

PLNT2530 - Unit 1

2024

Plant Regeneration Through Tissue Culture

Reference: Chapter 2, Plant Biotechnology, A Slater, N Scott and M Fowler, Oxford Press 2003

Chapter 5 Plant Biotechnology and Genetics, C Neal Stewart Jr., John Wiley and sons, 2008

Plant Biotechnology - Rationale

What traits do we want in cultivated plants?

How can we leverage the biology of plants to breed varieties that have desired traits?

Outline – how we make plant biology work for us

In the lab part of the course, we will learn the techniques by which plant cells are grown in culture, and plants regenerated from cells.

Phenotypic plasticity and totipotency

Plant tissue culture

Hormonal regulation of plant growth and differentiation

Types of culture

Sterile technique

Development in animals follows a fixed program

Example: development of avian embryo

Basic body plan is determined very early in development
All subsequent developmental steps elaborate on the basic plan



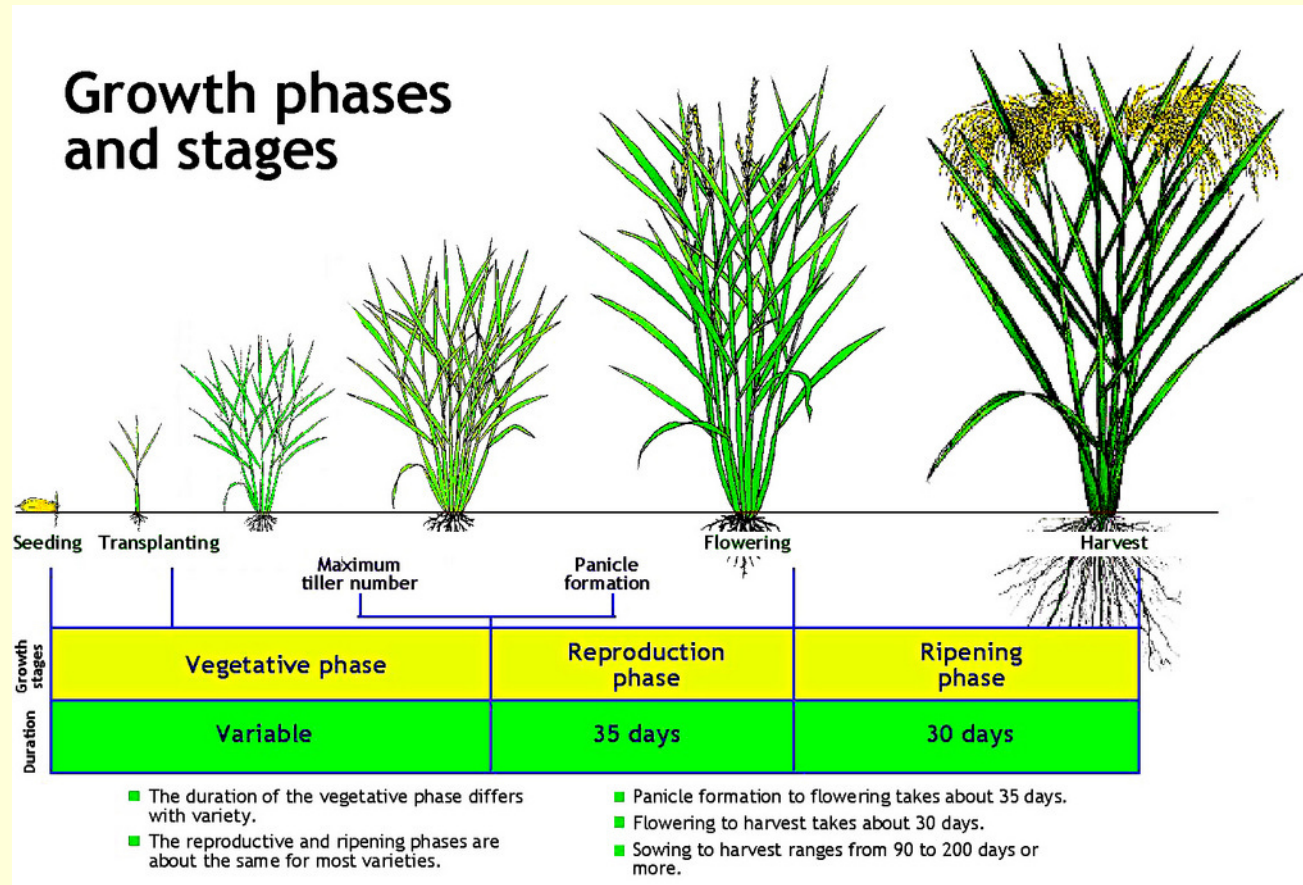
From: http://www.darwinwasright.org/developmental_biology.html

Development in plants is much more flexible than in animals

Example: development of a rice plant

Basic body plan is determined very early in development. Subsequent developmental steps are strongly-influenced by the environment.

Result: the precise body plan differs from plant to plant. eg. number, shape and size of leaves, and their arrangement, will vary from plant to plant.



Phenotypic Plasticity and Totipotency

All living systems have developed ways of adapting to environmental changes for the purpose of survival. Because they are sessile, plants have developed various ways of adapting to harsh environments as well as to predation from animals and insects.

Many of the processes that are a part of growth and development can be altered in response to the environment.

eg phototropism

The ability of a plant to alter its metabolism, growth and development to adapt to its environment is referred to as plasticity.

Plant biotechnology takes advantage of this property of plant development.

Particularly important is the ability of plants to regenerate lost organs and tissues. This involves the plant being able to initiate cell division from almost any tissue and have these new cells differentiate into any tissue in the plant.

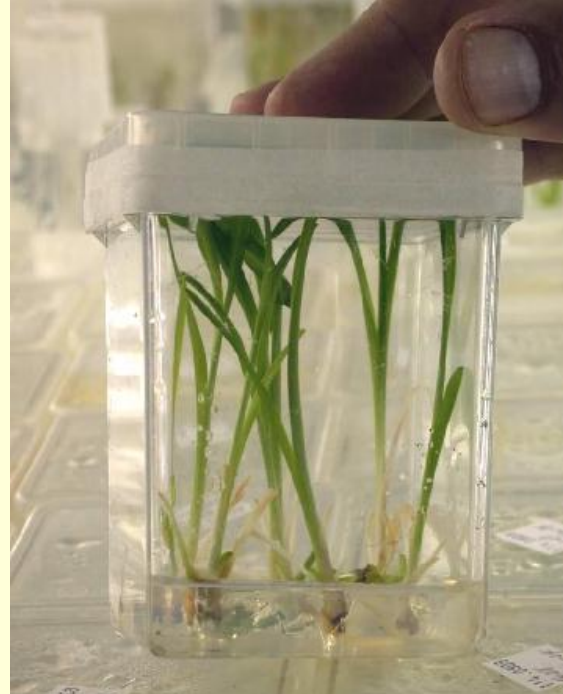
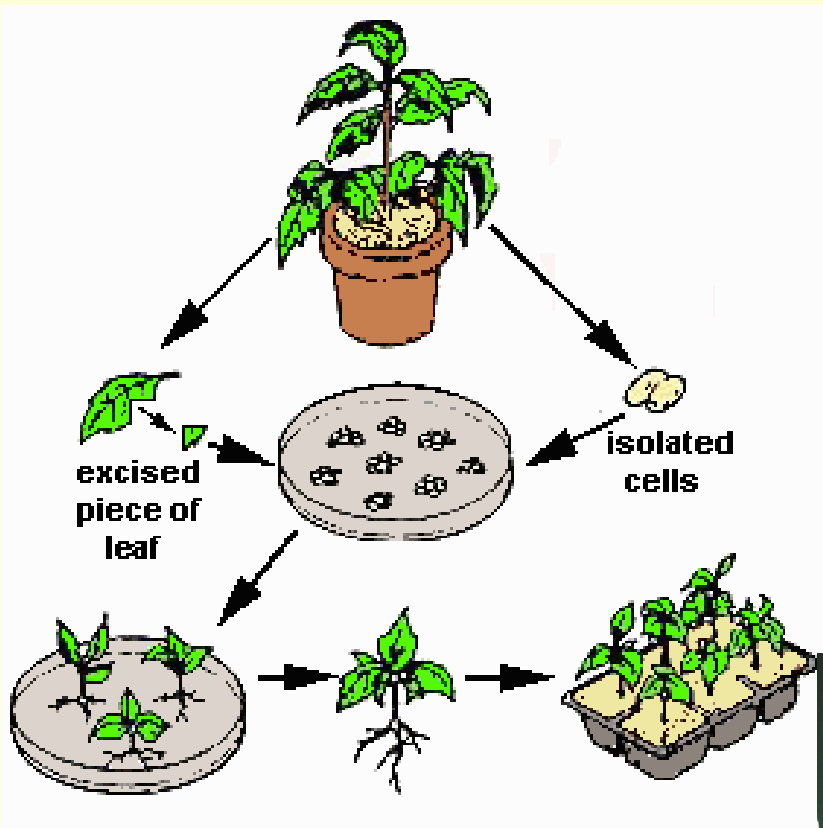
Siberian elm illustrates plasticity. Limbs and branches can be repeatedly cut off, and new branches will extend from wound points, or elsewhere.



This Norfolk Island Pine (which doubles as a Christmas tree) illustrates another aspect of phenotypic plasticity.

Note that three main shoots extend from the main trunk. Each shoot sends off branches opportunistically, in such a way as to maximize the area that captures light. In this way, the tree “fills in” to adapt to its environment.





Plantlets grown from culture

Plants can be regenerated in tissue culture either from tissue explants or from isolated cells.



华翠棕榈园

This regenerative aspect of plasticity is particularly important to the science and technology associated with tissue culture and plant regeneration.

When plant cells and tissues are cultured *in vitro*, in most cases they exhibit a very wide range of plasticity.

Regeneration of the whole plant from any single cell depends on **the concept that each cell, if given the appropriate stimuli, has the genetic potential to divide and differentiate into all types of tissues.**

This genetic potential by plant cells is referred to as **totipotency**

Tissue and Cell culture (applications)

- production of **doubled-haploid plants** to speed up production of homozygous plants
- combining of distantly related species by **protoplast fusion** with tissue culture regeneration (overcome some forms of reproductive incompatibility)
- **micropropagation** of plants using embryo, shoot or callus cultures (clonal propagation produces uniform plant progeny)
- **Genetic transformation** occurs at the cellular level and can be used to introduce altered genes into the host genome. (Cells must be regenerated into plants to recover the transgenic plant)
- **production of secondary metabolites** by plant cells

Tissue culture

-is the science of maintaining cells and/or tissues *in vitro* in an environment which regulates specific growth and development patterns.

Culture conditions requiring control include:

-Physical conditions (controlled with environmental chamber)

light

temperature

gaseous environment

-Chemical conditions (controlled by the culture media)

all essential nutrients, minerals, etc.

pH

water availability

Plant cell/tissue culture media

(The media may be liquid or 'solid')

3 basic components in media

- 1) **essential elements** (mineral ions) (as a mixture of salts)
- 2) **organic supplement** with vitamins and/or amino acids
- 3) **source of fixed carbon** (usually supplied as sucrose)

This media must supply all of the components required for plant cell growth and development.

The essential elements can be subgrouped for practical purposes into:

- a) macroelements,
- b) microelements and
- c) iron.

Plant cell/tissue culture media

Macroelements

- Nitrogen (N) -proteins, nucleic acids
- Potassium (K) -regulates osmotic potential, main inorganic cation
- Calcium (Ca) -cell wall structure, membrane function, signaling
- Magnesium (Mg) -enzyme cofactor, component in chlorophyll
- Phosphorus (P) -nucleic acids, energy transfer, req'd in respiration and photosynthesis
- Sulfur (S) -component of 2 amino acids and several cofactors

Microelements (required at low levels or as cofactors of variety of enzymes)

- Chlorine (Cl)
- Manganese (Mn)
- Cobalt (Co)
- Copper (Cu)
- Zinc (Zn)
- Molybdenum (Mo)

Iron is a microelement (quantitatively) but is identified separately because of the care required to avoid complexing with other essential elements. Normally added with EDTA (ethylenediamine tetraacetic acid) which forms a reversible complex with Fe^+ allowing it to become available gradually.

Organic supplements include B vitamins (thiamine-HCl and myoinositol) as well as amino acids. Various amino acids are recommended in specific cases (sometimes a casein hydrolysate is used)

Carbon source is required as the major component of all biomolecules synthesized, since photosynthesis is not occurring at the early stages of tissue culture and may not occur at all in certain tissues being cultured. Commonly sucrose is used because it is cheap, available and pure.

The most widely used plant culture media is MS media developed by Murashige and Skoog

Other media such as Gamborg's B5 media and the Schenk and Hildebrandt SH media are based on the fundamentals established in the MS media.

MS Media composition

<u>Element</u>	<u>Conc. in medium</u>
<i>Macroelements</i>	(mg/liter)
NH ₄ NO ₃	1650
KNO ₃	1900
CaCl ₂ ·2H ₂ O	440
MgSO ₄ ·7H ₂ O	370
KH ₂ PO ₄	170
<i>Microelements</i>	
KI	0.83
H ₃ BO ₄	6.2
MnSO ₄ ·4H ₂ O	22.3
ZnSO ₄ ·7H ₂ O	8.6
Na ₂ MoO ₄ ·2H ₂ O	0.25
CuSO ₄ ·5H ₂ O	0.025
CoCl ₂ ·6H ₂ O	0.025

<u>Element</u>	<u>Conc. in medium</u>
<i>Iron source</i>	(mg/liter)
FeSO ₄ ·7H ₂ O	27.8
NaEDTA·2H ₂ O	37.3
<i>Organic supplement</i>	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.5
Glycine	2
<i>Carbon source</i>	
Sucrose	30,000

Gelling Agent

When the plant cells/tissues in culture need to grow on a surface, 'solid' media is used. A gelled media produced by addition of agar can be used but quality can vary among suppliers. (Agar is produced from seaweed and is variable in quality.)

Linear polymer of galactose but may contain variable levels of acidic groups

Purified form of agar, agarose (low in acidic units), is widely used, as are several more expensive microbial generated gellan gums because of their higher consistency and reproducibility.

Gel provides a solid media which holds and releases water as required to the tissues.

Laminar flow hood

In the lab, air flow tends to be turbulent, which would result in bacterial and fungal contamination in culture.

Laminar flow hoods move air in layers, so that particles will tend to move parallel to the surface. Hoods that employ negative pressure also filter particles out of the air.

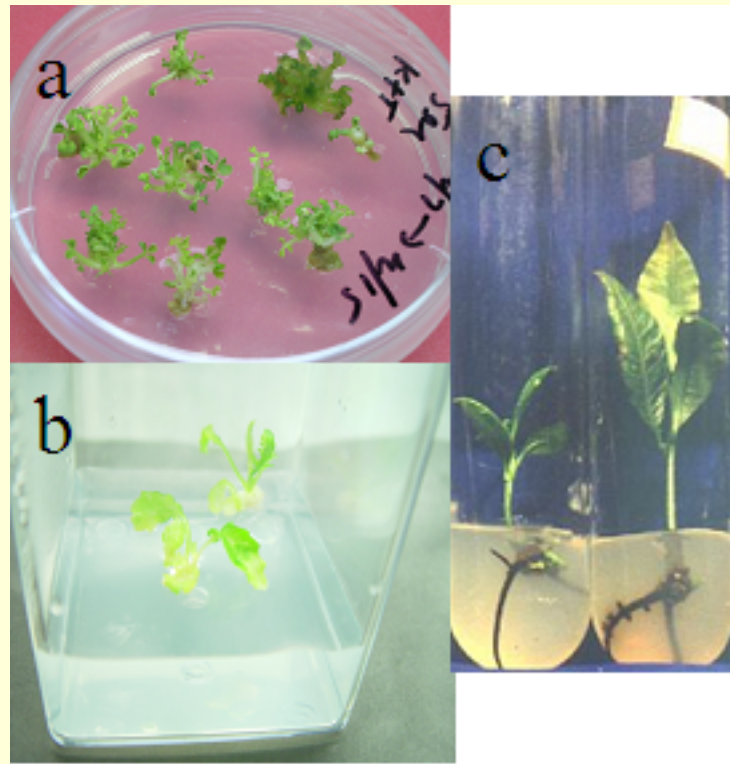


air flow



Plantlets in culture are typically grown in controlled environments. At left, plantlets are grown at room temperature in jars, with flourescent lights. At right is a more sophisticated growth cabinet that controls temperature and diurnal cycle.

Plantlets at different stages of regeneration.



Next, we'll look at how plant hormones are used to control the process of regeneration.

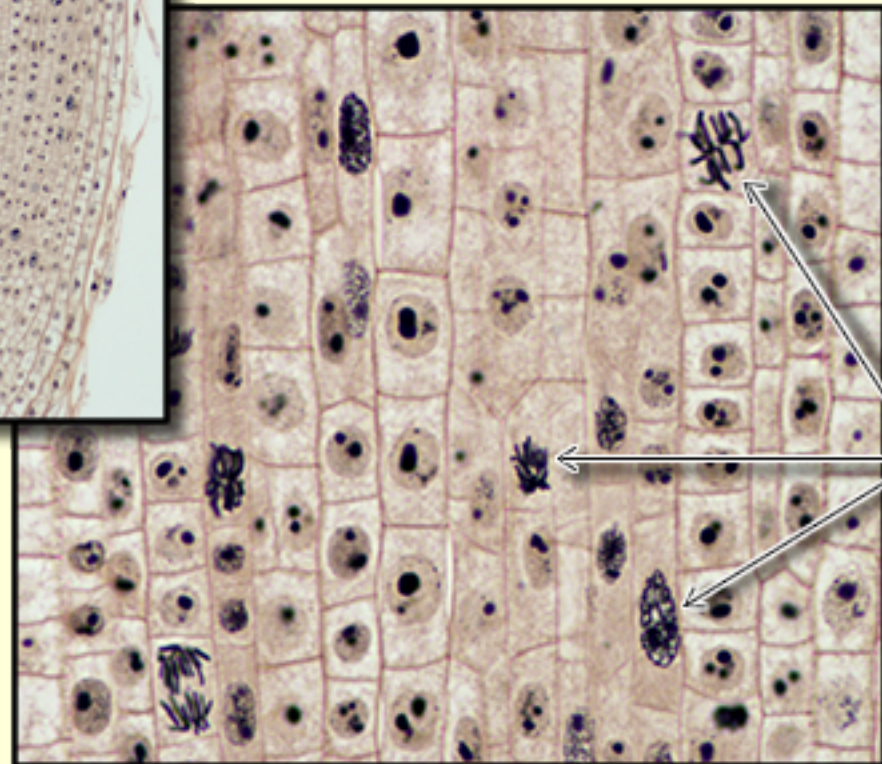
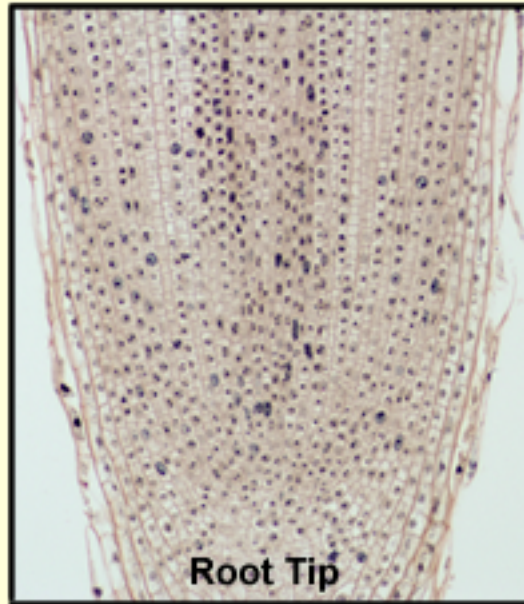
Hormones influence both cell Division and cell Elongation

Cell division - The complete replication of cellular contents, including the chromosomal complement, through mitosis or meiosis. Cell division occurs as organs grow and differentiate.

Cell elongation - The growth of a cell by increasing cellular mass and volume. Cell elongation occurs as organs mature and grow to their full size.

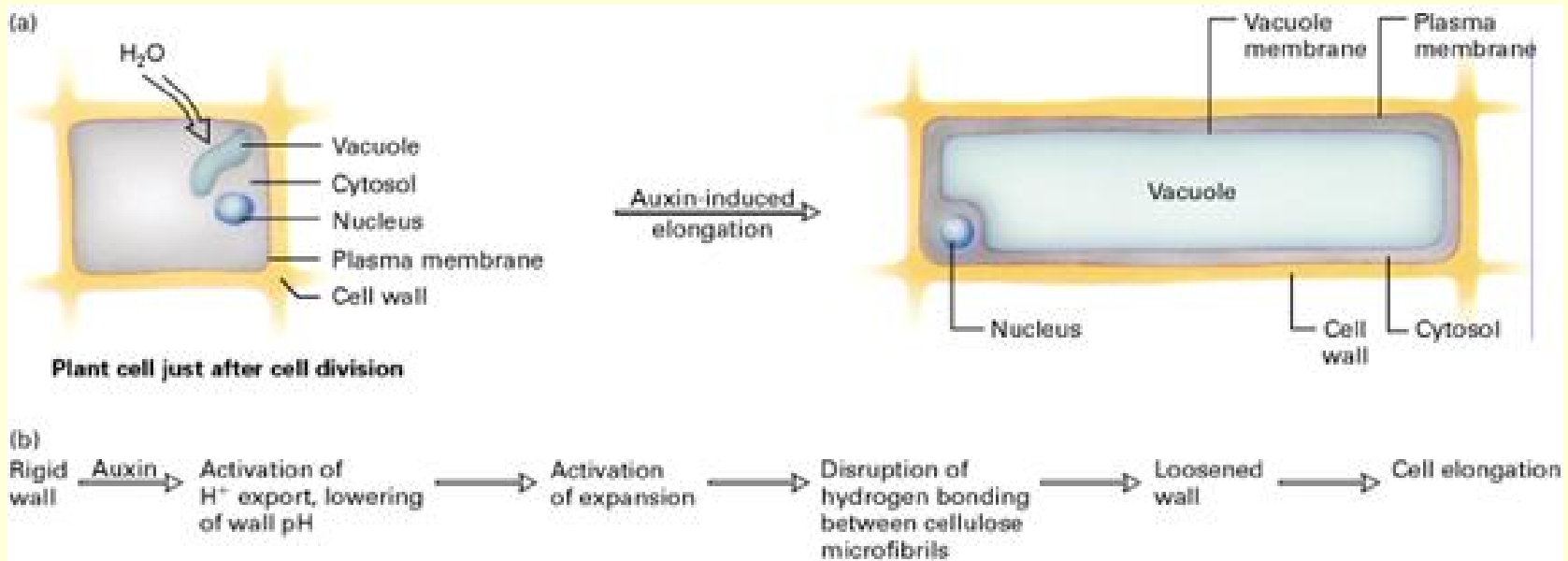
Cell Division

Plant Cell Division - Onion Root Tip



Initiation and growth of plant organs usually begins with cell division

Cell Elongation



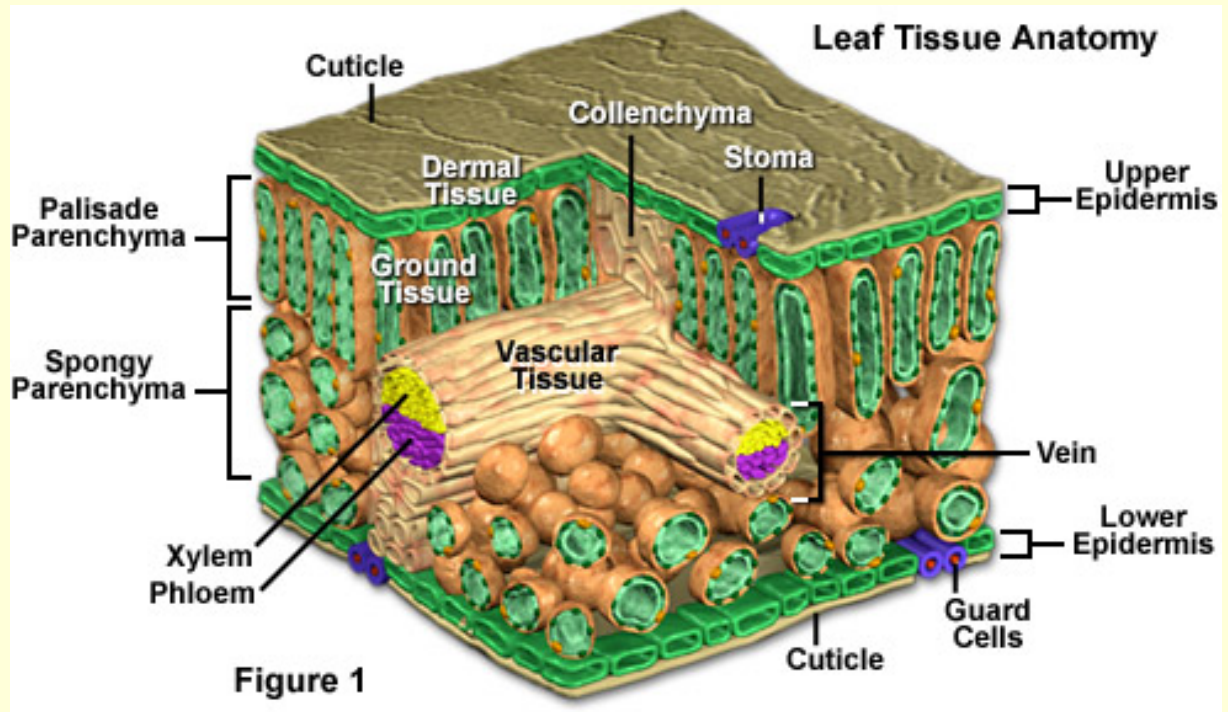
After a period of rapid division, many organs expand by cell elongation. Cell elongation is a process distinct from mitotic division.

- Cell does not undergo mitosis
- Characteristic of more mature tissues
- Cell wall loosens
- Cell volume is increased mostly through turgor pressure
- Most of the increase in volume is in the vacuole
- Cytoplasm usually doesn't increase as much

Differentiation of cells

As plants mature, cells specialize for different purposes. This process is known as cellular differentiation.

Cells differentiate with respect to size, shape, cell wall structure and composition, as well as with respect to biochemical pathways that are active.



<http://micro.magnet.fsu.edu/cells/leaftissue/leaftissue.html>

Plant Growth Regulators

In normal plant development endogenous hormones induce developmental changes in cells to create specific tissues.

- Five classes of plant growth regulators (hormones)
 - Auxins - promote both cell division and cell growth
 - Cytokinins - promote cell division
 - Gibberellins - play a role in cell elongation
 - Abscisic acid - inhibits cell division
 - Ethylene - controls fruit ripening in climacteric fruits (continue to ripen after harvesting)

(Latter 3 are not widely used in tissue culture)

In tissue culture added hormones are used to induce both growth and developmental changes according to what is desired.

Auxins and Cytokinins - Main Plant Growth Regulators

Commonly used auxins (all based on HOAc)

2,4-D 2,4-dichlorophenoxyacetic acid

2,4,5-T 2,4,5-trichlorophenoxyacetic acid

NAA 1-naphthylacetic acid

IAA Indole-3-acetic acid naturally occurring auxin

(not used in tissue culture due to its instability to both heat and light)

Commonly used cytokinins (purine derivatives)

BAP 6 benzylaminopurine

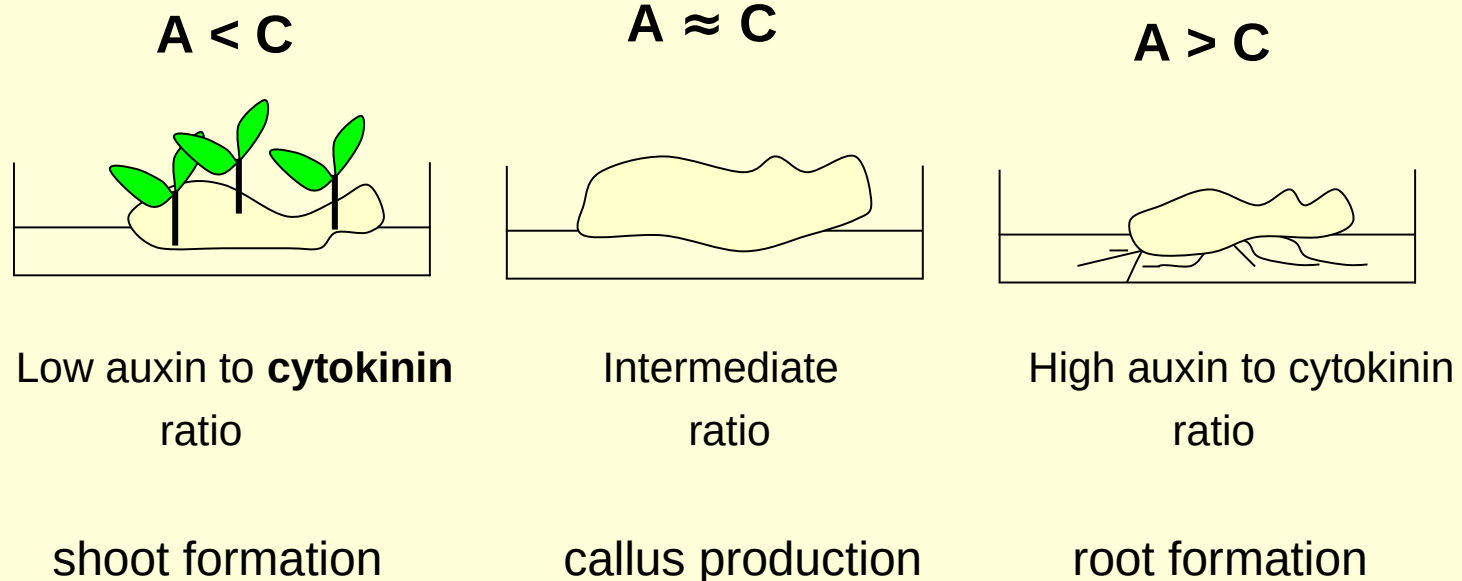
2iP N⁶-(2-isopenyl)adenine

Kinetin (6-furfurylaminopurine) and zeatin (2 of several natural cmpds)
(expensive and unstable)

Plant growth regulators and tissue culture

The effects of growth regulators have been ascertained by observation. Different plant species, different cultivars within a species, and even different plants within a cultivar may vary in response,

With auxins and cytokinins for many plants the following behaviors hold:



cells are de-differentiated

Pieces of tissue used to initiate plant tissue culture are referred to as explants.

Explants can be pieces of root, shoot, leaf, cotyledon, etc.

Young, rapidly growing tissue normally forms the most effective explants.

Genotypes of source material used for explants can vary widely in its ability to be successfully cultured and in the ability of cultured tissues to regenerate into whole plants.

Culture types

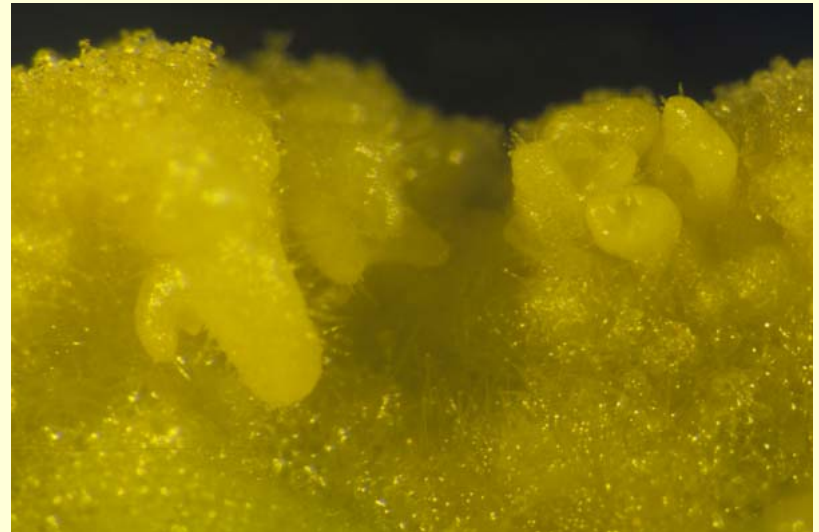
- 1) Callus culture - explants are maintained on media with auxin and cytokinin resulting in an unorganized, growing and dividing mass of cells.

This mass of cells is referred to as callus.

2 types of callus

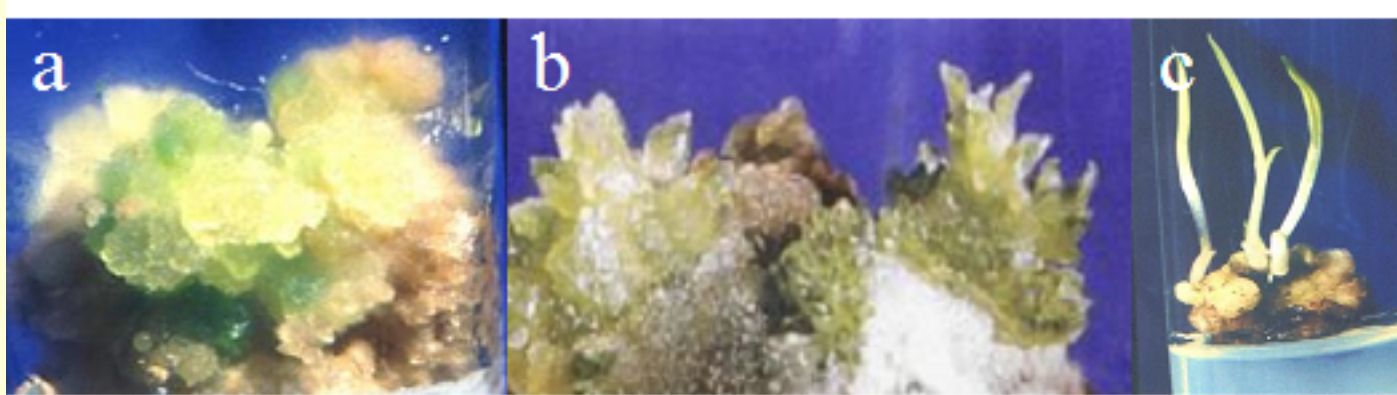
Compact callus - densely packed cells,

Friable callus - loosely associated cells.



Organized tissues arise from the compact callus during plant regeneration.

Shoots regenerated from callus culture. In shooting media, cells differentiate into the leaves and other components of the shoot.

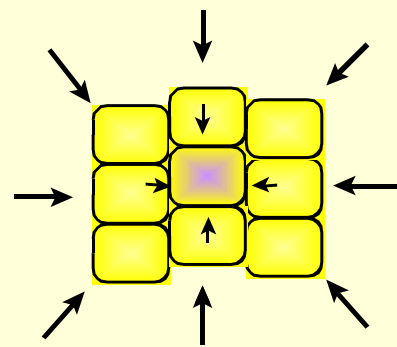


Culture types

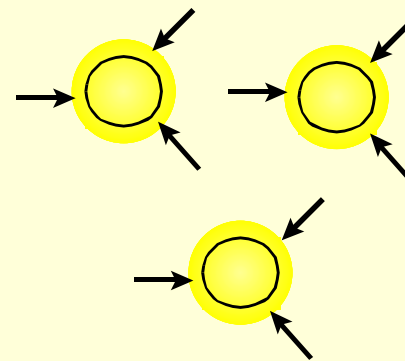
2) Cell-suspension culture – cells are maintained and continue to divide as individual cells or small clumps of cells.

- most readily initiated from friable callus because the loosely associated cells can be readily shaken free.

- cell-suspension cultures useful where a synchronous induction or treatment of cells is required.



Clumps of cells



Individual cells

Culture types

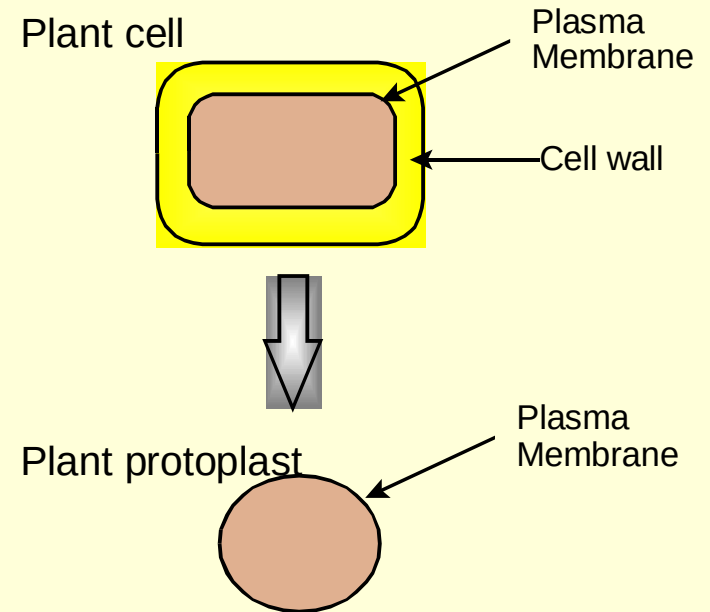
3) Protoplasts – plant cells with their cell walls removed.

- walls removed by a mixture of wall-degrading enzymes (cellulase, hemicellulase, pectinase)

- protoplast media requires an osmoticum to prevent protoplasts bursting.

- can be transformed directly with plasmids

- very fragile, care must be taken in handling until the cell wall are regenerated and the cells begins to divide.



Culture types

- 4) Embryo culture – embryos are widely used as the explants to create callus cultures or somatic embryos with monocot species.

- 5) Microspore culture – can be generated from anthers or pollen as the explant. When the explants are pollen grains (microspores), the resulting tissues and derived plants may be haploid.

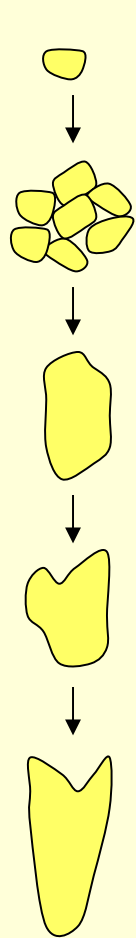
In breeding, homozygous plants can be produced from haploid plants derived from the pollen.

Plant Regeneration

Whole plants are regenerated from culture via two different processes:

- somatic embryogenesis** - in which cells and tissues develop into a bipolar structure containing both root and shoot axes with a closed vascular system (essentially - the type of embryogenesis that occurs in a seed)
- organogenesis** – in which cells and tissues develop into a unipolar structure, namely a shoot or a root with the vascular system of this structure often connected to parent tissues

Somatic embryogenesis



Single cell

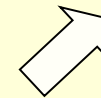
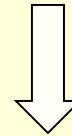
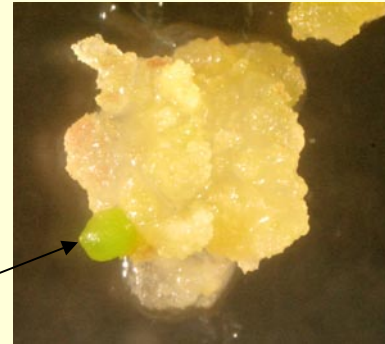
Group of cells

Globular embryo

Heart-stage embryo

Torpedo-stage embryo
(with cotyledons)

Post-torpedo stage



Somatic Embryogenesis - Differentiation of the plant body plan

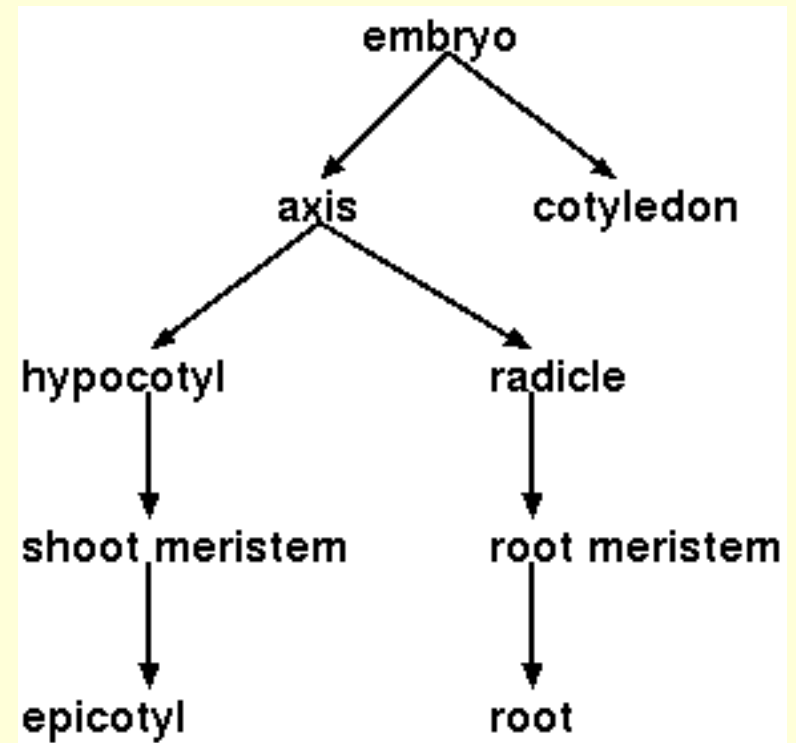
Two critical events:

i) regions along the longitudinal apical-basal axis must differentiate from each other and generate embryonic organ systems. The embryo first differentiates into the axis and cotyledon.

ii) the three primordial tissue layers of the embryo are determined: epidermal, vascular, and cortical layers.

After the critical heart embryo stage, the shoot meristem is determined. The shoot meristem will give rise to the epicotyl, which in turn, will mature into the bulk of the plant body.

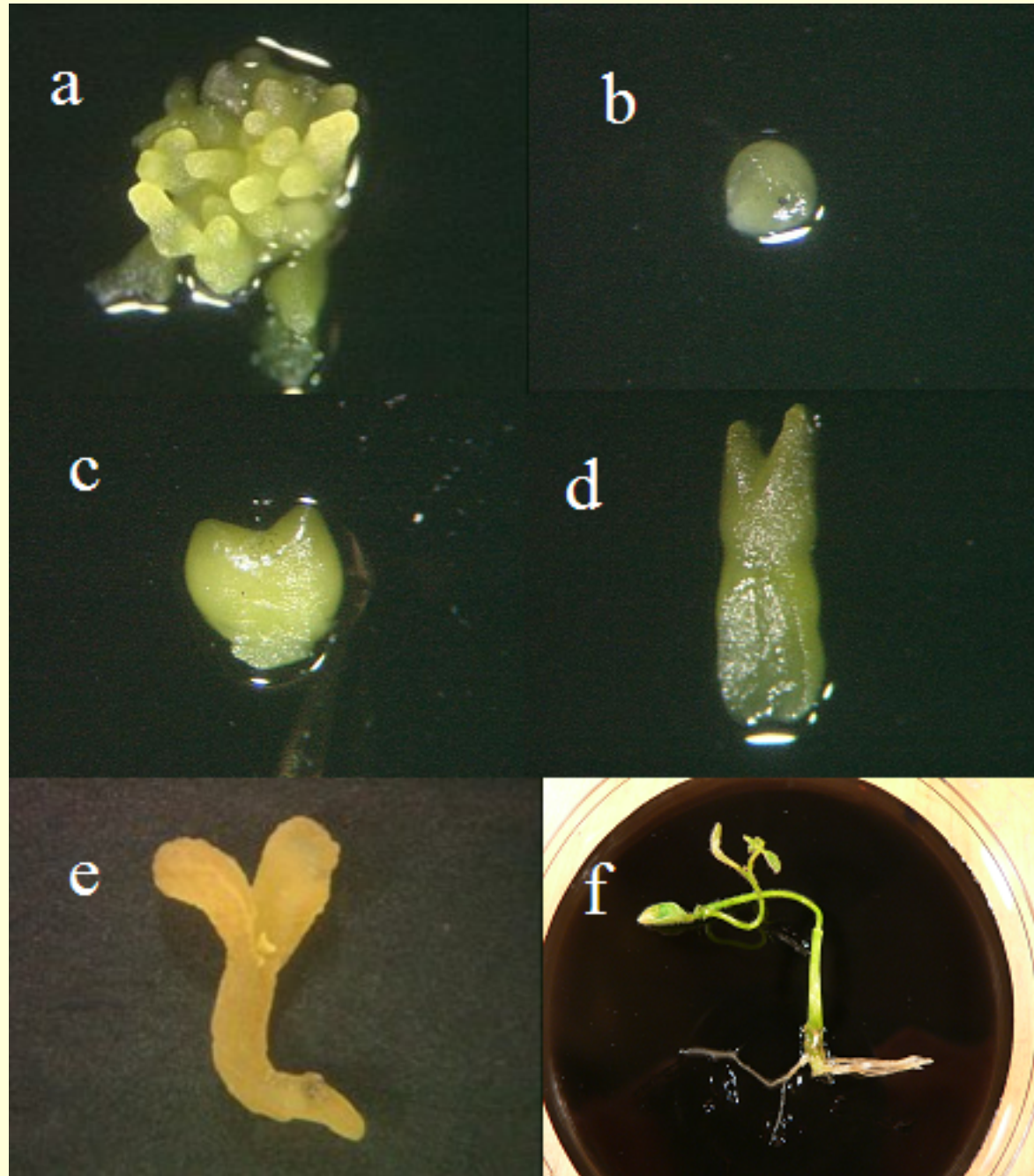
Most of embryo is devoted to formation of the root.



Development of dicot somatic embryos from undifferentiated callose to a complete embryo, with cotyledons, root and shoot.

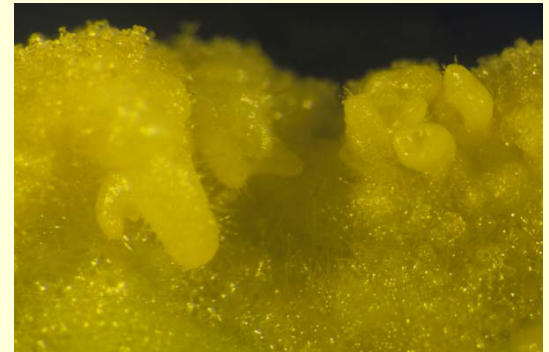
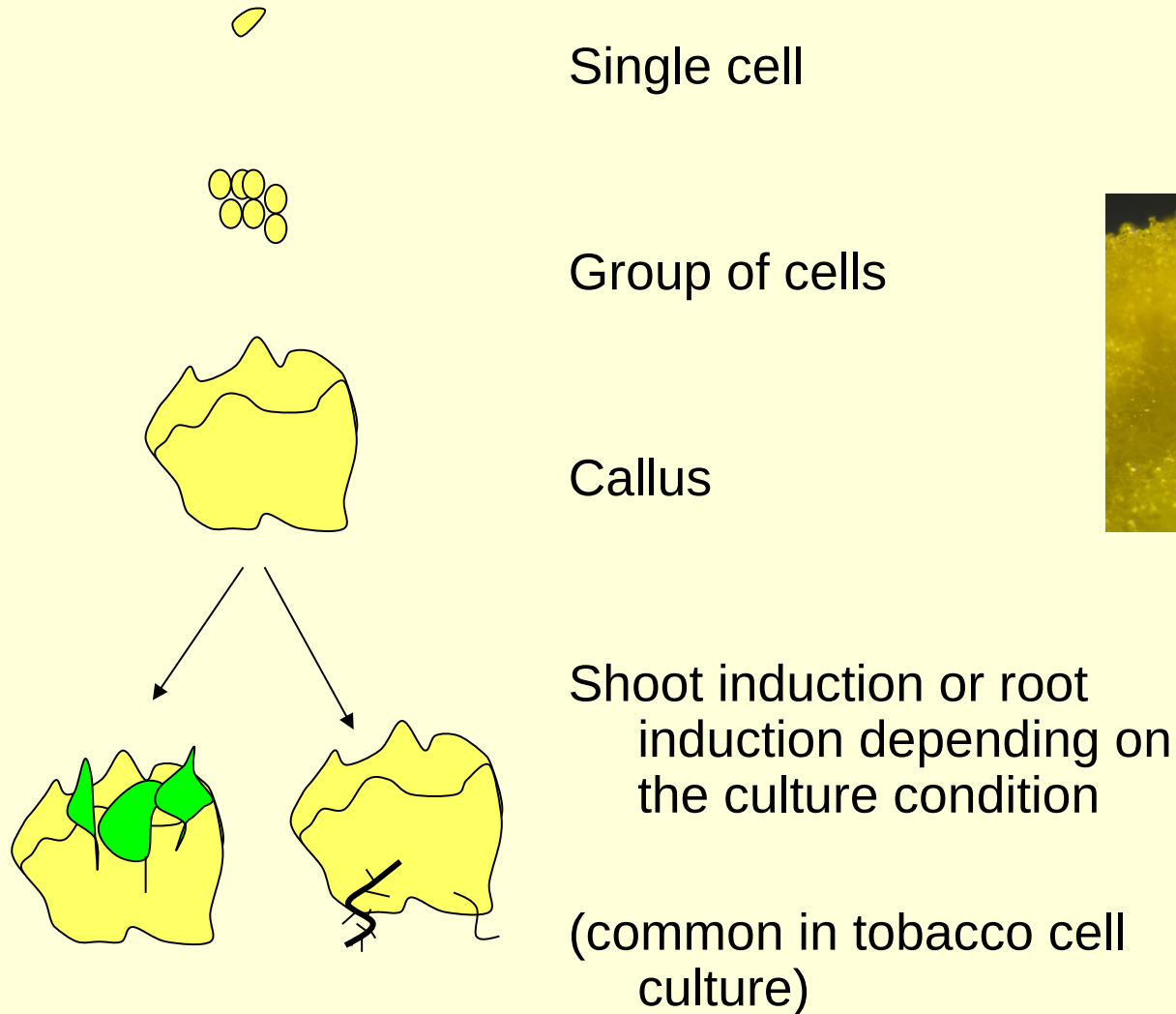
The cotyledons are storage organs that provide nutrition to the root and shoot until they are self-supporting through photosynthesis.

Cotyledons will undergo senescence and die off as the plant matures.



Organogenesis from callus

(but can also occur directly from the explant)





Organogenesis in wheat

Explant sterilization

The trick: Kill bacteria or fungi without killing the plant cells

Chemical Surface Sterilizing Agents:

		Conc'n	Duration	Effectiveness
Calcium hypochlorite	$\text{Ca}(\text{ClO})_2$	9-10%	5-30 min	VG
Sodium hypochlorite	NaClO	1-2%	5-30 min	VG
Hydrogen peroxide	H_2O_2	10-12%	5-15 min	G
Silver nitrate	AgNO_3	0.2-1%	5-30 min	G
Mercuric chloride	HgCl_2	0.01-0.05%	2-12 min	G

- Sodium hypochlorite is cheap and very effective.
- Commercial bleach is ~5% NaClO.
- Tissue exposure time depends on degree of contamination and tissue sensitivity to the hypochlorite.

Media and equipment sterilization

Liquid media

Heat stable media sterilized by autoclaving (120C, 15 psi, 20 min)

-time autoclaved depends on the volume of media

Heat labile media components sterilized by filtration through a 0.45 μm (or 0.22 μm) filter into a sterile container

- added to other autoclaved media after it has cooled sufficiently

Equipment

Heat stable materials can either be autoclaved (120C, 15 psi, 20 min) or baked for several hours at (>120C)

Surfaces sterilized by ultraviolet (UV) irradiation

Filter sterilization vessel

Non-sterile media is poured into the top compartment.
The lid is attached, and a vacuum pulls the media through
a filter with sub-micron sized pores



Maintaining sterility in the media

ANTIBIOTICS

-may be added to the media to reduce the risk of microbial contamination

The selected antibiotic(s) should:

- 1) be **bactericidal** not bacteristatic
- 2) be **broad spectrum** in its antibiotic properties
- 3) have **minimal phytotoxicity** for tissue

Example: Carbenicillin

Most important precaution to maintaining sterility:

Good aseptic technique!