DEVELOPMENT OF COST-EFFECTIVE MICROPROPAGATION TECHNOLOGY FOR APPLE ROOTSTOCKS

By

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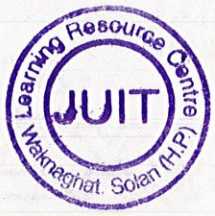

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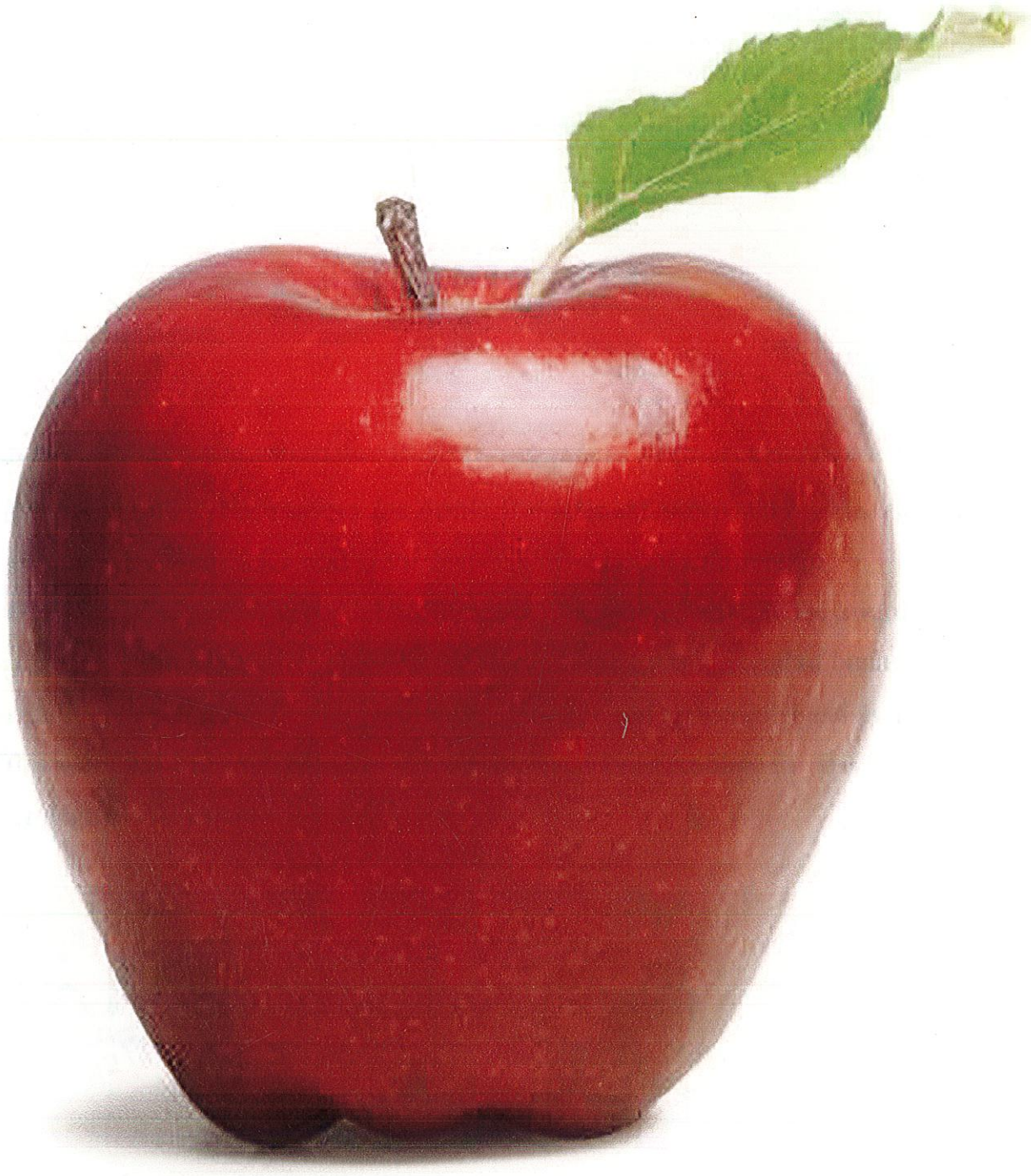

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CERTIFICATE

This is to certify that the thesis entitled **"Development of Cost -Effective Micropropagation Technology for Apple Rootstocks"** submitted by **Ms. Anu Bhatt** and **Ms. Shubhi Kansal** to the Jaypee University of Information Technology, Waknaghat in partial fulfillment of the requirement for the award of the degree of **Bachelor of Technology in Biotechnology** is a record of bona fide research work carried out by him under my guidance and supervision and no part of this work has been submitted to any other university or institute for this or any other degree or diploma.

22/5/10

(Dr. (Mrs.) Hemant Sood)

DECLARATION

We hereby declare that the work presented in this thesis has been carried out by me under the supervision of Dr. (Mrs.) Hemant Sood, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan-173215, Himachal Pradesh, and has not been submitted for this or any degree or diploma to any other university or institute. All assistance and help received during the course of the investigation has been duly acknowledged.

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Needless to say, errors and omissions are ours.

(Anu Bhatt)

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
°C	Degree Celsius
µM	Micro molar
BAP	Benzyl Amino Purine
cv.	Cultivars
gm	Gram
HCl	Hydrochloric Acid
IBA	Indole -3Butyric Acid
Kg	Kilogram
K lux	Kilo lux
KN	Kinetin
l / lt.	Litre
LCM	Low Cost Media
m	Meters
mg	Milligram
MS media	Murashige and Skoog Media
NaOH	Sodium Hydroxide
ppm	Parts per Million
rpm	Rotations per Minute
STM	Standard Media

ABSTRACT

Apple *(Malus spp.)* is an important fruit crop in the North-Western Himalayan Region of India at an altitude of 3000-4300 meters. It is a delicious fruit along with a wide range of health benefits which include psychological, cardiovascular and systemic benefits along with a decreased cancer risk on regular consumption. The low yield of apples can be amounted to the uncertainties of the monsoon and the weather and dependence over the old cultivars. This warrants the development of a cost effective micropropagation technology for its rapid multiplication. Culture of axillary shoot tips on MS media supplemented with different combinations of growth hormones resulted in the identification of MS +BAP (2mg/l) + KN (3mg/l) + IBA (1mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) as the best medium for multiple shoot formation with 59.7% shoot apices forming approximately 22 shoots/explant and MS + IBA (3mg/l) + KN (1mg/l) as that for root induction with 68.3% of explants forming approximately 20 roots/explants. In order to reduce the cost of media components for commercial production of planting material of apples, sucrose was replaced with table sugar and agar-agar was omitted completely. Comparative performance of nutrient media containing sucrose or table sugar as the carbohydrate source showed no considerable change in *in-vitro* multiple shoot formation per ex-plant, relative growth and vigor of shoots and frequency of root formation in shoots. The *in vitro* regenerated plantlets were transferred to pots and the best results were obtained on potting mixture containing vermiculite: perlite: cocopeat: sand: soil (1:1:1:1:1). The substitution of sucrose with table sugar and omission of agar-agar from the medium resulted in reduction of cost of per liter medium to the tune of $1/80^{th}$ of the cost of original nutrient medium. Other cost reducing alternatives used were autoclavable polybags for sub-culturing and use of tap water in media formation.

CHAPTER 1: INTRODUCTION

Apple is an important temperate fruit crop of India with regard to production, economic value, above all popularity. Because of some of its inherent characteristics such as high productivity, good storage life, attractive appearance and excellent flavor, it has become a favorite fruit of the people. It is the most important fruit crop of the temperate regions and ranks fifth among all fruits in India. The annual production of the apples is around 2,76,680 tones and the economy of H.P.(India) largely depends upon apple industry. The area under the cultivation of apples amounts to about 78,292 hectares (in H.P.). India being the seventh largest producer of apples in the world makes them a very important part of our economy.

Apart from being a delicious juicy fruit, apples also have a multitude of health benefits which led to people believing that "An apple a day, keeps the doctor away". Apples are known to provide psychological, cardiovascular and systemic benefits along with a decreased cancer risk on regular consumption. They help in prevention of dementia, in maintaining low cholesterol and in the prevention of overweightness along with decreasing the risks of lung cancer, colon cancer and prostate cancer.

The low yield of apples in India is a reflection of apple crop being cultivated on mountainous terrain, exposed to vagaries of monsoon, and dependent on almost century-old cultivars of the delicious variety. This warrants that it be multiplied rapidly on a large scale, and planted in its natural habitat. Micropropagation is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

Apple trees in an orchard are not grown from the seeds. Although they can be used, the fruit of the trees grown from the seeds will not be the same as that of the parent tree. In fact, it is unlikely that an apple tree grown from seed will bear good quality fruit. That's why to multiply apple trees we use vegetative propagation. The trees in this method are made up of two parts: the rootstock which controls the size of the tree and the scion or cultivar which determines the variety or kind of fruit that grows on the tree. Conventionally these two parts (scion or cultivar and rootstock) are joined together by grafting or budding. A rootstock is a part of the plant (at

times just the stump), which has already an established, healthy root system, used for grafting a cutting or budding from another plant. Scion is the tree part being grafted onto this rootstock. It has the properties desired by the propagator, and the rootstock is the working part which interacts with the soil to nourish the new plant. After a few years, the tissues of the two parts will have grown together, producing a single tree. However, genetically it always remains two different plants. The use of rootstocks (most commonly associated with fruiting plants and trees) is the only way to mass propagate many types of plants that do not breed true from seed or are particularly disease susceptible when grown on their own roots. Rootstocks are selected for traits such as vigor, tree size, precocity, resistance to drought, root pests, and diseases. The rootstock can be a different species from the scion, but must be closely related.

The three main categories of rootstocks are standard, semi-dwarf and dwarf; they decide only the size of the plant not the fruit. Apple trees planted on standard rootstocks will produce large, full-sized trees that may grow more than 25 feet tall. They are very hardy and can be planted in a wide range of soils and climates. They are sturdy, long-lived and very productive, but they take longer to bear fruit. Trees planted using semi-dwarf rootstocks will reach a height of 15 to 20 feet. They may not be as hardy as full-sized trees and are productive, however, they are not as long-lived as full-sized trees. Apple trees planted on dwarf rootstocks will grow 10 or 12 feet tall. They will be less hardy than full-sized or semi-dwarf trees. Because their roots tend to be rather shallow and not very strong, they need to be tied to a sturdy stake. They do not have a very long life but begin bearing fruit in two or three years and are not very productive as compared to the other two types.

Micropropagated rootstock are ideal for establishing mother stocks, especially when *in vitro* propagation has been coupled with virus and bacteria certification programs. It has been proved that rootstock plants which originated from tissue culture are more vigorous and produce more layers and cuttings which, due to juvenility effect, root better even in the case of difficult-to-root species such as *Pyrus communis*. The possibility of rapid propagation of rootstock released from breeding programs and plants which have been imported and passed quarantine procedures, is an additional advantage of micropropagation.

There are reports on tissue culture of other plant species, wherein the components of tissue culture media have been modified or replaced with low cost substitutes such as sucrose with table sugar (Kaur *et al.* 2005), omission of agar–agar (Mehrotra *et al.* 2007) and use of sunlight and tubular skylight (Kodym *et al.* 2007). The use of shake cultures utilizing liquid culture medium alone (Weathers and Giles, 1988) or in combination with solid culture medium (Debergh and Maene 1981; Aitken-Christie and Jones 1987) have also been developed and used by various workers (Earle and Langhans 1975; Takayama and Misawa 1981; Takayama 1991; Paque *et al.* 1992; Chu *et al.* 1993). This practice has been quite encouraging in reducing the cost of tissue culture media by 50 times or more.

The current study explored the feasibility of reducing the cost of *in vitro* multiplication of apple rootstocks and their hardening under glass house conditions, while in such a way that the quality of planting material does not compromise and also the technology can be up-scaled with ease. The current study has following objectives:

1) To optimize a cost-effective nutrient medium and cost effective components for large-scale multiplication of apple rootstocks.

2) Hardening of *in vitro* grown plantlets under glasshouse conditions.

CHAPTER 2: REVIEW OF LITERATURE

Review is discussed in the light of available literature relevant to the research problem both on apple as well as on other plants.

General micropropagation

Large scale commercial propagation by tissue culture has been used widely in the apple industry. In addition to private laboratories, government laboratories are also playing an important role e.g. The TERI Institute, Department Of Biotechnology etc

Tissue culture is the most widely and successfully used technology by the commercial industry for the mass production of horticultural crops. Tissue culture has been applied effectively in the clonal propagation of many plant species including ornamental and other crop plants. The use of meristem and shoot tip cultures for the recovery and establishment of pathogen free plants has also become common practice in the production of virus free stocks of vegetatively propagated plants in many commercial nurseries in the developed world.

Culture conditions

Medium

Murashige and Skoog (MS) medium (1962) supplemented with agar-agar, sucrose and hormones at desired levels are most commonly used for micropropagation of apple. Neculae *et al.* (1991) reported that the type and concentration of auxin present in the culture medium have a strong influence on the ability to form roots, and also on their growing in length. According to Najim *et al.* (1983)the highest micropropagation rate in MM106 apple rootstock can be obtained on the media lacking auxin, provided that BA at a concentration of 8.8uM is present. Damiano (1978) reported that addition of 1-2 g of activated charcoal per litre of medium promotes elongation of both shoots and roots.

Modified media

In vitro propagation of Malling Merton apple rootstock has been carried out in liquid medium by Snir and Erez (1980). They obtained intense proliferation when small shoots were cultured in liquid medium in orbital shaker. Bhagyalakshmi and Singh (1995) used liquid medium, static liquid medium and agar-gelled medium for *in vitro* shoot cultures of banana on MS medium

supplemented with BA (2.2uM) + IBA (0.98 µM). They obtained much better shoot multiplication on liquid medium, whereas agar-gelled medium supported maximum *ex vitro* survival of *in vitro* raised shoots. Alvard *et al.* (1993) reported that performance of banana *cv. Cavendish* in liquid medium was quiet significant.

Ganapathi *et al.* (1995) made an attempt to reduce the cost of culture media by using cheaper components like tap water, commercial grade sugar and minimizing salt components for *in vitro* production of banana plants. They obtained 4-6 shoots per explants on modified media consisting of simple sugar and tap water as cost effective components of the culture medium while on standard medium, the number of shoots was 2-3. Balachandran *et al.* (1990) reported significant increase in number of shoots and roots of turmeric and ginger on MS media containing tap water and table sugar in place of distilled water and sucrose, respectively.

Sharma *et al.* (1992) developed a cost effective protocol for micropropagation of 'colt', a rootstock of cherry by replacing sucrose with table sugar. Guleria(1993) used tap water and table sugar for successful micropropagation of MM111, Red Spur and Tydeman's Early Worcestor. Sharma and Singh (1995) developed a procedure for cost effective micropropagation of ginger on media containing tap water and simple sugar as cost effective components. They also reported good rooting percentage on media containing potato extract and simple sugar. Chandra *et al.* (1990) reported significant effect of sugar on micropropagation and microtuberization in potato.

Peterson *et al.* (1999) reported the significance of different carbon sources like glucose, fructose, maltose or mixture of glucose and fructose in medium for callus induction and plant regeneration of *Miscanthus x orgiformis*. Glucose, fructose, a mixture of monosaccharide was found to be better than sucrose in stimulating the formation of embryos in anther culture of many genotypes of *Triticum aestivum* (Chu *et al.* 1990); Last and Brettle, 1990: Orshinky *et al.*,1990). Kaur et al (1999) studied the possibility of eliminating some of the expensive organic supplements like NaH_2PO_4 nicotinic acid, Pyroxidine HCl so that Gerbera micropropagation becomes cheaper without sacrificing multiplication rate. Kaul (1998) investigated that expensive media components like sucrose and distilled water could be replaced with table sugar and tap water to make micropropagation of kiwi fruit a cost effective venture.

Nene *et al.* (1996) suggested that cost of micropropagation of chick pea could be reduced by replacing purified agar with tapioca. Flick and Evans (1984) reported the regeneration of tobacco by replacing agar-agar with tapioca (100 g/l) from MS medium and they obtained extensive shoot proliferation. Tiwari and Rahimbaev (1992) reported barley starch to be a better gelling agent than agar for barley anther culture.

Light

Duration, intensity and quality of light also influence the growth of cultures. Sobczykiewicz (1979) and Van Hoof (1974) used 3000 lux, Mullin *et al.* (1974) reported that 1000-1500 lux sufficient for the growth of *in vitro* cultures. The studies aiming at establishing the influence of light during the stage of *in vitro* micropropagation of the MM106 apple rootstock have shown that the light period can be reduced from 16 hours to 10-12 hours without any negative effect on the multiplication rate and quality of obtained plants, provided that the light intensity is maintained at 3000-4000 lux (Neculae *et al.*). By reducing the day light period to 10-12 hours during the stage of micropropagation, an up to 20% saving to the cost of energy became possible.

Sterilization of explants

Seemuller and Merkle (1984) used a complex bean flour medium for detection of *Phytophthora fragariae* in tissue cultures and regenerated plants were tested before planting in the field according to techniques of Duncan (1980).

Sangwan *et al.*(1987) sterilized shoot tips of some plants like chrysanthemum, solanum and Antirrhinium for 10 minutes in 5 per cent (w/v) calcium hypochlorite and were rinsed thrice in sterilized water. Damiano (1980) reported the use of 0.5 per cent merthiolate for sterilization. The use of Difco antibiotic disks have also been reported to result in control of bacterial contamination (Shirvin, 1981).

***In vitro* root induction**

Damiano (1978) reported addition of 1-2 g of activated charcoal per litre of medium promotes elongation of both shoots and roots. Comparing with IBA, IAA, NAA. Hunter *et al.* (1984a) found that IBA was the most desirable additive for root formation, the optimum concentration

being in the range of 0.75 mg/l to 1.0 mg/l. James (1979) studied that *in vitro* rooting was reduced on a culture medium supplemented with phloroglucinol.

According to Moncousin (1991), during rhizogenesis high concentration of sucrose had negative effects on rooting potential. Also the ratios between nitrogen and sucrose (Hyndman *et al.* 1962: Driver and Suttle, 1987), auxins and sucrose (Welander, 1976) highly influenced the rooting process. Jeong *et al.* (1996) reported 63 to 89 per cent of root formation frequencies in the shoots of five different cultivars of strawberry grown in liquid medium.

Kaul (1998) obtained rooting percentage (40.19) on rooting medium containing tap water and table sugar and did not differ significantly from the rooting percentage (40.74) obtained on standard rooting medium supplemented with distilled water and sucrose.

***Ex vitro* rooting**

Robert and smith (1990) reported direct rooting of micro propagated shoots under mist or in high humidity was usually more economical than rooting *in vitro*. Direct rooting of micropropagated shoots were more commercial and quicker as compared to rooting *in vitro* reported by Deberg and Maene (1981). Simmonds (1983) directly transplanted micropropagated shoots of M26 apple rootstock into the rooting mixtures as miniature cuttings where root regeneration and hardening of plants were carried out simultaneously.

Borkowska *et al.* (1999) reported direct *ex vitro* rooting of micropropagated strawberry shoots. They took micropropagated shoots from the multiplication phase and rooted in rockwool substrate and upto 96 per cent of plantlets moved into other field directly from multiplicates survived. Barbieri and Morini (1987) used one month old callus derived shoots and dipped in concentrated solution of IBA (750 ppm), transferred to pest-perlite mixture in glass jars for *ex vitro* rooting phase and acclimatization was achieved gradually by opening the jar lid everyday. Fasola and Predieri (1988) and Pedroso *et al.* (1992) reported successful in vivo rooting of 'Hayward', the most important kiwifruit (*Actinidia chinensis*) cultivar.

Acclimatization and Hardening

Zimmerman (1988) reported a variety of soilless media like sphagnum peat, perlite, vermiculite or mixture of two components for hardening of woody species. Shen *et al.* (1990) suggested

that rooted microcuttings of kiwifruit were transferred to plastic containers containing mixture of sterilized perlite and vermiculite for hardening. The plantlets were grown initially under glasshouse conditions with satisfactory survival percentage of kiwifruit. Sharma and Singh (1995) successfully hardened the micropropagated plants of ginger on unsterilized potting mixtures. Sharma *et al.* (1992), Mahajan (1997) and Kaur *et al.* (1999) used cheaper mixture of FYM: sand: soil in place of expensive perlite, vermiculite and cocopeat for hardening of 'Colt' rootstock cherry, strawberry and Gerbera, respectively. Kaul (1998) obtained 85.70 per cent survival rate of micropropagated plants of kiwifruit after hardening in soilrite and vermiculite potting mixtures.

CHAPTER 3: MATERIALS AND METHODS

Selection of plant material and establishment of axenic cultures

The apple plantlets were procured from the Fruit Breeding Department of University of Horticulture and Forestry, Nauni, H.P., India. The rootstocks obtained were M9 and M7. M9 is a dwarf rootstock which produces trees up to 2 m in height. They are very precocious and have a high productivity under correct conditions. The anchorage however is poor and they require support due to the brittle roots and high fruit: wood ratio. Good soil drainage is required; absence of it may adversely affect the hardiness of the plants. M7 on the other hand is a semi dwarf rootstock about twice the size of M9. They are somewhat precocious and have a moderate to low productivity in most conditions. They have a good anchorage and are free-standing when matured; hardiness is highly questionable and is probably less than M9. Shoot tips of pot grown plants were washed with sterile water to remove dirt and debris and surface sterilized in 0.5% Bavistin and 0.1% Mercuric Chloride followed by 4-5 washings in sterile water. The sterile shoot apices were cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinins.

Cleaning of Glassware

All the glassware to be used was washed with a dilute solution of labolin with the test tube brush and then rinsed thoroughly with tap water. Next it was wiped with 70% ethanol.

The glassware with contaminated cultures was first autoclaved for 15 mins at 121 lbs. and the molten media was disposed off. The glassware was then cleaned as mentioned above before reusage.

Preparation of media

Modifications of MS media supplemented with different concentrations and combinations of BAP, IBA and KN were prepared (Murashige and Skoog, 1962). The pH of the media was adjusted to be between 5.5 - 5.7 using 0.1N HCl and 0.1N NaOH, sucrose 3% (w/v) was added and finally agar-agar 0.8% (w/v) was added as a gelling agent. The media were autoclaved at 121°C and 15 lb/in^2 pressure for 15-20 minutes. The autoclaved media were kept in the Laminar Air Flow hood for 1-2 days before inoculations to screen for inherent contamination.

Multiplication of shoot cultures

For the purpose of shoot multiplication, we tried 10 different combinations and concentrations of KN, IBA and BAP. One set of cultures was incubated at 16/8hr light/dark cycle at 25 ± 2^0 C in plant tissue culture chamber. The other set of cultures was incubated at low temperature 15 ± 2^0 C with the same light and dark cycle. Data were collected on days to multiple shoot formation, number of shoots per explants and per cent shoot apices with multiple shoots. The data were analyzed for test of significance. The cultures were subcultured after every 4 weeks on shoot proliferation media for approximately 2 months ,so as to obtain good growth, and highly proliferated shoots.

Induction of roots in shoots

Individual shoots were excised from the parent cultures and transferred onto MS media supplemented with different concentrations and combinations of IBA and KN for root induction. The cultures were incubated under the same culture conditions as mentioned above. Data for days to root initiation, number of roots/shoot and per cent rooting were recorded and analyzed for test of significance.

Hardening and acclimatization of plantlets

Once the initial formation of roots could be seen, the rooted shoots were gently removed from the culture vessels, washed under running tap water, treated with 0.5% Bavistin and transferred to pots containing different combination and concentrations of sand, soil, vermiculite, perlite and cocopeat in the glasshouse conditions for acclimatization and hardening.

Vermiculite

Vermiculite is a micaceous mineral that is expanded in a furnace, forming a lightweight aggregate. Handled gently, vermiculite provides plenty of air space in a mix. Handled roughly, vermiculite compacts and loses its ability to hold air. Vermiculite holds water and fertilizer in the potting mix. It also contains calcium and magnesium and has a near-neutral pH.

Perlite

Perlite is an amorphous volcanic glass that has relatively high water content, typically formed by the hydration of obsidian. In horticulture perlite can be used as a soil amendment or alone as

a medium for hydroponics or for starting cuttings. It is sterile and pH-neutral. When added to a soil mix, perlite can increase air space and improve water drainage. Its pieces create tiny air tunnels that allow water and air to flow freely to the roots. Perlite will hold from three to four times its weight in water, yet will not become soggy. It helps prevent water loss and soil compaction.

Cocopeat

Coco peat, also known as coir pith, coir fiber pith, coir dust, or simply coir, is made from coconut husks, which are byproducts of other industries that use coconuts. Raw coconuts are washed, heat-treated, screened and graded before being processed into coco peat products of various granularity and denseness, which are then used for horticultural and agricultural applications and as industrial absorbent. It is used as a soil additive. It holds water well, re-wets well from dry, holds around 1000 times more air than soil and is mixed with sand, compost and fertilizer to make good quality potting soil.

Once the plants are transferred, they are kept covered with jars and poly bags for 10 – 15 days for preservation purposes and after that are exposed by uncovering for a period of 10 – 15 mins every day for the first few days during early mornings or late evenings. The exposure time is gradually increased over time.

Cost-effective micropropagation

Once a MS medium supplemented with IBA (1mg/l) + BAP (2mg/l) + KN (3mg/l) and IBA (3mg/l) + KN (1mg/l) was found suitable for *in vitro* shoot multiplication and root induction of apple cultures, respectively, modifications were made so as to cut down on the cost factor of the media. The alternatives adopted were replacing the sucrose with table sugar, distilled water with the normal tap water, use of autoclavable poly-bags and omission of agar-agar, to see the effect on shoot proliferation and growth. Data were recorded for all parameters as given above and statistically analyzed.

Media with Table Sugar

Sucrose acts as a carbon source in the media and provides plants with an energy source. The easy availability of the source and the pathway used for the utilization of this source in the plant by its cellular machinery determines the growth rate of the plant and its survival. If

sucrose is to be replaced with an alternate source of carbon for the plants, the new source needs to be functionally, structurally and chemically similar to it. Purified sucrose comes at a cost as high as Rs. 386/kg. Table sugar however, costs at just about Rs. 40/kg. Sucrose being a highly purified form of table sugar, there is no considerable change in the growth and survival rate of the plantlets.

Media with Tap Water

Tap water can be used instead of distilled water for the media formulation. This reduces the cost of the media by omitting the cost incurred during the distillation process which turns out to be about Rs.1.3/l of distilled water. The possible contamination problems are omitted during the autoclaving of the media which sterilizes the tap water. Since while autoclaving the water gets decontaminated, and does not affect the cultures, the growth obtained can be comparable to the ones in the media containing distilled water.

Liquid Cultures

The gelling agent or the agar used to solidifying the culture media comprises of about 70% of its total cost and hence by using liquid media we cut down on a major part of the incurred costs by omitting the agar component of the media. The agar costs around Rs. 3432/Kg and we use 8gms of agar per liter of media; the cost of the media is decreased by this factor. Also in liquid cultures the nutrients are more readily available for the plantlets as they are in the ionized form and can be more easily absorbed by the plantlets. As a result the growth rate on liquid media is higher than that in the solid media. Aeration was provided by placing the culture vessels on the shaker set at the rate of 120rpm, this prevented the plantlets from submerging in the liquid media and made fresh air available to them.

Polybags

Autoclavable poly bags were used to subculture the plantlets instead of the jam bottles being used before. Though the jam bottles were in themselves a cost cutting alternative, the poly-bags @ 95 bags / Rs. 100 proves to be even more efficient than the jars which come @ Rs. 12/jar. Along with this one poly-bag could accommodate 3-4 plantlets and provided optimum use of the incubation-shelf space available. The amount of media needed per plantlet was also reduced because of the greater holding capacity.

Ex-Vitro Rooting

Plantlets growing on shooting media were transferred directly to the potting mixtures and given hormone treatment to induce rooting in them. The shoots were potted on a mixture of vermiculite: perlite: coco peat: sand: soil (1:1:1:1:1) and given IBA dip to induce rooting in them. This step is highly cost efficient as it combines the root induction and hardening steps and thus saves the cost of preparation of rooting media as well as the time and labor spent on sub-culturing and growth on rooting media.

CHAPTER 4: RESULTS AND DISCUSSIONS

Establishment of cultures for multiple shoot formation

The surface sterilized shoot apices were cultured on MS media containing IBA (0 - 2 mg/l), BAP (0 – 2 mg/l) and KN (0 - 4 mg/l) in different combinations. Through these procedures we could obtain a response only from the M9 rootstocks and maintain their survival; the M7 rootstocks could not survive as well as the M9 ones. So to continue the experiments we used only the M9 rootstocks. Out of the different media combinations tested for multiple shoot formation from shoot apices, MS medium containing KN (3mg/l) + BAP (2mg/l) + IBA (2mg/l) + sucrose 3% (w/v) was found to be the best with 59.7 % of shoot apices proliferating into multiple shoots within 6-7 days of inoculation (Table 1). The same medium was found to be the best for obtaining maximum shoots (25) per explant. As concentration of IBA was further increased there was increase in root formation in shoots which were very slender in nature. With the increase in concentration of BAP in the medium, the shoot proliferation increased but the shoots were slanderous in nature. The cultures were incubated at 25±2 ^{0}C with a 16/8hr light/dark cycle.

Since apple grows at high altitudes where temperatures are low, we thought that incubating *in vitro* cultures at low temperature might help to provide better growth .The shoot apices were also cultured and incubated at low temperature (15±2°C) with the same light and light/dark cycle in order to see whether better shoot growth can be obtained. The shoot apices proliferated into multiple shoots within 5-6 days of culture with no significant differences with respect to number of shoots per explant as observed at 25±2° C (Table 3).

Induction of roots *in vitro* grown shoots

The shoots formed from *in vitro* grown cultures of axillary shoot tips were transferred MS media supplemented with different concentrations and combinations of IBA (0 – 3 mg/l) and KN (0 – 2 mg/l) for root induction. Data were recorded for days to root initiation, number of roots/ shoot and per cent shoots forming roots (Table 2). Root induction was best observed in MS + IBA (3mg/l) + KN (1mg/l) and occurred in 9-10 days of culturing with 68.3% of shoots forming roots. The same medium was found suitable for more number of roots (19.6) per shoot. The cultures were incubated again at both 25±2° C and 15±2° C with the same 16/8 hr light/dark

Table 1: Effect of different MS media compositions on *in vitro* shoot multiplication in Apple

MS + Growth Hormones			Days to multiple		Percent Shoots forming
KN	BAP	IBA	Shoot formation	Shoot/Explant	multiple Shoots (%)
0	0	0	21 – 23	5.4	19.7
0.5	1	0	18 – 19	12	37.2
1	1.5	0	15 – 16	15.3	41.8
1	2	1	12 – 13	17.9	53.3
2	1	0.5	12 – 13	17.2	52.1
2	1.5	1	9 – 10	19.8	55.4
2	2	2	9 – 10	21.7	57.3
3	1	0.5	8 – 10	22.1	55.6
3	2	1	6 – 7	25	59.7
4	0	2	6 – 7	23.8	45.8

Table 2: Effect of different MS media compositions on *in vitro* rooting in Apple shoots

MS + Growth Hormones		Days to multiple		Percent Shoots forming
IBA	KN	Root formation	Root/Explant	Roots (%)
0	0	NA	NA	NA
1	0	15 – 16	8.9	42.6
1	0.5	13 – 15	8.5	42.8
1	1	13 – 15	5.7	40.3
2	0	12 – 13	13.3	49.6
2	1	10 – 12	14.1	50.1
2	1.5	10 – 11	12.8	47.6
3	0	10 – 11	16.5	65.2
3	1	9 – 11	19.6	68.3
3	2	9 – 11	19.2	62.5

Table 3: Effect of different temperatures on growth and development of Apple plantlets

Temperature		Shoot Length		Root Length
(°C)	Shoots/Explant	(cm)	Roots/Explant	(cm)
25±1	18.6	9.5	20.7	8.2
15±1	19.1	9.3	21.1	8.9

Fig 1: Establishment of apple shoots apices in different culture media.

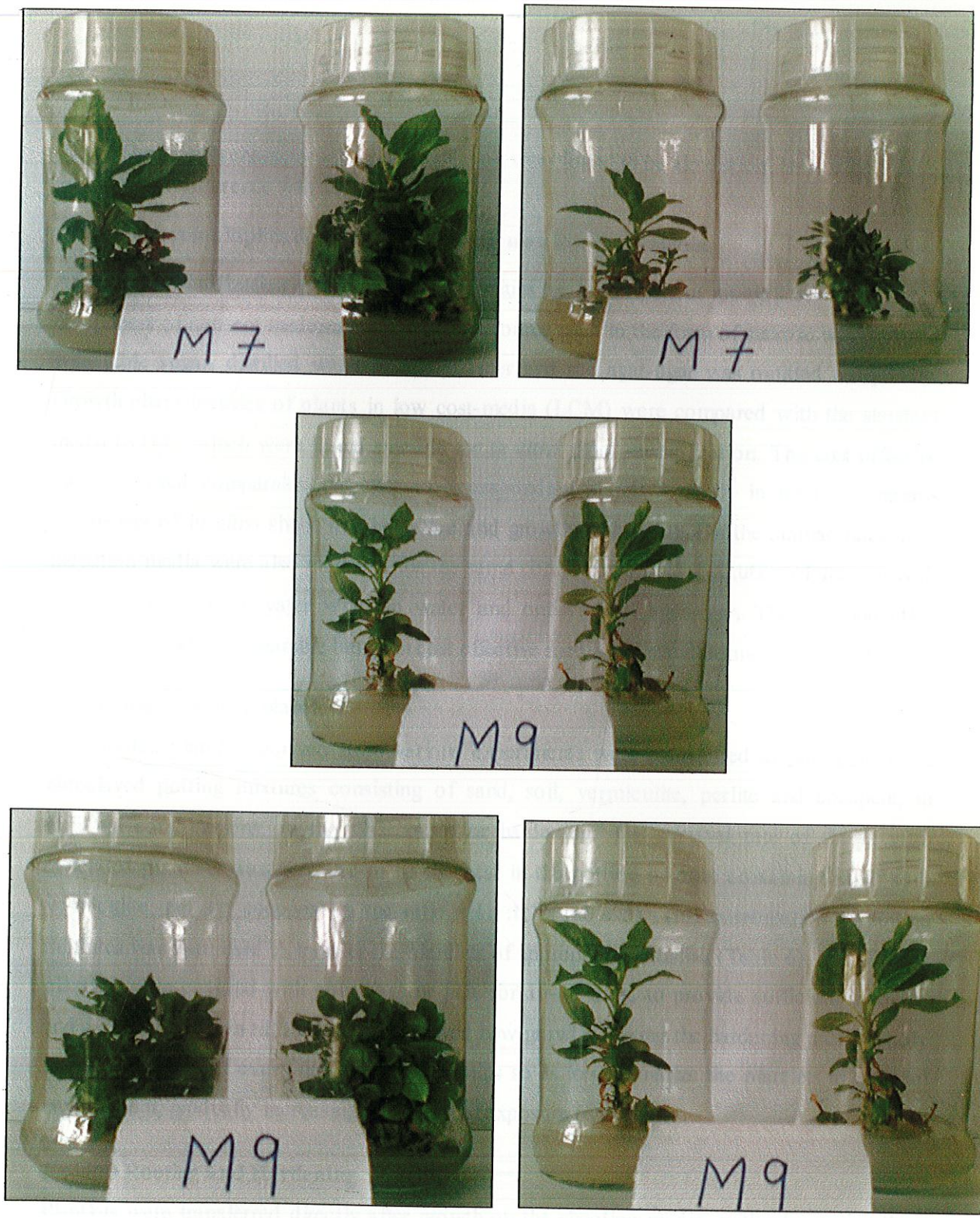


Fig 2: *In vitro* shoot multiplication after 3 weeks of sub-culturing

cycle. The growth characteristics on comparison were found to be almost similar and no considerable difference was detected (Table 3).

In vitro shoot multiplication and root induction on low- cost media

After the standardization of a suitable MS medium for *in vitro* shoot multiplication, the major component of nutrient medium such as carbon source used in the form of sucrose was replaced with table sugar, distilled water with tap water and the agar-agar was omitted completely. Growth characteristics of plants in low cost-media (LCM) were compared with the standard media (STM), which were found suitable for *in vitro* shoot multiplication. The cost effective media showed comparable response to corresponding standard media in terms of various parameters of *in vitro* shoot multiplication and growth (Table 4a). On the similar lines, root induction media were also modified for the same components i.e. substitution of sucrose with table sugar, distilled water with tap water and omission of agar-agar. The root induction response was also comparable between cost effective media and standard media (Table 4b).

Hardening of _in vitro_ plantlets

Well rooted plantlets derived from various experiments were transferred to pots containing autoclaved potting mixtures consisting of sand, soil, vermiculite, perlite and cocopeat, in different combinations, in the glasshouse for hardening. The survival rate of the *in vitro* generated plantlets was observed to be the best in the potting mixture containing sand: soil: vermiculite: perlite: cocopeat in the ratio 1:1:1:1:1 (Table 5). This combination of potting mixtures was then used to transfer the plantlets of appropriate statistics (Table 6). Initially, the plantlets were covered with poly bags or jars, for 10-15 days, to provide sufficient humidity and avoid desiccation till the plantlets showed new growth. During the hardening process, poly bags were taken off every day for 10 – 15 mins so as to acclimatize the plantlets to external environment, gradually increasing the length of exposure over time.

***Ex vitro* Rooting and Hardening**

Plantlets were transferred directly after growth on the shooting media, without growing them on root induction media. Instead, the plants were treated with IBA dip to promote root induction *ex vitro*. The survival rate of the plant observed was not as good as that of the plants transferred from the rooting media. However, if favorable conditions can be provided without

Table 4a: Comparison of shoot growth in LCM and STM

Type of Media	Days to multiple shoot formation	Shoots/explant	% of explants forming shoots
LCM	6 - 7	24.3	59.4
STM	6 - 7	25	59.7

Table 4b: Comparison of root growth in LCM and STM

Type of Media	Days to multiple root formation	Roots/explants	% of shoots forming roots
LCM	9 - 11	19.7	67.9
STM	9 - 11	19.6	68.3

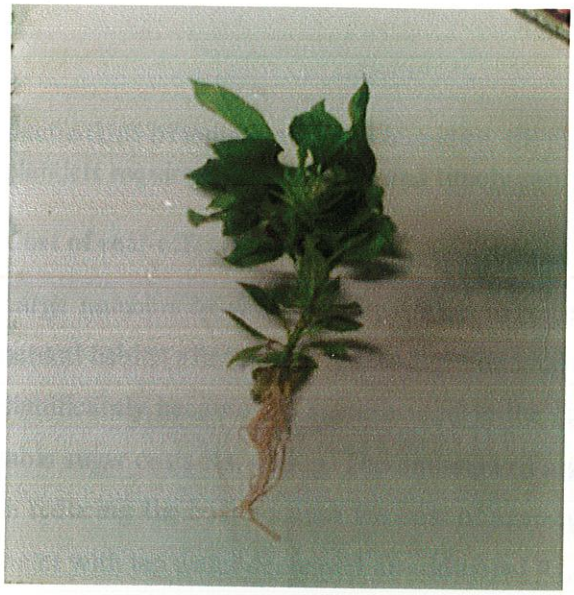
Table 5: Survival of the *in vitro* miltiplicated Apple plantlets in different composition of the potting mixes in the glasshouse

Composition of Potting Mix (ratio)	Percent survival of plants (%)
Sand: Soil: Vermiculite (1:1:1)	47.6
Sand: Soil: Perlite (1:1:1)	34.8
Sand: Soil: Cocopeat (1:1:1)	50.3
Sand: Soil: Vermiculite: Perlite: Cocopeat (1:1:1:1:1)	60.5

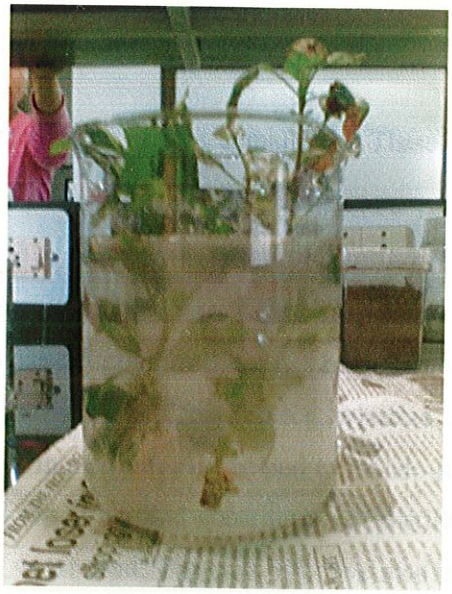
Fig 3: *In vitro* shoot multiplication in poly bags

Fig. 4.1: Shoot multiplication in liquid medium.

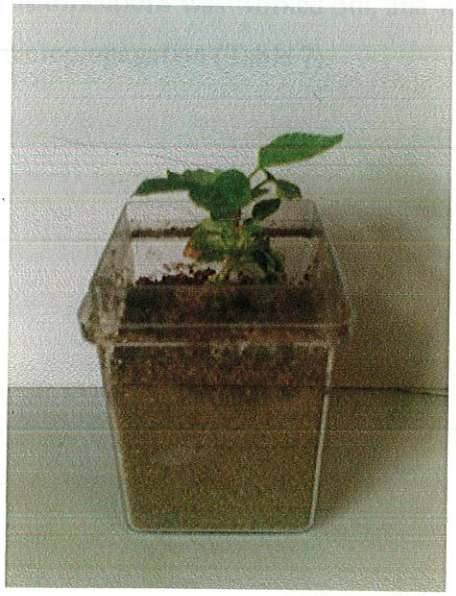
Fig. 4.2: Shoots in liquid cultures after 2 weeks of growth



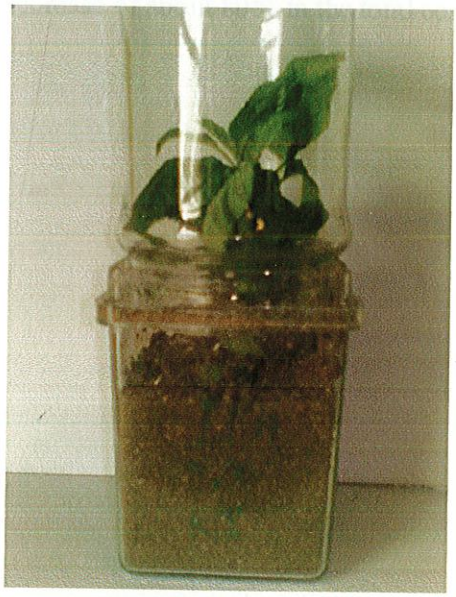
A: *In vitro* grown plantlet for transplantation



B: Bavistin (Antifungal) Treatment



C: Transfer of plantlets in potting mixtures



D: Plantlet covered with a jam jar.

Fig 5: Transplantation of *in vitro* grown apple plantlets.

fluctuations of temperature and humidity, the survival rate can be increased manifold. These plantlets require more intensive and timely care system.

Cost of cost-effective medium

Large numbers of well rooted plantlets have been regenerated, which are being transferred to natural habitat after proper hardening. The total cost of cost-effective medium has been reduced significantly because the sucrose used in the standard medium costs Rs. 386/Kg, whereas the table sugar costs Rs. 40/Kg. The omission of agar-agar from medium also helped tremendously in reducing the cost because the cost of agar-agar is Rs. 3432/Kg and substitution of distilled water with tap water decreased an additional Rs. 1.3/l of water used. The cost-effective medium composition identified in the current study holds great promise in not only rapid multiplication of thousands of plantlets of apple for reclamation in natural habitat but also of genetically superior strains of apple. The total percentage decrease in the cost of micropropagation with all the modifications was worked out, and it amounted to a decrease of about 80% in the total cost of micropropagation (Table 7).

Table 6: Statistics of the Apple plantlets at the time of transfer to potting mixes in the glasshouse

S. No:	Shoot length (cm)	No: of leaves	Root length (cm)	Roots/Shoot	Biomass of the shoot (gm)
1	6.5	20	4.6	8	0.92
2	7.3	7	7.3	12	0.67
3	7.7	8	6.8	5	0.93
4	8.2	18	9	14	1.15
5	8.7	15	10.5	17	1.22
6	9	19	9.5	23	1.37
7	9.6	16	10.7	27	1.43
8	10	23	9.4	35	1.67
9	10.4	27	9.5	29	1.98
10	11.2	24	11.5	31	2.45
Avg.	8.86	17.7	8.88	20.1	1.379

Table 7: Percentage decrease in the cost of micropropagation

S. No:	Original Component (Rs.)	Substitute (Rs.)	%age Decrease
1	Sucrose (Rs. 382/kg)	Table Sugar (Rs.40/kg)	89.53%
2	Distilled Water (Rs. 1.3/l)	Tap Water (NA)	~68%
3	Agar (Rs. 3432/kg)	Liquid Culture (NA)	~70%
4	Micropropagation	Micropropagation - Rooting	87.80%
5	Jam Jars (Rs. 12/jar)	Bags (Rs. 0.95/bag)	~93%
6	STM	LCM	~80%

Fig 6: *Ex vitro* rooting of plantlets

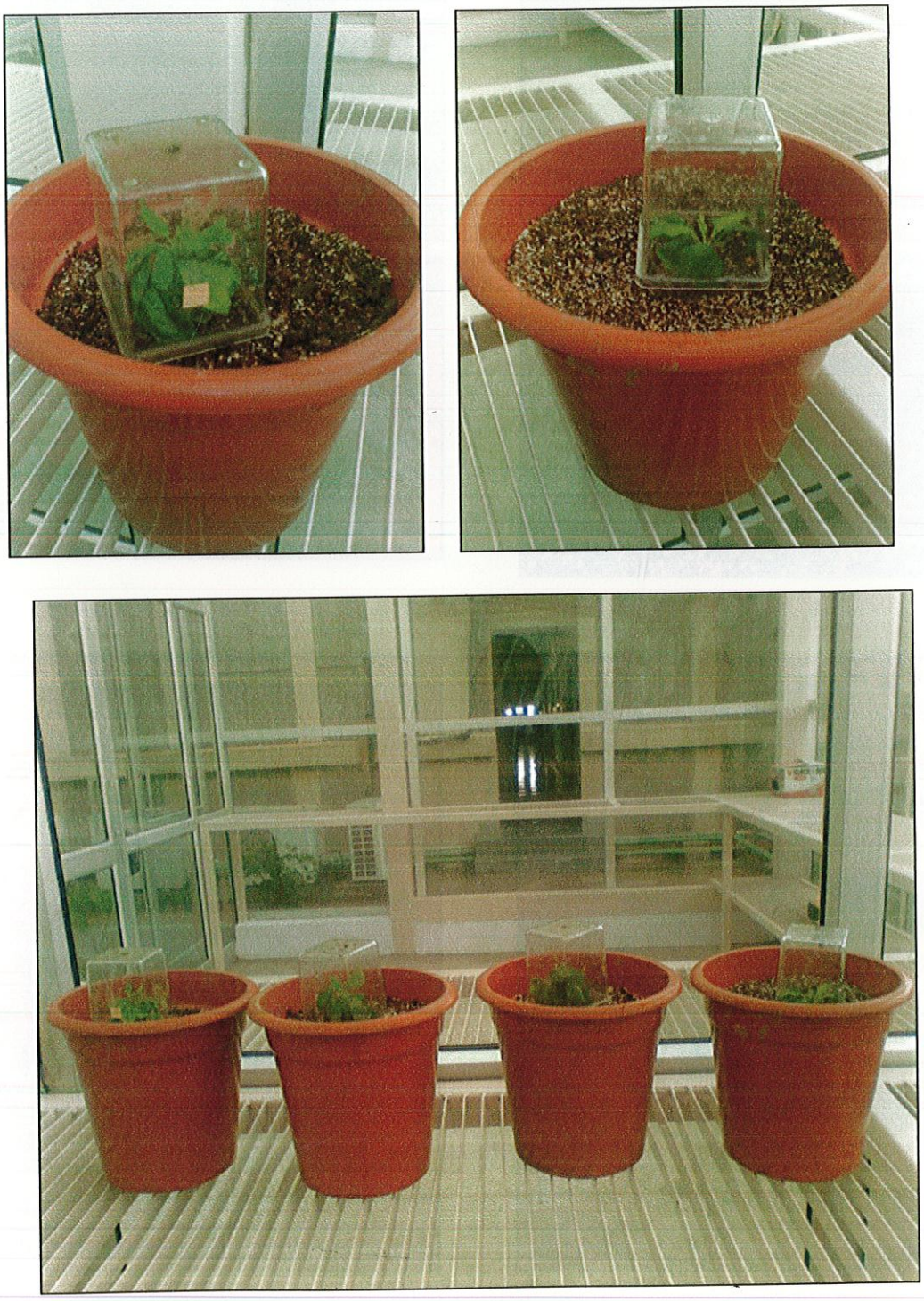


Fig 7.1: Hardening of plantlets in glasshouse

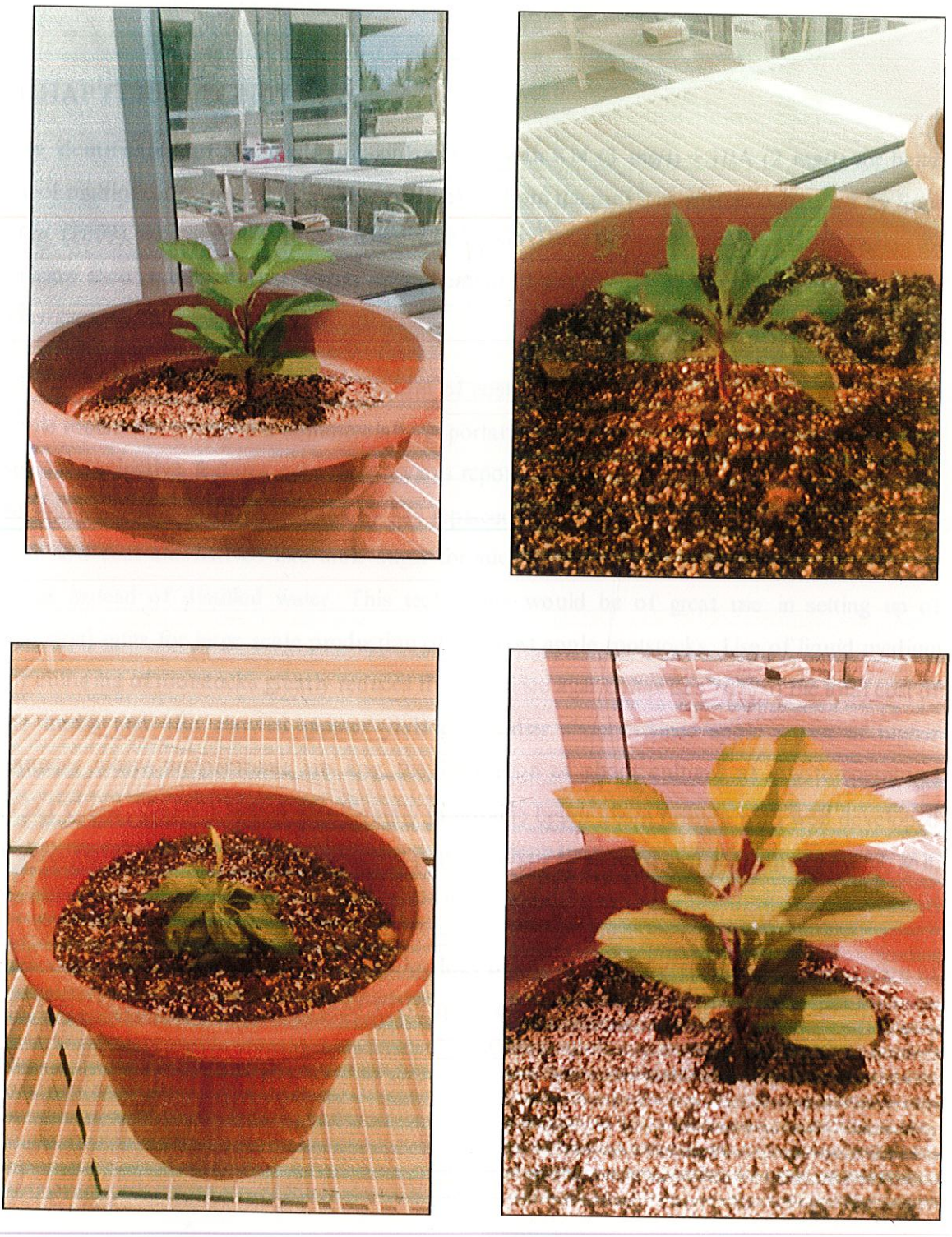


Fig 7.2: Hardened plantlets after 4 weeks

CHAPTER 5: CONCLUSION

The identification of MS medium supplemented with KN (3 mg/l) + IBA (2 mg/l) for better shoot multiplication on apple is not very different from the previous studies conducted by Sood *et al.* (2009), who have identified kinetin with auxin (IBA) as the best medium combination for in-vitro shoot multiplication. Rapid multiplication of shoots took place with no occurrence of vitrification and fasciated shoots.

The current study reports the development of cost effective micropropagation technology for apple rootstocks which is a commercially important species. The cost effective technology has several distinctive features over the previous reports of low cost tissue culture alternatives in several plant species. Our technology has replaced several components of the nutrient media with low cost alternatives like table sugar for sucrose, omission of agar-agar and use of tap water instead of distilled water. This technology would be of great use in setting up of industrial units for large scale production of low cost apple rootstocks. Use of liquid medium for culturing of rootstocks greatly reduces the cost since agar comprises 70% of the total cost of the media and also showed greater yield of sturdier shoots. Since apple grows at higher altitudes where temperatures are low, the incubation of shoot cultures at low temperatures (15±1°C and 25±1°C) helped significantly to provide better shoot growth with well developed leaves. Hardening of plants using several potting mixtures showed that a mixture of sand, soil, vermiculite, coco peat and perlite (1:1:1:1:1) is the best.

This cost effective micropropagation technology can help cost effective clonal propagation of apple rootstocks for commercial production and distribution to farmers.

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APPENDIX – I

Media components (Inorganic)

Components		Amount (gm/lt.)
STOCK A [10X]		
	KNO_3	19.00
	$MgSO_4.7H_2O$	3.70
	KH_2PO_4	1.70
STOCK B [20X]		
	NH_4NO_3	33.00
STOCK C [100X]		
	$CaCl_2.2H_2O$	44.00
STOCK D [100X]		
	Na_2 EDTA	3.726
	$FeSO_4.7H_2O$	3.785
STOCK E [100X]		
	KI	0.083
STOCK F [100X]		
	H_3BO_3	0.62
	$CoCl_2.6H_2O$	0.0025
	$ZnSO_4.7H_2O$	0.86
	$CuSO_4.5H_2O$	0.0025
	$MnSO_4.4H_2O$	2.23
	$Na_2MoO_4.2H_2O$	0.025

APPENDIX – II

Media components (Organic)

Components		Amount (gm/lt.)
STOCK G [100X]		
	m – Inositol	10.00
	Glycine	0.20
STOCK H [100X]		
	Pyridoxin HCl	0.05
	Nicotinic Acid	0.05
	Thiamine HCl	0.01