankful for the help rendered by Ms. Mahima Bansal, Mr. Sanjog Garyali, Ms. Amanpreet Kaur and Mr. Diwakar Aggarwal (Ph. D scholars) throughout my project period without which it would have been difficult for me to achieve my goals.

I am highly thankful to the Tissue Culture staff with special mention Mr. Khem Raj, Mr. Rajesh, Mr. Sandeep, Mr. Bhupinder and Mr. Baljeet Singh who always helped me to understand minor details of the work.

The whole contribution for my achievements goes to my parents and brother, who always stood by me during thick and thin, providing me the moral support and resources to finish my work. Their unwavering faith in me has been a source of constant inspiration for me.

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ABSTRACT

In vitro propagation of rose has played a very important role in rapid multiplication of cultivars with desirable traits and production of healthy and disease-free plants.

Shoot multiplication of *Rosa hybrida* cv. ‘First Red’ was initiated by supplying BAP and GA_3 to obtain wide number of shoots. BAP and GA_3 were found to be most effective growth hormones for shoot multiplication. To induce shoot organogenesis leaf explants were inoculated on different concentrations of auxins and cytokinins. Among the different auxins and cytokinins studied and over all explant types, (BAP- 12.5 µM and NAA- 25 µM; BAP- 25 µM and 2, 4-D – 12.5 µM) promoted the highest frequency of callus production for shoot regeneration. But unfortunately no shoot regeneration was obtained. Rooting was obtained on 50 µM IBA concentration for a short exposure of 12 hrs.

Among biochemical parameters, carbohydrates, phenols and amino acids were found in the sequence of first increasing and then gradually decreasing following IBA treatment. The content of carbohydrates were seemed to be increasing from the 1^{st} day of inoculation after IBA pulse treatment and then decreased as the root started emerging. Therefore, interpretation can be made that carbohydrates, phenols and amino acids may be involved in adventitious root growth.

Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) analyses indicated clonal uniformity of the newly formed *in vitro* raised plants, and these were also found to be true-to-type.

Abbreviations

%	Percentage
µl	Microlitre
2, 4-D	2, 4-Dichlorophenoxyacetic acid
BAP	6-Benzyl amino purine
DNSA	Dinitrosalicylic acid
GA_3	Gibberellic acid
HCl	Hydrochloric acid
Hr	Hour
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KOH	Potassium hydroxide
M	Molar
mg	Milligram
mg/g	Milligram per gram
Mins	Minutes
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
ºC	Degree centigrade
PCA	Perchloric acid
PCR	Polymerase Chain Reaction
PGR	Plant Growth Regulator
v/v	Volume by volume
w/v	Weight by volume

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CHAPTER 1

INTRODUCTION

Floriculture is a fast developing multi-billion dollar trade in national and international market and the demand for the new and exotics are ever increasing. Cut flowers and ornamental young plants are very important export products for several developing countries around the world. Consumption is on the rise, mainly in emerging trade groups like Eastern Europe, Russia, China, India and East Asia.

Flowers are symbol of beauty and tranquility, they form the soul of garden and convey the message of nature to mankind, especially, the rose since which is the world's most favourite and unchallenged 'Queen of flowers', belonging to family Rosaceace. It is being grown for various purposes, such as garden flowers, for aesthetic value, cut flowers and many more.

Rose is one of the most economically important flowers worldwide. Genetic improvement of rose through conventional breeding is limited by several factors such as polyploidy and highly heterozygous nature of existing cultivar. Vegetative propagation of the developed cultivar by traditional methods is limited by season and availability of propagules. The technique of *in vitro* propagation can be useful for large scale propagation from limited resources and is also prerequisite for genetic engineering.

Rosaceae

Rosaceae is the 19th largest family of plants (AWP 2007). It includes genera from 95 to 100 and 2830−3100 species (Judd et al. 1999; Mabberley 1987). The name is derived from the type genus *Rosa*. The largest genus by far is *Prunus* (plums, cherries, peaches, apricots and almonds) with about 430 species.

Roses are generally perennial shrubs. Most species are deciduous, but some are evergreen. They have a worldwide range, but are most diverse in the northern hemisphere. Several economically important products come from Rosaceae, including many edible fruits like apples, plums, cherries, peaches, pears, raspberries, strawberries, almonds and ornamental trees and shrubs.

Leaves are generally arranged spirally, but have an opposite arrangement in some species. They are pinnately compound. The leaf margin is most often serrate. The stipules are sometimes adnate to the petiole.

Flowers of rose family are generally described as "showy". They are actinomorphic and almost always hermaphrodite. Rosaceae generally have five sepals or multiple of five, five petals and many spirally arranged stamens. The bases of the sepals, petals, and stamens are fused together and forms a cup-like structure called *hypanthium*.

The **fruits** come in many varieties and were once considered the main characters for the definition of subfamilies amongst Rosaceae, giving rise to a fundamentally artificial subdivision. They can be follicles, capsules, nuts, achenes, drupes (*Prunus*) and accessory fruits, like the pome of an apple, or the hip of a rose (Phillips and Rixx 1988). Many fruits of the family are edible.

Rosa hybrida

Rose is one of the world's most important ornamentals for long time commonly known as 'Hybrid Tea Rose'. It is generally propagated by vegetative methods like cutting, layering, budding and grafting. Roses are found in almost all parts of India. These species are mostly native to Asia with smaller numbers native to Europe, north-west Africa and North America. They are widely grown in the Valley of Flowers and Mahim National Park (American Heritage Dictionary of the English language). Seeds are used for propagation of species, new cultivars and for production of rootstocks (Horn 1992).

A rose is a woody perennial plant belonging to genus *Rosa*, with 2800 species out of which around 100 species are wild (Phillips and Rix 1988). Flowers are large and showy, ranging in various colours like white, red, pink, orange, etc.

The genus *Rosa* is comprised of hundreds of species of prickly shrubs which may have a habit of climbing. The leaves are pinnate and may present in a number from 3-10 and basal stipules. Leaflets usually have a serrated margin and some thorns on the underside of the stem.

Taxonomy

Rosa hybrida (rose) is not a species in the botanical sense, but it describes most cultivated rose cultivars. These cultivated have been derived over centuries through complex crosses involving a number of species of the genus *Rosa* (Phillips and Rix 1988). The species are very variable and hybridize freely (Zielinski et al. 2004). The chromosome number of rose varies from 2n=2x=14 to 2n=8x=56, with most species being diploid or tetraploid. Commercial rose cultivars (*Rosa hybrida)* tend to be either triploid or tetraploid (Rout et al. (1999).

History

Roses have been cultivated for thousands of years, with centuries old records of medicinal use. In ancient Persia, roses were cultivated and used as remedies. In ancient Rome, Pliny listed 32 health conditions that rose preparations would effectively treat. Roses were cultivated in China, where varieties that had repeated blooms all season were developed (Mabberley 1997). Indian researchers found petrified fossils of rose (*Rosa* spp.) 35 million years old. During the later part of the 18^{th} century, the old European roses were crossed with different varieties of China roses. Breeding during late nineteenth century developed roses that flowered twice or perhaps three times in a season. This class was known as the Hybrid Pertpetuals (Ross 1991).

Economic importance

Plants from the family *Rosaceae* yield fruits and flowers that are of immense economic importance. The value of fruits, rose plants, cut flowers and other products derived from the plant support a wide variety of industries, farms and thus help in generation of employment.

Rose Tea: Medicinal tea can be made from rose hips, petals, leaves or combinations. Petals and leaves brewed as a tea can bring down fever. Petal and leaf tea has a cleansing effect, working as a diuretic to flush toxins from the body.

Rose bushes & Cut flowers: Rose bushes are used ornamental plants and cut roses are the favourite flower of wedding bouquets and floral gifts.

Rose water: Rose water is produced by distilling the petals of, and is used for skin treatments to smooth and moisturize the skin, and to relieve skin irritations. Rose water has antiseptic properties and is sometimes used as an eye wash to treat eye irritation. Rose water is also used as a skin refresher after bath.

Rose Hips: Rose hips are the ‘fruit’ of rose. The hip develops at the base of a pollinated flower and contains seeds. Hips are used to add flavour as well as nutrition to food. Besides the high vitamin C content, rose hips contain vitamins A, B_3, D and E. Hips are a source of bioflavonoids, flavonoids, fructose, citric acid and zinc.

Micropropagation

Micropropagation has emerged as a powerful technique with the great potential not only for rapid clonal propagation of plant species but also for the conservation of rare and endangered species (Fay 1992). The most significant advantage offered by micropropagation is that, in a relatively short time and space, a large number of plants can be produced starting from a single explant (Zimmerman 1996; Jain 1997; Hassanein et al. 2003).

Rosa hybrida is propagated through cuttings which are available during the months of December every year. About 4-5 thousand plants for planting at a distance of 1.5 m are ideally required for one hectare of land. Once planted, it gives economic yields for about 15 years and thereafter, plantations need to be rejuvenated either by deep pruning and heavy manuring or by fresh batch of plants in a cyclic manner. These cuttings are then planted in nursery beds till rooting takes place. Such plants are available for transplantation in the field during July-August.

Regeneration

The phenomena of regeneration in plants have been known from very long time. It is important for cuttings of twigs and the leaves of many plants to become independent individuals. Reports on regeneration of roses emerged in early 1990s and similarly there have been reports of induction of somatic embryogenesis from a variety explants including leaves (De Wit et al. 1990; Rout et al. 1991). Induction of adventitious shoots and regeneration from callus cultures are of importance for somaclonal variations and therefore, for breeding. Hill

(1967) reported the formation of shoot primordial on calli derived from stem explants in a cultivar of Hybrid Tea rose.

Biochemical analysis of Rose

It is been suggested that most cutting with leaves should be rooted in an environment that is not only cundusive to photosynthesis (Davis 1988), but should also enhance photosynthetic rate in order to synthesize some organic substances for rooting. Photosynthesis could be required to provide carbohydrates, auxin or other substances to the base of the cuttings involved in rooting.

A high level of endogenous auxin is required during the root inducing stage (Michniwicz and Kriesel 1990), while a reduced level of endogenous auxin has been implicated in the failure of rooting in a number of species of plant cuttings (Smith and Wearing 1972). The investigation conducted by Brunner (1978) demonstrated that the levels of endogenous IAA and IBA at the rooting region of control and auxin treated hypocotyls in *Phaseolus vulgaris* L. increased markedly during the first 24 hours after excision of the hypocotyls. In apple microcuttings, following treatment with endogenous levels of free IAA increased markedly during the first 3 hours and subsequently decreased slowly (Noiton et al. 1992).

Clonal fidelity

Modern *Rosa hybrida* cultivars are the result of a long history of rose development through many centuries. They are selected for defined traits such as flower bud and flower qualities (shape, colour, fragrance, stem length, and vase life). Breeding to modify a single characteristic generally results in changes to other characters as well. Thus current crop improvement strategies incorporate molecular biology techniques to improve *Rosa x hybrida* (Rout et al. 1999).

Molecular markers consist of DNA sequences at specific positions on a chromosome, or their immediate products like enzyme molecules. There is a number of molecular techniques available for characterization of the variation at the DNA level, e.g. RFLP (restriction fragment length polymorphism), which is a hybridization-based methodology using locus-specific probes, and the PCR (polymerase chain reaction) based markers like e.g. RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) and ISSR (inter simple sequence repeats).

For genetic diversity studies, the RAPD technique (Williams et al. 1990) shows some important advantages. Like the, prior knowledge of the DNA sequence is not needed, which makes RAPDs very suitable for investigation of species that are with less known genome. Unspecific primers are used to amplify non-coding as well as coding regions of the DNA. The method is fast and easy to perform but has, unfortunately, a problem with reproducibility since small changes in the PCR conditions may lead to unspecific amplification of fragments (Williams and Clair 1993). However, this method has been efficiently used for studying the clonal fidelity of micropropagated plants (Kumar et al. 2010).

The working principle of ISSR PCR is similar to that of RAPD except that ISSR primer sequences are designed from microsatellite regions such as (AGTG) 4 or (AG) 8 who distribute widely in genome as good targets for a PCR-based fingerprinting technique. The ISSR markers are is more stable than the RAPD. ISSR markers have great potential for studying molecular genetics natural populations (Wolfe et al. 1998).

Keeping in view the importance, the present study was aimed at the following objectives:

- To study the possibility of shoot regeneration from leaf explants.
- To study the factors affecting shoot regeneration.
- Rooting of micro shoots with a pulse treatment of IBA and acclimatization of plants.
- Biochemical changes during rooting of microplants.
- To study the clonal fidelity of micropropagated plants.

REVIEW OF LITERATURE

Micropropagation has emerged as a powerful technique with the great potential not only for rapid clonal propagation of plant species but also for the conservation of rare and endangered species (Fay 1992). The most significant advantage offered by micropropagation is that, in a relatively short time and space, a large number of plants can be produced starting from a single explant (Zimmerman 1996; Jain 1997; Hassanein et al. 2003).

The genus *Rosa* is well associated with the culture, religion and socio-economic aspects of life. Rose came earlier as a source of fragrance, as a cure for illness and as a symbol of beauty but not as a popular bush for the garden. Roses are generally multiplied vegetatively by grafting buds on stem of wild rose and by cuttings (Roy et al. 2004).

Rose is an important perennial flower shrub or vine of the genus *Rosa*. Reports on *in vitro* multiple shoot formation and flower induction of *Rosa hybrida* L. cv. 'Red Marterpiece' with maximum number of 5 shoots per explant on MS medium supplemented with 3 mg/L BA and 1 mg/L kinetin, followed by flower induction on MS medium containing 2 mg/L for 9 weeks was reported by Kanchanapoom et al. (2009a). The cultures were initiated by using nodal explants.

A few reports in *in vitro* propagation of hybrid ornamental roses (Hill 1967; Jacobs et al. 1969; Elliott 1970; Hasegawa 1980; Skirvin and Chu 1979; Martin et al. 1981; Avramis et al. 1982) using different explants viz. axillary and apical buds, nodal segments, leaf , stem segments are available.

Micropropagation of *Rosa damascena* Mill. from mature bushes using nodal explants was reported by Kumar et al. (2001). The effect of Thidiazuron (TDZ) has been compared with that of BAP on *in vitro* shoot proliferation. Rooting was achieved in these micro shoots by pulse treatment of indole-3-butyric acid (IBA) and followed by culturing on PGR- free medium.

Nodal explants containing lateral buds of actively field grown 'Perfume Delight' rose were used for micropropagation. These was exercised and cultured on MS basal medium containing different concentrations of BA and NAA. Later the study regarding *in vitro* flowering was done (Kanchanapoom et al. 2009b).

In vitro multiplication of Rose (*Rosa hybrida.* cv. Baccara) was performed to investigate the effects of BAP, NAA, IBA and GA_3. Young shoots containing axillary buds were taken and initiated on above mentioned medium to obtain high growth and multiplication of rose. Rooting of shoots was achieved by half strength of MS medium containing 2 mg/L IBA with upto 90 % rooting (Jafari et al. 2011).

Regeneration from long-term embryogenic callus of the *Rosa hybrida* cultivar 'Kardinal' was achieved on Schenk and Hildebrandt's basal salts medium (SH) variously supplemented with PGRs was reported by Jones et al. (2004). Media components used for three stages of development: (1) callus maintenance, (2) maturation of embryos, and (3) conversion of embryos to plants were shown to affect regeneration of plants from commercially important Red Rose cultivar 'Kardinal'.

Somatic embryogenesis, which allows the production of plants from somatic cells, is an important approach for plant regeneration in woody species. Somatic embryos have a bipolar morphology and the regenerated plants possess complete root and shoot systems. Thus, such a regeneration pathway provides a more integrated and resilient material than organogenesis-obtained plants (Rout et al. 1991).

Development of somatic embryos which was initiated from leaf explants of rose cultured on MS basal medium supplemented with auxin followed by several subcultures on MS basal medium supplemented with cytokinin was reported (Byrne et al. 2004). Embryos were initiated and maintained in two of the five genotypes demonstrating genotype specificity.

A study described a plant regeneration system for *Rosa hybrida* cv. 'Samantha', via direct somatic embryogenesis (Bao et al. 2012). Somatic embryogenesis was induced from leaf explants, achieving a frequency of 7.5% following 8 weeks of culture on a Murashige and Skoog (MS) medium supplemented with 3.0 mg/L 2, 4-D and 30 g/L glucose.

To optimize the efficiency of direct organogenesis and indirect shoot organogenesis in different genotypes of *Rosa hybrida* cv. 'Apollo' cultures were supplied with different combinations of PGRs. The cultures were initiated on MS medium supplemented with BA, TDZ and 2, 4-D (Habashi et al. 2012). Similarly shoot regeneration in *Rosa damascena* shoot regeneration via callusing from stem and leaf segments was also achieved (Ishioka and Tanimoto 1990).

Somatic embryogenesis has the potential to be a very efficient method for rose micropropagation. Induction of somatic embryogenesis in *Rosa* species has been demonstrated from calli derived from leaf explants (De Wit et al. 1990; Hsia and Korban 1996). Similar results were reported from mature leaves of rose (*Rosa* sp.) via somatic embryo development on medium supplemented with plant growth regulators like BAP, 2, 4-D, GA_3, 3-IAA, Kinetin and NAA. (Manos et al.1999).

An assessment of genetic fidelity of rose through molecular markers was also studied (Senapati et al. 2012). Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) molecular marker techniques were employed to validate the genetic fidelity in three varieties of *Rosa hybrida* multiplied *in vitro* by axillary multiplication. The results obtained were that axillary multiplication is the safest mode for multiplication of true to type plants without any somaclonal variation.

RAPD and ISSR markers have been used to determine genetic relatedness of siblings, for cultivar identification, phylogenetic analysis within and among *Rosa* species (Debener and Mattieshch 1999) and mapping rose genes to powdery mildew or black spot. Molecular markers have also been used to identify the source of crown gall disease in roses, which was found in many cases to be transmitted via infected rootstock material.

Random amplified polymorphic DNA (RAPD) markers were utilized for screening the clonal fidelity of in vitro-raised bulblets of *Lilium* sp. Asiatic hybrids produced through adventitious mode of propagation (Varshney et al. 2001). Analysis of individual primers revealed the RAPD patterns produced were all shared by *in vitro*-raised bulblets and the mother bulb. There was no variation observed within the tissue culture-raised progenies.

RAPD and ISSR markers has earlier been used effectively used for the testing of clonal fidelity of the micropropagated plants of *Cholorophytum borivillianum* (Kumar et al. 2010).

MATERIALS & METHODS

3.1 Plant material

The cultures of *Rosa hybrida* cv. 'First Red' were kindly provided by Dr. Rajinder Kaur, Associate Professor, Y.S. Parmar University of Horticulture and Forestry, Solan. These cultures were successfully maintained on MS medium containing 3% sucrose and 0.7% agar supplemented with BAP and GA_3. These cultures were used for further experimentation and were source of explants.

3.2 *In vitro* Studies

(A) Chemicals and Glass wares

All routine chemicals were purchased from HiMedia laboratories, Mumbai, Plant growth regulators (PGR's) were purchased from Sigma chemicals Co. St. Louis, USA. Unless otherwise mentioned all experiments were conducted in 300 ml culture bottles (Kasablanka, Mumbai).

(B) Culture Media

Murashinge and Skoog medium (MS 1962. detail in Annexure 1) with sucrose 3% (w/v) and agar (0.7 % w/v) was used as basal medium throughout the experiment. Growth regulators viz., cytokinins and auxins were added to the basal medium either singly or in various combinations, especially as mentioned with each experiment. The concentrated stock solutions of all the ingredients were prepared and stored under refrigeration. Similarly stock solutions of plant growth regulators (PGRs) were also prepared. All plant growth regulators (PGR's) were dissolved in few drops of NaOH (1N) or HCl (1N) and made to desired volume (2.5 mM) with distilled water and stored at 4 ºC.

Medium was prepared by adding required quantities of all the ingredients in the conical flask. After adding all the ingredients in required amounts, final volume was made up with the help of distilled water. The pH of medium was adjusted to 5.8 using 1N NaOH or 1N HCl (Cyber scan 510, Eutech Instruments, Singapore). After adjusting the pH, medium (50 ml) was poured in culture bottles and capped with plastic caps. It was then autoclaved (Equitron, Medica Instruments, India) at 121 ºC for 20 minutes at 15 psi.

(C) Callus induction and regeneration

The expanded leaves from micro-shoots were used as explants. These were inoculated on to basal MS medium containing sucrose (3% w/v), agar (0.7% w/v) and supplemented with various combinations and concentrations of plant growth regulators, 2-4-D (0-12.5µM) and BAP (0-12.5µM). The pH of the medium was adjusted to 5.8 before autoclaving at 121 ºC for 20 min. All the cultures were examined periodically and visual observations were recorded.

(D) Culture Conditions

All culture were incubated under a photosynthetic photon flux density (PPFD) of 42 µ mol $m^{-2}s^{-1}$, 16/8 hour photoperiod and incubated at 25 ± 1 ºC.

3.3 Shoot multiplication

Shoot cultures were excised and sub-cultured on MS medium supplemented with 6-Benzyl amino purine (BAP) (20 µM) and Gibberellic acid (GA_3) (14.4 µM). These cultures were regularly sub cultured on the same medium with same concentrations of PGRs as mentioned above. After inoculation bottles were sealed with cling film and were incubated.

3.4 Regeneration

Fully developed leaf explants were harvested and inoculated (adaxial side down) on a MS basal medium supplemented with 3% sucrose as carbon source with different PGRs [6-Benzyl amino purine (BAP), 2, 4-Dichlorophenoxy acetic acid (2, 4-D), α-Naphthalene acetic acid (NAA)] and solidified with 0.7% agar. Leaf explants were inoculated on medium with 12 different combinations of BA (1µM, 5µM and 12.5 µM) and NAA (2.5 µM, 5 µM, 12.5 µM and 25 µM). In another experiment, cultures were inoculated on medium supplemented with combinations of BAP (1, 5 and 12.5 µM) and 2, 4-D (2.5, 5, 12.5 and 25 µM). A total of 30 leaf explants were inoculated onto each medium combination.

3.5 Rooting of shoots

Rooting efficiency of shoots subjected to a pulse treatment of Indole-3-butyric acid (IBA) supplemented medium was studied. The shoots (2-3 cm tall) were transferred to media containing different concentrations (50 µM and 100 µM) of IBA at a short exposure of 12, 24 and 36 hr and transferred to MS basal medium devoid of plant growth regulators (PGRs).

3.6 Acclimatization

For acclimatization rooted shoots were transferred to pots filled with a mixture of soil, sand and coco peat and kept in a mist chamber (green house) for hardening. After 15 days of hardening, the plants were scored for survival and subsequently transferred to a greenhouse without misting.

3.7 Biochemical changes during rooting of microshoots

Plant material

The shoots developed on MS medium containing BAP (20 µM) and GA_3 (14.4 µM) were subcultured on rooting medium as mentioned above. Each day a sample (1 g) was taken and dried in hot air oven at 80±2 ºC for 24 hr. After drying, the sample was crushed and proceeded for biochemical analysis.

Analysis of carbohydrates

a) Extraction of carbohydrates

Dried powder (50 mg) was extracted with 80% ethanol for one hour and centrifuged at 10,000 X g at 25ºC for 60 minutes. The pellet was re-extracted with 80% ethanol and centrifuged. The two supernatants were pooled and the volume was reduced to 25 ml which was used for the extraction of total soluble sugars, total reducing sugars and total non-reducing sugars and amino acid contents.

The pellet was used to estimate the starch content by first washing it with 80% ethanol and then with water. It was then dissolved in 2 ml water and to this 2 ml of 50% PCA (v/v) was added and hydrolysed at room temperature (25ºC) for 20 minutes. The mixture was then centrifuged at 10,000 X g at 25ºC for 30 minutes and the supernatant made to 40 ml with water which was used for starch estimation.

Preparation of reagents

a) **Phenol reagent:** 10 ml distilled water was mixed with 90 ml phenol solution (90 % w/v).

b) **Dinitro salicylic acid (DNSA):** 0.5 g of DNSA was mixed with 50 ml distilled water containing 0.8 g sodium hydroxide (NaOH). To this solution, 15 g of sodium potassium tartarate or di-sodium tartarate was added and the total volume was made to 50 ml with distilled water.

c) **Anthrone reagent:** Prepared by dissolving 200 mg anthrone in 100 ml cold concentrated sulphuric acid.

d) **Ninhydrin reagent:** 8 g of Ninhydrin was dissolved in 100 ml of acetone.

b) Estimation of total soluble sugars

The method of Dubois et al. (1956) was used for the estimation of total soluble sugars. The extract (0.1 ml) was taken and made upto 2 ml with distilled water and to this 0.05 ml of phenol reagent was added. The color was developed by rapidly adding 5 ml of concentrated sulphuric acid (H_2SO_4) and allowed to stand at room temperature for 30 minutes. Optical density was taken at 485 nm. The concentration of total soluble sugar was calculated using a Standard curve prepared by taking different concentrations of D-glucose.

c) Estimation of total reducing sugars

Reducing sugars were determined following the method of Sumner (1935) using DNSA reagent. The ethanolic extract was used for the determination of total reducing sugars. To 0.5 ml of this extract, 0.5 ml water and 1 ml of DNSA reagent were added. The reaction mixture was boiled for 10 minutes and after cooling at room, temperature, optical density (OD) was recorded at 560 nm. The concentration of reducing sugar was calculated using a standard curve prepared by taking different concentrations of D-glucose.

d) Estimation of total non-reducing sugars

This was calculated by subtracting the value of total reducing sugar from that of total soluble sugars and the results were expressed as mg/g dry weight.

e) Estimation of the starch

The starch content was determined by the method of Mc Cready et al. (1950). To 0.1 ml of extract, 0.9 ml water was mixed followed by the addition of chilled anthrone reagent (5 ml).

The mixture was heated in the boiling water bath for 10 minutes and then allowed to cool down to room temperature. Optical density was taken at 630 nm against anthrone reagent as blank. The concentration of starch was calculated using a standard curve prepared with D-glucose.

f) **Estimation of Phenols**

To determine phenol content in the plant, samples were extracted in 0.3 N HCl and methanol (2:1). Then allowed it to dry. 0.5 ml of this extract was taken after 12hr and made to a final volume of 7 ml with distilled water. The extract was shaken and kept in diffused light for some time. To it 0.5 ml of phenol reagent was added followed. Then kept undisturbed for 20-25 minutes. Then added 10 ml of 35 % Na_2CO_3 (sodium carbonate). Again shaken and kept for 1 minute. Optical density was recorded at 630 nm. The concentration of phenols was calculated using the standard curve prepared with Tannic acid.

g) Estimation of amino acids

Amino acid was determined by taking 0.1 ml of ethanolic plant extract and then distilled water was added to it making volume upto 4 ml. Then 1 ml of ninhydrin reagent was added to test tubes (5 ml). The contents were mixed and few glass beads were dropped in each tube to avoid bumping and covered with aluminium foil. The mixture was heated in the boiling water bath for 15 minutes and then allowed to cool down to room temperature. After cooling 1 ml of ethanol was added to the mixture and mixed well. The optical density was recorded at 570 nm.The concentration of amino acid was calculated using a standard curve prepared with Glycine.

3.8 Evaluation of Clonal fidelity

(A) DNA Isolation

Total genomic DNA was extracted from the leaf samples taken from each micropropagated plant and mother plant by the Cetyl- trimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987).

Procedure

- 3.0 g of fresh tissue was washed with distilled water, dried and grinded in liquid nitrogen to fine powder, followed by immediate transfer to 50 ml centrifuge tube. To this was added pre-warmed CTAB extraction buffer to make slurry and incubated at 60 °C for 1 h in water bath.

- 0.2 % of β-mercaptoethanol was added to each sample and mixed and incubated at 60 ºC for 20-30 minutes.
- Equal volume of chloroform and isoamylalcohol (24:1 v/v) was added to the above slurry and mixed for about 3 mins, followed by centrifugation at 8000 rpm for 10 mins at 4 ºC.
- Aqueous phase was removed with the help of wide-bore pipette and transferred to clean tube. Chloroform extraction step was repeated again in case extract was colored.
- DNA was precipitated with 0.66 volume of cold isopropanol followed by incubation for 1 h at -20 °C.
- After centrifugation (10,000 X g for 15 mins) the supernatant was discarded and the pellet was dissolved in 1 ml TE buffer and transferred to microfuge tube.
- To the above solution 2µl of pre heated RNase solution (10 mg/ml stock) was added and incubated at 37°C for 30-40 minutes.
- Equal volume of phenol, chloroform and isoamylalcohol was added (25:24:1) followed by gentle shaking and centrifuged (10000 X g for 10 mins).
- Upper aqueous layer was collected and equal volume of chilled isopropanol was added and incubated for 1 hr at -20 °C.
- Following incubation, centrifugation was carried out at 10000 X g for 10 mins. The pellet was retained, dried and 500 µl of 70 % ethanol was added.
- Centrifugation was again carried at 10,000 rpm for 10 mins. The pellet was retained and suspended in 30 µl TE buffer and stored at -20 ºC.

Reagents required:

- CTAB buffer (100 ml)

2 % CTAB	20 g CTAB
20 mM EDTA	40 ml EDTA stock (0.5 M)
100 mM Tris-HCL pH 8.0	100 ml Tris –HCL stock (1M)
1.4 M NaCl	280 ml NaCl stock (5M)

Volume made up to 1 liter with distilled water, pH 7.5- 8.0 and autoclaved.

Mercaptoethanol (0.2% v/v) was added into buffer just before use.

Other reagents:

- Isopropanol
- Chloroform
- Isoamyl alcohol
- Saturated phenol
- Sodium acetate 3 M
- TE buffer

20 mM EDTA	EDTA stock (0.5)
100 mM Tris-HCl pH 8.0	Tris- HCl stock (1M)

(B) RAPD and ISSR Analysis

The components of RAPD and ISSR PCR amplifications were: 25 ng of template DNA, 10 mM of each dNTPs (Biogene, USA), 10 µM of primer (Integrated DNA technologies and Operon molecules for life, USA), 1 U of Taq DNA polymerase (Geneaid, USA) and 1x reaction buffer (Geneaid, USA) in a total volume of 20 µl.

Table 1. Details of 2 RAPD primers used in present investigation**.**

Primer	Nucleotide Sequence (5´-3´)	Amplification
RAPD 21	CAGGCCCTTC	+
RAPD 22	TGCCGAGCTG	+

Table 2. Details of 2 ISSR primers used in present investigation**.**

Primer	Nucleotide Sequence (5´-3´)	Amplification
ISSR 22	$(GA)_8C$	+
ISSR 23	$(GA)_8CT$	+

Preparation of reaction mixture

The stocks were mixed by inversion and spin to collect solution. Reaction mixtures were prepared by mixing the following components in PCR tubes.

Table 3. Reaction mixture for PCR analysis.

Components	Stocks	Vol/ Rxn
Sterile water dNTPs	-	13.0 µl
PCR buffer with $MgCl_2$	10 X	2.0 µl
dNTPs	2.5 mM each	1.5 µl
Taq Polymerase	5 µl	0.4 µl
Primer	10 µM	1 µl
DNA	25 ng/µl	2 µl

PCR reactions

PCR tubes were placed in thermal cycler (Applied Biosystems 2700 thermal cycler) and amplified using the following temperature profile.

Table 4. PCR conditions regarding RAPD and ISSR molecular markers.

Temperature (ºC)	Time (mins)	No. of cycles
94	5	1
94	1	
35/55*	1	40
72	1.5	
72	5	1

*Annealing temperature for RAPD was 35 ºC for 45sec.

Annealing temperature for ISSR was 55 ºC for 1 min.

(C) Agarose gel electrophoresis

Amplified products were separated in 1.2% agarose gel containing ethidium bromide (EtBr) using 1x TAE buffer. A constant voltage of 45 was provided for 4-5 hr. DNA fragments were visualized under UV transluminator. The patterns were photographed using Gel Doc system and stored as digital pictures. The reproducibility of the amplification was confirmed by repeating each experiment three times.

RESULTS

4.1 Shoot multiplication

Shoot multiplication was observed on MS medium supplemented with BAP and GA_3 within 2 weeks. The cultures were inoculated in the form of bunches as well as single shoots to initiate growth (Fig.1).

4.2 Regeneration and shoot organogenesis

The culturing of leaves for about a week or 3 weeks on MS medium supplemented with different concentrations of BAP and NAA resulted in some changes in the morphology of explants, such as explant swelling and the suberization of cut ends with slight callusing. These were again cultured on same medium and no shoot regeneration was observed. The highest percentage of callus was observed at a concentration of BAP (12.5 µM) and NAA (25 µM) having a morphology of dark green and compact type (Table 5). Other morphologies which were observed are light green, greenish yellow, dark green and hard and compact (Table 5).

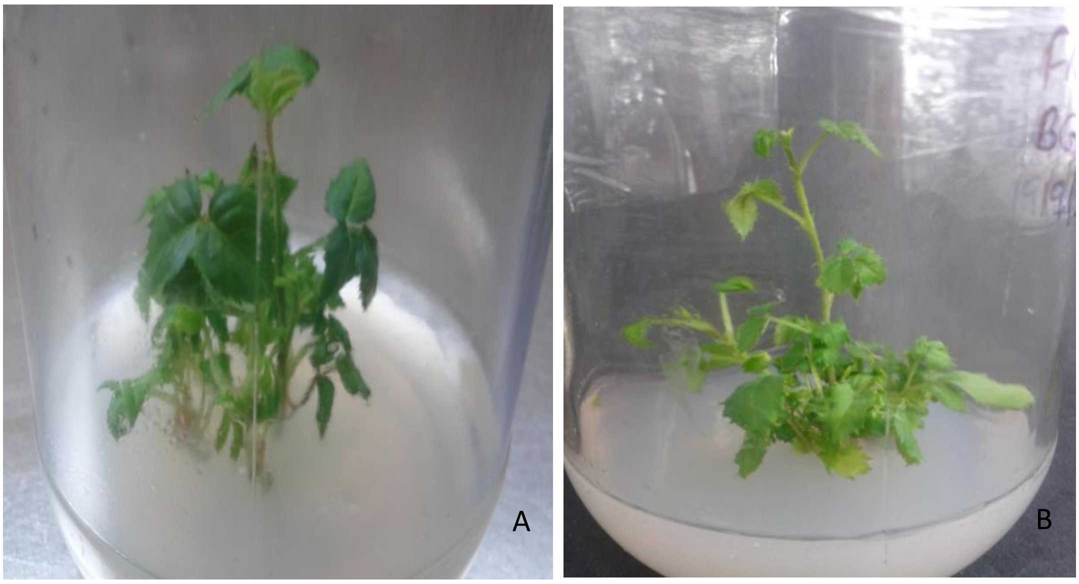

Fig.1. Multiplication of microshoots.

(A-B) Shoot multiplication on MS medium supplemented with BAP and GA_3.

Table 5. Effect of different concentrations of BAP and NAA on callusing in *Rosa hybrida* cv. 'First Red'.

BAP (µM)	NAA (µM)	% of explants -showing callus	Mean ± SD	Colour and morphology of callus
1.0	2.5	75	3.0 ± 0.0	Light green and compact
1.0	5.0	66.7	2.6 ± 0.5	Light green and compact
1.0	12.5	66.7	2.6 ± 0.5	Greenish yellow and hard
1.0	25	83.3	3.3 ± 1.1	Greenish white and compact
5.0	2.5	75	3.0 ± 1.0	Greenish yellow and hard
5.0	5.0	75	3.0 ± 1.0	Light green and hard
5.0	12.5	83.3	3.3 ± 0.5	Greenish white and compact
5.0	25	75	3.0 ± 1.0	Greenish white and compact
12.5	2.5	75	3.0 ± 0.0	Light green and compact
12.5	5.0	75	3.0 ± 1.0	Light green and compact
12.5	12.5	66.7	2.6 ± 0.5	Dark green and hard
12.5	25	91.6	3.6 ± 0.5	Dark green and compact

Similarly, leaf explants cultured on MS medium supplemented with different concentrations of 2, 4-D and BAP also differentiated callus. Callus like structures were observed but no shoot regeneration was seen (Fig.2). The highest percentage of callus was observed at a concentration of BAP (25 µM) and 2, 4-D (12.5 µM) having a morphology of greenish white and hard type. Other morphologies which were observed are light green, greenish yellow, dark green and hard and compact (Table 6).

Table 6. Effect of different concentrations of BAP and 2, 4-D on callusing in *Rosa_hybrida* cv. 'First Red'.

BAP (µM)	2,4-D (µM)	% of explants showing callus	Mean ± SD	Colour and morphology of callus
1.0	2.5	66.7	2.6 ± 0.5	Dark green and hard
1.0	5.0	75	3.0 ± 1.0	Light green and compact
1.0	12.5	83.3	3.3 ± 0.5	Light green and compact
1.0	25	75	3.0 ± 1.0	Light green and compact
5.0	2.5	75	3.0 ± 0.0	Greenish white and compact
5.0	5.0	75	3.0 ± 1.0	Pale green and hard
5.0	12.5	66.7	2.6 ± 0.5	Pale green and hard
5.0	25	83.3	3.6 ± 0.5	Greenish white and hard
12.5	2.5	75	3.0 ± 0.0	Greenish white and friable
12.5	5.0	66.6	2.6 ± 0.5	Light green and compact
12.5	12.5	66.6	2.6 ± 0.5	Dark green and hard
12.5	25	83.3	3.3 ± 1.1	Dark green and hard
25	2.5	75	3.0 ± 1.0	Dark green and hard
25	5.0	75	3.6 ± 0.5	Pale green and compact

25	12.5	91.6	3.3 ± 0.5	Greenish white and hard
25	25	83.3	3.3 ± 1.1	Greenish white and hard

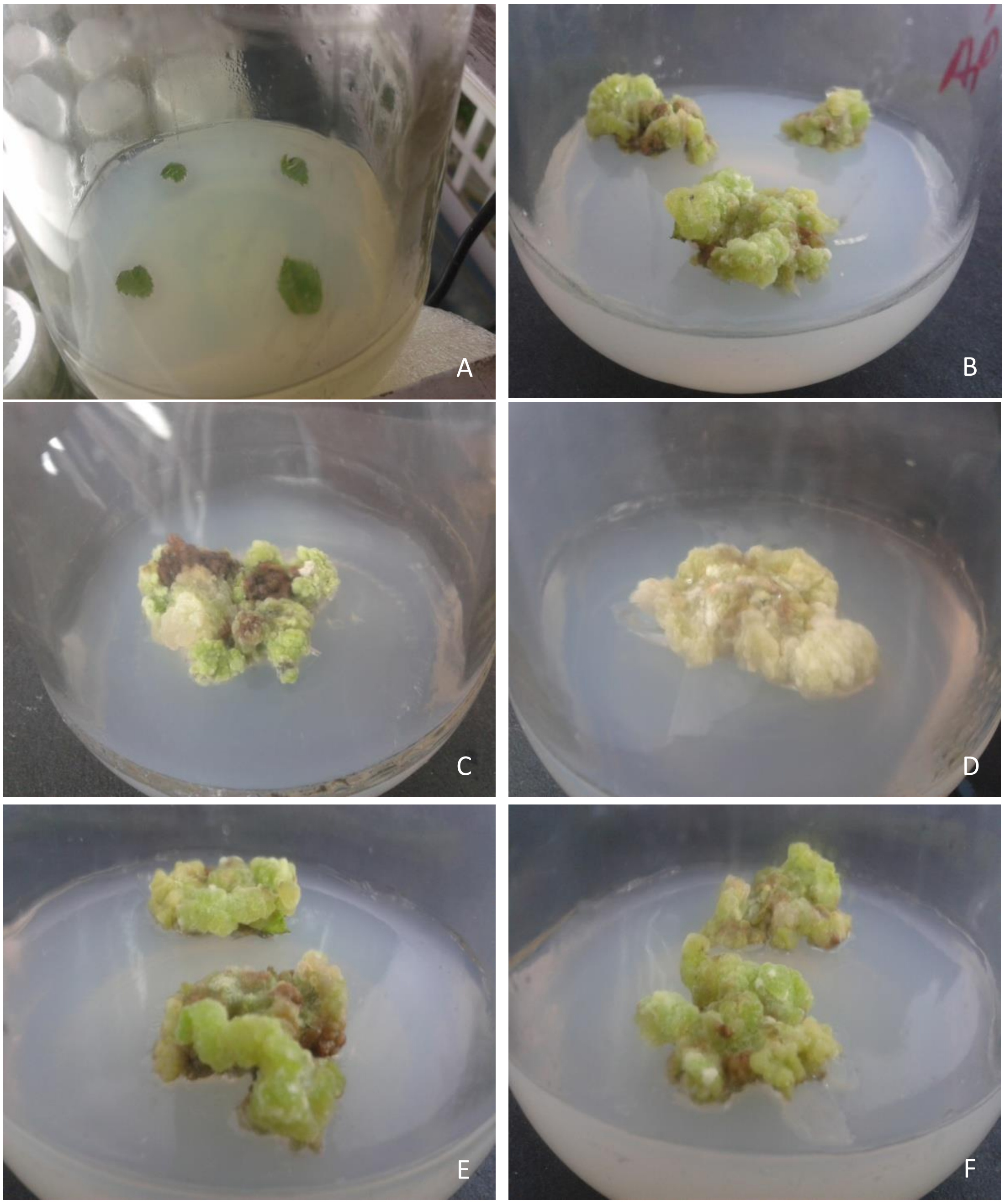

Fig.2. Callus initiation of *Rosa hybrida* was cultured on MS medium supplemented with different concentrations of auxins and cytokinins.

(A) Leaf explants inoculated on the regeneration medium. (B) Leaf explants showing green coloured hard callus, (C-D) Leaf explants showing whitish-green friable callus, (E-F) Leaf explants showing light green and compact callus.

4.3 Rooting

Rooting of microshoots was induced by the pulse treatment of IBA in MS medium. Different concentrations (50 and 100 µM) of IBA media was provided for a short period of span (12, 24 and 36 hr) and incubated in dark at 25 ºC. After the short exposure of IBA the cultures were transferred to MS basal media and incubated in light at 25 ºC. Maximum rooting was observed in MS media supplemented with 50 µM for 12 hr exposure with a percentage rooting of 94.3 %. With an increase in exposure period at 50 µM the percentage rooting gradually decreased. With an exposure of 100 µM concentration the rooting percentage was reduced to 72.3 % which continued to decrease with an increase in exposure period (Table 7; Fig.3a & b).

Table 7. Effect of MS medium supplemented with IBA on root induction of *Rosa hybrida.*

Concentration (µM)	IBA exposure time to shoots	No. of days taken	No. of roots per shoot	% of shoots rooting	Mean ± SD
50	12	9	11	94.3	5.6 ± 0.5
	24	10	9	83.3	5.0 ± 1.0
	36	12	9	72.2	4.3 ± 0.5
100	12	11	8	72.3	4.3 ± 0.5
	24	13	8	55.5	3.3 ± 0.5
	36	14	7	55.5	3.3 ± 0.5

4.4 Acclimatization of plants

After 5-6 weeks of culturing on rooting media, the plantlets were shifted to plastic pots for their hardening prior to final transfer to soil in natural conditions. After 15 days of hardening in the green house, plants were shifted to the earthen pots (Fig. 3 c & d).

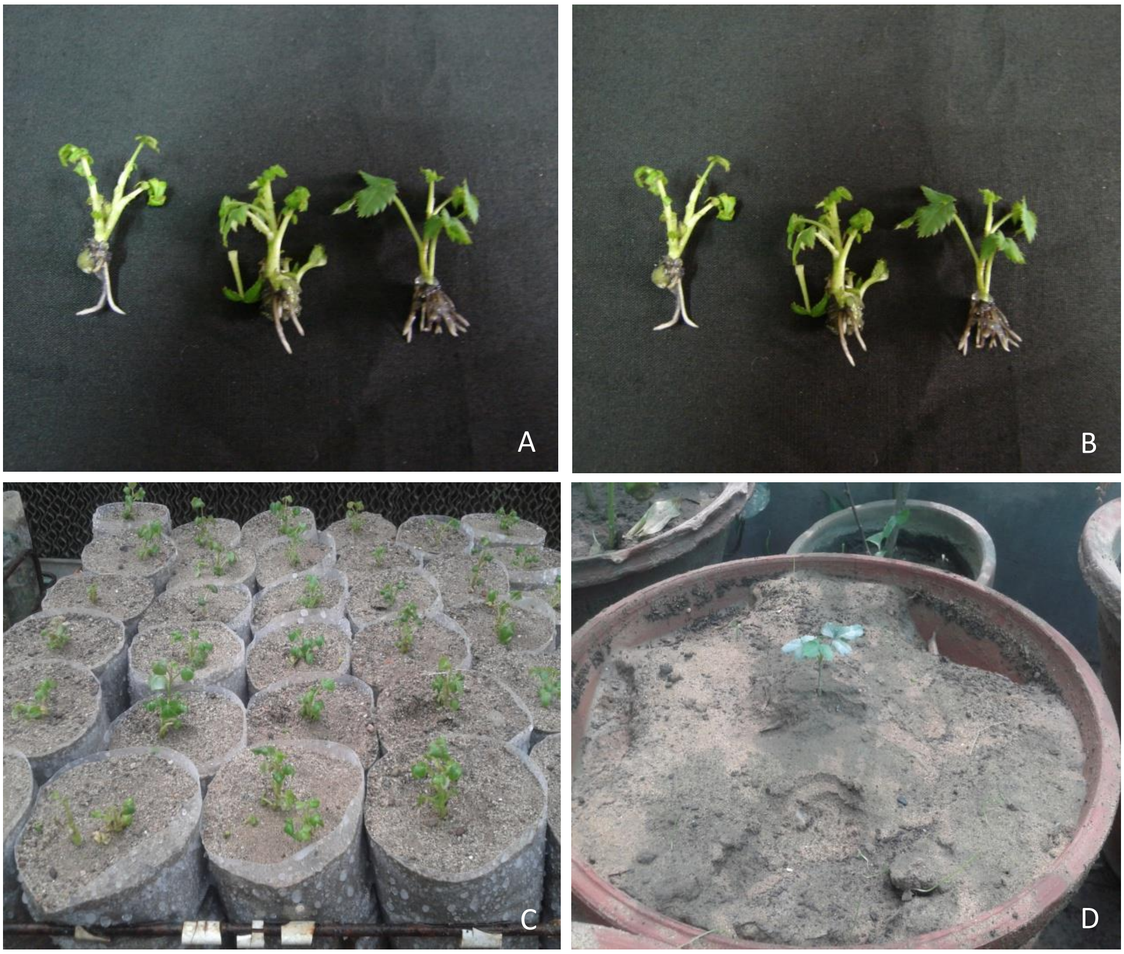

Fig.3. Rooting and acclimatization of microshoots after IBA treatment.

(A-B) Rooting of microshoots on MS medium supplemented with IBA.

(C) Acclimatization of rose plant in plastic pots. (D) Acclimatization of rose plant in earthen pot.

4.5 Changes in carbohydrates during rooting following IBA treatment

IBA (50 µM) was provided to the cultures for a short period of 12 hrs. After treatment with IBA shoots were inoculated to MS basal medium, it was observed that there was sharp increase in the amount of total soluble sugars, total reducing sugars and total non reducing sugars from control till the 7th day of inoculation. But on the 8th day the concentration decreased as the root emergence took place. The highest concentration of all three parameters was observed on the 7th day of exposure time (Table 8).

Table 8. Changes in soluble carbohydrates on MS basal medium following pulse treatment with 50 µM concentration of IBA for 12 hours leading to root formation.

Incubation time after exposure to IBA	Total soluble sugar (Mean ± SD)	Total reducing sugar (Mean ± SD)	Total non-reducing sugar (Mean ± SD)
Control	31.3 ± 3.0	28.6 ± 9.8	4.0 ± 3.1
Day 1	55.0 ± 50.7	27.4 ± 16.0	54.0 ± 20.7
Day 2	72.6 ± 9.2	23.4 ± 16.1	49.2 ± 10.2
Day 3	89.6 ± 15.8	29.2 ± 22.5	60.4 ± 20.3
Day 4	109.0 ± 45.5	20.8 ± 5.2	88.2 ± 45.5
Day 5	140.6 ± 30.0	29.0 ± 21.1	111.6 ± 28.5
Day 6	148.0 ± 8.7	31.2 ± 26.0	116.8 ± 7.9
Day 7	202.0 ± 14.4	45.8 ± 18.4	156.1 ± 14.0
Day 8	110.6 ± 3.0	34.2 ± 9.8	76.4 ± 2.7

Control: No exposure to IBA, Day 1 to Day 8: different periods of exposure after the IBA treatment.

The starch content was observed continuously varying in all treatments. The starch was high in control and it decreased on 1st and 2nd day of inoculation. On 3rd day, starch again increased to the maximum till 7th day. On the 8th day, starch content again dropped.

Similar condition was seen in case of phenols and amino acids. The phenol content was less in control and increased on 1st day of inoculation after IBA treatment and continues to increase till the end of experiment i.e., 7th day after IBA treatment phenol content again raised to the maximum and decreased as the root emerges.

In case of amino acids, the amount of amino acid decreased from control to the 1st day of inoculation, which again consistently increased till 7th day and reaches to the maximum value. On 8th day, the amount decreased as root emerges out (Table 9).

Table 9. Changes in the starch, phenol and amino acid contents on MS basal medium following pulse treatment with 50 µM concentration of IBA for 12 hrs leading to root formation.

Incubation time after exposure to IBA	**Total starch content (Mean ± SD)**	**Total phenol content (Mean ± SD)**	**Total amino acid content (Mean ± SD)**
Control	113.3 ± 10.0	55.3 ± 7.6	4.26 ± 1.1
Day 1	83.0 ± 10.5	53.3 ± 5.5	3.26 ± 8.7
Day 2	86.3 ± 5.8	44.6 ± 1.5	3.7 ± 5.2
Day 3	97.3 ± 32.8	46.6 ± 0.5	3.8 ± 4.0
Day 4	103.6 ± 13.6	47.3 ± 19.8	4.0 ± 0.5
Day 5	117.3 ± 1.1	48.6 ± 0.5	4.7 ± 3.0
Day 6	125.3 ±22.0	54.3 ± 1.1	5.0 ± 1.1
Day 7	127.0 ± 6.2	64.6 ± 13.3	5.1 ± 4.1
Day 8	88.6 ± 7.2	38.0 ± 0.0	4.2 ± 1.1

Control: No exposure to IBA, Day 1to Day 8: different periods of exposure after the IBA treatment.

4.6 Clonal fidelity of micropropagated shoots

The RAPD and ISSR profiles of plants derived from newly formed shoots and those of the mother plant were similar, indicating the clonal nature of these plants. A total number of 10 primers were used (5 each of ISSR and RAPD) out of which 2 ISSR and 2 RAPD primers resulted in the amplification of DNA fragments. These primers produced a maximum of 8 and a minimum of 1 band. Of the total of 25 markers obtained, 11 were scored using ISSR and 14 were scored using RAPD (Table 10). The size of the amplified markers ranged from

250 to 1,500 bp. This similarity in the banding profiles of the ISSR and RAPD markers in plants derived from micropropagated plants and the mother plant established the clonal nature of these shoots (Fig.4).

Table 10. Details of ISSR and RAPD primers and amplified bands of all the DNA samples.

Primer	**Sequences**	**Annealing temp. (ºC)**	**No. of scorable band**
ISSR 21	CAGGCCCTTC	55	6
ISSR 22	TGCCGAGCTG	55	5
RAPD 22	$(GA)_8C$	35	8
RAPD 23	$(GA)_8CT$	35	6

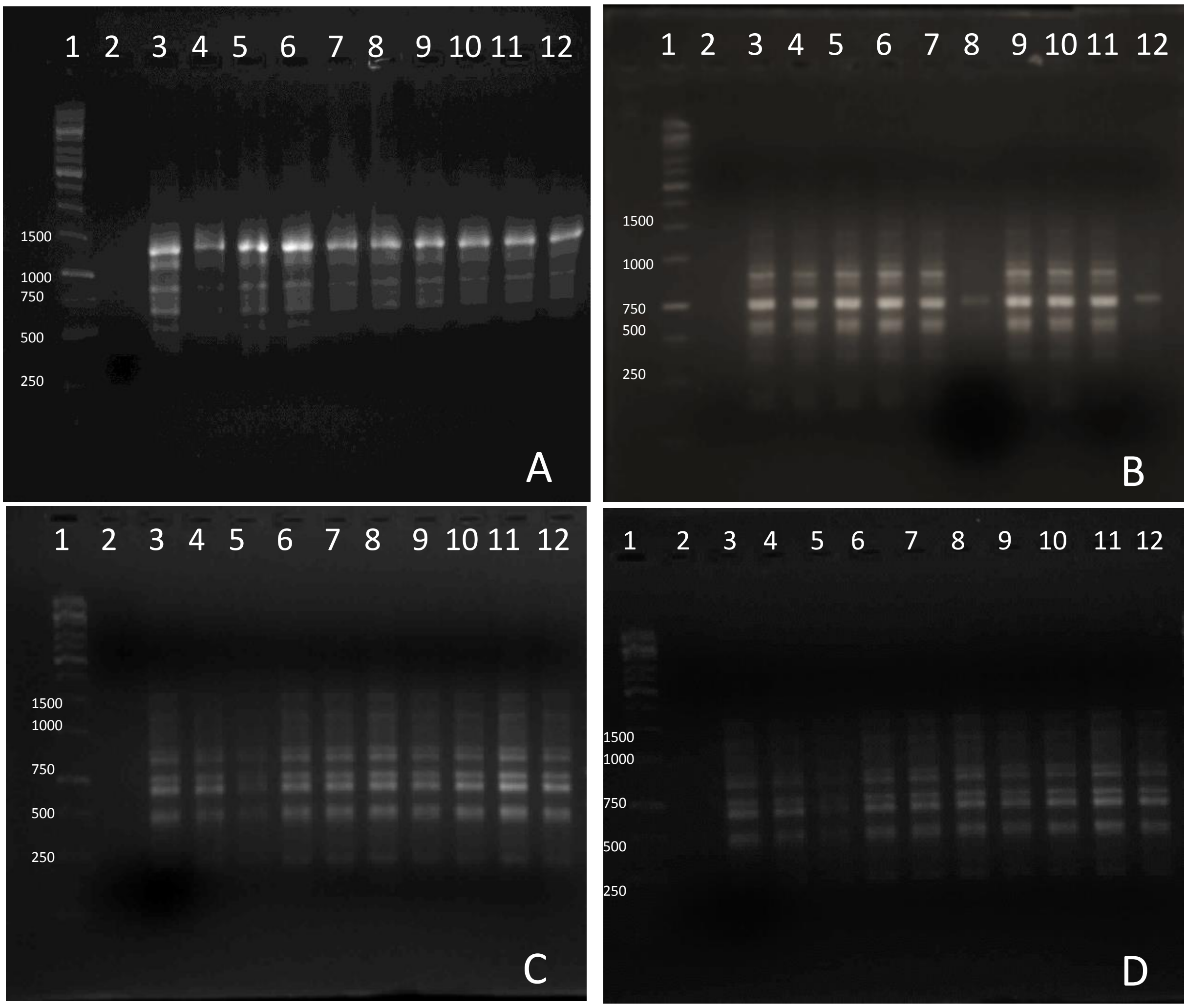

Fig.4. Gel electrophoresis of amplification products obtained using RAPD and ISSR primers. Profiles of micropropagated plantlets and the mother plant of *Rosa hybrida* cv. 'First Red'. Lane 1 Marker (1 Kb), Lane- 2 Negative control, Lane 3- mother plant and Lane-4 to 12 DNA from micropropagated plants.

A: Primer ISSR 21; B: Primer ISSR 22; C: RAPD 22: D: RAPD 23.

DISCUSSION

Rose is one of the most important commercial crops. It is generally propagated by vegetative methods like cutting, layering, budding and grafting. Seeds are used for propagation of species, new cultivars and for production of rootstocks (Horn 1992). Although propagation by vegetative means is a predominant technique in roses, yet it does not ensure healthy and disease-free plants. Moreover, dependence on season and slow multiplication rates are some of the major limiting factors in conventional propagation. So, in present investigation, *Rosa hybrida* cv. 'First Red' was successfully propagated under *in vitro* conditions. Factors effecting shoot multiplication, rooting, estimation of carbohydrates, starch, phenols and amino acids during rooting of microshoots and clonal fidelity were studied.

The success of a micropropagation protocol, to a large extent, depends on the shoot multiplication. For multiplication, the media formulations containing cytokinins as a major PGR is crucial, whereas, in some cases, low concentrations of auxins and GA_3 were also used (Pati et al. 2006). In our study, shoot multiplication of *Rosa hybrida* cv. 'First Red' was initiated by supplying BAP (20 µM) and GA_3 (14.4 µM) to obtain large number of shoots. As BAP is the most effective growth regulator and helps in stimulating shoot proliferation (Vijaya et al. 1991; Rout et al. 1999). BAP has approximately been used as an important and basic PGR in all the previous researches about micropropagation of rose. But it has been proved as a basic and important PGR in all the previous researches about micropropagation of rose. But with increase in the concentrations of BAP, the shoot length gets inhibited because of increase in the ethylene levels in the plants, which is known to block the basi-petal transport of endogenous auxin in the shoots resulting in inhibition of elongation of shoot length (Ahmad et al. 2003).

Addition of GA_3 to the medium did not significantly affect the proliferation but it can reduce shoot survival and leaf expansion (Skirvin et al. 1990). There are few reports available on incorporation of GA3 in the media for micropropagation of shoots (Skirvin et al. 1990) Nevertheless Rout et al. (1991) reported that incorporation of gibberellins at low concentrations (0.1-0.25mg/l) in the BAP supplemented medium improved shoot growth. But

BAP + GA_3 resulted in early bud break within 2 weeks with enhanced rate of shoot multiplication (Hasegawa 1980; Wulster et al. 1980).

Callus induction in our study were obtained from the leaf explants of *Rosa hybrida* cv. 'First Red' when maintained on MS medium provided with BAP, NAA or 2, 4-D. The callus was obtained within 2-3 weeks of inoculation of leaves giving rise to green and compact callus. The growth rate of callus was observed more on media having higher concentrations of auxins than cytokinins (BAP- 12.5 µM and NAA- 25 µM; BAP- 25 µM and 2, 4-D- 12.5 µM) (Table 5 & 6). The highest percentage of callus of 'First Red' was 91.3%. The inclusion of 2, 4- D alone or in combination with other plant growth regulators in the culture medium has been reported to be necessary for inducing somatic embryogenesis in roses (Rout et al. 1991; Hsia and Korban 1996). However, no such morphogenetic event was recorded in the present study.

Zieslinski et al. (1987) studied interaction of calli from different roses grown in close proximity. Khosh-Khui and Sink (1982) studied parameters involved in the establishment of friable callus but could not induce differentiation in these calli. These results are in line with present observations. In many cases callus formation in the presence of 2, 4-D and BAP has been reported, but no differentiation took place. In another cultivar of *Rosa hybrida* L., shoot and somatic embryo regeneration was reported in half strength MS medium supplemented with BAP (10 µM), NAA (0.05 µM), GA_3 (4.0 µM) and 600 mg/L L-proline (Rout et al. 1991). Efforts were made to regenerate the plant, but regeneration could not be achieved as it is difficult to initiate shoots from the leaf explants of rose (Kumar et al. 2010). Studies are still in progress to obtain shoot regeneration.

For any micropropagation protocol, successful rooting of microshoots is a pre-requisite to facilitate their establishment in soil. In our study, the rooting percentage was markedly enhanced when shoots were given a short exposure to IBA (50 µM; 12 hr) following this treatment direct rooting was observed. Exposure to IBA is known to induce roots in microshoots (Zimmerman and Broome 1981; Kumar et al. 2001). Short treatment of IBA has also been found useful in root induction in pigeon pea (Geetha et al. 1998). Such rooting response is considered better for field establishment of *in vitro* plants. As, IBA increases the cambial growth at the base of micro shoots resulting in the differentiation of root primordial (Haq et al. 2009). Roots were induced from excised mature microshoots on MS medium supplemented with low concentrations of auxins (Senapati and Rout 2008). A low percentage of rooting in medium devoid of IBA indicated the role of auxin along with some root inducing factors might occur naturally within the micro cuttings in the development of primordia (Haq et al. 2009).

In this study, some of biochemical changes were studied during root initiation after the IBA pulse treatment. Changes in total soluble sugars, total reducing sugars, total non-reducing sugars, total starch content, total phenol content and total amino acid content till root emerges were studies. After the IBA pulse treatment the high concentration of total soluble sugars, total reducing and non reducing sugars was recorded. This concentration kept on increasing till the 7th day, as during this period root induction, differentiation and emergence takes place. This suggests the involvement of sugars during adventitious rooting in rose. All through root induction to emergence the total soluble sugars were found to increase. An increase in carbohydrates has been observed during root induction following auxin treatment (Haissig 1986).

Earlier auxin treatment leading to root induction has been shown to increase the reducing sugar contents in the cuttings (Haissig 1986). These results are in line with these earlier findings. Moreover, reducing sugars on the 8th day in the hypocotyls might be consumed for growth, or even basipetally lost to the medium as the process of rooting is energy demanding. The importance of carbohydrate metabolism during rooting has been recognised by many researchers (Nanda et al. 1972). Activity of the key enzymes of carbohydrate metabolism through Embden Meyerhof Parnas (EMP) and pentose phosphate (PP) pathways was found to be higher during rooting of cuttings (Haissig 1982). Therefore, the level of sugar content dropped. However, IBA may regulate the metabolism of the cutting (Moncousin et al. 1988) indicated that a transient rise in the levels of carbohydatres occurs before the root initiation but the levels fall prior to subsequent cell division. Free auxin was also found to increase in the terminal cuttings until such time when the first adventitious roots become visible. Increased transport of carbohydrates from the leaves to the rooting zone has been observed in auxin treated cuttings (Nanda et al. 1972, Patrick and Wareing 1978).

Starch, phenols and amino acid contents in the shoots were measured in order to examine, whether IBA treatment contribute to accumulation of these contents. These contents increased during the first 7 days after the treatment with IBA, in which root primordia were formed and starch, phenol and amino acids were present in the basal hypocotyls. This decrease could be ascribed to it being consumed during root initiation and development. While starch, phenol and amino acid in the control increase. A further determination shows that starch, phenol and amino acid content after IBA pulse treatment at 7th day were different from that of 8th day. With this it can be concluded that all these contents get consumed as the root emerges.

The banding profiles of the RAPD and ISSR markers indicate that all of the newly micropropagated plants were similar to each other and to the mother plant, thereby indicating their clonal nature. The utility of RAPD and ISSR markers for testing the clonal fidelity of micropropagated plants has been well documented (Chandrika et al. 2008; Joshi and Dhawan 2007; Kumar et al. 2010; Martins et al. 2004). RAPD and ISSR markers have also been successfully used for the genetic analysis of *Eucalyptus* (Rani and Raina 1998). In our study, micropropagated plants showed a high degree of genetic uniformity that may be due to the stability of the genome to aseptic manipulations and culture pressures during shoot multiplication.

CHAPTER 6

CONCLUSION

In present time, biotechnology has become an important and indispensable part in breeding of rose and propagation programs as it eliminates the sterility problems through embryo rescue, decreases the breeding cycles, create variations by *in vitro* mutagenesis and leads to cultivar development through somaclonal variation. Tissue culture protocols play a vital role in commercial by producing disease-free plants.

Some protocols to induce shoot proliferation are developed which can lead to faster multiplication of shoot cultures. In order to meet the present day requirements of rose plants new protocols for rooting are developed which are very effective. The *in vitro* propagation of rose and direct regeneration protocols using leaf explants offers a great potential of rapid multiplication. The conclusions drawn from present study includes:

- *Rosa hybrida* cv. ‘First Red’ was successfully propagated by supplementing BAP and NAA under *in vitro* conditions.
- The multiplication of cultures was initiated on MS basal medium supplemented with BAP and $GA_{3.}$
- The leaf explants resulted in the callus induction when initiated on MS medium supplemented with BAP, NAA and 2, 4-D. But unfortunately no shoot regeneration obtained.
- Rooting was initiated by transferring shoots on MS basal medium following pulse treatment with 50 µM concentration of IBA for 12 hrs.
- Following IBA treatment total soluble sugars (reducing and non reducing sugar) were observed to increase during root induction.
- Acclimatization of rose plantlets were successfully done in fields.
- The clonal fidelity of micropropagated plants and those of mother plant were similar and revealed no variations.

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ANNEXURE 1

Media Composition

• Murashige and Skoog (1962) Medium

1. Macronutrients (Hi Media) mg/L

NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170

2. Micronutrients (Hi Media) mg/L

$MnSO_4.4H_2O$	16.90
$FeSO_4.7H_2O$	27.80
$ZnSO4.7H_2O$	8.60
H_3BO_3	6.20
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4.7H_2O$	0.025
$FeEDTA.2H_2O$	30.00

3. Vitamins (Hi Media)	mg/L
Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCL	0.5
Thiamine HCL	0.1

4. Sugar 30 g/L

5. Agar 7 g/L

ANNEXURE 2

1. CTAB Buffer

2% CTAB

20mM EDTA

100Mm Tris-Cl (pH 8)

1.4M NaCl

0.2% ß-mercaptoethanol

2. TE Buffer

1M Tris-Cl (pH 7.4)	10 ml (10mM)
0.5M EDTA (pH 8)	2 ml (1mM)

Adjust the volume to 1 lt by distilled water.

3. 10X TBE Buffer (Tris-borate-EDTA)

Tris Base	108 g
Boric Acid	55 g
0.5M EDTA (pH 8)	40 ml
Final pH	8.0

Make up the volume to 1 litre. Sterilize by autoclaving.