TFRI Publication No. 10

PLANT TISSUE CULTURE

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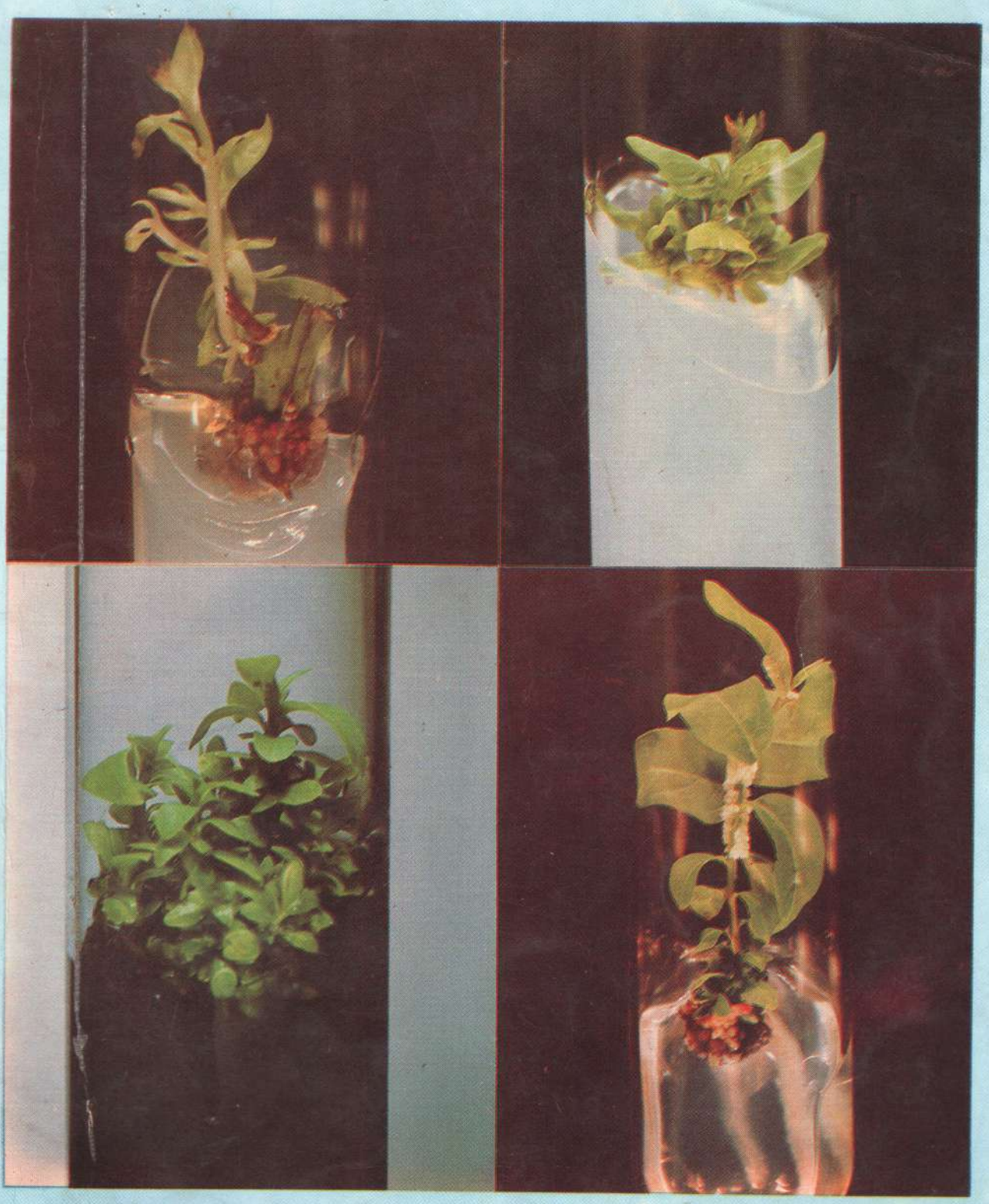

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1997

CONTENTS

Acknowledgement

The authors are grateful to Dr. B.N. Gupta, Director, Tropical Forest Research Institute, Jabalpur, Dr. A. K. Mandal, Head and Dr. S.A. Ansari, Scientist SD, Genetics and Plant Propagation Division for their suggestions and encouragement. The financial assistance provided by the World Bank FREE project for undertaking the publication of the technical bulletin is gratefully acknowledged.

1. Introduction and Importance

Plant tissue culture is used as blanket term for protoplast, cell, tissue and organ culture under aseptic (microorganism free) conditions. Basically, tissue culture is based on the principle of totipotency, which means that every living cell has the genetic potential to reproduce into entire organism which it has originally been derived from. The term micropropagation refers to *in vitro* vegetative propagation of plants, using tissue culture techniques starting with very small plant parts, the so called "explants" grown aseptically in a test tube or other container. In practice, the terms, micropropagation, tissue culture and *in vitro* culture are used interchangeably to mean any plant propagation procedure utilizing aseptic culture.

Tissue culture has acquired many practical applications in agriculture, horticulture and forestry. These include :

- an efficient, safe and often economical method of plant propagation for high value genotypes (e.g. elite trees) with high multiplication rate in reduced space
- Shoot-tip culture to raise virus-free plants from those infected with virus is being widely practiced
- International transport of pathogen-free plants in sterile cultures offers a method of bypassing the time-consuming quarantine requirements
- There is growing evidence that it may be possible to store valuable plant germplasm in cultures, under low temperature, more economically and safely than by conventional methods
- More recently, the possibility of growing single cells, fusing isolated protoplasts (cells without cell walls) of completely unrelated species and direct DNA-transfer to cells or protoplasts under *in vitro* conditions have emerged entirely new approach to plant improvement.

Micropropagation may be achieved by axillary budding, adventitious budding or somatic embryogenesis.

1.1 Axillary budding

In axillary budding, shoot proliferation is obtained from buds on the axil of leaf. This is the most widely used technique so far and safest as regards true-to-type propagation. However, multiplication rates are slower than other micropropagation techniques but are effective when compared to other vegetative propagation techniques.

1.2 Adventitious budding

The principle of adventitious budding is, dedifferentiation of mature cells and their subsequent regeneration. In simple terms, the cells of the plants differentiated in the form of buds, shoots, leaves on other plants parts are regenerated to an undifferentiated state, most of the time to callus stage, and then shoot or root regeneration takes place. The multiplication rates by this method are potentially high, however, risk of genotypic variation is there, especially when regeneration is obtained through callus formation. This technique is not very useful for clonal forestry as chances of variation are very high.

1.3 Somatic Embryogenesis

In contrast to axillary and adventitious budding, somatic embryos are originally derived from a bipolar structure (shoot apical and root apical poles developing at the same time), but produced by vegetative cells. Somatic embryogenesis thus assures the high potential multiplication rates with preservation of genotypic identity. However, favourable and promising responses can be elicited so far from embryonic or young seedling material of certain tree species only. Therefore, this technique has a limited application in forestry.

Keeping in view these facts, axillary budding remains the safest, most practical and efficient method of micropropagation and is being practised in most of tissue culture laboratories in India and abroad for clonal propagation.

2. Laboratory facilities, Equipments and General Techniques

The size of tissue culture set-up and extent to which it is equipped are governed by the nature of project undertaken and the funds available. However, a standard tissue culture laboratory should provide facilities for washing and storage of glassware; preparation, sterilization and storage of nutrient media; aseptic manipulation of plant material; maintenance of cultures under controlled conditions of temperature and light; and observation of cultures.

2.1 Washing area

The washing area needs to be very close to media room, should be provided with brushes of various sizes and shapes, a washing machine (if possible), a large sink, and running hot and cold water. It should also have steel or plastic buckets to soak the glassware to be washed, ovens or hot air cabinet to dry the washed labware and a dust-proof cupboard to store them.

2.2 Media preparation room

The usual facilities required for the preparation of medium includes, large cement benches or tables that can be cleaned easily, with large and deep enough sinks; special stable and quiet places for high precision balances; a refrigerator to store various chemicals, plant materials, short-term storage of stock solution etc., a hot plate cum-magnetic stirrer for dissolving chemicals, a pH meter, microwave oven or gas stove for melting agar and an autoclave for media sterilization.

2.3 Transfer area

This place is devoted to all tissue culture operations and manipulation, i.e. inoculation, transfer and subculturing in aseptic conditions under laminar flow hoods. Laminar flow hood is a special device which provides microorganism free air for aseptic manipulations. Transfer area is considered as "the heart" of the laboratory and the key requirement is that it needs to be as clean and contamination free as possible. In order to risk away any contamination, the area may be equipped with an ozonizer or

overpressure may be maintained with filtered air so that non-sterile air from outside does not enter.

2.4 Culture room

The room for incubating cultures is maintained at a controlled temperature and light conditions. Usually air conditioners and heaters are used to maintain the temperature around 25±2°C. Cultures are generally grown in diffuse light (less than 1 K lux). Diurnal control of illumination of the lights (fluorescent tubes/lamps) can be achieved using automatic timers. The culture room is provided with specially designed shelves to store cultures. While flasks, jars, and petridishes can be placed directly on the shelf or trays of suitable sizes, to support culture tubes some sort of racks are required. It is desirable to have emergency power points attached to a generator, to maintain both light and temperature in the culture room.

2.5 General Techniques

2.5.1 Glassware washing : Detergents especially designed for washing laboratory glassware should be used. After soaking in detergent solution for a suitable period (preferably overnight) the apparatus is thoroughly washed first in tap water and then rinsed in distilled water. To recycle the glasswares with contaminated tissue or media, it is extremely important to autoclave them without opening their closures so that all the microbial contamination is destroyed. The washed apparatus is dried in an oven or hot-air cabinet at about 75°C and stored in dust proof cupboard.

2.5.2 Sterilization : Plant tissue culture media containing a high concentration of sucrose, support the growth of many microorganisms (like bacteria or fungi). On reaching the medium, these microbes generally grow much faster than the cultured tissue and finally kill it. It is, therefore, absolutely essential to maintain a completely aseptic environment inside the culture vessels. The vials or containers containing medium are autoclaved (steam heating under pressure) at 15 lb (1.06 kg cm^{-2}, 121°C) for 15-40 min from the time the medium attains the required temperature. The exposure time varies with the volume of medium to be sterilized. Glass culture vials are mostly sterilized together with the medium. For pre-sterilized

nutrient medium the glasswares may be sterilized by autoclaving or dry heating in an oven at 160-180°C for 3h. The instruments used for aseptic manipulations, such as forceps, scalpels, needles, and spatulas are normally sterilized by dipping in 70 % ethanol followed by flaming and cooling.

Surface of the plant parts carry a wide range of microbial contaminants. To avoid this source of infection the tissue must be thoroughly surface sterilized before putting it on the nutrient medium. To disinfect plant tissues various sterilizing agent viz., Calcium hypochlorite (9-10 %), Sodium hypochlorite (2 %), Hydrogen peroxide (10-12 %), Bromine water (1-2 %), Silver nitrate (1 %), Mercuric chloride (0.1-1.0 %), antibiotics (4-5 mgl^{-1}) may be used. The duration of the treatment may vary from 5-30 min for most of the plant tissues.

2.5.3 Asepetic transfer of explants/subculturing/inoculation : It is very essential that all precautions are taken to prevent the entry of any contaminant into the culture vial when its mouth is opened either for subculture or for inoculating fresh tissue. To achieve this all transfer operations are carried out under laminar flow hood. The surface of laminar flow cabinet is swabbed with 70% alcohol, and all the required items (media, instruments, spirit lamp, match box, sterilized distilled water, sterilized empty flasks, etc.) transferred onto it. After spraying the chamber with 70% alcohol the door is tightly closed and the UV light switched on. For achieving a satisfactory degree of aseptic environment inside the cabinet, 20-30 min exposure to UV light is enough. Exposure to UV light builds up a high concentration of ozone (a toxic gas) inside the chamber. It is therefore, advisable to start the work 15-20 min after switching off the UV light. The mouth of culture vials is heated on spirit lamp flame to kill any microorganism spores sticking to the mouth. The instruments are sterilized by dipping in 70 % alcohol followed by flaming and cooling. All dissections are carried out in sterilized petriplates.

3. Tissue Culture Medium

A tissue culture medium usually consists of inorganic salts, a carbon source (generally sucrose), some vitamins and growth regulators. Other components added for specific purposes include nitrogenous organic compounds, tricarboxylic acid compounds and agar.

3.1 Inorganic nutrients : Mineral elements are very important in the life of a plant. For example, magnesium is a part of chlorophyll molecules; calcium, a constituent of cell wall; and nitrogen is an important part of amino acids, vitamins, proteins and nucleic acids. Besides C, H, and O, 14 other elements are known to be essential for plant growth, viz., nitrogen, phosphorus, sulphur, calcium, potassium, magnesium, iron, manganese, copper, zinc, boron, molybdenum, cobalt and chloride. Of these, the first six elements are required in comparatively large quantities and, therefore, termed as macro or major elements. the other elements are required in small amounts and are called trace or micro or minor elements.

3.2 Organic nutrients

3.2.1 Nitrogenous substances : Most cultured plant cells are capable of synthesizing all essential vitamins but, apparently in sub-optimal quantities. To achieve best growth of tissue it is often essential to supplement the medium with one or more vitamins and amino acids. Of these, thiamine (vitamins B_1) has generally proved to be an essential ingredient. Other vitamins, especially pyridoxine (vitamin B_6), nicotinic acid (vitamin B_3) and calcium pantothenate (vitamin B_5), and inositol are also known to improve the growth of cultured plant material. Various standard media show wide differences in their composition with respect to vitamins and amino acids (Appendix I).

3.2.2 Carbon source : The most commonly used carbon source is sucrose, at a concentration of 2-5 %. Glucose and fructose are also known to support good growth of some tissues. Autoclaving seems to bring about hydrolysis of sucrose into more efficiently utilizable sugars, such as fructose.

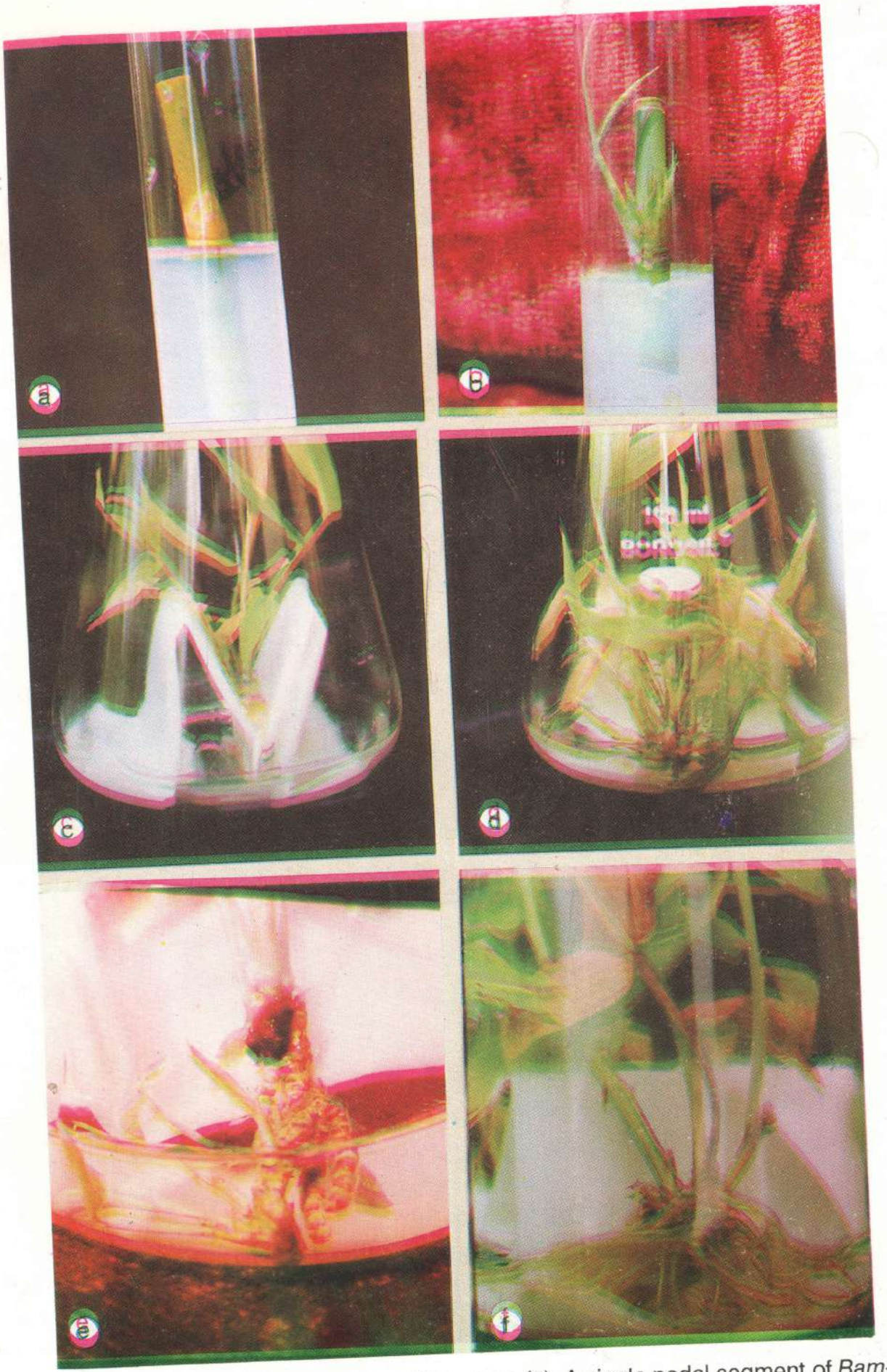

Fig. 1 Micropropagation of Bamboo (a), A single nodal segment of *Bambusa nutans*. (b), Bud break after one month of inoculation. (c), A cluster of shoots developed from a single nodal segment, (d) Shoot proliferation on liquid medium. (e), *In vitro* rhizome formation. (f), Rooted shoots of bamboo.

3.3 Growth hormones : In addition to the nutrients, it is generally necessary to add one or more growth substances, such as auxins, cytokinins, gibberellins, to support good growth of tissues and organs.

3.3.1 Auxins : In tissue culture, auxins have been used for cell division and root differentiation. The auxins commonly used in tissue culture are IBA (indole-3-butyric acid), NAA (napthaleneacetic acid), 2,4-D (2,4 dichlorophenoxyacetic acid), and 2,4,5-T (2,4,5 trichlorophenoxyacetic acid). Of these, IBA and NAA are widely used for rooting and, in interaction with cytokinin, for shoot proliferation.

3.3.2 Cytokinins : These hormones are required for cell division, modification of apical dominance, and shoot differentiation etc. In tissue culture media, cytokinins are incorporated mainly for cell division and differentiation of adventitious shoots from callus and organs. These compounds are also used for shoot proliferation by the release of axillary buds. Most commonly used cytokinins are BAP (6-benzylamino purine), kinetin (6-furfurylamino purine), and 2-ip (2-isopentenyl-adenine). Cytokinins are generally dissolved in dilute NaOH solution.

3.3.3 Gibberellins : There are over 20 known gibberellins. Of these, generally GA_3, is used. As compared to auxins and cytokinins, gibberellins are used very rarely.

3.4 Agar : In static cultures if liquid medium is used the tissue would get submerged and die due to the lack of oxygen. To avoid this, the tissue culture media are gelled with agar, a polysaccharide obtained from seaweeds, and tissue inoculated on the surface of the medium. Agar is generally used at a concentration of 0.8-1.0 %. However, agar is not an essential component of the nutrient medium. Single cells and cell aggregates can be grown as suspension cultures in liquid medium with gentle agitation. For certain system liquid medium may prove superior to agarified medium. In such cultures, filter paper bridges may be used to provide physical support to explants (Fig. 1c & 1d).

3.5 pH : The pH of the medium is usually adjusted between 5.0 and 6.0 before autoclaving. In general pH higher than 6.0 gives a fairly hard medium and a pH below 5.0 does not allow satisfactory gelling of the agar.

3.6 Media Preparation : The most simple method of preparing media today is to use commercially available dry powdered media containing inorganic salts, vitamins, and amino acids. The powder is dissolved in distilled water, and sugar, agar, and other desired supplements, the final volume is made up with distilled water. The pH is adjusted, and the medium autoclaved. Powdered media may be useful for routine purposes, such as micropropagation of plant species for which composition of medium required is well established. However, in experimental work where it is necessary to make major qualitative and quantitative changes, a series of concentrated solutions are prepared. For example, to prepare Murashige and Skoog's basal medium, four different stock solution may be prepared (Table 1); (a) major salts (20 x concentrated); (b) minor salts (200 x concentrated); (c) Iron (200 x concentrated); (d) Organic nutrients except sucrose (200 x concentrated). For the preparation of stock solution (a) to (d) each component should be separately dissolved to the last particle and then mixed with the others. Separate stock solutions are prepared for each growth regulator by dissolving in very minute quantity of the appropriate solvent and making up final volume with distilled water.

The sequence of steps involved in preparing medium is as follows :

- Required quantities of agar and sucrose are weighed and dissolved in water, about 3/4 the final volume of the medium, by heating them on gas stove or in microwave oven.
- Appropriate quantities of the various stocks solutions, including growth regulators and other supplements are added.
- The final volume of the medium is made with distilled water.
- After mixing well, the pH of the medium is adjusted with 0.1 N NaOH and 0.1 N HCl.
- The medium is poured into desired culture vessels which are plugged with non-absorbent cotton.
- The culture vessels containing medium are covered with aluminium foil to check wetting of plugs during autoclaving, and sterilized by autoclaving at 121^{o}C for 15 min.
- The medium is allowed to cool at room temperature and is stored at 4^{o}C.

Table 1 : Stock solutions for Murashige and Skoog's medium (MS)

	Constituents	Amount (mg l^{-1})
(a)	**Stock solution I**(20X*)	
	NH_4NO_3	33000
	KNO_3	38000
	$CaCl_2.2H_2O$	8800
	$MgSO_4.7H_2O$	7400
	KH_2PO_4	3400
(b)	**Stock solution II**(200X)	
	KI	166
	H_3BO_3	1240
	$MnSO_4.4H_2O$	4460
	$ZnSO_4.7H_2O$	1720
	$Na_2MoO_4.2H_2O$	50
	$CuSO_4.5H_2O$	5
	$CoCl_2.6H_2O$	5
(c)	**Stock solution III**(200X)	
	$FeSO_4.7H_2O$	5560
	$Na_2.EDTA.2H_2O$	7460
(d)	**Stock solution IV**(200X)	
	Inositol	20000
	Nicotinic acid	100
	Pyridoxine HCl	100
	Thiamine HCl	100
	Glycine	400

* indicates the original concentration of the nutrient solution.

4. General Techniques of Micropropagation

Micropropagation is the true-to-type propagation of a selected genotype, using *in vitro* culture techniques. Most often micropropagation is also associated with mass production at a competitive price.

Micropropagation generally involves five stages : 0, the preparative stage; 1, initiation of culture; 2, multiplication; 3, Elongation and root induction, and 4, Transfer to greenhouse condition (Fig. 2 & 3).

4.1 Stage 0, the preparative stage : Originally, stage 0 was introduced to be remedy for contamination problem. Raising mother plants under hygenic conditions can reduce some of them, especially those related to fungi. However, it is more difficult to interpret the results with respect to bacterial contamination, since most often we cannot distinguish between exogenous and endogenous bacteria.

To yield more hygenic explants, stock plants can be grown in greenhouse. For tropical and subtropical species it is advised to maintain a relatively high temperature (25°C) and a low humidity (75 %). It is important that plants do not receive water by overhead irrigation. All devices that provide water directly in pot or by capillarity are useful.

The reactivity of explant in stage 1 can be controlled by an appropriate treatment to the stock plant, the source of explants or the explant itself with plant growth regulators. Pulse treatment of the primary explants in solutions containing cytokinins has resulted in proliferation equivalent to that attained by placing the cytokinin in the medium.

4.2 Stage 1, initiation of culture : The purpose of stage 1 is to initiate aseptic cultures.

4.2.1 The explant : The nature of the explant to be used for *in vitro* propagation is governed by the method of shoot multiplication to be adopted. For enhanced axillary branching only such explants are suitable which carry a preformed vegetative bud (Fig. 1a). When the objective is to produce virus free plants from an infected individual it

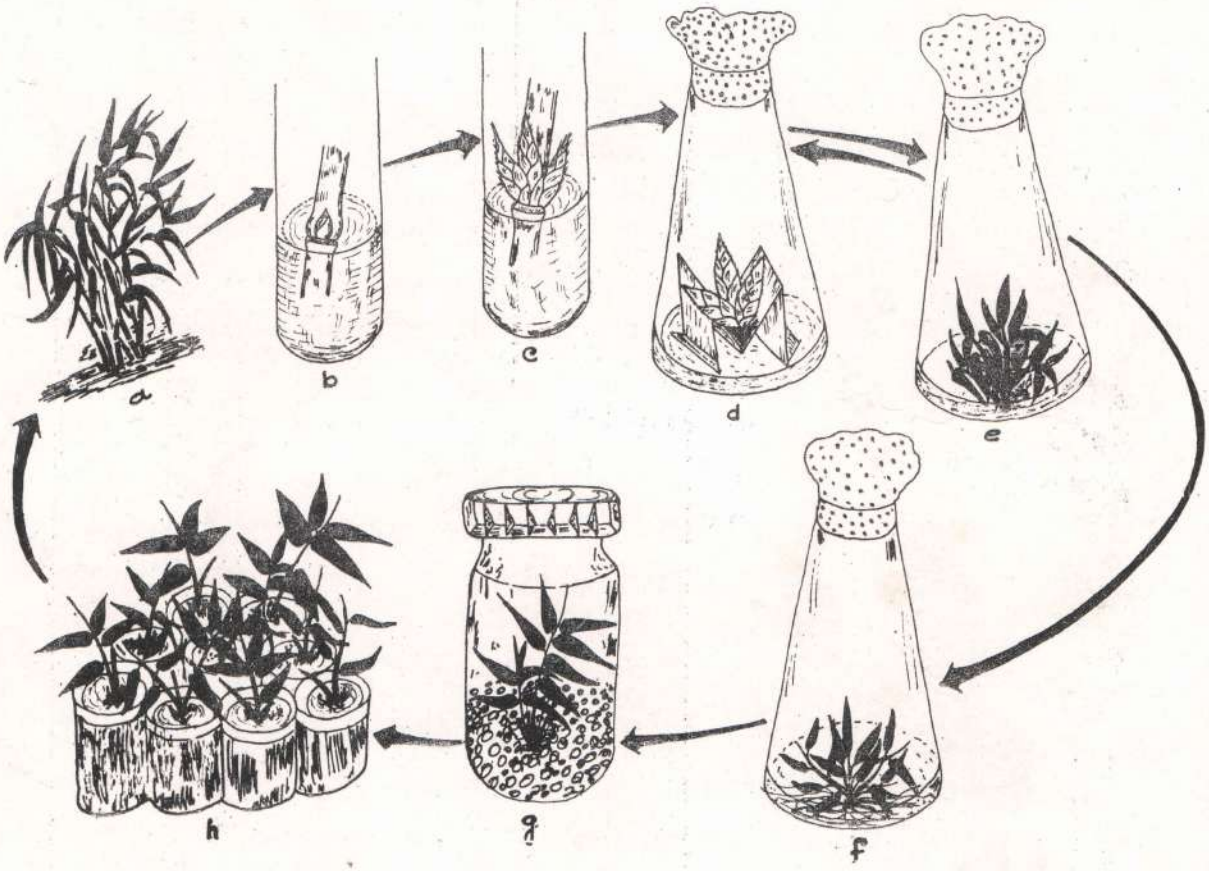

Fig. 2 Diagrammatic summary of the steps involved in micropropagation of bamboo through enhanced axillary branching. Stage 0, (a) Bamboo clump in field for the collection of nodal segments. Stage 1 (b-c); (b) Single nodal segment, (c) bud break. Stage II (d-e); (d) Shoot developed from single nodal segment, (e) Shoot multiplication on liquid medium, Stage III (f) *in vitro* rooting. Stage IV (g-h), (g) *in vitro* hardening, (h) Tissue culture raised plantlets established in potting mix.

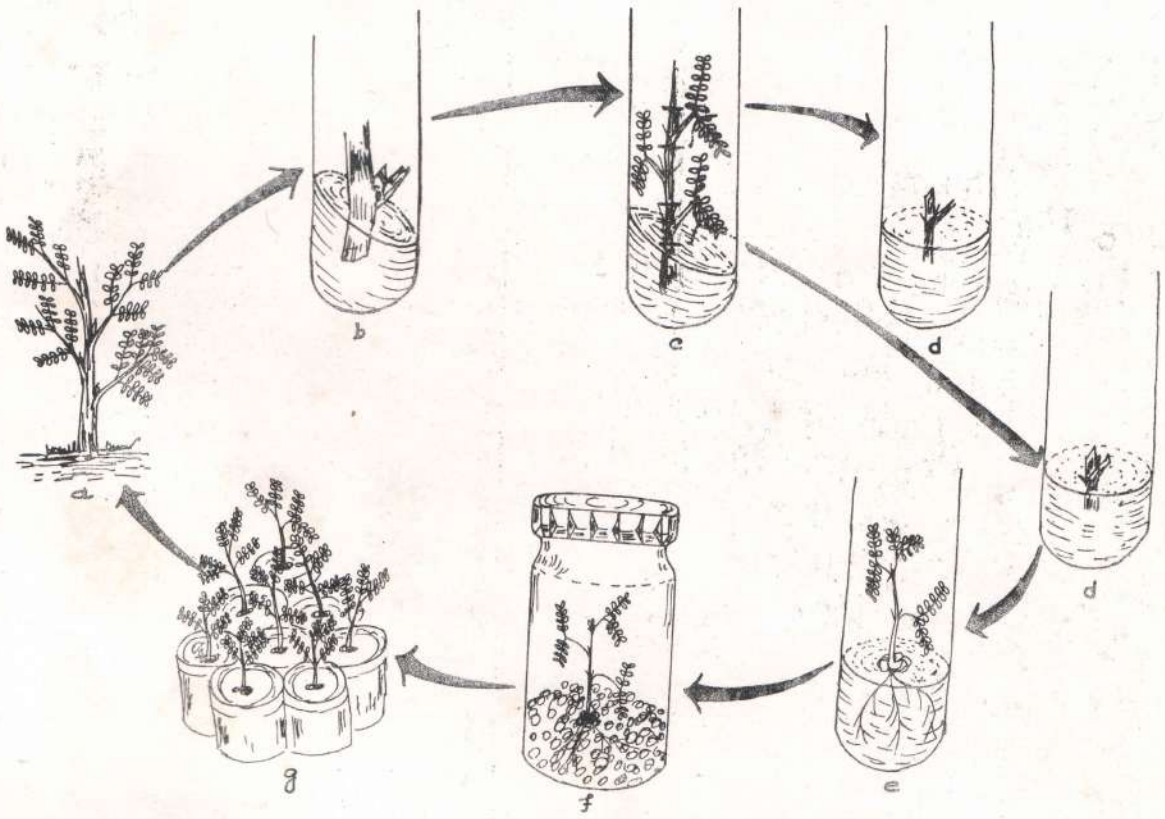

Fig. 3 Diagram showing steps involved in micropropagation of safed siris (*Albizia procera*) through nodal multiplication. Stage 0; (a) collection of nodal segments from selected tree. Stage 1 (b) Single nodal segment with axillary bud. Stage 2 (c-d), (c) Elongated shoot with three nodal segments, (d) Each segment used as propagule for further multiplication, Stage 3 (e) Rooted plantlet of safed siris. Stage 4 (f-g) *in vitro* hardening and transfer to shade house.

becomes obligatory to start with shoot tips. Small shoot-tip explants have a low survival rate and show slow initial growth. For shoot multiplication through adventitious bud formation, with or without callusing, pieces of root, stem, leaf etc. are used (Fig. 4a & 4b). Choice of the organ used as source of explant is governed by its natural capacity to form adventitious buds. It is a common experience that sub-terminal and old segments withstand the toxic effects of sterilizing agents much better than the terminal cuttings. The physiological state of the parent plant at the time of explant excision has a definite influence on the response of the buds. Explants from actively growing shoots at the beginning of growing season generally give the best results.

4.2.2 Sterilization : Standard methods for aseptic culture of plant tissue and organs described under general techniques should generally be adequate. Washing the plant material in running tap water for 20-30 min reduces the contamination. This may further be improved by washing in soap solution or cetavlon. A quick dip in 70 % ethanol before sterilization is useful in some species. Chances of contamination are much higher in the cultures of terminal cuttings and whole buds than that in the cultures of 0.5-1 mm shoot tips excised after removing several layers of older leaves.

4.3 Stage 2, multiplication : In a classical stage 2, the culture may supply shoots, for subsequent propagation as well as the material that is required to maintain the stock. The tissue can remain in stage 2 for several tissue culture generations without producing any shoot that can be directly rooted.

The conventional method of vegetative propagation by stem cuttings utilizes the ability of axillary buds to take away the function of the main shoot in the absence of terminal bud. The number of cuttings that can be taken from a selected plant in a year is extremely limited. In cultures, the rate of shoot multiplication by enhanced axillary branching can be substantially enhanced by growing shoots in a medium containing suitable cytokinin at an appropriate concentration. Due to continuous availability of cytokinin, the shoot formed by the bud present on the explant develops axillary bud which may grow directly into shoot. This process may be repeated several

times and the initial explant transformed into a mass of branches (Fig. 1c, 1d & 2e). At this stage, miniature shoots are excised and planted on a fresh medium of the same composition. The shoot multiplication cycle can be repeated a number of times. This process can go on independently, and can be maintained through out the year.

4.4 Stage 3, elongation and root development : The major cost in plant propagation by tissue culture is manual labour. This is especially pronounced in the latter stage (elongation and rooting) when individual shoots are manipulated. For this reason it is recommended to maintain cultures as cluster of shoots as long as possible. It is preferable that all manipulations of separated shoots, such as in the rooting stage, be performed under non aseptic conditions (*in vivo* rooting). For most plants elongation is still obtained by transfer of culture from the propagation medium to an appropriate elongation medium (Fig. 3c & 4c). This can be achieved by transplanting isolated shoots to a medium devoid of cytokinin. However, from an economic viewpoint, it is advisable to transplant cluster of shoots to a medium that allows good elongation of all of them or to add liquid elongation medium to established cultures instead of transplanting to a fresh medium.

To obtain full plants, the elongated shoots are transferred to a rooting medium which is different from the shoot multiplication medium especially in its hormonal composition. The number of shoot multiplication cycles after which rooting excercise is to be started is governed by the number of plants to be produced through micropropagation and the available nursery facilities. For rooting treatment, individual shoots measuring 1.5-2.5 cm in length are excised and transferred to rooting medium (Fig. 1f & 4d). In some plants, it has been possible to treat the shoots formed in cultures as minicuttings and root them out of culture. The basal cut end of the shoot is treated with auxin solution (pulse treatment) and planted in potting mix. The process is called *in vivo* rooting. When possible, rooting under non-sterile conditions should reduce the cost of plant production by cutting down a step in aseptic manipulation.

4.5 **Stage 4, transfer to greenhouse condition :** The transfer of plants from the culture vessel to soil requires a careful, step- wise procedure. The roots of *in vitro* raised plantlets are gently washed to remove the agar medium sticking to them. The most essential requirement for successful transplantation is to maintain the plant under a very high humidity (90-100 %) for the first 10-15 days, which is gradually reduced. After few weeks under high humidity, the plants are moved to shadehouse for 3-4 weeks (Fig 2h, 3g, 5a & 5b). Thereafter, plants may be kept under normal nursery conditions.

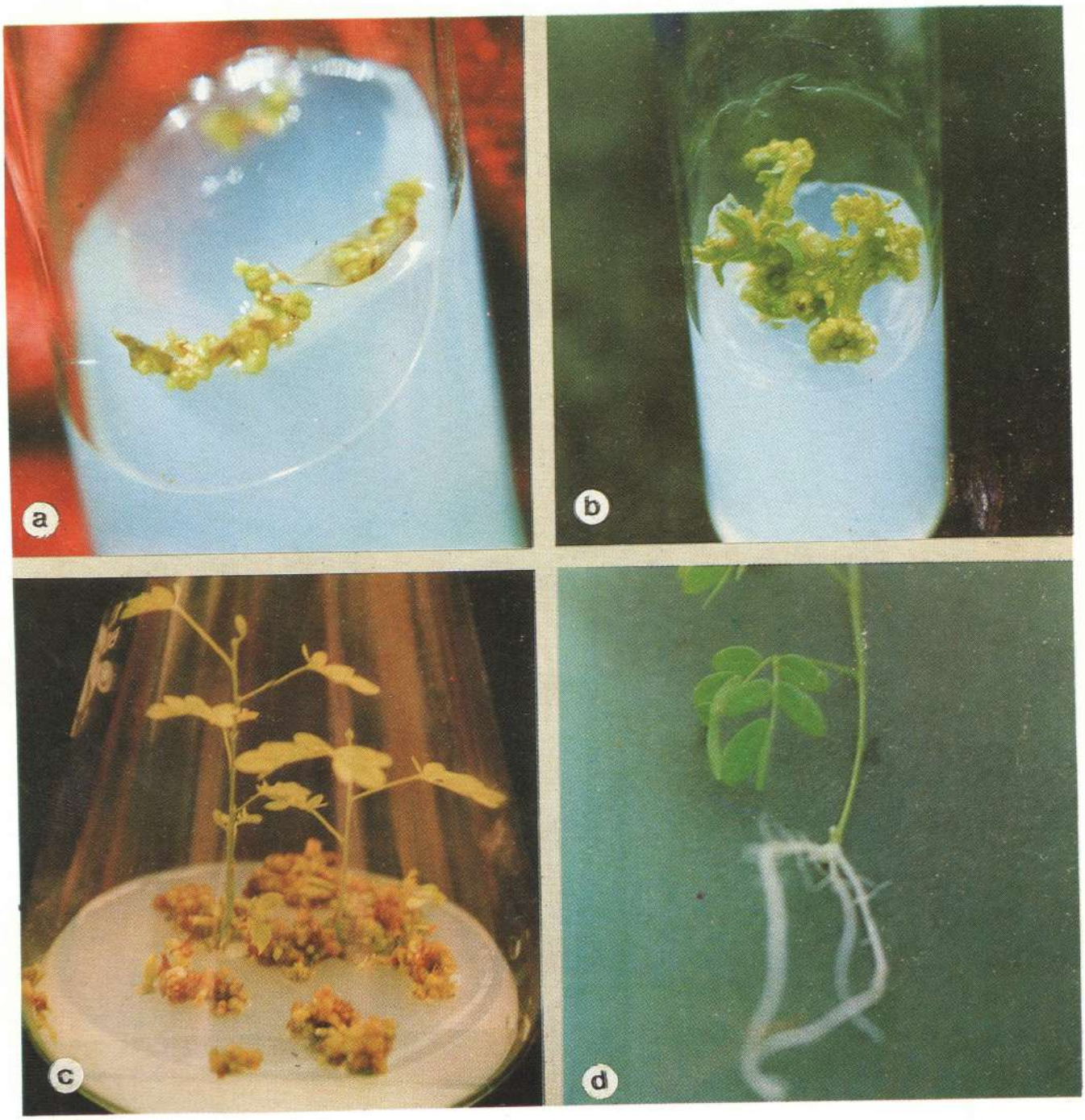

Fig. 4 Tissue culture of *Albizia procera*, (a-b), Direct regeneration from leaf disc and stem segment, (c), Shoot elongation (d), Rooted shoot from culture.

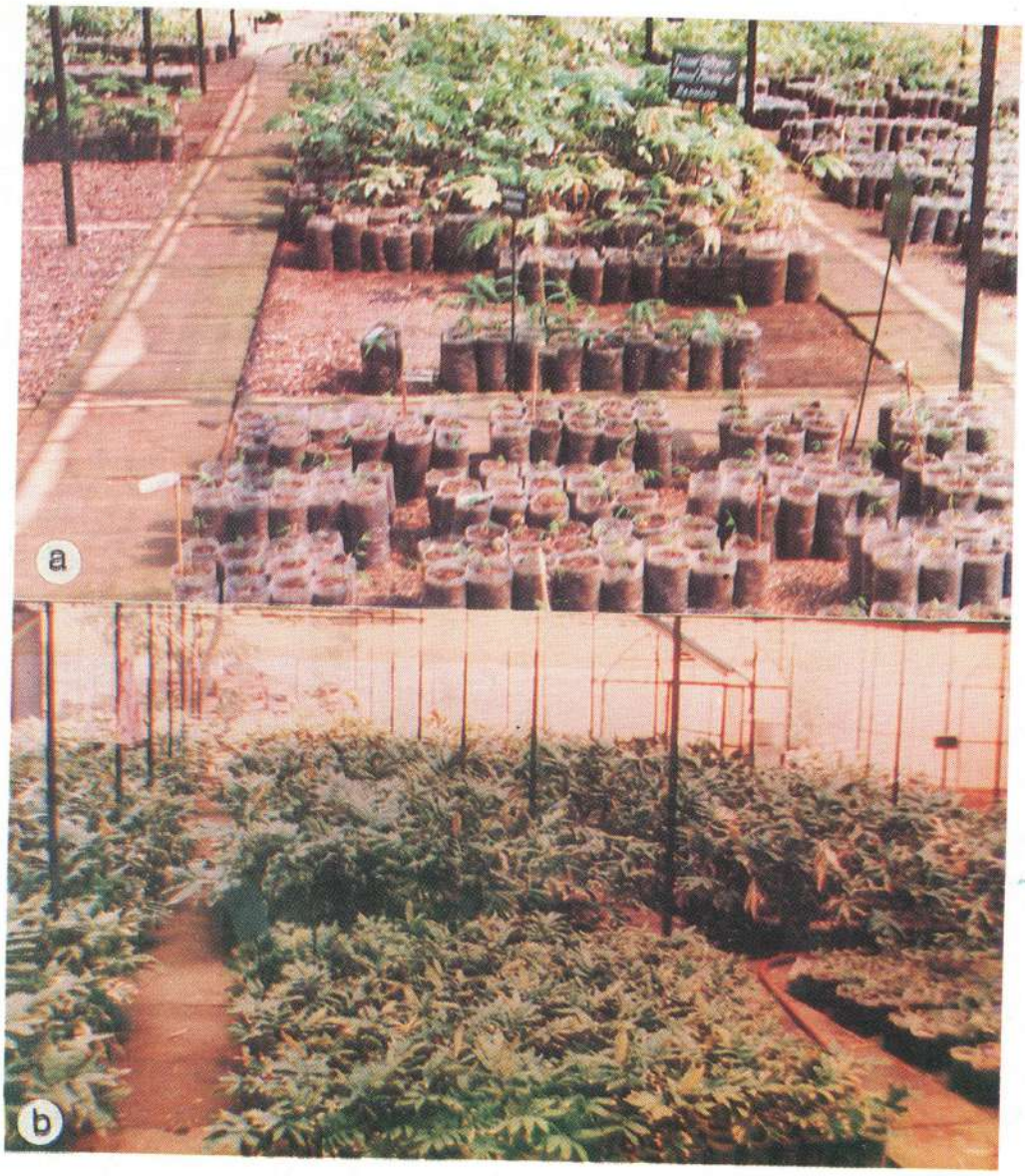

Fig. 5 (a & b) Tissue culture raised plantlets established in shade house.

5. Economic Considerations

The ultimate goal of tissue culture operation is to yield profit as it is the case with any economic activity. It is possible to start a tissue culture work in a small scale, where a few thousands of plants can be produced with an initial cost of a few lakhs of rupees. A rough estimate of initial investiment for small tissue culture unit producing 75,000 - 80,000 plants per annum is given in table 2a. However, this small scale setup may not be a viable unit today. Currently, the use of tissue culture material is so integrated and has assumed such proportions, and competition, that only larger and well managed enterprises have a fair chance to survive. As a consequences more money has to invested in facilities and managers, and now an investiment of 80 - 100 lakhs of rupees is a more realistic starting capital. This will certainly need to be doubled within five years, provided that firm survives the crucial five years.

For all practical purposes, to start a tissue culture production business, it is most desirable to have investors with an agricultural and/or biotechnological background. Thus a deeper understanding of problems involved in working with living material and research results that need scaling up can be expected. The risk factor being high, investor may loose patience sooner than five years bench mark and profits are unrealized.

It is of great importance to know the production cost of tissue culture plant. Keeping records of all media, labour and overhead costs makes it possible to calculate this figure. However, recording all these data and transforming them into an usable tool for the final calculation is not practical, because it is too labour intensive. An attempt has been made to calculate the cost of plantlet in a small scale lab producing 70,000 - 80,000 plants per annum (table 2b). It is worthwhile to mention that the production cost Rs. 3.53 per plant is calculated for *in vitro* operations only, hardening and further establishment cost should be added for the final cost.

Table 2 : Rough estimate for the establishment of Tissue culture unit

a. Infrastructural and Equipments

Item	Cost (000, Rs.)
Hot plate with Magnetic Stirrer	7.00
Autoclave (2 nos.)	25.00
pH meter (2 nos.)	5.00
Balances	15.00
Hot air oven	10.00
Distillation apparatus	25.00
Drying and draining racks	3.00
Laminar flow benches (2 nos.)	50.00
Refrigerator	10.00
Castor racks (5 nos.)	150.00
Sequential timers	30.00
Temperature controllers	30.00
Airconditioners (3 nos.)	90.00
Miscellaneous	50.00
Total	**500.00**

b. Recurring

Chemicals (media)	35.00
Electricity	30.00
Glassware	35.00
Labour	40.00
Supervision	50.00
Equipment	
(i) Maintenance (5 %)	25.00
(ii) Depreciation (10 %)	50.00
Total	**265.00**

Total No. of plants produced = 75,000

* Cost per plant = 265,000/75,000 = **Rs. 3.53**

* includes *in vitro* operation cost only

6. General Remarks

In vitro clonal propagation as a commercial technique is being practiced for a wide range of herbaceous and woody species by nurserymen all over the world. However, many important tree crops, including gymnosperms, whose propagation by conventional methods is difficult have so far remained problematical even in cultures. Considerable research is being done to overcome this limitation. Once a method has been developed and aseptic cultures established it may be tempting to continue to propagate them for many generations. This may lead to bulking up of any off-types that might have arisen in cultures at an early stage. To offset this real problem, it is suggested that only a few thousand plants are raised from a single explant taken from the selected plant.

There has been growing concern about the genetic uniformity of plants produced by tissue culture. If the conservative approach of enhanced axillary branching is adopted for shoot multiplication the chances of the appearance of off-types should be extremely low, if any. It must also be emphasized here that every time a slight change is observed in plants from tissue culture it should not automatically be ascribed to *in vitro* technique as some variability occurs in nature also. Finally, any comparison of the *in vitro* raised plants with *in vivo* propagated individuals should be done after a certain period after putting them out of cultures. The last stages of micropropagation may have a lingering effect on the growth and development of plants.

Micropropagation or clonal propagation may be incorporated wherever possible into existing crop and forestry systems as another tool for multiplying plant material. Commercial propagation of forest trees will be involved with the multiplication of elite genotypes and rare and endangered plant species, where a large number of plants can be produced from a few seeds in a relatively short time. Several woody species like poplars, eucalypts, wild cherry, red wood and radiata pine are now commercially propagated in laboratories, and other species like sandalwood, birch, teak and loblolly pine show promise. With time, micropropagation will become more routine, and will be integrated with forest nursery operations.

Using micropropagation, it is possible to exploit total genetic variance rather then just additive portions. While conventional breeding allows genetic gain in subsequent generations, tissue culture exploits genetic variation within a given generation through the cloning of outstanding individuals for testing and mass propagation. However to gain the maximum benefits, there has to be interaction between breeders in established tree improvement programms and tissue culture scientists.

The cost of generating plantlets *in vitro* varies with species and is dependent on the techniques used. In general *in vitro* multiplication has a higher unit cost than conventional propagation. Currently, the most advanced micropropagation technology avaialble is based on the multi - staged organogenic process which is extremely labour and cost intensive. Therefore, this process can only be profitable for species that cannot be multiplied conventionally, where the cost of macropropagation is high, or in cases where a high return is expected from the resulting genetic gain, disease free stock etc., that will offset the initial high cost.

Studies done on production costs in New Zealand, France, and Canada indicated that tissue culture plants cost three to ten times as much as seedlings, and labour costs represented 60-80 % of the total. Therefore, for *in vitro* techniques to be cost effective, the process has to be partially or fully automated. Also the effeciences of the techniques, namely, shoot multiplication by axillary or adventitious budding, and conversion of somatic embryos and production of artificial seed need to be improved. To aid this process, automatic tissue culture systems are being developed.

Throughout the world, the markets for wood and wood products are expanding. Hardwoods such as oak, walnut, cherry, etc., are viable economic commodities, while the markets for pulp, newsprint, paper and lumber from softwoods are increasing rapidly. Further, in the future, forest management will be viewed as another form of intensive cropping, differing from other agricultural crops mainly in length of the rotation and the nature of the economic end products. To fully exploit the potential for intensive cropping system only superior trees should be utilized for afforestation or reforestation. Undoubtedly, the application of micropropagation technology and other aspects of biotechnology will play a major role in producing the commercial planting stock necessary for the future.

APPENDIX I : Composition of some plant tissue culture media[a]

Constituents	Media (amounts in mg l^{-1})[b]						
	White's[c]	Heller's[d]	MS[e]	ER[f]	B_5[g]	Nitsch's[h]	NT[i]
Inorganic							
NH_4NO_3	-	-	1650	1200	-	720	825
KNO_3	80	-	1900	1900	2527.5	950	950
$CaCl_2.2H_2O$	-	75	440	440	150	-	220
$CaCl_2$	-	-	-	-	-	166	-
$MgSO_4.7H_2O$	750	250	370	370	246.5	185	1233
KH_2PO_4	-	-	170	340	-	68	680
$(NH_4)_2SO_4$	-	-	-	-	134	-	-
$Ca(NO_3)_2.4H_2O$	300	-	-	-	-	-	-
$NaNO_3$	-	600	-	-	-	-	-
Na_2SO_4	200	-	-	-	-	-	-
$NaH_2PO_2.H_2O$	19	125	-	-	150	-	-
KCl	65	750	-	-	-	-	-
KI	0.75	0.01	0.83	-	0.75	-	0.83
H_3BO_3	1.5	1	6.2	0.63	3	10	6.2
$MnSO_4.4H_2O$	5	0.1	22.3	2.23	-	25	22.3
$MnSO_4.H_2O$	-	-	-	-	10	-	-
$ZnSO_4.7H_2O$	3	1	8.6	-	2	10	-
$ZnSO_4.4H_2O$	-	-	-	-	-	-	8.6
$Zn.Na_2.EDTA$	-	-	-	15	-	-	-
$Na_2MoO_4.2H_2O$	-	-	0.25	0.025	0.25	0.25	0.25
MoO_3	0.001	-	-	-	-	-	-
$CuSO_4.5H_2O$	0.01	0.03	0.025	0.0025	0.025	0.025	0.025
$CoCl_2.6H_2O$	-	-	0.025	0.0025	0.025	-	-
$CoSO_4.7H_2O$	-	-	-	-	-	-	0.03
$AlCl_3$	-	0.03	-	-	-	-	-
$NiCl_2.6H_2O$	-	0.03	-	-	-	-	-
$FeCl_3.6H_2O$	-	1	-	-	-	-	-
$Fe_2(SO_4)_3$	2.5	-	-	-	-	-	-
$FeSO_4.7H_2O$	-	-	27.8	27.8	-	27.8	27.8
$Na_2.EDTA.2H_2O$	-	-	37.3	37.3	-	37.3	37.3
Sequestrene 330Fe	-	-	-	-	28	-	-

Constituents	Media (amounts in mg l^{-1})[b]						
	White's[c]	Heller's[d]	MS[e]	ER[f]	B_5[g]	Nitsch's[h]	NT[i]
Organic							
Inositol	-	-	100	-	100	100	100
Nicotinic acid	0.05	-	0.5	0.5	1	5	-
Pyridoxine HCl	0.01	-	0.5	0.5	1	0.5	-
Thiamine HCl	0.01	-	0.1	0.5	10	0.5	1
Glycine	3	-	2	2	-	2	-
Folic acid	-	-	-	-	-	0.5	-
Biotin	-	-	-	-	-	0.05	-
Sucrose	2 %	-	3 %	4 %	2 %	2 %	1 %
D-Mannitol	-	-	-	-	-	-	12.7 %

[a] Growth regulators and complex nutrient mixtures described by various authors are not included here.

[b] Concentrations of mannitol and sucrose are expressed in percentage.

[c] White

[d] Heller

[e] Murashige and Skoog

[f] Eriksson

[g] Gamborg et al.

[h] Nitsch

[i] Nagata and Takebe

Printed By : Ravi Printing Press, Jabalpur, Phone 21684