Tissue culture techniques

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Overview

Background

- In vitro = axenic = sterile culture
- Plant tissue culture is generally used for the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions in vitro.
- First approach made by Henri-Louis Duhumel du Monceau in 1756 (observed callus formation in plants)
- Development of cell theory by Schleiden and Schwann during 1830s
- Theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells.

History

- The first true plant tissue cultures were obtained by Gautheret (1934, 1935) from cambial tissue of *Acer pseudoplatanus*.
- The first plant growth hormone indoleacetic acid (IAA) was discovered in the mid-1930s by F. Kogl and his coworkers.
- In 1934 Professor Philip White successfully cultured tomato roots.
- In 1939 Gautheret successfully cultured carrot tissue.
- Later in 1955 Carlos Miller and Folke Skoog published their discovery of the hormone kinetin, a cytokinin.
- In 1962, Toshio Murashige and Skoog published the composition a plant tissue culture medium known as MS (named for the first letters of their last names) medium.
- Murashige was a doctoral student in Professor Skoog's lab, and they developed the now-famous MS medium working with tobacco tissue cultures.

Tissue culture laboratory setup

Media preparations/culture evaluation

- Bench
- Gas outlet
- Hot plate and magnetic stirrer
- Analytical and top-loading balances
- pH meter
- Refrigerator, freezer
- Water purification and storage system
- Dish-washing area
- Storage facilities-glassware, chemicals
- Autoclave (pressure-cooker will work for small media volume)
- Low bench with inverted light and dissecting microscopes (avoid locating next autoclaves or other high-humidity areas)
- Fume hood
- Desk and file cabinets
- Desktop centrifuge, spectrophotometer, microwave (transformation studies and protoplast isolation)

Aseptic transfer area

1. Laminar air flow transfer hood and comfortable chair
2. Dissecting microscope
3. Gas outlet
4. Vacuum lines
5. Forceps, spatulas, scalpel, and disposable blades

Aseptic transfer area

Figure 1: Working in the laminar flow hood

Environmentally Controlled Culture Area

1. Shelves with lighting on a timer and controlled temperature
2. Incubators—with controlled temperature and light
3. Orbital shakers

Conditions

- High humidity in culture room should be avoided.
- Most cultures can be incubated at $25 - 27\ ^{\circ}C$ under a 16:8-h light:dark photoperiod controlled by clock timers.
- Light conditions can be manipulated by mounted fluorescent lamps with light readings (by quantum radiometer-photometer) of 40-200 foot candles (full sun is approximately 10000 foot candles).

Basal media components and preparation

Inorganic salts

- The Murashige and Skoog (MS) (1962) formulation is the most widely used formulation.
- The MS formulation was developed to insure that no increases in cell growth in vitro were due to the introduction of additional salts from plant tissue extracts which were being tested at that time.
- The MS formulation insured that the inorganic nutrients were not limiting to tobacco cell growth and organic supplements such as yeast extract, coconut milk, casein hydrolysate, and plant extracts were no longer essential sources of the inorganic salts.

Inorganic salts

- The distinguishing feature of the MS inorganic salts is their high content of nitrate, potassium, and ammonium in comparison to other salt formulations.
- These salt stocks are prepared at 100 times the final medium concentration, and each stock is added at the rate of 10 ml per 1000 ml of medium prepared. The NaFeEDTA stock should be protected from light by storing it in a bottle that is amber colored or wrapped in aluminum foil. Concentrated salt stocks enhance the accuracy and speed of media preparation.
- Salt stocks are best stored in refrigerator and are stable for several months.

Inorganic salts

TABLE 3.1 Composition of the Five Inorganic Salt Stocks of the Murashige and Skoog Inorganic Formulation

Chemical	Concentration (g/liter stock)
Nitrate stock	
Ammonium nitrate (NH_4NO_3)	165.0
Potassium nitrate (KNO_3)	190.0
Sulfate stock	
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	37.0
Manganous sulfate ($MnSO_4 \cdot H_2O$)	1.69
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	0.86
Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	0.0025
Halide stock	
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	44.0
Potassium iodide (KI)	0.083
Cobalt chloride ($CoCl_233 \cdot 6H_2O$)	0.0025
PBMo stock	
Potassium phosphate (KH_2PO_4)	17.0
Boric acid (H_3BO_3)	0.620
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.025
NaFeEDTA stock	
Ferrous sulfate ($FeSO_2 \cdot 7H_2O$)	2.784
Ethylenediamineteraacetic acid, disodium salt (Na_2EDTA)	3.724

Figure 2: Composition of the five inorganic salt stocks of the Murashige and Skoog Inorganic formulation

Inorganic salts

TABLE 3.2 Inorganic Salt Formulation of Several Commonly Used Basal Salts for Plant Tissue Culture in Milligrams per Liter of Medium[a]

Chemical	White (1963)	B5[b]	N6[c]	WP[d]
NH_4NO_3				400
$(NH_4)2SO_4$		134	463	
$MgSO_4 \cdot 7H_2O$	720	246	185	370
KCl	65			
KNO_3	80	2528	2830	
KH_2PO_4			400	170
K_2SO_4				990
$NaH_2PO_4 \cdot H_2O$	19	150		
Na_2SO_4	200			
$CaCl_2 \cdot 2H_2O$		150	166	96
$Ca(NO_3)_2 \cdot 4H_2O$	300			556
$Na_2EDTA \cdot 2H_2O$		37.2	37.2	37.2
$FeSO_4 \cdot 7H_2O$		27.8	27.8	27.8
$Fe_2(SO_4)_3$	2.5			
H_3BO_3	1.5	3	1.6	6.2
$CoCl_2 \cdot 6H_2O$		0.025		
$CuSO_4 \cdot 5H_2O$	0.001	0.025		0.25
$MnSO_4 \cdot H_2O$		10		
$MnSO_4 \cdot 4H_2O$	7		4.4	22.3
MoO_3	0.0001			
$Na_2MoO_4 \cdot 2H_2O$		0.25		0.25
KI	0.75	0.75	0.8	
$ZnSO_4 \cdot 7H_2O$	3	2	1.5	8.6

[a] *Owen and Miller (1992).*
[b] *B5, Gamborg et al. (1968).*
[c] *N6, Nitsch and Nitsch (1969).*
[d] *WP, Lloyd and McCown (1980).*

Growth regulators

TABLE 3.3 Common Plant Growth Regulators Used in Plant Tissue Culture

Plant growth regulator	Abbreviation	MW
Abscisic acid	ABA	264.3
Indole-3-acetic acid	IAA	175.2
Naphthaleneacetic acid	NAA	186.2
2,4-Dichlorophenoxyacetic acid	2,4-D	221.0
Indole-3-butyric acid	IBA	203.2
6-Furfurylaminopurine	Kinetin	215.2
6-Benzly-aminopurine	BA	225.2
N^6 (^{2}-isopentenyl)-adenine	2iP	203.3
Trans-6-(4-hydroxyl-3-methylbut-2-enyl) amino purine	Zeatin	219.2
Gibberellic acid	GA_3	346.4
Thidiazuron	TDZ	220.2
or 1-phenyl-3-(1,2,3-thiadiazol-5YL)-urea		

Figure 4: Common plant growth regulators used in plant tissue culture

PGRs

- Type and concentration will vary according to the cell culture purpose. List is provided in Figure 4.
- Auxin (IAA, NAA, 2,4-D or IBA) is required by most plant cells for division and root initiation.
- 2,4-D is widely used for callus induction.
- IAA, IBA and NAA are used for root induction.
- Auxins can be dissolved either in alkali or in ethanol (latter is toxic to plants).
- IAA stocks have a working duration of 1 weeks when kept away from light, they are thermostable, however.

PGRs

- Cytokinins (kinetin, BA, zeatin and 2iP) promote cell division, shoot proliferation, and shoot morphogenesis.
- Cytokinins are prepared in a fashion alike Auxins, except that 1 N HCL and a few drops of water are used to dissolve the crystals.
- They can be stored for longer terms in the refrigerator and are thermostable.
- Normally, gibberellin is infrequently used in plant cell culture due to callus growth and auxin induced adventitious root inhibition.

PGRs

- Since stocks are highly susceptible to isomeric conversions, it should be made up fresh before addition to the medium by filter sterilization.
- Abscisic acid (ABA), a plant hormone involved in leaf and fruit abscission and dormancy, is useful in embryo culture. Its stock solutions can be prepared in water. It is heat stable but light sensitive (conversion of cis to trans form in presence of light results in lesser biological activity).
- Addition of ethylene biosynthetic inhibitors such as silver nitrate is beneficial.

PGRs

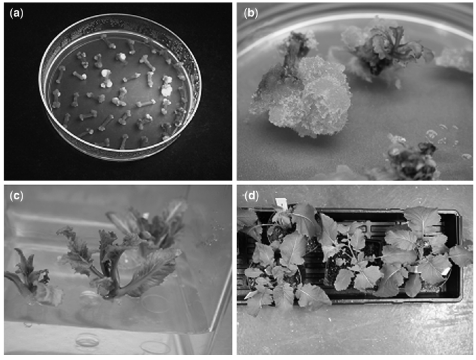

Figure 5: *Brassica juncea* plants produced from hypocotyl explants. Shoots are produced when a combination of auxin and cytokinin is used. (a) Callus from hypocotyl explants (note the green fluorescent protein fluorescent sectors on some of the calli); (b) shoots from callus; (c) shoots elongating; (d) whole plantlets transferred to soil.

Vitamins

- Catalytic functions in enzyme reactions.
- Thiamine (B1), Nicotinic acid (B3) and Pyridoxine (B6) are added to cell culture media.
- Stocks best stored in freezer.
- 10-ml aliquots used per liter of medium prepared.
- Vitamin stocks used in these exercises contain 5 mg of nicotinic acid and 5 mg pyridoxine-hydrochloride per 100 ml of water.
- The thiamine stock has 40 mg thiamine-hydrochloride in 1000 ml.

Vitamins

- **Common formulations**:
  - White (White 1963); in milligram-per-liter: 0.5 nicotinic acid, 0.1 pyridoxine-hydrochloride, and 0.1 thiamine-hydrochloride;
  - B5 Gamborg (Gamborg et al., 1976) with in milligram-per-liter medium: 100 inositol, 1.0 nicotinic acid, 1.0 pyridoxine-hydrochloride, and 10.0 thiamine-hydrochloride;
  - Murashige and Skoog (1962) with in milligram-per-liter medium: 100 inositol, 0.5 nicotinic acid, 0.5 pyridoxine-hydrochloride, and 0.1 thiamine-hydrochloride.

Carbohydrates

- Green cells require carbohydrate source (as sucrose, glucose, fructose or even starch at 2-5% (w/v)) until they are photosynthetically active.
- Embryo and anther culture require higher levels of carbohydrates than that by protoplast culture.
- Sugars undergo caramelization if autoclaved too long.

Gelling agent

- Provide stationary support
- Can include filter paper, cotton, cheesecloth, vermiculite, special membrane rafts with a liquid medium.
- Must include purified agar.
- Gelrite is transparent in appearance and is a polysaccharide produced from fermentation from *Pseudomonas* sps.
- Gelling should be done slowly in motion, while heating (in flask).
- Medium is then dispensed in measured amounts in the culture container.
- The agar can also be melted in the autoclave in a foil-capped Erlenmeyer flask for 15 min at $121^{\circ}C$, 15 psi.

Amino acids

- Important in morphogenesis.
- L-forms are natural forms, which is also the only detectable form.
- L-tyrosine can contribute to shoot initiation, L-arginine can facilitate rooting, L-serine can be used in microspore cultures to obtain haploid embryos.
- Casein hydrolysate, an enzymatic digest of milk protein (caution: do not use the acid digest of milk proteins), was a common ingredient in many early media formulations as it provided a mixture of amino acids to enhance tissue response.

Antibiotics

- Incorporation of bactericides and fungicides is to overcome contamination.
- Generally not desired because they can be toxic to explants.
- Transformaiton experiments using agrobacterium make it necessary to incorporate antibiotics (e.g. Timentin, carbenicillin (500 mg/liter), cefotaxime (300 μg/ml)).
- Should be added to medium after autoclaving.

Natural complexes

- Antioxidants, absorbents (charcoal), coconut endosperm, yeast extract, malt extract, tomato juice, etc.
- Activated charcoal may be growth promoting (promotes morphogenesis and absorbs inhibitory compounds)

Media pH

- Agar will not gel properly above 6.0 (too firm gel).
- pH naturally drops by 0.6-1.3 units after autoclaving and after cetain period of culturing.
- Medium pH is adjusted with 1.0 or 0.1 N HCL or NaOH by using a medicine dropper

Explant preparation

Explant age

- Younger tissue is more responsive *in vitro*.
- Older tissues might not form callus.

Season

- Spring season is appropriate time for good response in culture.
- Tissues that have met dormancy requirements are useful.
- Artificial chilling might also help enable break dormancy.
- Contamination is associated with summer season.

Explant size

- Smaller the explant, the harder it is to culture.
- Internal differences in hormone balance in the tissue can result in varying in vitro responses.

Plant Quality

- Healthy, unstressed, virus free.

Goal

- If clonal propagation is the goal, then the explant will usually be a lateral or terminal bud or shoot.
- For callus induction, pieces of the cotyledon, hypocotyl, stem, leaf, or embryo are usually used.
- Excellent explants for callus induction are seedling tissues from aseptically germinated seeds or imma ture inflorescences.
- Leaf tissue from the aseptically germinated seed is a good source of tissue for protoplast isolation.
- To produce haploid plants or callus, the anther or pollen is cultured.

Explant source

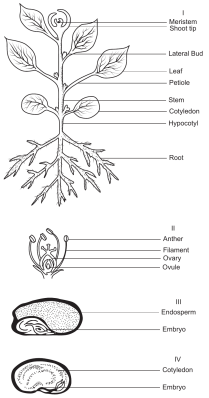

Figure 6: Schematic drawings (from top to bottom) of a plant, a flower, and monocotyledenous and dicotyldenous seeds indicate potential explant tissues.

Other factors

- Genotype: The ability to form regenerable callus in sorghum is heritable, and acted as a dominant trait (Ma et al., 1987). Also, rice regeneration is under control of both nuclear and cytoplasmic genes.
- Aseptic technique
- Asceptic germination of seeds
- Contamination

Processes and mechanism

Growth progression

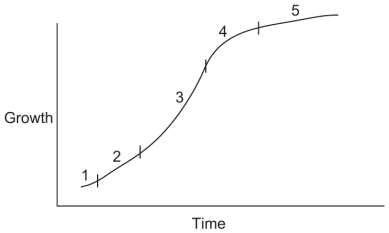

Figure 7: The rate of growth of callus tissue usually has five stages: (1) a lag phase in which cells prepare to divide; (2) a period of exponential growth in which cell division is maximal; (3) a period of linear growth in which division slows down and cells enlarge; (4) a period of decelerating growth; and (5) a stationary or no-growth period in which the number of cells is constant.

Callus induction

- Explants may be an aseptically germinated seedling or surface-sterilized roots, stems, leaves, or reproductive structures.
- *Callus* is a wound tissue produced in response to injury. It is a proliferation of cells from the wounded or cut region of an explant. Callus is generally made up of friable, large, vacuolated cells that are highly differentiated, but are unorganized.
- Callus can be hard and compact, and can contain regions of small meristematic cell clusters.
- Generally meristematic, undifferentiated cells are competent to regenerate via somatic embryo or organ initiation.
- Culture media (level of growth regulators) and conditions (temperature, light, solid vs. agar media) are important for callus formation and development.

Regeneration and morphogenesis

- Organogenesis
  - Direct organogenesis: Without callus phase
  - Indirect organogenesis: Via a callus phase
- Somatic embryogenesis

Technology

Haploid plants from anther culture

- Culture of anthers or isolated micropsores to produce haploid plants is known as anther culture or microspore culture.
- Embryos can be produced via a callus phase or be a direct recapitulation of the developmental stages characteristic of zygotic embryos.
- late uninucleate to early binucleate microspores are the best explants for embryogenesis.
- Somatic embryos develop into haploid plants.
- Chromosome doubling can be further used to produce doubled haploids (homozygous).
- In addition, haploid embryos are used in mutant isolation, gene transfer, studies of storage product biochemistry, and physiological aspects of embryo maturation.

Embryo culture/rescue

- Embryos are isolated from immature ovules or seeds are cultured in vitro.
- Useful tool for direct regeneration in species where seeds are dormant, recalcitrant, or abort at early stages of development.
- Embryo **rescue** also finds use in the production of interspecific hybrids between inviable crosses, whose seeds are traditionally condemned and discarded because of their inability to germinate.
- Generally, embryo culture goes hand in hand with in vitro control of pollination and fertilization to ensure hybrid production.
- Immature embryos can be used to produce embryogenic callus and somatic embryos or direct somatic embryos.

Meristem culture for virus free plants

- For the production of pathogen-free plants
- Apical meristem tips is used
- aka. meristem culture, meristem tip culture, or shoot tip culture
- Apical meristems in plants are suitable
- Meristem culture in combination with thermotherapy has resulted in successful production of virus-free plants.

Protoplast isolation and fusion/Somatic hybridization

- Using protoplast, it is possible to regenerate whole plants from single cells
- Following enzymatic digestion of cell wall (by pectinase, cellulase) and then of cell membrane.
- Isolated from cellular debris using filtering mesh and then floatation (in sucrose) technique.
- Cultured in high osmotic medium (solid or liquid); if solid also embedded in an alginate matrix.
- Fusion is accomplished by use of PEG (polyethylene glycol)

Agrobacterium mediated transformation

Bibliography