DEVELOPMENT OF *IN VITRO* REGENRATION PROTOCOL FOR APPLE ROOTSTOCKS M7 AND M9.

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By

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CERTIFICATE

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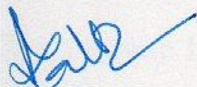

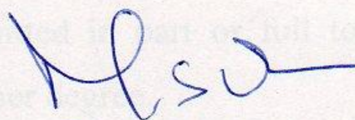

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I hereby declare that the work which is being presented in this dissertation entitled"- Development of *in vitro* regeneration protocol for apple rootstocks M7 and M9" submitted by the undersigned in partial fulfilment of the requirement for the award of Degree of Master of Sciences 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 Environmental Sciences, Thapar University, Patiala, India. The 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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ABSTRACT

This study was taken up for the *in vitro* propagation, regeneration and rooting of apple rootstocks M7 and M9. Murashige and Skoog's (1962) medium was used throughout the study. Maximum shoot multiplication was achieved when basal medium supplemented with 2.5 µM 6-Benzylamino purine (BAP) and 0.5 µM Naphthalene acetic acid (NAA). Rooting of microshoots was achieved on MS medium supplemented with 5.0 µM IBA. Shoot regeneration was attempted on MS medium supplemented with various concentrations of BAP and 2, 4-dichlorophenoxyacetic acid (2, 4- D) or NAA. Although initially no shoot regeneration occurred but shoots were regenerated when nodular calli developed on MS medium variously supplemented with BAP and NAA. Shoots were cultured on MS medium supplemented with 1.0 – 5.0 µM NAA and 1.0 – 5.0 µM BAP in both rootstocks. Histological analysis of regenerating tissue revealed that the developed structures were shoot buds. The clonal fidelity of regenerated plantlets was established using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Specific Repeats (ISSR) markers.

List of Abbreviations

%	Percent
µl	Microlitre
BAP	6-Benzylamino purine
NAA	Naphthaleneacetic acid
IBA	Indole-3- butyric acid
2, 4 –D	2, 4-dichlorophenoxyacetic acid
TBA	Tertiary butyl alcohol
h	hour
Kan	Kanamycin
M	Molar
µM	Micromolar
mM	Millimolar
mg	Milligram
mins	Minutes
ml	Mililitre

L	Litre
MS	Murashige and Skoog medium
ºC	Degree centigrade
w/v	Weight by volume
PGR(s)	Plant growth regulator(s)
SE	Somatic embryogenesis
ELS	Embryo like structures
RAPD	Random Amplified Polymorphic DNA
ISSR	Inter Simple Specific Repeats
dNTPs	deoxynucleotide triphosphates
PCR	Polymerase Chain Reaction

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INTRODUCTION

The apple (*Malus domestica)* is the pomaceous fruit belonging to family- Rosaceous. It is a widely cultivated fruits tree and well known amongst members of genus *Malus*. The tree originated in Western Asia, where its wild ancestor "the Alma" is still found. Different cultivars vary in their yield and ultimate size of tree, even when grown on same rootstock.

The apple tree was earliest to be cultivated, and its fruits have been improved through selection over thousands of years. There are more than 7,500 known cultivars of apples (Elzebroek and Wind 2008). Its economic value is due to its large scale consumption in food and beverage industry (Elzebroek and Wind 2008)**.**

Apples are very popular throughout the world because of great variety of food products that can be made from it like jams, jellies, beverages (like apple juice, wine) etc. (Downing 1989). Extremely sweet apples with barely any acidic flavour are popular in Asia and especially India (Tarjan et. al., 2006).

Although, India is not a major apple producing country yet it is ranked sixth in world with the average production of 1.4 million tons during 2002-04. Area under apple orchard in India is estimated to be second largest in the world, with its average yield of about 5.5 tons per hectare.

Apples are propagated vegetatively by grafting. In order to have fruits of uniformly good quality, most apple trees are propagated by grafting the scion of desired cultivar (which determines the fruit variety) on selected rootstock.

Apple cultivars

There are more than 7,500 known cultivars of apples. Different cultivars are available for temperate and subtropical climates. One large collection of over 2,100 apple cultivars is housed at the National Fruit Collection in England. Most of these cultivars are bred for eating fresh (dessert apples), though some are cultivated specifically for cooking (cooking apples) or producing cider (Tarjan et. al., 2006).

Commercially, popular apples are soft but crisp. Other desired qualities in modern commercial apple breeding are colour, absence of russeting, ease of shipping, longer shelf life, higher yield, disease resistance and flavour.

Modern apples are generally sweeter than older, as taste liking for apples have varied over time. Extremely sweet apples with barely any acid with flavour are popular in India. At least 55 million tonnes of apples were grown worldwide in 2005, with a value of about $10 billion. 35% of this total produce was from China. The United States ranked second with more than 7.5% of world production.

Apple rootstocks

Rootstocks influencing tree size and other characteristics have been used in apple propagation for over 2,000 years. Dwarfing rootstocks were probably discovered by chance in Asia. Alexander the Great, sent samples of dwarf apple trees back to his teacher, Aristotle, in Greece. They were maintained at the Lyceum, a centre of learning in Greece.

Most modern apple rootstocks were bred in the 20th century. Research leading to the selection of existing rootstocks was carried out at East Malling Research Station, Kent, England. Scientists at Malling research station worked in collaboration with the John Innes Institute and Long Ashton to produce a series of rootstocks, influencing characteristics such as disease resistance tree sizes, longevity and tree vigor, which have been used all over the world.

The rootstock influence many traits such as resistance to biotic and abiotic stresses, longevity, size of the tree and quality of fruit (Soumelidou et. al., 1994; Kamboj et. al., 1999; Michalczuk 2002). Other desirable characteristics imparted to the plant by rootstocks are disease resistance, fruiting at earlier age and improved fruit quality (Welander 1988).

Thus rootstock is an important part of the apple tree and development of good quality rootstocks is as much important as developing good quality scion. A good quality rootstock should possess certain characteristics like: easy to bud or graft, good root system which can provide proper anchorage to trees; should be able to induce early and heavy cropping. Rootstock should be resistant to most of pests and diseases such as black root rot (caused by *Xylaria Mali*), Rosellinia root rot. (Hemmat et. al., 1970).

Besides these qualities, rootstock should also have some other characteristics like winter hardiness and ability to grow in acidic, alkaline or water logged soil. Now a days there is a range of apple rootstocks available which vary in their characteristics like height, vigor and fruit bearing capacity of tree, but still there are a very few rootstocks which are resistant to biotic and abiotic stress.

Some of the commonly used rootstocks are M9, MM11, MM106, and M7.

M 7 (Malling 7) ROOTSTOCK

This rootstock was developed from a series of French rootstocks, "Doucin Reinette," known as the Doucin group, dating back to 1688. It is a semi dwarf rootstock, sturdy, disease resistant, winter hardy, uniform, productive rootstock that adapts well and transplants easily. It does best on deep, fertile, well-drained soils that retain consistent moisture. Anchorage problems with some varieties and heavy suckering are its most serious problems. It is resistant to Collar Rot but susceptible to wooly aphids.

M9 (Malling 9) ROOTSTOCK

It is probably the most widely-planted of all rootstocks. There are a very large number of clones of M9, with varying qualities and vigours. Its disadvantage is susceptibility to fire blight, which is endemic to some parts of North America. Apple trees on M9 are very productive and come into bearing within 2-3 years of planting; the tree reaches full size after about 5 years. M9 is also an excellent choice for the smaller garden or community orchard.

These rootstocks are propagated vegetatively. There is a huge demand of the planting material by this grower. In order to meet the requirement of healthy plants of rootstock in large number, faster methods of vegetative propagation such as micropropagation are required to be standardized. Further, there is a requirement of trait specific modification of these rootstocks through genetic transformation.

For a successful genetic transformation an efficient regeneration system is a prerequisite. Therefore, the present study was aimed at the following objectives.

The key objectives of our study are -

- To achieve shoot regeneration (direct or indirect) from leaf segments.
- To establish the mode of regeneration (shoot or somatic embryos) through histological studies.
- To induce rooting of micro shoots and establishment in the field.
- To study clonal fidelity of regenerated apple plants.

LITERATURE REVIEW

2.1 APPLE ROOTSTOCK

Apple like other fruit trees is not propagated by seeds. The plants are produced by grafting the scion from a good quality bearing apple tree on the suitable rootstock with desirable characters (like early and heavy fruiting, pest and disease resistance, ability to grow an alkaline and acidic soil etc.). Thus commercially available apple trees have dual genetic system formed as result of combination of rootstock and scion. However, graft incompatibility occurs frequently in these combinations (Errea et. al., 2001).

Economically it is useful to develop high density orchards for which dwarfing rootstocks are used (Awasthi et. al., 2001). Dwarfing rootstocks are used for commercial production as it give rise to high tree density, early fruit production, less expenditure on pesticides and high labour efficiency (Falahi et. al., 1997; Lakso et. al., 1999 and Welander 1988). Dwarf rootstocks give rise to fruit trees of small sizes which are easier and cheaper to manage, more productive and often bear fruits of better quality than the traditional large trees. In addition, dwarf trees allow the use of nets covering to protect them from damage caused by birds (Balmer 1998; Meland and Skjervheim 1998; Sitarek et. al., 1999; Sitarek 2006; Webster 1998).

2.2 MICROPROPAGATION OF APPLE

Apple is plant of great commercial value. Micropropagation of apple has played an important role in the production of healthy, disease-free plants and in the rapid multiplication of scions and rootstocks with desirable traits (Dobranszki and Silva 2010).

In vitro propagation of apple to produce high quality plants of uniform genetic makeup will be of great commercial value.

Tissue culture methods have been successfully applied for the propagation of *Malus* sp. (Lane 1992). During the last few decades, many reliable protocols have been developed for both rootstocks and scions from a commercial point of view (Silva et. al., 2008). Successful micropropagation of apple using pre-existing meristems (culture of apical buds or nodal segments) is influenced by several internal and external factors including *ex vitro* and *in vitro* conditions (Dobranszki and Silva 2010). Selection of a high quality rootstock is a cumbersome process, thus in order to use the high quality rootstock, it must be cloned to obtain a large number of plants. Therefore, micropropagation is the method of choice for the large scale cloning of selected rootstocks (Ciccotti et. al., 2008). However, it has been reported by Webster and James (1989) that different cultivars and rootstocks respond differently during micropropagation and *in vitro* rooting.

Apple proliferation (AP) is a serious disease of apple in Europe and was reported by Zimmerman and Fordham (1985). Its natural resistance was found in *Malus sieboldii*-derived genotypes which can be used as rootstocks. These genotypes are difficult to propagate by conventional vegetative propagation, thus micropropagation was attempted to multiply the material on larger scale. Micropropagation protocol was developed for culture establishment, multiplication and rooting of eleven interesting AP resistant genotypes (Webster et. al., 1989). They tested four different media formulations: MS- (Murashige & Skoog, 1962), QL - (Quorin & Lepoivre, 1977); WPM - (woody plant medium, Lloyd & McCown, 1981); DKW - (Driver and Kuniyuki, 1984) for standardization of multiplication medium. Phytohormones (0.25 µM IBA, 4.44 µM BAP and 0.28 µM GA3) and vitamins (MS modified for thiamine at 2.96 µM) were established for the propagation of *M. domestica* and were also suitable for the propagation of *M. sieboldii*-genotypes. The MS medium yielded the highest proliferation rates and the best shoot growth. Significantly better growth on the MS medium was also observed by replacing Fe-EDTA by Fe-EDDHA as the iron source. Amongst different treatments, a significantly higher percentage of rooting was observed when induction was carried out in dark with a pulse treatment of 25.0 µM IBA either in liquid or agar gelled medium. The time required for root formation on PGR free medium varied among different genotypes and three classes low, medium

and high of rooting efficiency was obtained. The acclimatisation method adopted in the greenhouse yielded survival rates between 90-100% for most of the genotypes.

Keul and Halmagyi (1992) had reported *in vitro* shoot multiplication of apples. Cultures were established on MS basal medium supplemented with Lee and Fossard (1977) (LF) vitamins, 10.0 µM BAP, 0.05 µM NAA, 30 g l^{-1} dextrose and 7 g l^{-1} agar. The highest shoot proliferation was obtained for all cultivars on medium supplemented with 5.0 µM BAP.

2.3 SHOOT ORGANOGENESIS OF APPLE

Regeneration of shoot buds from apical meristem was reported by Lane (1978). Plants of apple were regenerated from proliferating meristem-tips grown on N6 nutrient medium. Only BAP, at concentration of 5.0 µM, was required for initial growth and development of meristem-tips which produced proliferating shoot cultures in high frequency. NAA at concentration of 1.0 µM was used to initiate roots. Plantlets were then transferred to a growth regulator free medium where roots were developed fully before potting. It was reported that temperatures below 28°C and high salt concentration decreased rooting efficiency.

Effect of juvenility of *M. hupehensis* on regeneration of shoot buds and genetic transformation was reported by Rehman et. al., (2009). They used *Malus* as a model to examine the competence of genetically identical mature and juvenile tissues for regeneration and to undergo genetic transformation using *Agrobacterium tumefaciens*. Thidiazuron (TDZ) 5.0 µM was added to the regeneration media and it enhanced the frequency of shoot organogenesis and mean number of shoots per explants. Explants of juvenile origin and those with the adaxial side in contact with the medium exhibited higher regeneration efficiency and mean number of shoots regenerating per explants were higher than explants of mature origin and explants with the abaxial side in contact with the medium.

Factors that affected leaf regeneration efficiency; and effect of antibiotics on morphogenesis were reported by Yepes and Aldwinekle (1994). These authors

examined several factors that affected the frequency of organogenesis from leaf explants of scion cultivars ‘Empire’, ‘Freedom’, ‘Golden Delicious’, ‘Liberty’, ‘McIntosh’, and ‘Mutsu’ and rootstocks Malling 7A and Malling 26. The main factors affecting morphogenesis were BAP concentration, basal medium; leaf explants origin and maturity, explant orientation, and photosynthetic photon flux. Depending on the genotype, optimal regeneration was obtained using either 22.2 or 31.1 μM BAP on N6 basal medium, with the exception of ‘Golden Delicious’, which regenerated better on MS medium. After 6 weeks, the average number of shoots per segment varied from 5 to 16 and the percentage of regeneration between 70 and 100%, depending on the genotype tested and maturity of the explants. They observed that regeneration capacity increased dramatically from the tip towards the base of the leaf, and was higher from the middle to the proximal end.

Optimizing *in vitro* regeneration from Iranian native dwarf rootstock of apple was reported by Rustaee et. al., (2007). They reported that regeneration potential was different among cultivars and various factors needed to be optimized for individual genotypes. Rustaee et. al., (2007) reported that shoot regeneration step is a critical factor in apple *Agrobacterium* mediated transformation. In this experiment, a protocol for direct organogenesis from leaf taken from microshoots was standardized for apple CV “Gami Almasi”. Two regeneration media *viz* MS, N6 supplemented with various combinations of BAP and NAA were tested. Also two leaves and callus tissue were used for regeneration studies. It was observed that MS medium was better than N6 for shoot regeneration and optimum phytohormons concentrations were 37.5 μM BAP and 10.0 μM NAA. Shoots that regenerated were obtained only from leaf; and callus did not regenerate any shoot.

The role of cytokinins in shoot organogenesis in apple was reported by Korban et. al., (1992). The regeneration has been obtained from explants of many apple rootstocks and cultivars but the efficient regeneration protocol is still not available for a large number of apple rootstocks. Effective regeneration is necessary precondition for the implementation of different biotechnological application to plant breeding. Numerous studies have reported regeneration from apple somatic tissues, and organogenesis has been proved to be influenced by several factors including mother shoots (genotype, size, type, and age of explant), *in vitro* conditions (dark period, light intensity, and quality), and others (wounding, orientation of leaf explants). However, one of the

most important factors before and during the regeneration process is the type and concentration of cytokinin applied. Korban et. al., (1992) revealed that TDZ and BAP are the most frequently used cytokinins in the regeneration systems, but their efficiency depends on genotype and other factors. Other cytokinins (e.g., zeatin and kinetin) have also been tested in several experiments and they were found in general to be less active (Korban et. al., 1992). They also observed that organogenic ability of explants can also be increased by a properly selected cytokinin pre-treatment. Cytokinins applied in the pre-treatments, can influence the leaf structure, which in turn can alter the regeneration capacity of the leaf explants.

2.4 ROOTING OF MICROSHOOTS

Rooting of microshoots is reported to be influenced by number of factors: Interactions among light, temperature, phloroglucinol and auxin was reported by Zimmerman (1984). Delicious apple and several of its strains, which have been difficult to root, were successfully propagated with rooting percentages up to 100%. The combination of treatments which was used to achieve this result include placing the shoots on rooting medium in the dark at 30°C for the first week of the rooting, then shifting these to a regime of 16 h light and 8 h dark at 25°C. This was reported that rooting medium contained half strength MS salts plus 1.2 µM thiamine HCl, 0.56 mM myo-inositol, 1 mM phloroglucinol (PG), 1.4 µM IBA, 1.3 µM GA_3, 87.6 mM sucrose, and 7 g l^{-1} Difco Bacto agar. It was observed that dark treatment applied during the proliferation stage (etiolation) was less effective than one applied at the beginning of the rooting stage. The optimum length of dark treatment during rooting was 4 to 7 days. Increasing the temperature from 25°C to 30°C and in the absence of PG, improved rooting of 'Delicious', 'Royal Red Delicious' and 'Vermont Spur Delicious' but generally had less effect in the presence of PG. They also experimented that further increase in temperature to 35°C stimulated rooting of 'Royal Red Delicious' but reduced rooting of 'Vermont Spur Delicious'. Authors summarised that, in general PG stimulated rooting of Delicious and its strains, but had no effect on 'Golden Delicious'.

In vitro rooting of the apple rootstock M 26 in adult and juvenile growth phases and acclimatization of the plantlets was reported by Welander (2006). In order to obtain optimum conditions for *in vitro* propagation of the apple rootstock M 26 (*M. pumila*) in adult and juvenile growth phases, several rooting experiments were performed. Supraoptimal concentrations of IBA were added to the rooting media resulted in profuse callus formation. Since extensive callus development is detrimental to the survival of the plantlets, modified culture conditions were established to reduce callus formation. It was observed that a reduction of the time of exposure to IBA to 5 days and, thereafter, transfer to a PGR-free medium did not eliminate callus production. Exposure to darkness during the root initiation phase increased rooting. When basal medium was Lepoivre instead of the MS, callus formation was reduced. Welander (2006) reported that optimum conditions for rooting were obtained at much lower concentration of IBA than earlier reported, being 1.25 µM for the juvenile and 0.5 µM for the adult growth. Anatomical studies revealed that root initials were formed after 5 days of IBA treatment. Therefore, shoots were transferred directly to non-sterile conditions after the root-inducing phase. This resulted in a 90% survival of the plantlets. Subculture on PGR-free medium can thus be eliminated when the optimum auxin concentration was used (Welander 2006).

Effect of some antioxidants on *in vitro* rooting of apple shoots was reported by Standardi and Romani (1990). *In vitro*-proliferated shoots of 'Delicious' and 'Starkspur Red' apples were subjected to 1 week to root induction in the dark with their basal parts dipped in liquid medium containing 1.5 µM IBA and 43.4 mM sucrose. Then these shoots were transferred to root initiation and root elongation medium which was made up of plugs wetted with half-strength Lepoivre salts.

It was observed that when antioxidants, such as PVP (polyvinylpyrrolidone), 2-Me (2-mercaptoethanol), and DIECA (diethyldithiocarbamate), were added to the liquid induction medium, the percentages of rooting usually decreased. In contrast, when PVP and citric acid were added in the initiation-elongation medium, the percentages of rooting increased.

Influence of carbon sources and their concentrations on rooting and hyperhydricity of apple rootstock MM.106 reported by Bahmani et. al., (2009). Influence of fructose, sucrose, glucose, sorbitol and maltose as carbon sources at various concentrations on rooting and hyperhydricity apple rootstock MM106 was reported (Bahmani et. al., 2009). Type and concentration of sugars showed a significant effect on rooting percentage, mean root number, mean root length, hyperhydricity of cultures, as well as survival rate. These authors observed that microshoots show higher rooting percentage, mean root number, mean root length, when grown on media containing 90 mM sucrose. Shoots failed to root when fructose and maltose were used. (Bahmani et. al., 2009). The percentage of hyperhydric shoots was significantly higher at 60 mM maltose and 30 mM sorbitol as compared to 90 or 120 mM sorbitol, 90 mM sucrose and fructose and 120 mM maltose. Regenerated plantlets were acclimatized and successfully transplanted to soil with 90% survival.

Direct rooting of micropropagated M26 apple rootstocks was reported by Simmonds (1983). *In vitro* root development of micropropagated M26 apple rootstocks was rooted on low-nutrient MS media. The most prolific rooting was obtained by lowering the sucrose concentration to 1% and the macronutrients to a quarter strength in the rooting medium, but this increased root development and did not improve plant establishment. Although *in vitro* rooting on standard MS rooting medium increased during the 6-week of culture period, highest plant establishment was obtained from 3-week cultures with relatively little root development at the time of planting. These procedures which enhanced *in vitro* rooting were of no advantage in propagation. It was observed that more than 80% plant establishment was obtained by substituting the *in vitro* rooting stage with rooting in mist. Microshoots dipped in commercial rooting powder were established in a peat: sand (1:1) substrate in the mist frame. This procedure would reduce labour and cost of commercial micropropagation programmes.

2.5 ESTABLISHMENT OF APPLES

Modgil et. al., (2008) reported the commercially feasible protocol for rooting and acclimatization of micropropagated apple rootstocks. This work was carried out to optimize a successful protocol for rooting and acclimatization of tissue culture raised apple rootstocks of 'Malling' series. Root induction on dark on IBA supplemented MS medium and root elongation on medium without IBA provided best results for *in vitro* rooting. Among various substrates tested for root elongation, agar was found better than perlite and sand. However, maximum rooting with good shoot quality was achieved, when liquid medium was used for root elongation. Rooted plantlets of about 5 cm long were subsequently transferred to different media for acclimatization. Plants grown in coco peat showed maximum survival as compared to those grown in soil containing medium. On the other hand, the *in vitro* elongated shoots could also be rooted *ex vitro* successfully in coco peat after inducing root initials in liquid medium in dark for simultaneous *ex vitro* root elongation and acclimatization. It was reported that nearly 95% hardening was achieved during the months of October to March in comparison to summer and rainy months. Plants transplanted to the field in March-April established more successfully as compared to rest of the period of the year. After six months, plants in the field showed satisfactory survival and growth.

2.6 CLONAL FIDELITY OF APPLES

Modgil et. al., (2004) reported molecular analysis and genetic stability of micropropagated apple rootstock MM106. They used RAPD markers to assess the genetic stability of 10 micropropagated plants regenerated through axillary buds of clonal apple (*Malus pumila*) rootstock MM106. Eleven random decamer primers were successfully used to analyse genomic DNA from mother plants and *in vitro* raised plants. A total of 129 fragments were amplified with an average of 11.73 bands per primer. Among them, it was observed that 99 were monomorphic and 30 were polymorphic with 23.2% polymorphism. Among these 30, 12 were found monomorphic across seven plants. Three plants could be regarded as off-types. Their results showed that RAPD markers could be used to detect the genetic similarities and dissimilarities in micropropagated material.

Pathak and Dhawan (2001) reported the molecular analysis of micropropagated apple rootstock MM111 using ISSR markers for ascertaining clonal fidelity. They used ISSR to assess the genetic stability of micropropagated plants regenerated through axillary buds of apple (*Malus* × *domestica* Borkh.) rootstock MM111 after twenty second passages. A total of 24 primers were initially screened and finally 15 were chosen to analyse genomic DNA from mother plants and *in vitro* raised plants. It was observed that a total of 147 bands were generated with an average of 10 bands per primer. The outlier M7 was also taken along with tissue culture raised progenies of MM111 to rule out the possibility that the invariant banding pattern was on account of inefficiency of ISSR primers in detecting variations. A homogenous amplification profile was observed for all the micropropagated plants. The results confirmed the clonal fidelity of the tissue culture-raised MM111 plantlets and corroborated the fact that axillary multiplication is the safest mode for multiplication of true-to-type plants.

MATERIALS AND METHODS

3.1 Plant material, chemicals and glassware

Explants were taken from young, disease free plants of selected rootstock M7 and M9. Those explants were used for shoot multiplication experiments. Leaves from micro- shoots were used for regeneration experiments (shoot regeneration and somatic embryogenesis).

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) covered with polypropylene cap.

3.2 Washing and sterilization of glassware

Cleaning of glassware was carried out by soaking these in chromic acid followed by washing with tap water and subsequent rinsing with distilled water. The washed glassware was dried in oven at 70ºC for 1 to 2 hours. All contaminated culture bottles were autoclaved (Equitron, Medica Instruments, India) and contents were discarded before washing.

3.3 Medium preparation

Murashige and Skoog medium (MS, 1962. detail in annexure 1) containing agar (0.70 % w/v) was used as basal medium throughout the experiment. Medium was supplemented with sucrose (3.0%, w/v) for shoot multiplication and rooting experiment and with (2%, w/v) sucrose for regeneration experiment.

Growth regulators BAP and NAA or 2, 4 -D were added to the basal medium either alone or in various combinations, as specified 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 by dissolving in a few drops of potassium hydroxide (1.0 N) or hydrochloric acid (1.0 N) and made to desired volume (2.5 mM) with distilled water and stored at 4ºC. 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 1.0 N KOH or 1.0 N HCl and was autoclaved at 121°C for 20 min.

3.4 Culture conditions

All cultures were incubated under fluorescent lights (light intensity of 42µmol/m^2/s) in 16 h light/ 8 h dark cycle and incubated at 25 ± 1ºC.

3.5 Shoot multiplication

Cultures were established from the nodal segment (1 to 2 cm in length) taken from young, disease free plant of rootstock M7 and M9 grown under specific conditions. The young shoots sprouting from tissue grown under specific conditions were selected and nodal segments were excised in laminar air flow aseptically. These nodal segments were cultured on MS medium containing 3% (w/v) sucrose, 0.7% (w/v) agar and supplemented with 2.5 µM BAP and 0.5 µM NAA. These nodal explants after shoot proliferation were subcultured 2-3 times on the same medium. In another experiment the effect of varying concentration of BAP (1.0 - 12.5 µM) with NAA (1.0 - 12.5 µM) on the shoot multiplication and size of leaves were studied.

3.6 Rooting

Microshoots with shoot tips (2-3cm long) were used for the root induction experiment. For the purpose of root induction, the microshoots were inoculated on MS medium containing 3% (w/v) sucrose, 0.7% (w/v) agar and supplemented with 5.0 µM IBA.

3.7 Regeneration of shoots from leaf explants

Experiment was conducted to achieve regeneration from the leaf segments. Leaves (6 -9 mm) from actively growing microshoots (35-45 days old) of good condition were used as explants.

The leaves were excised under aseptic conditions in laminar flow cabinet and cut into transverse segments (2-3mm wide) and inoculated on medium with their adaxial surface towards medium. The effect of different concentration of BAP (0–12.5 µM) in combination with different concentration of NAA (0-12.5 µM) or 2, 4-D (0-12.5 µM) was studied for regeneration.

3.8 Histological observation

For histological studies, leaves explants cultured for 6 weeks on basal MS medium containing 1.0 µM NAA and 5.0 µM BAP were fixed in formaline/glacial acetic acid/ 50% ethanol in the ratio of 5 : 5 : 90 (v/v). Fixed tissues were dehydrated with tertiary butyl alcohol (TBA) series (Table- 1) and embedded in paraffin wax. Sections of 10.0µm thickness were cut with the microtome and stained with (1% w/v) safranin fast green combination prior to microscopic observation. The details of procedure are as below:- Plant material were fixed in FAA (formaldehyde: acetic acid: 50% ethanol, 5:5: 90 v/v) for one week and subsequently this tissue was stored in 70% ethanol. Processing is carried out following the method given below:

• Dehydration steps carried out in TBA (tertiary butyl alcohol) series as given below -

TABLE-1: Composition of TBA series used for the dehydration of fixed tissues.

S.No.	Rectified Alcohol (ml)	TBA (ml)	Water (ml)	Time (h)
A	30	20	50	3-4
B	50	20	50	3-4
C	50	35	15	3-4
D	45	55	-	3-4
E	25	75	-	3-4
F	-	100	-	3-4

To each grade keep the material for 3-4 hr, except for the "C" where it can be kept or longer periods (12 hr).

1. Waxing – material was kept in an oven at 60ºC and slowly wax was added to the material in TBA. So that TBA is replaced with wax, material is kept in oven till the traces of TBA were evaporated.

2. Block making – block of the material is made in wax in proper orientation.

3. Section cutting – 10.0µm thick sections were cut with the help of microtome.

4. Mounting and stretching – stretch the section in tap water over hot plate at 60ºC, then allow the section to dry for about 5-6 days.

5. Dewaxing – slides were dipped in pure xylol for 1-2 hrs (till wax is dissolved), then these were kept in each of the following grades for 2-3 min, unless otherwise specified.

S.No.	Xylol (ml)	Water (ml)	Time (hr)
1	100	0	till complete wax is dissolved
2	75	25	2-3min
3	50	50	2-3min
4	25	75	2-3min
5	0	100	2-3min

6. Rectified alcohols (90%)

7. 75% alcohol

8. 50% alcohol

9. 25% alcohol

10. Safranine in 25% alcohol

11. 25% alcohol

12. 50 % alcohol

13. 75% alcohol

14. 90% alcohol

15. Rectified alcohol

16. Absolute alcohol- I

17. Absolute alcohol- II

18. Clove oil 25% + alcohol 75%

19. Clove oil 50% + alcohol 50%

20. Fast green in 50 % clove oil in alcohol

21. Clove oil 50% in xylol (50%)

22. Clove oil 25% in xylol (75%)

23. Xylol - I

24. Xylol – II for 30 mins

Mount in DPX.

Care was taken that the slides were completely submerged in each grade.

3.9 Molecular characterization

I. DNA Isolation

Total genomic DNA was extracted from the leaf tissues of the plants by the Cetyl-trimethyl ammonium bromide (CTAB) method.

Procedure

• 3.0 g of fresh tissue was washed with distilled water, dried and grinded in liquid N_2 to fine powder, followed by immediate transfer to 50 ml centrifuge tube. To this was added prewarmed CTAB extraction buffer to make slurry and incubated at 60°C for 1 h in water bath.

• 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 5000 rpm for 10 mins.

• 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 coloured.

• DNA was precipitated with 0.66 volume of cold isopropanol followed by incubation for 1 h at -20°C.

• After centrifugation (10,000 rpm 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 pre heated 2 µl RNase solution (10 mg/ml stock) was added and incubated at 37°C for 1 h.

• Equal volume of phenol and chloroform was added (1:1v/v) followed by gentle shaking and centrifuged (10,000 rpm for 10 mins).

• Aqueous layer was retained. To this aqueous solution 0.3 volume of 3M sodium acetate and 0.6 volume of chilled isopropanol was added and incubated for 1 h at -20 °C.

• Following incubation centrifugation was carried out at 10,000 rpm for 10 mins. The pellet was retained, dried and dissolved in T.E Buffer and stored at -20°C.

Reagents needed:

1. CTAB buffer –

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)

Made upto 1 litre with distilled water, pH 7.5- 8.0 and autoclaved mercaptoethanol (0.2 % v/v) was added into buffer just before use.

2. Isopropanol

3. Chloroform

4. Isoamyl alcohol

5. Saturated phenol

6. Sodium acetate 3 M

7. TE buffer -

20 mM EDTA EDTA stock (0.5)

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

II.PCR Analysis

Following are the details of all the 6 primers used in present investigation-

Primers	Nucleotide sequence (5´-3´)
RAPD 1	AGCGCCATTG
RAPD 2	CTTCCCCAAG
RAPD 3	AGGGCGTAAG

Primers	Nucleotide sequence (5´-3´)
ISSR 1	CACACACACACACACACG
ISSR 2	GAGAGAGAGAGAGAGACG
ISSR 3	GAGAGAGAGAGAGAGATC

III. RAPD and ISSR Analysis

The components of RAPD and ISSR were: 40 ng of template DNA, 10 mM of each dNTPs (Biogene, USA), 10 µM of primer (Integrated DNA technologies and Operon molecules for life, USA), 5 U of Taq DNA polymerase (Geneaid, USA) and 10x reaction buffer (Geneaid, USA) in a total volume of 20 µl.

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:

Components	Stock concentration	Vol/ Rxn
dNTPs	10 mM each	2.0 µl
PCR buffer	10 X	2.0 µl
Mg_2Cl	25 mM	2.0 µl
Taq polymerase	5 u/ µl	0.3 µl
Primer	10 µM	1.0 µl
Sterile H_2O	----	11 µl
DNA	40 ng/µl	2.0 µl

PCR conditions

PCR tubes were placed in thermal cycler (Applied Biosystems, Model Gene Amp2700 USA) and amplified using the following temperature profile:

Temperature (^{o} C)	Time	No. of cycles
94	4 min	1
94	1 min	41
35 /55*	1 min	41
72	1:30 min	41
72	5 min	1

*Annealing temperature for ISSR is 55 ºC.

*Annealing temperature for RAPD is 35 ºC.

IV. Agarose gel electrophoresis

Amplified products were separated in 1.5% agarose gel containing ethidium bromide using 1x TAE buffer. A constant voltage of 55 was provided for 4 - 5 h. DNA fragments were visualized under UV light. The patterns were photographed using Geldoc system (Vilber Loumart, France) and stored as digital pictures. The reproducibility of the Amplification was confirmed by repeating each experiment three times.

RESULTS

4.1 Shoot Multiplication

Shoots multiplied from nodal segments were subcultured on MS basal medium for 2-3 cycles to build up the cultures. To check the effect of varying concentrations of BAP on shoot multiplication and on the size of the leaves, experiments were carried out, in which MS medium containing 2% (w/v) sucrose and 0.7 % (w/v) agar with different concentrations of BAP (5.0 µM, 2.5 µM and 0.1 µM) and 0.5 µM NAA were used. Each treatment consists of three replicates with five shoots in each flask and experiment was repeated two times.

It was observed that the shoot multiplication of apple rootstock M7 was maximum (28 shoots per culture vessel) on MS medium supplemented with 2.5 µM BAP and 0.5 µM NAA and the same was minimum (19 shoots per culture vessel) on MS medium supplemented with 5.0 µM BAP and 0.5 µM NAA. The average leaf size of apple rootstock M7 was maximum (6 - 9 mm) on MS medium supplemented with 2.5 µM BAP and 0.5 µM NAA (Table 2) (Figure 1.A).

TABLE 2. Effect of different concentrations of BAP and NAA in MS basal on shoot multiplication of apple rootstock M7.

BAP (µM)	NAA (µM)	Average number of shoots per culture vessel	Average Shoot Length(cm)	Average Leaf Size
0.1	0.5	20	4.0	++
2.5	0.5	28	5.2	+++
5.0	0.5	19	3.3	++

Each treatment considered of three tissue culture bottles with 5 nodal segments in each and data recorded after 4 weeks of subculture.

Leaves size: + (1-3mm in length and width), ++ (3-6mm in length and width), +++ (6-9mm in length and width).

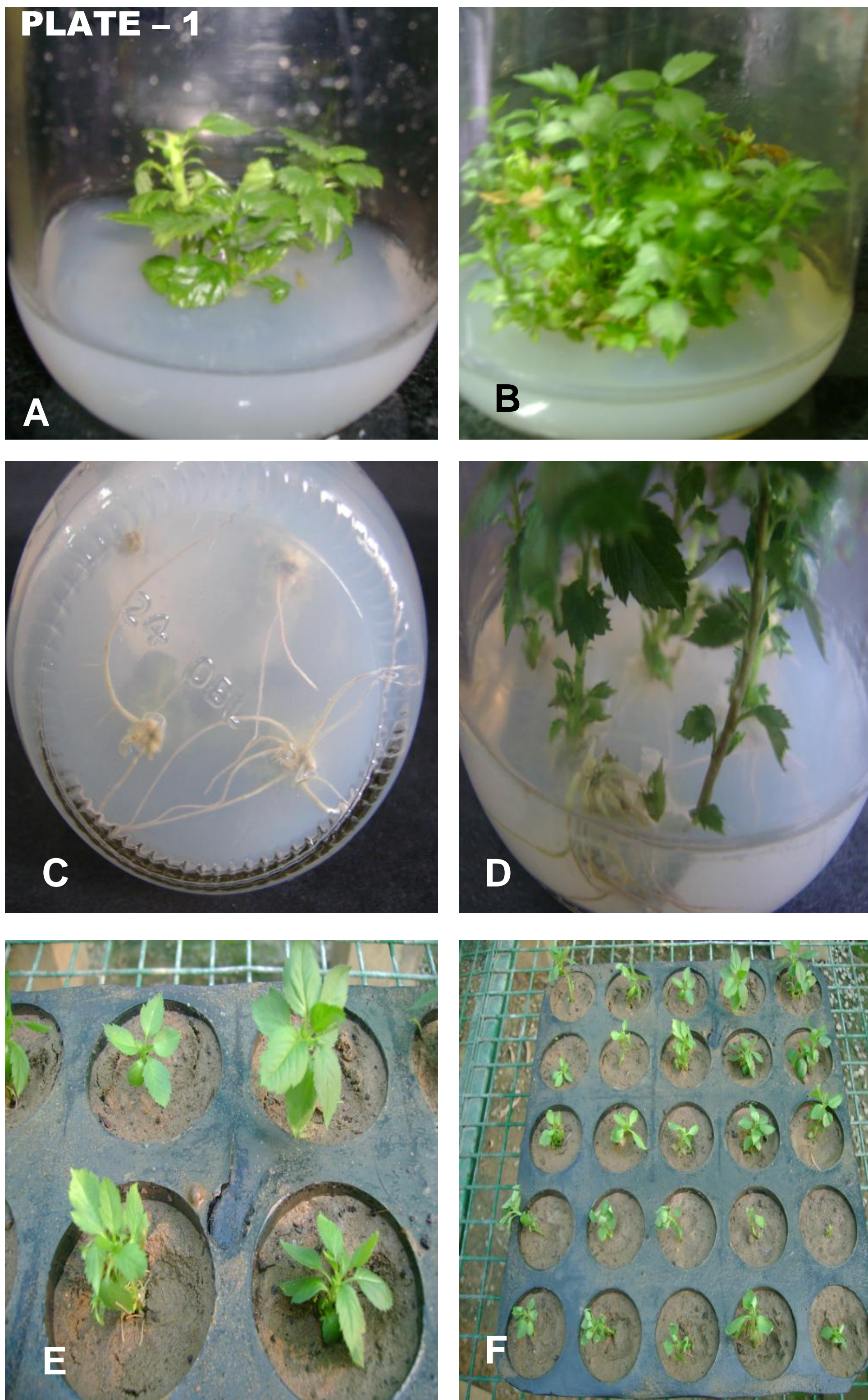

FIGURE 1: (A and B) Shoot multiplication of apple rootstocks M7 and M9 on MS medium supplemented with 2.5 µM BAP and 0.5 µM NAA respectively. (C and D) Rooting of microshoots of M7 and M9 apple rootstocks on MS media supplemented with 5.0 µM IBA respectively. (E and F) Acclimatization of M7 and M9 apple plants in the fields respectively.

TABLE 3. Effect of different concentrations of BAP and NAA on shoot multiplication of apple rootstock M9.

BAP(µM)	NAA(µM)	Average number of shoots per culture vessel	Average Shoot Length(cm)	Average Leaf Size
0.1	0.5	18	2.4	++
2.5	0.5	22	4.3	++
5.0	0.5	20	3.2	+++

Each treatment considered of three tissue culture bottles with 5 nodal segments in each and data recorded after 4 weeks of subculture.

Leaves size: + (1-3mm in length and width), +++ (3-6mm in length and width), +++ (6-9mm in length and width).

In rootstocks M9, the minimum shoot multiplication (18 shoots per culture vessel) was observed on MS medium supplemented with 0.1 µM BAP and 0.5 µM NAA and the same was maximum (22 shoots per culture vessel) on MS medium supplemented with 2.5 µM BAP and 0.5 µM NAA (Figure 1.B). The average leaf size was maximum in rootstock M9 on MS medium supplemented with higher concentration of BAP (5.0 µM) and 0.5 µM NAA (Table 3).

4.2 Shoot organogenesis

Experiments were carried out to achieve shoot organogenesis from leaves taken from microshoots of both M7and M9 rootstocks. MS medium containing 2% (w/v) sucrose and 0.7 % (w/v) agar was supplemented with different concentrations of BAP and NAA. Morphogenesis in the tissue was observed and it was found to vary with the different combinations of BAP and NAA or 2, 4 – D. In first week, there were no notable changes in the leaves cultured on the MS media containing 2% (w/v) sucrose, 0.7% (w/v) agar and different concentrations of BAP and NAA, but after three weeks of culture visible changes

like formation of globular structures and callus started to appear from the explants (Figure 3.A and 3.B).

Shoot regeneration was observed to be a two step process. The callus developed from cultured leaf segment on MS medium supplemented with different concentrations of BAP and NAA or 2, 4-D. The developed callus was subcultured on MS medium supplemented with various concentrations of BAP and NAA and shoot regeneration was observed in some of the combinations. Leaf segments of both the rootstocks cultured on MS medium supplemented with plant growth regulators (PGRs) developed callus. However, the texture and morphology of the callus varied on different media combinations in both the rootstocks. The colour of the callus varied from white, yellow, pale green and green (Table 4 – 7). The texture of the callus was compact, nodular and friable in different combinations.

TABLE 4. The effect of different concentrations of BAP and NAA on callusing for apple rootstock M7.

BAP(µM)	NAA(µM)	Percentage of leaves showing callusing	Colour and morphology of callus
0.0	0.0	0.0	-----
1.0	1.0	47.50	Pale green , nodular
1.0	5.0	60.83	Greenish , smooth
1.0	12.5	55.00	Pale green, smooth
5.0	1.0	35.83	Green , nodular
5.0	5.0	45.83	Dark green, nodular
5.0	12.5	69.16	Green-white, smooth
12.5	1.0	25.32	Yellow, smooth
12.5	5.0	40.00	Greenish, nodular
12.5	12.5	37.50	Yellow, smooth

MS Medium with 2% (w/ v) sucrose and 0.7% (w/v) agar was used as basal medium.

For each combination three bottles were used and in each bottles four leaves were inoculated.

TABLE 5. The effect of different concentrations of BAP and 2, 4-D on callusing for apple rootstock M7.

BAP(µM)	2, 4-D(µM)	Percentage of leaves showing callusing	Colour and morphology of callus
0.0	0.0	0.0	-----
1.0	1.0	25.83	Pale green, nodular
1.0	5.0	69.16	Green, nodular
1.0	12.5	57.75	White, smooth
5.0	1.0	34.16	Yellow, smooth
5.0	5.0	60.00	Green, nodular
5.0	12.5	65.83	White, nodular
12.5	1.0	20.00	Yellow, smooth
12.5	5.0	32.50	Pale green, smooth
12.5	12.5	47.50	Green, nodular

MS Medium with 2% (w/ v) sucrose and 0.7% (w/v) agar was used as basal medium.

For each combination three bottles were used and in each bottles four leaves were inoculated.

TABLE 6. The effect of different concentrations of BAP and NAA on callusing for apple rootstock M9.

BAP(µM)	NAA(µM)	Percentage of leaves showing callusing	Colour and morphology of callus
0.0	0.0	0.0	----
1.0	1.0	44.16	Pale green, nodular
1.0	5.0	25.00	Green, smooth
1.0	12.5	45.83	White, nodular
5.0	1.0	32.50	Green, nodular
5.0	5.0	60.83	Dark green, smooth
5.0	12.5	65.00	Yellow, smooth

12.5	1.0	52.40	Green, smooth
12.5	5.0	44.16	Green, nodular
12.5	12.5	27.50	Green-white, smooth

MS Medium with 2% (w/ v) sucrose and 0.7% (w/v) agar was used as basal medium.

For each combination three bottles were used and in each bottles four leaves were inoculated.

TABLE 7. The effect of different concentrations of BAP and 2, 4-D on callusing for apple rootstock M9.

BAP(µM)	2, 4-D(µM)	Percentage of leaves showing callusing	Colour and morphology of callus
0.0	0.0	0.0	----
1.0	1.0	57.50	Green, nodular
1.0	5.0	45.83	Pale yellow, smooth
1.0	12.5	24.16	White, smooth
5.0	1.0	65.00	Yellow, nodular
5.0	5.0	69.16	Green, nodular
5.0	12.5	60.00	Greenish, smooth
12.5	1.0	39.16	Dark green, nodular
12.5	5.0	34.16	Green, smooth
12.5	12.5	28.33	Yellow, smooth

MS Medium with 2% (w/ v) sucrose and 0.7% (w/v) agar was used as basal medium.

For each combination three bottles were used and in each bottles four leaves were inoculated.

The calli obtained on these media were transferred to MS medium supplemented with 5.0 µM each of BAP and NAA (Figure 2.A). Shoot start to appear from the calli of both M7 and M9 rootstocks after two weeks of inoculation on this medium and complete shoot growth was observed after three weeks (Figure 2.B). Maximum regeneration of shoots for both M7 and M9 apple rootstocks were obtained when leaves placed on MS Medium containing 2% (w/v) sucrose and 0.7% (w/v) agar, supplemented with 5.0 µM Beach of BAP and NAA (Figure 2.C).

PLATE - 2

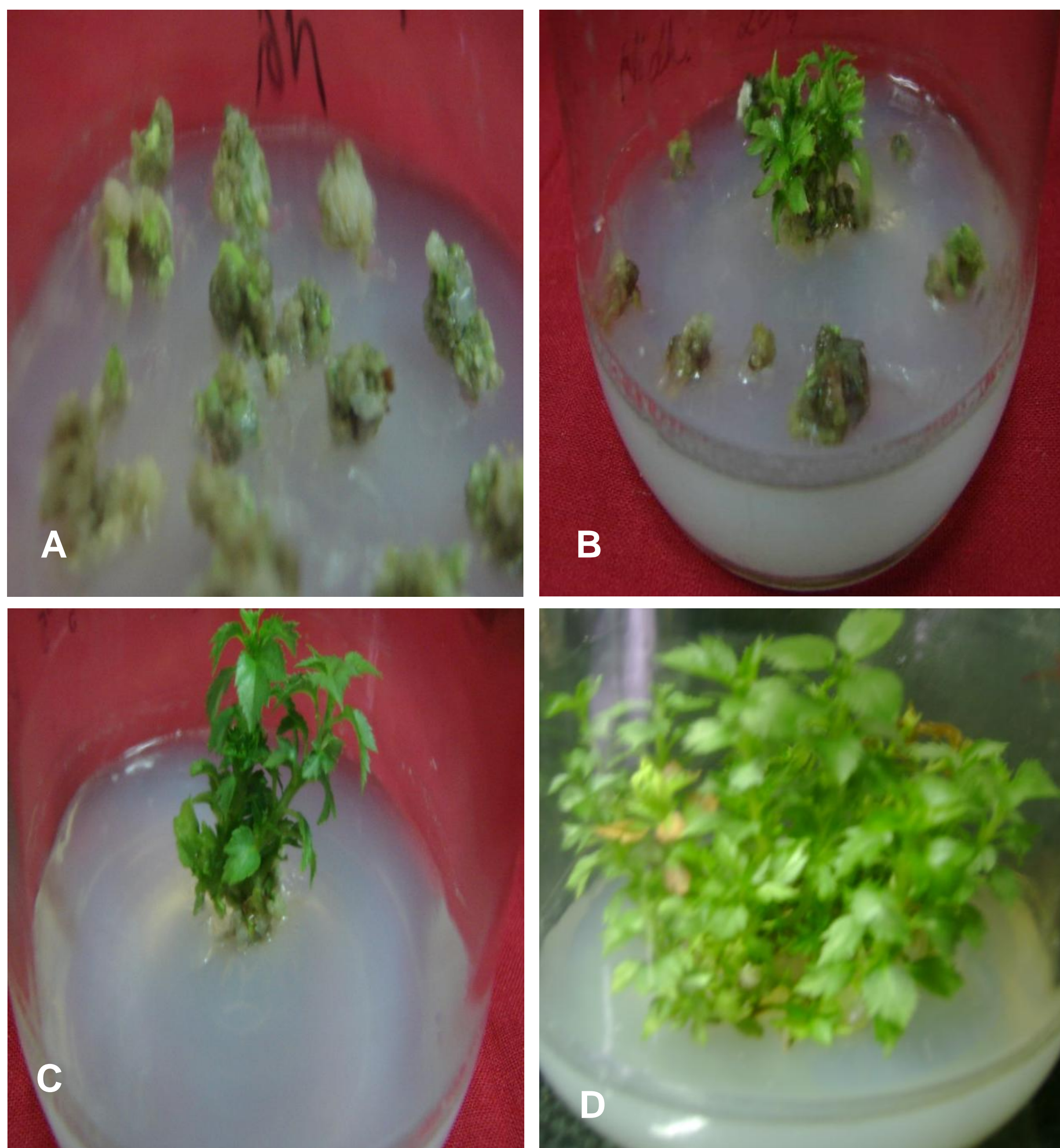

FIGURE 2: (A) Initiation callus when leaf segments of apple rootstock M9 cultured on MS media supplemented with 2.5 µM BAP and 5.0 µM NAA. (B) Nodular callus differentiated into shoot buds when MS media was supplemented with 5.0 µM BAP and 5.0 µM NAA. (C and D) Shoot formation in apple rootstock M9 on MS media was supplemented with 5.0 µM BAP and 5.0 µM NAA.

TABLE 8. The effect of different concentrations of BAP and NAA on shoot regeneration for apple rootstock M7.

BAP(μM)	NAA(μM)	Calllus showing shoot regeneration	Percentage of callus showing shoot regeneration
0.0	0.0	No	----
1.0	1.0	Yes	20
1.0	5.0	No	----
1.0	12.5	No	----
5.0	1.0	Yes	40
5.0	5.0	No	----
5.0	12.5	No	----
12.5	1.0	No	----
12.5	5.0	No	----
12.5	12.5	No	----

MS Medium with 2% (w/ v) sucrose and 0.7% (w/v) agar was used as basal medium.

For each combination two bottles were used and in each bottles five callus were inoculated.

The nodular callus differentiated from combinations of 1.0 μM BAP and 1.0 μM NAA and 5.0 μM BAP and 1.0 μM NAA in both rootstocks M7 and M9 resulted in shoot regeneration.

The nodular callus differentiated from leaf explants on different media combinations and subcultured on MS medium supplemented with different concentrations of BAP and NAA differentiated shoot buds (Figure 3.C). In 20 to 30 days after subculture, shoot differentiation was observed on MS medium supplemented with BAP (1.0 μM) and NAA (1.0 and 5.0 μM) (Figure 3.D). Higher percent calli showed shoot regeneration in rootstocks M7 than M9 (Table 8 & 9).

PLATE – 3

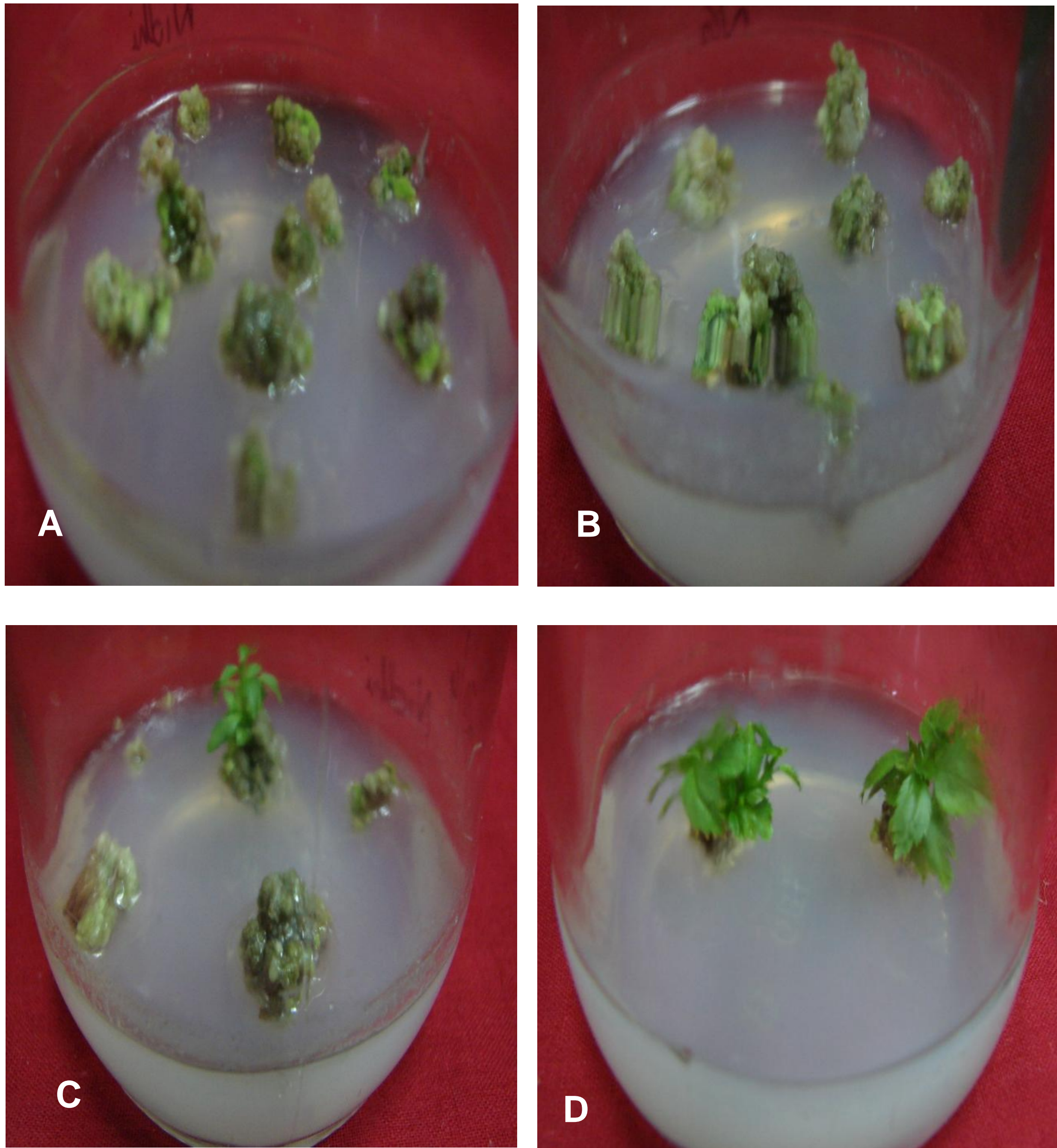

FIGURE 3: (A and B) Initiation and formation of callus when leaf segments of apple rootstock M7 cultured on MS media supplemented with 1.0 µM BAP and 5.0 µM NAA. (C) Nodular callus differentiated into shoot buds on MS media supplemented with 5.0 µM BAP and 5.0 µM NAA. (D) Shoot formation in apple rootstock M7 on MS media supplemented with 5.0 µM BAP and 5.0 µM NAA.

TABLE 9. The effect of different concentrations of BAP and NAA on shoot regeneration for apple rootstock M9.

BAP(µM)	NAA(µM)	Calllus showing shoot regeneration	Percentage of callus showing shoot regeneration
0.0	0.0	No	----
1.0	1.0	Yes	20
1.0	5.0	No	----
1.0	12.5	No	----
5.0	1.0	Yes	30
5.0	5.0	No	----
5.0	12.5	No	----
12.5	1.0	No	----
12.5	5.0	No	----
12.5	12.5	No	----

MS Medium with 2% (w/ v) sucrose and 0.7% (w/v) agar was used as basal medium.

For each combination two bottles were used and in each bottles five callus were inoculated

4.3 Histological observations

The leaves explants of apple rootstock M7 placed on MS medium with different growth regulators for shoot regeneration undergoes many morphological changes. Initial changes involve swelling or the development of globular structures at the terminal of the inoculated leaves. After four weeks shoot like structures start to appear on the developing tissue. These changes were hard to determine by simple observation of the tissue. In order to find out the mode of regeneration of the shoots and to collect information about the ongoing cellular changes in the tissue, histological studies of the cultured tissue on MS medium with 2% (w/v) sucrose and 0.7% (w/v) agar, supplemented with 5.0 µM BAP and 5.0 µM NAA were carried out. Serial sectioning and the examination of slides revealed various features of shoot organogenesis. The histological study of the tissue revealed anticlinal cell division in some of

the epidermal cells of explants (Figure 4.A) which were found to grow into globular and shoot like structures (Figure 4.B and 4.C). The structures then grew into shoots (Figure 4.D). The shoots were found to have direct vascular connections with the parent tissue.

PLATE - 4

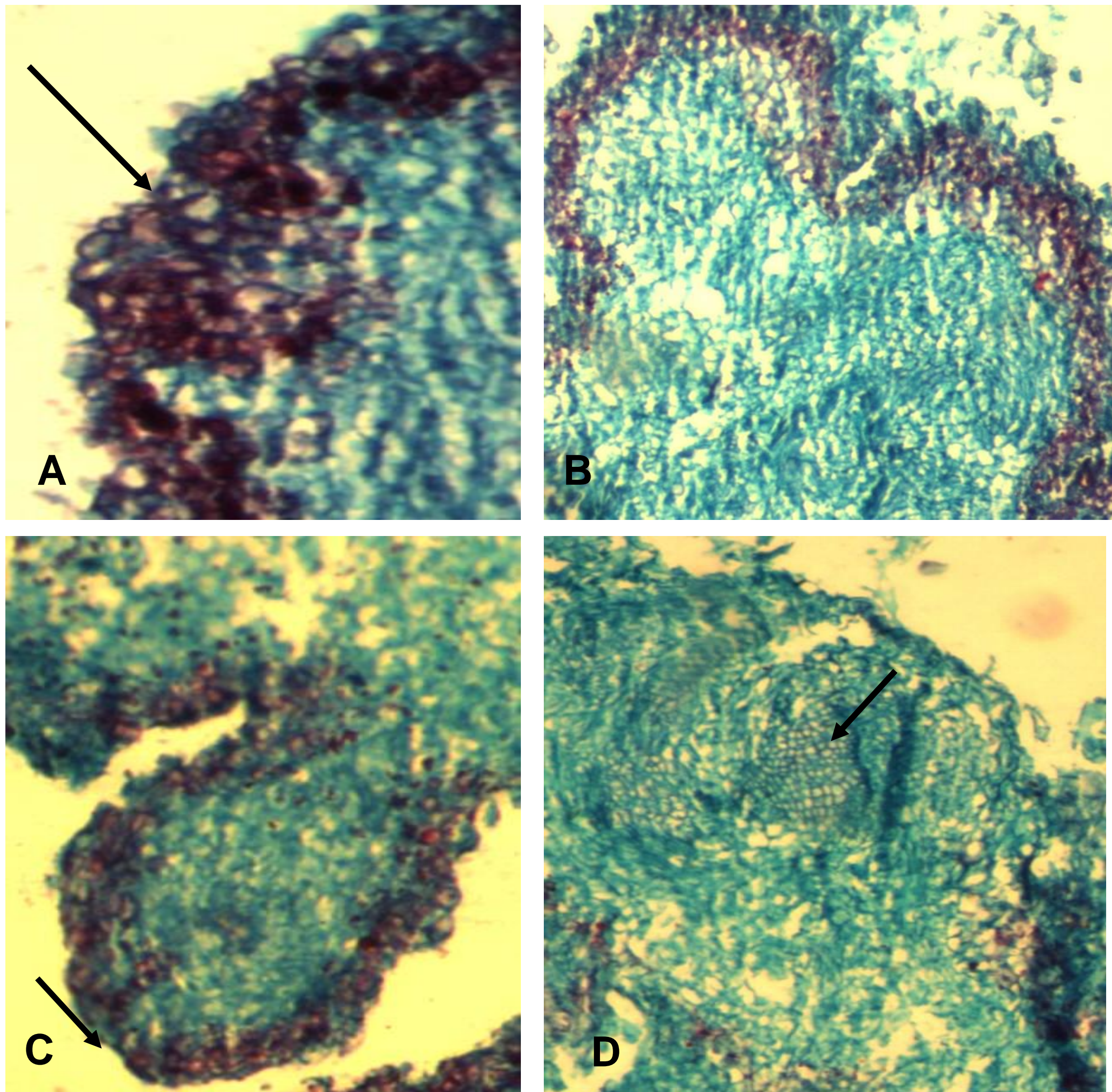

FIGURE 4: (A) Initiation of shoot organogenesis in apple rootstock M9.

(B) Initiation of shoot bud primodia in apple rootstock M9.

(C) Formation of shoot bud primodia in apple rootstock M9.

(D) Formation of shoot bud in M9 rootstock.

4.4 Rooting of micro shoots

Microshoots of apple (3-4 cm long) were used for the root induction. For the purpose of root induction, the microshoots were inoculated on MS medium solidified with 0.7 % (w/v) agar and supplemented with 5.0 µM IBA. Successful rooting of microshoots of M7 and M9 rootstocks were observed on MS media supplemented with 5.0 µM IBA (Figures 1.C and 1.D).

4.5 Acclimatization of plants

After 15 days of culture on rooting media, the plantlets were shifted to plastic pots for their hardening prior to final transfer to soil to natural conditions. For hardening of plants, plants with newly formed roots were taken out from the culture bottles with the help of forceps with utmost care to prevent any damage to newly formed roots and dipped in warm water not hot to remove the any traces of solidified agar media. After removing media, plants were dipped in 1% w/v solution of Bavistine to prevent any fungal infection to newly developed plants. After Bavistine treatment the plantlets were carefully planted in plastic pots containing 1:1 mixture of soil and farmyard manure. The plants were thoroughly watered and kept under polyhouse having 80% humidity and 31°C temperature for ten days (Figures 1.E and 1.F). In-between the ten days plants were thoroughly watered with the help of sprinkler to maintain required level of humidity. Then the plants were shifted to shade house with less humidity level and indirect sunlight. In shade house also plants were watered two times a day i.e. morning and evening to prevent wilting (if any).

4.6 Clonal fidelity

The clonal fidelity of the regenerated shoots from the leaf explants revealed no variations. Out of the three, each primer of RAPD and ISSR a total number of 32 markers were amplified. None of the marker was found to be polymorphic indicating the clonal uniformity of the micropropagated plants (Figure 5.A and 5.B).

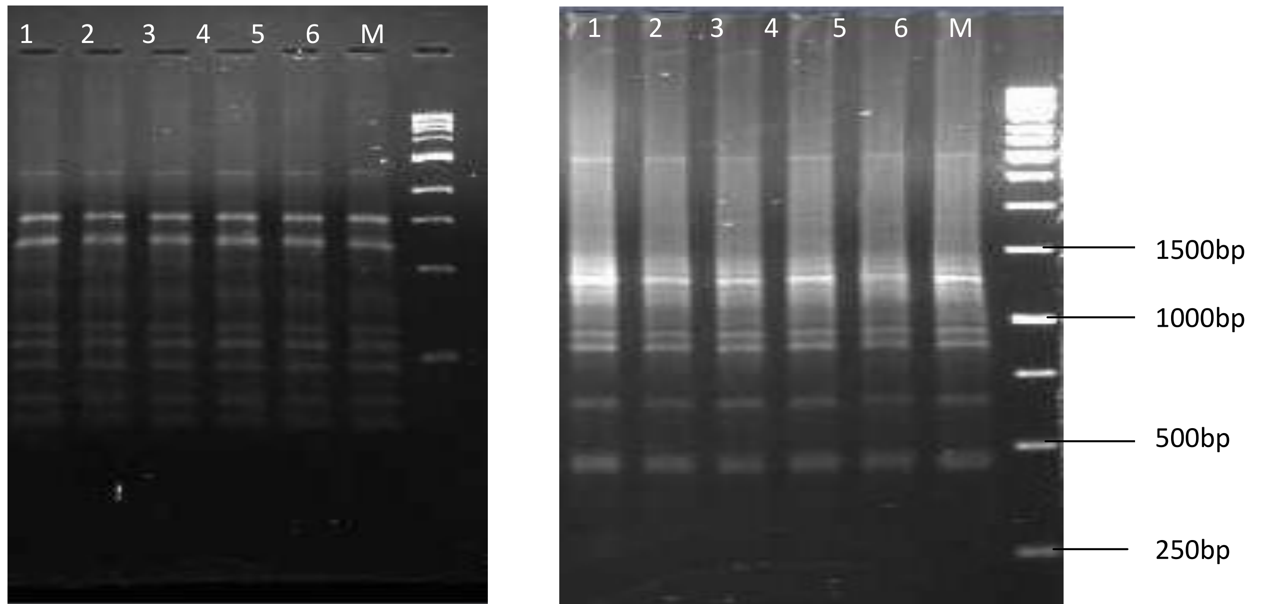

FIGURE 5: (A.) RAPD analysis of Apple rootstock M9 with primer RAPD- 2. (B.) ISSR analysis with primer ISSR- 3, Lane-1 Mother Plant; Lane 2-6 Regenerated plants; Lane M- 1 kb Molecular weight markers.

DISCUSSION

Plant tissue culture is a practice used to propagate plants under sterile conditions, often to produce clones of a plant. It is an important component of plant biotechnology and includes micropropagation, somatic embryogenesis, regeneration of plants etc. Plant micropropagation is an important constituent of plant cell and tissue culture.

Micropropagation has been successfully used for the large scale production and release of new clones. Many plant varieties and the number of species produced *in vitro* by micropropagation have been steadily increasing over the last few decades (Harry and Thorpe 1999).

Most temperate fruit trees like apple are characterized by a long reproductive cycle and are generally highly heterozygous, out breeding species. Genetic improvement of such species by conventional methods is a time-consuming and difficult task (Hofer 2005). So in order to alter the characters of these species, the protocol for their regeneration and somatic embryogenesis must be developed. In India micropropagation protocols of many trees species have been developed (Datta et. al., 1982; Devi et. al., 1994; Rai and Chandra 1989; Shekhawat et. al., 1993).

Therefore, the present study was carried out to investigate the various factors involved in *in vitro* propagation of apple rootstocks. These include study of micropropagation, regeneration, rooting, somatic embryogenesis, establishment in fields and clonal fidelity of selected apple rootstocks.

The main aim of the study was to standardize and develop more efficient protocol for the micropropagation and shoot regeneration from clones of M7 and M9 rootstocks of apple, because it has been observed that clone specific variation occurs in the regeneration and micropropagation (Aggarwal et. al., 2010).

In this study, the effects of PGRs on the apple rootstocks M7 and M9 were observed on shoot multiplication. For this purpose basal MS medium having 3.0% (w/v) sucrose and0.7% (w/v) agar was supplemented with different concentrations of BAP, NAA and 2, 4-D. About 80% of the nodal explants multiplied when shoots inoculated on MS medium supplemented with 2.5 µM BAP and 0.5 µM NAA.

The effect of different cytokinins and auxins, and their different concentration on micropropagation and shoot proliferation is well documented (Akbas et. al., 2010). Cytokinin when added in appropriate concentration regulates shoot proliferation, cell division and differentiation (Gross and Partiner 1994). A range of cytokinins (Kinetin, Zeatin and BAP) have been used for the purpose of micropropagation (Bhojwani and Razdan 1992).

Currently BAP is the most widely used cytokinin in the micropropagation industry due to its effectiveness and affordability (Bairu et. al., 2007). BAP played a significant role in shoot formation of *Malus zumi*. However, BAP at higher concentrations not only reduced the number of shoots but also resulted in stunted growth of the shoots (Xu 2008).

The main objective of regeneration study was to develop the efficient shoot organogenesis protocol for M7 and M9 rootstocks. In present study, leaves of both M7 and M9 rootstocks were placed on basal MS medium containing 2.0% (w/v) sucrose and 0.7% (w/v) agar supplemented with different concentrations of BAP and NAA or 2, 4 –D. However, initially no shoot regeneration was observed. Shoot organogenesis for rootstock M7 was achieved when callus developed from the leaves, placed on MS medium containing 2.0% (w/v) sucrose and 0.7% (w/v) agar, supplemented with 5.0 µM BAP, 5.0 µM NAA. Regeneration efficiency was higher for the callus grown on MS medium supplemented with 5.0 µM each of BAP and NAA. Regeneration for rootstock M9 was achieved when callus developed from the leaves on MS medium containing agar supplemented with different concentrations of BAP and NAA was cultured on medium supplemented with BAP and NAA.

In this study an analysis of regeneration; many morphological changes like development of globular structures on the inoculated leaves were observed.

Somatic embryogenesis is closely resembles to its *in vivo* form, zygotic embryogenesis (Yeung et. al., 2001). It is also assumed that it passes through the same characteristic stages (Menendez-Yuffa and Garcia 1997) the globular, heart and torpedo phases. Different features of the developing somatic embryos were clearly visible like initiation of somatic embryogenesis, formation of globular and heart shaped embryonic like structures.

Histological studies have been reported on shoot regeneration through organogenesis in tree species like *Eucalyptus camaldulensis* (Dibax et. al., 2005; Ho et. al., 1998; Mullins et. al., 1997; Muralidharan and Mascarenhas 1987) and in *E. tereticornis* (Subbaiah and Minocha 1990).

In present study, an attempt was made to set an experiment for the root induction in apple. For this, the micro shoots were inoculated on MS medium solidified with 0.7 % (w/v) agar and supplemented with 5.0 µM IBA. Successful rooting of micro shoots of M7 rootstock were observed on MS media supplemented with 5.0 µM IBA. The beneficial effect of IBA on rooting has been observed in many plant species (Amri et. al., 2010; Cheniany et. al., 2010; Linh 2001).

Clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species. A major problem often encountered with the *in vitro* cultures is the presence of somaclonal variation amongst subclones of one parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissues, or organs. Palombi and Damiano (2002) suggested the use of more than one DNA amplification technique as advantageous in evaluating somaclonal variation while working on micropropagated using a combination of two types of markers that amplify different regions of the genome (Martins et. al., 2006). Hence, in the present study, two PCR-based techniques, RAPD and ISSR, were adopted for evaluation of clonal fidelity in apple plantlets. Randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) (Williams 1990; Zietjiewicz et. al., 1994) markers have proven to be efficient in detecting genetic variations at molecular level.

In the present study RAPD and ISSR analyses of the regenerated plants showed similarity with mother plant indicating the clonal nature of the propagated material. In all 6 primers used (3 RAPD and 3 ISSR) for amplification, banding patterns were similar in regenerated plants to that of mother plant. Both RAPD and ISSR

markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plants (Martins et. al., 2006; Aggarwal et. al., 2010). The clonal fidelity of *in vitro* regenerated plants of apple rootstock MM106 was tested using RAPD markers (Modgil et. al., 2004). In present 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 micropropagation stages.

CONCLUSIONS

Micropropagation of apple rootstock, an important commercial horticulture crop belonging to family- Rosaceous, was achieved through axillary shoot proliferation. Cultures were successfully multiplied on MS media supplemented with 2.5 µM BAP and 0.5 µM NAA and further rooting of microshoots of rootstocks M7 and M9 on MS medium supplemented with 5.0 µM IBA were achieved and these microshoots were then acclimatize in the fields for hardening.

Besides micropropagation, efficient shoot organogenesis protocol has been developed from plants of Apple rootstocks M7 and M9. Callus formation achieved on MS medium supplemented with different combinations of BAP and NAA or 2, 4 –D; and shoots were regenerated from callus when these were shifted on MS medium supplemented with 5.0 µM each of BAP and NAA. The differentiated shoots/plants were found to be genetically uniform and identical to the mother plants. This protocol can be successfully exploited to undertake the improvement of these clones through trait specific plant genetic manipulations.

The following conclusion can be drawn from the present study:

The multiplication of culture was done successfully at high percentage on MS medium supplemented with 2.5 µM BAP and 0.5 µM NAA.

About 80% nodal explants of both M7 and M9 rootstocks, shown multiplication on MS medium supplemented with 2.5 µM BAP and 0.5 µM NAA.

The number of shoots multiplied, depend on the concentration of BAP mainly.

The leaf size of M7 rootstock was maximum when nodal explants were inoculated on MS medium supplemented with 0.1 μM BAP and 0.5 μM NAA.

The callus was obtained on MS media containing 2% sucrose on different combination of BAP, NAA and 2, 4-D. Further regeneration of shoots was achieved from these calli.

Shoot regeneration in both rootstocks were obtained when callus developed from the leaves, placed on MS medium containing 2% sucrose supplemented with 5.0 μM BAP and 5.0 μM NAA.

Histological studies revealed the formation of shoots.

Roots of nodal segments of M7 were achieved, when the explants were inoculated on MS media supplemented with 5.0 μM IBA.

Hardening of apple plantlets in the fields was done successfully.

The clonal fidelity of the regenerated shoots from leaf explants revealed no variations from the mother plant.

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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**
$MnSO_4.H_2O$	16.90
$FeSO_4.7H_2O$	27.80
$ZnSO_4.7H_2O$	8.60
H3BO3	6.20
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4.7H_2O$	0.025
$Na_2EDTA.\ 2H_2\ O$	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
Sugar	3000
4. Agar	7000 mg/l