Plant Propagation PLS 3223/5222

Guest Web Lecture
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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**
- Small propagule
- Aseptic conditions
- Controlled environment
- Heterotrophic growth
- Rapid multiplication
- Greater initial costs

**Macropagation**
- Larger propagule
- Non-aseptic conditions
- Less environmental control
- Photoautotrophic growth
- Slower multiplication
- 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
In Vitro Culture: Early Attempts

Haberlandt 1902
Innate potential of cells

- 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

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

<table>
<thead>
<tr>
<th>Category</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foliage Plants</td>
<td>63,695,000</td>
</tr>
<tr>
<td>Greenhouse Flowers</td>
<td>11,297,000</td>
</tr>
<tr>
<td>Perennials</td>
<td>9,448,000</td>
</tr>
<tr>
<td>Trees &amp; shrubs</td>
<td>15,294,000</td>
</tr>
<tr>
<td>Vegetables</td>
<td>12,862,000</td>
</tr>
<tr>
<td>Fruits</td>
<td>3,721,000</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>4,545,000</td>
</tr>
</tbody>
</table>

Total: 120,862,000

(Zimmerman, 2001)
USA Commercial Micropropagation
Laboratory Costs

- Labor: 46%
- Supplies & Overhead: 27%
- R&D: 13%
- Sales: 9%
- Admin.: 4%
- Interest: 1%

Commercial Micropropagation: A Global Industry

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

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**

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

3. Non-zygotic Embryogenesis

- Production of non-zygotic embryos from single cells

- Direct Non-zygotic Embryogenesis
- Indirect 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

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**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 diagram]

Developing leaf
Apical meristem
Developing lateral bud
First mature xylem
Lateral bud
Note vascular strands going to leaves
Important Discovery


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

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

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
Stage 0. Donor Plant Selection & Preparation

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

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

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**Meristem-tip isolation**

**3 wks**

**Single shoot (9 wks)**

**Clean**

**Culture indexing for pathogens**

**Rooting**

**Acclimatization**

**Shoot culture**
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 (shaken)

Three five-minute sterile water rinses
Shoot-tip Isolation

STAGE I. Culture Initiation

Inoculation  Day 0  Day 28

4 Weeks
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

Many times what you “see” is not what you get!

The “EBD”

- Need to screen (index) for the presence of cultivable 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

Multi-node stem
Basal meristem
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

1. Isolation phase
2. Stabilization Phase (physiological adjustment)
3. Production Phase

STAGE I. Culture Stabilization

- Rapid, uniform shoot growth
- Periodic slow growth
- No growth

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

**STAGE II. Shoot Multiplication**

[Diagram of micropropagation process]

- **Stage I**: Establishment of axenic culture
- **Stage II**: Proliferation of axillary shoots
- **Stage III**: Rooting
- **Stage IV**: Transfer to natural environment
- **Stage V**: Nursery
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)

- $\alpha$-indole-3-acetic acid (IAA)
- 1- naphthaleneacetic acid (NAA)
- indolebutyric acid (IBA)
STAGE II. Shoot Multiplication

Addition of NAA does not promote shoot production
Addition of NAA promotes shoot elongation

Subculture shoot clusters at 4 - 5 week intervals

3 - 8 fold increase in shoot numbers (4.3 x 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 Microcutting

Ex vitro 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

<table>
<thead>
<tr>
<th>STIMULATORY MEDIUM COMPONENTS</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. AUXINS</strong></td>
<td></td>
</tr>
<tr>
<td>indole-3-acetic acid [IAA]</td>
<td>Dosage effect (Conc. x time)</td>
</tr>
<tr>
<td>indole-3-butyric acid [IBA]</td>
<td>0.05 - 10 mg/liter for (days - weeks)</td>
</tr>
<tr>
<td>α-naphthaleneacetic acid [NAA]</td>
<td>or 50 - 100 mg/liter (sec - hours)</td>
</tr>
<tr>
<td><strong>2. HIGH SUGAR/NITROGEN RATIO</strong></td>
<td>Effect depends on mineral medium</td>
</tr>
<tr>
<td><strong>3. PHENOLS</strong></td>
<td>May stimulate rooting (<em>species dependent</em>)</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td></td>
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### Factors Important To In Vitro Rooting

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<th>Stimulatory Medium Components</th>
<th>Comments</th>
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<tr>
<td><strong>4. Activated Charcoal</strong></td>
<td>May reduce light in medium or absorb inhibitory compounds</td>
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### Factors Important To In Vitro Rooting

<table>
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<th>Inhibitory Medium Components</th>
<th>Comments</th>
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<tbody>
<tr>
<td><strong>1. Cytokinins</strong></td>
<td>Common observation. Eliminated in rooting medium</td>
</tr>
<tr>
<td><strong>2. Gibberellins</strong></td>
<td>Inhibit root formation</td>
</tr>
<tr>
<td><strong>3. High Ionic Strength of Medium</strong></td>
<td>Confounded by effects of individual nutrients</td>
</tr>
<tr>
<td><strong>4. Agar</strong></td>
<td>Exact cause unknown; agar may be impure and variable in content</td>
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STAGE III. Pretransplant (Rooting)

Auxin type & concentration used dependent on:

- Percent (%) rooting, root number and length

### Auxin Effects on In Vitro Stage III Rooting¹

<table>
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<th>Treatment IBAs (mg/L)</th>
<th>% Rooting</th>
<th>Root Number</th>
<th>Root length (mm)</th>
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<tr>
<td>0</td>
<td>37</td>
<td>2.4</td>
<td>23.3</td>
</tr>
<tr>
<td>0.05</td>
<td>43</td>
<td>3.5</td>
<td>18.1</td>
</tr>
<tr>
<td>0.1</td>
<td>55</td>
<td>4.1</td>
<td>14.2</td>
</tr>
<tr>
<td>0.5</td>
<td>71</td>
<td>5.7</td>
<td>6.5</td>
</tr>
<tr>
<td>1.0</td>
<td>84</td>
<td>7.1</td>
<td>4.3</td>
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¹Rooting responses of 10 mm microcuttings of *Aronia arbutifolia* after 28 days.
### Auxin Effects on In Vitro Stage III Rooting

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

### Stage III Rooting

![Images of plants with varying IBA concentrations](image)
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 \textit{in vitro} to \textit{ex vitro} conditions.

High humidity & low light \textit{In vitro}  
Lower humidity & high light \textit{Ex vitro}

STAGE IV. Transfer to Natural Environment

ACCLIMATIZATION:

- Process whereby plants physiologically and anatomically adjust from \textit{in vitro} to \textit{ex vitro} cultural and environmental conditions

Two reasons micropropagated plants may be difficult to re-establish \textit{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

"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

3. Other Factors Affecting Acclimatization & Quality

Container/Medium/Plug Size Considerations

<table>
<thead>
<tr>
<th>MICROCUTTING TYPE</th>
<th>SINGLE SHOOT</th>
<th>SHOOT CLUSTER</th>
</tr>
</thead>
<tbody>
<tr>
<td>SINGLE SHOOT</td>
<td>Acclimatized Plants</td>
<td></td>
</tr>
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<td>SHOOT CLUSTER</td>
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<td></td>
</tr>
</tbody>
</table>

Planting Stage III rooted microcuttings
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

4. Acclimatization Structures

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

**ADVANTAGES**
- Flexibility
- Maintains high humidity
- Easy to use

**DISADVANTAGES**
- Heat buildup
- Labor intensive

Humidity Tent

**ADVANTAGES**
- Inexpensive
- Maintains high humidity
- Easy to construct

**DISADVANTAGES**
- Heat buildup
- Must be monitored
**Automatic Mist System**

**ADVANTAGES**
- Automatic Misting
- Adjustable misting
- Lower labor input

**DISADVANTAGES**
- Nutrient leaching
- Algae/fungal buildup

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**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