Laboratory WORKS ON discipline “**Basics of biotechnology”**

***Module 2. Plant Biotechnology***

Lab. 6 Biotechnology Laboratory Requirements and Safety

The following facilities should be there in a Biotechnology laboratory.

1. Store room: for storing chemicals and glassware.

2. Cleaning and washing room: for general cleaning purposes.

3. General laboratory: for routine laboratory experiments.

4. Specialized rooms: preparation and sterilization room, laminar flow, sterile storage and culture rooms.

5. General instrumentation room: for PCR machine, gel documentation system, electrophoresis units, centrifuge, pH meter, balance, laminar flow, freezers, ice machine etc.

**6. Proper disposal of media, cultures etc.**

A biology laboratory include good computer attached with a printer, autoclaves, sinks, water distillation units, deionizers, hot air ovens, radiation shields, incubators, temperature control or cold room, dark room for development of photographic films, -20oC freezers, refrigerators, water baths, refrigerated centrifuges, micro centrifuges, trans illuminator with camera, scintillation counters to monitor radioactivity, blotting apparatus, hot plates, micro wave ovens, and many other miscellaneous items.

The laboratory needs a variety of glassware including reagent bottles, beakers, measuring cylinders, Erlenmeyer flasks, test tubes, pipettes, glass rods, petri dishes, and other culture vessels. Disposable sterile Petri dishes can be used for the culture of plant tissue. Other requirements include liquid nitrogen containers, syringes, needles, forceps, scalpels, membrane filters, magnetic stirrers, orbital shakers, nylon or nitrocellulose membranes, Para film, aluminium foil, marker pens, Whatman 3 M paper, ice bucket, latex gloves, plastic boxes, plastic bags and UV goggles, good quality chemicals, uninterrupted power supply and water supply.

**Plant Tissue Culture Laboratory Organization**

An ideal tissue culture laboratory should have at least two big rooms and a small room. One big room is for general laboratory work such as preparation of media, autoclaving, distillation of water etc. The other big room is for keeping cultures under controlled light, temperature and humidity. The small room is for aseptic work and for keeping autoclaved articles.

Instruments

Table 1. Tools and Instruments used in a biotechnology laboratory

Equipment Features

1. Autoclave

General Description: An autoclave is a large pressure cooker. It is a moist sterilization unit.

Principle: It operates with the principle of steam under pressure as the sterilizing agent. High pressures enable steam to reach high temperature, thus increasing its heat content and killing power. Most of the heating power of steam comes from its latent heat of vaporization (the amount of heat required to convert boiling water to steam).

Steam is able to penetrate objects with cooler temperatures because once the steam contacts a cooler surface; it immediately condenses to water, producing a concomitant 1, 870 fold decrease in steam volume. This creates negative pressure at the point of condensation and draws more steam to the area. A condensation continues so long as the temperature of the condensing surface is less than that of steam; once temperatures equilibrate, a saturated steam environment is formed. Achieving high and even moisture content in the steam-air environment is important for effective autoclaving. The ability of air to carry heat is directly related to the amount of moisture present in the air. The more moisture present, the more heat can be carried, so steam is one of the most effective carriers of heat. Steam therefore results in the efficient killing of cells and the coagulation of proteins.

Moist heat is thought to kill microorganisms by causing coagulation of essential proteins. Another way to explain this is that when heat is used as a sterilizing agent, the vibratory motion of every molecule of a microorganism is increased to levels that induce the cleavage of intramolecular hydrogen bonds between proteins. Death is therefore caused by an accumulation of irreversible damage to all metabolic functions of the organism. Death rate is directly proportional to the concentration of microorganisms at any given time. The time required to kill a known population of microorganisms in a specific suspension at a particular temperature is referred to as thermal death time (TDT). All autoclaves operate on a time/temperature relationship; increasing the temperature decreases TDT, and lowering the temperature increases TDT.

Standard temperatures/pressures employed are 115oC/10 p.s.i., 121oC/15 p.s.i., and 132oC/27 p.s.i. (psi=pounds per square inch).

Working: Steam enters the chamber jacket, passes through an operating valve and enters the rear of the chamber behind a baffle plate. It flows forward and down through the chamber and the load, exiting at the front bottom. A pressure regulator maintains jacket and chamber pressure at a minimum of 15 psi, the pressure required for steam to reach 121oC (250oF).Overpressure protection is provided by a safety valve. The conditions inside are thermostatically controlled so that heat (more steam) is applied until 121oC is achieved, at which time the timer starts, and the temperature is maintained for the selected time.



Preparation of Solutions

Preparation of Solutions

(a) Calculation of Molar, %, and "X" Solutions

(i) A molar solution is one in which 1 litre of solution contains the number of grams equal to its molecular weight.

Example. To make up 100 ml of a 5M NaCl solution = 58.456 (mw of NaCl) g × 5 moles × 0.1 liter = 29.29 g in 100 ml sol mole liter.

(ii) Percent solutions

Percentage (w/v) = weight (g) in 100 ml of solution

Percentage (v/v) = volume (ml) in 100 ml of solution.

Example. To make a 0.7% solution of agarose in TBE buffer, weigh 0.7g of agarose and bring up the volume to 100 ml with the TBE buffer.

(iii) "X" solutions. Many enzyme buffers are prepared as concentrated solutions,

e.g., 5 X or 10 X (5 or 10 times the concentration of the working solution), and are then diluted so that the final concentration of the buffer in the reaction is 1 X.

Example. To set up a restriction digestion in 25 ml, one would add 2.5 ml of a 10 X buffer, the other reaction components, and water for a final volume of 25 ml.

(b)Standard NaOH Solution (0.1M/0.1N)

Take 0.4g NaOH and dissolve gradually with 100ml sterile distilled water.

(c) Standard HCl Solution (0.1M/0.1N)

Dilute 9 ml of pure concentrated HCl to 1.0 l with distilled water in volumetric flask. Invert several times and transfer to a clean, dry bottle.

(c) Steps in Solution Preparation

(i) Refer to the laboratory manual for any specific instructions on preparation of the particular solution and the bottle label for any specific precautions in handling the chemical.

(ii) Weigh out the desired amount of chemical(s). Use an analytical balance if the amount is less than 0.1 g.

(iii) Pour the chemical(s) in an appropriate size beaker with a stir bar.

(iv) Add less than the required amount of water. Prepare all solutions with double-distilled water.

(v) When the chemical is dissolved, transfer to a graduated cylinder and add the required amount of distilled water to achieve the final volume.

An exception is when preparing solutions containing agar or agarose. Weigh the agar or agarose directly in the final vessel.

(vi) If the solution needs to be at a specific pH, check the pH meter with fresh buffer solutions and follow the instructions for using a pH meter.

(vii) Autoclave, if possible, at 121°C for 20 minutes. Some solutions cannot be autoclaved; for example, SDS. These should be filter-sterilized through a 0.22 mm filter. Media for bacterial cultures must be autoclaved the same day it is prepared, preferably within an hour or two. Store at room temperature and check for contamination prior to use by holding the bottle at eye level and gently swirling it.

Basics of Plant Tissue CulturePlant tissue culture is the science of growing plant cells, tissues or organs isolated from healthy, superior

quality mother plant to artificial medium.

Culture of Organized Structures

Organ culture is used as a general term for those types of culture in which an organized form of

growth can be continuously maintained. It includes the aseptic isolation from whole plants of such definite

structures as leaf primordia, immature flowers and fruits, and their growth in vitro. The most important kinds of organ culture are:

1. Meristem cultures: They are grown as very small excised shoot apices, each consisting of the apical

meristematic dome with or without one or two leaf primordia. The shoot apex is typically grown to

give one single shoot.

2. Shoot tip or shoot cultures: It starts from excised shoot tips or buds, larger than the shoot apices

employed to establish meristem cultures, having several leaf primordia. These shoot apices are

usually cultured in such a way that each produce multiple shoots.

3. Node cultures: The separate lateral buds, each carried on a small piece of stem tissue or stem section

carrying either single or multiple nodes can be cultured. Each bud is grown to provide a single shoot.

4. Isolated root cultures: The growth of roots, unconnected to shoots through which a branched root

system may be obtained.

5. Embryo cultures: They were fertilized or unfertilized zygotic (seed) embryos which are dissected out

of developing seeds or fruits and cultured in vitro until they have grown into seedlings. Embryo

culture is quite distinct from somatic embryogenesis.

Fig. 20. A diagrammatic section through a bud showing location and approximate relative sizes of a

meristematic dome, the meristem tip and shoot tip explants.

**Lab. Work 7.** Cultivation of mature wheat embryos in vitro on MS medium.Plant growth medium

**Murashige and Skoog medium** (or ***MSO*** or ***MS0*** *(MS-zero)*) is a plant growth medium used in the laboratories for cultivation of plant [cell culture](https://en.wikipedia.org/wiki/Cell_culture). MSO was invented by plant scientists [Toshio Murashige](https://en.wikipedia.org/wiki/Toshio_Murashige) and [Folke K. Skoog](https://en.wikipedia.org/wiki/Folke_K._Skoog) in 1962 during Murashige's search for a new [plant growth regulator](https://en.wikipedia.org/wiki/Plant_growth_regulator). A number behind the letters MS is used to indicate the sucrose concentration of the medium. For example, MS0 contains no sucrose and MS20 contains 20 g/l sucrose. Along with its modifications, it is the most commonly used medium in [plant tissue culture](https://en.wikipedia.org/wiki/Plant_tissue_culture) experiments in the laboratory.[[1]](https://en.wikipedia.org/wiki/Murashige_and_Skoog_medium#cite_note-1)

As Skoog's doctoral student, Murashige originally set out to find an as-yet undiscovered growth hormone present in tobacco juice. No such component was discovered; instead, analysis of juiced tobacco and ashed tobacco revealed higher concentrations of specific minerals in plant tissues than were previously known. A series of experiments demonstrated that varying the levels of these nutrients enhanced growth substantially over existing formulations. It was determined that [nitrogen](https://en.wikipedia.org/wiki/Nitrogen) in particular enhanced growth of tobacco in tissue culture.

**Major components of MS medium**

**Major salts (macronutrients)/ 1L**[Ammonium nitrate](https://en.wikipedia.org/wiki/Ammonium_nitrate) (NH4NO3) **1,650 mg/l**

* [Calcium chloride](https://en.wikipedia.org/wiki/Calcium_chloride) (CaCl2 · 2H2O) **440 mg/l**
* [Magnesium sulfate](https://en.wikipedia.org/wiki/Magnesium_sulfate) (MgSO4 · 7H2O) **370 mg/l**
* [Monopotassium phosphate](https://en.wikipedia.org/wiki/Monopotassium_phosphate) (KH2PO4) **170 mg/l**
* [Potassium nitrate](https://en.wikipedia.org/wiki/Potassium_nitrate) (KNO3) **1,900 mg/l**.

These are major salts present in MS media .

**Minor salts (micronutrients)/ 1L**[Boric acid](https://en.wikipedia.org/wiki/Boric_acid) (H3BO3) **6. 2 mg/l**

* [Cobalt chloride](https://en.wikipedia.org/wiki/Cobalt_chloride) (CoCl2 · 6H2O) **0.025 mg/l**
* [Ferrous sulfate](https://en.wikipedia.org/wiki/Ferrous_sulfate) (FeSO4 · 7H2O) **27.8 mg/l**
* [Manganese(II) sulfate](https://en.wikipedia.org/wiki/Manganese%28II%29_sulfate) (MnSO4 · 4H2O) **22.3 mg/l**
* [Potassium iodide](https://en.wikipedia.org/wiki/Potassium_iodide) (KI) **0.83 mg/l**
* [Sodium molybdate](https://en.wikipedia.org/wiki/Sodium_molybdate) (Na2MoO4 · 2H2O) **0.25 mg/l**
* [Zinc sulfate](https://en.wikipedia.org/wiki/Zinc_sulfate) (ZnSO4·7H2O) **8.6 mg/l**
* [Ethylenediaminetetraacetic acid ferric sodium](https://en.wikipedia.org/wiki/Ethylenediaminetetraacetic_acid) (NaFe-EDTA) constituting **5 ml/l** of a stock solution containing **5.57 g** [FeSO4.7H2O](https://en.wikipedia.org/wiki/Iron%28II%29_sulfate) and **7.45 g** [Na2-EDTA](https://en.wikipedia.org/wiki/Ethylenediaminetetraacetic_acid) per litre of water.
* [Copper sulfate](https://en.wikipedia.org/wiki/Copper_sulfate) (CuSO4 · 5H2O) **0.025 mg/l**

**Lab 8.** **Somatic Embryogenesis**

**Major Steps of Tissue Culture (Plants)**

**Initiation Phase (Stage 1)**

The initiation phase is the first phase of tissue culture. Here, the tissue of interest is obtained and introduced and sterilized in order to prevent any microorganism from negatively affecting the process. It is during this stage that the tissue is initiated in to culture

**Multiplication Phase (Stage 2)**

The multiplication phase is the second step of tissue culture where the in vitro plant material is re- divided and then introduced in to the medium.

The medium is composed of appropriate components for growth including regulators and nutrients.

 These are responsible for the proliferation of the tissue and the production of multiple shoots.

\*This step is often repeated several times in order to obtain the desired number of plants

Root formation (Stage 3)

It is at this phase that roots are formed.

Root formation hormones are required in order to induce rooting, and consequently complete plantlets

**Plant Tissue Culture**

Tissue culture is applied in plant research for such purposes as the growing of new plants, which in some cases undergo genetic alterations.

The plant of interest is taken through the tissue culture process and grown in a controlled environment.

**The Process of Plant Tissue Culture**

This process involves the use of small pieces of a given plant tissue (plant of interest). Once the tissue is obtained, it is then cultured in the appropriate medium under sterile conditions so as to prevent various types of microorganisms from affecting the process

The following is a general procedure for plant tissue culture

Medium preparation

The appropriate mixture (such as the MS mixture) is mixed with distilled water and stirred while adding the appropriate amount of sugar and sugar mixture. Here, sodium hydroxide or hydrochloric acid is used to adjust the pH - Contents used here will depend on the plant to be cultured and the number of tissues to be cultured.

Agar is added to the mixture, heat and stirred to dissolve

After cooling, the warm medium is poured into polycarbonate tubes (to a depth of about 4 cm)

With lids sitting on the tubes, the tubes are placed in a pressure cooker and sterilized for 20 minutes

**Lab. Work 9. Use of vitamins and organics/ 1L**

Preparation of modify MS medium for induction of organogenesis in carrot parenchyma tissue culture

* [Myo-Inositol](https://en.wikipedia.org/wiki/Myo-Inositol) **100 mg/l**
* [Nicotinic Acid](https://en.wikipedia.org/wiki/Nicotinic_Acid) **0.5 mg/l**
* [Pyridoxine](https://en.wikipedia.org/wiki/Pyridoxine) · [HCl](https://en.wikipedia.org/wiki/HCl) **0.5 mg/l**
* [Thiamine](https://en.wikipedia.org/wiki/Thiamine) · [HCl](https://en.wikipedia.org/wiki/HCl) **1.0 mg/l**
* [Glycine](https://en.wikipedia.org/wiki/Glycine) **2 mg/l**
* Edamin™ or [tryptone](https://en.wikipedia.org/wiki/Tryptone) **1 g/l** (optional)
* [Indole Acetic Acid](https://en.wikipedia.org/wiki/Indole_Acetic_Acid) **1-30 mg/l**(optional)
* **0.04-10 mg/l**(optional)
* Thiamine. Thiamine (Vit. B

Myo-inositol. Myo-inositol (also sometimes

[Myo-Inositol](https://en.wikipedia.org/wiki/Myo-Inositol) described as meso-inositol or i-inositol) is the only one of the nine theoretical stereoisomers of inositol which has significant biological importance.

Medically it has been classed as a member of the Vitamin B complex and is required for the growth of

yeast and many mammalian cells in tissue culture.

Rats and mice require it for hair growth and can develop dermatitis when it is not in the diet. Myo-

inositol has been classed as a plant ‘vitamin’, but note that some authors think that it should be regarded as a supplementary carbohydrate, although it does not contribute to carbohydrate utilization as an energy source or as an osmoticum.

Thianeurine) in the form of thiamine pyrophosphate, is an essential co- factor in carbohydrate metabolism and is directly involved in the biosynthesis of some amino acids. It has been added to plant culture media more frequently than any other vitamin. Tissues of most plants seem to require it for growth, the need becoming more apparent with consecutive passages, but some cultured cells are self sufficient. The maize suspension cultures of Polikarpochkina et al. (1979) showed much less growth in passage 2, and died in the third passage when thiamine was omitted from the medium

**Pantothenic acid.** Pantothenic acid plays an important role in the growth of certain tissues. It favoured callus production by hawthorn stem fragments and stimulated tissue proliferation in willow and black henbane. However, pantothenic acid showed no effects with carrot, vine and Virginia creeper tissues which synthesize it in significant amounts (ca. 1 μg/ml).

Lab 9. Subcultivation of carrot calli tissue. Analysis first results of experiment N2

The influence of explant type as well as of the type of growth regulators and concentration on callus induction processes and somatic organogenesis of shoots was studied in vitro on four tomato genotypes of Russian breeding. Cytological study of callus tissue was conducted. It was established that tomato varieties possess a substantially greater ability to indirect shoot organogenesis compared with the F1 hybrid. The highest frequency of somatic organogenesis of shoots, as well as their number per explant, was observed for most of the genotypes studied during the cultivation of cotyledons on Murashige-Skoog culture medium containing 2 mg/l of zeatin in combination with 0.1 mg/l of 3-indoleacetic acid. An effective protocol of indirect somatic organogenesis of shoots from different explants of tomato varieties with a frequency of more than 80% was developed.

To explore the effects of different hormonal combinations on induction, proliferation and differentiation of Orostachyis fimbriatae callus culture. Aseptic seedling leaves were used as explants,the different concentrations of IAA,NAA, 6-BA and KT on induction proliferation of callus were optimized by orthogonal test to explore the optimum medium for differentiation of callus by tissue culture techniques. The best medium for induction was MS + IAA 1.0 mg/L + NAA 0.5 mg/L + KT 1.0 mg/L, and the best hormonal combination for proliferation was MS + IAA 0.5 mg/L + 6-BA 0.5 mg/I. + KT 1.0 mg/L. The best medium for differentiation was MS + IAA 0.1 mg/L + KT 2.0 mg/L, and 1/2MS + IAA 0.2 mg/L was the optimum medium for rooting culture. The system of regeneration of Orostachyis fimbriatae is establishd by tissue culture techniques in this study.

**Lab. Work 10.** Use of Four media (PESI solid, MS liquid, MS solid and ASP-C-I solid medium) to induce callus from excised tissues .

Four media (PESI solid, MS liquid, MS solid and ASP-C-I solid medium) were used to induce callus from excised tissues of the kelp Laminaria japonica. Only PESI solid medium and MS solid medium produced calli. Modified MS solid medium supplemented with mannitol (3%,W/V), yeast extract (0.1%, W/V), VB2 (0.5 mg/ml), VB12 (0.5 mg/ml), kinetin (0.108 Î¼g/ml) and NAA (1.860Î¼g/ml) showed much better effect on callus induction than non-modified MS solid medium. After 24 days of induction 75.5% of tissues in PESI solid medium showed callus formation. For modified MS solid medium, after three months of induction 67.3% of tissues dedifferentiated into calli. No callus could be found after five months of induction in either MS liquid or ASP-C-I solid medium. When calli were squashed and cultured in N-P enriched autoclaved seawater, MS liquid medium and ASP12-NTA liquid medium (both modified with kelp extract), differentiation of cells and regeneration of sporophytes were only observed in ASP12-NTA medium supplemented with kelp extract. Gametophyte-like filaments formed first, then eggs were released. It was suggested that sporophyte formation could be a process of parthenogenesis. Sterilization techniques in tissue culture of L. japonica were also tested in this study.

MS media supplemented with combination of high cytokinin to low auxin were prepared by the lab technician as follows: NAA:Kinetin ratios (2:0, 0.5:1, 1:0.5, and 0:2) and NAA:BAP ratios (2:0, 0.5:1, 1:0.5, and 0:2). Additional of two control plate with MS media that supplemented with same ratio of auxin and cytokinin were also prepared by the lab technician.

Plant tissue culture (PTC) is the techniques used to grow plant from any of the plant segment, tissues or cell in a contaminated free environment media such as MS media (Singh & Kumar, 2009). PTC techniques is important plant biotechnology aspect in which it facilitates the production of genetically modified plants and induced rapid multiplication of difficult-to-propagate plant species. Besides that, the ability to produce totipotent plant cell using PTC techniques has significant impact on crop improvement via genetic engineering.

There are two different processes, which involve explant differentiation and growth in PTC, which are organogenesis growth and adventititous roots or shoots growth directly from the explants. Organogenesis is the process of forming a specific organ from non-specific mass of meristem or parenchyma cell known as callus. Meanwhile, for formation of adventitious roots or shoots means the roots or shoots structure arise from the explants that have been excised This situation does not usually happen if the plant sample are cultured in a medium with the same ratio of auxin and cytokinin.

The presence of plant growth regulators (PGR) such as auxin, cytokinin, gibberellins, abscisic acid and ethylene has significant impact in the process of plant growth and differentiation. Gibberellins responsible for growth, seed germination and promote fruits growth. As for ethylene helps in controlling of fruit ripening as well as controlling cell division and cell elongation. Meanwhile, abscisic acid act on seed maturation and give the ability to the seeds to response during stress in undergoing dormancy period (Davies, 2010). However, in this experiment only, auxin and cytokinin are involve, in which auxin hormones responsible for growth of roots, phototropism and gravitropism, while, cytokinin helps in inducing the growth of shoots and regulates auxin action. In this experiment, NAA will be used as synthetic auxin and kinetin and BAP as synthetic cytokinin.

This experiment was conducted in order to determine the effect of PGR, auxin and cytokinin on the organogenesis in carrots and petunia leaves.

 The same methods were implied to the carrot. However, the carrot sample was cut into 27 pieces about 0.5cm thick on the surface of sterile ceramic tile. After soaking the carrot samples in sodium hypochlorite and rinsed for three times, 3 carrot pieces was transferred into petri dish with different ratios of NAA:BAP and NAA:Kinetin. The result recorded based on table 1 and table 2.

Culture condition

The transferred petunia leaves and carrot pieces in the 18 petri dishes were incubated for 28days in 25ÂÂ±2ÂÂ°C temperature, and photoperiod of 16hours in light & 8hours in dark as provided in plant culturing room in Monash University Sunway Campus. In addition, for every 4 days, the tissue cultured was checked to whether there are presents of contamination and to transfer the samples into new MS plates.