Plant Propagation PLS 3223/5222

Guest Web Lecture
Dr. Michael Kane
Environmental Horticulture Department

PLANT MICROPROPAGATION

Micropropagation

- ***Rapid clonal in vitro ("in glass") propagation of plants from cells, tissues or organs cultured aseptically on defined media contained in culture vessels maintained under controlled conditions of light and temperature***

Student Learning Objectives

- Recite the plant tissue culture principles and concepts related to the commercial micropropagation, specifically by shoot culture
- Outline the critical procedures to successfully optimize each micropropagation stage in a commercial laboratory setting

Micropropagation

In vitro propagation

Tissue culture propagation

MICROPROPAGATION	MACROPROPAGATION
Small propagule	Larger propagule
Aseptic conditions	Non-aseptic conditions
Controlled environment	Less environmental control
Heterotrophic growth	Photoautotrophic growth
Rapid multiplication	Slower multiplication
Greater initial costs	Nominal costs

Plant Tissue Culture: Historical Perspective

How did it all begin?

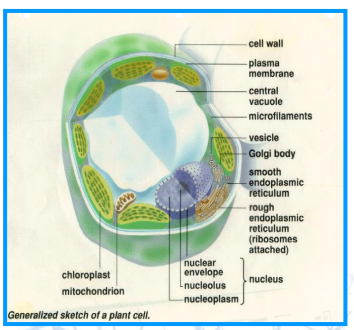
Historical Perspective

Schleiden 1838
Schwann 1839 → **Cell Theory**

Cell is the basic unit of life

Totipotency Concept

- **Each living cell of a multicellular organism should be capable of independent development if provided with the proper external conditions**

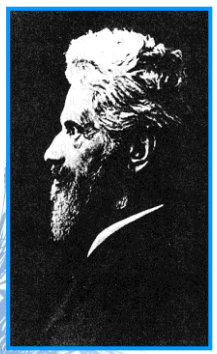


Plant Cell

In Vitro Culture: Early Attempts

Haberlandt 1902

Innate potential of cells



Haberlandt

Attempted culture of isolated leaf cells

Formulated plant tissue culture principles

Culture Medium: mineral salts & glucose

Unsuccessful results

Eichhornia crassipes

In Vitro Culture: Early Attempts

Knudson → **1920s** → **Asymbiotic orchid seed germination & culture**

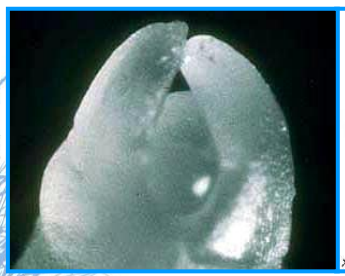
Concept of in vitro plant production

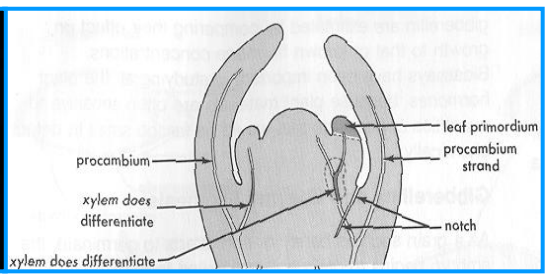
Knudson

Orchids | Orchid Seedlings | Seedling Culture

Toward Commercial Micropropagation
1950s

Morel & Martin	1952	Meristem-tip culture for disease elimination





Commercialization of Micropropagation
1960s

Morel	1960	Disease eradication
Wimber	1963	in vitro production of orchids

Commercialization of Micropropagation
1970s & 1980s

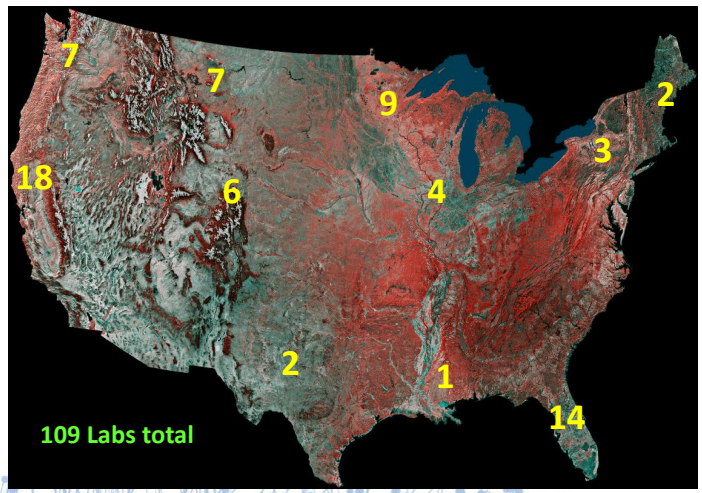
Murashige → 1974 → Broad commercial application

Dr. Toshio Murashige
University of California

Micropropagation:
Advantages for Plant Production

- Rapid & efficient propagation
- Year-round production
- Precise crop production scheduling
- Reduce stock plant space
- Long-term germplasm storage
- Production of difficult-to-propagate species

Commercial Micropropagation Labs (2000)

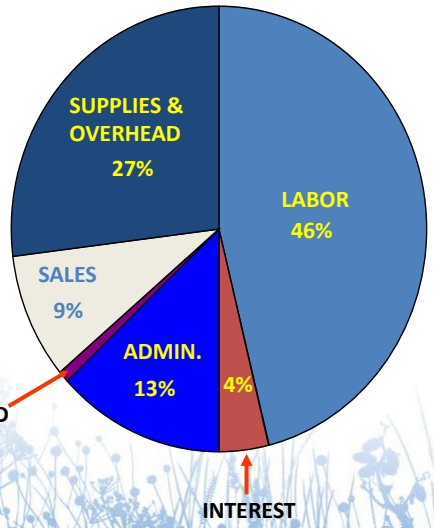


Micropropagation Production in the United States

Foliage Plants	63,695,000
Greenhouse Flowers	11,297,000
Perennials	9,448,000
Trees & shrubs	15,294,000
Vegetables	12,862,000
Fruits	3,721,000
Miscellaneous	4,545,000
Total:	**120,862,000**

(Zimmerman, 2001)

USA Commercial Micropropagation Laboratory Costs



Commercial Micropropagation: A Global Industry

- Israel
- Japan
- India
- Malaysia
- Mexico
- Central America
- South America

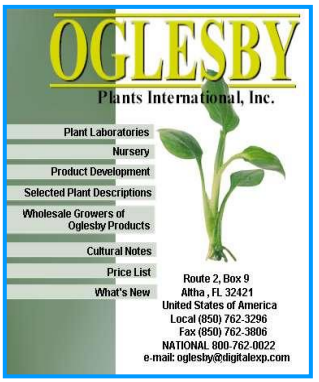
Bangkok Flower Center
Thailand

Strive to reduce labor costs!

Oglesby Plants International, Inc.

1985

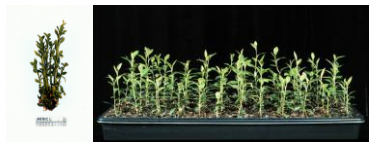
- **Lab built in Altha, FL**
- **12,000,000 plants/yr**



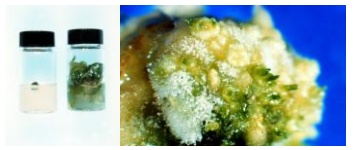
Oglesby Plants International, Inc.

Micropropagation Methods

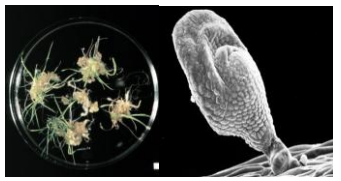
Shoot culture



Shoot organogenesis



Non-zygotic embryogenesis



Micropropagation Methods

1. Shoot Culture

- **Production of axillary shoots followed by rooting of individual shoots (pre-existing meristems on explants)**

Shoot Culture

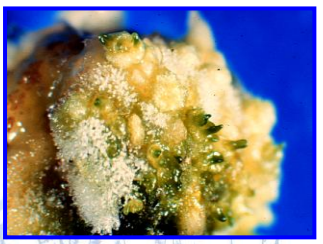
Micropropagation Methods

2. Shoot Organogenesis

- Production of adventitious shoots followed by rooting of individual shoots (shoot production does not originate from pre-existing meristems on the explants)

- Direct Shoot Organogenesis
- Indirect Shoot Organogenesis

Direct Shoot Organogenesis



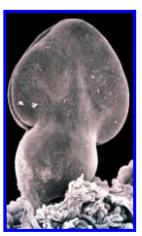
Indirect Shoot Organogenesis

Micropropagation Methods

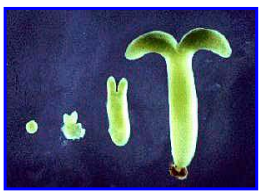
3. Non-zygotic Embryogenesis

- Production of non-zygotic embryos from single cells

- Direct Non-zygotic Embryogenesis
- Indirect Non-zygotic Embryogenesis



Grape non-zygotic embryo



Non-zygotic embryogenesis

Shoot Culture

Method Overview

- ***Clonal in vitro propagation by repeated enhanced formation of axillary shoots from shoot-tips or lateral meristems following culture on media supplemented with plant growth regulators, usually cytokinins. Shoots produced are either rooted first in vitro or rooted and acclimatized ex vitro***

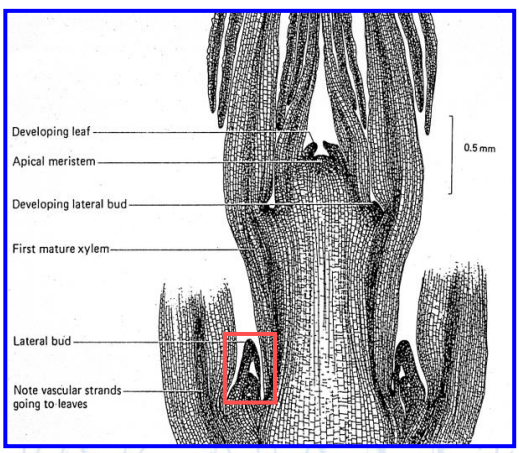
Concept

- **Micropropagation from pre-existing meristems**

Shoot Meristems

Background

- **Axillary bud in leaf axil**
- **Each encloses a shoot-tip**
- **Each bud has potential to develop into a shoot**
- **Lateral bud outgrowth suppressed (apical dominance)**
- **Hormone interactions**
- **Pathogens often not present in apical meristems**



Generalized Shoot-tip

Important Discovery

Wickson, M. and K.V. Thimann. 1958. The antagonism of auxin and kinetin in apical dominance. Physiologia Plantarum 11:62-74.

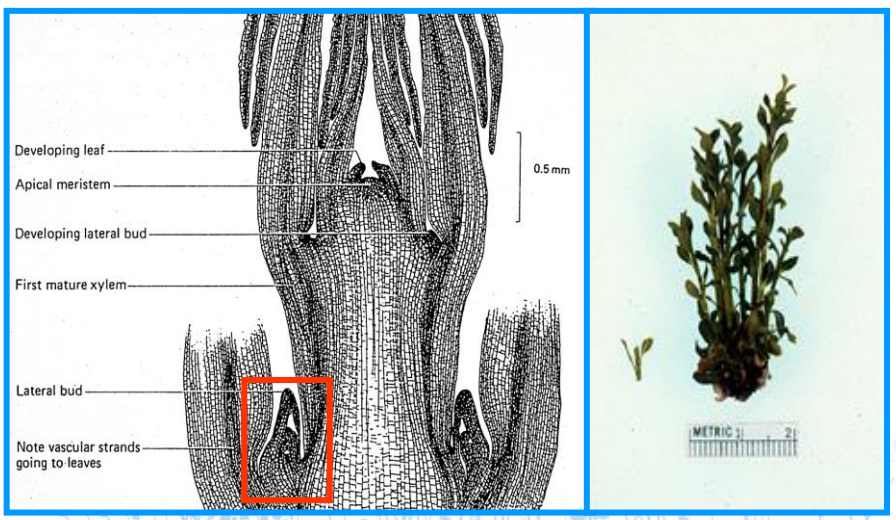
Apical dominance along pea stems could be suppressed by application of cytokinin

Axillary branching enhanced by high doses of cytokinins

Basis for micropropagation via enhanced axillary branching (shoot culture)

Cytokinin in medium disrupts apical dominance and enhances outgrowth

Shoot Culture



Cytokinin-enhanced outgrowth of lateral meristems

Shoot Culture

Most widely used method for commercial micropropagation

Relatively high genetic stability in the plants produced

Shoot Culture

Advantages

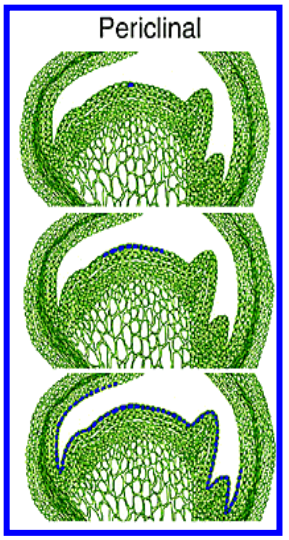
- Reliable rates and consistency of shoot multiplication
- 3 - 8 fold multiplication rate per month
- Pre-existing meristems are least susceptible to genetic changes

Shoot Culture

Advantages

- **Periclinal chimeras can be propagated**

Pinwheel African Violet (chimera)



Shoot Culture

Disadvantages

- **Less efficient than organogenesis or non-zygotic embryogenesis**
- **Sometime difficulties in rooting shoots produced**
- **Axillary shoot production not enhanced by cytokinins in some species**
- **Very labor intensive**

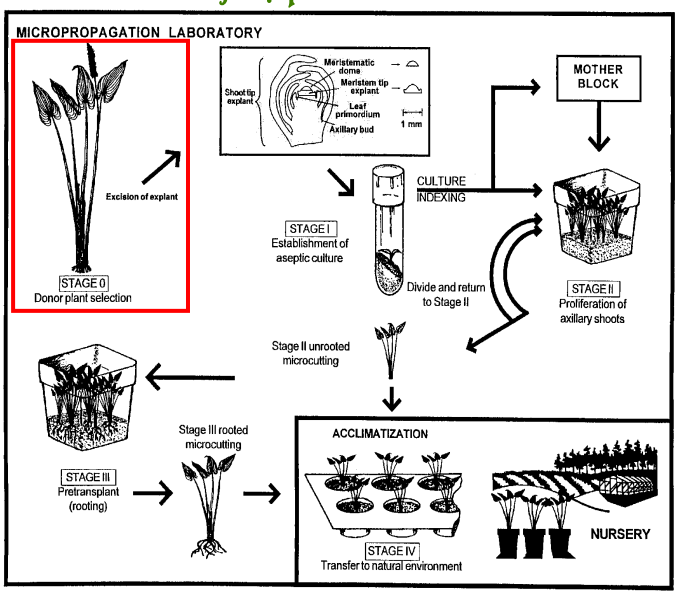
Micropropagation Stages

Shoot Culture

- **Stage 0.** • Donor Plant Selection
- **Stage I.** • Establishment Of Sterile Culture
- **Stage II.** • Shoot Multiplication
- **Stage III.** • Pretransplant (rooting)
- **Stage IV.** • Transfer To Natural Environment

Five stages to successfully produce plants via micropropagation

STAGE 0. Donor Plant Selection & Preparation



Shoot Culture

Stage 0. Donor Plant Selection & Preparation

- **Explant quality & responsiveness in vitro influenced by phytosanitary/physiological conditions of donor plant**

STAGE 0. Donor Plant Selection & Preparation

Donor Plant Preparation Tips

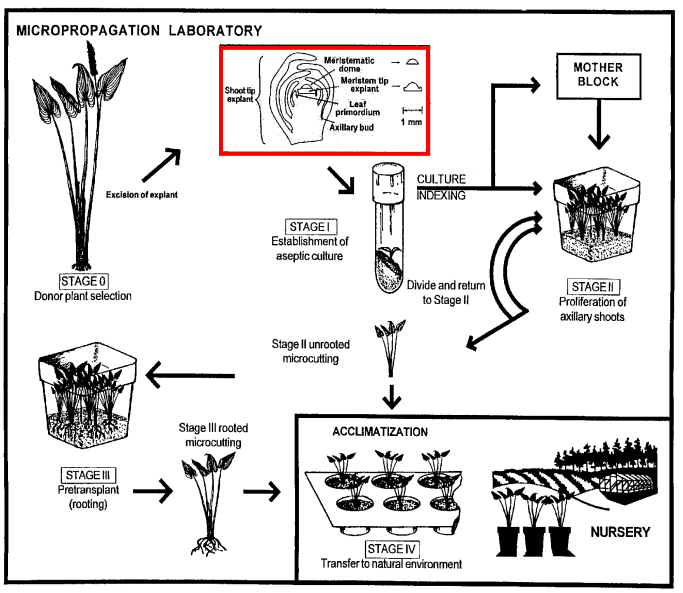
- **Maintain specific pathogen-tested stock plants**
- **Clean controlled conditions allowing active growth**
- **Low humidity, drip irrigation, antibiotic sprays**

STAGE 0. Donor Plant Selection & Preparation

Donor Plant Preparation

- Modification of physiological status
  - Trim to stimulate lateral shoots
  - Pretreat with cytokinins or gibberellic acid
  - Use forcing solution: 2% sucrose, 200 mg/l 8-hydroxyquinoline citrate and growth regulators
  - Light/temperature pretreatments

STAGE I. Establishment of Aseptic Culture



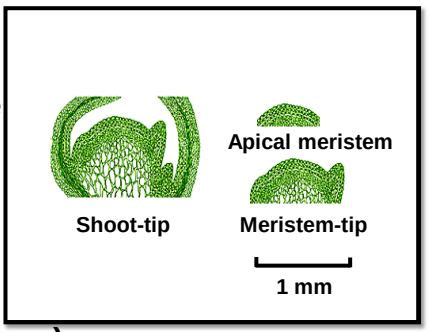
Meristem and Meristem-tip Culture

Techniques used specifically to produce *pathogen eradicated plants* not directly used for propagation

Meristem Culture

Culture of apical meristem dome

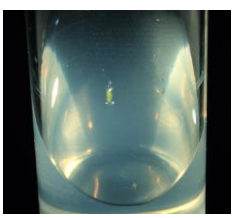
0.1 - 0.2 mm diameter
0.2 mm in length



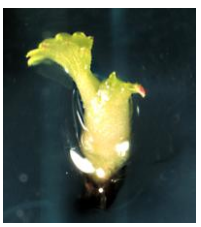
Meristem-tip Culture

Culture of larger (0.2 - 0.5 mm long) meristem-tip explants that include apical meristem plus several subtending leaf primordia

Meristem and Meristem-tip Culture



Meristem-tip isolation



3 wks

Single shoot (9 wks)

Culture indexing for pathogens

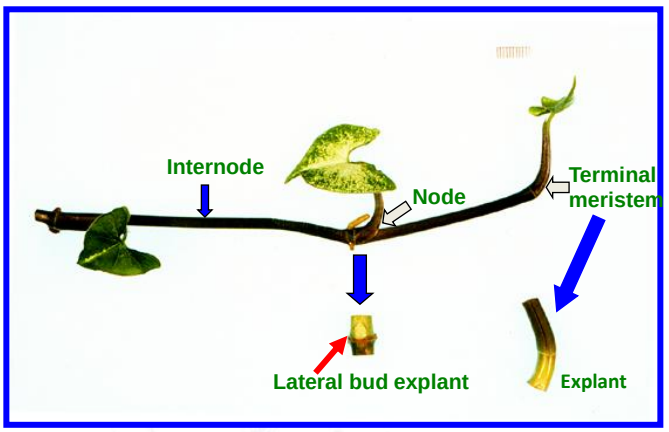
Clean

Rooting

Shoot culture

Acclimatization

Shoot Culture



Syngonium

Surface Sterilization

10 - 15 minute rinse in tap water

↓

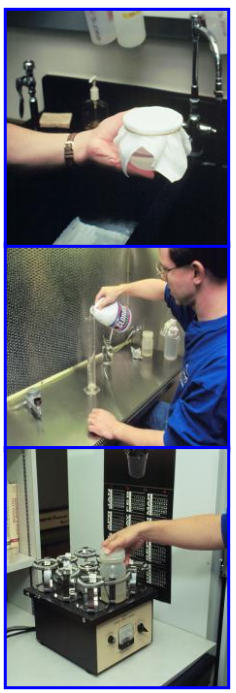
1 - 2 minute soak in 50 - 70% ethanol

↓

8 - 15 minutes in 0.1 - 1.2% sodium hypochlorite containing 2 drops Tween-20/ 100 ml (shakened)

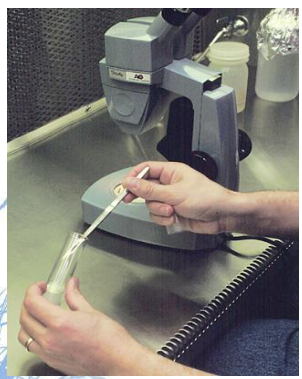
↓

Three five-minute sterile water rinses



Shoot-tip Isolation

STAGE I. Culture Initiation



Inoculation

Day 0

4 Weeks

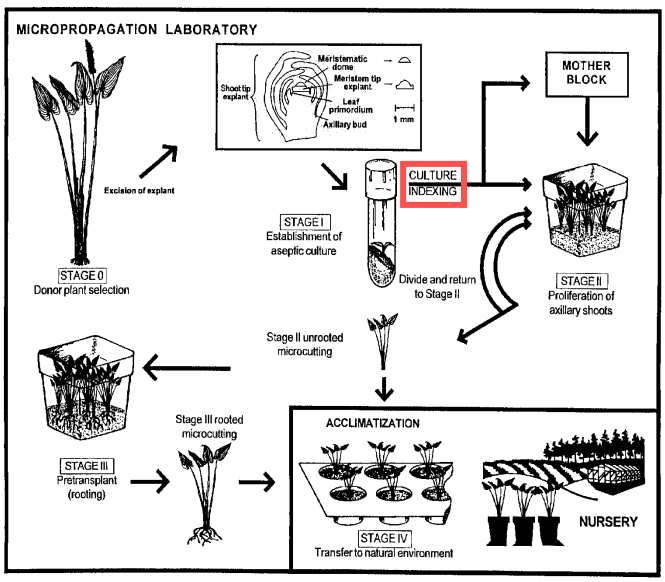
Day 28

STAGE I. Culture Medium

Murashige & Skoog mineral salts
30 g/l sucrose
100 mg/l myo-inositol
0.4 mg/l thiamine
0.5 mg/l cytokinin (2-iP)
0.1 mg/liter auxin (IAA)
7 g/l agar or Phytagel
pH = 5.7

Smaller explants require more complex medium

STAGE I. Culture Indexing



STAGE I. Culture Contamination

Bacterial **Fungal**

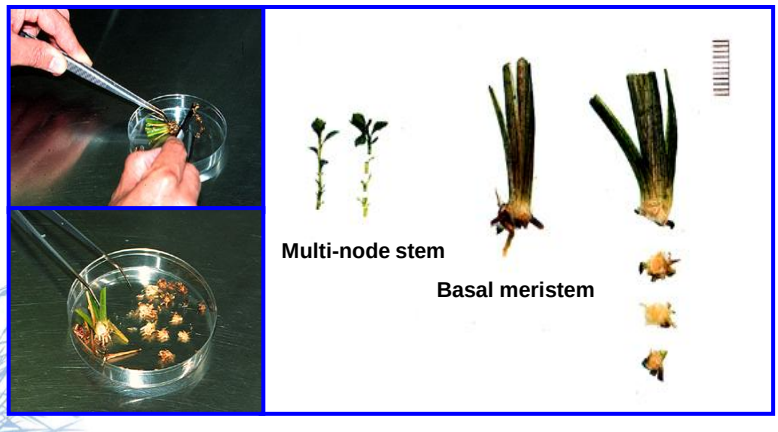
STAGE I. Culture Contamination

Many times what you "see" is not what you get!

The "EBD"

- Need to screen (index) for the presence of <u>cultivable</u> contaminants

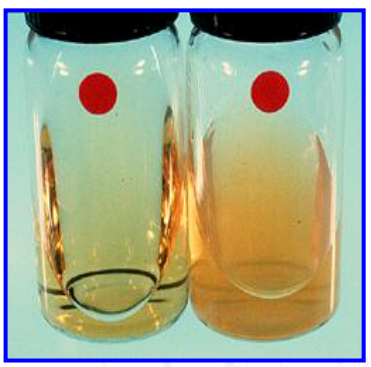
STAGE I. Culture Indexing



STAGE I. Culture Indexing Medium

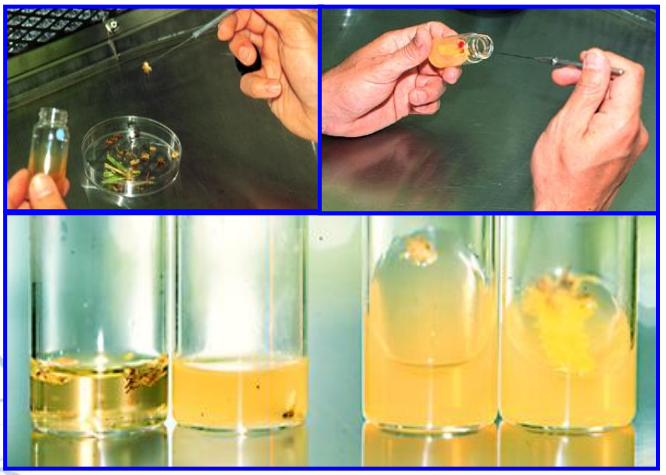
Liefert & Waites Sterility Test Medium

- Beef Extract
- Glucose
- Lab-Lemco Powder
- Murashige & Skoog Medium
- Peptone
- Sodium Chloride
- Sucrose
- Yeast Extract



Liquid Solid

STAGE I. Culture Indexing Medium



Liquid Medium **Solid Medium**

"Stab & Streak" Method

STAGE I. Establishment of Aseptic Culture

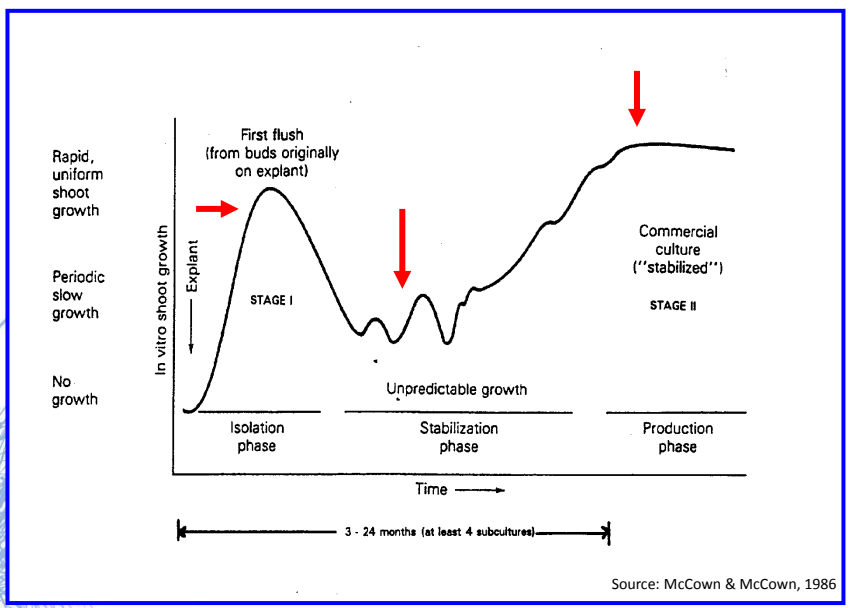
So now we have a sterile (indexed) Stage I culture

STAGE I. Establishment of Aseptic Culture

Misconception that shoot multiplication occurs rapidly immediately following inoculation of explant *in vitro*

Three important phases of explant establishment

STAGE I. Culture Stabilization



Source: McCown & McCown, 1986

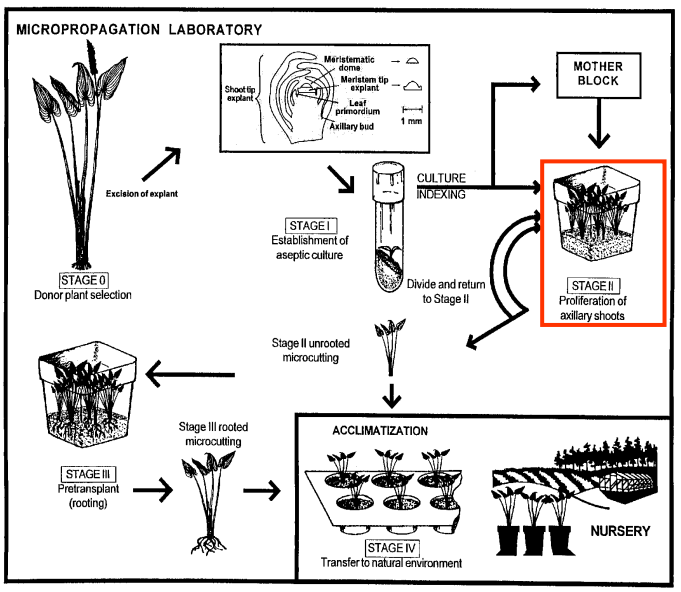
Mother Block Concept

Mother Block

- A **slowly multiplying, indexed** and **stabilized** set of cultures
- Serve as source of cultures (explants) for Stage II multiplication

Mother Block Room

STAGE II. Shoot Multiplication



STAGE II. Shoot Multiplication

Cytokinin-enhanced axillary shoot production

STAGE II. Shoot Multiplication

Repeated enhanced axillary shoot production

Presence of higher cytokinin level in medium to disrupt apical dominance

- **2-isopentenyladenine (2-iP)**
- **Benzyladenine (BA)**
- **Kinetin (KIN)**
- **Thidiazuron (Dropp®)**

STAGE II. Shoot Multiplication

Stage II selection of cytokinin type and concentration determined by:

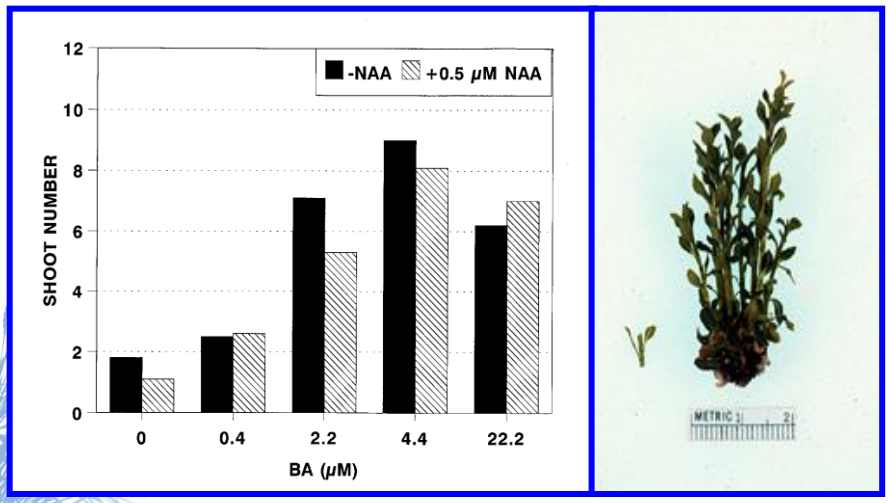
- **Shoot multiplication rate**
- **Length of shoot produced**
- **Frequency of genetic variability**
- **Cytokinin effects on rooting and survival**

STAGE II. Shoot Multiplication

Auxin may be added to enhance shoot production/elongation (graph)

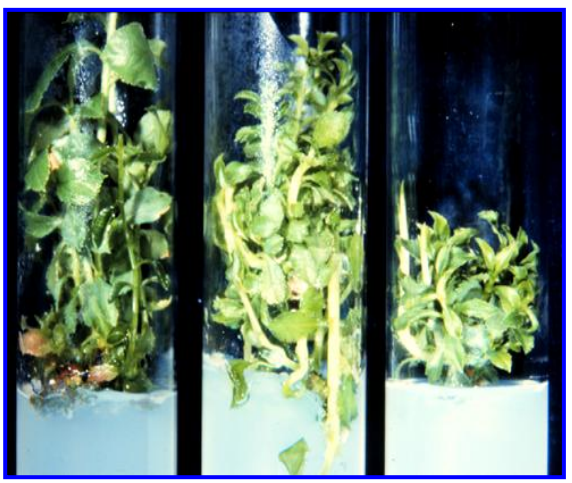
- **α-indole-3-acetic acid (IAA)**
- **1- naphthaleneacetic acid (NAA)**
- **indolebutyric acid (IBA)**

STAGE II. Shoot Multiplication



Addition of NAA does not promote shoot production

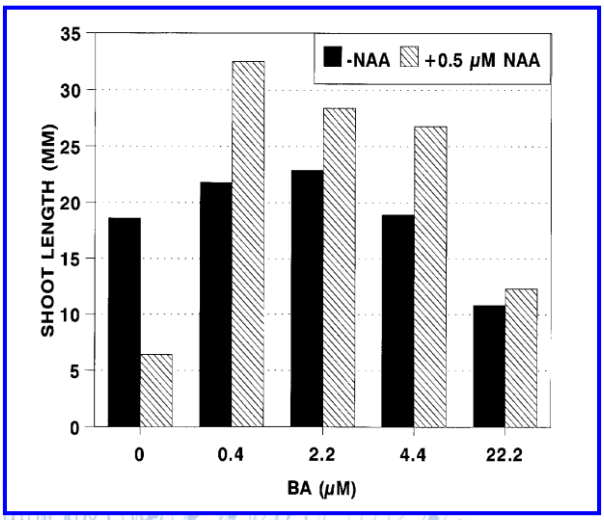
STAGE II. Shoot Multiplication



0.4 **2.2** **4.4**

μM BA

STAGE II. Shoot Multiplication



Addition of NAA promotes shoot elongation

STAGE II. Shoot Multiplication

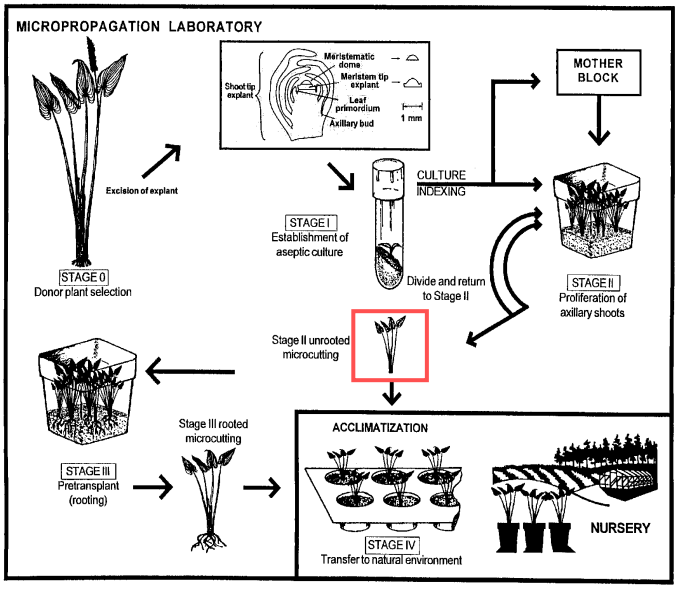
Subculture shoot clusters at 4 - 5 week intervals

3 - 8 fold increase in shoot numbers (4.3×10^7 shoots/explant/year)

Number of subcultures possible is species/cultivar dependent:

- Frequency of genetic variability
- Some subcultured 8 - 48 months
- Boston fern 3 subcultures maximum
- Adventitious shoot formation (mixed culture)

STAGE II. Shoot Microcuttings

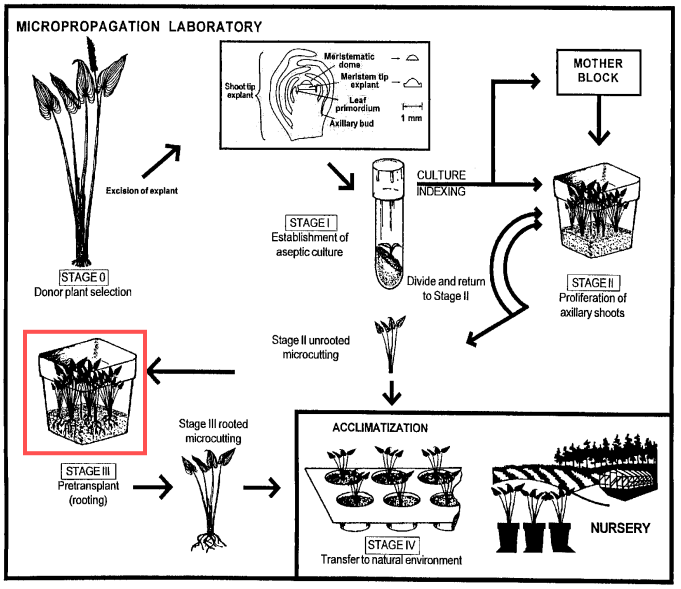


STAGE II. Shoot Microcuttings

Ex vitro **rooting**

Stage II Microcutting

STAGE III. Pretransplant (Rooting)



STAGE III. Pretransplant (Rooting)

Preparation of Stage II shoots/shoot clusters for transfer to soil (prehardening)

Elongation of shoots prior to *ex vitro* rooting

Fulfilling dormancy requirements of storage organs

STAGE III. Pretransplant (Rooting)

✓ **Adventitious rooting of individual shoots or clusters *in vitro***

Stage III rooting is not usually desirable

- **Very expensive 35 - 75% of total production cost**
- ***In vitro* formed roots not well-developed**
- **Roots easily damaged during transplanting**
- **Numerous factors influence *in vitro* rooting**

Factors Important To In Vitro Rooting

STIMULATORY MEDIUM COMPONENTS	COMMENTS
1. AUXINS	
indole-3-acetic acid [IAA] indole-3-butyric acid [IBA] α-naphthaleneacetic acid [NAA]	Dosage effect (Conc. x time) 0.05 - 10 mg/liter for (days - weeks) or 50 - 100 mg/liter (sec - hours)
2. HIGH SUGAR/ NITROGEN RATIO	Effect depends on mineral medium
3. PHENOLS Phloroglucinol	May stimulate rooting (species dependent)

Factors Important To In Vitro Rooting

STIMULATORY MEDIUM COMPONENTS	COMMENTS
4. ACTIVATED CHARCOAL	May reduce light in medium or absorb inhibitory compounds

Factors Important To In Vitro Rooting

INHIBITORY MEDIUM COMPONENTS	COMMENTS
1. CYTOKININS	Common observation. Eliminated in rooting medium
2. GIBBERELLINS	Inhibit root formation
3. HIGH IONIC STRENGTH OF MEDIUM	Confounded by effects of individual nutrients
4. AGAR	Exact cause unknown; agar may be impure and variable in content

STAGE III. Pretransplant (Rooting)

Auxin type & concentration used dependent on:

- **Percent (%) rooting, root number and length**

Auxin Effects on In Vitro Stage III Rooting[1]

Treatment IBA (mg/L)	% Rooting	Root Number	Root length (mm)
0	37	2.4	23.3
0.05	43	3.5	18.1
0.1	55	4.1	14.2
0.5	71	5.7	6.5
1.0	84	7.1	4.3

[1]Rooting responses of 10 mm microcuttings of *Aronia arbutifolia* after 28 days

Auxin Effects on In Vitro Stage III Rooting[1]

Treatment IBA (mg/L)	% Rooting	Root Number	Root length (mm)
0	37	2.4	23.3
0.05	43	3.5	18.1
0.1	55	4.1	14.2
0.5	71	5.7	6.5
1.0	84	7.1	4.3

[1]Rooting responses of 10 mm microcuttings of *Aronia arbutifolia* after 28 days

Stage III Rooting

0 0.05 0.1 0.5 1.0

IBA (mg/L)

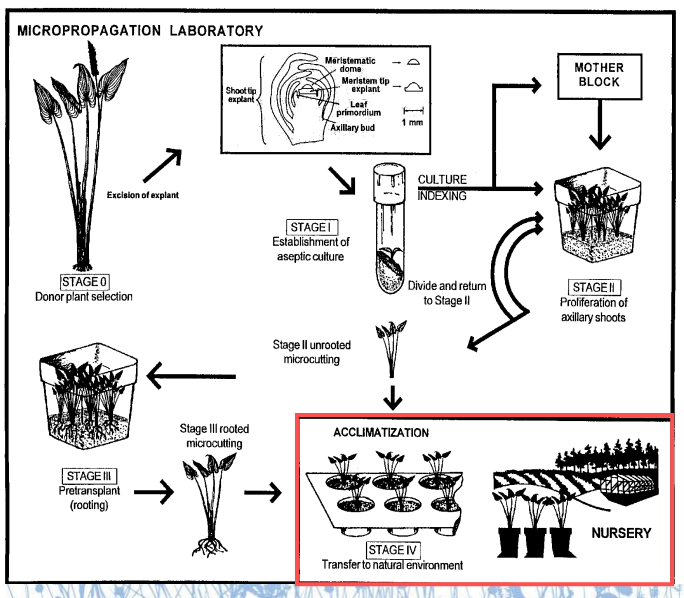
DAY 28

STAGE III: Pretransplant (Rooting)

Auxin type and concentration used dependent on:

- Percent (%) rooting, root number and length
- Auxin effects on post-transplant growth
  - NAA used in Stage III may retard Stage IV growth

STAGE IV. Transfer to Natural Environment



STAGE IV. Transfer to Natural Environment

Ultimate success of shoot culture depends on ability to re-establish vigorously growing quality plants from *in vitro* to *ex vitro* conditions

High humidity & low light
In vitro

Lower humidity & high light
Ex vitro

STAGE IV. Transfer to Natural Environment

ACCLIMATIZATION:

- *Process whereby plants physiologically and anatomically adjust from in vitro to ex vitro cultural and environmental conditions*

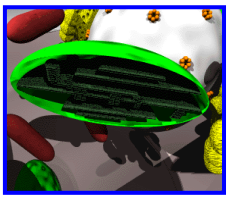
Two reasons micropropagated plants may be difficult to re-establish *ex vitro*:

- **1. Low photosynthetic competence (heterotrophic nutrition)**
- **2. Poor control of water loss**

STAGE IV. Transfer to Natural Environment

1. Low Photosynthetic Competence

- Plants largely heterotrophic (may be photomixotrophic)
- Poorly differentiated leaf structure
- Poorly developed chloroplasts
- Poor CO_2 fixation



STAGE IV. Transfer to Natural Environment

"Lifeboat Effect"

- Need for carbohydrate reserve (starch) in stems and leaves during initial acclimatization

Example

Cauliflower begins carbon fixation 7 days post-transplant

14 days required for positive carbon balance

STAGE IV. Transfer to Natural Environment

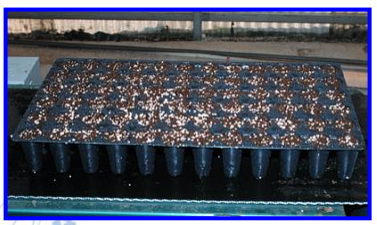
2. Poor Control of Water Loss

- Reduced cuticle (wax) development
- Abnormal stomata development and function
- Non- or marginally functional roots

STAGE IV. Transfer to Natural Environment

3. Other Factors Affecting Acclimatization

- Plant Quality
  - Culture medium carry over effects
  - Bacterial/fungal contamination
- Soil Mix Selection
  - Pasteurized
  - Well drained
  - Dilute fertilizer (150 mg/L N)



STAGE IV. Transfer to Natural Environment

Planting Stage III rooted microcuttings

STAGE IV. Transfer to Natural Environment

3. Other Factors Affecting Acclimatization & Quality

Container/Medium/Plug Size Considerations

Single **Shoot Cluster**

MICROCUTTING TYPE

12-cell pack **4-cell pack** **12-cell pack** **4-cell pack**

SINGLE SHOOT **SHOOT CLUSTER**

STAGE IV. Transfer to Natural Environment

3. Factors Affecting Acclimatization & Quality (cont.)

- **Light & Temperature Control**
  - **Light (photoperiod & intensity)**
  - **Move plants through one or more intermediate light levels (2-fold increase every 6 - 14 days)**
- **Humidity & Moisture Control**
  - **Near 100% humidity *in vitro***
  - **Humidity gradually decreased**

STAGE IV. Transfer to Natural Environment

4. Acclimatization Structures

- **Propagation dome**
- **Humidity tent**
- **Automatic mist system**
- **Fog system**

Propagation Dome

ADVANTAGES	DISADVANTAGES
Flexibility	Heat Buildup
Maintains high humidity	Labor intensive
Easy to use	

Humidity Tent

ADVANTAGES	DISADVANTAGES
Inexpensive	Heat Buildup
Maintains high humidity	Must be monitored
Easy to construct	

Automatic Mist System

ADVANTAGES
- Automatic Misting
- Adjustable misting
- Lower labor input

DISADVANTAGES
- Nutrient leaching
- Algae/fungal buildup

Fog System

ADVANTAGES
- 100% humidity
- No nutrient leaching
- Decrease heat buildup
- Lowers light levels

DISADVANTAGES
- Expensive
- High maintenance

STAGE IV. Transfer to Natural Environment

Fully acclimatized *Syngonium*

Micropropagation Videos

1. *Laboratory Procedures for Tissue Culture: A Beginner's Guide*
2. *Handling Tissue Culture Plants in the Nursery*