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Low-cost Tissue Culture Procedures for Micropropagation of Apple Root Stocks

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ABSTRACT

The low yield of apples (*Malus* spp.) can be amounted to the uncertainties of the monsoon, dependence over the old cultivars and prone to pathogen infestation. This warrants the development of a cost-effective micropropagation technology for the rapid multiplication of commercially important root stocks which can be utilized for re-plantation in apple orchards. Axillary shoot tips of M9 rootstock formed most multiple shoots on Murashige and Skoog (MS) medium supplemented with 3 mg/l 6-benzyladenine (BA), 2 mg/l kinetin (KN), 3% (w/v) sucrose and 0.8% (w/v) agar-agar, with 85.7% of shoot apices forming multiple shoots. To reduce the cost of media components for commercial production of the planting material from root stocks, sucrose was replaced with table sugar and agar-agar was completely omitted. The low-cost medium combination of MS liquid medium supplemented with 2 mg/l BA, 3 mg/l KN and 3% (w/v) table sugar was best, resulting in 22 shoots/explants. There were no significant differences in multiple shoot formation/explant, relative growth and vigor of shoots and frequency of root formation in shoots independent of whether medium contained sucrose or table sugar. *In vitro* regenerated plantlets were successfully hardened and transferred to the field. The substitution of sucrose for table sugar and the omission of agar-agar from the medium reduced the cost/liter medium by approximately 80-fold when other cost-effective alternatives such as tap water and autoclavable polybags were used, assisting in easier and more effective commercialization.

Keywords: apple liquid cultures, low-cost medium, micropropagation
Abbreviations: BA, 6-benzyladenine; **IBA**, indole-3 butryic acid; **KN**, Kinetin **MS**, Murashige and Skoog

INTRODUCTION

Apple (*Malus domestica*) is an important temperate fruit crop of India with regard to production, economic value and above all popularity. Because of some of its inherent characteristics, such as high productivity, good storage life, appearance and flavor, it has become a favorite for many. It is the most important fruit crop of the temperate regions and ranks fifth among all fruits in India. The annual production of apples is around 276,680 tones and the economy of Himachal Pradesh (India) largely depends upon the apple industry. The area under the cultivation of apples amounts to about 78,292 ha (H.P.) (National Horticulture Mission).

The low yield of apples in India is a reflection of an apple crop typically cultivated on mountainous terrain being exposed to monsoons, and is dependent on almost century-old cultivars of var. 'Delicious'. 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 (Dalal and Faroqui 2004).

Apple trees in an orchard are generally not grown from apple seeds. Although apple trees can be grown from seeds, their fruit will not be the same as that from the parent tree. In fact, it is unlikely that an apple tree grown from seed will have good quality fruit. That is why most apple trees 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 the two parts (scion or cultivar and rootstock) are joined together by grafting or budding. A rootstock is a plant, and sometimes just the stump, which already has an established, healthy root system, used for grafting a cutting or a budding from another plant. The tree part being grafted onto the rootstock is usually called the scion. The scion is the plant which 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 although genetically it always remains two different plants. The use of rootstocks is most commonly associated with fruiting plants and trees but 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 (Hartmann *et al*. 1997).

Micropropagated rootstock are ideal for establishing mother stocks, especially when *in vitro* propagation has been coupled with virus and bacteria certification programs (Snir and Erez 1980). 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* (Dalal *et al*. 2006). At present there are a lot of methods for the micropropagation of apple via rootstocks and scions. However, somaclonal variations or methylation changes during tissue culture threaten the clonal stability of the plants produced (Teixeira da Silva and Dobránszki 2010). It was noted that one of the most important factors for the regeneration of the apple plant tissue was the use of cytokinins. The type and the amount of cytokinins were found to be important and there was an effect of cytokinin pre-treatment on the organogenic ability of the leaf explants depending on their genotype (Dobránszki *et al*. 2010).

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Research Note

There are reports on the tissue culture of apple root stocks (Dalal *et al*. 2006) but no effort was reported on lowering the cost of *in-vitro* production of apple root stocks and its successful transplantation by using cost-effective media components, culturing vessels and potting mixtures. There are successful reports where 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) in *Fragaria ananassa*, omission of agar–agar (Mehrotra *et al*. 2007) in pineapple and use of sunlight and tubular skylight in micropropagation of banana (Kodym and Zapata-Arias 2001). 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 scientists for carrying out large-scale multiplication in different plant species (Earle and Langhans 1975, in carnation; Takayama and Misawa 1981 in begonia; Takayama 1991 and Paque *et al*. 1992, in conifer).

The current study explored the feasibility of reducing the cost of *in vitro* multiplication of apple rootstocks by using cost effective media components, glassware 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.

MATERIALS AND METHODS

Selection and establishment of explants

The apple rootstocks (M9) were procured from the Fruit Breeding Department of University of Horticulture and Forestry, Nauni, H.P., India. M9 is one of the most widely used rootstocks in commercial apple orchards 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. They are somewhat precocious and have a moderate to low productivity in most conditions. Axillary shoot tips (0.5-1.5 cm long) excised from pot grown plants were washed in 2% (v/v) detergent solution Teepol (Qualigen, India) and surface sterilized for 2-3 min in 0.5% bavistin (BASF, India) and for 30 sec in 0.1% (w/v) mercuric chloride (Merck, India) followed by 4-5 washes in sterile distilled water. The sterile shoot apices were cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinins.

Preparation of media

Modifications of MS (Murashiege and Skoog 1962) media supplemented with different concentrations and combinations of IBA (indole-3-butyric acid), KN (kinetin) and BA (6-bezyladenine) were prepared. The pH of the media was adjusted to 5.5-5.7 using 0.1N HCl and 0.1N NaOH, 3% (w/v) sucrose was added and finally 0.8% (w/v) agar-agar was added as the gelling agent. The media were autoclaved at 121°C and 15 lb/in^2 pressure for 15-20 min in 150-ml Erlenmeyer flasks (Borosil, India) by dispensing 40 ml of molten media into each flask plugged with non-absorbent cotton wrapped in one layer of muslin cloth. The autoclaved media were kept for 1-2 days before inoculations to screen for inherent contamination.

Multiplication of shoot cultures

For shoot multiplication, MS with IBA (0-2 mg/l), BA (0-2 mg/l) and KN (0-4 mg/l) in different combinations was attempted. 10 different combinations and concentrations of KN, IBA and BA were tested. One set of cultures was incubated at 16h photoperiod provided by cool fluorescent light (3000 lux) at 25 ± 1°C in plant tissue culture chamber. 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 to obtain good growth, and highly proliferated shoots.

Induction of adventitious roots

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 in culture tubes (25 × 150 mm) containing 10 ml of medium. The pH of medium was adjusted to 5.7 prior to autoclaving. The cultures were incubated under the same photoperiod 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 (1: 1: 1), perlite and cocopeat in the glasshouse conditions for acclimatization and hardening.

Cost-effective micropropagation

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

Table 1 Measures implemented to lower the cost of apple rootstock micropropagation.

	Medium composition
Low cost medium (LCM)	
LCM1	MS + table sugar (2%) + agar-agar (0.8% w/v)
LCM2	MS + table sugar (2%) without agar- agar
Standard medium (STM)	
STM 1	MS + sucrose (2%) + agar-agar (0.8% w/v)
STM 2	MS + sucrose (2%) without agar-agar

1. 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.

2. 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.

3. 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 8 g of agar/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 120 rpm, this prevented the plantlets from submerging in the liquid media and made fresh air available to them.

4. 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 at Rs. 100 for 95 bags proves to be even more efficient than the jars which cost Rs. 12/jar. In addition, one poly-bag could accommodate 3-4 plantlets 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.

5. *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 dipped in IBA (2 mg/l; overnight dip) to induce rooting. 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.

Cost of low-cost medium

The cost of MS medium/l was calculated with and without modification in its components. The cost of sucrose used in the standard medium was compared with the cost of table sugar with and without the use of agar-agar to find out reduction in cost of per liter medium (**Table 1**).

RESULTS AND DISCUSSION

Establishment of cultures for multiple shoot formation

The surface sterilized shoot apices of M9 rootstocks were cultured on MS media containing and out of the different media combinations tested for multiple shoot formation from shoot apices, MS medium containing KN (3 mg/l) + BA (2 mg/l) + IBA (2 mg/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 2**). The same medium was found to be the best for obtaining maximum shoots (25/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 BA in the medium, the shoot proliferation increased but the shoots were slender. However, a combination of KN (3 mg/l) + IBA (2 mg/l) gave best shoot growth. As in other studies people have used this combination for shoot proliferation and to obtain good growth (**Table 2, Fig. 1A, 1B**).

Table 2 Effect of different growth hormones on *in vitro* shoot multiplication in M9 apple rootstock.

MS + PGRs			Days to multiple shoot formation	No. shoots/explant	Shoots forming multiple shoots (%)
KN	BA	IBA		* (Mean ± SE)	* (Mean ± SE)
0	0	0	21 – 23	5.4 ± 0.12	19.7 ± 0.19
0.5	1	0	18 – 19	12 ± 0.11	37.2 ± 0.92
1	1.5	0	15 – 16	15.3 ± 0.21	41.8 ± 0.11
1	2	1	12 – 13	17.9 ± 1.11	53.3 ± 0.18
2	1	0.5	12 – 13	17.2 ± 0.98	52.1 ± 0.42
2	1.5	1	9 – 10	19.8 ± 0.45	55.4 ± 0.62
2	2	2	9 – 10	21.7 ± 0.54	57.3 ± 0.52
3	1	0.5	8 – 10	22.1 ± 0.66	55.6 ± 0.71
3	2	1	6 – 7	25 ± 0.46	59.7 ± 0.52
4	0	2	6 – 7	23.8 ± 0.22	45.8 ± 0.52

* Data represents mean of 20 replicates per treatment in three repeated experiments. PGR = plant growth regulator

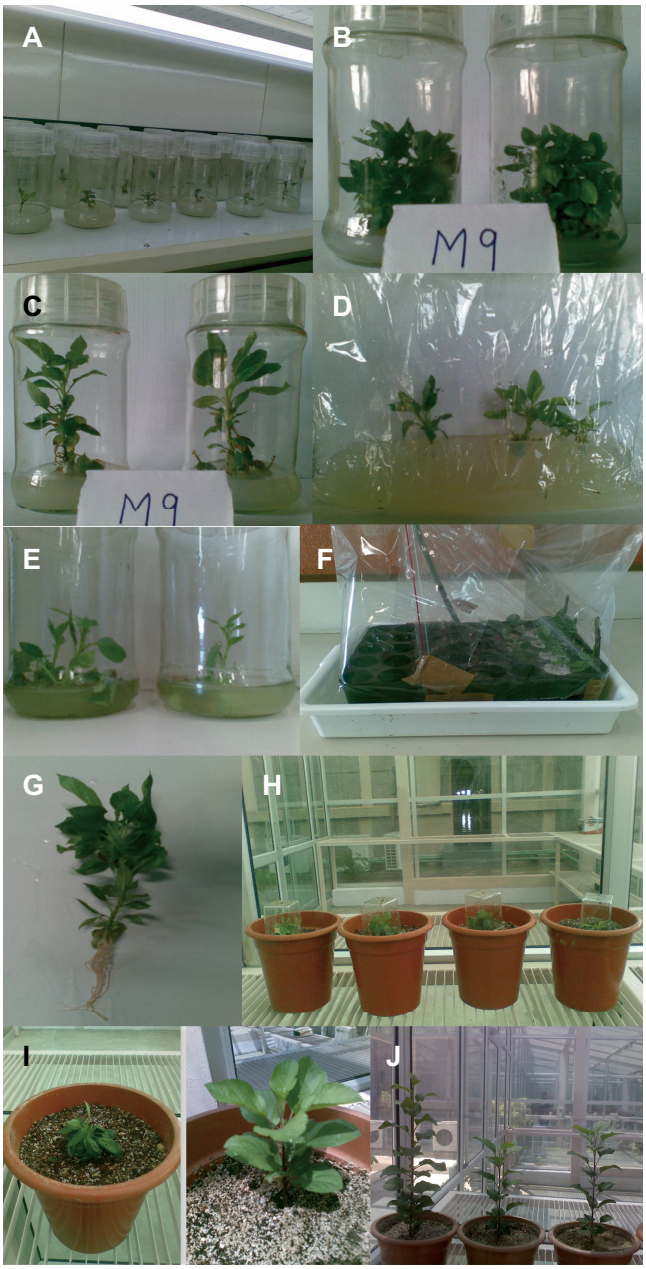

Fig. 1 (**A**) Establishment of axenic cultures by culturing shoot apices in different MS media. (**B**) *In vitro* shoot multiplication. (**C**) *In vitro* root induction. (**D**) *In vitro* shoot multiplication in polybags. (**E**) Shoot multiplication in liquid medium. (**F**) *Ex vitro* root induction. (**G**) Plantlet ready for transplantation. (**H**) Hardening of plants in glasshouse. (**I**) Hardened plant after 4 weeks. (**J**) Hardened plant after 14 weeks.

Induction of adventitious roots from *in vitro* 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 percentage of shoots forming roots (**Table 3**). Root induction was best observed in MS + IBA (3 mg/l) + KN (1 mg/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/shoot (19.6) (**Table 3; Fig. 1C**). Temperature has no effect on shoot or root growth (**Table 4**).

Table 3 Effect of different MS media compositions on *in vitro* rooting in apple shoots.

MS + PGRs		Days to multiple root formation	No. roots/explant	Shoots forming roots (%)
IBA	NAA		* (Mean ± SE)	* (Mean ± SE)
0	0	NA	NA	NA
1	0	15 – 16	8.9 ± 0.17	42.6 ± 0.12
1	0.5	13 – 15	8.5 ± 0.21	42.8 ± 0.17
1	1	13 – 15	5.7 ± 0.11	40.3 ± 0.51
2	0	12 – 13	13.3 ± 0.42	49.6 ± 0.77
2	1	10 – 12	14.1 ± 0.76	50.1 ± 0.59
2	1.5	10 – 11	12.8 ± 0.41	47.6 ± 0.13
3	0	10 – 11	16.5 ± 0.54	65.2 ± 0.54
3	1	9 – 11	19.6 ± 0.34	68.3 ± 0.49
3	2	9 – 11	19.2 ± 0.27	62.5 ± 0.13

* Data represents mean of 20 replicates per treatment in three repeated experiments. PGR = plant growth regulator

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 were compared with the standard media, 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 5A**). 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 5B**, **Fig. 1D, 1E)**

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 plants was not as good as that of plants transferred from rooting media. However, if favorable conditions can be provided without fluctuations in temperature and humidity, the survival rate can be increased. These plantlets require more intensive and timely care (**Fig. 1D**).

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 to a glasshouse for hardening, the best ratio being 1:1:1:1:1 (**Table 6**). This ratio of potting mixtures was then used to transfer the plantlets (**Table 7**). Initially, the plantlets were covered with poly-bags or jars for 10-15 days to provide sufficient humidity and to avoid desiccation until the plantlets showed new growth. During the hardening process, poly-bags were removed daily for 10-15 min to acclimatize the plantlets to the external environment, gradually increasing the length of exposure time (**Figs. 1E-H**).

Table 5A Comparison of shoot growth in LCM and STM.

Type of media	Days to multiple shoot formation	No. shoots/explant	% of explants forming shoots
		* (Mean ± SE)	* (Mean ± SE)
LCM	6 – 7	24.3 ± 0.13	59.4 ± 0.14
STM	6 – 7	25 ± 0.33	59.7 ± 0.23

*Data represents mean of 20 replicates per treatment in three repeated experiments.

Table 5B Comparison of root growth in LCM and STM.

Type of media	Days to multiple root formation	No. roots/explant	% of shoots forming roots
		* (Mean ± SE)	* (Mean ± SE)
LCM	9 – 11	19.7 ± 0.33	67.9 ± 0.15
STM	9 – 11	19.6 ± 0.43	68.3 ± 0.27

*Data represents mean of 20 replicates per treatment in three repeated experiments.

Table 6 Survival of the *in vitro* multiplied apple plantlets in different composition of the potting mixes in the glasshouse.

Composition of potting mix (ratio) and their labeling	Percent survival of plants (%) * (Mean ± SE)
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

* Data represents mean of 20 replicates per treatment in three repeated experiments.

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 8**).

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Table 4 Effect of different temperatures on growth and development of apple plantlets.

Temperature (°C)	No. shoots/explant	Shoot length (cm)	No. roots/explant	Root length (cm)
	* (Mean ± SE)	* (Mean ± SE)	* (Mean ± SE)	* (Mean ± SE)
25 ± 1	18.6 ± 0.47	9.5 ± 0.63	20.7 ± 0.38	8.2 ± 0.17
15 ± 1	19.1 ± 0.98	9.3 ± 0.32	21.1 ± 0.88	8.9 ± 0.26

* Data represents mean of 20 replicates per treatment in three repeated experiments.

Table 7 Statistics of the apple plantlets after hardening in different potting mixes (1-4) tried in the glasshouse.

Potting mixtures	Shoot length (cm)	No: of leaves	Root length (cm)	No. roots/shoot	Shoot biomass (g)
	* (Mean ± SE)	* (Mean ± SE)	* (Mean ± SE)	* (Mean ± SE)	* (Mean ± SE)
1	7.5 ± 0.22	15.1 ± 0.24	7.7 ± 0.44	8.2 ± 0.36	0.92 ± 0.76
2	6.3 ± 0.43	7.2 ± 0.63	7.3 ± 0.54	5.1 ± 0.76	0.67 ± 0.09
3	9.5 ± 0.31	19.6 ± 0.52	9.5 ± 0.29	23.7 ± 0.46	1.37 ± 0.21
4	11.2 ± 0.21	24.4 ± 0.23	11.5 ± 0.54	31.6 ± 0.43	2.45 ± 0.23

Table 8 Percentage decrease in the cost of micropropagation.

Original component (Rs.)	Substitute (Rs.)	% Decrease
Sucrose (Rs. 382/kg)	Table sugar (Rs.40/kg)	89.53
Distilled water (Rs. 1.3/L)	Tap water (NA)	~68
Agar (Rs. 3432/kg)	Liquid culture (NA)	~70
Micropropagation	Micropropagation - rooting	87.80
Jam Jars (Rs. 12/jar)	Bags (Rs. 0.95/bag)	~93
STM	LCM	~80

* Data represents mean of 20 replicates per treatment in three repeated experiments. STM = optimized tissue culture medium for apple root stock culture; LCM = modified low cost medium for apple root stock culture.

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