PROJECT REPORT ON PLANT TISSUE CULTURE

PART A: PRIMER ON PLANT TISSUE CULTURE

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PART A: PRIMER ON PLANT TISSUE CULTURE

1. 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.

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.

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.

2. Process of Plant Tissue Culture

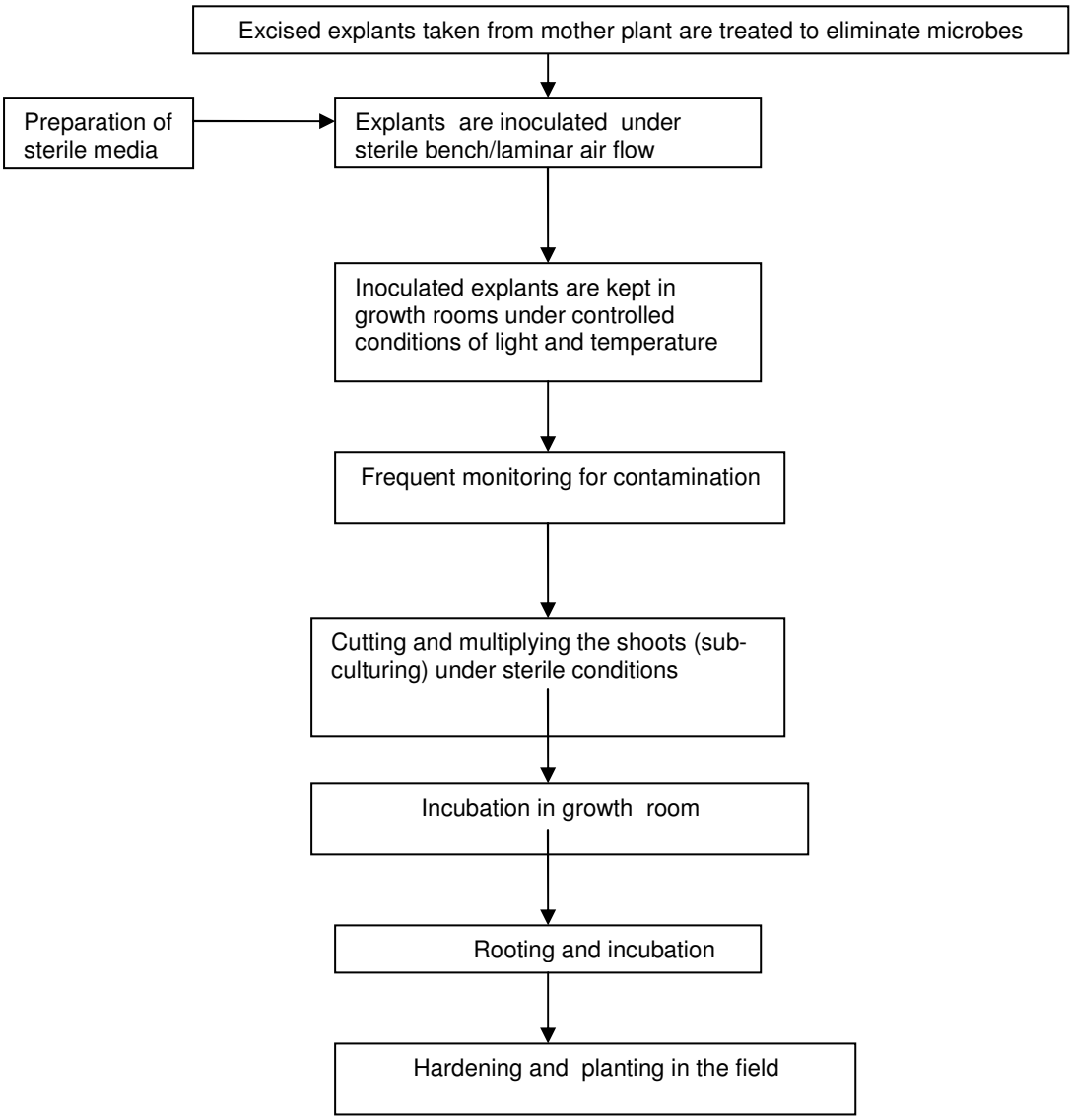

Figure I: Production process of Tissue Culture Plants (TCPs)

3. Stages of Tissue Culture Process

3.1 Preparation of nutrient medium

A semi-solid medium is prepared in double distilled water containing macro elements, micro elements, amino acids, vitamins, iron source, carbon source like sucrose and phyto-hormones. The medium is heated for dissolving the agar and 25 to 50 ml is dispensed into each wide mouth bottles. The vessels containing culture media are then sealed and sterilized by autoclaving.

3.2 Establishment of aseptic culture

The starting material for the process is normally an actively growing shoot tip of axiliary or terminal bud or shoot tip of a plant. The process of tissue culture starts from the selection of mother plants having the desired characteristics. Ex-plant preferably the meristematic tissue of the selected mother plant is isolated. The excised tissue/explant is washed with water and then rinsed with a disinfectant such as savlon or detol solution followed by a sterile-water wash. The tissue is then dipped in 10% bleach solution for ten minutes for disinfecting the plant tissue material, killing most of the fungal and bacterial organisms. Sterilization process of explants depends on the plant species and types of explants

3.3 Inoculation

Inoculation is carried out under aseptic conditions. In this process explants or micro shoots are transferred on to the sterilized nutrient medium.

Fig II: Inoculation of excised micro shoots

3.4 Development of plants in growth room

After the inoculation of the plant tissue, the bottles are sealed and transferred into growth room to trigger developmental process under diffused light (fluorescent light of 1000-2000 lux) at 25 ± 2^{O}C and 50 to 60% relative humidity. Light and temperature requirements vary from species to species and 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, are discarded. The healthy cultures grow into small shoot buds. These are sub-cultured on the fresh medium after 4 weeks. The number of subcultures required is specific to the plant species, which are standardized. The shoots generally develop after 4 weeks. After enough number of shoots is developed in each container (10 to 15), to a minimum height of 2 cm they are transferred to another medium for initiating the process of rooting. The constituent of rooting medium for each plant species are specific. Roots are generally formed within 2 to 4 weeks. Plants at this stage are delicate and require careful handling.

Fig III: In vitro rooting of micro shoots

3.5 Hardening of micro plants

Due to very high humidity inside the culture vessel and artificial conditions of development, the plantlets are tender and are therefore are not ready for coping up with the filed conditions. The plants removed from the sterile medium are washed and are maintained under intermittent mist or are covered with clean transparent plastic. After 10 to 15 days under high humidity, the plants are transferred to green house and maintained for another 4 to 6 weeks. They are then ready to be transferred to net house or the field. Normally, the tissue culture plants are sold either as ex-agar plants or hardened plants from the green house.

3.5.1: Ex-agar plants

Depending on the parameters such as location/the site of planting, soil quality and the climatic conditions defined by the customer, the ex-agar plant for sale could be *in vitro* rooted plants or only the shoots. When the tissue culture plants are sold at this stage, the plants are washed in sterilized water to remove the agar medium.

The washed plants are sorted into 2 to 3 grades and packed in corrugated plastic boxes lined with sterilized tissue paper as per specifications of the Plant Quarantine Authority, Government of India for exports. The number of plants per box depends on the customer's requirement. Depending on the final destination and the preference of the customer, the plants are treated with specific fungicides and antibiotics to avoid infection.

The ex-agar plants are preferred for export or for destinations where hardening facility are available. The plants after being removed from nutrient media should preferably be transplanted within 72 hours.

Fig IV: Ex agar plants ready for packaging and dispatch

3.5.2: Hardened plants

The plants are transferred to net pots/ pro tray for acclimatization after they fully develop shoots and roots in the bottles. The rooted plantlets are transferred to pots filled with suitable substrate and are watered. This operation is carried out on an open bench. These pots are then transferred to the green house for 4 to 6 weeks. During this process, they are given fertilizers and treated like plantlets obtained by any other means of propagation. After the plants are acclimatized fully, they are transferred to poly-bags. At this stage the plants are completely hardened and are ready to be planted in the field for cultivation. Hardening units can be set up in sites away from the micropropagation unit.

Fig. V: Hardening of plants in green house

4. Advantages of Micro-propagation Technology

Micro-propagation has several advantages over conventional methods of propagation such as:

1. **Rapid multiplication :**

   Micro-propagation offers rapid multiplication of desired plant speceis.

2. **Requirement of only limited number of explants** :

   Small pieces of plant (explants)/tissue can be used to produce a large number of plants in a relatively small space.

3. **Uniform or true to type plants :**

   Micro-propagation provides a high degree of phenotypic/physical uniformity. Since the production cycle takes place under controlled conditions, proper planning and scheduling based on the market demand is possible. The resulting product has very high degree of uniformity compared with traditionally propagated plants.

4. **Germplasm storage:**

   Plants can be stored *in vitro* in a small space and less labour is 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 is possible to index virus of mother plant/explant which is to be used for mass multiplication.

6. **Growth manipulation:**

   Nutrient levels, light, temperature and other factors can be more 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.

8. For species that have long generation time, low levels of seed production, or seeds that do not readily germinate, rapid propagation is possible through tissue culture.

9. The time required is much shortened, no need to wait for the whole life cycle of seed development.

5. Commercially propagated plants through micro-propagation in India

The plants in each category which are commercially propagated are as follows

Plant category	Plants
Fruits	Banana, Pineapple, Strawberry,
Cash crops	Sugarcane, Potato
Spices	Turmeric, Ginger, Vanilla, Large cardamom, Small Cardamom

Medicinal plants	Aloevera, Geranium, Stevia, Patchouli, Neem
Ornamentals	Gerbera, Carnation, Anthurium, Lily, Syngonium, Cymbidium
Woody plants	Teak, Bamboo, Eucalyptus, Populus
Bio fuel	Jatropha, Pongamia

6. Mitigating Risks of commercial plant tissue culture

The utilization of plant tissue culture for commercial production is limited by two major risks viz., spread of diseases especially those caused by viruses, and variations. The movement of plants also involves accidental risk of introducing plant disease. Pathogens that are often symptom less, such as viruses, pose a risk. The risk of distribution of inferior micropropagated plants has posed a major threat to the ever-increasing agribusiness industry. In order to prevent these risks, effective testing (indexing) procedures are required prior to bulking up culture for commercial propagation. Standard procedure 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 of cycles of multiplication, grading of cultures as well as plants, insect, pest monitoring in hardening area etc.

7. Need for Certification of tissue culture raised plants

Micropropagation is effectively used for producing quality planting material free from disease. Yet there is threat of inadvertent propagation of virus

infected plants which will not only result in loss or poor performance of the crop but also spread of virus. Further failure to used standard crop specific guidelines can lead to variations in the plants produced. The most deleterious variants in tissue culture raised plants are those that affect yield through somaclonal variations and carry viruses and other pathogens which are difficult to diagnose. This is an area of great concern and requires a well structured system to support the tissue culture industry to ensure virus free quality planting material for commercial production.

PART B: TECHNO-COMMERCIAL FEASIBILITY

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1. MARKET SCENARIO

Demand for tissue cultured plantlets is growing rapidly. India, with its low cost skilled labour as well as scientific manpower (both of which are essential for tissue culture) has a natural advantage. Additional favourable factors are the wide 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 conservative estimates it is around Rs. 200 crores with an annual growth rate of 20%. There are more than 70 established commercial tissue culture units. Their production capacity ranges between 0.5 million to 10 million plants per annum with an aggregate production capacity of about 200 million plantlets per year. The protocols have either been developed in-house or transferred through the various research institutions and universities engaged in development of the protocols 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 raised plants in improving yield and quality, their domestic consumption is also increasing optimistically. The major consumers of tissue culture raised plants are the State Agriculture Department, Agri Export Zones (AEZs), State agencies such as Spice Board, sugar industry and private farmers. The paper industry, medicinal plant industry and State Forest Departments are using tissue culture raised plants in a limited scale. Also a number of progressive farmers and nurseries in the states are the major consumers of Tissue culture plants particularly for flowers, banana, sugarcane and medicinal plants.

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 amounts should not be purchased in large quantities as they may lose their activity, pick up moisture or get contaminated. Such problems can be overcome by purchasing small lots on a regular basis.

Washing and Media Preparation Room:

The glassware washing area should be located near the sterilization room. This area should have at least one large sink but two sinks are preferable with running tap water. Adequate workspace is required on each sides of the sink; this space is used for glassware soaking and drainage. Plastic netting can be placed on surfaces near the sink to reduce glassware breakage and enhance water drainage. The outlet pipe from the sink should be of PVC to resist damage from acids and alkalis. Both hot and cold water should be available and the water still and de-ionisation unit should be located nearby. The washing room should be swapped periodically. Mobile drying racks can be used and lined with cheesecloth to prevent water dripping and loss of small objects. Ovens or hot air-cabinets should be located close to the glassware washing and storage area. Dust-proof cabinets and storage containers should be installed to allow for easy access to glassware. When culture vessels are removed from the growth area, they are often autoclaved to kill contaminants and to soften semi-solid media. It should be possible to move the vessels easily to the washing area. The glassware storage area should be close to the wash area to expedite storage 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 room

but within the local fire safety regulations. Media preparation area should be equipped with both tap and purified water. An appropriate system for water purification must be selected and fitted after careful consideration of the cost and quality. A number of electrical appliances are required for media preparation; hence, it is essential to have safety devices like fire extinguisher, fire blanket and a first aid kit in the media preparation room. A variety of glassware, plastic ware and stainless steel apparatus is required for measuring, mixing, and media storage. These should be stored in the cabinets built under the worktables and taken out for use as and when required. The water source and glassware storage area should be in or near the media preparation area. The workbench tops should be made with plastic laminate surfaces that can tolerate frequent cleaning. Media storage room should have capacity to storage the media for at least 7 days. Sterility Class 1,00,000 is desirable for media storage room.

Inoculation Room

The most important work area is the Inoculation room where the core activity takes place. The transfer area needs to be as clean as possible with minimal air disturbance. Walls and floors of the Inoculation room must be smooth to ensure frequent cleaning. The doors and windows should be minimal to prevent contamination, but within local safety code. There is no special lighting requirement in the transfer room. The illumination of the laminar airflow chamber is sufficient for work. Sterilization of the instruments can be done with glass-bead sterilizers or flaming after dipping in alcohol, usually ethanol. The culture containers should be stacked on mobile carts (trolleys) to facilitate easy movement from the medium storage room to the transfer room, and finally to the culture room. Fire extinguishers and first aid kits should be provided in the transfer room as a safety measure. Special laboratory

shoes and coats should be worn in this area. Ultraviolet (UV) lights are sometimes installed in transfer areas to disinfect the room; these lights should be used only when people and plant material are not in the room. Sterility Class 1,00,000 is desirable for inoculation room which can be achieved through installation of pressurized air module or air handling unit.

Growth Room

Culture room is an equally important area where plant cultures are maintained under controlled environmental conditions to achieve optimal growth. It is advisable to have more than one growth room to provide varied culture conditions since different plant species may have different requirements of light and temperature during *in vitro* culture. Also, in the event of the failure of cooling or lighting in one room, the plant cultures can be moved to another room to prevent loss of cultures. In the growth room, the number of doors should be minimal to prevent contamination. The culture containers can be placed on either fixed or mobile shelves. Mobile shelves have the advantage of providing access to cultures from both sides of the shelves. The height of the shelves should not exceed 2m.

The primary source of illumination in the growth room is normally from the lights mounted on the shelves. Overhead light sources can be minimized, as they would be in use only while working during the dark cycle. Plant cultures may not receive uniform light from the conventional downward illumination. Lights directly fitted to the racks create uneven heat distribution. Sideways illumination is an alternative, which requires less number of lights, and provides more uniform lighting. But care has to be taken not to break the lights while moving the cultures across the shelves. Sterility Class 1,00,000 is desirable for growth room.

3. PROJECT DETAILS

A: PROFILE OF A SELF CONTAINED UNIT:

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

1.	Banana	*Musa acuminata*
2.	Sugarcane	*Saccaharum officinarum*
3.	Ginger	*Zingiber officinale*
4.	Medicinal plants	*Chlorophytum borovillianum* (Safed musli), *Aloe barbadensis*
5.	Ornamental plants	Carnation-*Dianthus caryophyllus,* Orchids-*Vanilla*

Location

The 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 of temperature. Extreme climatic condition adds to the cost of maintenance.

Project Cost

A. Fixed asset

S. No.	Head	Cost (Rs. In lakhs)
1.	Land	5.00
2.	Land development	5.60
3.	Building	35.20
4.	Utilities	16.00
5.	Equipment	69.40

6.	Green and shade house	30.00
7.	Miscellaneous fixed asset	2.75
	Total	**163.95**

Land: Approximate 5 acres land should be adequate for setting up a TC unit with the above capacity. Cost of land is assumed at Rs. 5.00 Lakhs

Building and civil works

The 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 house

A green house of 7500 sq.ft. and a shade house of 80,000 sq.ft. have been assumed at a cost of Rs. 22.00 lakhs and 8.00 lakhs (total Rs. 30 lakhs) respectively. The greenhouse should be provided with heating equipment, fans and cooling systems.

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 include meristems of elite and disease free plants, ready to use culture medium, sucrose and agar.

(II) Manpower

The unit with the proposed capacity may need 40-50 people at various positions including managerial, supervisory, skilled and unskilled

(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**

Recurring expenses (per annum) Rs. 69.12 lakhs

CAPITAL INVESTMENT

	(Rs. lakhs)
Fixed assets	163.95
Technology knowhow	15.00
Working Capital (3 months)	17.28
Total	**196.23**

MEANS OF FINANCE

Particulars		(Rs. in Lakhs)
1.	Debt	117.73
2.	Equity	78.50
Total		**196.23**
Debt: Equity	-	3:2
Interest	-	16%

FINANCIAL ANALYSIS

(I) Cost of Production

	(Rs. lakhs)
Recurring cost (per annum)	69.12
Depreciation (@10%)	17.87
Interest (@16% Pa)	18.83
Total	**105.82**

(II) Turnover

Average selling price	-	Rs. 7 per plant
Total no. of plants	-	30 lakhs
Total turnover	**-**	**Rs. 210 lakhs**

(III) Profitability

Net profit	-	Rs. 104.18 lakhs
% Profit on sales	-	49%
IRR	-	26%
Return on investment	-	53%

(B) ECONOMICS OF STARTING PLANT TISSUE CULTURE BUSINESS WITH THE MINIMAL INVESTMENT

Micro propagation business can be started by entrepreneurs interested in venturing into this area, with smaller investment by setting up a hardening unit to start with. Such entrepreneurs can procure primary hardened tissue culture plantlets from established micro propagation units and undertake secondary hardening in the facility and sell it to the farmers. Once the market is established, a full-fledged micro propagation unit could be set up. The following profile provides an overview of profitability for a hardening facility for handling 3 lakh plantlets per annum.

HARDENING FACILITY

Project Details

Capacity

3 lakh plantlets / annum

Land

1 acre

Project Cost

A. FIXED ASSETS

S. No	Heads	Rs in lakhs
1.	Land and site development	1.00
2.	Green House	8.00
3.	Electrical fittings	0.60
4.	Furniture and fixtures	0.60
Total	`	**10.20**

B. Recurring expenses (per month)

	(Rs. lakhs)
Raw Material (Rs 4.00/explant)	1.00
Manpower	0.15
Utilities {power (500 units), water}	0.15
Contingencies	0.05
Total	**1.35**

Annual Recurring Expenses (per annum) Rs. 16.20 lakhs

C. CAPITAL INVESTMENT

	(Rs. lakhs)
Fixed Assets	10.10
Working capital (3 months)	3.63
Total	**13.73**

D. MEANS OF FINANCE

1.	Debt	10.25
2.	Equity	3.63
	Total	**13.73**

Debt : Equity	-	3:2
Rate of interest on loans	-	16%

E. FINANCIAL ANALYSIS

Sl. No.	**Particulars**	**(Rs. Lakhs)**
1.	Debt	10.25
2.	Equity	3.63
Total		**13.73**

(I) Cost of Production (per annum)

		(Rs. lakhs)
Recurring cost		14.52
Depreciation (@10%)		0.80
Interest (@16%)		1.31
Total		**16.63**
Debt : Equity	-	3:2

(II) Turnover

Total plantlets		3 lakhs
Selling price		Rs. 8 per plant
Total turnover		**Rs. 24 lakhs**

(III) Profitability

Net profit	**-**	**Rs. 7.37 lakhs**
% Profit on sales	-	30%
IRR	-	19%
Return on investment	-	56%