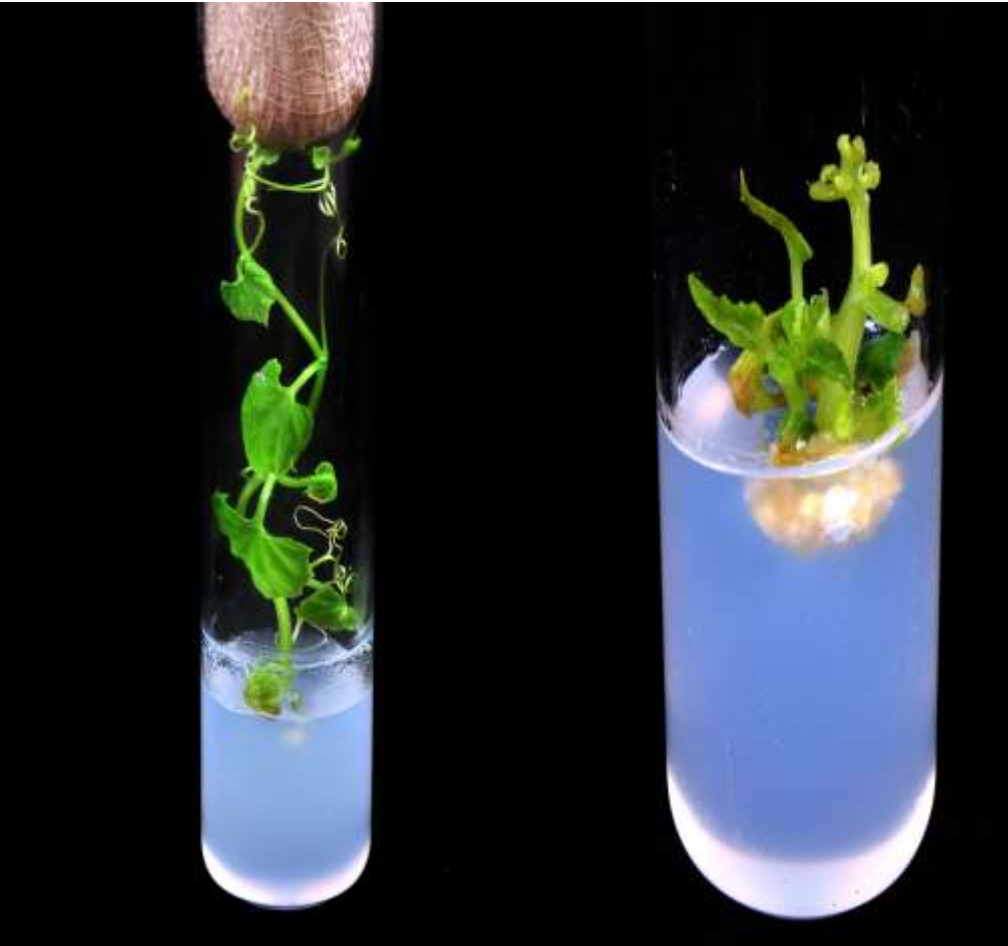


Plant Tissue Culture



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Professor

What Is plant tissue culture?

Or *in vitro* culture?

Or *in vitro* propagation?

Or Micropropagation ?

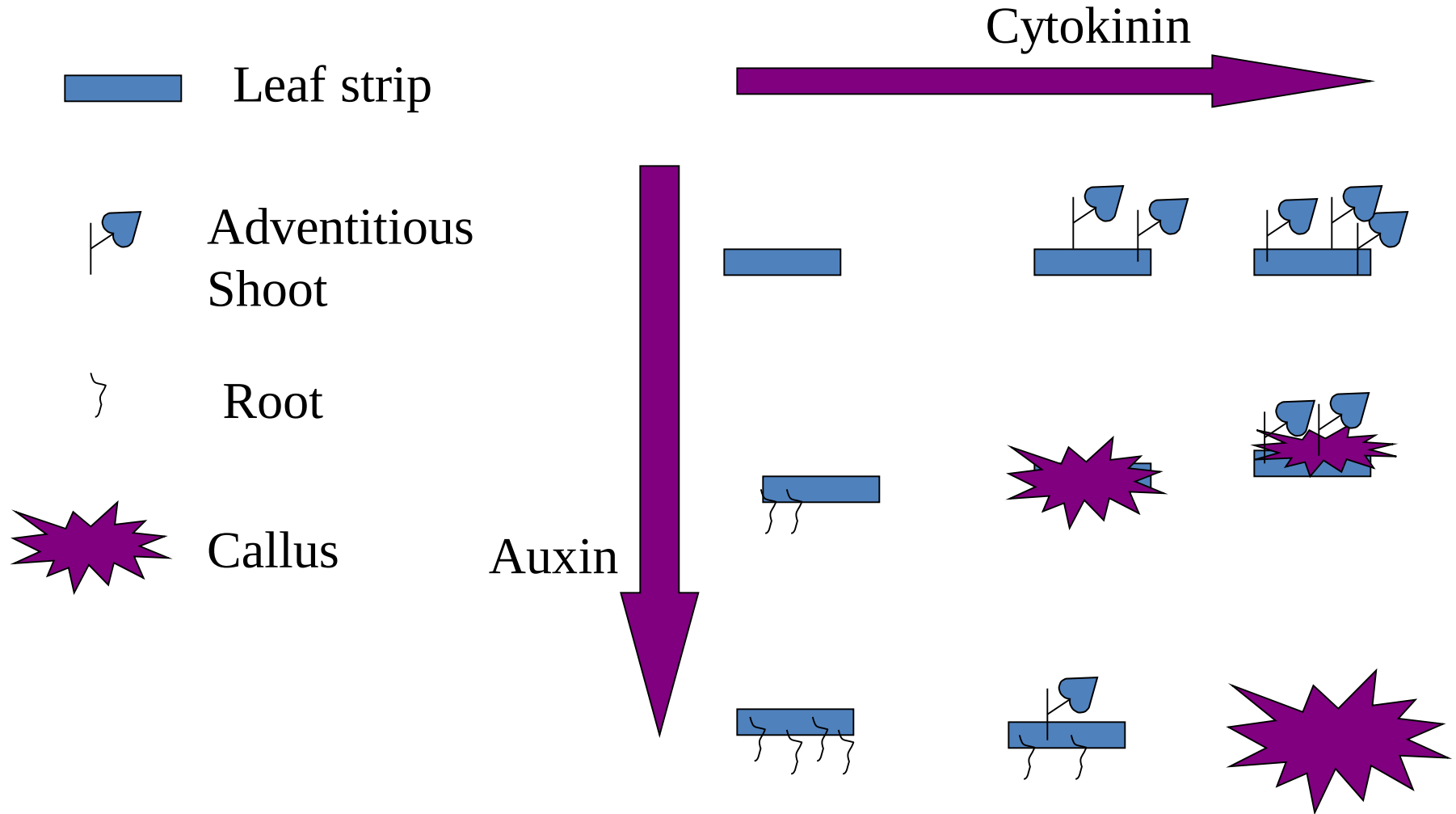
Definition

the culture of plant seeds, organs, explants, tissues, cells, or protoplasts on nutrient media under sterile conditions.

Basis for Plant Tissue Culture

- Two Hormones Affect Plant Differentiation:
 - Auxin: Stimulates Root Development
 - Cytokinin: Stimulates Shoot Development
- *Generally*, the ratio of these two hormones can determine plant development:
 - $\uparrow$ Auxin $\downarrow$ Cytokinin = Root Development
 - $\uparrow$ Cytokinin $\downarrow$ Auxin = Shoot Development
 - Auxin = Cytokinin = Callus Development

Control of in vitro culture



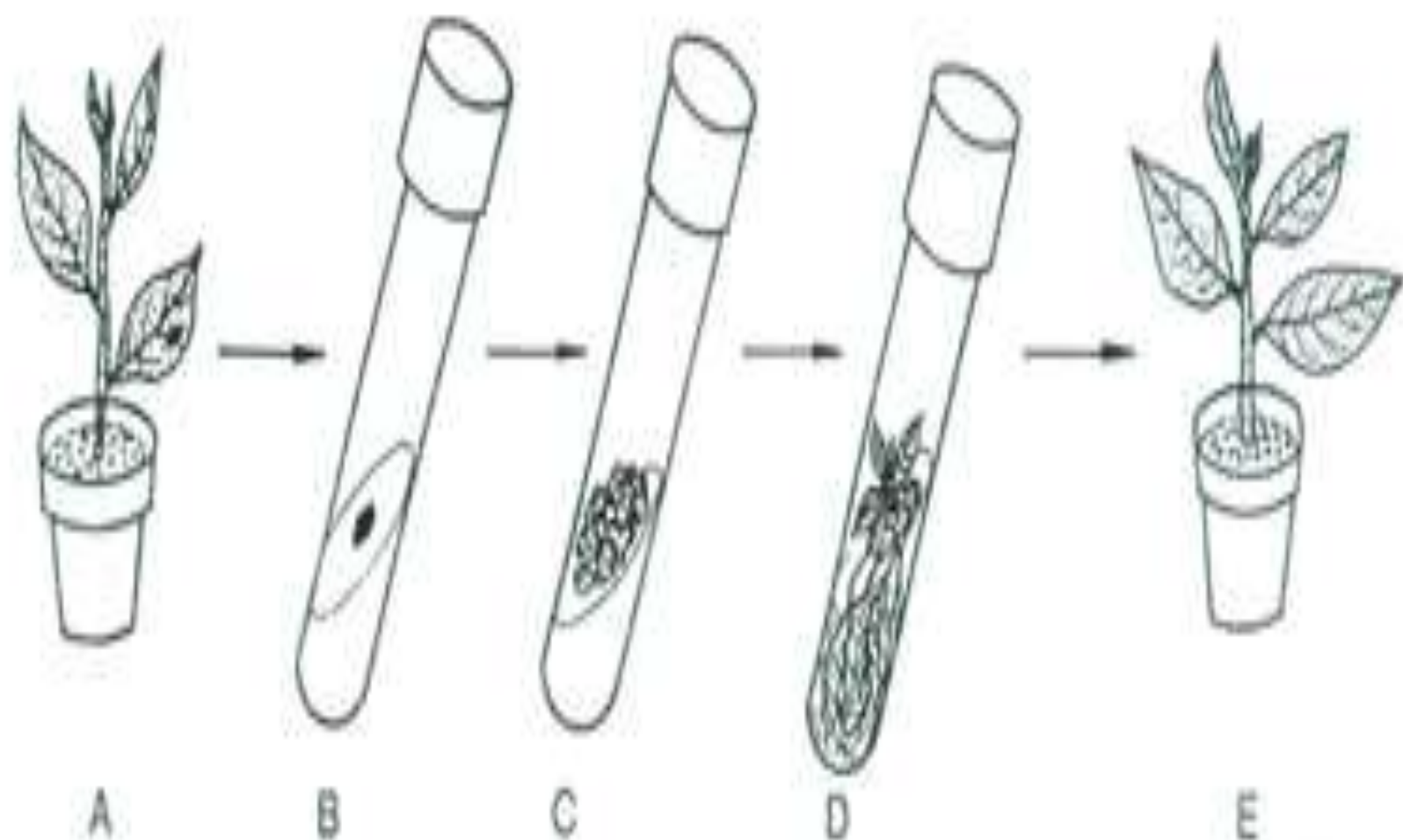


FIG. 8.1. In Vitro regeneration of plants through tissue culture, (A) Source of tissue explant. (B) Explant culture on agar medium. (C) Callus production. (D) Plantlet regenerated. (E) Plantlet transplanted into sterile soil.

Factors Affecting Plant Tissue Culture

- **Growth Media**
 - Minerals, Growth factors, Carbon source
- **Environmental Factors**
 - Light, Temperature, Photoperiod
- **Explant Source**
 - Types

Usually, the younger, less differentiated the explant, the better for tissue culture
 - Genetics
 1. Different species show differences in amenability to tissue culture
 2. In many cases, different genotypes within a species will have variable responses to tissue culture; response to somatic embryogenesis has been transferred between melon cultivars through sexual hybridization

Choice of explant

Desirable properties
of an explant

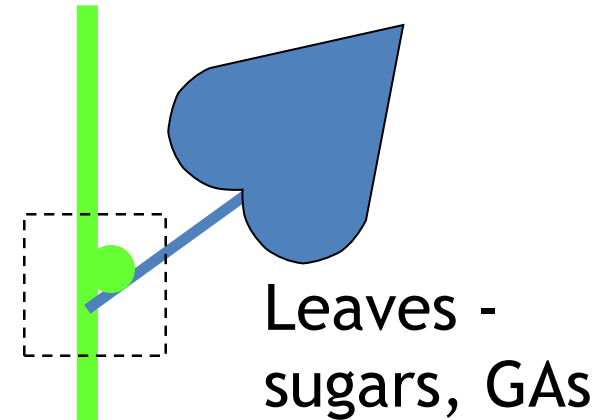
- ✓ Easily sterilisable
- ✓ Juvenile
- ✓ Responsive to culture

- ✓ Shoot tips
- ✓ Axillary buds
- ✓ Seeds
- ✓ Hypocotyl (from germinated seed)
- ✓ Leaves

Media

- ✓ When you make an explant like an axillary bud, you remove it from the sources of many chemicals and have to re-supply these to the explants to allow them to grow.

Shoot tip - Auxins and Gibberellins



Roots - water, vitamins
mineral salts and cytokinins

Medium constituents

- ✓ Inorganic salt formulations
- ✓ Source of carbohydrate
- ✓ Vitamins
- ✓ Water
- ✓ Plant hormones - auxins, cytokinins, GA's
- ✓ Solidifying agents
- ✓ Undefined supplements

Carbohydrates

- ✓ Plants in culture usually cannot meet their needs for fixed carbon. Usually added as sucrose at 2-3% w/v.
- ✓ Glucose or a mixture of glucose and fructose is occasionally used.
- ✓ For large scale cultures, cheaper sources of sugars (corn syrup) may be used.

Photoautotrophic culture

- Growth without a carbon source. Therefore need to boost photosynthesis.
- High light intensities needed (90-150mMole/m²/s) compared to normal (30-50).
- Usually increase CO₂ (1000ppm) compared to normal 369.4ppm.
- Much reduced level of contamination and plants are easier to transfer to the greenhouse.

Inorganic salt formulations

- ✓ Contain a wide range of Macro-elements ($>\text{mg/l}$) and microelements ($<\text{mg/l}$).
- ✓ A wide range of media are readily available as spray-dried powders.
- ✓ Murashige and Skoog Medium (1965) is the most popular for shoot cultures.
- ✓ Gamborgs B5 medium is widely used for cell suspension cultures (no ammonium).

Vitamins

- ✓ A wide range of vitamins are available and may be used.
- ✓ Generally, the smaller the explant, the more exacting the vitamin requirement.
- ✓ A vitamin cocktail is often used (Nicotinic acid, glycine, Thiamine, pyridoxine).
- ✓ Inositol usually has to be supplied at much higher concentration (100mg/l)

Plant hormones

(Growth regulators)

- ✓ Auxins
- ✓ Cytokinins
- ✓ Gibberellic acids
- ✓ Ethylene
- ✓ Abscissic Acid
- ✓ “Plant Growth Regulator-like compounds”

Auxins

- ✓ Absolutely essential (no mutants known)
- ✓ Only one compound, Indole-3-acetic acid.
Many synthetic analogues (NAA, IBA, 2,4-D, 2,4,5-T, Pichloram) - cheaper & more stable
- ✓ Generally growth stimulatory. Promote rooting.
- ✓ Produced in meristems, especially shoot meristem and transported through the plant in special cells in vascular bundles.

Cytokinins

- ✓ Absolutely essential (no mutants known)
- ✓ Single natural compound, Zeatin. Synthetic analogues Benzyladenine (BA), Kinetin.
- ✓ Stimulate cell division (with auxins).
- ✓ Promotes formation of adventitious shoots.
- ✓ Produced in the root meristem and transported throughout the plant as the Zeatin-riboside in the phloem.

Gibberellins (GA's)

- A family of over 70 related compounds, all forms of Gibberellic acid.
- Commercially, GA3 and GA4+9 available.
- Stimulate etiolation of stems.
- Help break bud and seed dormancy.
- Produced in young leaves.

Abscissic Acid (ABA)

- Only one natural compound.
- Promotes leaf abscission and seed dormancy.
- Plays a dominant role in closing stomata in response to water stress.
- Has an important role in embryogenesis in preparing embryos for desiccation. Helps ensure 'normal' embryos.

‘Plant Growth Regulator-like substances’

- ✓ Polyamines - have a vital role in embryo development.
- ✓ Jasmonic acid - involved in plant wound responses.
- ✓ Salicylic acid.
- ✓ Not universally acclaimed as plant hormones since they are usually needed at high concentrations.

Undefined supplements

- ✓ Sources of hormones, vitamins and polyamines.
- ✓ e.g. Coconut water, sweetcorn extracts
- ✓ Not reproducible
- ✓ Do work.

Fundamental abilities of plants

✓ Totipotency

the potential or inherent capacity of a plant cell to develop into an entire plant if suitable stimulated.

It implies that all the information necessary for growth and reproduction of the organism is contained in the cell

✓ Dedifferentiation

The capacity of mature cells to return to meristematic condition and development of a new growing point, followed by redifferentiation which is the ability to reorganize into new organs

✓ Competency

the endogenous potential of a given cell or tissue to develop in a particular way

Type of in vitro culture

- ✓ Culture of intact plants (Seed orchid culture)
- ✓ Embryo culture (embryo rescue)
- ✓ Organ culture
 1. shoot tip culture
 2. Root culture
 3. Leaf culture
 4. anther culture
- ✓ Callus culture
- ✓ Cell suspension and single cell culture
- ✓ Protoplast culture

Breeding Applications of Tissue Culture

- ✓ Micropropagation
- ✓ Germplasm preservation
- ✓ Somaclonal variation
- ✓ Embryo culture
- ✓ Haploid & dihaploid production
- ✓ *In vitro* hybridization – protoplast fusion
- ✓ Plant genetic engineering

Micropropagation

- Embryogenesis
 - Direct embryogenesis
 - Indirect embryogenesis
- Organogenesis
 - Organogenesis via callus formation
 - Direct adventitious organ formation
- Microcutting
 - Meristem and shoot tip culture
 - Bud culture

Somatic Embryogenesis

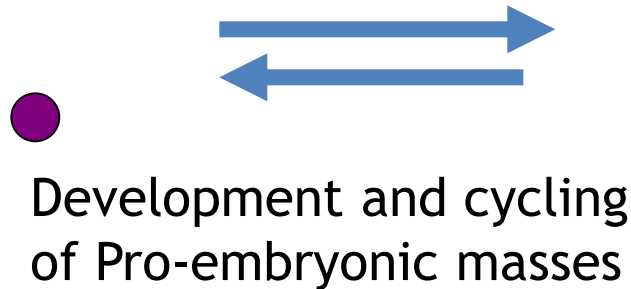


Somatic Embryogenesis

- ✓ The process of initiation and development of embryos or embryo-like structures from somatic cells
- ✓ The production of embryos from somatic or “non-germ” cells.
- ✓ Usually involves a callus intermediate stage which can result in variation among seedlings
- ✓ Not a common micro-propagation technique but is currently being used to produce superior pine seedlings

Somatic embryogenesis from Pro-embryonic masses (PEMs)

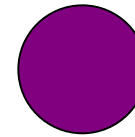
+ Auxin leads to high [Putrescine]



Single cells sloughed off the surface



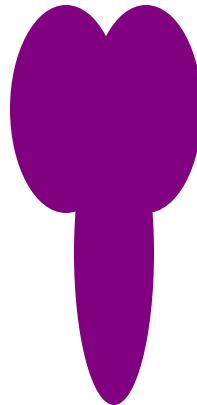
Putrescine to Spermidine



Spermidine to Spermine

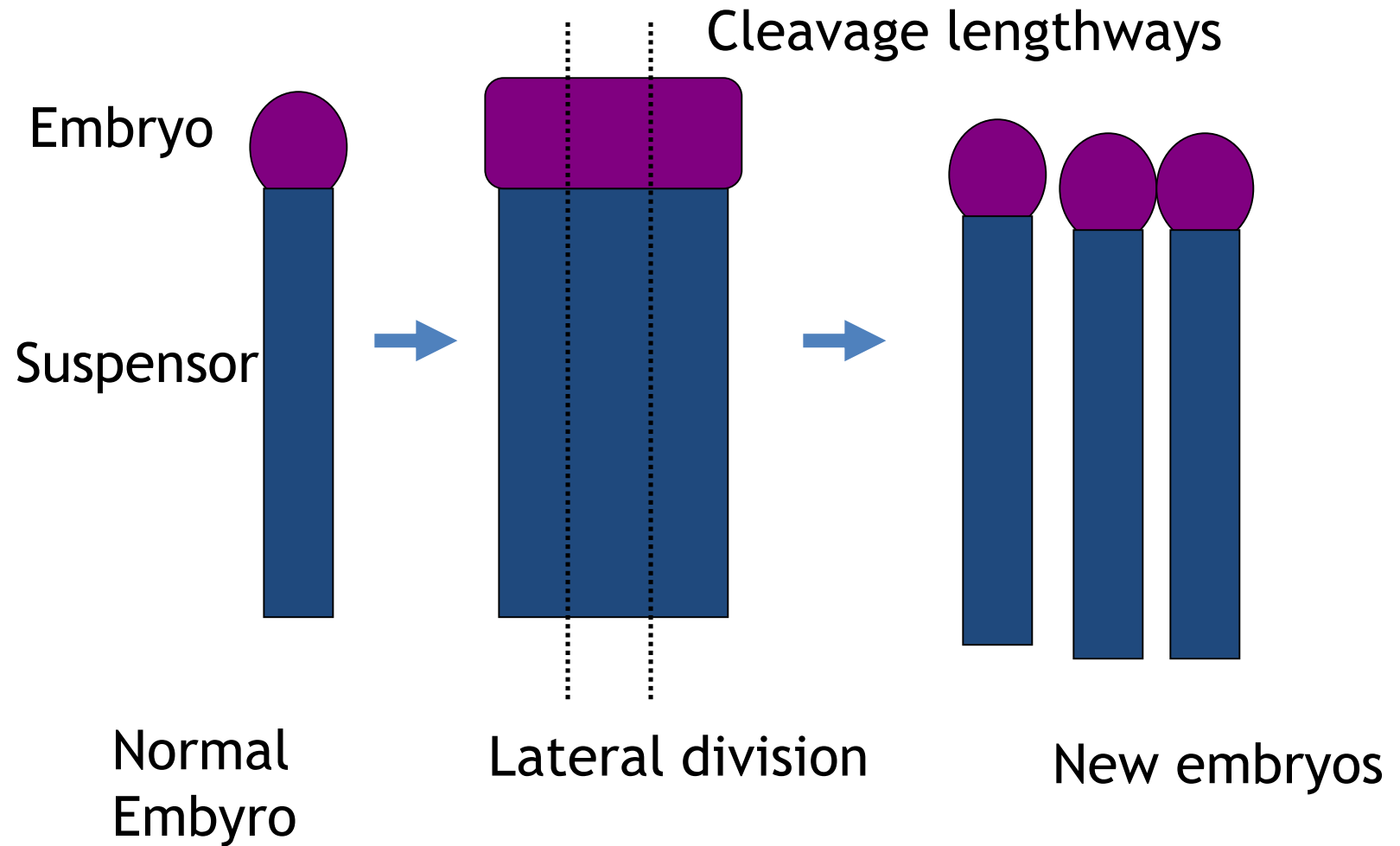


Remove
Auxin
Polyamine
Inter-convesions



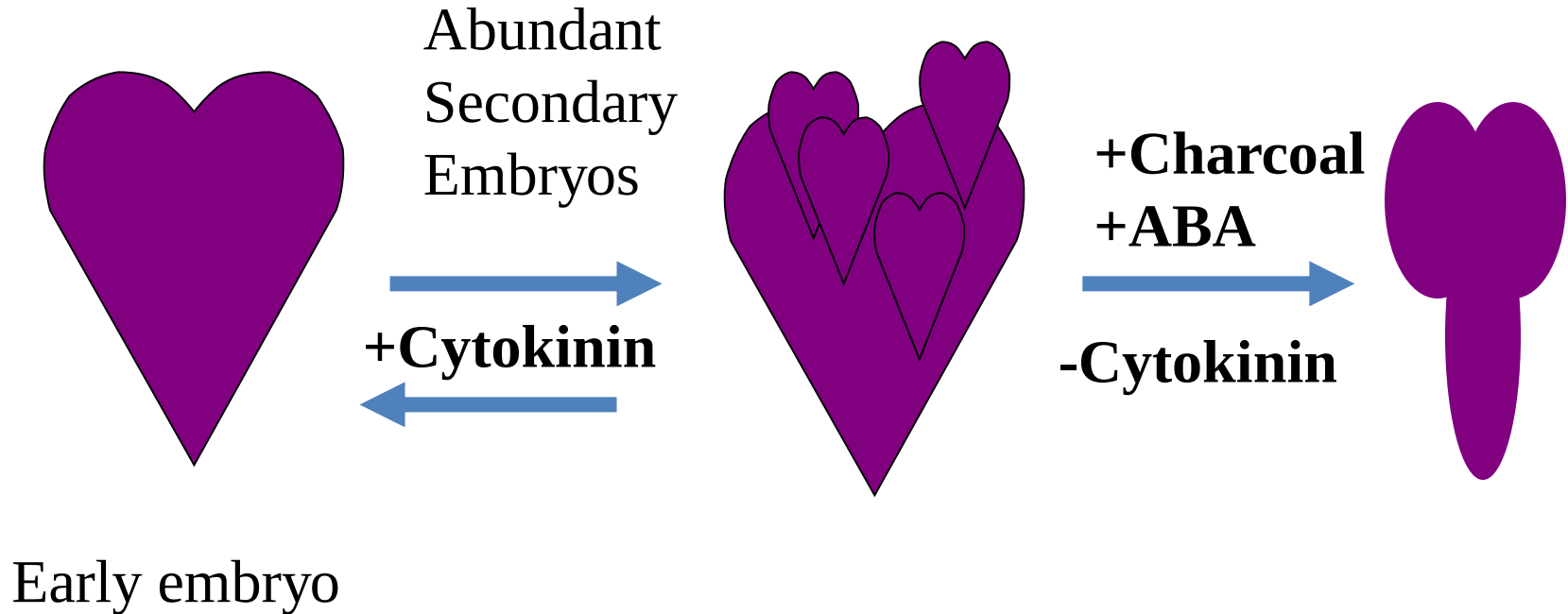
*E.g. Carrot,
Monocots, some
conifers*

Cleavage Polyembryony- conifers



Secondary embryo formation

- Most dicots



Embryo Fermentations

- ✓ Somatic Embryos may be produced profusely from leaves or zygotic embryos.
- ✓ For micropropagation, potentially phenomenally productive.
- ✓ Shear sensitivity is a problem.
- ✓ Maturation in liquid is a problem.

Somatic Embryos

- ✓ Tissue culture maintains the genetic of the cell or tissue used as an explant
- ✓ Tissue culture conditions can be modified to cause to somatic cells to reprogram into a bipolar structure
- ✓ These bipolar structures behave like a true embryo - called somatic embryos

Organogenesis



Organogenesis

- ✓ The process of initiation and development of a structure that shows natural organ form and/or function.
- ✓ the ability of non-meristematic plant tissues to form various organs *de novo*.
- ✓ the production of roots, shoots or leaves.
- ✓ These organs may arise out of pre-existing meristems or out of differentiated cells.
- ✓ This, like embryogenesis, may involve a callus intermediate but often occurs without callus.

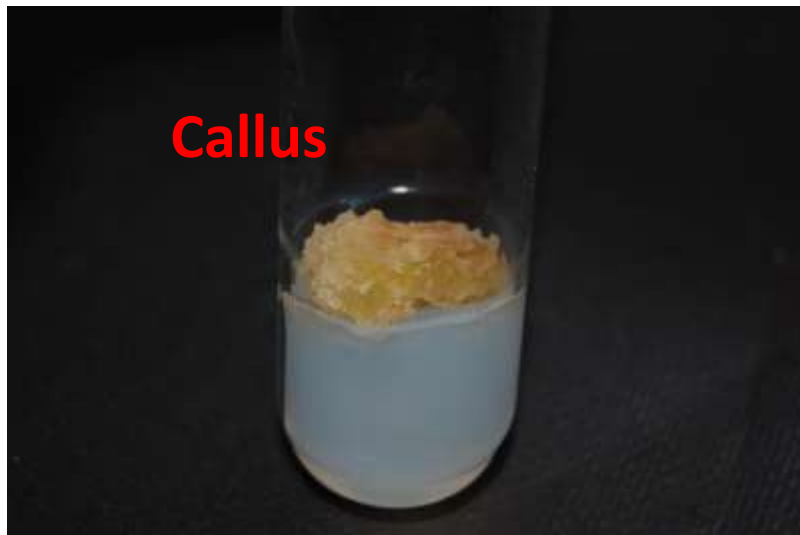
Plant Organogenesis

✓ Indirect:

- This pathway includes a callus stage.
 - Callus: Undifferentiated tissue that develops on or around an injured or cut plant surface or in tissue culture.

✓ Direct:

- It bypasses a callus stage. The cells in the explant act as direct precursors of a new primordium
 - An organ or a part in its most rudimentary form or stage of development



Organogenesis

- ✓ Adventitious shoot formation is the de-novo development of shoots from cell clusters in the absence of pre-existing meristems.
- ✓ In some species (e.g. Saintpaulia), many shoots can be induced (3000 from one leaf).
- ✓ In other species (e.g. coffee), it may be necessary to induce an un-organised mass proliferation of cells (callus) prior to adventitious shoot formation.

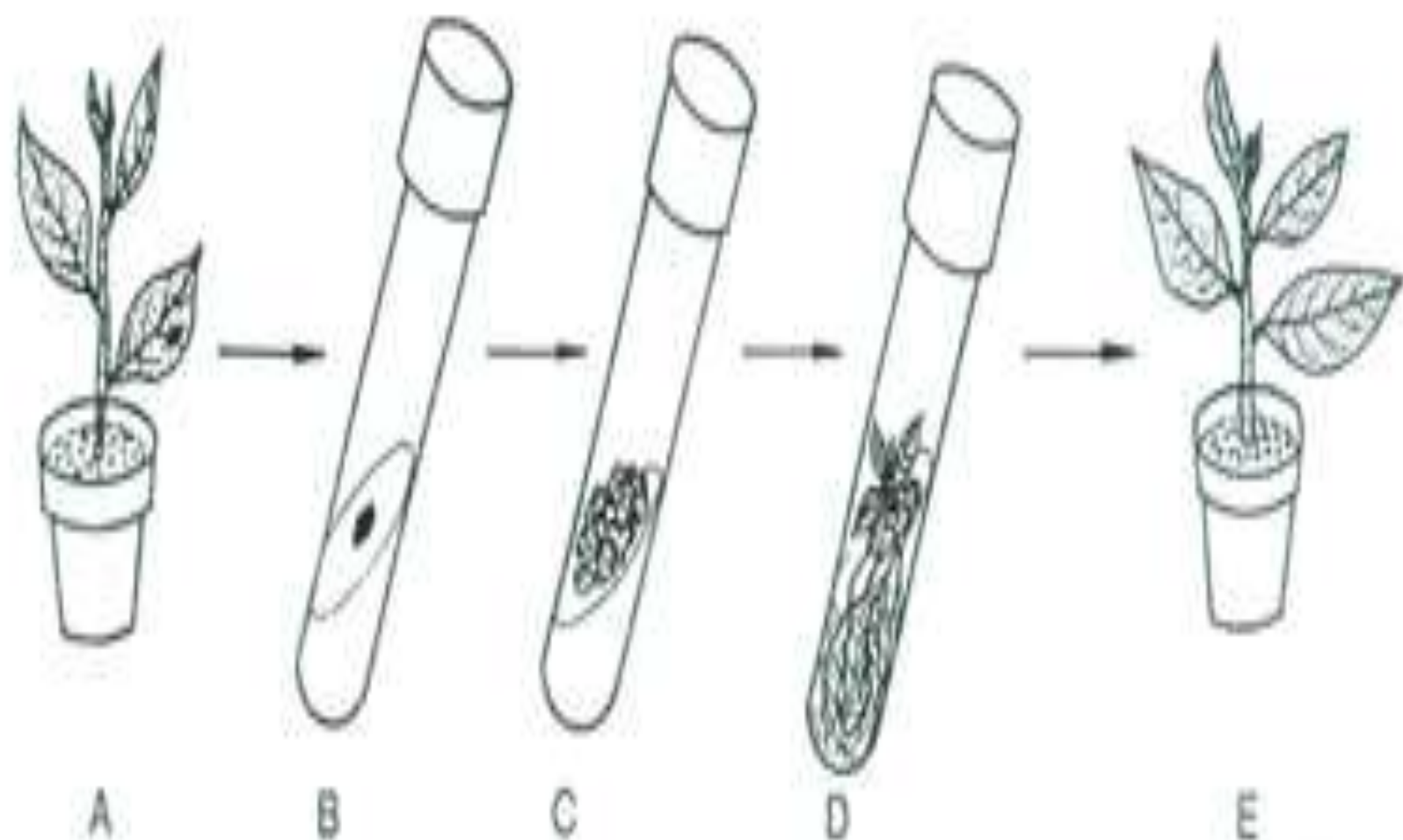


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Somatic Embryogenesis and Organogenesis

- Both of these technologies can be used as methods of micro-propagation.
- Not always desirable because they may not always result in populations of identical plants.
- The most beneficial use of somatic embryogenesis and organogenesis is in the production of whole plants from a single cell (or a few cells).

Microcutting

- This is a specialized form of organogenesis
- It involves the production of shoots from pre-existing meristems only.
- Requires breaking apical dominance
- Microcuttings can be one of three types:
 - Nodal
 - Shoot cultures
 - Clump division

Micropropagation

- The art and science of plant multiplication *in vitro*
- Usually derived from meristems (or vegetative buds) without a callus stage
 - Tends to reduce or eliminate somaclonal variation, resulting in true clones
- Can be derived from other explant or callus (but these are often problematic)

Steps of Micropropagation

- Stage 0 – Selection & preparation of the mother plant
 - sterilization of the plant tissue takes place
- Stage I - Initiation of culture
 - explant placed into growth media
- Stage II - Multiplication
 - explant transferred to shoot media; shoots can be constantly divided
- Stage III - Rooting
 - explant transferred to root media
- Stage IV - Transfer to soil
 - explant returned to soil; hardened off

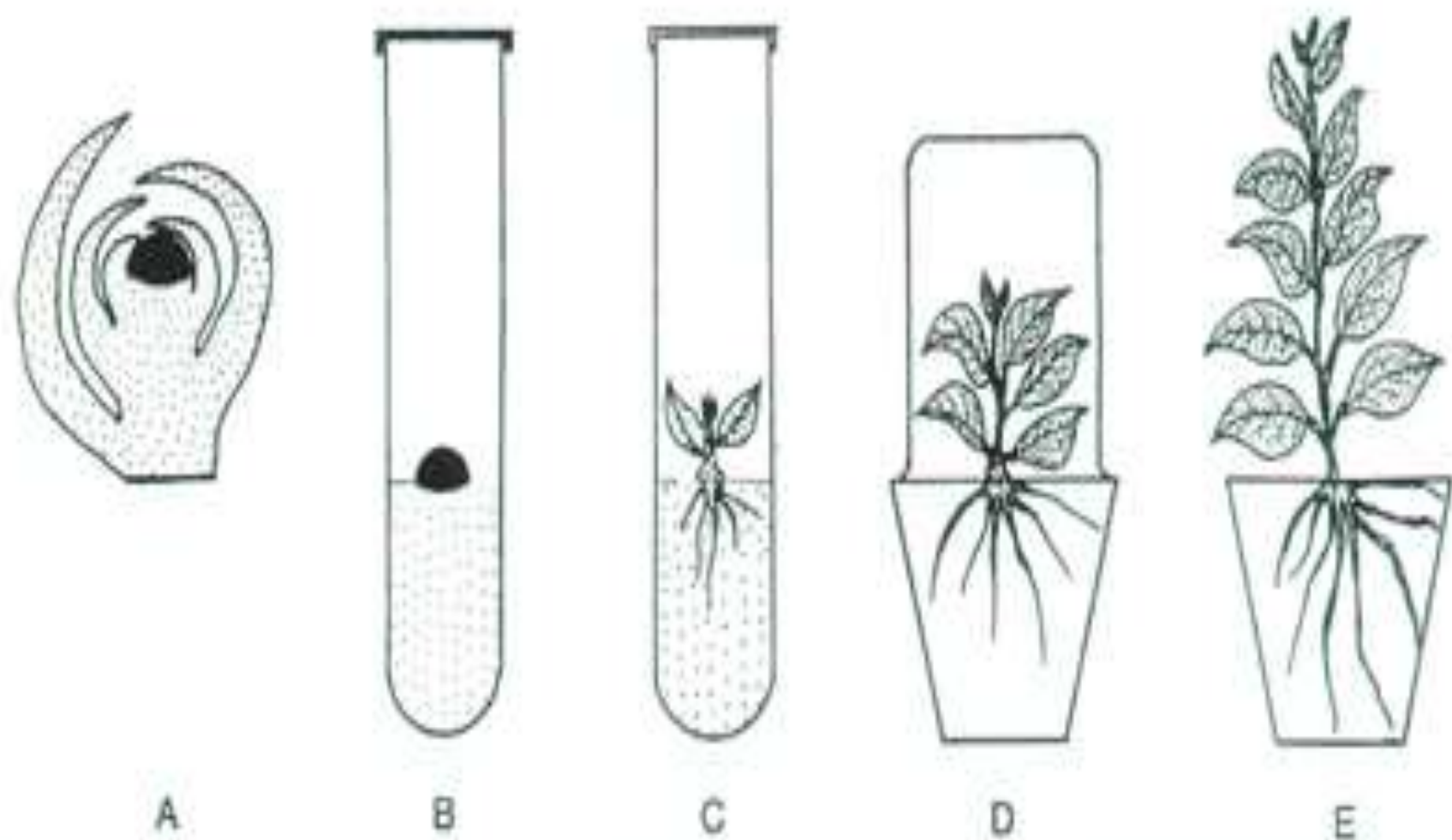


FIG. 8.3. Meristem-tip culture. (A) Apical meristem showing section to be excised. (B) Excised meristem tip cultured on agar medium. (C) Plantlet regenerated from excised meristem tip. (D) Plantlet transferred to sterile soil. (E) Virus-free plant growing in soil.

Features of Micropropagation

- Clonal reproduction
 - Way of maintaining heterozygosity
- Multiplication Stage can be recycled many times to produce an unlimited number of clones
 - Routinely used commercially for many ornamental species, some vegetatively propagated crops
- Easy to manipulate production cycles
 - Not limited by field seasons/environmental influences
- Disease-free plants can be produced
 - Has been used to eliminate viruses from donor plants

Somaclonal Variation

A general phenomenon of all plant regeneration systems that involve a callus phase

- two general types of Somaclonal Variation:
 - Heritable, genetic changes (alter the DNA)
 - Stable, but non-heritable changes (alter gene expression, epigenetic)

Somaclonal Breeding Procedures

- Use plant cultures as starting material
 - Idea is to target single cells in multi-cellular culture
 - Usually suspension culture, but callus culture can work
- Optional: apply physical or chemical mutagen
- Apply selection pressure to culture
 - Target: very high kill rate, you want very few cells to survive, so long as selection is effective
- Regenerate whole plants from surviving cells

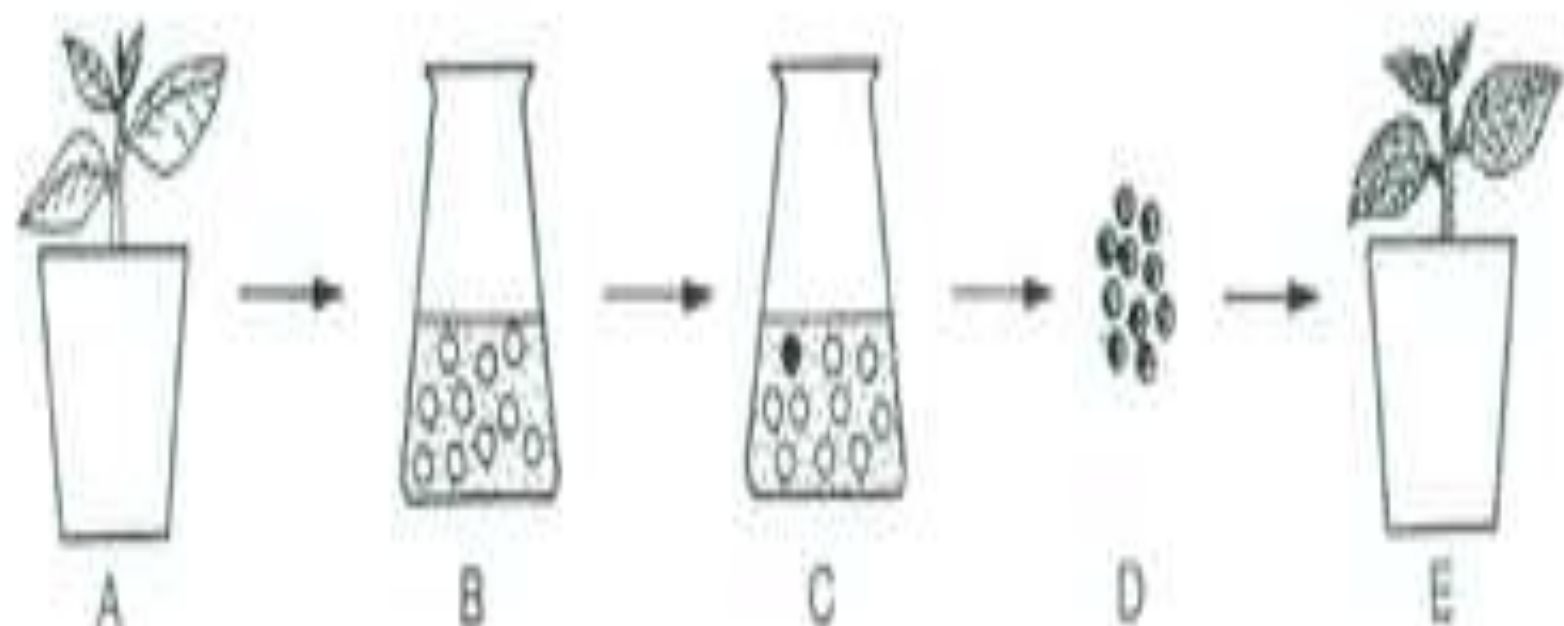


FIG. 8.8. Somaclonal variation. (A) Haploid plant from which cultured tissue was obtained. (B) Cell suspension culture from haploid plant. (C) Mutant cell in suspension culture. (D) Aggregate of mutant cells. (E) Haploid plantlet that exhibits the mutant character. Culturing cells from haploid plants permits either dominant or recessive mutations to be exhibited in the regenerated plantlet. If tissue from diploid plants is cultured, only dominant mutations will be immediately expressed but recessive mutations will be expressed in some progeny plants as a result of segregation.

Requirements for Somaclonal Breeding

- Effective screening procedure
 - Most mutations are deleterious
 - With fruit fly, the ratio is ~800:1 deleterious to beneficial
 - Most mutations are recessive
 - Must screen M_2 or later generations
 - Consider using heterozygous plants?
 - But some say you should use homozygous plants to be sure effect is mutation and not natural variation
 - Haploid plants seem a reasonable alternative if possible
 - Very large populations are required to identify desired mutation:
 - Can you afford to identify marginal traits with replicates & statistics? Estimate: ~10,000 plants for single gene mutant
- Clear Objective
 - Can't expect to just plant things out and see what happens; relates to having an effective screen
 - This may be why so many early experiments failed

Embryo Culture

- ✓ Embryo culture developed from the need to rescue embryos (embryo rescue) from wide crosses where fertilization occurred, but embryo development did not occur
- ✓ These techniques have been further developed for the production of plants from embryos developed by non-sexual methods (haploid production discussed later)

Embryo Culture Uses

- Rescue F1 hybrid from a wide cross
- Overcome seed dormancy, usually with addition of hormone to media (GA)
- To overcome immaturity in seed
 - To speed generations in a breeding program
 - To rescue a cross or self (valuable genotype) from dead or dying plant

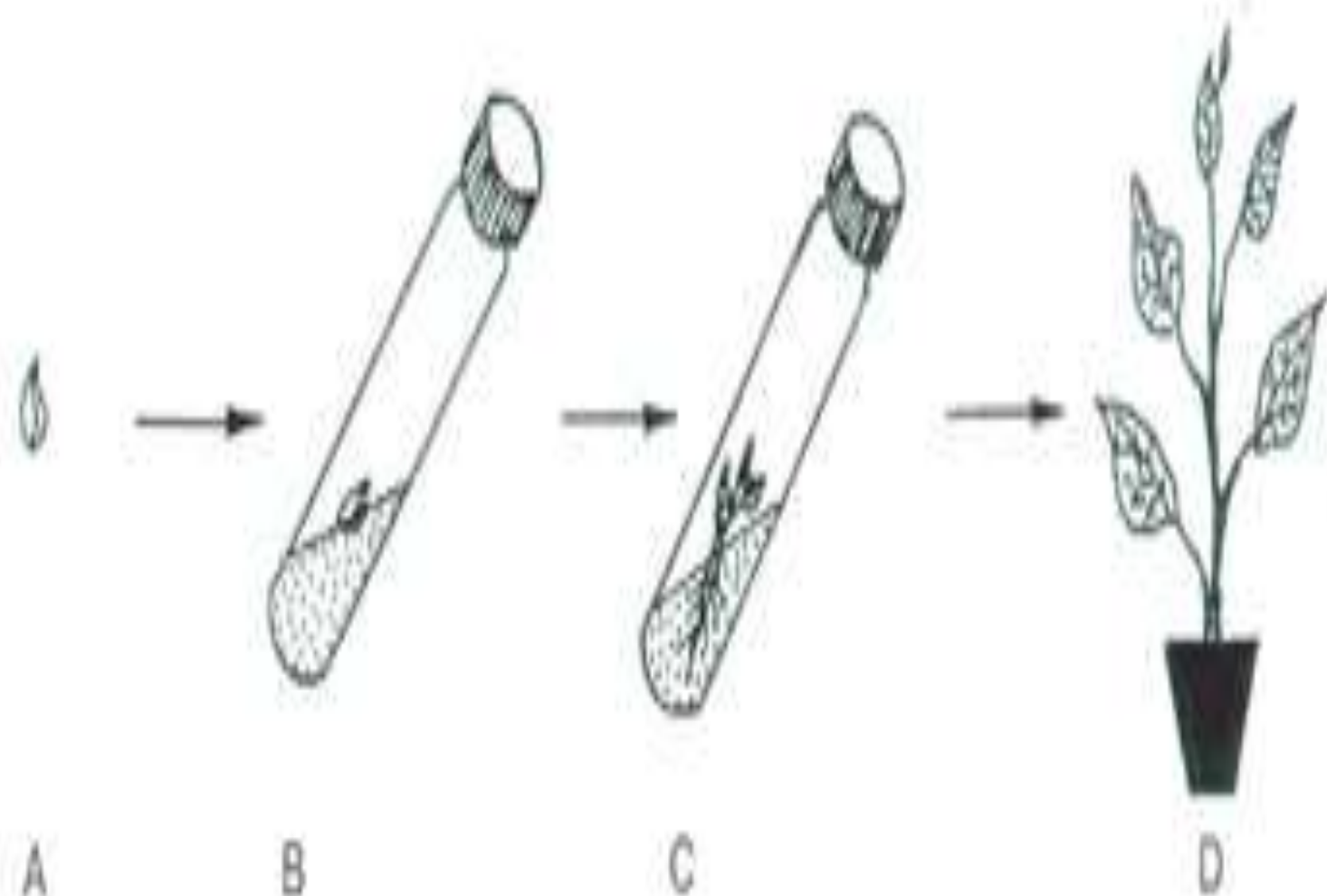
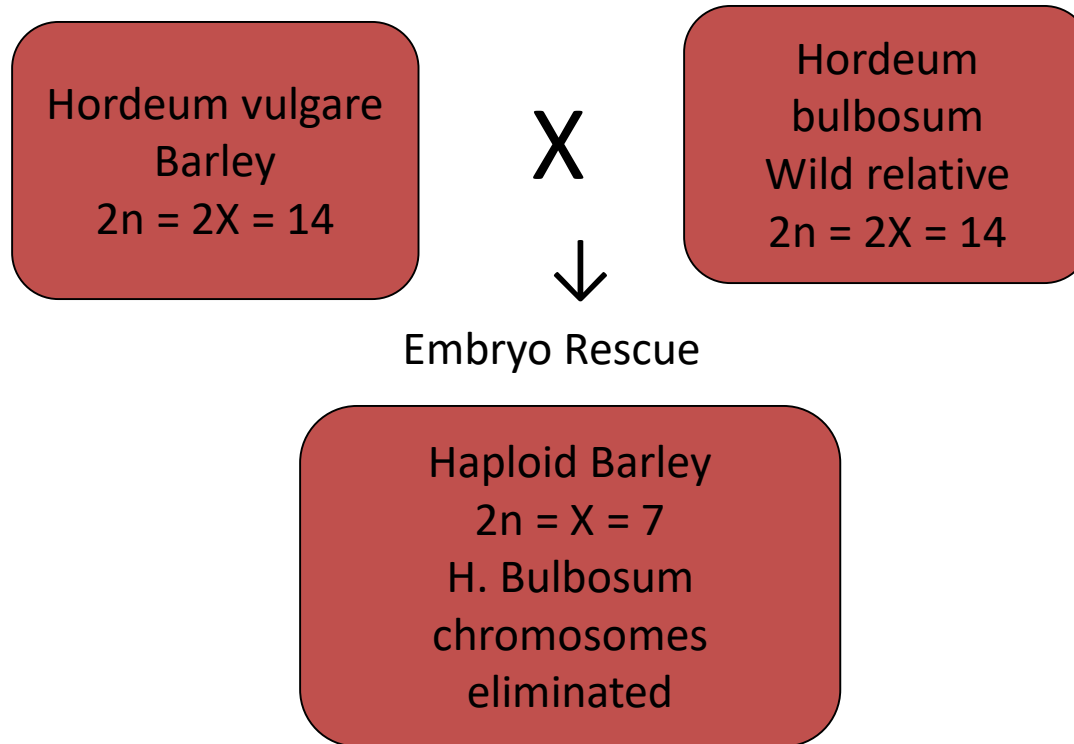


FIG. 8.4. Embryo culture. (A) Proembryo dissected 3 to 5 days after pollination. (B) Proembryo cultured on solid agar medium. (C) Plantlet developing from embryo. (D) Plantlet transplanted into soil.

Haploid Plant Production

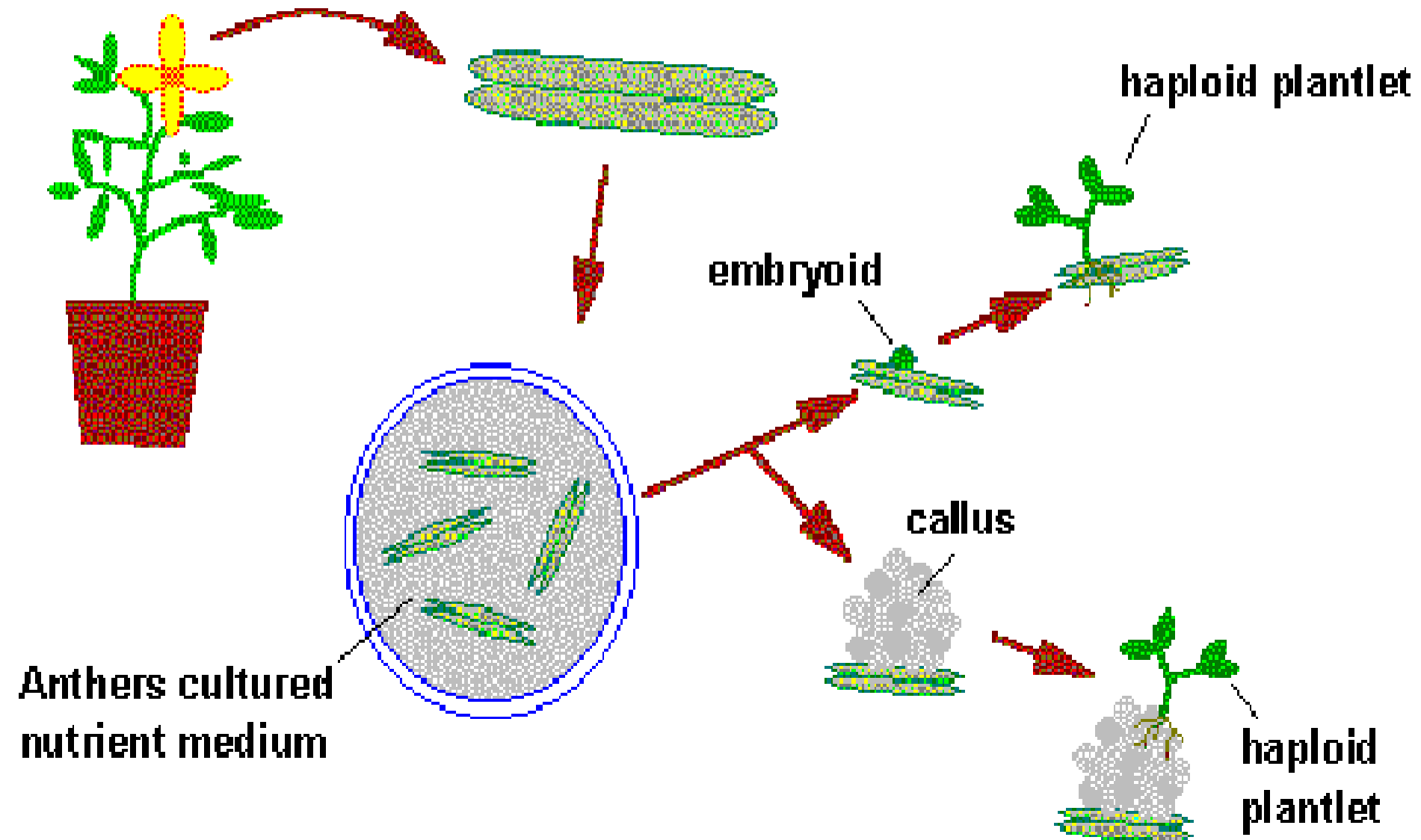
- Embryo rescue of inter-specific crosses
 - Creation of allopolyploids (e.g. triticale)
 - Bulbosum method
- Anther culture/Microspore culture
 - Culturing of Anthers or Pollen grains (microspores)
 - Derive a mature plant from a single microspore
- Ovule culture
 - Culturing of unfertilized ovules (macrospores)
 - Sometimes “trick” ovule into thinking it has been fertilized

Bulbosum Method



- ✓ This was once more efficient than microspore culture in creating haploid barley
- ✓ Now, with an improved culture media (sucrose replaced by maltose), microspore culture is much more efficient (~2000 plants per 100 anthers)

Anther/Microspore Culture



Anther/Microspore Culture Factors

- Genotype
 - As with all tissue culture techniques
- Growth of mother plant
 - Usually requires optimum growing conditions
- Correct stage of pollen development
 - Need to be able to switch pollen development from gametogenesis to embryogenesis
- Pretreatment of anthers
 - Cold or heat have both been effective
- Culture media
 - Additives, Agar vs. 'Floating'

Ovule Culture for Haploid Production

- Essentially the same as embryo culture
 - Difference is an unfertilized ovule instead of a fertilized embryo
- Effective for crops that do not yet have an efficient microspore culture system
 - e.g.: melon, onion
 - In the case of melon, you have to “trick” the fruit into developing by using irradiated pollen, then x-ray the immature seed to find developed ovules

What do you do with the haploid?

- Weak, sterile plant
- Usually want to double the chromosomes, creating a di-haploid plant with normal growth & fertility
- Chromosomes can be doubled by
 - Colchicine treatment
 - Spontaneous doubling
 - Tends to occur in all haploids at varying levels
 - Many systems rely on it, using visual observation to detect spontaneous di-haploids
 - Can be confirmed using flow cytometry

Specific Examples of DH uses

- Evaluate fixed progeny from an F_1
 - Can evaluate for recessive & quantitative traits
 - Requires very large di-haploid population, since no prior selection
 - May be effective if you can screen some qualitative traits early
- For creating permanent F2 family for molecular marker development
- For fixing inbred lines (novel use?)
 - Create a few di-haploid plants from a new inbred prior to going to Foundation Seed (allows you to uncover unseen off-types)
- For eliminating inbreeding depression (theoretical)
 - If you can select against deleterious genes in culture, and screen very large populations, you may be able to eliminate or reduce inbreeding depression
 - e.g.: inbreeding depression has been reduced to manageable level in maize through about 50+ years of breeding; this may reduce that time to a few years for a crop like onion or alfalfa

Protoplasts



- Created by degrading the cell wall using enzymes
- Very fragile, can't be pipette
 - ✓ Mechanical method
 - ✓ Enzymatic method

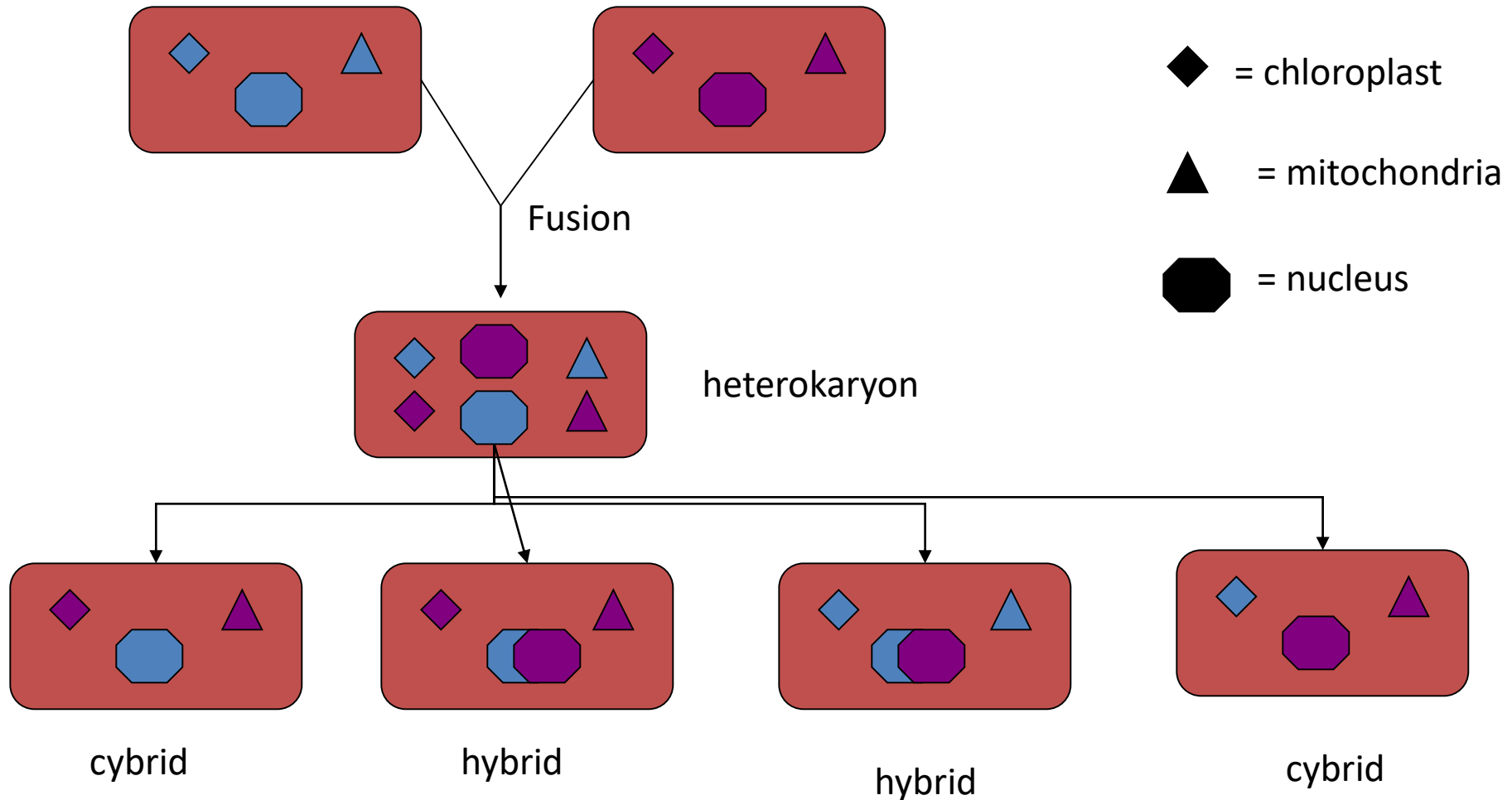
Protoplast Fusion

- Protoplast can be induced to fuse with one another:
 - **Electrofusion:** A high frequency AC field is applied between 2 electrodes immersed in the suspension of protoplasts- this induces charges on the protoplasts and causes them to arrange themselves in lines between the electrodes. They are then subject to a high voltage discharge which causes their membranes to fuse where they are in contact.
 - **Polyethylene glycol (PEG):** causes agglutination of many types of small particles, including protoplasts which fuse when centrifuged in its presence
 - **Addition of calcium ions at high pH values**

Uses for Protoplast Fusion

- Combine two complete genomes
 - Another way to create allopolyploids
- Partial genome transfer
 - Exchange single or few traits between species
 - May or may not require ionizing radiation
- Genetic engineering
 - Micro-injection, electroporation, Agrobacterium
- Transfer of organelles
 - Unique to protoplast fusion
 - The transfer of mitochondria and/or chloroplasts between species

Possible Result of Fusion of Two Genetically Different Protoplasts



Identifying Desired Fusions

- Complementation selection
 - Can be done if each parent has a different selectable marker (e.g. antibiotic or herbicide resistance), then the fusion product should have both markers
- Fluorescence-activated cell sorters
 - First label cells with different fluorescent markers; fusion product should have both markers
- Mechanical isolation
 - Tedious, but often works when you start with different cell types
- Mass culture
 - Basically, no selection; just regenerate everything and then screen for desired traits

Example of Protoplast Fusion

- ✓ Protoplast fusion between male sterile cabbage and normal cabbage was done, and cybrids were selected that contained the radish mitochondria and the cabbage chloroplast
- ✓ Current procedure is to irradiate the cytoplasmic donor to eliminate nuclear DNA – routinely used in the industry to re-create male sterile brassica crops

Industrial Applications

- Secondary metabolites produced by plants
 - Alkaloids, Terpenoids, Steroids, Anthocyanins, Anthraquinones, Polyphenols
- Often unclear function in the plant
- Often restricted production (specific species, tissue or organ)
- Many are commercially valuable
- Cell culture techniques allow large-scale production of specific secondary metabolites

Cell culture systems

- ✓ Callus
- ✓ Cell suspension culture



Callus

- An unorganised mass of cells
- Equimolar amounts of auxin and cytokinin stimulate cell division

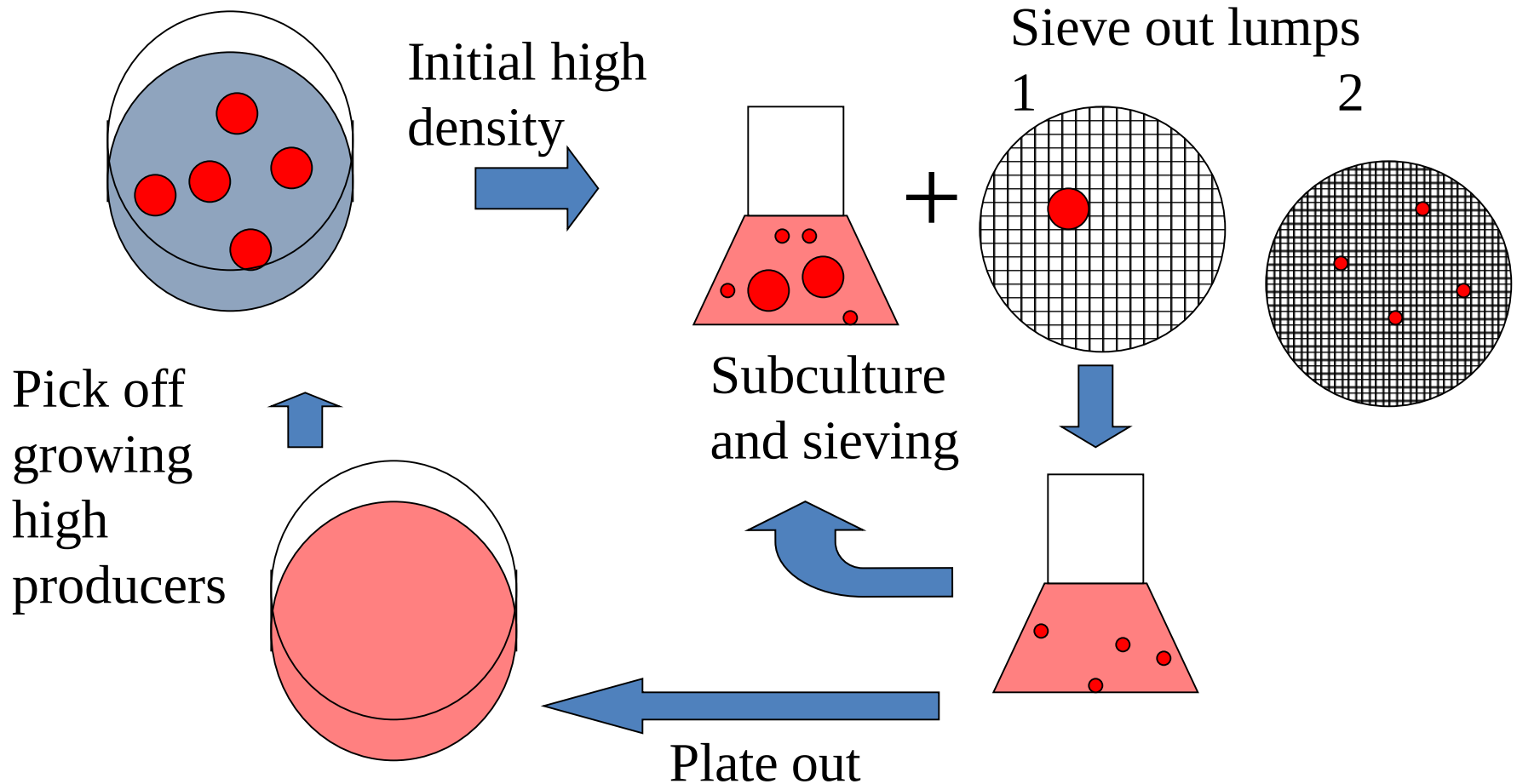
Cell suspension culture

- ✓ When callus pieces are agitated in a liquid medium, they tend to break up.
- ✓ Suspensions are much easier to bulk up than callus since there is no manual transfer or solid support.

Introduction of callus into suspension

- ✓ 'Friable' callus goes easily into suspension.
 - 2,4-D
 - Low cytokinin
 - semi-solid medium
 - enzymic digestion with pectinase
 - blending
- ✓ Removal of large cell aggregates by sieving.
- ✓ Plating of single cells and small cell aggregates - only viable cells will grow and can be re-introduced into suspension.

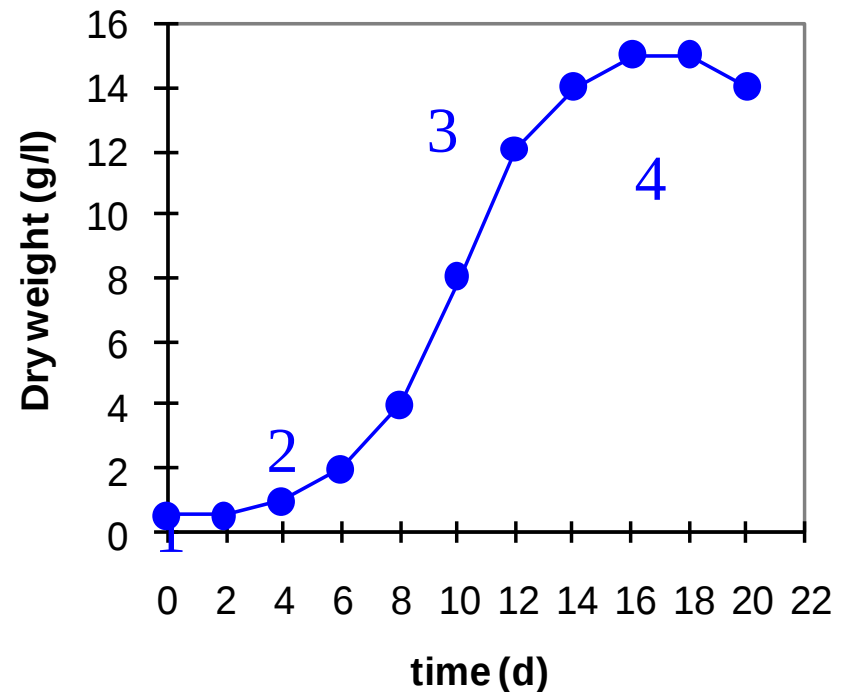
Introduction into suspension



Growth kinetics

- ✓ Initial lag dependent on dilution
- ✓ Exponential phase (dt 1-30 d)
- ✓ Linear/deceleration phase (declining nutrients)
- ✓ Stationary (nutrients exhausted)

Plant Cell Suspension typical Growth curve



Characteristics of plant cells

- Large (10-100 μm long)
- Tend to occur in aggregates
- Shear-sensitive
- Slow growing
- Easily contaminated
- Low oxygen demand
- Will not tolerate anaerobic conditions
- Can grow to high cell densities (>300g/l fresh weight).
- Can form very viscous solutions

Thank You