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Lesson: Tissue Culture Applications- Part I

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Learning Outcomes

The chapter is aimed at enabling the readers to learn:

- The basic principle behind tissue culture techniques of micropropagation, endosperm culture and virus elimination.
- Major discoveries that led to the development of these techniques.
- Different methods and applications of these techniques.
- The importance of tissue culture in context of both, scientific research and commercial applications.

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Chapter: Tissue Culture Applications – Part I

Introduction

As you would be well aware, there is much talk today about 'Genetically Modified' crops or transgenic crops which are genetically transformed to express desirable traits. This was a remarkable feat in the field of biology. Do you know how many years of basic scientific and technological research it took to achieve this possibility of plant transformation? It took about 88 years. See the Table ahead to get a glimpse of the major discoveries that led to it.

This feat was possible due to active research in three different areas, conducted in the early twentieth century:

- Tissue culture of plants,
- Plant regeneration from single somatic cells,
- Study of crown gall disease.

In this Chapter, we shall focus on the applications of first of the above mentioned research areas i.e. 'Plant Tissue Culture'. It is defined as a system in which cells satisfy two main requirements – (1) remain undifferentiated (2) capable of infinite growth (White, 1939a). We would mainly discuss how the different techniques of plant tissue culture have found applications in different spheres of Plant Biology.

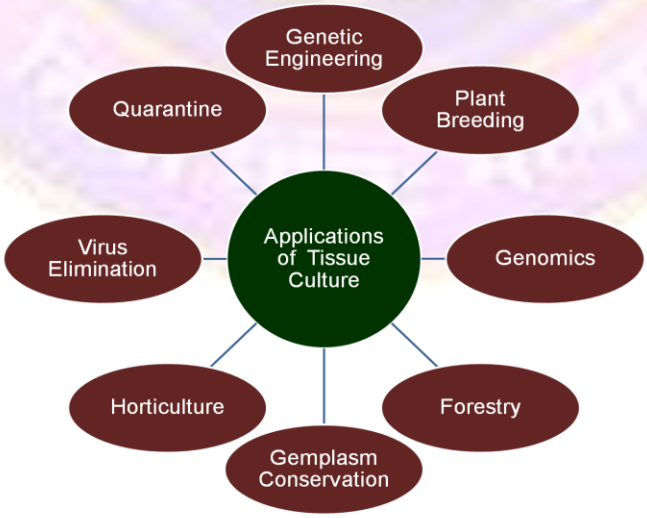


Figure: Various applications of plant tissue culture.

Developed by: Author

1990 — 1st Maize transformation (Gordon-Kamm et al., 1990)		
1980		• Gene gun transformation (Klein et al., 1987; Sanford, 2000) • Chimeric genes (Herrera-Estrella et al., 1983b; Fraley et al., 1983; Bevan et al., 1983) • Binary vectors (Hoekema et al. 1983) • Disarmed plasmids (Willmitzer et al., 1983; Joos et al., 1983) • *tms, tmr, tml* identified (Klee et al.,1984; Barry et al., 1984; Akiyoshi et al., 1984)
1970	•Single cells to somatic embryos (Backs-Hüsemann and Reinert, 1970)	• T-DNA nuclear location (Chilton et al., 1980; Willmitzer et al., 1980) • T-DNA in plasmid (Chilton et al., 1977) • Plasmids in *Agrobacterium* (Zaenen et al., 1974)
1960	•Single cells to plantlets (Vasil and Hildebrandt, 1965a, b)	• Bacterial virulence transferred (Kerr, 1969)
• Controlled Organogenesis (Skoog and Miller, 1957) 1940		• TIP named ((Braun and Mandle, 1948) • Autonomous crown gall growth (Braun, 1943) • 1º vs. 2º tumors (White and Braun, 1941, 1942)
• First true plant tissue culture (White, 1939a). 1920		
1900	•Attempted single cell culture (Haberlandt, 1902)	•Bacterial cause of crown gall (Smith and Townsend, 1907)
PLANT TISSUE CULTURE	**REGENERATION OF WHOLE PLANTS FROM SINGLE SOMATIC CELLS**	**CROWN GALL DISEASE**

Figure: Chronological achievements in Plant Biotechnology.

Source: Sussex, Ian M. "The scientific roots of modern plant biotechnology." *The Plant Cell Online* 20.5 (2008): 1189-1198.

Micropropagation

Principle

Micropropagation is a technique that involves manipulation of small segments of plant material, under aseptic conditions, favorable to the formation of new plants. The plants produced are genetically uniform and free from associations with other organisms.

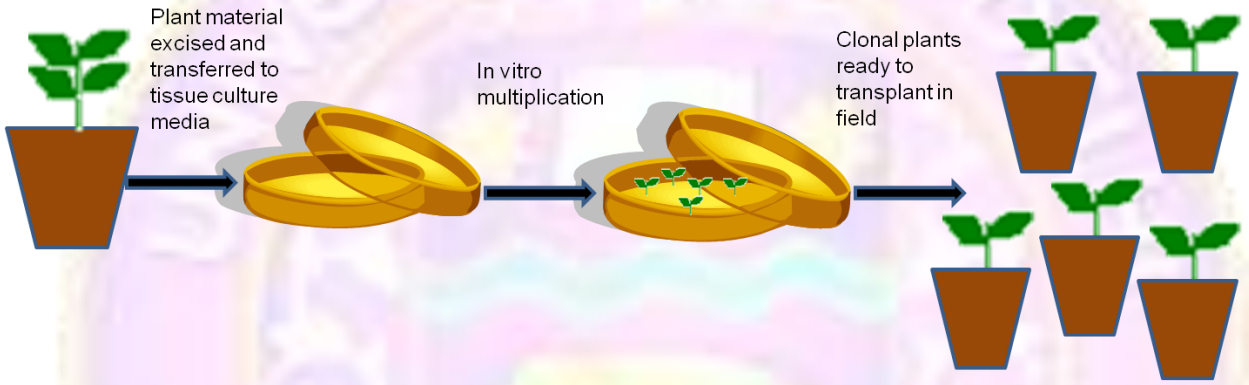


Figure: General overview of micropropagation.

Developed by: Author

Plants may have sexual or asexual mode of reproduction as explained in figure ahead. Propagation of genetically similar progenies of a plant by asexual reproduction is referred to as clonal propagation and a population of plants derived in this way constitutes a clone.

The methods of vegetative reproduction have been used by horticulturists for clonal multiplication of many cultivars. For crop plants, such as banana, chrysanthemums etc. which do not produce viable seeds or produce less seeds, vegetative propagation is the only method for multiplication.

Video link

http://www.cornell.edu/video/history-of-plant-cloning-6-micropropagation-or-plant-cloning

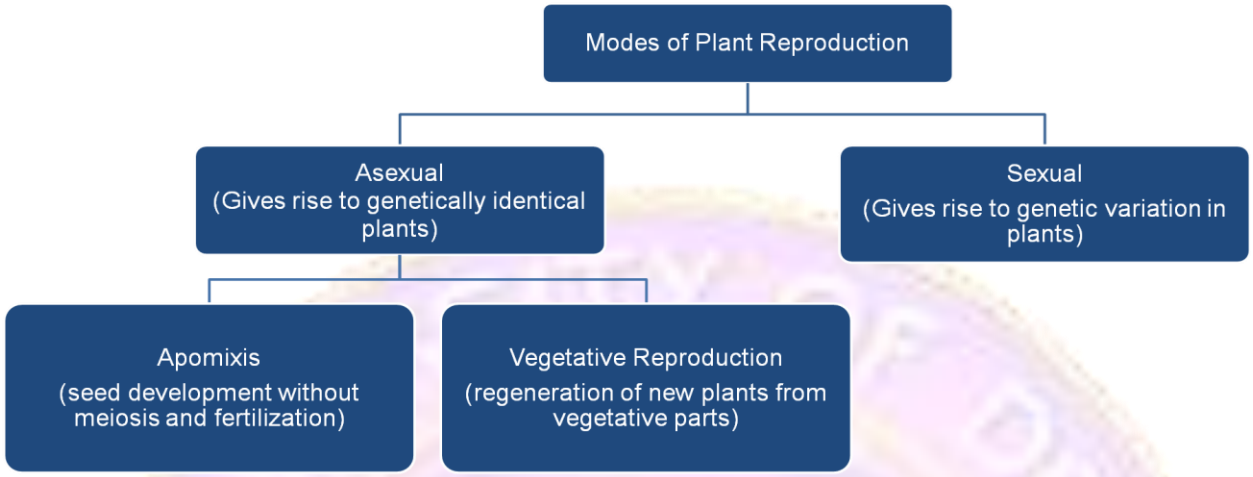


Figure: Modes of plant reproduction

Developed by: Author

Micropropagation is a popular technique for propagating plants vegetatively. Micropropagation is advantageous over the traditional methods as a many plants can be produced from a single explant in a relatively short time and space.

Discovery

While there are many significant achievements in the 'story of development of the technique of plant tissue culture', we should know at least some important discoveries in context of micropropagation. Philip White (1939) showed that excised tomato root tips could grow for potentially infinite time in a liquid medium. Gautheret and Nobe'court(1939) showed culture of carrot root tips. But Skoog and Miller (1957) were the first to demonstrate organogenesis from tobacco callus culture. The credit for initial demonstration of application of tissue culture for clonal propagation of plants goes to G. Morel (1972) who used this technique in orchids.

Methodology

Micropropagation is generally classified into five steps – Stages 0 to 4.

Stage 0: Preparative stage for donor plant

This is the preliminary stage in which the donor plant is prepared such that good explants can be obtained from it, for using in Stage 1. It involves

- Growing of plants in a glasshouse.
- Irrigation in a way to avoid direct overhead watering.
- The stock plants are exposed to culture conditions which are suitable for it.

Such pretreated plants show better quality of explants and reduced contamination problem and need a less harsh sterilization treatment in the next stage.

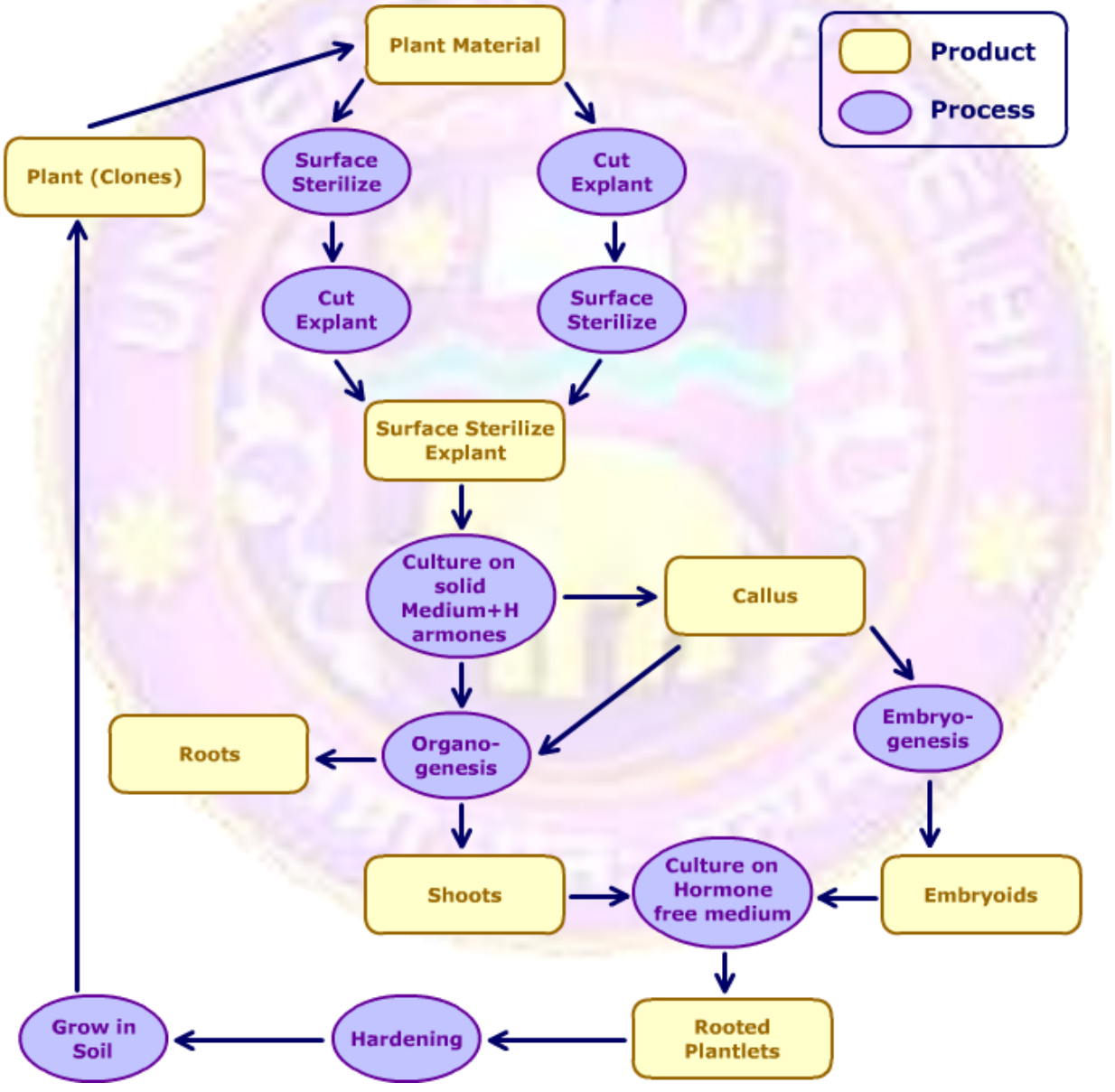


Figure: Flow chart showing steps in tissue culture.

Source: Hussain, Altaf, et al. "Plant tissue culture: Current status and opportunities." *Recent advances in plant in vitro culture* (2012): 1-28.

Stage 1: Initiation of cultures

This stage involves excision of plant material, sterilization of plant material, tissue culture media, instruments and work space to be used followed by transfer of the explants to culture media to begin micropropagation.

(i) **Explant**

Shoot tips, nodal or stem cuttings, leaves or even embryos can be used as explants for *in vitro* propagation depending on the technique to be followed for shoot multiplication.

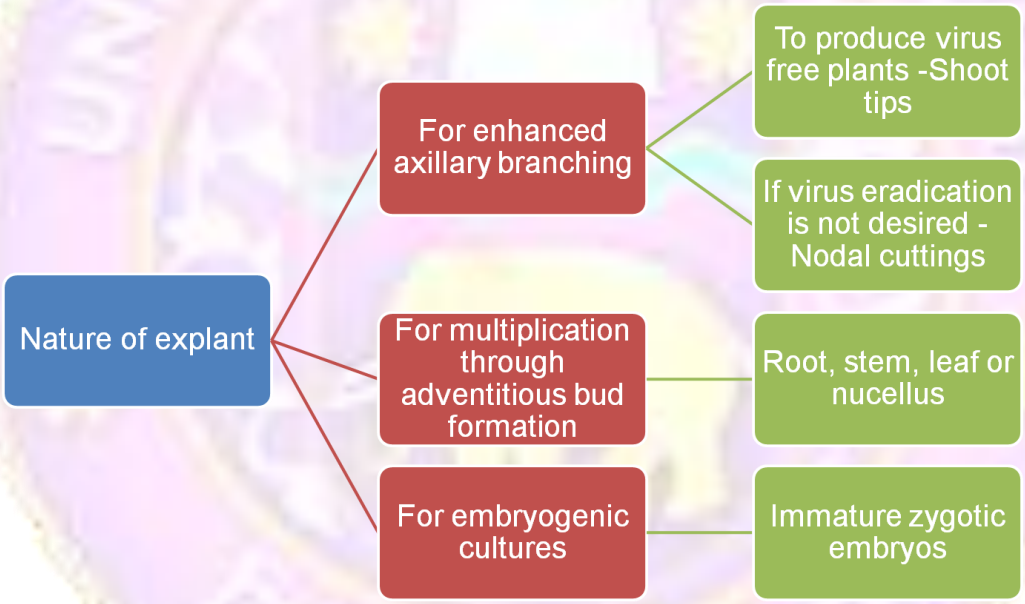


Figure: Nature of explants used depends on the basis of method of shoot multiplication desired.

Developed by: Author

Factors affecting response of explants

Different factors may affect the response of explants to tissue culture like,

- Physiological condition of the donor plant at the time of explant excision.

- Stage of growth of plant - Explants taken at the start of the growing season which show active growth, generally give best results.
- Seasonal fluctuations – Their effect may be decreased by maintaining the parent plant in controlled conditions in glasshouse or growth cabinets.

(ii) **Sterilization**

a) **Tissue culture media** contains nutrient which are favourable for growth of microbes also. Therefore, there are chances that it becomes contaminated with microorganisms. These contaminants may release substances which are toxic for the growth of plant and the plant may be killed by these contaminants. Therefore, it is important to maintain aseptic culture conditions.

b) **Instruments** should be sterilized before starting tissue culture experiments and also while the experiment is being carried out.

c) **Plant material** may carry a wide range of microbial contaminants. Therefore, the explant itself should be decontaminated by surface sterilization before planting it on the culture medium. The concentration of the sterilizing agent and the duration of treatment should be such that the plant tissue is minimally harmed by the treatment as surface sterilization is also toxic to the plant tissue.

d) **Transfer area** To prevent the entry of any contaminant into the culture vessel when it is opened for inoculation or subculture, transferring steps are carried out under aseptic environment i.e. in laminar air flow chambers.

How does a laminar flow hood function?

The main parts in a laminar flow hood are - filter pad, a fan and a HEPA (High Efficiency Particulates Air) filter. The air is sucked by the fan through the filter pad which traps the dust. The prefiltered air passes the HEPA filter which removes contaminating fungi, bacteria, dust etc. The sterile air now flows into the working area where work can be done without risk of contamination.

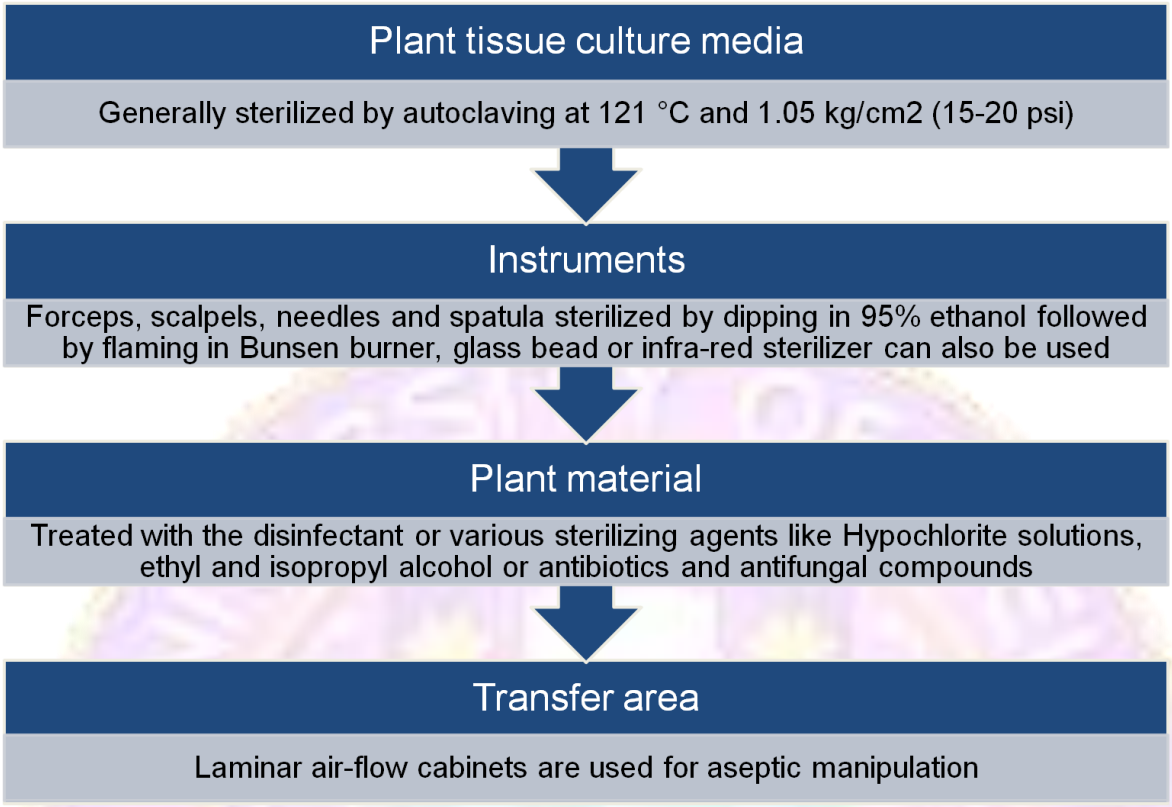


Figure: Schematic representation of procedure of sterilization.

Developed by: Author

Stage 2: Multiplication

This is the most important step in micropropagation. Mainly, three methods are used to carry out *in vitro* multiplication.

(i) **By callusing** - Differentiation of plants from cultured cells may occur via

a. Organogenesis or

b. Somatic embryogenesis

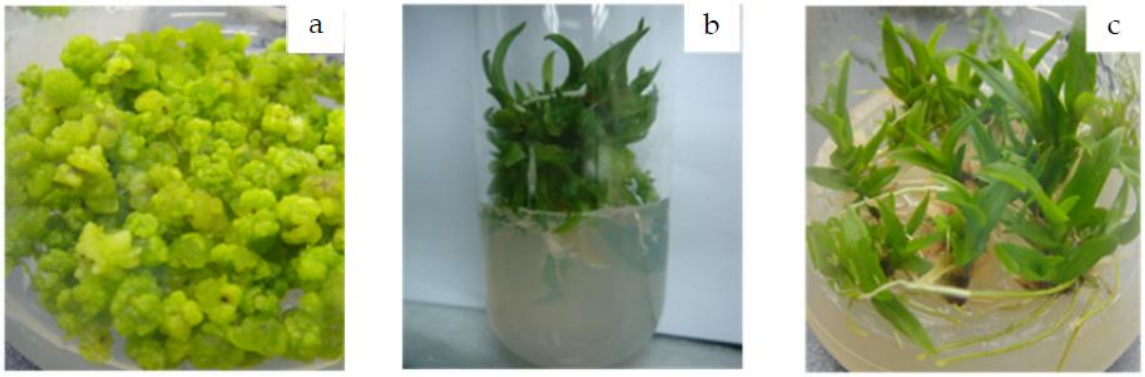


Figure: Orchid micropropagation (a) callus culture (b) regeneration of shoots(c) rooting of plantlets

Source: http://cdn.intechopen.com/pdfs-wm/40180.pdf

Somatic embryos are bipolar structures, with well defined root and shoot meristem. Therefore, somatic embryogenesis once established lends itself to better control than organogenesis. Unlike microcuttings, somatic embryos be stored by dessicating and preserving them at low temperatures, to use later. Because of these characteristics somatic embryogenesis is considered a better system for micropropagation than organogenesis.

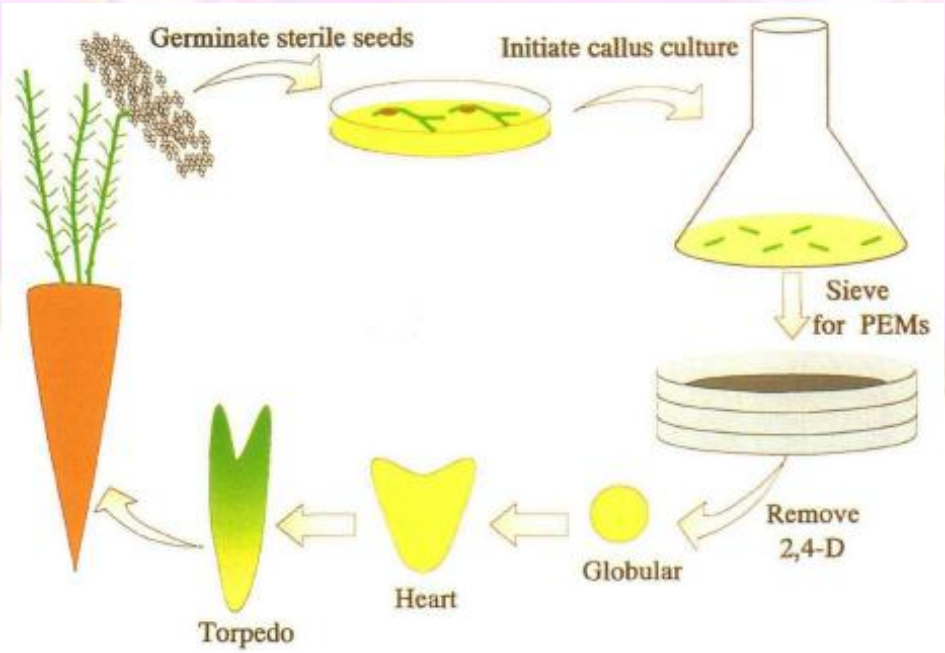


Figure: Summary of the Culturing of Carrot Somatic Embryos.

Source: Zimmerman, J. Lynn. "Somatic embryogenesis: a model for early development in higher plants." *The Plant Cell* 5.10 (1993): 1411.

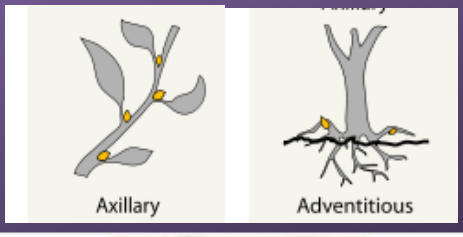
(ii) **Adventitious bud formation** – Adventitious buds are those which arise from any place different from the leaf axil. These adventitious buds can be induced to form on leaf or stem explants by using appropriate phytohormones. This method can thus be used to carry out micropropagation in plants which do not otherwise follow vegetative mode of propagation.

(iii) **Enhanced axillary branching** - Axillary buds are those which are present in the axil of leaf, and can develop into a shoot. These axillary buds may stay dormant for various durations in different species. In species with a strong apical dominance, the terminal bud needs to be removed to stimulate the next axillary bud to form a shoot. In tissue culture, the rate of multiplication of shoots by axillary branching can be increased by growing shoots in culture medium with appropriate concentration of the hormone cytokinin. The shoots originally regenerated from the buds on explants develop new axillary buds. These axillary buds may directly give rise to shoots or they may further give rise to more axillary buds. At this stage, shoots can be cut and transferred to a fresh medium where the shoot multiplication cycle can be carried out again.

This method is the most frequently used one for micropropagation since it enables the production of uniform plantlets without any genetic variation. This technique is also called multiple-bud induction. It has been used to propagate many economically important plants.

What is the difference between an axillary and an adventitious bud?

Axillary buds are those which are located in the axil of a leaf while adventitious buds are those which occur elsewhere, for example on trunk or on roots. Visit link - http://upload.wikimedia.org/wikipedia/commons/e/eb/Plant_Buds_clasification.svg



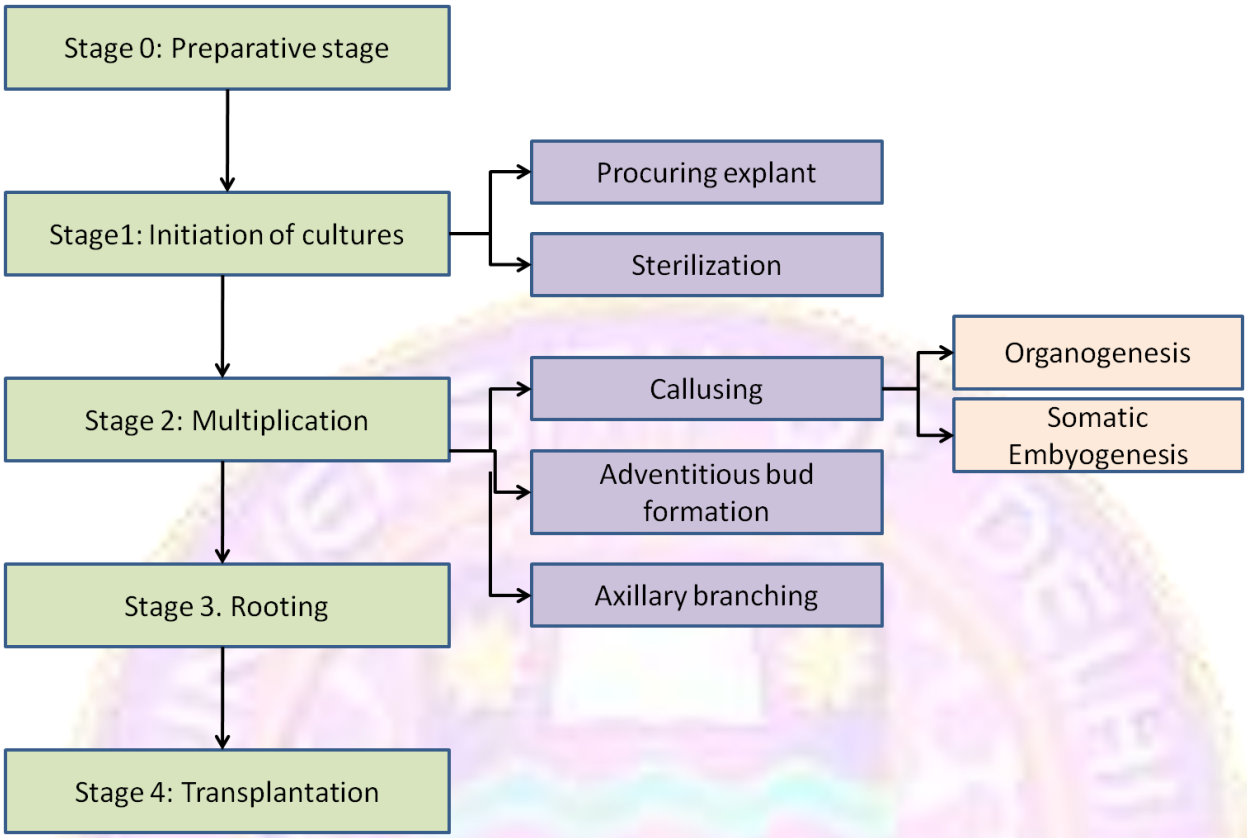


Figure: Different stages involved in micropropagation.

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Stage 3: Rooting of shoots

Somatic embryos carry a radical and plumule but the shoots regenerated from axillary or adventitious buds need additional treatment so as to become capable of normal germination. This step involves transfer of these shoots to a culture medium suitable to induce rooting.

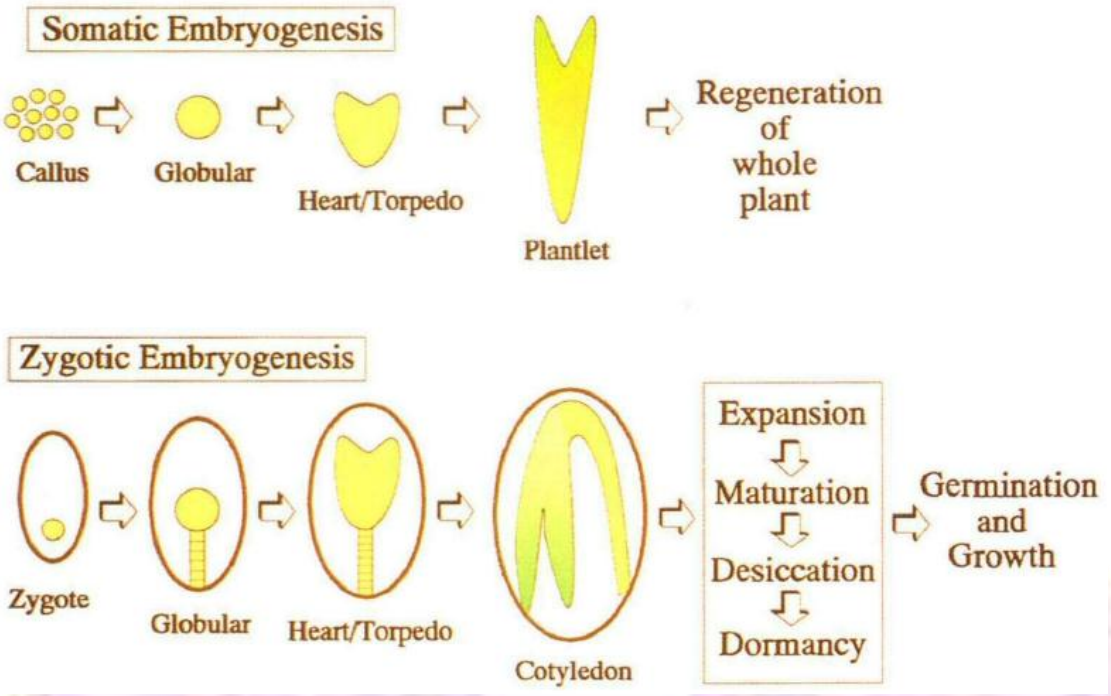

Figure: A Comparison of Somatic and Zygotic Embryogenesis.

Source: Zimmerman, J. Lynn. "Somatic embryogenesis: a model for early development in higher plants." *The Plant Cell* 5.10 (1993): 1411.

Figure: *In vitro* culture of *Jatropha curcas* (a) Callus (b) regeneration of shoots (c) induction of roots.

Source: http://cdn.intechopen.com/pdfs-wm/40180.pdf

Stage 4: Transplantation

The plantlets raised *in vitro* face unique conditions in terms of nutrients, light, humidity and gaseous environment. They are raised rapidly but they may suffer from various structural and physiological abnormalities, which may make them unsuitable if they are exposed to natural environmental conditions.

They may suffer from mainly two kinds of deficiency:

(i) They exhibit poor control over water loss.

(ii) Their mode of nutrition becomes heterotrophic.

Therefore, they require a process of gradual acclimatization which prepares them for the transition from laboratory conditions to the field conditions. This stage involves following steps:

- The lower parts of the explants are washed gently for the removal of culture medium.
- The individual shoots or plantlets are then transferred to pots which contain soil with some inorganic nutrients. These pots should be well irrigated.
- For the initial 10-15 days, high humidity (90-100%) is maintained around the plants, to maintain a similar environment as is present in *in vitro* conditions.
- The humidity is gradually decreased to a normal level over a period of two to four weeks.
- After a period of four to six weeks, the plants are moved from low light conditions to high light conditions.

By this time the plants generally become acclimatized and are shifted to a net house or field for better growth. During this phase, the soil in which plants are growing is irrigated regularly and the supply of inorganic nutrients is maintained by fertilizers.

Video Power point presentation link

http://irrecenvhort.ifas.ufl.edu/Propagation/modules/module6/chapter-17-18/player.html

Applications

Micropropagation can be used to produce, maintain, propagate and transport pathogen-free plants safely and economically.

1. **Agriculture**

   a) Development of new cultivars

   Desirable genotypes can be produced through genetic transformation in plants. Genes from other species or even other organisms can be transferred into any crop of our interest. Also, in tissue culture, plants can be propagated throughout the year. Since plants can be multiplied at a fast pace through tissue culture, this can lead to reduction in the duration of selection and release of newly developed cultivars.

   b) Mass clonal multiplication

   It is currently the most popular application of micropropagation. Through this method, millions of plants can be generated starting from a small plant tissue, within a year. Such a fast rate of plant propagation cannot be achieved by *in vivo* methods. An advantage of tissue culture propagation is that the shoot multiplication cycle is very short (few weeks), each cycle producing an exponential increase in the number of shoots.

   c) Quarantine

   Micropropagation can be used to quarantine the new crops introduced from outside a country.

   d) Pathogen elimination

   Micropropagation is also an effective way to eliminate viruses and other pathogens. In fact, the first plants to be mass-produced by micropropagation were virus-free clones of *Cymbidium* orchids. Potato is another plant that is often troubled with virus infections. Potatoes are normally propagated vegetatively by buds, or "eyes," on the tubers, and any virus infection is readily carried from one generation to the next. The most effective way to eliminate potato viruses is by micropropagation of virus free lines through shoot-tip cultures.

   e) Stock maintenance

In commercial nurseries *in vitro* methods can be used to decrease the growing space usually provided for the maintenance of stock plants. Several thousand million plants can be maintained inside culture vials on a shelf space.

Figure: *In vitro* storage of banana germplasm at Musa International Transit Centre, France.

Source: http://www.bioversityinternational.org/research-portfolio/conservation-use-of-bananas-tree-crops/international-musa-germplasm-transit-centre/

f) Importance for dioecious plants

Vegetative propagation is extremely important in the case of dioecious species where seed progeny yields male and female plants in a ratio of 1:1, but the economically one of the sexes may be more desirable than the other. For example, in *Asparagus officinalis*, male plants have more economic importance than their female counterparts but their vegetative propagation is not possible. However, *in vitro* methods can be used for its multiplication. In the case of papaya, female plants are preferred for commercial purposes but male and female plants can only be distinguished at the flowering stage. Therefore, micropropagation can be done to produce female plants.

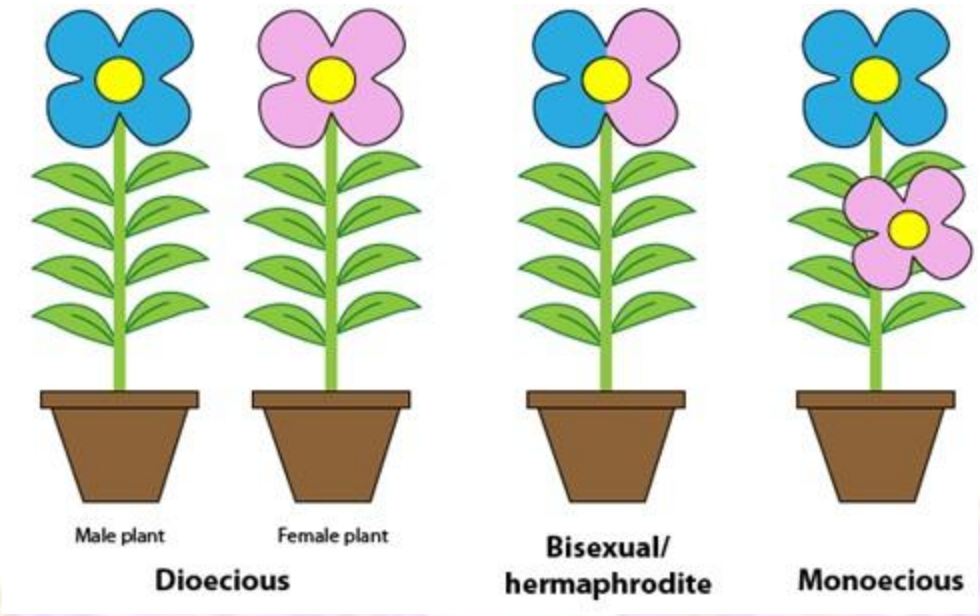

Figure: Cartoon showing monoecious and dioecious conditions. Blue flowers represent male flowers and pink flowers are the female ones.

Source: http://plantbreeding.coe.uga.edu/index.php?title=4._Plant_Reproductive_Systems

2. Horticulture

Many horticultural and plantation crops are propagated *in vitro* on commercial scale. In the floral industry, it is commonly used to produce clones of cultivars (varieties under cultivation) that are particularly popular because of flower color or other characteristics.

Recognized Tissue Culture Production Units in India Till date

88 commercial Tissue Culture Production Units have been recognized by the Department of Biotechnology (DBT), Govt. of India under the "National Certification System for Tissue Culture Raised Plants (NCS-TCP)".

http://dbtncstcp.nic.in/html/content/Recognized_TCPU%27s.pdf

3. Conservation of biodiversity and threatened plants

In vitro conservation and propagation of endangered plants is another important application of this aseptic technique. (Refer to the chapter Tissue Culture Applications – Part III for details)

4. Forestry

In vitro clonal propagation is commercially used for many herbaceous and woody plant species. The technique is used extensively in the production of forest tree species for planting in tree plantations and reforestation efforts because it avoids the tedious process of collecting and germinating seeds. Moreover, trees with superior characteristics can be cloned for higher productivity. On the other hand, cloning trees for commercial pulp and logging industries does reduce the genetic diversity of the forest, which could have long-term detrimental effects. Genetic diversity enables some members of a population to survive stresses that might damage others. In a cloned forest, however, if the clone turns out to be particularly susceptible to drought or a new disease, the entire forest is at risk. For example, micropropagation has been used on a large scale for conservation of *Dalbergia* species in India.

Limitations of micropropagation

- **Hyperhydration**

  Hyperhydration (earlier called vitrification) refers to the morphological, physiological and metabolic abnormalities affecting *in vitro* culture. It occurs during intensive shoot multiplication stage. Besides the common abnormalities shown by *in vitro* growing plants, hyperhydrated shoots have short internodes and appear thicker, brittle, and water-soaked. They generally exhibit slow growth and develop necrosis and may ultimately die.

  The chances of hyperhydration to occur may be decreased by taking some precautions like: (1) increasing the concentration of agar (2) using an agar which has a higher gelling strength (3) improving the aeration of the container (4) overlaying of medium with paraffin (5) using a desiccant such as CaSO4 and silica gel and (6) bottom cooling of the culture vial.

Figure: Hyperhydric Vs Healthy Plant

Source: "Hyperhydric Vs Healthy Plant" by Mariyana Ivanova and Johannes Van Staden - Influence of gelling agent and cytokinins on the control of hyperhydricity in *Aloe polyphylla*.

- **Off types**

  In vitro raised plant populations may show presence of many off-types. The abnormalities induced *in vitro* are influenced by

  - ✓ The choice of explant and the constituents of culture medium, especially the concentration of phytohormones.
  - ✓ Duration of culture.
  - ✓ Genotype of the donor plant.

  Generally, the plants raised through callus culture show maximum aberrations. Therefore, the callus phase, unless unavoidable, must be minimized.

- **Contamination**

  Contamination is a serious problem in tissue culture and it can cause great losses if it occurs at an advanced stage of the production. There are many causes of contamination:

  - ✓ Slow growing bacteria may be present in the explants and the infection may be visible only at a later stage.
  - ✓ Fungal spores may be carried to the culture through air or even the workers, if proper precautions are not undertaken
  - ✓ Vectors like mites and thrips may be present in the culture vessels and if overlooked they may become contaminating agents.

  It is, therefore, extremely important to maintain high standards of aseptic conditions in and around the working area.

- **Oxidative browning**

The injury caused due to excision of explants, some phenolic compounds are leached out from cells, which readily oxidize to produce quinines, thus causing discoloration. Such phenol oxidation products may be toxic and result in necrosis and ultimately, death of the explant. To overcome this problem, quick transfer of explants within the same vessel or to fresh medium at short intervals can be carried out.

- **Recalcitrance of adult-trees**

  The desirable traits in trees can only be observed when they reach an adult stage. But at this stage they become recalcitrant for tissue culture. There are two ways to overcome this problem:

  ✓ The most juvenile tissue can be selected as explants from the adult trees.

  ✓ The parts of the desired plants may be rejuvenated by treatments like cytokinin spray on selected branches. The explants may then be used from such newly derived parts.

- **High costs**

  Although there are many crops for which tissue culture has been adopted at a commercial scale, but it still remains an expensive proposition as compared to methods of vegetative propagation. Therefore, its use is justified only in cases where the desired objective cannot be met by conventional methods.

Virus elimination

Plants too, like animals and humans, can get infected by bacteria, fungi and viruses. How to cure a plant if it gets a viral infection? Elimination of viruses and other pathogens is important to optimize yield and also to facilitate the transfer of living plant material across countries. If the plants are infected with bacteria or fungi, chemical compounds are available for their treatment, but there are no chemicals available for virus-infected plants.

If the complete population of cultivar is not infected it is possible to develop healthy stock by selecting the uninfected plants and multiplying them by vegetative propagation, but what is the way out if the entire population carries the infection? Well, the only solution left to obtain pathogen-free cultivar stock is complete removal of the pathogen from a vegetative part of the plant followed by regeneration of complete plants from it. Once a pathogen-free

plant is obtained it can be used for vegetative propagation. Why and how is it possible through tissue culture? We shall discuss it in this section.

Principle

A unique property of virus infected plants which enables us to rescue them from infection is the uneven distribution of viruses in plants. The apical meristem is usually free or carries a very low titer of the viruses. In older tissues the concentration of virus generally increases with the distance from the meristem tips. The reasons for this uneven distribution may be:

- Viruses move in a plant through xylem and phloem which are not present in the meristematic region.
- Virus may alternatively move from cell to cell through plasmodesmata, which is a rather slow way to keep pace with the actively growing shoot apex.
- Actively dividing meristem cells have high metabolic activity which does not allow virus multiplication.
- The 'virus inactivating immune systems' in the plant body, if any, are more active in the meristematic region.
- Shoot apices have a high level of endogenous which may inhibit multiplication of viruses.

Therefore, meristem tip culture can be used for virus elimination.

Discovery

The knowledge of the distribution gradient of virus in the shoot tip enabled Holmes (1948) to obtain virus-free plants from infected plants of Dahlia through shoot-tip cuttings. Morel and Martin (1952) also used the same principle to develop the technique of meristem-tip culture for *in vivo* virus elimination.

Methodology

The apical meristem of a shoot is defined as the portion distal to the youngest leaf primordium. The apical meristem together with 1 - 3 young leaf primordial constitutes the shoot apex.

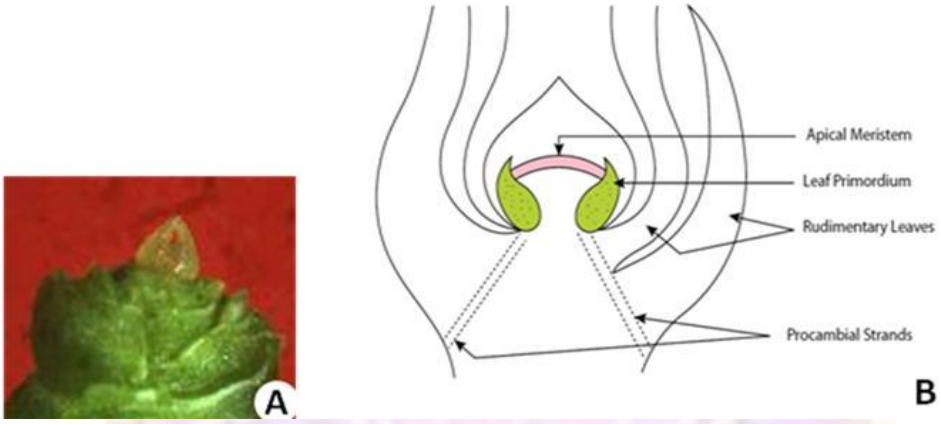

Figure: A. A shoot-tip explant; B. A cross section of shoot-tip meristem

Source: http://nptel.ac.in/courses/102103016/7

Virus-free plants are raised by culturing 100-1000/~m long explants from the shoot apex, called as 'meristem tips'.

Virus indexing

After subjecting the meristem-tips to *in vitro* culture, only a proportion of the cultures yield virus free plants. Therefore, all plants are tested for specific viruses before being used as mother plant to produce virus-free stock. The following tests can be performed for virus testing:

- Physical examination of leaves and stem for visible symptoms characteristic of the virus.
- Sap transmission test/bioassay test'/'infectivity test' - Leaves are ground in phosphate buffer to release sap which is used to swab the leaves of the indicator plant (a plant very susceptible to specific viruses). It is checked whether the symptoms appear on the indicator plants. It is a sensitive test but is a slow process requiring several days to months.
- Enzyme-linked immunosorbant assay (ELISA) – It is a serological test which allows quick detection of important viruses. It relies on the use of antibodies prepared against the viral coat protein.

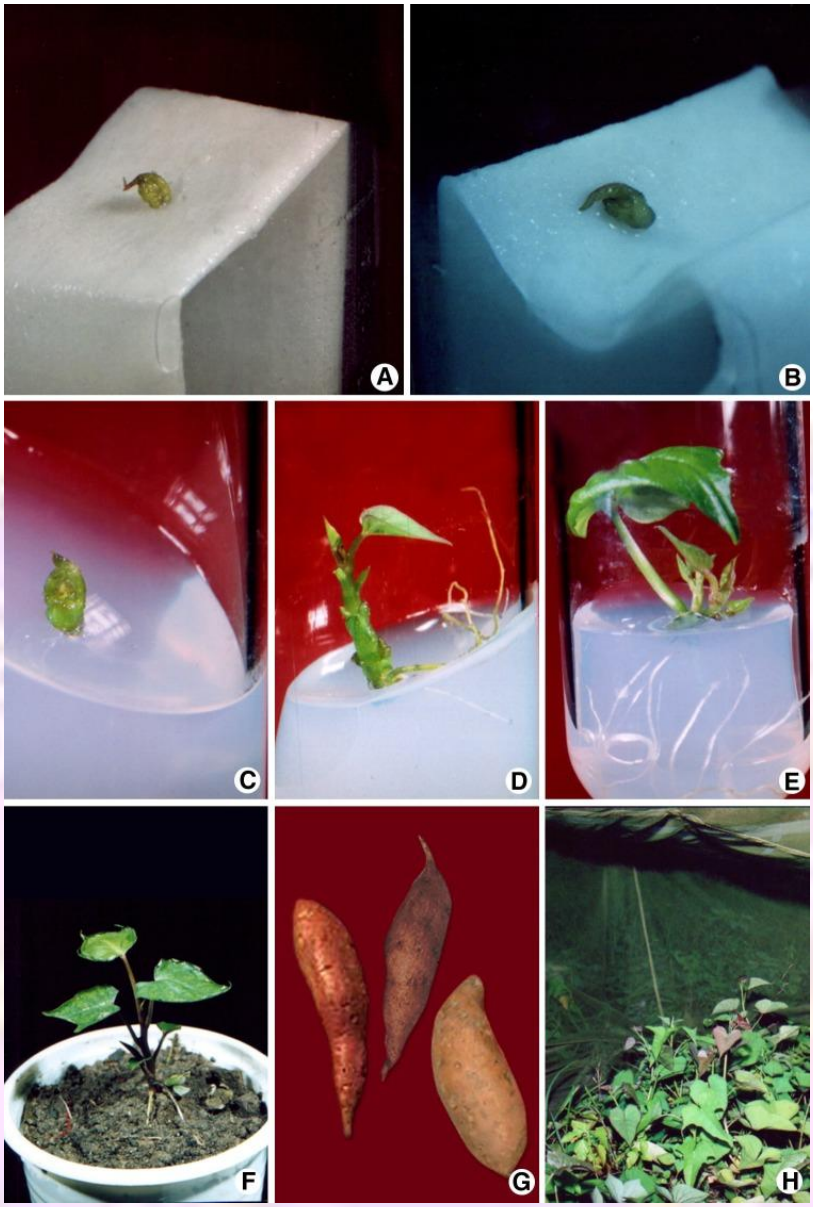

Figure: Meristem culture for Viral elimination in sweet potato. a Isolated apical meristem (6 days old) was developed on filter paper placed in liquid MS medium. b Shoot initiation after 14 days Shoot with a primary leaf developed after subculture in semisolid medium. d Complete plantlet with root after transfer to a semisolid medium. e Multiplication of plantlets using nodal explant. f Acclimatization of plantlets. g Storage root developed in plants derived from meristem-tip culture. h Meristem-derived plantlets in greenhouse.

Source: Alam, Iftekhar, et al. "Elimination and detection of viruses in meristem-derived plantlets of sweet potato as a low-cost option toward commercialization." *3 Biotech* 3.2 (2013): 153-164.

What is ELISA?
ELISA is a tool used for diagnosis of antigens, in medicine, plant and animal pathology, and also as a quality-control method in various industries. Antigens from the sample to be tested are attached to a surface where a specific antibody is applied which binds with the antigen. This antibody is then linked to an enzyme. Finally, a substance which contains the enzyme's substrate is added. The subsequent reaction produces a signal which can be detected, it is most commonly a color change in the substrate. Video link - www.youtube.com/watch?v=Tp61S-2F2B4

Thermotherapy

Often, apical meristems are not always free of virus and it can't be considered as a universal occurrence. For example, Tobacco Mosaic Virus (TMV), Potato Virus X (PVX) and Cucumber Mosaic Virus (CMV) invade the meristematic region of the shoot tips and interrupts growth. In such cases, virus-free plants have been obtained by combination of meristem-tip culture and thermotherapy. In this technique, the mother plants are exposed to heat treatment before excising the meristem-tips or, alternatively, shoot-tip cultures are grown in high temperature regimes (35°C-40°C) for certain duration (6h to 6 weeks) to obtain virus free plants.

Chemotherapy

Chemotherapy is the treatment by chemicals to kill micro-organisms. It will not eradicate the virus completely. However, a large number of antibiotics, growth regulators, amino acids, purines and pyrimidines can be tested for inactivation of viruses. The efficacy of the compound may vary with the virus and the host genotype. A nucleotide analogue ribavirin has been found to be the most efficient for eliminating plant viruses.

Species	Procedure	Virus	Reference
Alstromeria sp.	meristem culture	*Alstroemeria mosaic virus* (AlMV)	Chiari and Bridgen, 2002
Chrysanthemum sp.	meristem culture	*Cucumber mosaic virus* (CMV)	Verma et al., 2004
Chrysanthemum morifolium cv. Regol Time	meristem culture, chemotherapy and thermotherapy	*Chrysanthemum B Carla virus* (CVB)	Ram et al., 2005
Chrysanthemum morifolium	meristem culture	mixed infection by CMV and *Tomato aspermy virus* (TAV)	Kumar et al., 2009
Chrysanthemum sp.	meristem culture	*Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Iris yellow spot virus* (IYSV)	Balukiewicz and Kryczynski, 2005
Dianthus gratianopolotanus	meristem culture	*Carnation mottle virus* (CarMV), *Carnation latent virus* (CLV), potyviruses	Fraga et al, 2004a
Lilium sp.	meristem culture	*Lily symptomless virus* (LSV)	Allen, 1975
L. x elegans	meristem culture and thermotherapy	LSV	Nesi et al., 2009
New Guinea Impatiens (*I. hawkerii*)	meristem culture	mixed infection by TSWV and CMV	Gera and Dehan, 1992
I. hawkerii	meristem culture	TSWV	Milošević et al., 2011
Phlox paniculata	meristem culture and thermotherapy	CLV, CarMV, CMV, *Tobacco mosaic virus* (TMV), *Tospoviruses* (subgroups I, II and III), *Potyviruses*	Fraga et al., 2004b
Viola odorata	meristem culture	*Viola mottle virus* (VMV), CMV, *Bean yellow mosaic virus* (BYMV)	Van Caneghem et al., 1997

Figure: Elimination of viruses from ornamental plants by shoot meristem culture or its combination with other biotechnological procedures

Source: Milošević, Snežana, et al. "Virus elimination from ornamental plants using in vitro culture techniques." Pesticidi i fitomedicina 27.3 (2012): 203-211.

Endosperm Culture

Principle

Endosperm is a triploid tissue formed by fusion of one male and two female nuclei (double fertilization) and it is present in developing seeds of more than 80% of angiosperms. *In vitro* culture of endosperm enables regeneration of triploid plants, which are of considerable commercial importance in many species.

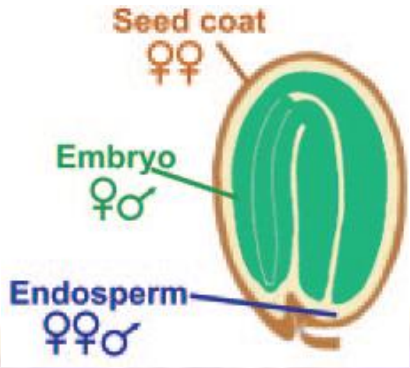

Figure: Schematic representation of Arabidopsis seed.

Source: Sundaresan, Venkatesan. "Control of seed size in plants" *Proceedings of the National Academy of Sciences of the United States of America* 102.50 (2005): 17887-17888.

Discovery

Johri and Bhojwani (1965) first demonstrated direct shoot formation from cultured mature endosperm of *Exocarpos cupressiformis*.

Methodology

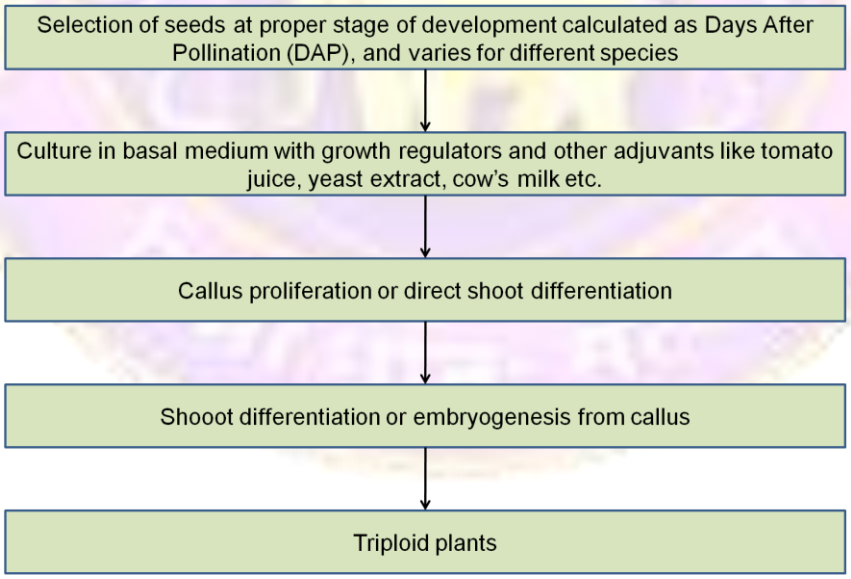

Figure: Flowchart showing basic steps in endosperm culture.

Developed by: Author

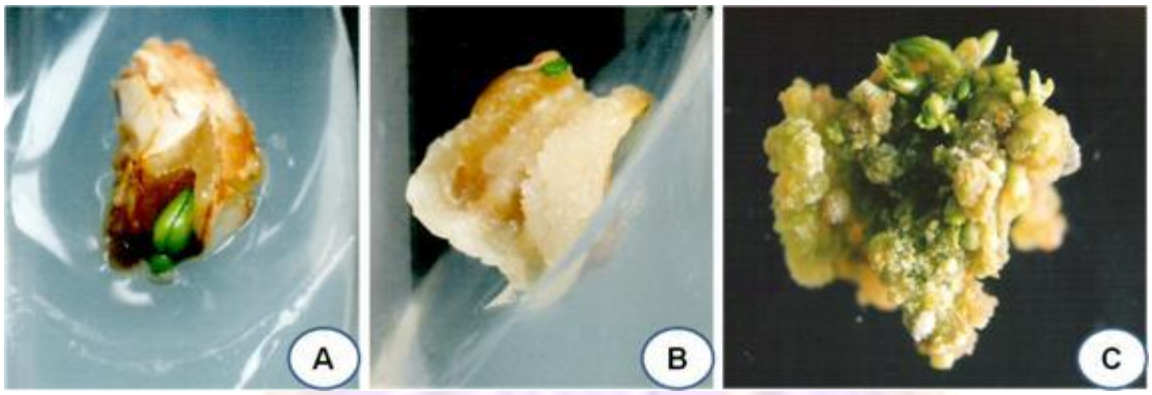

Figure: Shoot regeneration from endosperm culture of *Azadirachta indica* : **A.** An immature seed was cultured It split open after 2 weeks to release the embryo (green) and callused endosperm , **B.** White endosperm callus can be seen in the fully opened seed (after 3 weeks). **C.** Subculture of endosperm callus (6 week old) showing differentiation of shoots and nodules.

Source: Thomas, Thuruthiyil Dennis, and Rakhi Chaturvedi. "Endosperm culture: a novel method for triploid plant production." *Plant Cell, Tissue and Organ Culture* 93.1 (2008): 1-14.

Applications

Conventionally, triploids are produced by hybridization between diploids and tetraploids (produced by chromosome doubling). Endosperm culture overcomes following limitations of traditional ways of triploid plant production –

- Hybridization is carried out by first producing tetraploids from diploids using colchicine treatment (refer chapter Tissue Culture Applications – Part II). But the rate of formation of tetraploids is generally low (7–22%).
- The process of hybridization is time consuming and laborious.
- The crossing of tetraploids and diploids may not always be successful.
- If the cross is successful, the rate of seed set, seed germination and seedlings survival is low.
- The triploids prepared in this way may not show uniform behavior because of their genotype (the genes would have segregated first at tetraploid level and subsequently after the cross with diploid).

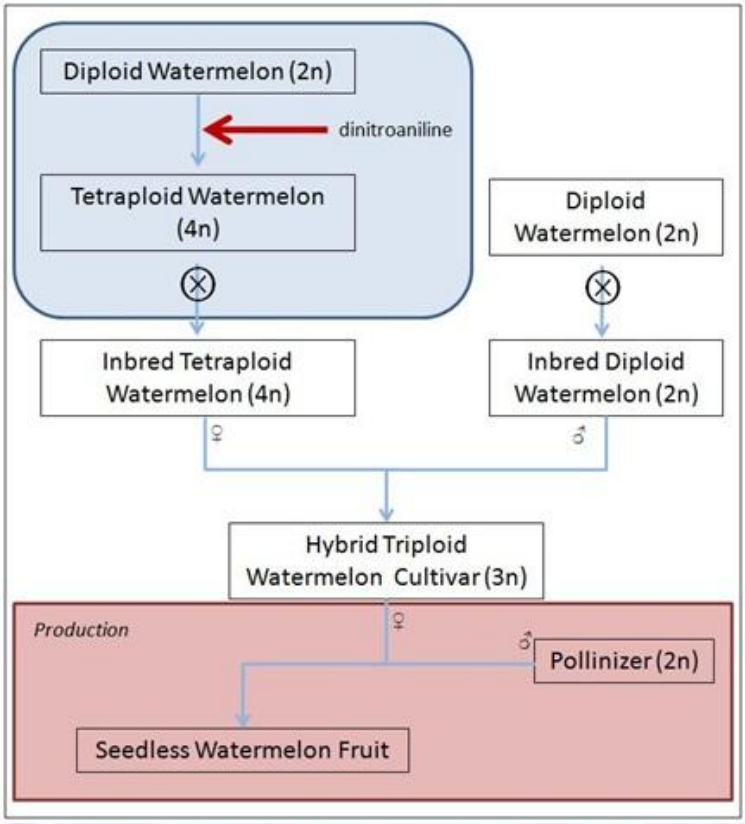

Figure: A flowchart depicting the crosses invoved in the production of triploid watermelon.

Source: http://plantbreeding.coe.uga.edu/index.php?title=5._Polyploidy

The triploid plants have following applications:

1. **Seedlessness of triploid plants**

   Usually the triploid plants are sterile, which is undesirable if seeds are of commercially important. But in plants where the seedlessness improves the quality of fruits like banana, papaya, grapes, citrus, watermelon etc. triploid plants can be very useful.

2. **Vigorous growth**

   Triploids generally grow more vigourously than the diploids. Hence, in plants where the vegetative parts are the economically important parts, triploids are very useful. For example, triploid *Populus tremuloides is* preferred over its diploid counterparts as it contains superior pulp quality. Triploid tomato produces larger and tastier fruits

than diploids. Triploid rice have been shown to have broader leaves, faster growth rate, and more tillers than the normal diploids.

Summary

Tissue culture is a term used to collectively refer to different methods which are used to maintain and propagate plant cells and/or organs *in vitro* under aseptic conditions. The property of plant cells which allows this is 'totipotency', which means that each living cell can give rise to an entire organism.

There are many techniques of tissue culture designed to fulfill different objectives of scientific research as well as commercial applications.

Micropropagation allows clonal propagation of plants in a laboratory. It involves various steps starting from procurement of explants from a donor plant and its culture in sterile conditions in tissue culture media culminating in its development into a complete plantlet through somatic embryogenesis or organogenesis through callus formation. This is a very useful technique as it is routinely used genetic engineering to raise transgenic plants and in other fields like germplasm conservation, forestry, horticulture etc.

Tissue culture techniques can also be used to raise virus free plants through callus culture of meristem tips which are known to evade viral invasion. This enables rescue of plants from viral infections. Shoot tip culture may also be used in combination with thermotherapy and chemotherapy for this purpose.

Endosperm culture is another important application in which endosperm is cultured in vitro to develop triploid plants which are highly desired in some species because of commercial importance.

Exercise

1. How can tissue culture be used to develop plant cultivars with desired properties?

2. What is the difference between organogenesis and somatic embryogenesis?

3. What are the factors that affect micropropagation?

4. What is the difference between a zygotic and a somatic embryo?

5. What is the use of developing triploid plants?

6. Why are shoot tips free from viral invasion? Is this a universal phenomenon?

7. How is micropropagation useful in agriculture?

8. Define the following:

- Shoot apical meristem
- Virus elimination
- Micropropagation
- Organogenesis
- Endosperm culture

Glossary

Androgenesis: Development of plants from male gametophytes.

Anther Culture: Culture of excised anthers, to obtain haploids.

Aseptic culture: Carrying out culture experiments from tissue which are made free of any micro-organisms, in a strile environment.

Clonal multiplication: Multiplication of plants, starting from a single individual, using asexual methods..

Culture: Growing of cells, tissues or organs in a medium containg organic and inorganic nutrients under aseptic conditions.

Differentiation: The process of acquiring different morphological and physiological characteristics by cells or tissues growing in culture.

Diploid: Having two copies of each chromosome characteristic for the species.

Embryo: An organized structure which follows pre-defined stages of development to develop into a complete plantlet.

Embryogenesis: The chain of events in which an embryo develops either in the seed or *in vitro.*

Endosperm: The nutritive tissue that surrounds the developing embryo in a seed.

Explant: A plant organ or piece of tissue used to initiate a culture.

Gametoclonal variation: Variation by expressed by gametoclones in phenotype, either genetic or epigenetic.

Growth regulators: Organic compounds like auxins, cytokinins, ethylene and gibberellins which, in small amounts, influence growth, differentiation and multiplication of plants.

Haploid: Having single copy per cell of each chromosome characteristic of the species (=n).

In vitro: Literally means 'in glass'; used to refer to any process carried out in aseptic cultures.

Indexing: Testing of plants for pathogens or contaminants.

Meristem: A group of actively dividing cells, usually localized in a small region of the developed tissues.The main types of meristems are: apical, lateral, and intercalary meristems.

Organogenesis: Differentiation of organs from cells or tissues.

Plantlet: A small plant formed after germination of the embryo.

Regeneration: A morphogenetic response leading to development of new organs, embryos or whole plants from explants in culture.

Somatic embryogenesis: The process of initiation and development of embryo from non-gametic cells.

Subculture: The process in which the tissue growing *in vitro* is subdivided and transferred to fresh medium.

Totipotency: Potential of a cell to differentiate into any other type of cell and produce a whole organism.

Transgenic plants: Plants which have been genetically modified for certain characteristics by introducing one or a few genes from another source.

Virus: Any of a group of submicroscopic agents which infect plants, animals or bacteria and are unable to reproduce outside the host. It consists of nucleic acid (DNA or RNA) surrounded by a protein coat.

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Web Links

http://www.plantcell.org/content/20/5/1189.full.pdf+html

http://www.cornell.edu/video/history-of-plant-cloning-6-micropropagation-or-plant-cloning

http://cdn.intechopen.com/pdfs-wm/40180.pdf

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC160372/

http://upload.wikimedia.org/wikipedia/commons/e/eb/Plant_Buds_clasification.svg

http://irrecenvhort.ifas.ufl.edu/Propagation/modules/module6/chapter-17-18/player.html

http://dbtncstcp.nic.in/html/content/Recognized_TCPU%27s.pdf

http://en.wikipedia.org/wiki/Hyperhydricity#mediaviewer/File:Hyperhydric_Vs_Healthy_Plant.jpg

http://nptel.ac.in/courses/102103016/7

www.youtube.com/watch?v=Tp61S-2F2B4

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3597136/pdf/13205_2012_Article_80.pdf

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http://bioscipub.com/journals/bbb/pdf/279-288.pdf