# MODULE

# TISSUE CULTURE TECHNOLOGY INTRODUCTION

**By**

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# CHAPTER I
# PRELIMINARY

## 1.1 Background

The increasing exports of agricultural commodities have a growing need for plant seeds. Plant seeds of a higher variety productions are very few, but the need is high. One factors contributing to the success of future agricultural development are the provision of quality plant seeds. The technology of tissue culture is one of the most frequently talked about technology which have proven successful. Plants can be replicated at any time due to the high propagation factor through tissue culture. The culture of tissue can develop plant seeds immediately from the superior varieties which are very small in number. Plant growth can benefit more by culturing tissue, as the plant's characteristics are identical to the parent (uniform) and seeds can in no time be produced in large quantities and without disease. Tissue culture is a means of vegetative propagation of sterile artificial media in a controlled environment by culturing cells, organs or plant parts. For almost every type of plant of high economic value, technology of tissue culture can be implemented. The section of the plant to be reproduced must have totipotential properties, namely the cell's ability to regenerate again in a whole plant.

On this basis the Tissue Culture Technology Introduction Module has been compiled.

## 1.2 Brief Description

This course will focus on defining tissue culture, the fundamental theory of tissue culture, the strengths and disadvantages of tissue culture, tissue culture lab, tissue culture stages, the limitations and the problem of tissue culture, tissue culture media, basic tissue culture media and growth regulators (ZPT).

## 1.3 Benefits for Participants in the Teaching Material

This course is intended to serve as a guide for people to explain the technology for plant tissue culture.

### 1.4 Objectives for Learning

1. Basic skills

   Ability to explain technology of Plant Tissue Culture correctly.

2. Indicators of Success

   1) Defining the culture of tissue;
   2) Explanation of Basic Tissue Culture Theory;
   3) Explaining tissue culture's pros and cons;
   4) Describing the Laboratory of Tissue Culture;
   5) Describing the tissue culture stages;
   6) Describing tissue culture restrictions and problems;
   7) Describing Culture Media of Tissue;
   8) Describing the Tissue Culture Media Basic Solution;
   9) Description of Growth Regulatory Substances.

### 1.5 Material

1) Tissue Culture Definition;
2) Tissue Culture Basic Theory;
3) Tissue Culture Benefits and Disadvantages;
4) Laboratory of Tissue Culture;
5) Tissue culture stages;
6) Tissue culture restrictions and problems;
7) Culture media of tissue;
8) Solution of the tissue culture media base;
9) Growth Regulatory Substances.

### 1.6 Instructions for Study

You can take the following steps to ensure that the learning process runs smoothly and the learning goals are achieved correctly:

1. Read the learning goals carefully and understand them.
2. Study each section in order.
3. Fulfilling the training.
4. Study material from other sources of knowledge and insight for enrichment.

# CHAPTER II
# TISSUE CULTURE DEFINITION

The growing need for seeds is one of the effects of the increased export of agricultural goods. There are very limited seeds from a superior variety produced while there are very high plant seeds needed. One of the factors determining future success in agricultural development is that good quality seeds are supplied. The tissue culture techniques are one of the most popular techniques of hope that have proved to be successful.

By means of tissue culture, the high multiplication factor allows the reproduction of plants at any time. The tissue culture can immediately develop seeds of superior varieties, which are very small in number. If successful, propagation of plants through tissue culture can be more profitable because they have the same characteristics as their parents (uniform), and the seed may be produced in large numbers without diseases in a short amount of time.

Tissue culture is a technology for plant propagation that isolates certain parts of the plant (organ, tissue, cells, anther and others) from sterile media with macro- and micro-nutrients and differentiates them again into perfect plant.

The underlying theory is that cells possess totipotential properties, i.e., that all living plant cells are fitted with full genetic information, and physiological instruments for growing and growing into a whole plant if their conditions match. This theory believes that every part of the plant can reproduce because all parts of the plant consists of living tissues. Plants can culture tissues if they have totipotential properties, namely cells' ability to regenerate into whole plants.

Tissue culture is known as *gewebe kultur* when it is translated into German, tissue culture in English, *weefsel cultureuur* in Dutch. Tissue culture is the isolation method in sterile culture bottles and aseptic conditions of parts of plants, including protoplasm, cells, tissues or organ, which can multiply and regenerate into sterile whole plants.

The attempt at a new plant is known as a cell culture or tissue culture from one single cell or tissue.

"Tissue culture" is known in global language, according to Suryowinoto (1991). Culture is cultivation and tissue as a group of the same form and function cells. Tissue culture therefore means cultivating a plant tissue into a small, property-

like plant. Tissue culture methods have been designed to help plants reproduce, especially for plants difficult for sexual reproduction.

Tissue culture is a type of vegetation that has the same basis as cutting. Plant parts cultured (explants), roots, springs, flowers, meristem, pollen, can be taken from plants.

There are three main principles for tissue culture according to Thorpe (1981):

- Are plant components isolated from entire plants (organs, roots, leaves etc.).
- Appropriate conditions of maintenance of plant parts in the appropriate environment.
- Maintenance in aseptic conditions.

# CHAPTER III
# CULTURE BASIC THEORY

a. Cells of a multicellular organism are actually the same as zygote cells everywhere, because they came from one cell (*omne cellula ex cellula*).
b. Theory of cell totipotency. The Swann and Schleiden cell theory (1898) states that cells possess potential characteristics, namely that each living plant cell has full genetic information, as well as physiological instruments for the production and development of a complete plant when the conditions are appropriate. This theory believes that every part of the plant can be reproductive because all parts of the plant contains living tissues.

The totipotency theory, which says that every plant cell can become a new plant, is the basis for tissue culture to be implemented. The cell and tissue component of the plant can be planted in a sterile, controlled environment in tissue cultures. As with the theory about totipotency, part of the plant culture in the media can actually grow and become new plants if the circumstances are right.

Tissue culture has several cultures, including:

a) Seed culture, culture of which the seed or seedling material uses.
b) Culture of organ (organ culture) using organic planters, such as, root tips, shootings of an axilla, petioles, blades of leaves, flower, young fruit, flowers, stalks, roots, etc.
c) Callus culture, is a tissue culture (a cell group), usually as parenchymal tissue as an explant.
d) A culture that uses a shaker with continuous shaking of liquid media and uses explants, usually in the form of calluses or tissues from meristems, as explants, cells or cell aggregates.
e) Culture of Protoplasm. The used expansion is the cell removed by means of enzymes from the cell's wall. Protoplasts are put on solid media and allow their cell walls to be separated and re-formed. Protoplastic culture is usually used for somatic or cell fusion (2 protoplast fusion, both intraspecific and interspecific).
f) A haploid culture is a culture which comes in the plant's reproductive parts: *Keralasari* / anther (former culture / microspore culture), *tepungsari* / polen manure, ovules (ovule culture) for the production of haploid plants.

# CHAPTER IV
# TISSUE CULTURE BENEFITS AND LOSSES

Culture of tissue has several advantages: 1) identical characteristics to the parent; 2) short-term reproduction; 3) the plant is free from fungi and bacteria. No need for a large nursery area; 4) not affected by season. While the disadvantages are: 1) tissue seedlings are highly susceptible to disease and air; 2) the tissue culture method is deemed expensive and difficult for some people; 3) a high initial capital investment is required for buildings, equipment and supplies (special laboratories); 4) the necessary reliable human resources to work on the propagation of tissue culture to achieve satisfactory results; 5) the product at the root of the tissue culture is less sturdy.

There are several differences between the natural propagation of plants and tissue culture. Nutrients are natural in soil propagation, plants can produce their own foods (autotrophs), and plant sources must be old enough, soil photosynthesis, rainy seasons and uncontrolled dry season. In tissue culture, the media consists of chemical nutrients, plants are not making their own food, and the plant sources are little affected by photosynthesis and the season.

The culture of tissues is a technique that needs a great deal of equipment. Instruments for tissue culture planting should be sterile. Autoclave can be used with metal and glass tools. Planting instruments such as: pins and cleaves can be sterilised either by burning or heat in a special scalpel bacticiner, the handle can be sterilised by heating, but when heated at high temperatures, the clover can become dull. Therefore sterilisation should be used by immersing the blades in the solution of alcohol or chlorine. Shear, scissors, scalpel handle, filter paper, petridishes, vacuum bottles, needles, pipettes; tooling for light culture that must be sterilised before planting. Factors for tissues culture: this tool is in the seed room, a room that should always be sterile, and is Laminar Air Flow Cabinet (LAFC). This instrument is used as a treatment stage for planting.

Entkas, the ancient form of Laminar Air Flow Cabinet (LAFC), has therefore the same function as (LAFC). Shaker is a shovel tool that can be adjusted to our wishes to adjust their rotation. This corner may be used for the culture of orchid explant appeals or for protocorming or often called plb from appeals of different plant tissues (protocorm like bodies). Autoclave, is a plant tissue culture medium and tools

sterilisation. These types of tools vary from analytical to scales, but it is important to use the scale to weigh up very small unites. This tool acts as an instrument for weighing tissue culture chemicals. Stirrer, this tools is used for stirring with heater. This tool works as both a stove and a shaker by using electricity. Erlenmeyer flask, this tool is used in culture of plant tissues to pot distilled water, media storage and planting of explants. Measuring cylinder is used to measure water and chemicals to be used to measure distilled water. Beaker glass used to pour or prepare chemicals in the tissue medium and distilled water. Petridish is a kind of glass of cup that is necessary for culturing the tissue. Tweezers and scalpels are used for holding, collecting or planting explants in explant slices. Spirits lamp, in a laminar air flow cabinet or at Entkas used for a sterilising dissecting kit (scalpel and tweezers) for seeding or subculture purposes. Protoplast insulation and chloroplast ionisation tubes are used when processing.

# CHAPTER V
# CULTURE LABORATORY

The laboratory facilities for tissue culture are divided into several sections, which work differently and have differing needs. Special design must be carried out in a tissue culture laboratory. Because there is a sterile or microbial free part or room. The tissue culture spaces are grouped in accordance with the activities as following:

A. Non-sterile room

1) Room for guests

In a tissue-cultural laboratory, a living room should also be installed, because usually guests who want to see facilities and the laboratory's environment and guests who are keen to purchase tissue-culture results always visit a tissue-cultural laboratory.

2) Room of management

The management room shall carry out every correspondence concerning the purchase of laboratory equipment, the purchase of tissue culture media, the sale of tissue cultivation seedlings, and research transactions or collaborative agreements.

3) Room for staff

Tissue culture laboratories require a large number of research staff, the aim is to divide the work by specialising in it. This employee room can also be used for discussions between employees when the meeting is held.

4) Toilet

To prevent contamination by microbes, the tissue culture room should be in a clean atmosphere. When people enter the seeding or incubatory room, they have to have clean, sweaty and dust-free, clean body and clean clothing.

5) Room for change

In order to avoid microbial contamination, employees must wear clean clothes in the tissue culture laboratory and wash them in a new sense. Thus, a changing room must be provided in the tissue culture room.

6) Chemical and glassware storage space

There are numerous types of chemicals that are media for tissue culture. Special arrangements are therefore needed for storage so that it is easy to find. Irregular storage will slow the work down, for example, it takes a long time to find just one media component. Cool rooms should store expensive chemicals like growth

hormones and protoplast insulation enzymes. In a separate coupling it is necessary to store glass tools such as aluminium bags, cups and other glassware.

7) Room for preparation

This room comes with the equipment and the lab equipment to be washed. Existing equipment contains freshly washed utensils' plastic baskets or anoher baskets that is not easy to rust.

8) Room for weighing and sterilisation

A number of web-cultured media are sold at relatively costly prices in packages. Laboratory personnel therefore prefer to compile the medium as they need. Therefore, all chemical components require latency. Taking macro and micro chemicals into accounts, for example.

9) Green House

A greenhouse is a building with a glass roof and top walls. A greenhouse has to be a place where seed pots can be planted.

B. Sterile Room

1) Plantlet Space

This room uses a cooling equipment (AC), so the room temperature can reach 25 degrees C, making this ideal for plantlet growth. There are hundreds of bottles that contain plantlets. Therefore, the aluminium rails must be furnished in this room, so that the bottles can be placed orderly and neatly.

2) Room of the incubator

The growth of plant explants must be monitored daily in tissue media. A special room more sterile than the seedling, namely the incubator room, is needed for this monitoring. It must be about 25 degrees C for the Incubator Room and equipped with fluorescent lamps since the explants grown in the incubator room require an adjustable temperature and light to suit the type of explant.

3) Room of Enkas and Shaker

Callus would produce the explants just planted and incubated in the incubator room. Should the callus be old enough, the explant or callus can be expanded by means of a liquid medium (media without the use of a compactor or agar) and shaken on a shaker. The result is a protocorm, or it is called plb in foreign terms (protocorm like bodies). The protocorm shape is solid and green round. When the protocorm is so, it will be transferred to solid media and will be cultured in seedlings. In the same room

are also the Enkas with a shaker, as the Laminar Air Flow Cabinet is used for sowing explants.

4) Seeding room

The seeding room is normally made of a not too large size, 2 to 3 m squares. The aim is not to need a long time and to not experience difficulties in the implementation of the room sterilisation. The sowing room walls are fitted with porcelain, which facilitates sterilisation. A 96 per cent sprayer is used to sterilise the room. Sterilise the floor with a mop of 96% alcohol. It is essential to do this sterilisation before use of the seeding room. If the prospective sower is about to enter the room, the ultra-violet light first must be disabled and the sower is permitted to enter the room. If you left the door open and close the door again, we suggest that you turn off the fluorescent light. Therefore, seeding room can be guaranteed as such a sterile room.

The method of tissue culture can be seen from media types and also explant origin and source. Tissue culture can be split into 2 methods with respect to medium type:

a. Solid Method

Media that is too dense will make it difficult for roots to grow, because roots are difficult to penetrate into the media. Meanwhile, media that is too soft will cause failure in work. Failures can involve sinking the planted explants. Immersed explants will not be able to grow into callus, because the location of the callus area, namely the incision (wound tissue), is covered by the medium. The solid method can be used for the cloning method, to grow protoplasts after isolation, to grow plantlets from muscular protocytes after removal from cell suspension, and to grow plantlets from fused (combined) protoplasts.

b. Method of liquidity

The use of the liquid method is less practical than the solid method because it is very hard to grow callus directly from explant, so that the success is very small. The use of liquid media for cell suspension is therefore emphasized, namely the culture of PLB (Protocorm Like Body). It can then be transported to a suitable solid medium into plantlet from this protocorm. The production of fluid media is much quicker than solid media because the agar does not have to be heated. Liquid media do not also need a compacting agent to remain a nutrient solution.

While the method of tissue culture is considered from the type of material used, the methods of tissue culture today include:

1) Culture of meristem.
2) Culture of anther.
3) Endosperm culture
4) Cell culture of suspension
5) Culture of protoplasts
6) Culture of embryos
7) Culture of spore
8) And others.

# CHAPTER VI
# TISSUE CULTURE STAGE

The application of the technique of tissue culture requires different preconditions to support the life of the cultured tissue. Sterile containers and growing media are extremely important. The medium is where the tissue grows and absorbs nutrients that support tissue life. The medium of growth offers the different materials that the tissue needs to live and replicate. Culturing media are classified as two: solid media and liquid media. Generally, solid media is a gel solid like agar. The nutrients are agar-mixed. Nutrients dissolved in water are the liquid media. Liquid media, depending on requirements, may be calm or in constant movement.

The executioner must work carefully and seriously, because every step of the work needs its own knowledge base to be handled independently. The following steps are:

a. Culture Start-Up

At this stage, the primary objective of in vitro propagation is to develop a culture from explants free of microorganisms and to initiate new growth (Wetherell, 1976). It promotes a culture of aseptic or accents. It also hoped that the cultured explants will start a new growth in this phase, so that selecting the plant parts which grow the strongest will be able to multiply in the following stages (Wetherell, 1976).

The problem often faced in this culture stage is browning or blacking of explants. It is caused by phenolic compounds caused by mechanical stress caused by injury during the parent plant's explant insulation process. The phenolic compounds are toxic, prevent growth, or even exterminate explants.

b. Sterilisation

Sterilisation is the microorganism release process. Sterilisation is aimed at creating conditions for sterile culture. Phases of sterilisation:

1. Sterilise in an autoclave at 121 degrees C, glass and stainless utensils
2. Plant material sterilisation (explants)

One of the steps to be taken before explants planted on planting medium is sterilisation. In anticipation of contamination, the sterilisation process is an obligatory procedure.

Tissue culture takes a very important step in creating a culture without contaminants, as the plants on the ground contain dust, pollution and various

contaminants on the surface. Fungi, bacteria, insects and their eggs, mites and spores can become living contaminants. When the source is not removed, fungi and bacteria are cultured on the media that contain sugars, vitamins and minerals. If the explant is contaminated, bacteria or fungi will die by toxic compounds.

Contaminants from plant tissues, especially bacteria, are also found in some plants. These inner contaminants are very difficult to treat, since the problem is not solved by surface sterilisation. Systemic antibiotics or bactericides should be used for plants than contain internal contaminants.

A different level of contamination of the surface depends on each plant material:

a. Plant Types
b. The section used for the plant
c. Surface morphology (e.g. hairy or not)
d. The environment for growth (green house or field)
e. Season taking (rainy or dry season)
f. Age of the plant (seedling or adult plant)
g. Condition of plant (healthy or sick)

This requires a standard sterilisation procedure for all plants to be identified. Standard procedures for a type of plant originating from another location are also difficult to determine. Preliminary tests must determine the sterilisation process for each plant.

The important things to be taken into consideration when sterilising plant material are: that both plant cells and contaminants are living. Contaminants must be removed without killing the plant cells.

Various kinds of materials for disinfectant sterilising plants:

| Material | Concentration | Time to Soak |
|---|---|---|
| Calcium hypochlorite | 1 - 10% | 5 - 30 minutes |
| Sodium hypochlorite | 1 - 2% | 7 - 15 minutes |
| Hydrogen peroxide | 3 - 10% | 5 - 15 minutes |
| Silver nitrate | 1% | 5 - 30 minutes |

| Material | Concentration | Time to Soak |
|---|---|---|
| Mercury chlorite ($HgCl_2$) | 0.1 - 0.2% | 10 - 20 minutes |
| Povidone iodine | 2.5 - 10% | 5 - 10 minutes |
| Fungicide | 2 g / l | 20-30 minutes |
| Antibiotics | 50 - 100 mg / l | ½ - 1 hour |
| Alcohol | 70% | 1 - 10 minutes |

Generally, the plant tissue is toxic to these sterilising materials. It is required to retract all the residual active ingredients that are still attached to the floreting surface after soaking the explants in a sterilisation agent solution many times.

Two or more sterilising agents are sometimes used during sterilisation. Soak first in alcohol, then sodium hypochlorite, and then rinse with sterile water, for example. Soak may also be started with a fungicide or antibiotic solution, followed by $HgCl_2$ and rinsed with sterile water. There must be preliminary trials to determine which procedure is effective.

c. Making media for culture

In most of the tissue culture, media is a determining factor. Depends on the type of plant to be propagated, the composition of media used. Usually used media are mineral salts, vitamins and hormones. More ingredients like agar, sugar and others also required. The added growth regulators (hormones) vary according to the purpose of the tissue culture, both in the type and in volume. Test tubes or glass bottles are placed with a finished medium. Media should also be heated with an autoclave to sterilise the used medium.

Stages of media production for culture:

- Material preparation
- pH measurement (5.7 – 5.8)
- Formulation
- Give gelatine and medium heating
- Media for sterilisation

d. Planting of the plantation

Plants are planted in a laminar air flow using sterilised instruments through subculture or transmission on the plantation medium.

Good explant requirements:

- A healthy, fertile parent
- A parent of known species
- Growing place in a good environment
- Optimum shooting height is around 5 cm
- Immediate shooting is processed as quickly as possible

Stages of subculture:

- Induction of shooting

The seed plants must be clear and free from pests and diseases, with clear types, species and varieties. In an in-vitro cultured or greenhouse the broodstock of the explant must be especially prepared for healthy and contaminant free growth of explants.

- Repeat of shootings / Multiplication

The activity to multiply future plants through the planting of explants in the media is multiplication. This is done to prevent contamination which leads to explant growth failure. The explant tubes are placed on racks and placed in a clean room at room temperature.

- Rooting Roots

Is the phase of root growth of explants which indicates that the culture process is beginning to run properly. Rooting is used with MS + NAA media. Observations are made every day in order to monitor root growth and development and any bacteria or fungal contamination. The process of rooting takes usually 1 month. Contaminated explants show symptoms like fungus-caused white or blue or red (caused by bacteria).

- Incubation

Explants in a controlled room / environment are placed in the incubation phase (to test their success). Appropriate temperature is 24-28 degrees C for culture growth. An air conditioner (AC) is installed in the room to adjust the incubation room to the desired temperature.

- Acclimation

The acclimation of plants from the internal to the exterior environment is the process of adaptation / transfer of plants (from a controlled environment to an uncontrolled environment). The move is done attentively and step-by-step with the provision of a hood. The hood is used for protecting seedlings against external air and plagues because tissue seedlings are highly susceptible to external plagues and diseases.

Once the seedlings are adaptable to their new environment, they will gradually remove the caps and treat the seedlings in the same way as generative seedlings. Typically, 2 weeks with plastic cover to 4 weeks without a plastic cover are acclimatised. At the time the seedlings are 20-25 cm high. In addition, the seeds are ready for polybag culture. The plants must then be grown in the nursery until they are 50-60 cm high and then taken to the farm.

Factors that affect regeneration

1) In vitro regeneration forms for Culture: axillary shoots, adventitious shoots, somatic embryos, formation of protocorm like body, etc.
2) Explant

It is one part of a plant, which is used as a base for the propagation of plants. Significant explant factors are genotype/variety, explant age, branch location and sex (male/female). Plant parts that may be used as explants include young shoots, young stem, young leaves, cotyledon, endosperm, young ovaries, anther, embryo, etc.

3) The Media of Growth

Composition of inorganic salts, growth regulators and the physical form of the media is included in the culture medium. In tissue culture there are 13 media compositions, for example: Murashige and Skoog (MS), Woody Plant Medium (WPM), Knop, Knudson-C, Anderson, etc. MS is the medium used frequently.

4) Regulators for Plant Growth

Concentrations, sequence of use and the induction period in certain cultures must be taken into account in the use of ZPT. Auxins like Indole Acetic Acid (IAA), Napthalene Acetic Acid (NAA), and 2,4-D, CPA are commonly used. Groups of cytokine, like kinetin, 2I-P, Zeatin, Thidiazuron, PBA, and Benziladenine (BA). Like

the GA3 from gibberellins. Growth inhibitor groups such as Ancymidol, Paclobutrazol, TIBA, and CCC.

5) Environmental growth

Temperature, radiation length, ray intensity, light quality and the size of the crop container form the growing environment which may affect plant regeneration.

# CHAPTER VII
# TISSUE CULTURE CONSTRAINTS AND PROBLEMS

Up to now, only some private entrepreneurs and government agencies concerned about agricultural technology research and development, and only a few universities, have been trying to implement the technique of tissue culture, since implementing tissue culture techniques requires specific skills and must have a background knowledge. It is clear, therefore, that ordinary farmers will find it very difficult to accept this. In addition, a special laboratory is absolutely essential to the implementation of tissue culture techniques, but it is still needs adequate equipment, although it can be done simply (in a small space). Another option is for farmers to be unwilling to work aseptically.

The work on tissue culture comprises: Media preparation, planting material isolation (explantation), explant sterilisation, inoculation of explants, acclimatization and effort to transfers to the field tissue culture plants. The executioner must work carefully and seriously, because every step of the work needs its own knowledge base to be handled independently. Because all work needs to be performed with care and patience. The costs of this in vitro reproduction are also high, unless we formulate the medium ourselves. It is obviously very expensive when we have to buy a ready-made medium (on packaging) as the finished medium still has to be imported abroad. In addition, we need to acquire isolation and protoplastic fusion suggestions, which will of course increase costs. Enzymes used in tissue culture, are still purchased abroad, such as Japan.

In addition to all the above-mentioned obstacles, we need to acknowledge the importance of tissue culture techniques, particularly for biotechnology development, for the science world.

There are many problems in tissue culture that appear as a nuisance and even cause the aims of culturally unsuccessful activities. Cultural disturbances can generally be caused by the planted material, by the cultural environment, or by people. There are culture problems which can be predicted in advance and some are hard to predict. The way it can be overcome cannot be preventative for the unpredictable, but it can be resolved when the case appears.

The problems in the culture of tissues, i.e.:

1) Contamination

In tissue culture activities, contamination is very common. The appearance of this disease is something very reasonable because of its enriched use, when understood in a basic way. This diversity can be seen from the contamination type. The phenomenon of contamination is quite different (bacteria, fungi, viruses, etc.).

Contamination prevention efforts:

- Use the various tools required in culture of tissue.
- Ensure a good and correct process of media sterilisation.
- Do planting when it is convenient and spare time.

2) Browning

Browning is a feature of the brunette or black appearance that often prevents explants from growing. In fact, the browning event is often a natural occurrence. Browning is usually a sign of explant physiology deterioration and often leads to explant death.

3) Vitrifying / Vitrification

A problem in culture: Growth appearance and abnormal growth are the characteristics of vitrification. Plants are short or stunted. The production is short. The growth of the stem tends to grow in diameter. The entire plant is very tumultuous. Palisade tissue is not present in the leaves.

4) Genetic diversity

Genetic variation is an obstacle when tissue culture is used in large quantities for uniform plant spread and not for plant breeding. The in vitro culture may cause genetic variation, which is the result of the uncontrolled repeated subculture and inappropriate techniques: A high multiplier rate, variations occurring. In callus cultures and cell suspension cultures, the most common genetic variations are due to the presence of chromosomal instability through culture, media or hormone techniques. It is certainly not easy to overcome the issue of genetic variation, because it must be notice to cultural aspects.

5) Development and growth

The main problem with the process of growth is if the planting explants are stagnant and do not die but do not grow from planting up to a certain amount of time. To avoid this, planting material which is not juvenile or thematic can be

prevented. Because explant growth begins with the active dividing young cells, or with the young old cells again. The media can also be the cause of growth stagnation, because a cell may or may not be encouraged to conduct its division or expansion process under the media conditions.

The stage of the formation of a callus must be continued with the promotion of embryo somatic induction of callus cells during the embryogenesis of tissue culture. There may be endogenous or exogenous incidents of somatic embryos.

6) Pre-treatment

Not only do the planting of explants and bottle growth and development cause the in vitro problem, but the demands of pre-treatment activities can be greatly influenced. If no pre-service activities are carried out, problems will arise. Pre-treatment for some purposes is usually carried out and generally obstacles must be removed. The barriers may be chemical, physical, biological. The introduction of active compounds, potential interference, reaction processes and alternative management must start from the barriers to the handling of chemicals.

7) Micro Environment

It is also a frequent problem that the environmental problem of an incubator cannot be ignored. The temperature of the incubator room determines greatly the growth of explants, while the growth and development of the explants can be affected by too low or high temperatures. However, the needs between plants and others differ, because incubator area of tissue culture laboratory space cannot generally be varied from one room to another. However, this solution is difficult to resolve. Thus the optimisation of growth between one culture and another cannot be expected to be the same.

# CHAPTER VIII
# PLANT TISSUE CULTURE MEDIA

Media is a major factor in propagation by tissue culture. The success of plant propagation and reproduction using tissue culture methods in general really depends on the type of media. The growth media in tissue culture has a very big influence on the growth and development of the explants and the seeds it produces. Therefore, various tissue culture media have been found so that there are quite a lot of them. The names of the growth media for these explants usually match the names of the discoverers. The growth medium for the explants contains almost the same qualitative chemical components, only slightly different in the amount of content for each compound. The basic media that is often used in Anthurium tissue culture itself is MS media and its modifications (Pierik et al.,1974; Pierik and Steegmans, 1976;Kunisaki, 1980; Kuenhle et al., 1992; Chen et al; Hamidah et al., 1997; Teng, 1997;2 ; Rachmawati, 2005), media Nitsch and its modification (Geir, 1986, 1987, 1988).

In general, the main composition of tissue culture growing media consists of hormones (growth regulators) and a number of elements that are usually found in the soil which are grouped into macro elements and micro elements. We will get better results if, into the media, add vitamins, amino acids, and hormones, media compactor (agar), glucose in the form of sugar or sucrose, distilled water, and additional organic matter (Gunawan, 1992).

The mineral nutrient requirements for in vitro cultured plants are basically the same as the nutrient requirements for plants grown in the soil. The nutrient elements needed by plants in the field are basic necessities that must be available in tissue culture media. Among other things, macro nutrients and micro nutrients. These nutrients are supplied in the form of mineral salts. The composition of the media and its development is based on the approach of each researcher (Gunawan, 1992).

Macronutrients are nutrients that are needed by plants in large quantities. These macro nutrients include, Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Sulfur (S), Magnesium (Mg), and Iron (Fe).

Micronutrients are nutrients needed in small amounts. These micro nutrients are components of plant cells which are important in metabolic processes and other physiological processes (Gunawan, 1992).

Among these micro nutrient elements are :

1. Chlorine (Cl), is given in the form of KI.
2. Manganese (Mn), is given in the form of MnSO4.4H2O.
3. Copper (Cu), is given in the form of CuSO4.5H2O.
4. Cobalt (CO), is given in the form of CoCl2.6H2O.
5. Molybdenum (Mo), is given in the form of NaMoO4.2H2O.
6. Zinc (Zn), is given in the form of ZnSO4.4H2O.
7. Boron (B), is given in the form of H3BO3.

The most commonly used vitamins in plant tissue culture media are thiamine (vitamin B1), nicotinic acid (niacin), pyridoxine (vitamin B6). Thiamine is an essential vitamin in plant tissue culture because thiamine affects cell growth and development. Vitamin C, such as citric acid and ascorbic acid, is sometimes used as an antioxidant to prevent or reduce browning of explants.

Sugar is used as a source of energy in culture media, because generally the parts of the cultured plant or explants are not autotrophic and have a low photosynthetic rate. Therefore, tissue culture plants require sufficient carbohydrates as an energy source. According to Gautheret in Gunawan (1992), sucrose is the best source of energy producing carbohydrates exceeding glucose, maltose, raffinose. However, if there is no sucrose, the carbohydrate source can be replaced with sugar. Granulated sugar fulfills the requirements to support culture growth. Apart from being a source of energy, sugar also functions as an osmotic pressure medium.

Cultured explants should always be in contact with or exposed to the medium. The most widely used media compactor is agar. Agar is a polysaccharide mixture obtained from several algae species. In the elemental analysis, data was obtained that the agar contains a few elements of Ca, Mg, K, and Na (Debergh, 1982 in Gunawan, 1992). The advantage of using agar is :

1. The agar freezes at 450C and thaws at 1000C so that within the culture temperature range the agar will be in a stable freezing state.
2. Not digested by plant enzymes.
3. Does not react with compounds that make up the media.

In general, tissue culture media can be divided into basic media and treatment media. A basic media recipe is a recipe for a combination of substances that contain essential nutrients (macro and micro), a source of energy and vitamins. In the tissue culture technique, there are dozens of basic media. The name of the basic media recipe is generally taken from the name of the discoverer or researcher who used it for the first time in a special culture and obtained an important result.

Some of the basic media that are widely used :

1. Murashige and Skoog's (1962) basic media which can be used for almost all types of culture, especially for herbaceous plants.
2. Basic mediaB5 for cell culture of soybeans, alfafa, and other legumes.
3. The basic medium of White (1934) is very suitable for root culture of tomato plants.
4. Vacin and Went basic media which are commonly used for orchid tissue culture.
5. The basic media of Nitsch and Nitsch are commonly used in pollen and cell culture.
6. Schenk and Hildebrandt (1972) basic media or SH media suitable for tissue culture of monocot plants.
7. Special medium for woody plants or Woody Plant Medium (WPM).
8. N6 media for cereals, especially rice.

# CHAPTER IX
# TISSUE CULTURE MEDIA BASIC SOLUTION

Stock solution is a solution whose concentration be elevated from the concentration of the media. Usually stated in multiples of media concentration, for example 10x, 20x, 100x, even 1000x media concentration.

The purposes of making stock solutions is to avoid repeated weighing each time when you make the media. In addition, sometimes a scale for weighing materials in very small quantities is not available in the laboratory.

Stock solutions should be stored in a low temperature and dark place such as refrigerator. The components of the stock solution can be divided into groups :

1) **Macro elements,** consisting of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S).
2) **Micro elements**, consisting of boron (B), cobalt (Co), copper (Cu), iodine (I), iron (Fe), manganese (Mn), molybdenum (Mo) and zinc (Zn).
3) **Vitamin** (vitamin B1) and myo-inositol, niacin and pyridoxine (B6) are added to some formulas of media.

Table 1. Concentration of Chemicals in MS Media (1962) and the Nutrient Elements contained

| No | Chemicals | Concentration (mg/l) | Elements Contained |
|---|---|---|---|
| **Macro Elements** | | | |
| 1 | $KNO_3$ | 1900 | K, N |
| 2 | $NH_4NO_3$ | 1650 | N |
| 3 | $CaCl_2.2H_2O$ | 440 | Ca |
| 4 | $MgSO_4.7H_2O$ | 370 | Mg, S |
| 5 | $KH_2PO_4$ | 170 | K, P |
| **Micro Elements** | | | |
| 6 | $MnSO_4.4H_2O$ | 16,9 | Mn, S |
| 7 | $ZnSO_4.7H_2O$ | 8,6 | Zn, S |
| 8 | $H_3BO_3$ | 6,2 | B |
| 9 | KI | 0,83 | K, I |
| 10 | $Na_2MoO_4.7H_2O$ | 0,250 | Mo |
| 11 | $CoCl_2.6H_2O$ | 0,025 | Co |
| 12 | $CuSO_4.5H_2O$ | 0,025 | Cu, S |
| **Iron Micro Elements(Fe)** | | | |
| 13 | $FeSO_4.7H_2O$ | 27,8 | Fe |
| 14 | $Na_2EDTA$ | 37,3 | |

The preparation of stock solutions is based on groupings, namely macro stock, micro stock , iron (Fe) stock, vitamin stock, hormone stock, especially if the stock solution is not stored for too long (soon to be used up). Hormone stocks can be stored for 2 – 4 weeks, while nutrients can be stored for 4 – 8 weeks. With the stock solution will make the media easier, just dilute it.

Table 2. Requirement for MS Media Stock Solution

| Stock code | Stock name | The material being weighhed (mg) | Volume of stock containers (ml) | Stock taken for 1 liter of media (ml) |
|---|---|---|---|---|
| **Macro Stock Solution** | | **10 x** | | |
| A | $KNO_3$ | 19000 | 1000 | 100 |
| | $NH_4NO_3$ | 16500 | | |
| | $CaCl_2.2H_2O$ | 4400 | | |
| | $MgSO_4.7H_2O$ | 3700 | | |
| | $KH_2PO_4$ | 1700 | | |
| **Micro Stock Solution 1** | | **100 x** | | |
| B | $H_3BO_3$ | 62 | 1000 | 10 |
| | $Na_2MoO_4.7H_2O$ | 2,5 | | |
| | $CoCl_2.6H_2O$ | 0,25 | | |
| | KI | 8,3 | | |
| | $MnSO_4$ | 169 | | |
| | $ZnSO_4.7H_2O$ | 86 | | |
| | $CuSO_4.5H_2O$ | 0,25 | | |
| **Micro Stock Solution 2** | | **100 x** | | |
| C | $FeSO_4.7H_2O$ | 278 | 1000 | 10 |
| | $Na_2EDTA$ | 373 | | |
| **Vitamin Stock** | | **100 x** | | |
| D | Glisin | 20 | 1000 | 10 |
| | Asam nikotin | 5 | | |
| | Piridoksin HCl | 5 | | |
| | Thiamin HCl | 1 | | |
| **Hormone Stock (according to the needs)** | | | | |
| E | BAP | 100 | 100 | 1 |
| | NAA | 100 | 100 | 1 |

In making stock solutions, you must notice to the storabiliy of the solution. Solutions that have precipitation can no longer be used. Stock solution precipitation generally occurs when the solution concentration is too high. Therefore, solution precipitation can be avoided by making a solution that is not too concentrated or does not use a mixed solution, namely by making one stock solution for only one type of material (especially for macro nutrients). The storage conditions also need to be considered, because there are some materials that cannot stand with high

temperatures or light. The stock solution is sometimes also overgrown with microorganisms, this contaminated stock solution can no longer be used.

**Calculation of Material Requirements**

**1) Expresses Concentration**

The concentration of the material in the medium or stock solution can be expressed in :

1. ppm (part per million)
2. mg/l
3. M (molarity) = mol/l = molecular weight (g/l)

Dimana : 1 ppm = 1 mg/l

1 M = $10^3$ mM = $10^6$ µM

1 M = molecular weight x 1000 mg/l

The concentration of a solution can be enlarged or also called concentrated and reduced or called diluted. Concentration of the solution can be done by increasing the solute per unit volume of the same or dissolving the same substance in a smaller volume of solution. In the case of concentrating the media concentration for the stock solution and dilution for the stock material, the equation can be used :

**Vstock x Cstock = Vmedia x Cmedia**

Where : Vstock = Volume stock solution (ml)

Cstock = Concentration of the stock solution (... x Cm)

Vmedia = Volume media solution (ml, liter)

Cmedia = Concentration media (mg/l, ppm, M)

**2) Calculation Example**

**Question 1**

There is a stock solution of $NH_4NO_3$ with a concentration of 10x the concentration of the media. If we make 2 liters of media, how many volumes of $NH_4NO_3$ stock solution are taken?

**Answer**

Vs x Cs = Vm x Cm

Vs x 10 Cm = 2000 ml x 1 Cm

Vs = 2000 ml x 1 Cm / 10 Cm = 200 ml

**Question 2**

How much growth regulator Benzyl Adenine (BA) chemical must be weighed to make 200 ml of BA stock solution with a concentration of 1 mM (BM BA = 225) ?

**Answer**

1 M BA = BM BA g/l = 225 g/l

1 mM BA = 225 mg/l = 225 mg/1000 ml

BA = 200 ml x 225 mg/1000 ml = 45 mg

**Question 3**

If we are going to make 2 liters of MS media with BA concentration of 5 µM, how many ml of the BA stock solution should be added to the media ?

**Answer**

The BA stock solution that must be added to the media is :

Vs x Cs = Vm x Cm

Vs x 1000µM = 2000 ml x 5 µM

Vs = 2000 ml x 5 µM / 1000µM = 10 ml

# CHAPTER X
# PLANT GROWTH REGULATOR

Growth regulators are supplements that are added to the tissue culture medium to regulate growth and development in tissue culture and plant organ cultures. Plant hormone is an organic compound that is synthesized in one part of the plant and transported to another part, which in very low concentrations can cause a physiological response.

Physiologically, plant hormones (phytohormones) are the messengers between cells needed to control the entire plant life cycle, including germination, rooting, growth, flowering and fertilization. In addition, plant hormones are produced in response to various environmental factors, such as excess nutrients, drought conditions, light, temperature and stress both chemically and physically. Therefore, the availability of hormones is very much influenced by the season and the environment.

Growth regulators are complex natural organic compounds synthesized by higher plants, which affect plant growth and development. In tissue culture, there are two very important classes of growth regulators, namely cytokinins and auxins. These growth regulators influence growth and morphogenesis in cell, tissue and organ cultures. The interaction and balance between growth regulators given in the media and those produced by cells endogenously determine the direction of development of a culture. The addition of auxins or exogenous cytokinins, changes the level of endogenous growth regulators of the cell. This level of endogenous growth regulators is then a triggering factor for growth processes and morphogenesis. Apart from auxins and cytokinins, gibberellins and other compounds are also added in certain cases.

There ae generally known five groups of plant hormones: ***auxins, cytokinins, gibberellins, abscisic acid and ethylene.***

## 1) Auxin

Auxins are widely used in tissue culture to stimulate callus, cell and organ suspensions.

Selection of auxin types and concentrations, depending on :

1. The desired type of growth.
2. Endogenous auxin levels.

3. The ability of the network to synthesize auxin.
4. Other added growth substances.

Natural auxin is Indole Accetic Acid (IAA), the level of auxin in the explants depends on the part of the plant taken and the type of plant. In addition, it is also influenced by the season and age of the plant. In some cases of in vitro culture there are cells that can grow and develop without auxins such as tumor cells. These cells are called habituated cells.

The effect of auxins on plant tissue growth be expected in two ways :

1. Induces the secretion of $H^+$ ions out the cell through the cell wall. The acidification of the cell walls causes $K^+$ to be taken up and this uptake reduces the water potential in the cell. As a result, water enters the cells and the cells enlarge.
2. Influencing RNA metabolism which means protein metabolism, possibly through transcription of RNA molecules. Synthetic auxins that are often used in plant tissue culture are listed in the table below.
3. Promote apical dominance.
4. In small amounts stimulate root growth.

**2) Cytokinins**

The cytokinin group is a derivative of adenine. This group is very important in regulating cell division and morphogenesis. Like auxins, there are natural and synthetic cytokinins. The first cytokinins discovered, were kinetins isolated by Skoog in the Botany laboratory at the University of Wisconsin. Kinetin was obtained from Herring fish DNA which autoclaved in an acidic solution. The compounds from the DNA when added to the medium for tobacco, in fact stimulate cell division and cell differentiation. The compound is then called kinetin. The function of cytokinins in plants among others, is :

1. Promote the formation of organogenesis and morphogenesis.
2. Promote cell division.
3. The combination of auxin and cytokinins will stimulate callus growth.

**3) Gibberellin**

The use of gibberellin in plant tissue culture, sometimes helps morphogenesis. But in callus cultures where growth was fast only with giving auxins and cytokinins,

so if added gibberellin will often inhibit. In general gibberellins, especially $GA_3$, inhibit rooting.

The positive effect of gibberellin was found in sugar beet culture, where $GA_3$ stimulated shoot formation from inflorescence pieces (Coumans et al., (1982 *in* Gunawan 1988). Potato shoot culture growth is also good when 0.01-0.10 mg/l $GA_3$ is combined with 0.5-5.0 mg/l kinetin (Goodwin *et al.,* (1980 *in* Gunawan 1988). The molecular weight of $GA_3$ is 346,38.

In general, geibberellin functions, among others :

a. Kill dormancy.
b. Promotes germination.
c. Push ahead the imbibition process.

**4) Abscisic acid**

Abscisic acid (ABA) is a growth inhibitor as opposed to gibberellins because this hormone forces dormancy, preventing seeds from germinating and causing the loss of leaves, flowers and fruit. Naturally, the high concentration of abscisic acid is triggered by environmental stress such as drought.

**5) Ethylene**

Ethylene is a growth hormone which is generally different from Auxin, Gibberellin, and Cytokinin. Under normal condition, ethylene form is gaseous and its chemical structure is very simple. In nature ethylene will play a role if there is a physiological change in a plant. This hormone will play a role in the fruit ripening process in the climacteric phase.

Research on ethylene was first conducted by Neljubow (1901) and Kriedermann (1975), the results show that ethylene gas can make changes to plant roots. Research result from Zimmerman et al (1931) shows that ethylene can support abscission in leaves, but according to Rodriquez (1932), these substances can support the flowering process in pineapple plants.

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