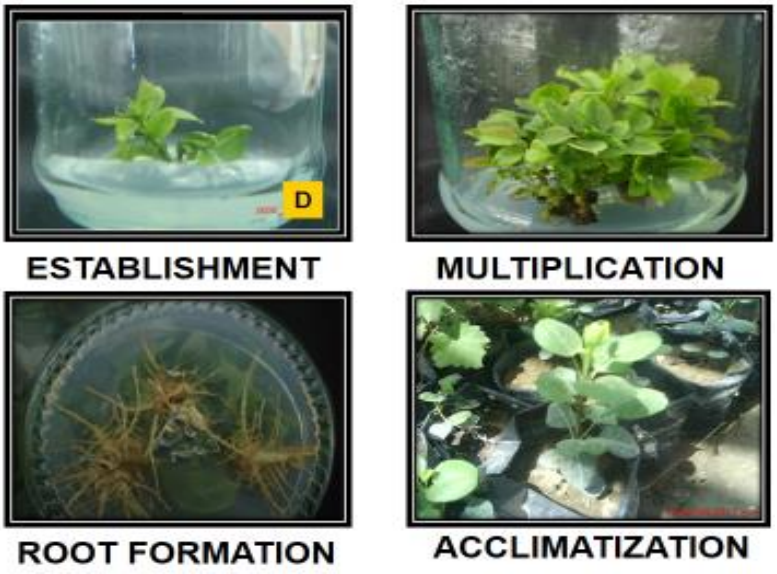
Introduction

Success of micropropagation is largely due to separating different developmental aspects of culture into stages, each of which is manipulated by media modification and environmental control. Four distinct stages are recognized for most plants:

- Stage I. Establishment
- Stage II. Multiplication
- Stage III. Root formation
- Stage IV. Acclimatization



Stage I. Establishment

The objectives for stage 1 are to successfully place the explant into the aseptic culture by avoiding contamination and then to provide an in vitro environment that promotes stable shoot production. Important aspects of the stage include:

- Explant source selection
- Explant disinfestations
- Culture medium
- Stabilization

Explant source selection

The selection and management of the source plant is an important aspect of successful micropropagation. Three aspects require particular attention:

A. Genetic and epigenetic (juvenile) characteristics of the source plants

B. Control of pathogens

C. Physiological conditioning of the plant to optimize its ability to establish in a culture

Explant source selection

A. Genetic and epigenetic (juvenile) characteristics of the source plants

B. Control of pathogens

Pathogen problems include:

Contamination by fungi, molds, yeasts, and bacteria on the surface of stems or lodged in cracks, and elsewhere;

Systemic viruses and virus-like organisms.

Explant source selection

- C. Physiological conditioning.

- growing plants in containers in the greenhouse with environmental controls to achieve the proper stage of shoot development in relation to seasonal patterns,
- producing healthy plants,
- and treatment with growth regulators.

Explants Disinfestation

- Disinfestation is the process of removing contaminants from the surface of the organ rather than from within the organ.
- Disinfestation requires the use of chemicals that are toxic to the microorganism but relatively nontoxic to plant material.

Explants Disinfestation

- Materials used to clean explants include

1. Sodium Hypochlorite
2. silver nitrate.
3. mercuric chloride.
4. Ethyl or Isopropyl alcohol
5. Calcium Hypochlorite

Explants Disinfestation

- A typical procedure for explant preparation would be to cut the tissue into short pieces several centimeters long.

- and wash in tap water with a detergent. For woody pieces, a quick dip in alcohol may be helpful.

- Plant parts are placed into the disinfecting solution (with a surfactant or detergent added) for 5 to 15 minutes.

Explants Disinfestation

- The solution is then poured off and the material rinsed two or three times with sterile water.
- Modifications of this basic procedure that may improve effectiveness include prewashing, mechanical agitation, vacuum infiltration, and multiple treatments.

Sodium Hypochlorite (NaOCl)

- Found in laundry bleach, is approximately a 5.25% (v/v) solution.
- Generally, the laundry bleach is diluted in water to 5 - 25% (v/v), with 2 drops of Tween-20 added/ 100 ml.
- The duration of treatment is usually from 5 to 30 minutes followed by three rinses with distilled water.
- It is best to use small counter of bleach and record on the bottle when it was opened.
- Over time the bleach will evolve chlorine gas and lose its effectiveness.

Calcium hypochlorite

It is used at 0.8%, (w/v) (8 grams of $Ca(OCl)_2$ in 1-liter water), stir for 10 to 15 minutes, allows to settle, decant solution, and mix with equal parts of water, add 2 drops of Tween-20/ 100 ml, treat tissue for 5 – 30 minutes.

Ethyl or Isopropyl alcohol

It is used at 70% (v/v) to swab the plant material prior to disinfestation, and it can be used to dip plant material for 1 – 5 minutes before or after sodium or calcium hypochlorite treatment.

Mercuric Chloride ($HgCl_2$)

It can be used at 0.1 – 1.0% for 2 – 10 minutes prior to sterile water rinse. It is toxic to plant tissue, therefore it is important to rinse the explant thoroughly.

Silver Nitrate ($AgNO_3$)

It can be used at a concentration 1% for 5 – 30 minutes. Bromide water is also used at a concentration of 1 – 2% for 2 – 10 minutes.

Culture Medium

Typically, the culture medium has lower amounts of hormones during establishment compared to the multiplication stage of micropropagation.

Stabilization

- If the explant is successfully established, several microshoots are produced within a few weeks, the number depending upon the apical dominance of the particular kind of plant.

- Establishment and stabilized cultures are ready for subculturing and can be moved along to Stage II of micropropagation.

Stage II. Multiplication

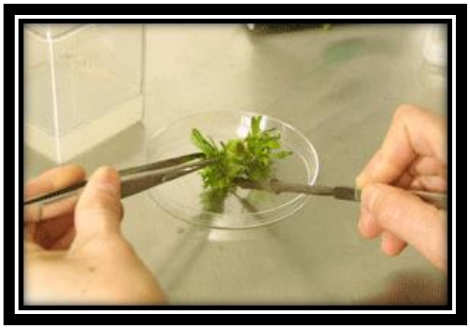
- The purpose of Stage II is to maintain the culture in the stabilized state and multiply the micro shoots to the number required for rooting.
- The basic medium of Stage II is similar to Stage I.
- One or more subcultures should occur between each test to equalize the test material.

Growth regulators

- Cytokinin and auxin are used to support a basic level of growth but are equally important to direct the developmental response of the tissues in culture.
- Shoot initiation is strongly supported by cytokinin concentration. In general, the minimum concentration of cytokinin that stimulates lateral shoot initiation is selected during the multiplication stage.
- Increased cytokinin levels may promote additional shoot proliferation, but can inhibit shoot elongation.

Subculturing

- After microshoots have reached an appropriate length, they are harvested and either used to start new cultures or transferred to Stage III for rooting.
- The original culture can be trimmed or separated and subcultured to new multiplication media.



Subculturing

- Subculturing frequency varies from two to eight weeks depending on the speed of shoot development.
- wakefulness is essential to maintain shoot proliferation and to establish optimum shoot multiplication rates.
- If subculturing is delayed too long, leaf yellowing and necrosis can develop.

It is time to transfer when:

- the medium is discolored
- leaves turn brown
- growth slow or stops
- tubes become crowded

Stage III. Root Formation

- The function of Stage III is to root microcuttings and in some cases, to prepare them for transplanting out of the aseptic protected environment of the test tube to the outdoor conditions of the greenhouse or transplant area.
- Rooting can take place in the *in vitro* or *ex vitro* environment.

In vitro rooting

- Microcuttings are moved to a root-inducing medium in which the growth regulator balance of the medium is changed to reduced (or no) cytokinin and increased auxin.
- The concentration of basal salts in the rooting medium is usually cut in half. Individual microcuttings may be placed into a root-inducing (often liquid) medium for a few days and then transferred to an auxin-free medium for rooting.

.

Ex vitro rooting

Many commercial, as well as experimental micropropagation systems, avoid *in vitro* rooting by treating microcuttings with auxin, increasing them directly in a soilless greenhouse rooting medium, and placing them under mist or high humidity conditions for rooting.

Stage IV. Acclimatization

- This stage involves the shift from a heterotrophic (sugar-requiring) to an autotrophic (free-living) condition and the acclimatization of the microplant to the outdoor environment.
- Keeping the shoot actively growing is important because acclimatization and development of autotrophic conditions depend on new growth after transfer from the test-tube environment.

- Immediately upon transplanting, rooted microplants should be kept in very high humidity, gradually exposing them to outdoor conditions to prevent dehydration.

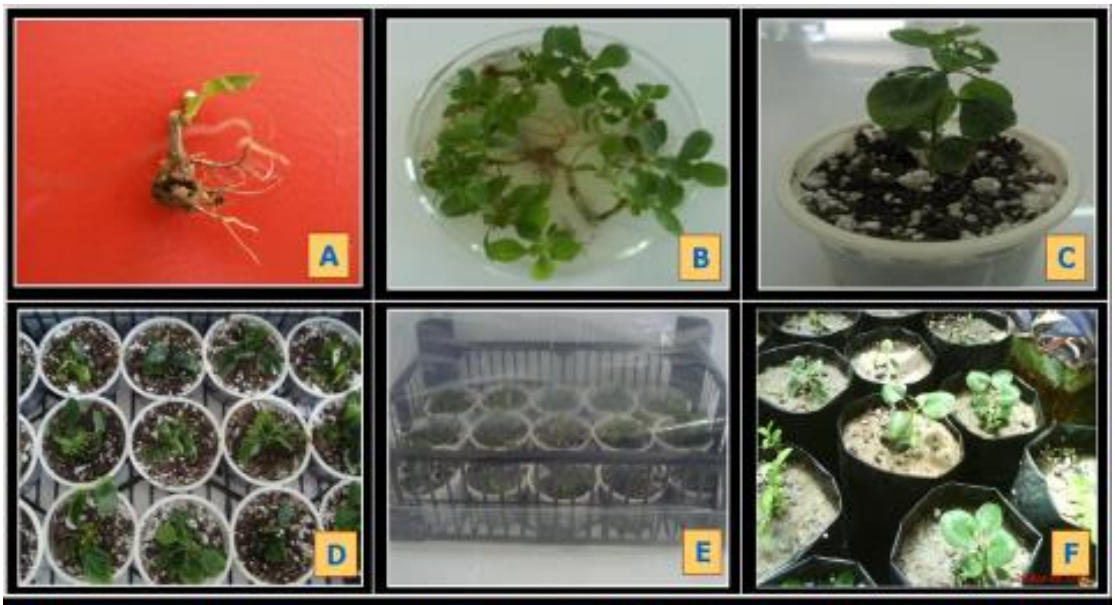


Figure (33): Acclimatization stage: **A: Just removed from nutrient medium and washed micrograf. B: Treating with Benlate fungicide (0.1% for ten minutes). C: Planted in sterilized medium mixture (peatmoss+ silt+ Styrofoam 1:1:0.5). D. Potted plantlets before covering. E. Covered plantlets with polyethylene. F: Transplanted into a plastic bag in the greenhouse.**