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Review Article

Plant Tissue Culture: A Review

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ABSTRACT

Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. The commercial technology is primarily based on micropropagation, in which rapid proliferation is achieved from tinystem cuttings, axillary buds, and to a limited extent from somatic embryos, cell clumps in suspension cultures and bioreactors. The cultured cells and tissue can take several pathways. The pathways that lead to the production of true-to-type plants in large numbers are the preferred ones for commercial multiplication. The process of micropropagation is usually divided into several stages i.e., prepropagation, initiation of explants, subculture of explants for proliferation, shooting and rooting, andhardening. These stages are universally applicable in large-scale multiplication of plants. The delivery of hardened small micropropagated plants to growers and market also requires extra care.

Keywords: multiplication of cells, micropropagation, pathways

INTRODUCTION

A whole plant can be regenerated from a small tissue or plant cells in a suitable culture medium under controlled environment. The plantlets so produced are called tissue-culture raised plants. These plantlets are a true copy of the mother plant and show characteristics identical to the mother plant. For example, if the mother plant is a high yielding plant the plantlets will also be high yielding. Many plant species are presently being propagated through tissue culture successfully. This capacity of a single cell to grow into a complete plant is termed as Totipotency, which was first put forward by a German Botanist Haberlandt in 1902. Tissue culture is the propagation of plants wherein a part/tissue of the plant is placed in nutrient media that favors the production of shoots, roots following which they are hardened and transferred to soil. Quality planting material of economically important species can be produced in a large scale/desired quantity through tissue culture[1] . Plant tissue culture can be initiated from almost any part of a plant however, for micropropagation or direct shoot regeneration, meristemetic tissue such as shoot tip is ideal. The physiological state of the plant does have an influence on its response to tissue culture. The mother plant must be healthy and free from obvious signs of disease or pest. The shoot tip explants being juvenile contain a higher proportion of actively dividing cells. It is important to use quality mother plant stock to initiate cultures[2]. The cultural conditions required to initiate and sustain plant cells in culture, or to regenerate intact plants from cultured cells, are different for each plant species. Each variety or clone of a species often have a particular set of cultural requirements.

REVIEW OF LITERATURE

Kato et al suggested that an agitation speed of 50 to 100 r.p.m. was most appropriate for the growth of tobacco cells in stirred-jar fermenters.it is true that culture plant cells are more fragile than bacterial cells,however,martin noted: "it seems obvious that cells lines differ in there resistance to shear effect and that a single optimum agitation speed that cannot bedesigned for all lines" [3] A roller-bottled system using a round flask was used by lamport in 1964 .a V-shape fermenter was proposed by veliky and martin. It is an inverted flask carrying two Teflon-coated stirring bars on a glass pin situated at the bottom of the flask a drain/sample port is also located at the bottom. The top of the flask is fitted with four standard taper penetration. Berlin et al .compared the highest cinnamoyl puttrescine producing, pflurophenylalamine resistance

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strainTX-4 or N. tabacum L. CV xanthi with a low producing srain for five enzymes of the biosynthetic pathway. As a result ,activities of these enzymes ,phenylalamine ammonia-lyase ,trans –cinnamate -4-hydroxylase,4-coumarate:Coa ligase,ornithine decarboxylase and argine decarboxylase were found to be 3 to 10 times higher in TX4 cells[4].

Davis et al recognised that addition of oxalate to the medium of gossypiumhirsutum suspension culture could reduce the amount of verticillium dahlia elicitor to be employed to stimulate metabolite synthesis.addition of a fungal elictor often inhibits the growth of plant cells but a combination of the elictor and oxalate did not reduce the cells mass of the plant ,therefore secondary metabolite synthesis was increased uptoten fold[5]. Dunlop and Curtis reported that a combination of phosphate limitation and fungal elicitation synergistically production of secondary metabolites. They found that either phosphate limitation or elicitation with a mycelial extract of the fungus,rhizoctoniasolani alone results in increased production of the sesquiterpenesolavetivone by agrobacterium rhizogenes-transformed hairy root cultures of hyoscyamusmuticus. In many species,somatic embryos are morphologically similar to the zygotic embryos,although some biochemical,physiological and anatomical differences have been documented.the synthetic auxin, 2,4-D is commonly used for embryo induction.in many angiosperm,e.g.,carrot and alfalfa subculture of cells from 2,4-D containing medium to auxin-free medium is sufficient to induce somatic embryogenesis.

Stages of Tissue Culture Process:

1. Preparation of nutrient medium: A semi-solid medium is prepared in double distilled water containing macroelements, micro elements, amino acids, vitamins, iron source, carbon sourcelike sucrose and phyto-hormones. The medium is heated for dissolving theagar and 25 to 50 ml is dispensed into each wide mouth bottles. The vesselscontaining culture media are then sealed and sterilized by autoclaving[6].

2. Establishment of aseptic culture: The starting material for the process is normally an actively growing shoot tipofaxiliary or terminal bud or shoot tip of a plant. The process of tissue culturestarts from the selection of mother plants having the desired characteristics.Ex-plant preferably the meristematic tissue of the selected mother plant isisolated. The excised tissue/explant is washed with water and then rinsed witha disinfectant such as savlon or detol solution followed by a sterile-waterwash. The tissue is then dipped in 10% bleach solution for ten minutes fordisinfecting the plant tissue material, killing most of the fungal and bacterialorganisms. Sterilization process of explants depends on the plant species andtypes of explants[7].

3.Inoculation: Inoculation is carried out under aseptic conditions. In this process explants ormicro shoots are transferredon to the sterilized nutrient medium.

4. Development of plants in growth room: After the inoculation of the plant tissue, the bottles are sealed and transferredinto growth room to trigger developmental process under diffused light (fluorescent light of 1000-2000 lux) at 25 ± 2 oC and 50 to 60% relativehumidity. Light and temperature requirements vary from species to speciesand sometimes during the various stages of developments.The cultures are observed daily for growth and any signs of infection/contamination. Cultures, that do not show good growth or infected, arediscarded. The healthy cultures grow into small shoot buds. These are sub-cultured on the fresh medium after 4 weeks. The number of subculturesrequired is specific to the plant species, which are standardized. The shootsgenerally develop after 4 weeks. After enough number of shoots is developedin each container (10 to 15), to a minimum height of 2 cm they are transferredto another medium for initiating the process of rooting. The constituent ofrootingmedium for each plant species are specific. Roots are generallyformed within 2 to 4 weeks. Plants at this stage are delicate and requirecareful handling[8].

5. Hardening of micro plants: Due to very high humidity inside the culture vessel and artificial conditions ofdevelopment, the plantlets are tender and are therefore are not ready forcoping up with the filed conditions. The plants removed from the sterilemedium are washed and are maintained under intermittent mist or arecovered with clean transparent plastic. After 10 to 15 days under highhumidity, the plants are transferred to green house and maintained foranother 4 to 6 weeks. They are then ready to be transferred to net house orthe field. Normally, the tissue culture plants are sold either as ex-agar plantsor hardened plants from the green house.

A: Ex-agar plants: Depending on the parameters such as location/the site of planting, soil qualityand the climatic conditions defined by the customer, the ex-agar plant for salecould be in vitro rooted plants or only the shoots. When the tissue cultureplants are sold at this stage, the plants are washed in sterilized water toremove the agar medium.The washed plants are sorted into 2 to 3 grades and packed in corrugatedplastic boxes lined with sterilized tissue paper as per specifications of thePlant Quarantine Authority, Government of India for exports. The number ofplants per box depends on the customer's requirement. Depending on thefinal destination and the preference ofthe

customer, the plants are treatedwith specific fungicides and antibiotics to avoid infection.The ex-agar plants are preferred for export or for destinations wherehardening facility are available. The plants after being removed from nutrientmedia should preferably be transplanted within 72 hours[9].

B: Hardened plants: The plants are transferred to net pots/ pro tray for acclimatization after theyfully develop shoots and roots in the bottles. The rooted plantlets aretransferred to pots filled with suitable substrate and are watered. Thisoperation is carried out on an open bench. These pots are then transferred tothe green house for 4 to 6 weeks. During this process, they are givenfertilizers and treated like plantlets obtained by any other means ofpropagation. After the plants are acclimatized fully, they are transferred topoly-bags. At this stage the plants are completely hardened and are ready tobe planted in the field for cultivation. Hardening units can be set up in sitesaway from the micropropagation unit[10].

5. Advantages of Micro-propagation Technology

Micro-propagation has several advantages over conventional methodsof propagation such as:

1. Rapid multiplication: Micro-propagation offers rapid multiplication of desired plantspeceis.

2. Requirement of only limited number of explants: Small pieces of plant (explants)/tissue can be used to producea large number of plants in a relatively small space.

3. Uniform or true to type plants: Micro-propagation provides a high degree ofphenotypic/physical uniformity. Since the production cycletakes place under controlled conditions, proper planning andscheduling based on the market demand is possible. Theresulting product has very high degree of uniformity comparedwithtraditionally propagated plants

4. Germplasm storage: Plants can be stored in vitro in a small space and less labouris required for maintenance of stock plants.

5. Disease free planting material: Plantlets produced by tissue culture are usually disease free.With proper diagnosis and treatments, elimination of fungus,bacteria and virus prior to large scale propagation is possible.With the help of seroloical and molecular technique it ispossible to index virus of mother plant/explant which is to beused for mass multiplication.

6. Growth manipulation: Nutrient levels, light, temperature and other factors can bemore effectively controlled to manipulate the growth,multiplication and regeneration.

7. Round the year production: Micro-propagation is independent of season. As micro -propagation could be carried out throughout the year;production cycle can be scheduled to meet peak demands. For species that have long generation time, low levels of seedproduction, or seeds that do not readily germinate, rapidpropagation is possible through tissue culture.The time required is much shortened, no need to wait for thewhole life cycle of seed development. Commercially propagated plants through micro-propagation in India[11]. The plants in each category which are commercially propagatedare as follows

Table 1. Type of plants.

Plant type	Name of plant
Medicinal plants	Aloevera, Geranium, Stevia, Patchouli, Neem
Ornamentals	Gerbera,Carnation,Anthurium, Lily,Syngonium,Cymbidium
Woody Plants	Teak,Bamboo, Eucalyptus,Populis
Bio fuel	Jatropha, Pongamia

6. Mitigating Risks of commercial plant tissue culture: The utilization of plant tissue culture for commercial production is limited bytwo major risks viz., spread of diseases especially those caused by viruses,and variations. The movement of plants also involves accidental risk ofintroducing plant disease. Pathogens that are often symptom less, such asviruses, pose a risk. The risk of distribution of inferior micropropagatedplantshas posed a major threat to the ever-increasing agribusiness industry. Inorder to prevent these risks, effective testing (indexing) procedures arerequired prior to bulking up culture for commercial propagation.Standardprocedure should be adopted such as:

• Carefully selection of mother plants

• Ensuring establishment of virus free culture through indexing of 100 %explants

• Proper package and practices to be adopted such as limited number ofcycles of multiplication, grading of cultures as well as plants, insect,pest monitoring in hardening area etc[12].

7. Need for Certification of tissue culture raised plants: Micropropagation is effectively used for producing quality planting materialfree from disease. Yet there is threat of inadvertent propagation of virus infected plants which will not only result in loss or poor performance of thecrop but also spread of virus. Further failure to used standard crop specificguidelines can lead to variations in the plants produced. The most deleteriousvariants in tissue culture raised plants are those that affect yield throughsomaclonal variations and carry viruses and other pathogens which aredifficult to diagnose. This is an area of great concern and requires a wellstructured system to support the tissue culture industry to ensure virus freequality planting material for commercial production.With the objective of production and

distribution of quality tissue cultureplanting materials Department of Biotechnology (DBT), Government of Indiahas established National Certification System for Tissue Culture Raised Plants (NCS TCP). For details about NCS-TCP, please refer the manual on "National Certification System for Tissue Culture Raised Plants (NCS-TCP):An Overview or log in to www.dbtncstcp.nic.in . DBT is the Certificationagency for the purpose for certification of Tissue culture raised plants/propagules up to laboratory level and to regulate its genetic fidelity as authorized vide the Gazette of India Notification dated 10 th March 2006 ofMinistry of Agriculture under section 8 of the Seeds Act.

PART B: TECHNO-COMMERCIAL FEASIBILITY:

1. MARKET SCENARIO: Demand for tissue cultured plantlets is growing rapidly. India, with its low costskilled labour as well as scientific manpower (both of which are essential fortissue culture) has a natural advantage. Additional favourable factors are thewide range of plant biodiversity in the country and favorable tropical climate(which enables greenhouses with low energy consumption)The potential for the domestic market is enormous and by conservativeestimates it is around Rs. 200 crores with an annual growth rate of 20%.There are more than 70 established commercial tissue culture units. Theirproduction capacity ranges between 0.5 million to 10 million plants per annumwith an aggregate production capacity of about 200 million plantlets per year.The protocols have either been developed in-house or transferred through thevarious research institutions and universities engaged in development of theprotocols through support of the Department of Biotechnology (DBT)Currently, the focus of the companies is mainly banana, floriculture,sugarcane and potato.With increasing awareness about the advantages of tissue culture raisedplants in improving yield and quality, their domestic consumption is alsoincreasing optimistically. The major consumers of tissue culture raised plantsare the State Agriculture Department, Agri Export Zones (AEZs), Stateagencies such as Spice Board, sugar industry andprivate farmers. The paperindustry, medicinal plant industry and State Forest Departments are usingtissue culture raised plants in a limited scale. Also a number of progressivefarmers and nurseries in the states are the major consumers of Tissue cultureplants particularly for flowers, banana, sugarcane and medicinal plants[13].

2. Establishment of Commercial Plant Tissue Culture Unit

Commercial plant tissue culture unit consists of the following components

Storage room for chemicals: It is advisable to have a separate area for storage of chemicals,apparatus and equipments. Chemicals required in small amountsshould not be purchased in large quantities as they may lose theiractivity, pick up moisture or get contaminated. Such problems can beovercome by purchasing small lots on a regular basis.Washing and Media Preparation Room:The glassware washing area should be located near the sterilizationroom. This area should have at least one large sink but two sinks arepreferable with running tap water. Adequate workspace is required oneach sides of the sink; this space is used for glassware soaking anddrainage. Plastic netting can be placed on surfaces near the sink toreduce glassware breakage and enhance water drainage. The outletpipe from the sink should be of PVC to resist damage from acids andalkalis. Both hot and cold water should be available and the water stilland de-ionisation unit should be located nearby. The washing roomshould be swapped periodically. Mobile drying racks can be used andlined with cheesecloth to prevent water dripping and loss of smallobjects. Ovens or hot air-cabinets should be located close to theglassware washing and storage area. Dust-proof cabinets and storagecontainers should be installed to allow for easy access to glassware.When culture vessels are removed from the growth area, they are oftenautoclaved to kill contaminants and to soften semi-solid media. It shouldbe possible to move the vessels easily to the washing area.Theglassware storage area should be close to the wash area to expeditestorage and access for media preparation.The media preparation room should have smooth walls and floors,which enable easy cleaning to maintain a high degree of cleanliness.Minimum number of doors and windows should be provided in this roombut within the local fire safety regulations. Media preparation areashould be equipped with both tap and purified water. An appropriatesystem for water purification must be selected and fitted after carefulconsideration of the cost and quality. A number of electrical appliancesare required for media preparation; hence, it is essential to have safetydevices like fire extinguisher, fire blanket and a first aid kit in the mediapreparation room. A variety of glassware, plastic ware and stainlesssteel apparatus is required for measuring, mixing, and media storage.These should be stored in the cabinets built under the worktables andtaken out for use as and when required. The water source andglassware storage area should be in or near the media preparationarea[14]. The workbench tops should be made withplasticlaminatesurfaces that can tolerate frequent cleaning. Media storage room shouldhave capacity to storage the media for at least 7 days. Sterility Class1,00,000 is desirable for

media storage room.InoculationRoomThe most important work area is the Inoculation room where the coreactivity takes place. The transfer area needs to be as clean as possiblewith minimal air disturbance. Walls and floors of the Inoculation roommustbe smooth to ensure frequent cleaning.

Profile of a self contained unit: The project profile of a micropropagation unit with an annual productioncapacity of 3 million plantlets is discussed below. A product mix of 5different plants has been assumed:

1. Banana Musa acuminata
2. Sugarcane Saccaharumofficinarum
3. Ginger Zingiberofficinale
4. Medicinal plants Chlorophytumborovillianum (Safedmusli), Aloe barbadensis
5. Ornamental plants Carnation-Dianthus caryophyllus, Orchids-Vanilla

LocationThe tissue culture laboratory should be preferably located in a moderated climate condition having uninterrupted supply of water and power. The tissue culture operations have to be carried out under controlled conditions oftemperature. Extreme climatic condition adds to the cost of maintenance.

Project Cost

A. Fixed asset

Table 2. Fixed asset of Tissue culture project

Head	Cost (Rs. In lack)
Land	5.00
Land development	5.60
Building	35.20
Utilities	16.00
Equipment	69.40
Green and shade house	30.00
Miscellaneous fixed asset	2.75
Total	163.95

Land: Approximate 5 acres land should be adequate for setting up a TCunit with the above capacity. Cost of land is assumed at Rs. 5.00 Lakhs Building and civil worksThe building of about 8800 sq.ft includes class 1000 clean rooms and areas with comfort AC for laboratory, growth rooms and office space.

The following facilities would be required in the building.

a) Storage room for chemicals
b) Washing and Media preparation room
c) Sterilization room
d) Inoculation room
e) Culture room

The total cost is estimated at Rs. 35.20 lakhs @ Rs. 400/sft.Green houseA green house of 7500 sq.ft. and a shade house of 80,000 sq.ft. have beenassumed at a cost of Rs. 22.00 lakhs and 8.00 lakhs (total Rs. 30 lakhs) respectively[15].

Equipment Major equipment and instruments required for the plant are as follows:

- Autoclave Laminar air
- Flow cabinet Equipment for sterilization
- Electronic weighing balance
- Water distillation apparatus
- Air handling units
- Refrigerator
- Air conditioners
- Stereomicroscope
- Digital pH meter
- Shelves / racks
- Green house material

WORKING CAPITAL REQUIREMENT

(I) Raw material

The basic inputs for the production of micropropagated plantlets includemeristems of elite and disease free plants, ready to use culturemedium,sucrose and agar.

(II) Manpower

The unit with the proposed capacity may need 40-50 people at variouspositions including managerial, supervisory, skilled and unskilled[16].

Table 3. Recurring expenses (per month)

(III) Recurring expenses (per month)	(Rs. lakhs)
Raw Material	2.50
Manpower	2.41
Utilities (power, water)	0.45
Contingencies (marketing, office expense, repair etc)	0.40
Total	5.76

(B) ECONOMICS OF STARTING PLANT TISSUE CULTURE BUSINESS WITH THE MINIMAL INVESTMENT: Micro propagation business can be started by entrepreneurs interested inventuring into this area, with smaller investment by setting up a hardening unitto start with. Such entrepreneurs can procure primary hardened tissue cultureplantlets from established micro propagation units and undertake secondaryhardening in the facility and sell it to the farmers. Once the market isestablished, a full-fledged micro propagation unit could be set up. Thefollowing profile provides an overview of profitability for a hardening facility forhandling 3 lakh plantlets per annum.

4. Government Schemes and Incentives: Various Central and State Government departments have framedfinancial schemes and announced incentives for assistance of tissueculture industry which are summarized below:a. Ministry of AgricultureThe Department of Agriculture and Cooperation under the Ministry ofAgriculture, Government of India

has the following programmes andschemes for promotion of horticulture[17].

(i) There is a provision for assistance of uptoRs. 21 lakhs and Rs. 10lakh for setting up tissue culture units in public and private sectorrespectively subject to a maximum of 20% of the project cost.

(ii) Under the Integrated Development of Fruits scheme assistance isgiven for purchase of planting material under the area expansionprogramme for the following crops: -

a) Rs. 7,000/hectare for plants of Guava, Amla, Date Palm, Plum Peach, Bes, Fig and citrus.

b) Rs.10,000/hectare for plants of mango, almond, pomegrante, apple,nuts, apricot, olive, papaya, litchi and sapota.

c) Rs. 30,000/hectare for plants for Bananas and pineapples.

d) Rs. 70,000/hectare for plants of grapes and strawberry.

In addition, 50% subsidy is given to the farmers for purchase of tissue culture banana by the Andhra Pradesh State Agriculture Departmentunder the Macro Management Scheme

b. Agricultural and Processed food products Export **Development Authority (APEDA)**

APEDA under the Ministry of Commerce and Industry has taken thefollowing initiatives for promoting tissue culture in the country.

(i) A state-of-the-art airfreight trans-shipment centre has been set up fortemperature sensitive perishables at Delhi, Mumbai and Bangaloreairports[18].

(ii) Airfreight subsidy is given for Tissue Culture Plants along with otherlive plants / bulb in category of perishable horticulture produce forexport. The rate of subsidy to West Asia and CIS countries is at the rate of Rs.10 per kg or 25% of the airfreight rate approved by IATA or 1/3 rdof the FOB value whichever is the least.

(iii) The rate of subsidy for export to Europe other than CIS countries,North America and Far East at the rate of Rs.25 per kg or 25% of the airfreight rate approved by IATA or 1/3 rd of the FOB value whichever is the least.

(iv) 50% subsidy is given for the development of infrastructure likerefrigerated van, packaging, export promotion, market development,consultancy services and feasibility studies, organization building and human resource development.

(v) Financial assistance is also given for strengthening quality control facilities and implementation of ISO 9000.c. National Horticulture Board (NHB)The mandate of NHB is to promote integrated development ofHorticulture and to help in coordinating, stimulating and sustaining the production and processing of fruits and vegetables.It also helps in establishing a sound infrastructure in the field ofproduction, processing and marketing with a focus on post harvestmanagement. For setting up of a new tissue culture lab there is a provision for back-ended capital subsidy not exceeding 20% of the project cost with a maximum limit of Rs. 25 lakh per project. NHB also has a scheme for providing subsidy for cultivation under controlled climate condition in poly houses, green houses, net houses, etc[19]. The units planning expansion in the domestic market by having a network of nurseries or additional hardening facilities can avail this scheme. The provision also exits for high quality commercial horticulture crops, Indigenous crops/produce, herbs, aromatic & medicinal plants, seed &nursery, bio-pesticide and establishmentof Horticulture Health Clinics Laboratory. In all these cases, the subsidy is routed through the involvement of a financial institution on the completion of the project. [20].

CONCLUSION

The current article combines the study of plant tissue culture performed by different scientistsworld wide. Plant tissue culture technique has brought revolution in the pharmacy field. This study unwinds different aspects of plant tissue culture technique and shows applicability of this tool for production of pharmaceuticals.It canbe concluded that plants are the wide source of medicines. Tissue culture technique can be utilized for production of such medicines. Tissue culture started a new era in the field of phytochemicals. Though many techniques are developed till date to improve yield and economy of tissue culture, more research is should be carried out for further development.

Fig II: Inoculation of excised micro shoots

Fig. 2. In vitro rooting of micro shoots.

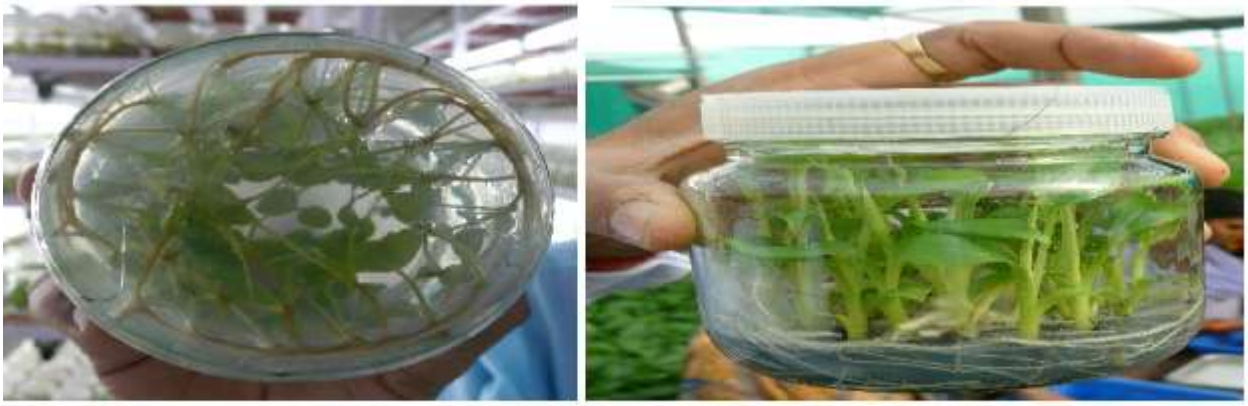

Fig III: In vitro rooting of micro shoots

Fig. 3. Ex Agar plants.

Fig IV: Ex agar plants ready for packaging and dispatch

Fig. V: Hardening of plants in green house

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