National Science Foundation Plant Genome
Cereal Plant Transformation Workshop
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Cereal Transformation
Laboratory Basics and Set-up

NSF Plant Transformation Workshop
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Establishing a Laboratory Capable of Plant Transformation and Tissue Culture

Requirements for Set-up

Equipment
Setting Up A Plant Transformation Laboratory

Equipment and Space

What is Required to Set up and Establish a Functioning Plant Transformation Laboratory
Setting Up A Plant Transformation Laboratory

Equipment and Space

Teaching
Research
Outreach
Cell and Tissue Culture Laboratory

Laminar Flow hoods
Dissecting Microscopy Equipment
DuPont Biolistics Gene Gun
Dissecting Light Microscopy Imaging in Aseptic Space
Student Cell Culture Laboratory DNA Extraction
Molecular Vector Construction Laboratory
Molecular Analysis Equipment and Space
Molecular Analysis Laboratory DNA Extraction
Tissue Culture Media Laboratory
Tissue Culture Laboratory *Agrobacterium* Isolation Area
Spectrophotometer
Several Rotary Incubators
Tissue Culture Laboratory Electroporation Equipment
Tissue Culture Media Laboratory
Walk-in Growth Room
Autoclave, Freeze Drier, -80 Freezer
GFP Fluorescence Light Microscopy
Photography
Videography
Several Stand up Growth Cabinets Equipped with Temperature and Light Control
Conference Room and Teaching Space
Laboratory Basics
The Importance of Plant Tissue Culture
A Plant Tissue Culture Facility for Plant Biotechnology
Introduction to Plant Biotechnology

• Goals of plant breeding and crop improvement are to develop germplasm that:
  Minimizes agronomic problems
  Maximizes yield and quality

• Major limitation with classical plant breeding
  Relies on the selection of chance genetic combinations drawn from a finite gene pool

• Goals of plant biotechnology
  Understand the biochemical processes and molecular biology underlying yield, disease resistance, and quality characteristics
  Develop technologies, including transformation, that allow planned genetic changes to modify these processes
  Expand the germplasm available to plant breeding programs
Overview of Plant Transformation:

• **Goals of plant transformation for crop improvement are to produce**
  - Produce fertile transgenic plants
  - At reasonable frequencies
  - From “elite” backgrounds

• **Components of any successful plant transformation system**
  - Delivery of DNA to the plant genome without compromising cell viability
  - Selection of transformed cells
  - Regeneration to produce intact fertile plants
  - Expression and inheritance of the introduced genes

*These components need to be developed in parallel*
Overview of Plant Tissue Culture:

• Plant tissue culture exploits the *in vitro* plasticity of plant growth and development
  Whole plants can be regenerated from a wide range of isolated plant cells (totipotency)

• Cell division and callus (dedifferentiated tissue) formation, embryogenesis and organogenesis can be induced using plant growth regulators
  Cytokinins, BAP, zeatin
  Auxins, 2,4-D, dicamba

• There are no universally applicable methods of plant tissue culture, transformation, selection and regeneration
  Protocols must be modified for each genus, species, variety and tissue
Morphogenetic Plasticity in Plant Tissue Culture
Overview of Plant Tissue Culture (Continued)

- Various factors have made cereals difficult to handle in tissue culture
  - Tissue physiology
  - Developmental characteristics
  - Tissue culture requirements

- Within individual cereal species the “elite” germplasm is usually least amenable to tissue culture

- Patent and FTO issues often influence the choice of tissue culture methods and/or drive the development of novel transformation technologies in cereal biotechnology
  - Ciba’s reliance upon protoplast electroporation systems for corn transformation
  - Northrup King’s reliance upon PEG-mediated transformation of protoplasts for corn
  - Zeneca’s development of silica fiber-mediated corn transformation
Overview of Plant Tissue Culture (Continued)

Somaclonal Variation

• Plants regenerated in tissue culture may differ from that of the plant from which the cells originated
  - Genotypic differences such as chromosomal rearrangements, transposon activation, DNA methylation and mutation
  - Phenotypic differences such as morphological abnormalities, albinism, reduced fertility and/or poor agronomic characteristics

• This somaclonal variation is associated with extensive periods in tissue culture (callus induction) either before or after transformation

• The most suitable explants for transformation are those which require the least amount of time in tissue culture
  - Cells that can rapidly enter embryogenesis, such as those from immature embryos cultured in high concentrations of auxin to generate somatic embryos
  - Meristem (germ line) transformation for tissue culture-free plant production
Laboratory Basics
Laminar Flow Hoods and Aseptic Technique
Tools of the Trade
Tools of the Trade

Bead Sterilization
Tools of the Trade

Disposable mats, paper plates of filter paper may be used for dissection under sterile conditions on these materials after they have been autoclaved.
Tools of the Trade

Containers
Tools of the Trade

Containers
Aseptic Technique
Maintain Proper Aseptic Technique Throughout the Procedure
EdgeGARD Laminar Flow Hoods for Plant Tissue Culture

Dissecting light microscope, gas, Bunsen burner or Bead Sterilizer
Receptacle (for microscope or other electrical), Lights and Blower switches are underneath to the right light. Blower is left on for routine use.
Tools for use routine autoclaved and stored until use

Forceps, scalpels, spatulas in a glass test tube, which can be used for the 95% alcohol used to flame or bead sterilize
95% Ethanol is used to flame sterile tools

Ethanol is stored in a Flame Proof cabinet for safety (not in the hood!). Note: Whatman filter paper, if need for some procedures is autoclaved wrapped in aluminum foil or in Glass Petri dishes.
70% Ethanol is used to decontaminate surfaces of the hood.

70% Ethanol is more effective than 95% Ethanol, because it hydrates spores and bacteria allowing for effective microbialcidal conditions. Also, it is relatively safe for humans (most commercial hand washes contain 70% ethanol) and is less flammable than 95% Ethanol.
Spray 70% Ethanol liberally on the hood surface
Use a paper towel to wipe the surfaces. Ignite Bunsen burner with flint lighter. (The pilot light is sufficient and safer).
Also use 70% Ethanol on a paper towel to decontaminate any equipment in the hood, such as the dissecting microscope (knobs base and fiber optic cord or any other surface which may have been touched previously)

Note: 70% Ethanol can also be used on micropipete racks, micropipeteters, tube racks, wiping down the light source knob, or any other surface which may be contaminated. Do not be complacent about sources of possible contamination.
Also use 70% Ethanol on a paper towel to decontaminate any equipment in the hood, such as the dissecting microscope (knobs base and fiber optic cord or any other surface which may have been touched previously)

70% Ethanol is more effective than 95% Ethanol, because it hydrates spores and bacteria allowing for effective microbialcidal conditions. Also, it is relatively safe for humans (most commercial hand washes contain 70% ethanol) and is less flammable than 95% Ethanol.
Subculture of Tissue Cultures:

Flame tools in 95% ETOH, wait until the tool cools, keep petri dishes in front (toward the back of the hood), never should the hand pass over an open container, transfer the tissue (either sideway or back to front) replace the cover, then wrap with Parafilm, micropore tape (3M 1530-1) or another appropriate tissue culture tape.
A Dissecting Light Microscope is often used when necessary to visually select for specific callus morphotypes or transformants under selection (i.e. embryogenic callus) for subculture.
Proper use of a Dissecting Light Microscope to maintain aseptic technique in the hood
A Dissecting Photo Light Microscope is used to image and record under sterile technique. ALWAYS record ALL images with proper publication quality (this is NOT just data collection!)

Tissues can be returned to culture so this is not destructive to samples
ALL images should be publication quality (this will save time later), and often a researcher may not always appreciate what samples are important.
Greenhouse Space

Field Space
The Chemistry of Plant Tissue Culture Media

Plant tissue culture media are composed of several components: Various salts, vitamins, amino acids, growth regulators, sugars, agar or gel rite and water.
Media Making: A Media Kitchen
Plant Tissue Culture Media
Comparison of MS and N6 Media

<table>
<thead>
<tr>
<th>Murashige and Skoog (MS)</th>
<th>Component (mg/L)</th>
<th>(N₆)</th>
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**Macronutrients**

| 1650 | Ammonium nitrate (NH₄NO₃) | 463 |
| 1900 | Potassium Nitrate (KNO₃) | 2830 |
| 440  | Calcium Chloride (CaCl₂)  | 125.33 |
| 180.7| Magnesium Sulfate (MgSO₄) | 90.37 |
| 170  | Potassium Phosphate (KH₂PO₄) | 400 |

**Micronutrients**

| 0.83 | Potassium Iodide (KI) | 0.8 |
| 6.2  | Boric Acid (H₃BO₃) | 1.6 |
| 16.9 | Manganese Sulfate (Mn SO₄ • 4H₂O) | 3.3 |
| 8.6  | Zinc Sulfate (Zn SO₄ • 4H₂O) | 1.5 |
| 0.25 | Molybdic Acid (Na₂Mo0₄ • 2H₂O) | / |
| 0.025| Cupric Sulfate (Cu SO₄ • 5H₂O) | / |
| 0.025| Cobalt Chloride (CoCl₂ • 6H₂O) | / |
| 27.8 | Ferrous Sulfate (Fe SO₄ • 7H₂O) | 27.85 |
| 37.26| Sodium EDTA (NaEDTA • 2H₂O) | 37.26 |
Micro- and macro-elements

Based on the need of the plant for these elements.

Not a guide-line for the importance of these elements for the plant.

Micro-elements: Fe, Cu, Mn, Co, Mo, B, I, NI, CI and Al

• Only the mineral part of its related salt is of importance to the plant. The anion is mostly not essential.

• Hard to recommend the minimal required amount of minerals to be added to a medium. Determined empirically in growth curve studies

Macro-elements: Mg, Ca, P, S, N and K

• In general, from the macro-elements both anions and cations are important: both K+ and NO3- are essential

• The concentration ammonium that can be supplied without harmful consequences for the plant, is sometimes sharply defined
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

Nitrogen (N), Nitrate (NO₃)⁻, and Ammonium (NH₄)⁺

Nitrate reductase
Nitrite reductase
Reduced nitrogen containing compounds
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

Nitrogen (N), Nitrate (NO₃)⁻, and Ammonium (NH₄)⁺

The major component of almost all media is inorganic nitrogen in the form of nitrate or ammonium. The salts which are mostly used are potassium nitrate (KNO₃), ammonium nitrate (NH₄NO₃) and calcium nitrate (Ca(NO₃)₂·4H₂O).

These compounds provide important inorganic nitrogen to synthesize complex organic molecules.

Ammonium is in the roots mainly stored as organic nitrogen. Nitrate can be transported via the xylem to other parts of the plant, where it participates in the nitrogen assimilation. Nitrate can be stored in the vacuoles of the cell and fulfills an important function in osmoregulation.
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

Nitrogen (N), Nitrate (NO₃⁻), and Ammonium (NH₄⁺)

Nitrate reductase
Nitrate cannot simply be used to synthesize organic molecules but has to be reduced to ammonia first.

Nitrite reductase
The reduction of NO₂⁻ to NH₃ by nitrite reductase is carried out in the leaves. Reduced ferredoxin supplies the electrons for the reduction of nitrite. Ferredoxin, reduced by electrons generated in photosystem I, supplies the electrons for the reduction of nitrite.

Ammonium and ammonia (NH₃ ~ NH₄⁺ + OH) are toxic for plants, even in low concentrations. Glutamine synthetase and glutamate synthase, both present in roots and shoots, are key enzymes in the conversion of ammonium (see also, phosphinothricin PPT)
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

Calcium (Ca)

• Location:
  Cell Wall
  Cell Membrane
  Enzymes
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

Calcium (Ca)

In contrast to the other macro-nutrients, calcium is largely bound to the cell wall and cell membrane.

Between two cell walls Ca²⁺ mainly binds to R-COO groups of polygalacturonic acids under formation of pectates.

In apple, 90% of the total amount of calcium in the cell can be stored as pectate. The high concentration of calcium in the cell wall and cell membrane mainly serves the strength of the cell wall and the regulation of the cell membrane structure.

Pectin is broken down by the enzyme polygalacturonase. However, calcium strongly inhibits the activity of polygalacturonase. A high enzyme activity is observed in absence of calcium, causing degradation of the cell wall. The result is a softening of the plant tissue. If sufficient calcium is available most pectin will be in the form of calcium pectate. In this way, the cell wall is highly resistant to destructive activity of polygalacturonase. The presence of Ca²⁺ is also important for the resistance against fungal infections. Calcium is important in cell and root multiplication and pollen tube growth.
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

Phosphorous (P)

- Nucleic acids
- Phospholipids
- Energy metabolism
- Phosphate pool location
- Enzymes
**Macro-elements**: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

**Phosphorous** (P)

Essential element to RNA and DNA backbone structure

Phospholipids in membranes; a phosphate-ester bond connects diglyceride with amines, amino acids or alcohol groups

Phosphorus is very important for the energy metabolism of the plant in forming energy rich phosphate esters (C-P), like in glucose-6-phosphate and adenosine tri-phosphate (ATP) Photosynthesis is used to synthesize ATP and this energy is liberated during the hydrolysis of ATP in ADP and Pi

The phosphate metabolite pool is mainly in the form of phosphate esters, and present in cytoplasm and mitochondria. The non- metabolic pool, mainly in the form of Pi, is present in the vacuole.

Pi has also a strong regulatory function in many metabolic processes in the plant

Phosphorus deficiency results in delayed growth and a dark green color of the leaves. For optimal growth 0.3 to 0.5gram phosphorus per g dry weight is required
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

Potassium (K)

- Enzymes
- Cell extension
- Ion Balance
**Macro-elements:** Calcium, Phosphorous, **Potassium,** Magnesium, Nitrogen, and Sulphur

**Potassium (K)**

The potassium ion is present in the highest concentration, in the cytoplasm between 100 en 200 mM and in the chloroplasts between 20 and 200 mM.

K+ is essential for the activation of many enzymes. More than 50 enzymes in the plant depend on, or are stimulated by potassium. K+ is essential for the activation of many enzymes. More than 50 enzymes in the plant depend on, or are stimulated by potassium.

The development of a large central vacuole in the cell is an important process in the cell extension by increasing osmotic potential

K+ is important in the maintenance of the ion balance.
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

Magnesium (Mg)

• Photosynthesis
• Enzymes
• Energy Metabolism
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulfur

Magnesium (Mg)

Mg²⁺ ions are very mobile and able to form a complex with strong nucleophilic ligands like phosphoryl groups.

Magnesium is the central atom in chlorophyll molecules of photosystem I and II which are parts of the photosynthesis. If magnesium is optimally available, 10 to 20% of the Mg²⁺ ions in the leaves will be localized in the chloroplasts.

Magnesium forms a bridge between both ribosome sub-units. In magnesium deficiency, the subunits will dissociate and protein synthesis stagnates.

Magnesium is required for the activity of RNA polymerases, enzymes involved in the synthesis of RNA. A shortage of Mg²⁺ will block RNA synthesis. In the leaves 25% of the total proteins is localized in chloroplasts. Magnesium is also important for Ribulose Biphosphate Carboxylase activity.

Magnesium is indispensable for the energy metabolism of the plant because of its importance in the synthesis of ATP

(ADP + Pi ~ ATP).
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulphur

Sulphur (S)

- Sulphur assimilation
- Proteins
- Metallathioeins
- Nonreduced sulphur
Macro-elements: Calcium, Phosphorous, Potassium, Magnesium, Nitrogen, and Sulphur

Sulphur (S)

Sulfur is taken up as $S_0^{2-}$ in the roots of the plant at a relatively low rates. Like nitrate, sulphate has to be reduced first before it can be used for the synthesis of reduced sulfur containing compounds like amino acids, proteins and enzymes. Sulfur is in the nonreduced form incorporated in sulphulipids and polysaccharides. The first step in the sulfur assimilation is activation of $SO_4^{2-}$ by the enzyme ATP sulfurylase, utilizing ATP.

Sulfur in protein is present in the amino acids cysteine and methionine.

Low-molecular sulfur containing compounds, the metallothioneins, are frequently found in plants. Most of these compounds contain cysteine.

Sulfur is in the nonreduced form a component of sulpho-tipids, which form a structural constituent of membranes. The characteristic odor of species like onions and garlic is mainly due to the presence of volatile sulfur containing compounds.
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Boron (B)

• Least Understood

• Cell Wall Structure
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

**Boron (B)**

Least understood: important to the cell wall
Taken up by roots and transported via the xylem

In the cell membrane it is mainly present as a borate ester.

There are no enzymes known that contain boron or which are activated by boron.

The functions of boron are mainly extracellular.

Boron is involved in the lignification of the cell wall and differentiation of the xylem.

A deficiency of boron immediately results in inhibition of the length & growth of primary and secondary roots
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Chloride (Cl)

• Chloroplast & Photosynthesis
• Osmotic potential
• ATPase
• Nitrogen metabolism
**Micro-elements:** Boron, **Chloride**, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

**Chloride (Cl)**

Varies from 70 to 700 mM per kilogram dry weight.

Taken up as Cl- and very mobile in the plant.

The main functions of the ion are osmoregulation and compensation of charges.

Chloride functions in the chloroplast as a cofactor in the oxygen generating manganese complex; role in photosystem II during the Hill reaction when H2O is split into O2 and 2H+.

The chloride ion regulates **osmotic potential** as in the opening and closing of stomata and regulation of the osmotic potential of vacuoles

ATPase and regulation of proton pumps

Chloride activates asparagine synthetase, an enzyme important in nitrogen metabolism.
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Iron (Fe)

• Hemoproteins

• Iron-sulphur proteins
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Iron (Fe)

Iron is mainly bound to chelators and complex compounds.

Free Fe^{2+} & Fe^{3+} levels are extremely low (10 mM).

Most plants only absorb Fe^{2+}. Fe^{3+} has to be reduced to Fe^{2+} at the root surface before it is transported to the cytoplasm (only grasses mainly take up iron in the 3+ form. Iron is mainly transported as an iron-carbohydrate complex

The iron-containing proteins can be separated into hemoproteins and iron sulphur proteins. The most well-known hemoproteins are the cytochromes. Catatases and peroxidases are also heme-iron containing enzymes.

Ferridoxin is the most common iron-sulphur protein and functions as carrier in the electron transport of reactions catalyzed by nitrite reductase, sulphate reductase,
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Copper (Cu)

• Photosynthesis
  Super Oxide Dismutase

• Mitochondrial
  Electron transport chain
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Copper (Cu)

Copper is a divalent cation and is taken up by the plant as CU2+ or as a copper chelate complex and is transported as a copper complex.

A shortage of copper immediately results in a decrease of the activity of many copper containing enzymes. About 50% of the copper present in chtoroptasts is bound to plastocyanin. Copper deficiency is very soon followed by a decrease in photosynthesis.

Copper is, in addition to zinc, part of the enzyme Super Oxide Dismutase (Ca-Zn.SOD). Superoxide is neutralized by SOD and the H2O2 is subsequently detoxified into oxygen and water by catalase.

Besides the Cu-Zn.SOD a manganese containing SOD is present in the cell as well. SOD detoxifies the reactive O2- radical into H2O2 and O2, thereby protecting the cell for the destructive capacity of this radical. Copper also plays an important role in the mitochondrial electron transport chain.
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc.

Cobalt (C0)

• Function: Unknown

• Probable Role in Nitrogen Fixation
### Micro-elements

Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

### Cobalt (C0)

The function of cobalt in plants is not known:

Cobalt is important in nitrogen fixation, such as root tubers of legumes of Rhizobium species. Cobalt is an essential component of the cobalamin enzyme. Three enzyme systems of Rhizobium bacteria are known to contain cobalamin.

Cobalt is required for bacterial methionine synthesis, ribonucleotide synthesis and synthesis of methylmalonyl-coenzyme A mutase. Methylmalonyl-coenzyme A mutase is necessary for the synthesis of leghemoglobin.
Micro-elements:  Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Manganese (Mn)

• Photosynthesis
  Hill Reaction Of Photosystem II
• Superoxide Dismutase
  Oxygen free radicals
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Manganese (Mn)

Manganese is taken up by the plant as bivalent unbound Mn²⁺

Mn is strongly bound to several metalloproteins, either as structural part of the binding site of the enzyme or as part of the [Mn(II)/Mn(III)] redox system

Mn is involved in the so-called Hill reaction of photo system II, in which water is split into oxygen and protons

Besides the Cu-Zn.SOD a manganese containing SOD is present in the cell as well. SOD detoxifies the reactive O₂⁻ radical into H₂O₂ and O₂, thereby protecting the cell for the destructive capacity of this radical.
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc

Molybdenum (Mo)

• Nitrogen Assimilation
  Nitrogenase
  Nitrogen Reductase
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Molybdenum (M0)

Molybdenum is in aqueous solutions mainly present as $\text{MoO}_4^{2-}$

A few enzymes are known to use Mo as a co-factor. The two most described molybdenum containing-enzymes are nitrogenase and nitrate reductase.

Molybdenum is directly involved in the reduction of N2. The nitrogen molecule is bound to the molybdenum atom in the nitrogenase complex

Nitrate reductase reduces nitrate into nitrite in nitrogen assimilation. Nitrate reductase contains a heme~iron molecule and two molybdenum atoms.

FAD, cytochromes (Fe(II)/Fe(III) molybdenum (Mo(V)/(VI)) are functional parts of the nitrate reductase complex and the electron transport chain
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc

Zinc (Zn)

- Enzyme Co-factor
- Protein synthesis
- Indol Acetic Acid Synthesis
Micro-elements: Boron, Chloride, Iron, Copper, Cobalt, Manganese, Molybdenum, Zinc,

Zinc (Zn)

Zinc is very important for protein synthesis. A shortage of zinc results in considerable reduction of protein synthesis. Zn is essential for the activity of RNA polymerase. RNA polymerase contains two Zn atoms which determine the proper structure of the enzyme.

Zinc plays a role in the synthesis of typophan, a precursor of IAA
Vitamins

Some common examples:

- Thiamine HCL
- Nicotinic Acid
- PridoxineHCL

Vitamins are added in several forms and concentrations.

Essential for many biochemical reactions.

In almost all media for plant cell culture, Thiamine (vitamin B1) is included: Linsmaier and Skoog

Myo-Inositol significantly stimulates the growth and development of plants; however, not essential for growth

The effect of vitamins on the development of the cell in vitro differs from species to species
Carbon Sources

Some Common Examples

- Sucrose
- Glucose
- Maltose
- Fructose
Media Addenda

Some Common Examples

- Coconut Water
- Casein Amino acids
- Myo-inositol
- Glycine
- Activated charcoal
- DDT
- Silver Nitrate
- Copper sulfate
- PVP
In plant and animal physiology, hormones denote substances that are synthesized in low amounts in one part of an organism and transported to target tissues in other parts where they exert an effect. In contrast with animal hormones, the synthesis of a plant hormone is often not localized in a specific tissue, but may occur in many different tissues.

Furthermore, plant hormones may be transported and act in distant tissues, but often they have their action at the site of synthesis. Another property of plant hormones is their lack of specificity: each of them influence a wide range of processes.

Auxin, for example, has been found to influence cell elongation, cell division, induction of primary vascular tissue, adventitious root formation, senescence, fruit growth, outgrowth of axillary buds and sex expression.

Some biologists prefer to use terms like 'plant growth substance' or 'plant growth regulator'.

In plant tissue and cell culture, two classes of plant hormones, cytokinins and auxins, are of major importance. Other hormones, in particular gibberellins, ethylene, abscissic acid, polyamines, or jasmonates, have been used only occasionally.
Auxins

**Types of auxins**
Various naturally occurring auxins are known, namely, IAA, IBA, 4-CI-IAA, PAA and conjugates of these auxins. In addition, many chemical analogues have been synthesized. Some of them are regularly used: NAA, 2,4-0, 2,4,5,- T, dicamba and 4-CPA. These synthetic auxins are in many cases more effective than the natural auxins.

**Effects of Auxins**
Auxins were discovered eighty years ago (1920s) by the Dutch plant physiologist F.W. Went. He observed that auxins produced in the tip of an Avena coleoptile influence the curvature of the coleoptile just below the tip. Shortly after that, the root-inducing capability of IAA was discovered and NAA and IBA were chemically synthesized.
Auxins in Tissue Culture

The role of auxin in tissue culture has been established by Skoog and Miller in 1957. They observed that pith tissues excised from tobacco stems form shoots at high cytokinin and low auxin concentration in the medium, roots at low cytokinin and high auxin concentration, or callus at intermediate concentrations of both plant hormones. A few years later, the formation of somatic embryos by treatment of tissues with the auxin 2,4-D was observed.

It should be noted that auxins are only required during the initial phases of adventitious root formation and somatic embryogenesis. After that, they become inhibitory and block the outgrowth of the newly formed organs and embryos. Auxin is also required for callus formation and it has been suggested that auxin triggers a specific step in the cell cycle. Auxin, either synthesized endogenously or applied exogenously, inhibits the outgrowth of axillary buds.

The rapid uptake of auxin results in depletion of the medium. When plant tissues are cultured in liquid medium, most of the auxin may have disappeared from the medium within a few days. Auxin depletion of solidified medium close to the explant may occur soon after sub-culturing, because replenishment from other parts of the medium is slow. From the crucial medium components, auxin seems to be the only one that is so very rapidly depleted.
Auxins in Tissue Culture

Uptake and metabolism

The epidermis of plants is relatively impermeable to auxin. In micro-cuttings most uptake occurs therefore via the cut surface. Auxin is taken up very rapidly. Uptake occurs 'by diffusion and by active uptake via a carrier. Diffusion depends on the dissociation of the molecule. Auxin is more protonated outside the plasmalemma (In the cell wall the pH is ca. 5.5; IAA is a weak acid with a pKa of 4.7) than inside the cell (the cytoplasm has a pH of ca. 7). The undissociated lipophilic auxin may diffuse easily through the plasmalemma into the cell. The anionic form that prevails in the cytoplasm, however, cannot easily diffuse out through the plasmalemma. So auxin is trapped within cells. Auxin is transported out of cells by efflux carriers.
Auxins in Tissue Culture

*Modulators of auxin transport, metabolism and action*

The endogeneous level of auxin and auxin action can be manipulated in various ways. In plant tissues, auxin is actively transported in a polar way.

TIBA and NPA block this transport, because these compounds bind to the efflux carrier.

The endogenous level of auxin can be increased by transforming plants with the auxin biosynthetic genes of Agrobacterium tumefaciens. The transformed plants show appropriate changes in their phenotype.

Phenolic compounds (e.g. ferulic acid or phloroglucinol) may inhibit oxidation of applied auxin. This is not specific inhibition of enzymatic oxidation: photooxidation is also inhibited by adding phenolic compounds to the medium. PCIB is a genuine anti-auxin and competes with auxin for the auxin binding place at the auxin receptor.
Cytokinins

*Types of cytokinins*

Cytokinins are the most complex class of plant hormones. The naturally occurring cytokinins are Z, ip, and DHZ and their ribosides ZR, iPA and OHZR. In addition, conjugated (non-active) and phosphorylated (active) cytokinins have been isolated from plant tissues.

For a long time, BAP has been considered to be a synthetic cytokinin, but has been recently shown to be a naturally occurring one. In addition to these cytokinins that are all of the purine type, nonpurine cytokinins have been reported such as thidiazuron (TOZ) and CPPU (4-PU-30). These compounds have a very high cytokinin activity and are particularly successful in woody plants. TOZ is used commercially as a cotton defoliant. In this case, it acts by inducing ethylene synthesis. Meta-topolin is a highly active aromatic cytokinin that was first isolated from Populus.

In tissue culture, BAP and the synthetic cytokinins kinetin and TOZ are most frequently used.
Cytokinins in Tissue Culture

Effects
The discovery of cytokinins is closely linked to tissue culture. In the early years of plant tissue culture, it was observed that malt, salmon sperm, coconut and yeast extracts promote both the growth and initiation of buds in vitro. Because these preparations all contain purines, nucleic acids were tested.

It was observed that autoclaving of nucleic acids strongly enhanced their effect. The active compound formed by autoclaving appeared to be kinetin, a hitherto unknown purine. In 1964, Letham isolated zeatin from immature corn.

Cytokinins promote cell division, but they likely influence another step in the cell cycle than auxins. Thus, addition of cytokinins is usually required to obtain callus growth. In micropropagation, cytokinins are used to promote axillary branching. High concentrations of cytokinin result in extreme, undesirable bushiness. Other applications of cytokinin in tissue culture are promotion of adventitious shoot formation, prevention of senescence, reversion of the deteriorating effect of auxin on shoots, and, occasionally, inhibition of excessive root formation (for example in germinating somatic embryos). Cytokinins inhibit root formation and are therefore left out from rooting media.
Auxins & Cytokinins

Fig. 4.1. Structural formulae of some auxins and cytokinins. Auxins include (a) indole-3yl-acetic acid, (b) \( \alpha \)-naphthaleneacetic acid, (c) 2,4-dichlorophenoxyacetic acid. Cytokinin activity is shown by (d) adenine, (e) kinetin, (e) and (f) \textit{trans-zeatin}.

(a) IAA  (b) NAA  
(c) 2,4-D  (d) Benzyl Adenine  
(e) Kinetin  (f) \textit{trans-Zeatin}

\textsuperscript{3} Both the \( \alpha \) and \( \beta \) isomers of NAA are commercially available, but the \( \alpha \) isomer is always used in culture media. The \( \beta \) isomer is a weak auxin with relatively little physiological activity.
Plant Morphogenesis and Tissue Culture
Callus production, isolation and maintenance

Explant Preparation

Surface Sterilization

Dissection

Inoculation
Callus production, isolation and maintenance

Explant Sources

Dicots

Surface Sterilization

Dissection

Inoculation
Callus production, isolation and maintenance

Explant Sources

Monocots

Surface Sterilization

Dissection

Inoculation
Callus production, isolation and maintenance

Callus Production

Auxin/Cytokinin
Callus production, isolation and maintenance

Callus Production

Auxin/Cytokinin

Undifferentiated cell growth?

Dedifferentiated cell growth?
Callus production, isolation and maintenance

Fig. 5.1. Growth response of a typical callus culture. This particular callus should be subcultured approximately at the time indicated by X.

Callus Production
Subculture Passage
Growth rate
Fresh wt
Volume
The Regulation of Organ and Callus Production in Response to the Ratio Of Auxin to Cytokinin in Tissue Culture Media
Suspension cultures

Suspension Culture of Fescue

- Embryogenic callus initiation
- Subculture of embryogenic callus
- Callus inoculum to liquid
- Suspension culture dispersed growth
- Regeneration to plants
Suspension Cultures As a Versatile Culture System
Suspension cultures

Batch Cultures

Continuous Cultures

Bioreactors
Suspension cultures

Growth Curves

Packed Cell Volume

Subcultures
Protoplast isolation and culture

Explant Preparation

Plasmolysis

Enzyme digestion
- Cellulase
- Macerozyme
- Hemicellulase
- Pectolyase
- Pectinase

Fig. 12.2. Basic technique for the isolation of protoplasts from an excised leaf. The leaf is surface sterilized, rinsed repeatedly in sterile distilled water, and the cells are plasmolyzed in a solution of mannitol. The lower epidermal layer is stripped from the leaf to enhance enzyme penetration into the mesophyll tissue. Following treatment with one or more wall-degrading enzymes, a crude suspension of mesophyll protoplasts is obtained.
Protoplast isolation and culture

Protoplast Purification

Sieving

Centrifugation

Resuspension

Plating

Nurse cultures
Protoplast isolation and culture

Nurse Cultures
Filters
Alginate
Protoplast isolation and culture

Protoplasts and Totipotency

Embryogenic suspensions as a source of protoplasts

DNA delivery
Protoplasts:

‘Naked Plant Cells’

Plant cells with their Cell wall removed.

Can be used to insert DNA
• Direct DNA Uptake
• Electroporation
• Microinjection

Serious Drawbacks
• Very Tedious
• Cultivar limitations
• Not effective
• Non-fertile plants
But…
DNA can be delivered to Protoplasts and transgenic Plants can be recovered

But…
It is a tough way to go.
Fig. 3.1. Growth response of a typical callus culture. This particular culture should be subcultured approximately at the time indicated by X.
Meristem, shoot tip, and bud cultures

Meristems as Explants
Meristem, shoot tip, and bud cultures

Meristems as Explants

Fig. 10.1. Micropropagation of potato (Solanum tuberosum). (a) Excised shoot tip grows to produce a plantlet. (b) In micropropagating culture multiple shoots are produced by outgrowth of axillary buds. (c) In vitro plantlet transferred to “jiffy” pot. (d) Plantlet derived from shoot tip ready for transfer to field.

Shoot Multiplication

Micropropagation

Virus Free

High Cytokinin
Meristem, shoot tip, and bud cultures

Meristems as Explants

Shoot Multiplication

Micropropagation

Virus Free

High Cytokinin
Meristem, shoot tip, and bud cultures

Meristems as Explants

Shoot Multiplication
Micropropagation
Virus Free
High Cytokinin
Organogenesis

Multiple shoots

A multi-celled events

High cytokinin
Microspore cultures and production of haploids

Microspores as a source for induction of haploid somatic embryos

Chromosome number can be doubled with colchicine
Microspore cultures and production of haploids

Microspores divide in culture

DAPI stained
Figure 2.1. Embryo development in Phlox drummondii. (a) First division of the zygote. (b–e) Stages of the embryogeny in the first four days after fertilization. (f–h) Later stages showing differentiation of protoderm, procambium, and cortical parenchyma. The shoot apex is first distinguishable in (g). Key: C, cortical parenchyma; Pc, procambium; Pd, protoderm; S, suspensor. ×225. (Miller and Wetmore, 1945.)
Figure 2.2. Embryo development in Phlox drummondii. (a–d) Dissected embryos showing globular, heart, and early torpedo stages. (e–h) Sections of embryos showing cellular detail of comparable stages. (e), (f), and (g) correspond to stages (f), (g), and (h) in Fig. 2.1. (i) Fully developed embryo showing shoot and root apices. Key: Cot, cotyledon; RA, root apex; RC, root cap; S, suspensor; SA, shoot apex; (a–d) ×60, (e–g) ×160, (h) ×95, (i) ×25. (Miller and Wetmore, 1945.)
Somatic Embryogenesis

Dicots

*In vitro* embryos go through the same stages of development as embryos *in planta*

Can be encapsulated

Artificial seeds
Somatic Embryogenesis

*In vitro* embryos go through the same stages of development as embryos *in planta*

Can be fused

Can form secondary embryos
Monocot Embryogenesis

Figure 2.4. Embryo development in Zea mays. (a) Three-celled embryo showing first division of terminal cell. (b) Six-day embryo showing embryo proper and suspensor. (c) Seven-day embryo showing delimitation of protoderm in embryo proper. (d) Nine-day embryo showing increased cytoplasmic density (on left) in region where meristems will arise. (e) Eleven-day embryo showing superficial position of shoot apical meristem and internal origin of root apical meristem. (f) Thirteen-day embryo showing shoot and root apical meristems, suspensor below and scutellum above. (g) Forty-four day, fully developed embryo. (a–d) ×140, (e) ×125, (f) ×42, (g) ×8.5. (Randolph, 1936.)
Somatic Embryogenesis
maize and other grasses
Type I And Type II Cultures

Fig. 1 Schematic representation of the 4 different pathways of regeneration in cultured maize embryos. (1) direct organogenesis, (2) indirect organogenesis, (3) direct somatic embryogenesis, (4) indirect somatic embryogenesis. The developmental pathway can be changed into another pathway, which is indicated by dashes. cl, coleoptile; ea, embryo axis; s, shoot; sb, scutellar body; sc, scutellum; 'sc', scutellum of somatic embryo; se, somatic embryo; sp, shoot primordium.
Somatic Embryogenesis
maize and other grasses
Type I And Type II Cultures

Figure 6.14 Embryogenic callus and plant regeneration. For most plants, the levels of two hormones, auxin and cytokinin, in the medium and the ratio of one to the other determine whether a small piece of tissue only forms callus (when both hormones are at high levels), sends out shoots (high cytokinin, low auxin), or sends out roots (high auxin, low cytokinin). 
(a) Proliferation of embryogenic callus derived from young leaf tissue. 
(b) Shoots begin to regenerate from pieces of callus after the biologist reduced the concentration of cytokinin in the medium. 
(c) When shoots have elongated, the biologist cut them off the callus and placed them on a media with a high auxin concentration. 
(d) Roots develop on media with a high auxin concentration. In this example, the plant is sugarcane. Source: Courtesy of James E. Irvine and T. Erik Milheir.
Organogenesis

roots

Multi-celled events

Root cultures

High auxin
Thank You