Studies on *in vitro* propagation and genetic transformation protocol of apple rootstock M7

Thesis submitted in partial fulfillment of the requirement for the award of

degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

By

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July 2012

Certificate

This is to certify that the thesis entitled "**Studies on *in vitro* propagation and genetic transformation protocol of apple rootstock M7** " submitted by Kirti Mehta (Roll No. 301001013) as a part of curriculum for Degree of Master of Science, Department of Biotechnology and Environmental Science, Thapar University, Patiala, is a record of student's own work carried under our guidance and supervision. This report has not been submitted for the award of any other degree or certificate in this or any other University.

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Candidate's Declaration

I, hereby declare that the work which is being presented in the dissertation entitled "**Studies on *in vitro* propagation and genetic transformation protocol of apple rootstock M7**", in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab; is an authentic record of my own work during a period of six months from January 2012 to June 2012, under the supervision of Dr. Anil Kumar, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University. 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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Acknowledgement

This is a golden opportunity for me to convey my sincere regards for all those people who enabled me to accomplish my dissertation work successfully. It is my privilege to express my sincere gratitude to Dr. Anil Kumar, Assistant Professor, TIFAC CORE, Thapar University, for his guidance, encouragement and valuable advice. It is his confidence imbibing attitude and splendid discussions and endless endeavours through which I have gained significant experience. My sincere thanks to Dr. M.S. Reddy, Head, Department of Biotechnology and Environmental Sciences, for his immense concern throughout the project work.

I am thankful for the help rendered by research fellows Mr. Diwakar Aggarwal, Ms. Mahima Bansal, Mr. Sanjog Garyali throughout my project period without which it would have been difficult for me achieve my goals. Special thanks to all faculty members and staff, DBTES and TIFAC CORE, for their constant encouragement and support throughout the project work. I am thankful for the help rendered by the Tissue Culture staff at TIFAC-CORE with special mention of Mr. Khem Raj, Mr. Rajesh, Mr. Sandeep and Mr. Bhupinder who were always there for me to understand the minor details of the work on a day-to-day basis. I feel lacunae of words to express my most heartfelt and cordial thanks to research scholars Ms. Navdeep, Ms. Gurdeep, Mr. Balwant and my friends Deepwinder, Rabia, Himani, Ranjan, Shivani, Tania, Himangi, Preetika who have always stood by my side during all the tough times.

The whole credit for my achievements during the project work goes to my family. It was their unshakeable faith in me that always helped me to proceed further.

In the end, I am thankful to Almighty for blessing me to complete this work successfully.

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Abstract

The present study was an attempt to develop the shoot regeneration and genetic transformation protocol for apple rootstock M7. Impact of medium composition (mineral nutrients, different combinations of plant growth regulators) on shoot multiplication, callusing, regeneration and rooting of apple rootstocks cultured on Murashige and Skoog (MS) medium were investigated. The best shoot prolification in terms of shoot number and shoot quality was obtained using 2.5 µM 6-benzylaminopurine (BAP) and 1.0 µM Naphthaleneacetic acid (NAA) during the shoot multiplication phase. The rooting of microshoots were induced by indole-3-butyric acid (IBA) and best rooting was achieved on MS medium containing 5 µM IBA. Shoot regeneration was observed 5-6 weeks after subcultured on MS medium supplemented with 5.0 µM BAP and 5.0 µM NAA.

In vitro produced leaves of the apple rootstock M7 were infected with *Agrobacterium tumefaciens* strains containing a binary vector carrying the *nptII* gene and the *uidA* gene on the T-DNA. The effect of different parameters on transient GUS expression after two days of co-cultivation with *Agrobacterium* was studied. Co-cultivation period of 2 days and a bacterial density of 0.6 OD_{600} resulted in higher transient GUS expression in explants.

The clonal fidelity of micropropagated shoots were established using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Specific Repeats (ISSR) markers.

List of Abbreviations

%	Percent
µl	microlitre
BAP	6- Benzylamino purine
°C	Degree centigrade
Carb	Carbenicillin
CTAB	Hexadecyltrimethylammonium bromide
dNTPs	deoxynucleotide triphosphates
2,4-D	2,4-dichlorophenoxyacetic acid
EDTA	Ethylenediaminetetraacetic acid
GUS	β-Glucuronidase
h	hour
IBA	Indole-3-butyric acid
ISSR	Inter Simple Specific Repeats
Kan	Kanamycin
L	litre
M	Molar
µM	Micromolar
mM	Millimolar

mg	Milligram
mins	Minutes
ml	Mililitre
MS	Murashige and Skoog (1962)
NAA	Naphthaleneacetic acid
Nos	Nopaline synthase
NptII	Neomycin phosphotransferase
OD_{600}	Optical Density at 600 nm
PCR	Polymerase Chain Reaction
PGR(s)	Plant growth regulator(s)
RAPD	Random Amplified Polymorphic DNA
TE	Tris EDTA
w/v	weight by volume
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

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CHAPTER 1

Introduction

Apple (*Malus domestica*), fruit of the genus *Malus* (about 25 species) belongs to the family Rosaceae. Apple is a pomaceous fruit and is the most widely cultivated fruit tree. Apple (*Malus domestica*) is one of the most consumed fruit in the world. The major production areas are in the temperate regions, however, because of its excellent storage capacity it is transported to distant markets covering the four corners of the earth.

Apple trees grow as small deciduous trees**.** Apples at harvest, vary widely in size, shape, colour and acidity. Depending upon cultures and environmental character, they are usually roundish, 50–100 mm (2–4 inches) in diameter, and some shade of red or yellow in colour. Apple trees can range in size from 6 to 30 feet in height, depending on variety and type of rootstock (dwarf, semi-dwarf, etc.). Apple trees have simple leaves that are arranged alternately along the stem. The leaves are bright to dark green in color and with toothed margins. Apples are 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).

There are more than 7,500 known cultivars of apples, with a range of desired characteristics. Different cultivars are bred for various tastes and uses, including cooking, fresh eating and cider production. The apple fruit is very nutritious, aromatic and rich in minerals and vitamins. Extremely sweet apples with barely any acidic flavor are popular in Asia especially India (Tarjan *et al.*, 2006). Apple trees are generally propagated by grafting, although wild apples grow readily from seed. Trees are prone to a number of fungal, bacterial and pest problems, which can be controlled by a number of organic and non-organic practices.

Apples, along with oranges and bananas, dominate the world market as one of the most popular fruits. World apple production has expanded considerably over the last 20 years, with total production getting increasingly dominated by China (Hassall and Associates, 2001). Apple is world's most important temperate fruit crop with a production of 63 million tons/yr.

Apple cultivars

There are more than 7,500 known cultivars of apples (Dobrazanski *et al.,* 2006). Cultivars vary in their yield and the ultimate size of the tree, even when grown on the same rootstock. Different cultivars are available for temperate and subtropical climates. Because of the chilling requirements, apples do not flower in tropical climates.

Some cultivars, if left unpruned, will grow very large, which allows them to bear more fruit, but makes harvest difficult. Commercially, popular apple cultivars are soft but crisp and other desired qualities in modern commercial apple breeding are a colorful skin, absence of russeting, ease of shipping, longer shelf life, flavor, high yields and disease resistance (Cummins and Aldwinckle, 1983; Momol *et al.,* 1998; Westwood, 1988).

Old cultivars are often oddly shaped, russeted and have a variety of textures and colours. Many of them have excellent flavor, but may have other problems that make them commercially unviable, such as low yield, prone to biotic and abiotic stress, or lesser shelf life. Fruit softening is an irreversible process of senescence and excessive softening of apple is undesirable because it results in short shelf life and lower sensory values (Abbott *et al.*, 1984; Harker *et al.,* 1997, Harker *et al.,* 2002; Jaeger *et al.,* 1998).

Apple rootstocks

Advancements in rootstock breeding and selection have revolutionized the manner in which apples are grown throughout the world. In modern production systems, selection of an appropriate rootstock is as important to the viability and success of a new plantations as the choice of fruiting cultivar. Rootstocks affect a number of horticultural attributes, including winter hardiness, fruit size, precocity, productivity, tree vigor, and resistance to both biotic and abiotic stresses. Depending on which rootstock is used, apple trees may attain size which can be in this following 4 categories: dwarf, semi-dwarf, semi-vigorous or semi-standard, and vigorous or standard size.

Dwarfing rootstocks significantly reduce tree size, facilitating an increase in planting density (Ferree *et al.,* 1993; Hampson *et al.,* 2002, 2004a, 2004b; Robinson *et al.,* 1991). Dwarfing rootstocks control wood production in the tree, directing its energy into fruit production. 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 center of learning in Greece. The number of rootstocks available commercially for dwarfing apple trees is steadily increasing. The important effects of dwarfing rootstocks are tree size reduction, early bearing and high yield efficiency (Faust, 1989; Lehman *et al.,* 1990; Rom *et al.,* 1987). Rootstocks also influence tree anchorage and root brittleness. Generally, more dwarfing rootstocks have shallower root systems and more vigorous rootstocks are deeper rooted. However, depth of rooting is also influenced by soil type, compaction and drainage.

Rootstocks affect the ultimate size of the tree and have been used in apple cultivation for over 2,000 years. Whilst "seedling" trees (grafted on to roots of the same variety grown from seed) were the norm until the 20th century, the technique of grafting a fruiting variety on to a rootstock to control the size, vigour and precocity of apple trees in particular had been known for several centuries. In the late nineteenth and early twentieth centuries researchers began to formalise the use of size-controlling rootstocks for apple trees and later for other fruit tree species. Although rootstocks are invariably given cryptic numeric reference codes, they are essentially fruit tree varieties in their own right. New rootstocks are developed using the same techniques that are used for developing fruit varieties - a combination of chance, observation, and scientific crossing of varieties with desirable characteristics. Most modern apple rootstocks were developed in 20th century. Beginning around 1912 researchers at East Malling Research Station in the UK were the first to classify rootstocks and develop new ones for specific purposes. The rootstock not only influences the size of the tree, it also provides other characteristics such as precocity (the age of the tree when it will first start to bear fruit), some disease resistance attributes, and resistance to harsh winters. Ferree and Carlson (1987) and Tukey (1964) characterize dwarfing rootstocks, especially M9 as intolerant of drought stress, while more vigorous rootstocks, particularly MM111, are considered more drought resistant.

The success of a rootstock is determined by its tolerance to prevailing conditions of soil, climate and disease. Standardized clonal apple rootstocks are required in India for uniform yield and quality. The trend in apple production has been towards high density plantings which gives higher yield per unit area of land. East Malling Research Station, in conjunction with some other UK research stations at Merton and Long Ashton developed a range of virus-free rootstocks of which M27, M9, M26, MM106, M7, MM111, and M25 are in widespread use. Work on rootstocks able to resist fireblight and cold was initially carried out at the New York Agricultural Experimental Station in Geneva, NY, and this led to the G-series rootstocks. These rootstocks are often better suited to the climate and disease regime of North America than the East Malling series - although M9 and M7 are also widely used in North America.

The famous M9 rootstock was one of the first released by this programme, and remains one of the most widely-planted commercial rootstocks. It is a standardised form of one of the French "Paradise" rootstocks, known as Jaune de Metz. (When young, these rootstocks have a yellowsheen to the bark). Genetic improvement of apple rootstocks by traditional breeding and selection methods is a very slow process due to the long life cycles. However, by applying genetic engineering, the improvement of apple rootstocks can be speeded up (Holefors *et al.*, 1998; Welander *et al.*, 1998; Zhu *et al.*, 1998).

M7 (Malling7) Rootstock

M7 (Malling7) is descended from a French tree, "Doucin Reinette" in around 1688. M7 is one of the most popular commercial semi-dwarf apple rootstocks. It is semi-vigorous, adapts to a wide range of soil types and climates and can withstand temporary drought conditions**.** It suckers extensively, prone to crown gall, resistant to fireblight and tolerant to water logged soils. It is one of the most popular apple rootstocks of the original Malling-series rootstock in North America. The planting of semi-dwarf apple tree has been a compromise between the big standard apple tree that requires much ladder work and the small dwarf tree that can be pruned and picked from the ground. Fruit production is relatively light for the first 10 years, after which ladders are needed to pick the fruit. As a rootstock, M7 has a history of poor anchorage, low uptake of potassium and frost-tender roots.

Malling7 has been recommended for commercial use in H.P. and J&K on the basis of growth attributes, fruit yield and quality and graft compatibility. It is susceptible to root rot but is less susceptible to collar rot.

Propagation of several apple rootstocks by the method of tissue culture was successfully developed including M7 (Werner and Boe, 1980; Martins and Pedrotti, 2001; Silveria *et al.*, 2001).

Genetic transformation

Transformation is a key to sustaining the demand—permitting the potential enhancement of existing cultivars as well as to investigate the development of new cultivars resistant to pest, disease and storage problems that occur in the major production areas.

Plant transformation was defined by Eede *et al*. (2004) as the stable incorporation and expression of foreign genes. Conventional breeding of woody species is a low efficiency process because of their long life cycle and the high heterozygosity of the parental lines.

In recent years, the adoption of new technologies such as plant tissue culture and recombinant DNA have advanced and opened up a new avenue for fruit tree improvement (Jain, 2001; Faheem *et al.,* 2008), which can shorten breeding period and breed varieties with stress tolerance (Zhuo *et al.,* 2007). Transformation techniques are aimed at the introduction of additional genes corresponding to beneficial properties into the otherwise unaltered genome of a cultivar, and may therefore speed up the breeding process. Genetic transformation of plants can be accomplished by two methods, either by direct gene transfer or *Agrobacterium*-mediated gene transfer. Direct gene transfer is commonly employed in monocot species that are not amenable to *Agrobacterium* transformation. Microprojectile bombardment and direct gene transfer to protoplasts are used commonly to transform a variety of monocotyledonous plants (Vain *et al.,* 1995). Further, *Agrobacterium*-mediated genetic transformation offers several advantages, such as defined integration of transgenes, low copy number and preferential integration into transcriptionally active regions of the chromosomes and hence is the most powerful tool for plant genetic transformations (Gelvin, 2003). *Agrobacterium tumefaciens* is a naturally occurring, soil-borne bacterium that causes crown gall disease in a wide range of dicot species. The bacterium contains a Ti plasmid (tumor inducing) that interacts with the plant cells to provide a convenient mechanism for gene transfer into cells. During bacterial infection of plant tissue the T-DNA (transfer DNA), a mobile segment of Ti plasmid, is transferred to the plant cell nucleus and integrated into plant chromosomes. Except for the T-DNA, the virulence (*vir*) region located on the Ti plasmid is also important for transformation (Zupan *et al*., 2000). Several reviews are available that describe *Agrobacterium* biology in detail (Gelvin, 2003; Zupan *et al.*, 2000; Zupan and Zambryski, 1997).

Many transformation procedures of herbaceous plants are based on explants derived from seed or seedling organs such as whole embryos, hypocotyls or cotyledons. However, as passage through a sexual stage results in a drastic reshuffling of the genome and hence alteration of cultivar properties, it is essential that transformation procedures of woody species are based on organ explants such as leaves or stems which are derived from vegetatively propagated plants. Apple leaf explants have previously been shown to regenerate shoots at efficiencies close to 100% (James *et al.*, 1988; Fasolo *et al.*, 1989; Sriskandarajah *et al.*, 1990; Welander and Maheswaran, 1992).

Today, many agronomically and horticulturally important species are routinely transformed using this bacterium, and the list of species susceptible to *Agrobacterium*-mediated transformation seems to be growing (Gelvin, 2003). As a genus, *Agrobacterium* can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot angiosperm species (Anderson and Moore, 1979; Decleene and Deley, 1976; Gelvin, 2003; Porter, 1991).

Genetic transformation of apple typically involves the inoculation of cut leaf blades with disarmed *Agrobacterium tumefaciens* strains containing plasmid binary vectors (De Bondt *et al.*, 1994; James *et al.*, 1989; Maheswaran *et al.*, 1992; Sriskandarajah *et al.*, 1994; Yao *et al.*, 1995).

Many of the selected apple rootstocks have problems with respect to biotic and/or abiotic stress and require modifications for their improvement. The improvement of the selected rootstock can effectively be taken up through genetic transformation methods, which can also help in retaining the desired genetic combination of the given cultivar. Therefore, the present study is an attempt to develop the genetic transformation protocol for the selected rootstock of apple i.e M7.

The key objectives of our study are-

- To optimize condition for shoot regeneration from leaf explant.
- To test the clonal fidelity of regenerated plants.
- To test the sensitivity of leaf explants to various selection markers.
- To study the factors influencing genetic transformation efficiency.

CHAPTER 2

Literature review

Apples are one of the oldest cultivated fruits, and are recorded in the writings of almost all of the classic pre-Christian era civilisations. Apple is one of the most important fruits in temperate zones and it is the third most important fruit crop (64.3 million t/year) in the world following bananas (81.3 million t/year) and grapes (66.3 million t/year) (FAO, 2009). Apples are usually multiplied by grafting the fruiting cultivar onto a rootstock. Mature woody plants are typically more difficult to propagate vegetatively than their juvenile counterparts (Hackett, 1985; Greenwood and Hutchison, 1993).

Among the temperate fruit tree species, apple (*Malus domestica)* was chosen for gene transfer studies because of its economic importance (Korban and Chen, 1992), long juvenile period, high level of self incompatibility and high heterozygosity (Brown, 1975).

2.1 Micropropagation of Apple

Micropropagation is defined as the culture of different somatic cells, tissues or organs of plants under controlled *in vitro* conditions with the aim of producing a large number of clonal plants, which are genetically identical to the stock plant, in a relatively short time compared to conventional propagation. This implies *in vitro* cloning based on the fact that different plant parts, buds, meristems, tissues and cells are capable of regeneration into whole plants under adequate *in vitro* conditions.

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 (Dobránszki and da Silva, 2010).

Different stages of apple micropropagation such as shoot multiplication, shoot elongation, rooting of microshoots, acclimatization and even regeneration were related to medium composition (Zimmerman and Debergh, 1991; Karp, 1995). It has been suggested that phenotypic changes following long-term micropropagation result from rejuvenation of mature tissues (Webster and Jones, 1989; Jones and Webster, 1992; Noiton *et al.*, 1992; Hammatt and Grant, 1993). In apple, Webster and Jones (1989) and Noiton *et al.*, (1992) concluded that such changes were a result of subculturing.

During the last few decades, many reliable methods of *in vitro* propagation have been developed for both rootstocks and scions from a practical, commercial point of view. 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* (e.g. genotype and physiological state) and *in vitro* conditions (e.g., media constituents and light) (Dobranszki and da Silva, 2010). A micropropagation procedure developed for an apple genotype on a specific medium could not always be extrapolated with the same success for another genotype (George and Debergh, 2008).

Micropropagation allows quick propagation of new varieties or breeding lines or variants for apple breeders. It is an essential step in the success of regeneration of transgenic lines and determines the effectiveness of a transformation protocol (Aldwinckle and Malnoy, 2009).

2.2 Shoot Organogenesis of Apple

Apple is an excellent model species because its regeneration *in vitro* is extremely well explored and studied (Dobranszki and da Silva, 2010; Magyar–Tabori *et al.*, 2010), despite being a hardwood species. Adventitious shoot regeneration is a prerequisite for successful application of different biotechnological methods in apple breeding (Korban and Chen, 1992; Ou *et al.,* 2008). Generation of adventitious shoots from apple (*Malus domestica)* leaf explants has been well documented (Liu *et al.*, 1983a, 1983b). Regeneration via organogenesis from leaf explants has been described for different apple rootstocks and scions (reviewed by Magyar-Tabori *et al.*, 2010; Dobranszki and da Silva, 2010). In most cases, the regeneration potential depends on explant type, genotype, and the composition of the cultivation medium (Rout and Lucas, 1996; Cheng *et al.*, 2003; Eudes *et al.,* 2003). Regeneration from plant tissues *in vitro* is largely dependent on explant choice, medium composition and control of the physical microenvironment (Thorpe and Patel, 1984; Brown and Thorpe, 1986). In apple, the most studied regeneration technique is adventitious shoot formation from leaves. Murashige (1974) identified several factors that should be considered in explants selection including organ chosen as the tissue source, the physiologic and ontogenetic age of the organ and the overall quality of the donor plant. Pretreatment of donor plants may also play an important role in determining the regeneration response (Klimaszewska and Keller, 1985; Khehra and Mathias, 1992; Nhut *et al.*, 2002). Manipulation of environmental and nutritional parameters such as temperature, light quality and intensity, and medium composition during *in vitro* or *in vivo* donor plant growth affected the regeneration response in several plant species.

The position of leaves along the shoot is one of the important factors influencing the success of regeneration from apple leaves; younger leaves regenerate more easily (Druart, 1990; Fasolo *et al.,* 1989; Famiani *et al.,* 1994; Modgil *et al.*, 2005) observed that the apical, expanded leaves showed a higher regeneration ability, i.e., more regenerated shoots per explant.

James *et al*. (1988) examined the factors affecting the frequency of plant regeneration from apple leaf tissues and found that the number of regenerated shoots was enhanced when leaves were cut into three or more strips rather than when cut into discs.

Out of several *in vitro* factors influencing organogenesis from apple leaf tissues, one of the most important is the hormonal balance of the regeneration and final proliferation media (Ferradini *et al.,* 1996; Dobranszki *et al.,* 2005; Magyar-Tabori *et al.,* 2010). 6 - Benzylaminopurine (BA) and thidiazuron (TDZ) are the most frequently used cytokinins in apple shoot multiplication and they were compared in several studies (Yepes and Aldwinckle, 1994).

2.3 Rooting of Microshoots and establishment of apples

Root formation is a difficult step in micropropagation of many woody plants (Custodio *et al.*, 2004) and is regulated by a number of physiological, biochemical and genetic factors

(Pawlicki and Welander, 1995). It is an important aspect for enhancing survival and growth during acclimatization and losses at this stage have considerable economic value from practical point of view (Ahmad *et al.,* 2003; Custodio *et al.,* 2004).

Thorpe (1982) indicated that root initiation and growth were high energy requiring processes that could only occur at the expense of available metabolic substrates, which were mainly carbohydrates. Various concentrations of indole-3-butyric acid (IBA) initiated rooting but maximum rooting percentage was found with 2.0 and 2.5 mg/l of IBA in M7 and with 1.0 mg/l of IBA in M 106 (Sharma *et al.,* 2007).

Microshoots of apple (*Malus domestica*) rootstocks G.65, G.30 and G.11 were transferred from stage II axillary shoot cultures to stage III rooting media containing 10 levels of indole-3-butyric acid (IBA) for four weeks to determine optimal conditions for rooting. Microshoots were inverted or left in an upright position. Rootstocks and microshoot position affected rooting and survival; the highest rooting was 30% for G.65 when planted inverted on a medium supplemented with 2 mg/l IBA, 100% for G.30 when planted upright with 3 mg/l IBA, and 100% for G.11 in an position inverted with 1 or 2 mg/l IBA.

Interactive effect of BAP and IBA on the root formation of apple was reported by De Klerk *et al*. (2001). Rooting percentage was not modified by the BAP content of the medium. The efficacy of different types of auxin such as indolebutyric acid (IBA), indoleacetic acid (IAA), and α-naphtalene acetic acid (NAA) was investigated on rooting of nine apple cultivars by Zimmerman and Fordham (1985).

Teresa (1992) studied the effect of L-arginine on rooting of microshoots of apple rootstock. The effect was studied in combination with other rooting factors such as: phloroglucinol, initial dark period, concentration of indolyl-3-butyric acid and inorganic nitrogen levels. In all treatments, arginine caused an increase in root number per rooted shoot and enlargement of the shoot base. Arginine was especially effective with low indolyl-3- butyric acid levels as well as without it, and with low or no inorganic nitrogen. The effect of arginine on root number interacted with dark treatment and with phloroglucinol. The most efficient amount of arginine was 200 mg/l. Well developed microshoots were rooted *ex vitro* by two hour treatment with aqueous solution of 100 mg/l IBA prior to their planting in substrate composed of soil and vermiculite (Xu *et al.*, 2008).

2.4 Clonal fidelity of apple

Clonal uniformity is an important aspect of micropropagation. When plant tissue passes through different stages of *in vitro* culture; many of the regenerated plantlets appear to be no longer clonal copies of their donor genotype. The broader utility of any micropropagation system may be limited due to occurrence of cryptic genetic changes and development of somaclones (Rani and Raina, 2000). Micropropagated plants regenerated from preformed structures like shoot tips, axillary buds and tissues of hard wood shoot cuttings have been reported to maintain clonal fidelity but plant growth regulators especially synthetic ones at sub- and super- optimal levels have been shown to be associated with induction of somaclonal variations (Martin *et al.,* 2006). Thus screening the micropropagated plants and identifying variants at an early stage is essential. Relatively little attention has been paid to test the genetic stability of micropropagated plants in apple. The detection of off types among micropropagated plants especially trees by morphological observations, karyotype analysis and isozymes has several limitations. On the other hand, DNA markers are more informative and can detect any DNA variations that may arise in tissue culture derived plants (Modgil *et al.,* 2009). Among the different DNA polymorphism detection techniques available, RAPD has widely been used to study cloanal integrity, detect genetic and somaclonal variations and identify cultivars in several crops (Martin *et al.,* 2006; Saini *et al.,* 2004).

In a rootstock micropropagation programme, it is of paramount importance to produce true-to-type planting materials, hence somaclonal variations of any kind, if induced may multiply very fast and lead to loss of the chief characteristics of the parent rootstocks. Furthermore, this genetic instability may be a risk associated with the application of *in vitro* culture techniques for handling and storage of germplasm (Ray *et al.,* 2006). Variation is understood to be generated via combination of genetic and/or epigenetic changes. A lack of any phenotypic variation among regenerants does not necessarily imply a concomitant lack of genetic (or epigenetic) change (Larkin and Scowcroft, 1981) and it is therefore of interest to assay *in vitro* raised plantlets at the genotypic level. The polymerase chain reaction (PCR) has previously been used in conjunction with short random amplified polymorphic DNA (RAPD) primers (decamer or ten-mer) to assess the genetic stability of micropropagated grape plantlets (Khawale *et al.*, 2006; Singh *et al.*, 2005), MM 106 apple rootstock (Modgil *et al.*, 2005), peach (Hashmi *et al.*, 1997) and strawberry (Boxus *et al.*, 2000).

Genetic Transformation

Genetic transformation offers an alternative approach for introducing desirable traits, such as resistance to herbicides, diseases, and insects; or developing new floral colors (Marrewijk, 1994). Genetic transformation of apple tissue was reported two decades ago using leaf explants (James *et al.*, 1989; Maheswaran *et al.*, 1992). *Malus* transformation has been achieved in some laboratories by various methods, including the direct uptake of naked DNA by protoplasts (Lee *et al.*, 1995), and the *Agrobacterium*-mediated transformation of leaf segments (De Bondt *et al.*, 1996; James *et al.*, 1989; Maheswaran *et al.*, 1992; Norelli *et al.*, 1994; Martin *et al.*, 1990 and Song *et al.*, 2000). In these early studies *Agrobacterium*-mediated transformation was used with leaf explants, and the effects of explant age, orientation, and genotype were explored (De Bondt *et al.*, 1994; Yepes and Aldwinckle 1994; Puite and Schaart 1996). Genetic transformation of apple typically involves the inoculation of cut leaf blades with disarmed *Agrobacterium tumefaciens* strains containing plasmid binary vectors (De Bondt *et al.*, 1994; James *et al.*, 1989; Maheswaran *et al.*, 1992; Sriskandarajah *et al.*, 1994; Yao *et al.*, 1995). T-DNA binary vectors have revolutionized the use of *A. tumefaciens* to introduce genes into plants (Gelvin, 2003). These plasmids are small and easy to manipulate in both *E. coli* and *A. tumefaciens* as they can replicate themselves easily in *E. coli* and in *A. tumefaciens*.

CHAPTER 3

Materials and Method

3.1 Plant material, chemicals, glassware and culture establishment

Explants are taken from young, disease free plants of selected rootstock M7. Those explants were used for shoot multiplication experiment. Leaves from micro-shoots were used for shoot regeneration and genetic transformation experiment.

All routinely used chemicals were purchased from HiMedia Laboratories (Mumbai, India), growth regulators and antibiotics were purchased from Sigma Chemical Co. (St Louis, MO, USA). Unless otherwise mentioned all experiments were conducted in 300 ml glass culture bottles (Kasablanka, Mumbai) covered with propylene cap containing 50 ml of medium.
The medium used contained Murashige and Skoog's medium salts, vitamins, 3% (w/v) sucrose and solidified with 0.65%-0.70% (w/v) agar (Solarbio, Beijing, China).

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 an oven at 70°C for 1 to 2 hours. All contaminated culture bottles were autoclaved (Equitron, Medica Instruments, India) and contents were discarded before washing.

3.3 Media preparation and culture condition

Stock cultures of apple rootstocks M7 were maintained on MS (Murashige & Skoog, 1962) medium consisting of MS macro and micro elements and supplemented with MS vitamins, 7.5 g/l agar and 30 g/l sucrose. Growth regulators BAP and NAA were added to the basal medium either alone or in various combinations, as specified with each experiment. All media were adjusted to pH 5.8 with 1 N NaOH or 1 N HCl before autoclaving at 120°C for

20 mins. All plant growth regulators were added before autoclaving. All cultures were incubated in growth room set at 25±2°C under a 16/8 h (day/night) photoperiod with a light intensity (42 µmol/m^2/s) provided by cool white fluorescent tubes. The concentrated stock solutions of all ingredients were prepared and stored under refrigeration. The stock solutions of plant growth regulators (PGRs) were also prepared by dissolving in a few drops of sodium hydroxide (1.0 N) or hydrochloric acid (1.0 N) and made to desired volume (2.5 mM) with distilled water and stored at 4°C. 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.

3.4 Shoot multiplication

Cultures were established from the nodal segment (1-2 cm in length) taken from young, disease free plant rootstock M7 grown under specific conditions. The young shoots sprouting from tissue grown under specific conditions were selected and nodal explants were excised in laminar air flow aseptically. These nodal explants were cultured on basal 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 prolification were subcultured 2-3 times on the same medium. In another experiment the effect of various concentration BAP (1.0-7.5 µM) with NAA (0.5-1.0 µM) on the shoot multiplication and size of leaves were studied.

3.5 Rooting

Microshoots with shoot tips (2-3 cm long) were used for the root induction. For the purpose of root induction, the microshoots were inoculated on MS medium containing 3% (w/v) sucrose, 0.7% (w/v) agar and various concentration of IBA (1.0-12.5 µM).

3.6 Regeneration of shoots from leaf explants

Experiment was conducted to achieve regeneration from the leaf segments. Leaves (7-9 mm) from actively growing microshoots (35-45 days) of good condition were used as explants.

The leaves were excised under aseptic conditions in laminar air flow cabinet and cut in to transverse segments (2-3 mm wide) and inoculated on medium with their adaxial surface towards medium. The effect of different concentration of BAP (1.0-15 µM) in combination with different concentration of NAA (1.0-12.5 µM) was studied on shoot regeneration.

3.7 Genetic Transformation

3.7.1 Bacterial strains and plasmids

The binary vector pBI121 having reporter gene *uid*A gene (β-glucuronidase) (GUS) and selection marker gene *npt* II (neomycin phosphotransferase II) was used for genetic transformation.

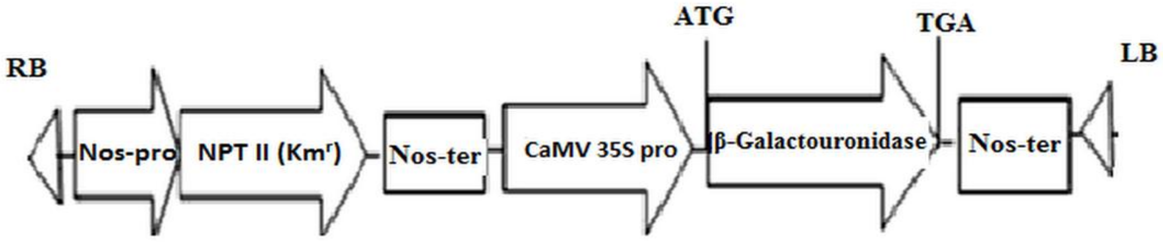


Figure 1: Schematic diagram of a part of the T-DNA region of binary vector pBI121. RB, right border; LB, left border; Nos-pro, nopaline synthase promoter; NPTII, gene for neomycin phosphotransferase; Nos-ter, terminator of nopaline synthase; CaMV35S pro, 35S promoter of cauliflower mosaic virus; β-glucuronidase, GUS reporter gene.

The genes encoding kanamycin resistance were on the T-DNA left and right borders under the control of CaMV 35S and nos promoters (Jefferson *et al*., 1987). Kanamycin was an efficient selective agent for selection. The transformed *A. tumefaciens* strains were cultured to log phase and were maintained at 28°C on yeast extract peptone (YEP) agar medium (10 g/1 bacto peptone, 10 g/l yeast extract, 0.5 g/l NaCl and 1.5 g/l agar pH-7) containing 15 µg/ml rifampicin and 50 µg/ml kanamycin and used for the genetic transformation experiments. When the bacterial density has reached about OD_{600} 0.5-1.0, these were used for the genetic transformation of leaves of apple rootstock M7.

3.7.2 Antibiotic sensitivity of explants

To test the effectiveness of antibiotic in selecting transformants, leaf segment explants were cultured on shoot induction medium (MS medium + 5.0 µM BAP + 5.0 µM NAA + 0.7% agar +30 g/l sucrose, pH 5.8), supplemented with different concentrations of kanamycin (25, 50, 75 mg/l), cefotaxime (100, 200, 400 mg/l), carbenicillin (100, 200, 400 mg/l) or sporodex (100, 200, 400 mg/l). After 4 weeks, the survival of leaves and callus formation was determined.

3.7.3 Optimization of conditions for transformation

Leaf explants were infected with a culture of *Agrobacterium.* Explants were removed blotted dried with sterile filter paper to remove excess bacteria and kept on MS medium for co-cultivation. Co-cultivation leads to the induction of virulence and gene transfer, and therefore, is an essential step for *A. tumefaciens* transformation. After co-cultivation, transient GUS expression could be observed from leaves and callus. There was no significant difference between 3 and 4 days of co-cultivation for apple leaves and callus, 4 days of co-cultivation caused overgrowth of *A. tumefaciens* which were difficult to completely eliminate by antibiotic and therefore were harmful to the leaves and callus.

3.7.4 Transformation, selection and plant regeneration

Overnight culture of *A.tumefaciens* having an OD_{600} of 0.5-1.0 was diluted 1:10 with YEP medium or liquid MS without plant growth regulators. The leaf explants were immersed in bacterial suspension for 10 mins with constant shaking. Explants were removed blotted dried with sterile filter paper to remove excess bacteria and kept MS medium for co-cultivation. Co-cultivation was carried out at 25ºC for 1-4 days in the dark. The infected cultures were washed and explants were cultured on selection medium (MS + 5.0 µM BAP + 5.0 µM NAA) containing 200 µg/ml cefotaxime to stop the growth of *A. tumefaciens* attached to the explants and 50 µg/ml kanamycin for the selection of transformed tissue for 10-15 days at 25 ± 1 °C with a 16/8- hrs (light/dark) photoperiod provided by cool white fluorescent tubelight.

Following incubation the GUS expression was assayed. A number of transformation parameters were studied in order to optimize the transient expression of reported gene. These parameters are as follows:

Co-cultivation period: To test the importance of the co-cultivation period, leaf explants which had been inoculated for 15 mins were transferred to co-cultivation medium and incubated for 1,2,3,4 days. At the end of the designated co-cultivation period, explants were washed and transient *gus* activity was recorded.

Pre-culture period: The effect of different preculture periods of explants on MS medium (0, 1, 2, 3 days) on transformation was evaluated. To test its importance, cultures were pre-cultured on MS medium without any growth regulator.

Infection time: The importance of inoculation period or infection time was tested by inoculating leaf explants with an *Agrobacterium* suspension for various period of 5, 10, 15, 20 mins. Following co-cultivation period, explants were washed and *gus* activity was assayed.

Mode of injury: The effect of various modes of injury to leaf explants (scalpel, needle, carborundum paper) on transformation was studied. The explants were injured with either surgical blade or pricked with needle or injured by rubbing with carborundum paper. After 15 mins of infection, explants were transferred to co-cultivation medium for 2 days, after which transient *gus* expression was assayed.

pH: Maintaining proper pH of the co-cultivation medium is important for the genetic transformation using *Agrobacterium tumefaciens*. Therefore, effect of different pH (5.5, 5.6, 5.7, 5.8) of media during co-cultivation was studied on transformation.

Bacterial density: Transformation was investigated based on concentration of *Agrobacterium* suspension culture at optical density (OD). The importance of *Agrobacterium* cell density was evaluated by inoculating leaf explants with *Agrobacterium* suspension which were supplied at various bacterial cell concentrations (i.e. OD_{600} 0.2, 0.4, 0.5, 0.6).

Photoperiod: Different light conditions during co-cultivation also have a strong effect on efficiency of transient expression of transformed gene. Therefore, effect of photoperiod (continuous dark, continuous light and 16 h light/8 h dark) on transformation was studied.

3.8 GUS assay

The *udiA* gene encoding the enzyme β-glucuronidase (GUS) appears promising as a genetic marker for early confirmation of successful plant cell transformation.

Transient histochemical GUS activity was assessed in infected leaves and callus. The system used in our current work exploits the *udi*A gene found in *Escherichia coli* encoding the GUS enzyme (Jefferson *et al.,* 1987). Plant tissues were incubated into GUS buffer (Jefferson *et al.,* 1987) with the substrate 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexyl-ammonium, Sigma) at 37ºC from 2 hrs to overnight. Stained tissues were washed and placed in 75% (w/v) ethanol (to remove the chlorophyll) before examining under the microscope.

Table 1: Preparation of solution for the *gus* activity:

Reagents	**for 1ml**	**final concentration**
1M Sodium phosphate buffer, pH 7.0	100µl	0.1M
0.25M EDTA, pH7.0	40µl	10mM
0.005M Potassium ferro-cyanide, pH7.0	100µl	0.5mM
0.005M Potassium ferri-cyanide, pH7.0	100µl	0.5mM
0.02M X-Gluc	50µl	1mM
10% Triton X-100	10µl	0.1%
Distilled water	600µl	

3.9 Molecular Characterization

3.9.1 DNA Isolation

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

Procedure

- 1.5 g of fresh leaves were taken from each microshoot grounded in liquid N_2 to fine powder, followed by immediate transfer to 50 ml centrifuge tube. To this prewarmed CTAB extraction buffer was added 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 x g 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 (10000 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.

- After incubation, equal volume of phenol and chloroform was added (1:1 v/v) was added followed by gentle shaking for 5 mins and then centrifuged at10,000 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
100 mM Tris-HCl pH 8.0	100 ml Tris-HCl stock (1M)
1.4 M NaCl	280 ml NaCl stock (5M)

Made up to 1 litre with distilled water, pH 7.5 – 8 and autoclaved mercaptoethanol (0.2 % v/v) was added into buffer just before use.

2. Isopropanol
3. Chloroform
4. Isoamyl alcohol
5. Saturated alcohol
6. Sodium acetate 3M
7. T.E buffer contain 20 mM EDTA and 100 mM Tris-HCl (pH 8.0) obtained by mixing required amount from the stocks: EDTA stock (0.5 M) and Tris-HCl stock (1M).

3.9.2 PCR Analysis

Table 2: Following are the sequences of all the 5 primers used in present investigation:

Primers	Nucleotide sequence (5’-3’)
RAPD 1	TTGGCACGGG
RAPD 2	GGGGTGACGA
RAPD 3	GAGAGCCAAC
ISSR 1	GAGAGAGAGAGAGAGACG
ISSR 2	GAGAGAGAGAGAGAGATC

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. Amplification with both RAPD and ISSR primers was carried out in a total volume of 20 µl.

Table 3: PCR reaction mixture comprises of following components:

Components	Stock solution	Vol/Rxn
dNTPs	10mM each	1.50 µl
PCR buffer	10 X	2.0 µl
Taq polymerase	5 U/µl	0.3 µl (1.5U)
Primer	10 µM	1.0 µl
Sterile H_2O	-----	13.20 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 temperature profile mentioned in Table 4.

Table 4: PCR cycling parameters:

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

*Annealing temperature for ISSR is 55°C

*Annealing temperature for RAPD is 35°C

3.9.3 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 gels were photographed using Geldoc system (Vilber Loumart, France) and stored as digital pictures.

CHAPTER 4

Results

4.1 Shoot multiplication

In vitro clonal multiplication of apple rootstock M7 using nodal segments was carried out. To study the effect of various concentrations of BAP and NAA on size of leaves and shoot multiplication. The microshoots were inoculated to shoot proliferation medium supplemented with various concentrations of BAP (1.0,2.5,5.0,7.5 µM) and NAA (0.5,1.0 µM) on MS medium. After two months of cultivation, number of shoots and mean length of shoots were recorded. The highest length and number of shoots were obtained for shoots grown in media containing 2.5 µM BAP and 1.0 µM NAA and the minimum average length was obtained on MS supplemented with 1.0 µM BAP and 1.0 µM NAA.

Table 5: Effect of different concentration of BAP and NAA in MS basal on shoot multiplication of apple rootstock M7:

BAP (µM)	NAA (µM)	Average number of shoots per vessel	Average shoot length (cm)	Average leaf size
1	0.5	20	4.0	++
1	1	18	3.6	++
2.5	0.5	17	4.3	++
2.5	1	28	5.3	+++
5	0.5	19	4.1	++
5	1	18	4.3	+++
7.5	0.5	16	3.8	++
7.5	1	15	4.3	++

Each treatment considered of three tissue culture bottles with 5 nodal segments in each and data recorded after 4 weeks of subculture. Leaves size: + (1-4mm in length and width), ++ (4-6mm in length and width), +++ (6-9mm in length and width).

Plate-1

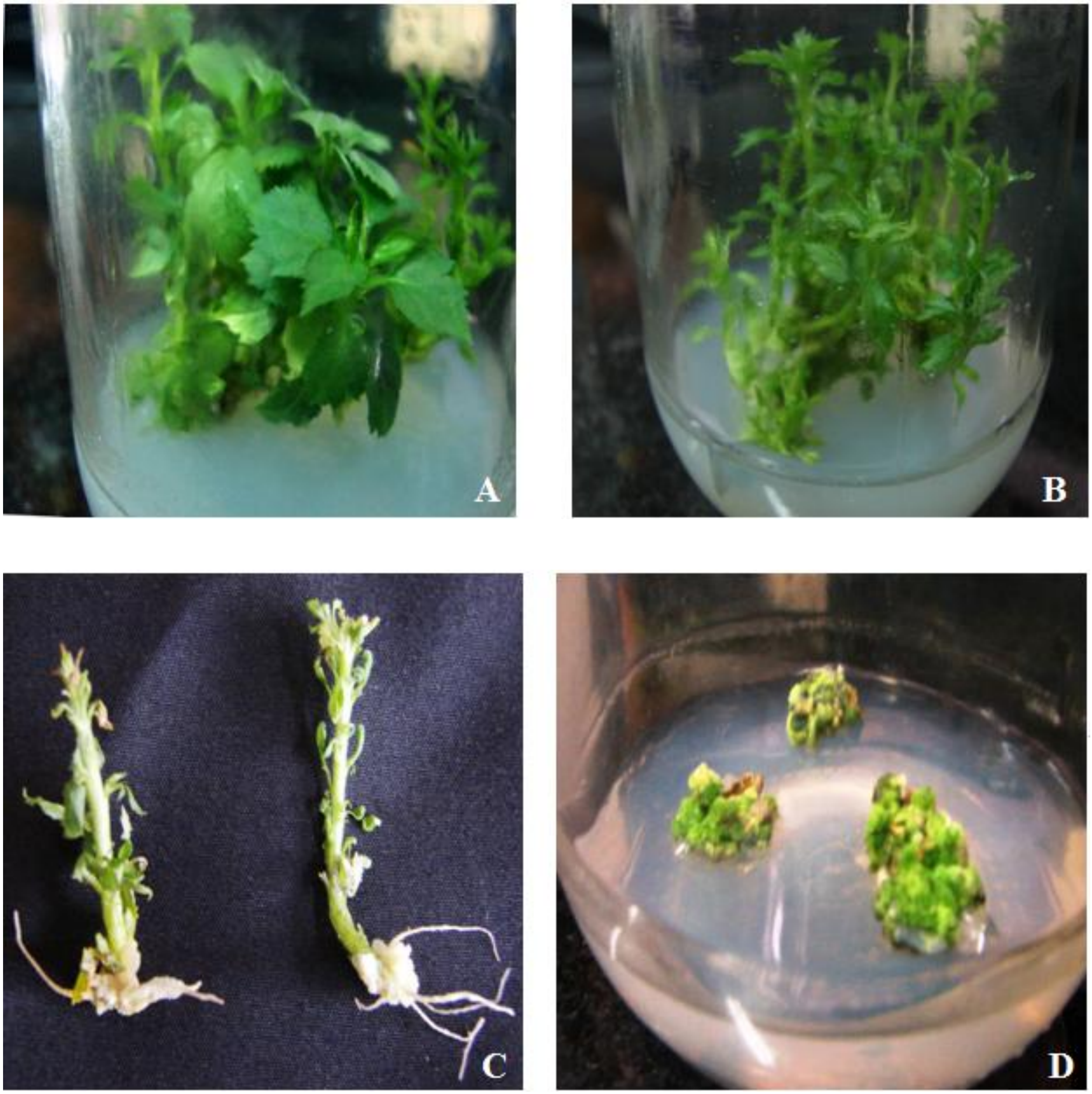


Figure: 2 (A and B) Shoot multiplication of apple rootstock M7 on MS medium supplemented with 2.5 µM BAP and 1.0 µM NAA. (C) Rooting of microshoots of M7 apple rootstock on MS medium supplemented with 5.0 µM IBA. (D) Callus initiation when leaf segments of apple rootstock M7 were cultured on MS medium supplemented with 1.0 µM BAP and 12.5 µM NAA.

4.2 Shoot organogenesis

Leaves were removed from 4-week-old green (non-etiolated) microshoots, wounded (two transverse cuts) and transferred (cut side up) to a regeneration medium. Morphogenesis in the tissue was observed and it was found to vary with the different concentrations of BAP and NAA. During the initial stage (1-2 weeks of incubation), there was some expansion and proliferation of cells at the cut surface but callus growth was limited. But after four weeks of culture visible changes like formation of globular structures and callus started to appear from the explants.

The callus developed from cultured leaf segment on MS medium supplemented with different concentrations of BAP and NAA. The callus developed was subcultured on MS medium supplemented with various concentrations of (0-15.0 μM) BAP and (0-12.5 μM) NAA and shoot regeneration was observed in some of the combinations. MS medium lacking plant growth regulators served as control. Data on average shoot number per explant and average shoot length (cm) was recorded after an interval of 4 weeks. Regeneration from the cut edge of the leaf lamina was usually associated with severed vascular tissue, most commonly the midrib, and was indistinguishable from that occurring at the petiolar stub. In this particular series of experiments, all leaf lamina surface for shoot regeneration occurred on explants that also regenerated at their proximal ends.

Leaf segments of M7 apple rootstock developed into callus when cultured on MS medium supplemented with plant growth regulators (PGRs).

However, the texture and morphology of the callus varied on different media combinations. The composition of growth regulators had great influence on the colour and texture of callus. The colour of the callus varied from white, yellow, pale green, creamish green, green (Table 6).

Plate 2

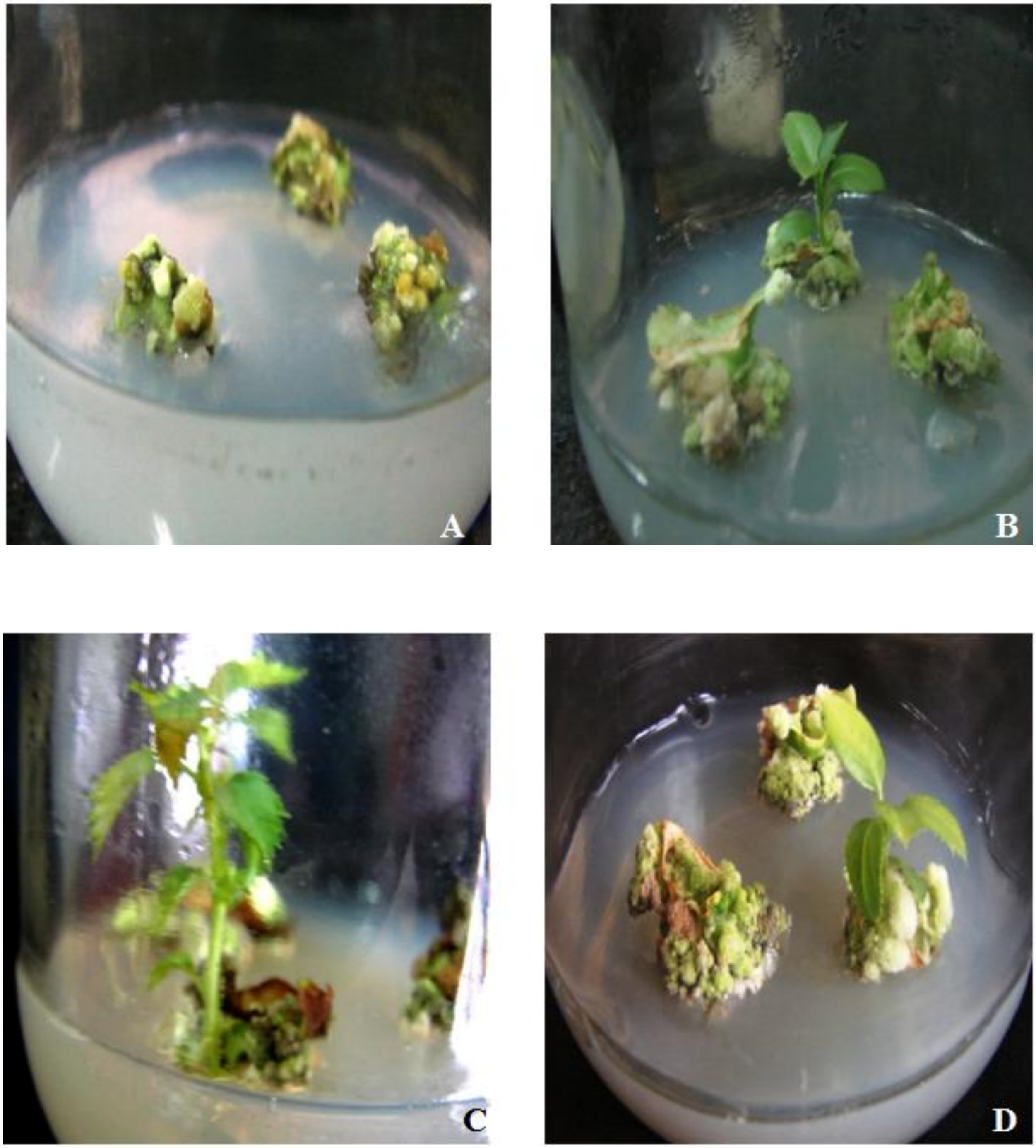


Figure 3: (A) Initiation of Shoot regeneration occurred from the nodular callus in apple rootstock M7. (B, C, D) Shoot regeneration occurred from the nodular callus differentiated from combinations of 1.0 µM BAP and 1.0 µM NAA and 5.0 µM BAP and 1.0 µM NAA in apple rootstock M7.

Table 6: Effect of different concentrations of BAP and NAA on callusing and shoot regeneration of apple rootstock M7:

BAP (µM)	NAA (µM)	% of leaves showing callus	Colour & morphology of callus	% of callus showing shoot regeneration
0	0	0	---	---
1	1	33.3	Green, nodular	---
1	2.5	50	pale green, smooth	25
1	5	40	Creamish green, smooth	---
1	12.5	83.3	whitish and pale green, smooth	---
5	1	42.8	Green, nodular	50
5	2.5	33.3	Whitish green, smooth	---
5	5	66.6	pale green, nodular	---
5	12.5	50	yellow, pale green, nodular	---
15	1	33.3	pale green, smooth	---
15	2.5	50	Green, smooth	---
15	5	66.6	pale green, smooth	---
15	12.5	66.6	Whitish, green, 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 bottle four leaves were inoculated.

The calli obtained on these madia were transferred to MS medium supplemented with 5.0 µM BAP and 5.0 µM NAA. After two weeks of inoculation shoots start to appear from calli of apple rootstock M7 on this medium and complete shoot growth was observed after four weeks.

Shoot regeneration occurred from 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 apple rootstock M7 (Table 6).

The nodular callus differentiated from leaf explants on different media combinations subcultured on MS medium supplemented with different concentrations of BAP and NAA differentiated shoot buds (figure 3). In 30-45 days after subculture, shoot differentiation was observed on MS medium supplemented with 1.0 BAP and 1.0 µM NAA and 5.0 µM BAP and 1.0 µM NAA.

4.3 Rooting of Microshoots

Rooting of microshoots (3-4 cm long) of apple rootstock was achieved on medium supplemented with IBA and NAA. All the IBA concentrations had significant effect on rooting of M7. For root induction, microshoots were inoculated on MS medium supplemented with various concentrations of IBA and NAA. The maximum rooting was observed with 5.0 µM of IBA which differ significantly from others. IBA at 1.0 µM and 2.5 µM also resulted in good rooting (Table 7).

Table 7: Rooting response of microshoots of apple rootstock M7 on MS medium supplemented with different concentrations of IBA or NAA:

PGRs IBA (μM)	% Shoots showing rooting	Average number of roots per shoot	Average root length(cm)
0	--	--	--
1.0	58	3.4	++
2.5	43	2.8	+++
5.0	67	4.5	+
12.5	--	--	--
NAA (μM)			
0.1	--	--	--
0.5	25	1.7	+
1.0	--	--	--

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 bottle four microshoots were inoculated.

Average shoot length: + (0.2-0.4) cm, ++ (0.5-0.7) cm, +++ (0.8-1.0).

4.4 Clonal fidelity

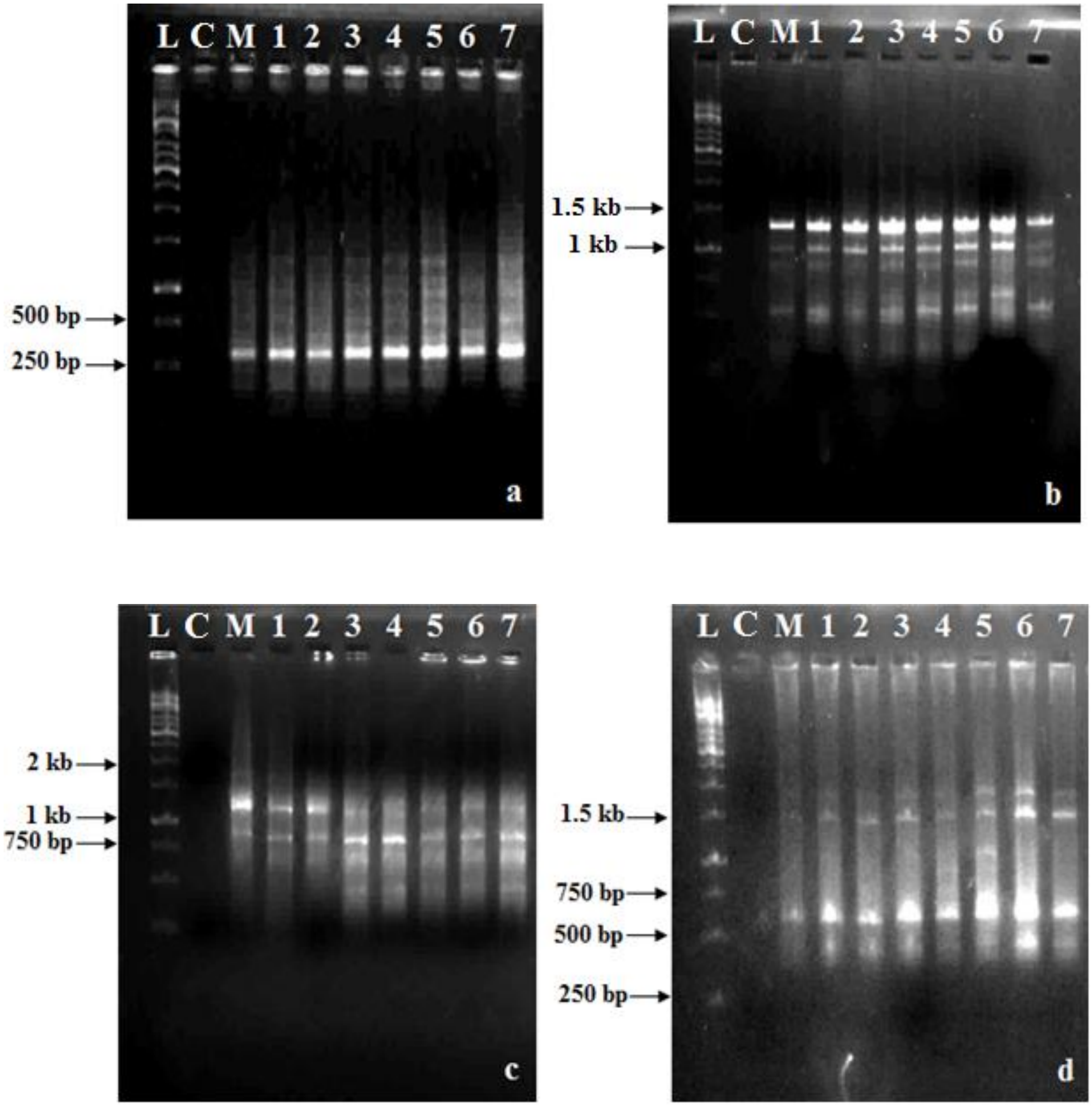
Micropropagated plantlets were subjected to randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) analyses in order to evaluate their genetic stability and/or detect likely existing variations among *in vitro* derived plantlets. Eight randomly selected microshoots of apple rootstocks of M7 were used for assessing the genetic fidelity. In the present study, we adopted the use of two PCR- based techniques, RAPD and ISSR, for the identification of genetic fidelity in apple rootstock M7 plantlets because of their simplicity and cost-effectiveness. Three RAPD (10-mer) and two ISSR (dinucleotide contained repeats) primers were used for PCR and reproducible band profiles were obtained.

The use of two types of markers, which amplify different regions of the genome, allows better chances for identification of genetic variation in the plantlets. All the amplified bands (both with RAPD and ISSR) were monomorphic (figure 9, a-d). Although this study has not detected any genetic variations, it is possible that some changes might have occurred that go undetected as there is a possibility of point mutations occurring outside of the priming sites.

Analysis of individual primers revealed that RAPD patterns were similar for both the *in vitro* raised plantlets and their respective mother plants (control plant), which indicated that there was no genetic variation in the regenerated plantlet population.

Table 8: RAPD and ISSR Analysis

Primers	No. of Bands Amplified
RAPD 1	1
RAPD 2	5
RAPD 3	4
ISSR 1	4
ISSR 2	6



M	Mother plant
L	Ladder (1kb)
C	Negative Control

Figure 4 : (a,b,c) RAPD profile of mother and micropropagated plants of apple rootstock M7 generated by random decamer primers.(d) Inter Small Sequence Repeats (ISSR) amplification pattern obtained for DNA of mother plant (lane M) and regenerated shoot cultures (lanes 2-7) generated by ISSR primer .

4.5 Genetic Transformation

Experiment was conducted to test the sensitivity of leaf explants in various concentrations of antibiotics kanamycin, sporodex, cephotaxime, carbenicilin. Less number of leaf explants survived when kanamycin used in concentration > 50 µg/ml. Therefore, the concentration of kanamycin was kept at 50 µg/ml in all experiments (unless otherwise mentioned).

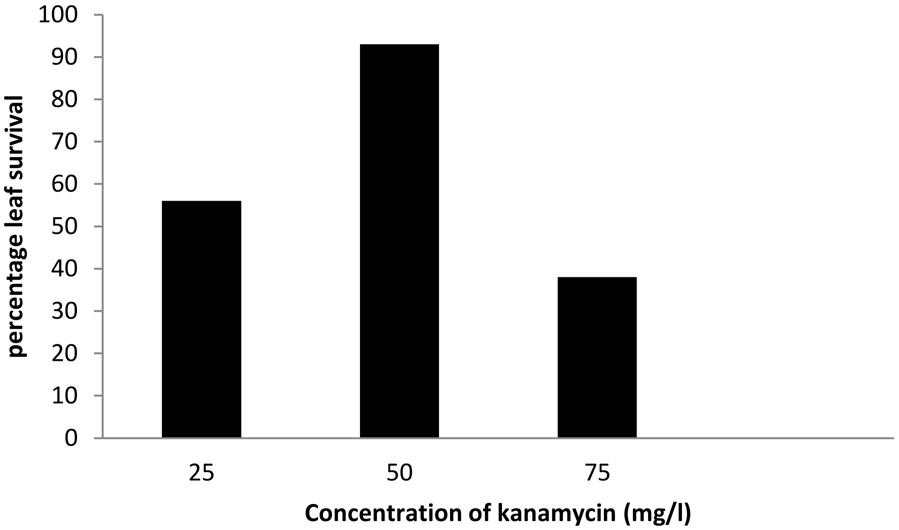


Figure 5: The effect of different concentrations of kanamycin in selection medium on explants survival of apple rootstock M7 after 3-4 weeks of inoculation.

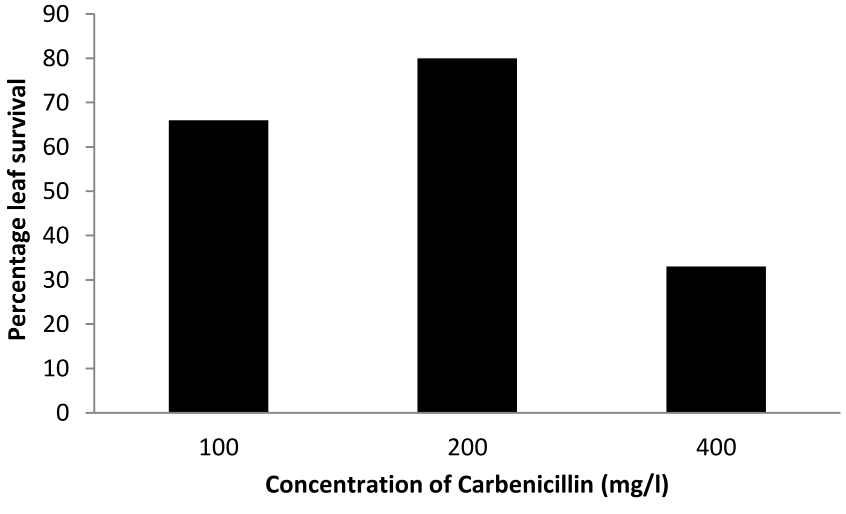


Figure 6: The effect of different concentrations of carbenicilin in selection medium on explants survival of apple rootstock M7 after 3-4 weeks of inoculation.

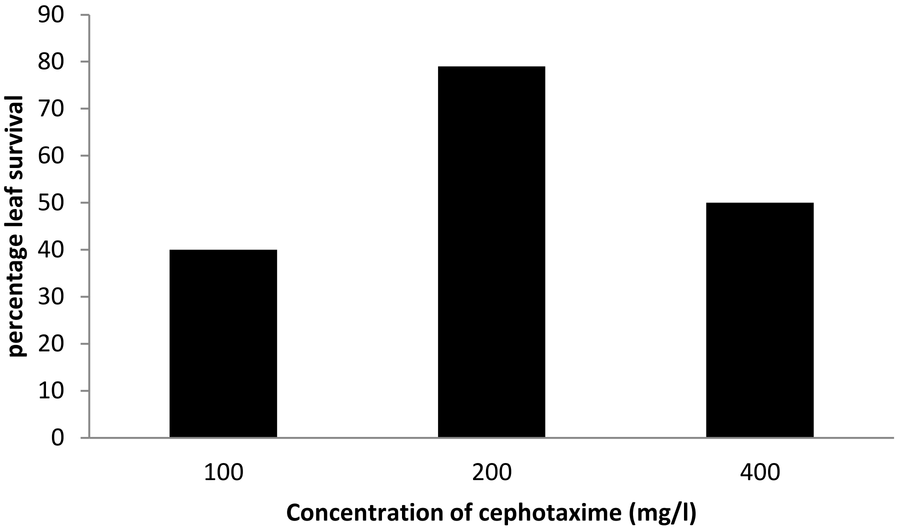


Figure 7: The effect of different concentrations of cephotaxime in selection medium on explants survival of apple rootstock M7 after 3-4 weeks of inoculation.

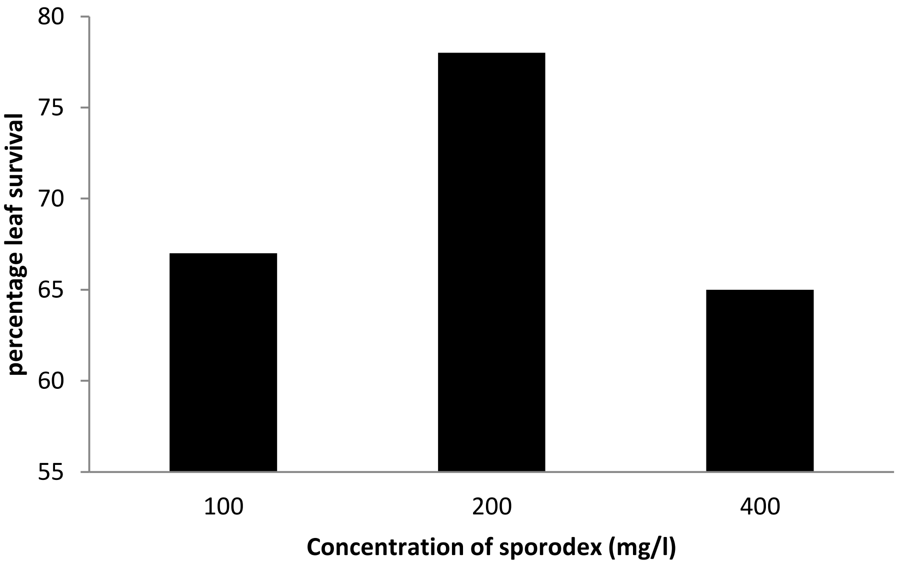


Figure 8: The effect of different concentrations of sporodex in selection medium on explants survival of apple rootstock M7 after 3-4 weeks of inoculation.

All of the pBI121-containing bacterial strains grew on kanamycin-containing luria agar plates, while nontransformed, wild-type strains failed to grow. The pBI121-containing strains of bacteria hydrolyzed X-gluc to form a blue pigment, whereas the wild-type strains did not hydrolyze X-gluc. The time for color formation varied from 2 to 20 hr.

The efficiency of two strains of *A. tumefaciens* namely, EHA105 and LBA4404 were tested for genetic transformation. Strain EHA105 induced significantly higher transient GUS activity as compared to strain LBA4404. Therefore, strain EHA105 was used in all subsequent experiments.

The effect of different parameters on transient GUS expression after two days of co-cultivation with *Agrobacterium tumefaciens* EHA 105 harbouring binary vector pBI121 was studied.

Higher percentage of GUS expression was observed when method of injury was needle, bacterial density at OD_{600} was 0.6, pre-culture period of 3 days. The transient expression was observed when leaf explants were infected with *Agrobacterium strain* EHA 105.

Plate 3

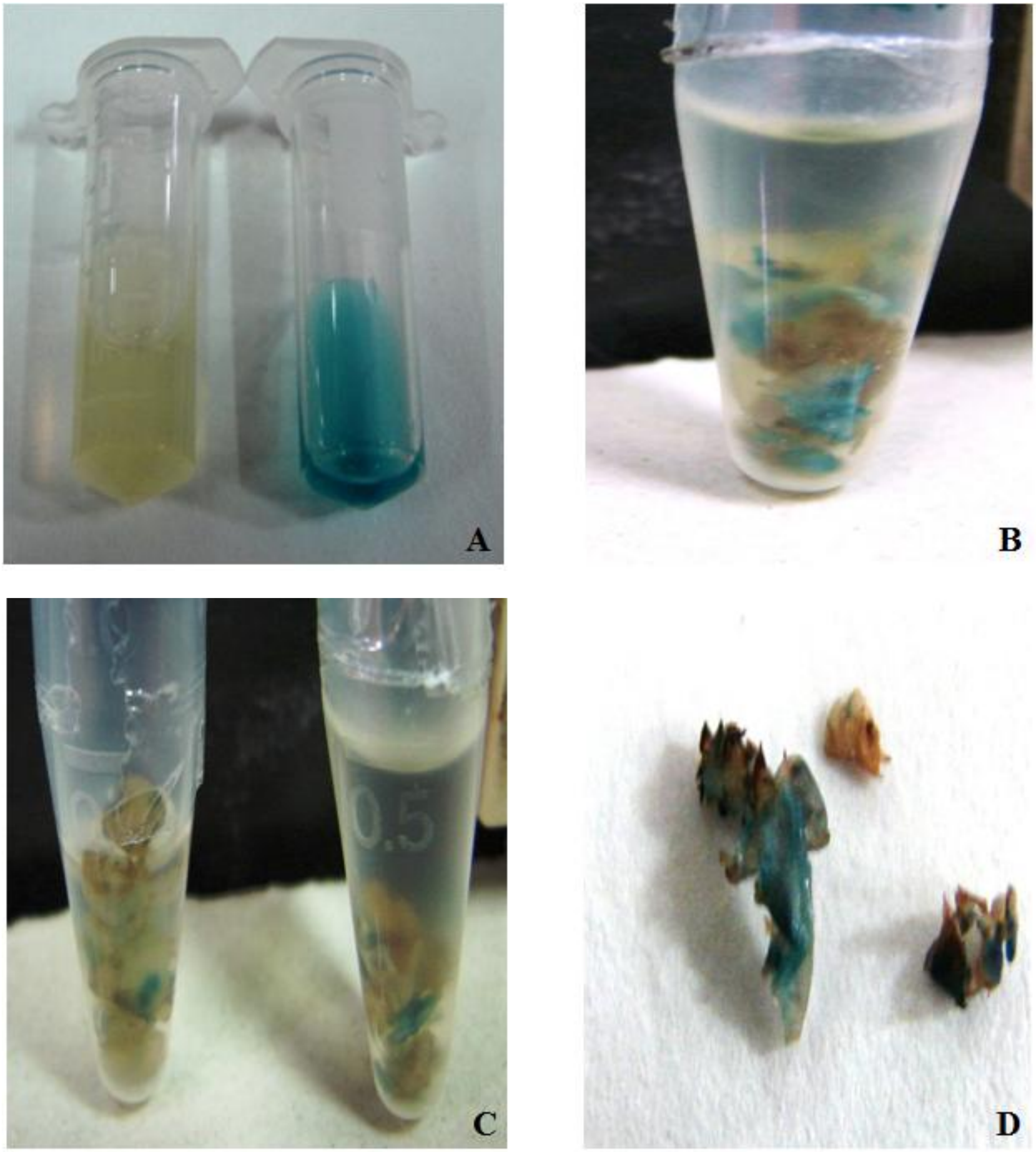


Figure 9: (A) Blue colour appeared in *Agrobacterium* culture showing positive GUS expression while no staining was seen in the control.(B,C,D) Transient GUS expression showing blue coloured patches on transformed leaves of apple rootstock M7.

Table 9: The effect of different parameters on transient GUS expression in leaf explants after two days of co-cultivation with *A. tumefaciens* EHA 105 harbouring binary vector pBI121:

Factors	Variable	% GUS expression
Pre-culture period (in days)	0	49.44
	1	61.22
	2	66.66
	3	65.50
Method of Injury	Scalpel	68.44
	Needle	73.66
	Carborundum	70.50
	Intact	53.33
Bacterial density (OD_{600})	0.2	57.50
	0.4	59.22
	0.5	63.44
	0.6	67.50
Infection time (in mins)	5	48.9
	10	51.50
	15	66.33
	20	53.44
Co-cultivation time (in days)	1	47.77
	2	63.33
	3	59.99
	4	33.33
pH	5.5	56.33
	5.6	68.33
	5.7	60.50
	5.8	59.77
Photoperiod	Continous dark	37.11
	Continuous light	68.22
	16 h photoperiod	72.66

CHAPTER 5

Discussion

Plant Tissue Culture techniques have become important for pursuing a wide range of fundamental and applied problems in research and development. The techniques encompass a variety of procedures used for specific purposes. It is an important component of plant biotechnology and includes micropropagation, somatic embryogenesis, regeneration of plants, etc. Micropropagation has a great commercial potential due to the speed of propagation, decreased production space requirement and the ability to multiply elite clones exhibiting superior growth (Garton and Mosses, 1985; Kane *et al*., 1989). A lot of research work has been done on nodal culture of several plants (Sanjaya *et al.,* 2005; Siddique *et al*., 2006). 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).

The present study was carried out to investigate the various factors involved in *in vitro* propagation of apple rootstocks. The study include micropropagation, regeneration, rooting, clonal fidelity and genetic transformation.

The main aim of the study was to standardize and develop more efficient protocol for the micropropagation and shoot regeneration from the M7 clone of apple rootstock.

Each aspect (shoot multiplication, shoot elongation, leaf production or rooting) of apple rootstocks were significantly influenced by medium composition. This has been described by several authors (Lane and McDougald, 1982; Caboni and Tonelli, 1999; Kovalchuk *et al*., 2009).

The success in the micropropagation of apple lies in the conditions in which parent plant survives, type of explant used, time of collection of explant, accuracy in sterilization procedure, composition of nutrient medium, type of hormones used, control over oxidative browning after inoculation and hardening of plants produced.

Various apple genotypes have been extensively studied with respect to regeneration and transformation.

Apple shoot meristems have been cultured aseptically to promote growth and proliferation of shoots. Regenerated shoots were rooted to produce plants by basal treatment with IBA. Although tissue culture work on apple was started more than half a century ago (Letham, 1958) and nearly 360 research papers have been published, some aspects have not yet been touched and many aspects need to be reinvestigated.

As the work was integrated in a breeding program to increase the agronomic value of these rootstock genotypes (Jarausch *et al*., 2007) it was important to define the best protocols for propagation and *in vitro* rooting of the different genotypes. Rooting ability improvement through tissue culture propagation has been widely used for establishing apple rootstock stool beds for getting high-quality (more roots per shoot) and more productive (more shoots per foot) beds.

In this study, the effect of PGRs on the apple rootstock M7 was observed on shoot multiplication. For this purpose basal medium having 3.0% (w/v) sucrose and 0.7% agar was supplemented with different concentrations of BAP, NAA. Maximum shoot multiplication occurred when shoots were inoculated on MS medium supplemented with 2.5 μM BAP and 1.0 μM NAA. The main objective of *in vitro* multiplication phase is to produce the largest number of plants in a short period of time. Apart from the plant growth regulators and their concentration, to obtain good results in this phase it is necessary that the culture medium supplies the essential substances for the *in vitro* plants growth. Gaspar *et al*. (1996) reported that when the nutritious medium composition was in agreement with the plant requirement, the multiplication key factor was the presence and concentration of the plant growth regulators, particularly the cytokinins, because *in vitro* multiplication rate was largely controlled by the genotype and cytokinin concentration interaction.

The effect of different cytokinins and auxins, and their different concentrations on micropropagation and shoot prolification is well documented (Akbas *et al*., 2010).

Ratio of cytokinin to auxin is an important factor especially in the establishment and prolification of cultures. Cytokinin when added in appropriate concentration regulates shoot prolification, cell division, differentiation (Gross and Partiner, 1994). A range of cytokinins (kinetin, BAP and Zeatin) have been used for the purpose of micropropagation (Bhojwani and Razdan, 1992).

The clonal propagation is the favorite method in many parts of the world, because it allows the production of more uniform offsprings.

Maximum rooting percentage (67%) was observed on medium supplemented with 5.0 µM IBA followed by 58% at 1.0 µM of NAA as shown in Table 7. Maximum rooting percentage is probably due to the reason that optimum concentration of IBA might be responsible to increase the cambial growth at the base of micro cuttings that result in differentiation of root primordial (Haq *et al.,* 2009). Achievement of minimum rooting on medium without IBA rather than complete inhibition of rooting indicated that, endogenous auxin along with some root inducing factors might occur naturally within the micro cuttings that may help for root primordia initiation (Haq *et al.,* 2009). Auxin is clearly involved in morphogenesis since it regulates plant cell division, elongation and differentiation (Chen, 2001). Furthermore, root inducing factors are believed to be essential for rooting, which combine with auxin to form a complex that directs RNA to activate enzymes that cause root initiation (Hartmann *et al.,* 2007). Thus, IBA is by far the most commonly used auxin to obtain root initiation.

An efficient regeneration system is a prerequisite for apple transformation (Skirvin *et al.,* 1986). Efficient regeneration and transformation are major prerequisites for the development of suitable expression system. Therefore, prior to an experiment, it would be appropriate to have a standardized protocol to maximize regeneration (shoot or embryo). The hormonal combination of 5.0 µM BAP and 1.0 µM NAA was found to be best for the shoot regeneration from callus. Important factors that influence regeneration include media composition, age, genotype and orientation of the explant; and incubation conditions (James *et al.,* 1988; Welander 1988; Fasolo *et al.,* 1989). The regeneration medium that has generally produced the greatest shoot regeneration is the MS medium (1962) supplemented with 2-3% sucrose and standard vitamins complements.

Transformation efficiency is affected by various factors. The various factors namely pre-culture, bacterial density, mode of injury, incubation conditions, acetosyringone etc., influenced transformation efficiency of leaf explants. Culture medium and incubation conditions of the explants, prior to *Agrobacterium* infection have been reported to enhance T-DNA delivery in some plant species (Ho *et al.,* 1998; Padmanabhan and Sahi, 2009; Yevtushenko and Misra 2010).

Method of injury to the tissue prior to bacterial infection was also observed to play an important role in T-DNA delivery. Histochemical observation of apple leaf pieces 2-4 days after inoculation with *Agrobacterium* plasmid binary vectors containing the *uidA* gene, encoding β-glucuronidase (GUS), indicated that the majority of GUS expression appeared to be associated with wound sites caused by needle during leaf manipulation. Thus, pricking of tissue using hypodermic needle enhanced transient GUS activity.

Wounding of tissue before infection could allow bacterial penetration deep into the tissue facilitating the accessibility of plant cells to *Agrobacterium* on one hand and may stimulate the induction of *vir* genes as a result of phenolics secretion on other (Stachel *et al*., 1986), which could be the main reasons for enhanced bacterial efficiency for T-DNA delivery (Binns and Thomashow, 1988).

The pH of medium during co-cultivation also influenced the efficiency of T-DNA delivery. Higher frequency of explants showed transient GUS activity when cultured on medium with pH of 5.6.

Low pH during co-cultivation was reported to be beneficial for *Agrobacterium* mediated transformation across the species by Godwin *et al*. (1991).

During the process of transformation, *A.tumefaciens* tends to colonize the explants. To limit the development of this bacterium, explants tissues were cultured on medium containing antibiotic cef (200 mg/l).

The density of bacterial suspension used for infection of the explant also influenced transient GUS activity. Maximum transient GUS activity was obtained in explants that were infected with the bacterial suspension having an OD_{600} of 0.6. At higher bacterial density, the decrease in transient GUS activity was observed. This could be due to increased production of toxic compounds due to bacterial overgrowth (Sonia *et al.,* 2007) resulting in the necrosis of the tissue.

A co-cultivation period following *Agrobacterium* infection influenced the expression of transient GUS activity. A maximum of explants showed transient GUS activity when these were co-cultivated for 2 days.

The rate of GUS activity in cut leaves also was significantly higher than in nonwounded *Agrobacterium*-treated leaves.

Stable plant transformation requires a considerable investment in time before the expressed proteins can be analyzed. In contrast, transient gene expression systems are rapid, flexible and straightforward. The transient expression assays described ensure that most errors and technical problems with gene expression can be identified and resolved before making stable transformants (Kapila *et al.*, 1996). It was found that none of the control plants gave positive result with the X-Gluc indicating the absence of any endogeneous GUS activity, whereas the transformed ones showed the presence of the blue spot indicative of the transient expression.

In commercial micropropagation, it is compulsory to check regularly the clonal fidelity or genetic uniformity of micropropagated plantlets (Khawale *et al*., 2006).

In the present investigation, two PCR-based techniques namely, RAPD and ISSR were used to test clonal fidelity because of their simplicity and cost effectiveness.

Furthermore, they require only a small quantity of DNA sample and do not need any prior sequence information and are simple to perform as well as fast (Lakshmanan *et al*., 2007). All RAPD and ISSR profiles from micropropagated plants were found to be monomorphic and analogous to those of their respective mother plants.

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CHAPTER 6

Conclusion

Tissue culture has played an important role in apple rootstock propagation.

Micropropagation was achieved readily with apple rootstock M7 with good shoot multiplication and rooting *in vitro*. For the apple rootstock M7 the best suited medium for propagation was defined following the criteria of good shoot proliferation combined with good shoot growth.

We have made an attempt to develop a protocol for shoot regeneration and *Agrobacterium*-mediated transformation from the leaf explants for apple rootstock M7.

Besides micropropagation, shoot organogenesis has been achieved from leaf explant of apple rootstock M7. Callus formation was achieved on MS medium supplemented with different combinations of BAP and NAA and shoots were regenerated from callus when these were shifted on MS medium supplemented with 5.0 µM BAP and 1.0 µM NAA.

The following conclusions can be drawn from the present study:

The multiplication of culture was done successfully on MS medium supplemented with 2.5 µM of BAP and 1.0 µM NAA. About 70-80 % of nodal explants showed multiplication with this medium combination. Shoot proliferation was also obtained on MS medium supplemented with cytokinin alone. With 2.5 µM BAP, maximum numbers of healthy and sizeable shoots were proliferated. The callus was obtained on MS medium containing 2% sucrose on different concentration of BAP and NAA. Callogenesis using leaf was initiated on MS medium supplemented with BAP and NAA. The callus thus produced was put in a medium having different concentrations of BAP and NAA for shoot organogenesis.

Well developed shoots were excised from cultures of proliferating shoots and inoculated on MS medium with different concentrations of IBA or NAA. For rooting of shoots 5.0 µM IBA was found better.

Transient GUS expression of explants (leaves and calli) following bacterial infection was determined.

The molecular data of this study clearly revealed that *in vitro* culture initiation from nodal segments may be performed to create true-to-the-type plantlets. There was no polymorphism observed in both RAPD and ISSR markers.Since there were no changes in the banding pattern observed in tissue culture plants as compared with that of mother plant, we conclude clonal uniformity of the propagated plants.

CHAPTER 7

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Annexure I

Media Composition

Murashige and Skoog (1962) Medium

1. **Macronutrients (Hi Media)**	mg/l
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
2. Micronutrients (Hi Media)	
$MnSO_4 \cdot 4H_2O$	16.90
$FeSO_4 \cdot 7H_2O$	27.80
$ZnSO_4 \cdot 7H_2O$	08.60
H_3BO_3	06.20
KI	00.83
$Na_2MoO_4 \cdot 2H_2O$	00.25
$CoCl_2 \cdot 6H_2O$	00.025
$CuSO_4 \cdot 5H_2O$	00.025
$Na_2EDTA \cdot 2H_2O$	30.00
3. Vitamins (Hi Media)	
Myoinositol	100.00
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sugar	3000
4. **Agar**	7000

Annexure II

Yeast Extract Peptone (YEP)

	g/l
Yeast Extract	10.0
Peptone	10.0
Sodium Chloride	5.0
Final pH	7.5

Luria Broth (LB)

	g/l
Casein enzyme hydrolysate	10.0
Yeast Extract	5.0
Sodium Chloride	5.0
Agar	15.0
Final pH	7±0.2