الجمهورية الجزائرية الديمقراطية الشعبية

People's Democratic Republic of Algeria

وزارة التعليم العالي والبحث العلمي

Ministry of Higher Education and Scientific Research

Higher School of Agronomy
Mostaganem
Martyr Mohamed el Amjed
Ben Abdelmalek

المدرسة العليا للفلاحة
بمستغانم
الشهيد محمد الأمجد بن عبد المالك

Second Cycle Department

Course Handout of:

In vitro culture and plant sanitation

Presented by:

Dr. KOUADRIA R.

For 4th year Students

Specialties: Plant Production and Plant Protection

Academic Year: 2023-2024

Preamble

This handout is the course materials of *in vitro* culture and plant sanitation, intended for the second year cycle 2 (4th year) students in the specialties: Plant Production and Plant Protection (methodology teaching unit, Coefficient: 2, Credits: 4).

This course has a semester time volume of 45 hours for 15 weeks of teaching and a weekly time volume of 1 hour 30 minutes of lessons and 1 hour 30 minutes of practical work. To assimilate this teaching, prior knowledge such as cell biology and morphogenesis notions are recommended. The method of evaluation of this subject is through continuous assessments as well as oral presentations by students and a semester exam.

Tissue culture refers to rapidly multiplying stock plant material to produce true-to-type plantlets using modern plant tissue culture methods. The power of tissue culture as a propagation tool in horticulture crops becomes especially apparent when the goal is to generate large numbers of propagules of these crops that are either rare or have some unusual feature that makes clonal multiplication highly desirable. Tissue culture has potential application for a number of purposes, including mass clonal propagation, genetic engineering, protoplast culture and production of potentially useful somaclonal variants. The micropropagation method enables a million-fold expansion per year of a desired plant. It has been applied to a wide range of fruit, vegetable and ornamental species. The dream of multiplying disease-free plants of horticultural crops on the specified time schedule in an industrial way has come true through micropropagation. Micropropagation can be either through embryogenesis or through organogenesis.

Program Content

Introduction……...1

Chapter I. Introduction to *in vitro* culture……..2

I.1. Introduction and History……...2

I.2. Definitions……3

I.3. *In vitro* plant tissue culture importance……5

I.4. *In vitro* plant tissue culture types……..6

1.5. Layout of an *in vitro* Plant Tissue Culture Laboratory……...7

1.6. *In vitro* culture aseptic conditions (sterilization)……9

I.6.1. Sterilization of glassware and instruments……9

I.6.2. Sterilization of culture media……9

I.6.3. Sterilization of the explant……9

I.7. Culture media……9

I.7.1. Culture media types……...9

I.7.2. Murashige and Skoog culture medium……10

I.7.2.1. Preparation of Media……10

I.7.2.2. Components of Tissue Culture Medium……...10

I.8. Roles of Plant Growth Regulators in organogenesis……..12

I.9. Factors affecting tissue culture……...14

Chapter II. *In vitro* culture techniques using somatic cells……15

Introduction……...15

II.1. Micropropagation Definition……15

II.2. Micropropagation Methods……...15

II.2.1. Production of virus-free plants……...16

a. Meristem culture……...16

b. Virus elimination through heat treatment: thermotherapy……16

1. Procedure……..16

2. Advantages……17

3. Disadvantages……...18

II.2.2. Somatic embryogenesis……...……...18

1. Definition……..……...18

2. Stages……..……...18

3. Types……..……....19

3.1. Immature embryo culture…….…….....19
3.2. Interspecific embryo rescue…….……..19
4. Advantages…….……...20
5. Disadvantages…….……..20
II.2.3. Protoplast culture and Somatic hybridization……20
A. Definition…….……20
B. Technique description……..21
1. Obtaining protoplasts……21
a. Mechanical Method……...21
b. Sequential Enzymatic Method……..21
c. Mixed Enzymatic Method…….21
2. Testing the viability of isolated Protoplasts……..22
3. Culturing Protoplasts…….23
4. Protoplasts Fusion…….23
a. Spontaneous fusion……...23
b. Chemical fusion……23
c. Electrical fusion: Electrofusion…….24
5. Fusion products diversity……..25
a. Nuclear genome…….25
b. Chloroplastic genome……...25
c. Mitochondrial genome……..25
6. Factors affecting protoplast yield……..25
7. Importance of protoplast isolation and culture…….25
8. Major limitations of protoplast culture…….26
II.3. Micropropagation stages……...26
II.4. Somaclonals variations……..29
II.4.1. Epigenetic variations (Non-heritable Variations)……...29
II.4.2. Genetic variations (Heritable Variations)……...30
II.4.3. Somaclonal Variants Detection and Isolation……31
II.4.4. Types of somaclonal variations……..32
II.4.5. Advantages of Somaclonal Variations……...32
II.4.6. Disadvantages of Somaclonal Variations…….33
II.4.7. Ways of reducing somaclonal variation…….33

Chapter III. *In vitro* culture techniques using reproductive cells……...34
Introduction……...34
III.1. Haploid production methods……34
III.1.1. Spontaneous haploids……34
a. Polyembryony……...34
b. Parthenogenesis……35
III.1.2. Haploid induction *in vitro*……35
a. Androgenesis (motherless plants)……35
b. Gynogenesis (fatherless plants)……36
III.1.3. Haploid induction *in situ*……...37
a. Interspecific crosses……..37
b. Crosses with irradiated (denatured) pollen……...37
III.2. Stages of haplodiploidization……...37
III.3. Advantages and uses of haploids……38
III.4. Limits of haplomethods……...38
III.4.1. Case of species recalcitrant to androgenesis……38
III.4.2. Problem of albinism……..39
III.4.3. Gametoclonal variations……...40
Chapter IV: Uses, advantages and disadvantages of *in vitro* cultures……...41
Introduction……...41
IV.1 Application of *in vitro* culture in agriculture……41
IV.1.1. Creating new plant varieties……41
IV.1.2. Plant propagation and selection……41
IV.2. Advantages of *in vitro* culture……42
IV.3. Disadvantages of *in vitro* culture……42
IV.4. Limits of *in vitro* culture……..43
Conclusion……44
Exercises……...45
Glossary……49
References……52

Introduction

Plant tissue culture is a technique in which fragments of tissues from plants **"explants"** are developed *in vitro* in an artificial medium under aseptic conditions. It involves culturing explants such as shoot tip, root tip, callus, seed, embryo, pollen grain, ovule or even a single cell; isolated from mother plant on a sterile nutrient medium which leads to cell multiplication and plant regeneration, then, they are hardened and transferred to soil (Kaya and Huyop, 2020; Vidyagina et *al*., 2021). The regenerated plantlets are a true copy of the mother plant and show characteristics identical to the mother plant. For example, if the mother plant is a high yielding plant the plantlets will also be high yielding. Many plant species are presently being propagated through tissue culture successfully (Sawant, 2021).

Plant tissue culture can be initiated from almost any part of a plant however, for micropropagation or direct shoot regeneration, meristemetic tissue such as shoot tip is ideal. The physiological state of the plant does have an influence on its response to tissue culture. The mother plant must be healthy and free from obvious signs of disease or pest. The shoot tip explants being juvenile contain a higher proportion of actively dividing cells. It is important to use quality mother plant stock to initiate cultures (Sawant, 2021).

The commonly used medium in plant tissue culture is Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with plant hormones which include auxins, cytokinins, abscisic acid, gibberellins, ethylene (Kaya and Karakütük, 2018).

The cultural conditions required to initiate and sustain plant cells in culture, or to regenerate intact plants from cultured cells, are different for each plant species. Each variety or clone of a species often have a particular set of cultural requirements. Plant tissue culture techniques are also central to innovative areas of applied plant science, including plant biotechnology and agriculture (Sawant, 2021).

The present course handout will help students to understand the process of Plant tissue Culture and it significant role in sustainable agriculture.

Chapter I. Introduction to *in vitro* culture

I.1. Introduction and History

The *in vitro* culture is one of the plant biotechnology techniques used to produce biomass from plant cells for plant improvement, production and protection. Production is based on culturing plant explants on artificial culture media under aseptic conditions.

The history of plants *in vitro* cultures can be summarized as follow (Sharma et *al*., 2015):

- 1838- The discovery of cells totipotency by **Schleiden** and **Schwann**.
- 1902- The first unsuccessful attempt of *in vitro* culture using monocots by **Haberlandt.** Moreover, he explained the concept of cell totipotency**.**
- 1904- The first attempt in Crucifers embryo culture by **Hannig.**
- 1922- The orchid seeds symbiotic germination by **Knudson.**
- 1922- The root tips *in vitro* culture by **Robbins.**
- 1924- A callus formation on carrot root explants using lactic acid by **Meyer**.
- 1934- A failed *in vitro* culture of cambial tissues of different trees and shrubs by **Guatheret.**
- 1934- The identification of the first plant hormone, Indole Acetic Acid "IAA", leading to cell enlargement by **Kogl.**
- 1941- The use of Coconut Milk for very young Datura embryos growth and development by **Overbeek.**
- 1942- The observation of secondary metabolites in plant callus cultures by **Gautheret**.
- 1943- The identification of tumor-inducing principle of crown gall tumors by **Braun**.
- 1944- The first *in vitro* culture of tobacco used to study adventitious shoot formation by **Skoog.**
- 1946- The first whole plants of *Lupines* and *Tropaeolum* from shoot tips by **Ball.**
- 1948- The formation of adventitious shoots and roots in tobacco by **Skoog**.
- 1957- The discovery that root or shoot formation in plant tissue culture depends on auxin/ cytokinins ratio by **Skoog** and **Miller.**
- 1958- The *in vitro* culture of excised ovules of *Papaver somniferum* by **Maheshwari**.
- 1958- The regeneration of somatic embryos from Citrus ovules nucleus by **Maheshwari and Rangaswamy**.
- 1962- The development of Murashige and Skoog "MS" medium by **Murashige and Skoog.**

- 1964- The first haploid plants from Datura androgenesis by **Guha and Maheshwari.**
- 1973- The aptitude of Cytokinins to break dormancy in *Gerberas* by **Pierik**
- 1978- The tomato and potato somatic hybridization resulting pomato by **Melchers.**
- 1978- The industrial scale fermentation of plant cells for shikonin production by **Tabata.**
- 1981- The introduction of the term "somaclonal variation" by **Larkin**.
- 1981- The auxotroph isolation by cell colony screening in haploid protoplasts of *Nicotiana plumbaginifolia* treated with mutagens by **Sidorov.**
- 1985- The leaf discs infection and transformation with *Agrobacterium tumefaciens* and regeneration of transformed plants by **Horsch.**
- 1985- The development of disarmed Ti-plasmid vector system for plant transformation by **Fraley**.
- 1985- The development of binary vector system for plant transformation.
- 1985- The use of electroporation for gene transfer in protoplasts of Dicot and Monocot plants.
- 1993- The *in vitro* fertilization with isolated single gametes resulting in zygotic embryogenesis and recovery of fertile maize plants by **Kranz.**
- 1993- The "Green Hairy roots" showing photoautotrophy due to development of photosynthetic ability by **Flores.**
- 1996- The development of 'agrolistic' method for plant transformation by **Hansen**.

I.2. Definitions

I.2.1. Biotechnology

Plant biotechnologies are biological techniques in which plant material is the raw material. These laboratory techniques cover interventions on plant organs, tissues, cells or DNA, in order to accelerate production and improve characteristics, for agricultural research or industrial production (Bentahar et *al*., 2023).

Depending on their field of application, biotechnologies are divided into several types (Bentahar et *al*., 2023):

- **Green biotechnology:** used in agriculture (plants - animals).
- **White biotechnology:** applied in industry (development of new sustainable energy sources).
- **Red biotechnology:** applied in medicine (production of vaccines - antibiotics).
- **Blue biotechnology:** applied in the sea (Production of biomaterials).

- **Yellow biotechnology:** applied to the environment (depollution).

I.2.2. *In vitro* culture

In vitro cultures are cultures of plant explants (leaves - roots - stems - protoplasts – meristems- anthers- roots tips and embryos), on a synthetic medium (water - mineral elements - vitamins - sugars - phytohormone and sometimes agar-agar), under sterile conditions (sterilization of equipment, culture medium, explant and work surface), in a controlled environment (temperature, light, hygrometry), and in a reduced space (small jars or test tubes). The aim of *in vitro* cultures is to regenerate a whole plant from plant explants, due to the property of **totipotency** (Guédira, 2016).

1.2.3. Totipotency

Totipotency forms the basis of successful plant tissue culture. The theory of Totipotency states that each cell has the ability to regenerate into a complete plant. Each somatic cell has the same genetic constitution (DNA sequence) as that of a zygote, and also has the potential of expressing all the properties of an organism. Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stems or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones. Since, handling a single cell is practically difficult, therefore, usually a tissue or an organ form the plant is used to initiate the tissue culture work and hence Plant Tissue Culture is often also called as Plant Cell, Tissue and Organ Culture. Totipotency is based on the ability to dedifferentiate: cells can revert to simple, unspecialized cells, and then differentiate into the various specialized cell types (Guédira, 2016).

1.2.4. Differentiation

The term differentiation is used in many different senses in biology. In broad sense, it is defined as the process by which meristematic cells are converted into two or more types of cells, tissues or organs which are qualitatively different from each other (Guédira, 2016).

1.2.5. De-differentiation

The term is used to denote the process of formation of unorganized tissues from the highly organized tissues (Guédira, 2016).

1.2.6. Explant

a. Definition

The excised piece of differentiated tissue or the organ which is used for culture is called as explant (Donar plant) e.g., embryos, young leaf, bud, etc. Tissue aseptically obtained and prepared from the donor plant for culture (Sharma et *al*., 2015: Sawant, 2021).

The health of this plant determines the nature of the explant (Sharma et *al*., 2021).

- If the mother plant is diseased, an explant consisting of meristematic cells (undifferentiated explant) must be harvested.
- If the mother plant is healthy, other types of explant can be taken: stem - leaf - root (differentiated explants).

b. Choice of explant

- ✓ The tissue obtained from a plant to be cultured is called an **Explant**.
- ✓ In a totipotent, explant can be collected from any part of the plant.
- ✓ Always young and healthy parts of plants are selected as explants.
- ✓ In many plants, explants of various organs vary in their rate of growth & regeneration.
- ✓ The choice of explant material also determines if the plantlets developed via tissue culture are haploid/diploid (Guédira et *al*., 2016).

I.3. *In vitro* plant tissue culture importance

According to Sharma et *al*. (2015), the importance of in vitro plant tissue culture is that:

- A large number of plantlets can be produced starting from the single explant in a relatively short time and space. The time required is much shortened, no need to wait for the whole life cycle of seed development. For species that have long generation time, low level of seed production, or seeds that readily do not germinate, rapid propagation is possible.
- An explant taking does not usually destroy the mother plant, so rare and endangered plants can be cloned safely.
- It is easy to select desirable traits directly from the culture setup (*in vitro)* thereby decreasing the amount of space required, for field trials.
- Once established, a plant tissue culture line can give a continuous supply of young plants throughout the year.
- *In vitro* growing plants are usually free, from the bacterial and fungal diseases. Virus eradication and maintenance of plants in virus free state. This facilitates movement of plant across international boundaries.

- Plant tissue banks can be frozen and then regenerated through tissue culture. It preserves the pollen and cell collections from which plants may be propagated.

I.4. *In vitro* plant tissue culture types

The *in vitro* culture types (Sharma et *al*., 2015) are illustrated in figure 01.

I.4.1. Callus culture: Callus culture may be defined as production and maintenance of an unorganized mass of proliferative cell from isolated plant cell, tissue or organ by growing them on artificial nutrient medium in glass vials under controlled aseptic conditions.

I.4.2. Organ culture: The organ culture refers to the *in vitro* culture and maintenance of an excised organ primordial or whole or part of an organ in way and function.

I.4.3. Single cell culture: Single cell culture is a method of growing isolated single cell aseptically on nutrient medium under controlled condition.

I.4.4. Suspension culture: Suspension culture is a type of culture in which single cell or small aggregates of cell multiply while suspended in agitated liquid medium. Suspension cultures are used in induction of somatic embryos and shoots, production of secondary metabolites, *in vitro* mutagenesis, selection of mutants and genetic transformation studies.

I.4.5. Embryo culture: Embryo culture may be defined as aseptic isolation of embryo (of different developmental stages) from the bulk of maternal tissue of mature seed or capsule and *in vitro* culture under aseptic and controlled physical condition in glass vials containing nutrient semisolid or liquid medium to grow directly into plantlet.

I.4.6. Anther culture: Androgenesis is the *in vitro* development of haploid plants originating from potent pollen grains through a series of cell division and differentiation.

I.4.7. Pollen culture: Pollen culture is the *in vitro* technique by which the pollen grains (preferably at the microscope stages) are squeezed from the intact anther and then cultured on nutrient medium where the microspores without producing male gametes.

I.4.8. Somatic Embryogenesis: Somatic embryogenesis is the process of a single or group of cells initiating the development pathway that leads to reproducible regeneration of non zygotic embryos capable of germinating to form complete plants.

I.4.9. Protoplast Culture: It is the culture of isolated protoplasts which are naked plant cells surrounded by plasma membrane which is potentially capable of cell wall regeneration, cell division, growth and plant regeneration on suitable medium under aseptic condition

I.4.10. Shoot tip and Meristem culture: The tips of shoots (which contain the shoot apical meristem) can be cultured *in vitro* producing clumps of shoots from either axillary or adventitious buds. This method can, be used for clonal propagation.

I.4.11. Explant Culture: There are variety of forms of seed plants viz., trees, herbs, grasses, which exhibit the basic morphological units i.e. root, stem and leaves. Parenchyma is the most versatile of all types of tissues. They are capable of division and growth.

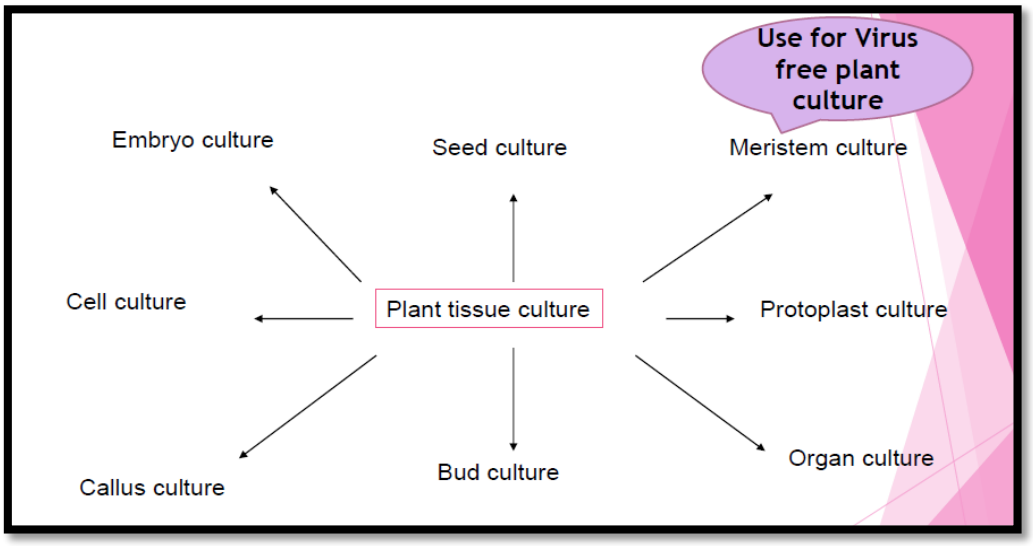

Figure 01: Types of *in vitro* culture

1.5. Layout of an *in vitro* Plant Tissue Culture Laboratory

Figure 02 shows the *in vitro* plant laboratory arrangement (Sharma et *al*., 2021).

a. Washing Room

- ✓ Adequate Water Supply
- ✓ Proper Drainage System
- ✓ Distill Water Unit (DDW, TDW, Deionized Water)
- ✓ Acid and Alkali Resistant sinks/ working platform
- ✓ Autoclave for used culture vessels disinfection
- ✓ Hot Air Oven (80^0C & 240^0C) for drying of Vessels and Sterilization
- ✓ Ventilator with exhaust fan

b. Media Preparation Room

- ✓ Sufficient working platform and sufficient storage space for the chemicals, culture vessels, glass wares, plastic wares, etc.
- ✓ Hot plates, magnetic stirrers, pH meter, balances, water baths, and media dispensing equipment, micropipettes.
- ✓ Refrigerators and freezers (-20, -80) to store stock solutions
- ✓ Reverse osmosis or double distilled water Unit.

c. Sterilization Room

- ✓ Adequate supply of tap water

- ✓ Ventilator with exhaust fan
- ✓ Thermo tolerant drainage pipes
- ✓ Autoclave

d. Media Store Room

- ✓ Fitted with UV Light for disinfection/ regular fumigation and cleaning

e. Inoculation Chamber:

- ✓ Laminar Air Flow (Horizontal flow)
- ✓ Air purifier
- ✓ Air Conditioner
- ✓ Bunsen Burner/Glass bead sterilizer, Portable Autoclave
- ✓ Dissecting Microscope/ Electronic top pan balance for callus subculture/ centrifuge for pollen culture or protoplast culture
- ✓ Fire extinguishers

f. Culture Room:

- ✓ Culture Racks fitted with fluorescent tube lights
- ✓ Two Air Conditioners (alternating working) with thermostat
- ✓ Rotatory shaker for suspension culture and somatic embryos production
- ✓ Hygrometer, Lux meter, Photoperiod controller
- ✓ Stereo microscope and Observation table.Dark Room.

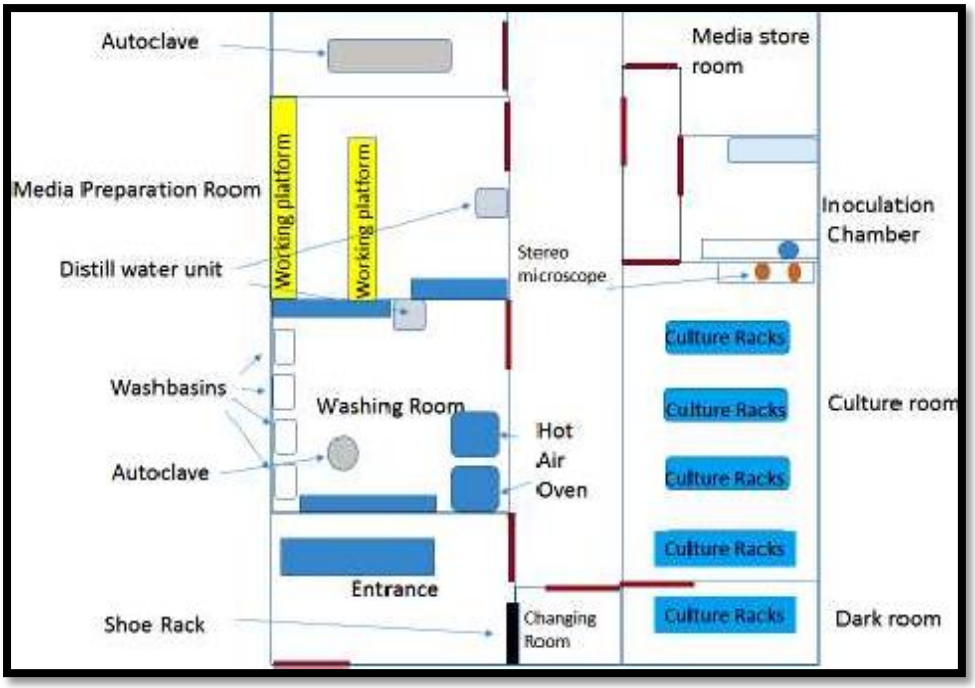

Figure 02: Layout of a Plant Tissue Culture Laboratory (Sharma et *al*., 2021)

1.6. *In vitro* culture aseptic conditions (sterilization)

I.6.1. Sterilization of glassware and instruments

- Sterilization by autoclave or oven.
- Instruments can also be sterilized by ethanol followed by flame treatment (Guédira, 2016).

I.6.2. Sterilization of culture media

- Steam sterilization of culture media by autoclaving at 120°C for 20 min. For effective sterilization, it is advisable to handle culture media in small quantities in several containers. The principal laboratory method used to sterilize solutions of heat labile materials, vitamins and phytohormones is filtration through filters capable of retaining microorganisms. Filters may have different sizes of pore diameters of about 0.2µm are effective in removing microorganisms from solution. After filtration, they are added to the autoclaved medium (Guédira, 2016).
- Antibiotics can be added to the culture medium to prevent bacterial growth (Guédira, 2016).

I.6.3. Sterilization of the explant

- Explants are sterilized by using different types of disinfectants like sodium hypochlorite (2%), silver nitrate (1%) calcium hypochlorite (9-10%), bromine water (1-2%), mercuric chloride (0.1-1%), hydrogen peroxide etc. and different types of antibiotics (Sharma et al., 2015; Guédira, 2016; Sawant, 2021).

I.7. Culture media

The success of *in vitro* plant tissue culture depends on the chemical composition of the culture media. The choice of medium composition depends on a number of parameters, including the nature of the tissue being cultured (bud, embryo, meristem, etc.) and the culture phase (initiation, multiplication, elongation or rhizogenesis). For best results, small quantities of amino acids, vitamins and growth regulators should be added to the culture media (Guédira, 2016).

I.7.1. Culture media types

Culture media has significant effect on plant regeneration from different parts of plant (Kumar and Reddy, 2011). Various types of nutrient media have been employed in micropropagation like : White medium, Nitsch and Nitsch medium, B5 medium and Gamborg medium (Diallo et *al*., 2008), but the most widely used culture medium is Murashige and Skoog (1962) (MS medium), because most of the plants respond favorably to MS medium,

since it contains all the nutrients essential for plant growth *in vitro*. Selection, strength and combination of media are also one of important parameter for optimizing the regeneration protocol (Diallo et *al*., 2008).

I.7.2. Murashige and Skoog culture medium

This was the first culture medium developed in 1962.

Murashige and Skoog (MS) medium is the most pertinent and frequently used medium for regeneration of plant from tissues and callus. This is a high salt medium owing to its additional content of potassium and nitrogen salts.

I.7.2.1. Preparation of Media

This is a very critical step for the experiment to be successful. While making the media taking individual constituents, each ingredient is separately weighed and dissolved before putting them together. After making up volume by water, pH is adjusted and then medium is autoclaved (Sharma et *al*., 2021).

Preferably, following four stock solutions are prepared:

Major salts (20X concentration), Minor salts (200X concentration), Iron (200X concentration) and Organic nutrients (200X concentration), separate stock solution for each growth regulator is prepared. Appropriate quantities are taken from stocks and mixed to constitute basal medium. Required quantity of agar, sucrose and organic supplements if needed are added separately (Sharma et *al*., 2021).

I.7.2.2. Components of Tissue Culture Medium

The composition of *in vitro* culture media is as follow (Murashige et Skoog, 1962; Sharma et *al*., 2021):

1. Water: necessary to prepare the different solutions. Distilled and filtered water.

2. Inorganic Nutrients: *In vitro* growth of plants moreover requires blend of macro and micronutrients in the vein of *in vivo* growth.

- **Macronutrients:** are requisite in concentration larger than 0.5 mM/l. They consist of nitrogen, potassium, phosphorus, calcium, magnesium and sulphur in form of salts in media. Nitrogen is usually supplied in form of ammonium ($NH4^+$) and nitrate ($NO3^-$) ions. Nitrate is superior to ammonium as the sole N source but use of $NH4^+$ checks the increase of pH towards alkalinity.

- **Micronutrients:** are required at a concentration below 0.05mM/l. These consist of iron, manganese, zinc, boron, copper and molybdenum. These inorganic elements though required in minute quantity are crucial for plant growth, most critical of them being iron

which is unavailable at low pH. Therefore, it is supplied as iron EDTA complex to make it available at wide array of pH.

3. Carbon Source: Sugar is vital part of nutrient medium as source of energy, since most plant cultures are unable to photosynthesize effectively in consequence of inadequately developed cellular and tissue development, be deficient in chlorophyll, inadequate gas exchange and carbon dioxide in tissue culture vessels etc. Consequently, they need external supply of carbon for energy. The most favored carbon or energy source is sucrose at a concentration of 20-60g/l. While autoclaving the medium, sucrose is hydrolysed to glucose and fructose which are then used up for growth. Fructose, if autoclaved is toxic. Other mono or disaccharide and sugar alcohols like glucose, sorbitol, raffinose etc. may be used depending upon plant species.

4. Organic Supplements:

- **Vitamins:** are organic substances required for metabolic processes as cofactors or components of enzymes. Therefore, for optimal growth, medium should be supplemented with vitamins. Thiamine (B1), nicotinic acid (B3), pyridoxine (B6), pantothenic acid (B5) are generally used vitamins of which thiamine (0.1 to 5mg/l) is essentially added to medium as it is involved in carbohydrate metabolism. Rest vitamins are promontory.
- **Amino acids:** Addition of amino acids to media is essential for stimulating cell growth in protoplast cultures and also in inducing and maintaining somatic embryogenesis. L-glutamine, L-sparagine, Lcystein, L-glycine are commonly used amino acids.
- **Complex organics:** are group of undefined supplements such as casein hydrolysate, coconut milk, yeast extract, orange juice, tomato juice etc. These compounds are often used when no other combination of known defined components produce the desired growth.
- **Activated charcoal:** acts both in promotion and inhibition of culture growth depending upon plant species being cultured. It is reported to stimulate growth and differentiation in orchids, carrot, ivy and tomato whereas inhibits tobacco, soybean etc. It absorbs brown-black pigments and oxidized phenolics produced during culture and thus reduce toxicity. It also absorbs other organic compounds like PGRs, vitamins etc which may cause the inhibition of growth. Another feature of activated charcoal is that it causes darkening of medium and so helps root formation and growth.

5. Plant Growth Regulators: stimulate cell division and hence regulate the growth and differentiation of shoot and roots on explants and embryos in semisolid or in liquid medium cultures. The four major PGRs used are auxins, cytokinin, gibberellins and abscissic acid.

- **Auxins:** induce cell division, cell elongation, apical dominance, adventitious root formation, somatic embryogenesis. When used in low concentration, auxins induce root initiation and in high, callus formation occurs. Commonly used synthetic auxins are 1-naphthaleneacetic acid (NAA). 2,4 dichlorophenoxyacetic acid (2,4-D), indole-3 acetic acid (IAA), indolebutyric acid (IBA) etc. Both IBA and IAA are photosensitive hence must be stored in the dark.
- **Cytokinins:** promote cell division and stimulate initiation and growth of shoots *in vitro*. Zeatin, 6- benzylaminopurine (BAP), kinetin, 2-iP are the frequently used cytokinins. They modify apical dominance by promoting axillary shoot formation. When used in more concentration, CK inhibits root formation and induces formation of adventitious shoot. If ck/auxin ratio is low, leads to embryogenesis, callus initiation and root initiation whereas if ck/auxin is high, it gives rise to axillary and shoot proliferation.
- **Gibbrellins and abscissic acid:** are lesser used PGRs. Gibbrellic acid (GA3) is mostly used for internode elongation and meristem growth. Abscissic acid (ABA) is used only for somatic embryogenesis and for culturing woody species.

6. Solidifying agents: are used for preparing semisolid tissue culture media to enable explant to be placed in right contact with nutrient media to provide aeration. Agar (0.5% to 1 %) is preferred over other gelling agents because it is inert and doesn't react with media constituents also not digested by plant enzymes. Mechanical support for cell or tissue growth can also be provided without using any gelling agent by filter-paper bridge, perforated cellophane and polyurethane foam etc.

7. pH: pH affects absorption of ions and also solidification of gelling agent.

✓ Optimum pH for culture media is 5.8 before sterilization.

✓ Values of pH lower than 4.5 or higher than 7.0 greatly inhibit growth and development *in vitro*.

✓ The pH of culture media generally drops by 0.3 to 0.5 units after autoclaving and keeps changing during the time of culture owing to oxidation together with differential uptake and secretion of substances by growing tissue.

I.8. Roles of Plant Growth Regulators in organogenesis

According to Guédira et *al*. (2016) and Sharma et *al*. (2021) the balance of growth regulators depends on the objective of the cultivation *in vitro* (as e.g. shoot, root, callus or suspension culture) and on the micropropagation phase considered (initiation, multiplication or rooting).

- In the multiplication phase, the level of cytokinins should be normally higher than of auxins.
- In the rooting phase, in turn, the use of cytokinin is, in some cases, not necessary and higher levels of auxins can be supplemented to the culture medium.

The auxin/cytokinin ratio determines tissue development in culture (Fig.03).

- High concentrations of auxins + low concentrations of cytokinins: promote rooting of leafy stems: **Rhizogenesis**.
- High concentrations of cytokinins + low concentrations of auxins: promote development of axillary or adventitious buds and thus plant multiplication: **Caulogenesis**.
- Balanced concentrations of Auxins and Cytokinins: anarchic proliferation (callus) of cells that cannot organize themselves into distinct tissues and organs: **Callogenesis**.

Rhizogenesis and **Caulogenesis:** involve direct organogenesis: organ formation directly on the surface of cultivated explants.

Callogenesis: is indirect organogenesis: plant organs are formed from the callus tissue of cultured explants.

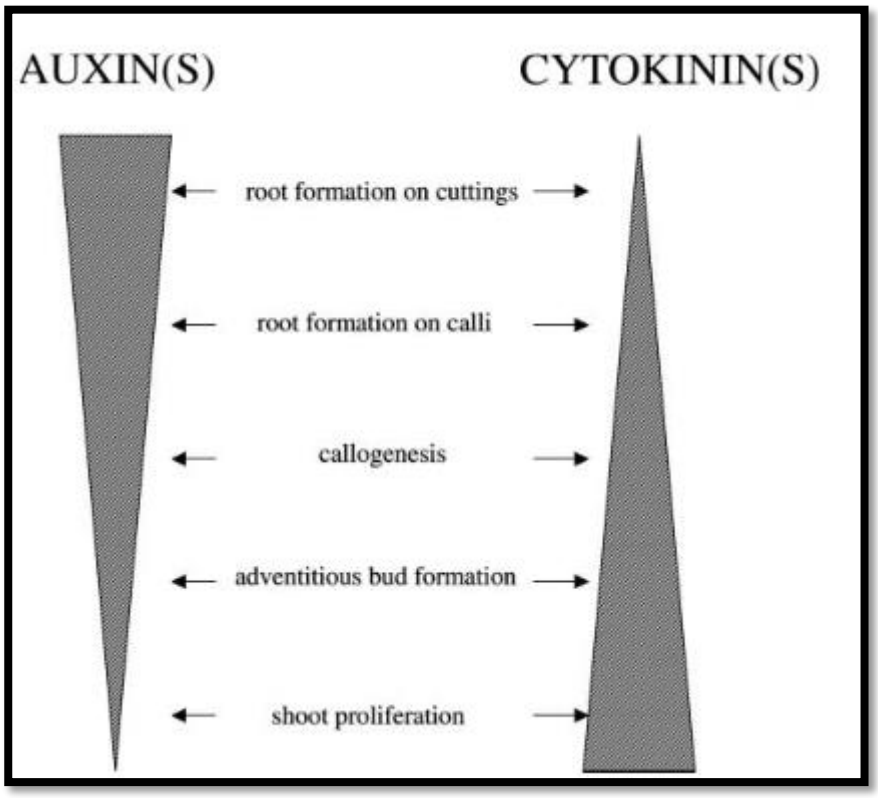

Figure 03: The control of different organogenic programs by the balance between auxins and cytokinins (Guédira et *al*., 2016).

I.9. Factors affecting tissue culture

The *in vitro* tissue culture can be affected by several factors (Guédira et *al*., 2016):

- **Growth media:** Minerals, carbon source, vitamins, hormones.
- **Environmental factors:** Light, temperature, photoperiod, humidity.
- **Explant source:** Usually younger, less differentiated, explant is used for tissue culture.
- **Genotype:** The regeneration capabilities of explants in culture differ depending upon the genotype of the plant. Therefore, the microprogation system developed for one particular cultivar will not automatically be applicable to another plant even within the same species

Chapter II. *In vitro* culture techniques using somatic cells

Introduction

In nature, plants propagate asexually (vegetative multiplication) to produce genetically identical plants. Multiplication of genetically identical copies of a cultivar via tissue culture is called micropropagation: **"the *art and science of multiplying* plants *in vitro*".**

Various *in vitro* plant culture techniques are used in the laboratory to produce and propagate plants identical to the mother plant, including meristem culture, somatic embryogenesis, haplo-diploidization by androgenesis or gynogenesis, and protoplast culture.

II.1. Micropropagation Definition

Micropropagation is a technique in which any vegetative (meristmatic) part of plant (shoot tip, shoot bud, etc.) is excised aseptically and cultured on sterile media under controlled conditions to give a complete plant, which is exact copy of its donor plant (Njukwe et *al*., 2007). It can be defined as clonal propagation *in vitro.* The new plant is genetically identical to its parent and can be called clone. Micropropagation is used to multiply a large number of plantlets without sexual reproduction or seed formation. Furthermore, it is used to produce virus-free plants (Sharma et *al*., 2015; Godoy et *al*., 2017).

Traditionally, it was done by using cuttings, budding, grafting, corms, tubers or other vegetative propagules. However, these traditional procedures are laborious, dependent on environmental conditions and their success frequency is low (Rajput et *al*., 2023).

Micropropagation is used to address the above-mentioned problems. It results in rapid multiplication of plants under *in vitro* conditions within a short period of time in a small space. Since it is performed under controlled environmental conditions, micropropagation is not season dependent. This method is useful for the multiplication of non-fertile plants, rare plants, endangered plants or other plants for which the character of choice cannot be maintained by sexual reproduction (elite plants). Micropropagation has been employed successfully in agriculture, horticulture and forestry like potato, banana, etc. (Rajput et *al*., 2023).

II.2. Micropropagation Methods

Several methods are used for plant tissue culture. The ordinarily used method for organ formation can occur directly from meristems, or indirectly from dedifferentiated cells (callus). The obtained cultures can then be employed to mass-produce plants (micro propagation) or to develop specific organs (e.g., roots) (Espinosa-Leal et *al*., 2018).

II.2.1. Production of virus-free plants

a. Meristem culture

Most of the crop plants are infested with viruses that result in huge damages in terms of productivity and yield. For vegetatively propagated plants, virus infection is a serious problem, as the entire clonal population will also be infected with viruses (Kumar and Reddy, 2011).

Therefore, production of virus free plants is important to maintain the yield and quality of vegetatively propagated plants. Interestingly, these virus particles have uneven distributions throughout the plant body (Sharma et *al*., 2015).

Generally apical or axillary meristems are free from virus particles. Consequently, very small part of apical meristems (less than 1 mm long) can be used as explant to initiate cultures for the production of virus free plants (Fig.04). This method is important and has been successfully used for clonally propagated crops, like sugarcane, banana and potato (Ahluwalia et *al*., 2016; Rajput et *al*., 2023). This technique can be combined with thermotherapy (culture at high temperatures), to help eliminate viruses (Guédira, 2016).

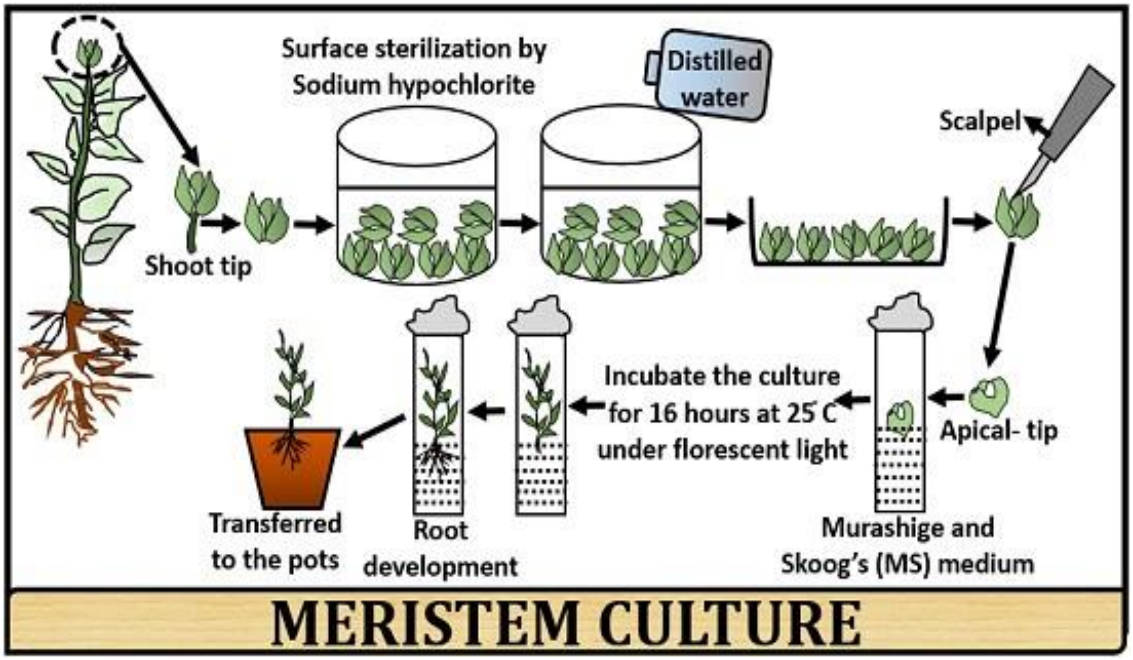

Figure 04: Meristem culture steps

b. Virus elimination through heat treatment: thermotherapy

1. Procedure

High temperature treatment has been widely used in production of virus free plants (30 to 40°C).

Preparation for therapy: surface sterilization & culture establishment.

Temperature treatment: after three weeks of subculture *in vitro*, shoots are transferred to a special growth chamber, and grown at an increased temperature regime for 14 to 21 days.

Regeneration and indexing: Regeneration of small plantlets occurs within a few weeks. After indexing virus free plants selected and multiplied further.

Acclimatization: Plantlets are rooted *in vitro* and transferred to the greenhouse. After careful hardening potted plants are re-tested for their pathogen-free status (Wang et *al*., 2018).

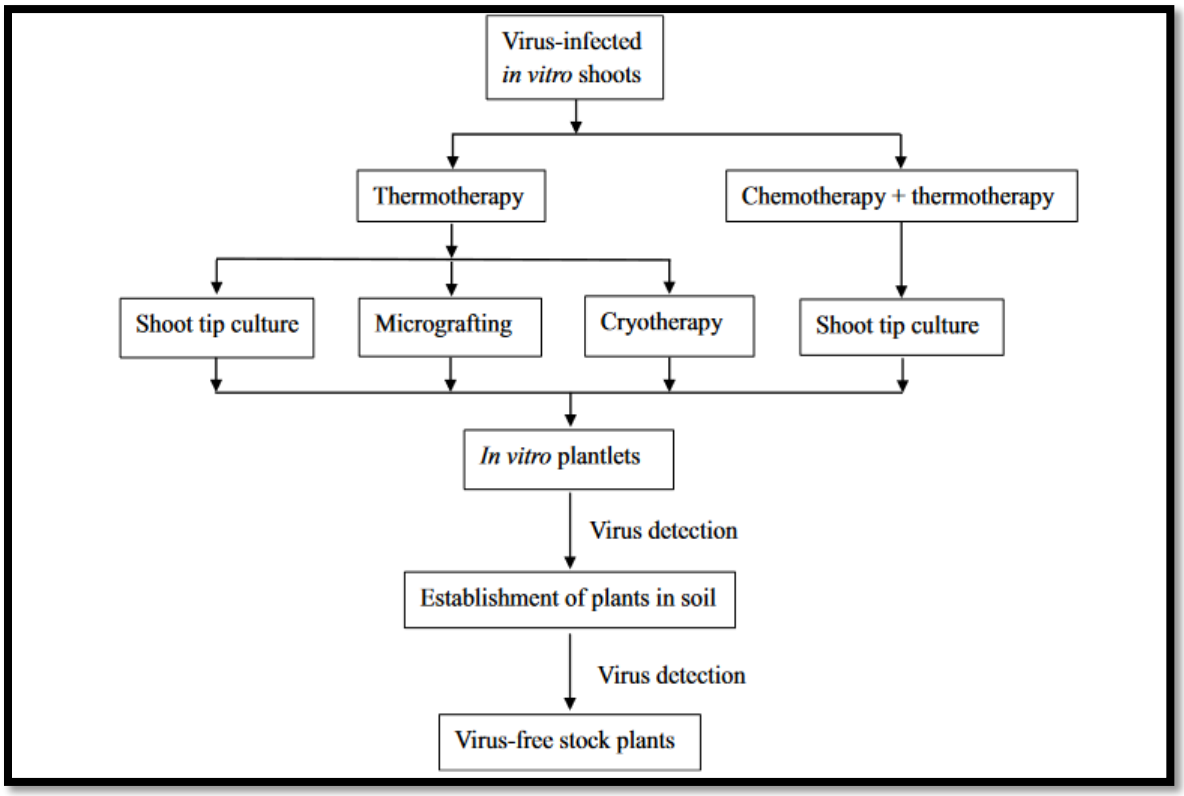

Figure 05: *In vitro* thermotherapy-based methods for production of virus-free plants (Wang et *al*., 2018).

2. Advantages

The advantages of virus-free plants production are (Guédira, 2016; Wang et *al*., 2018):

- Completely healthy mother plants are obtained as a basis for the propagation of various varieties.
- Sanitized plants have increased growth vigour and restored flowering and fruiting qualities.
- The obtained varieties are conform to the original variety and can be multiplied in large quantities, with homogeneous production.
- It can also be used to save endangered varieties.

3. Disadvantages

- The obtained plants are free from virus, but have not become virus-resistant. Therefore, there are possibilities of obtaining infected plants (no regeneration of the trait) (Guédira, 2016).

II.2.2. Somatic embryogenesis

1. Definition

Somatic embryogenesis is the process of creating embryos from somatic plant cells in order to produce a whole plant. (Fig.06). It can either be direct or indirect, depending on whether the process begins with the original explants or with previously produced callus (undifferentiated callus cell) (Gupta et *al*., 2020).

2. Stages

The different steps of somatic embryogenesis (Long et *al*., 2022):

1. Initiation of embryo cultures by culturing zygotic embryos extracted from immature seeds (to produce embryonic cultures consisting of somatic embryos). The explants are cultured on a medium containing growth regulators, mainly auxin and often cytokinins. The explant cells differentiate and divide, becoming competent to initiate an embryogenic program. An embryo culture consists of somatic embryos remaining in a highly undifferentiated state. It can be easily preserved in liquid nitrogen: cryoconservation, for a long time, requiring very little human intervention.
2. Proliferation on a solid or liquid medium with the same composition of growth regulators as in the previous stage.
3. Pre-maturation of somatic embryos on a medium devoid of growth regulators, which inhibits proliferation but stimulates embryo formation and development.
4. Maturation of embryos by culture on medium containing ABA. This stage enables the accumulation of reserve substances and the establishment of the structures needed for germination. Following maturation, somatic embryos can be dehydrated to condition them for short- or medium-term storage, enabling production of a given number of plants over a long period.
5. Plant germination and development are achieved by transferring cotyledonary somatic embryos to a simplified culture medium devoid of growth regulators.
6. Once the seedlings have been acclimatized and begun to grow actively, they can be transferred to conventional greenhouse and nursery conditions. The transition period, during

which light, temperature and humidity conditions in the greenhouse, must be precisely controlled.

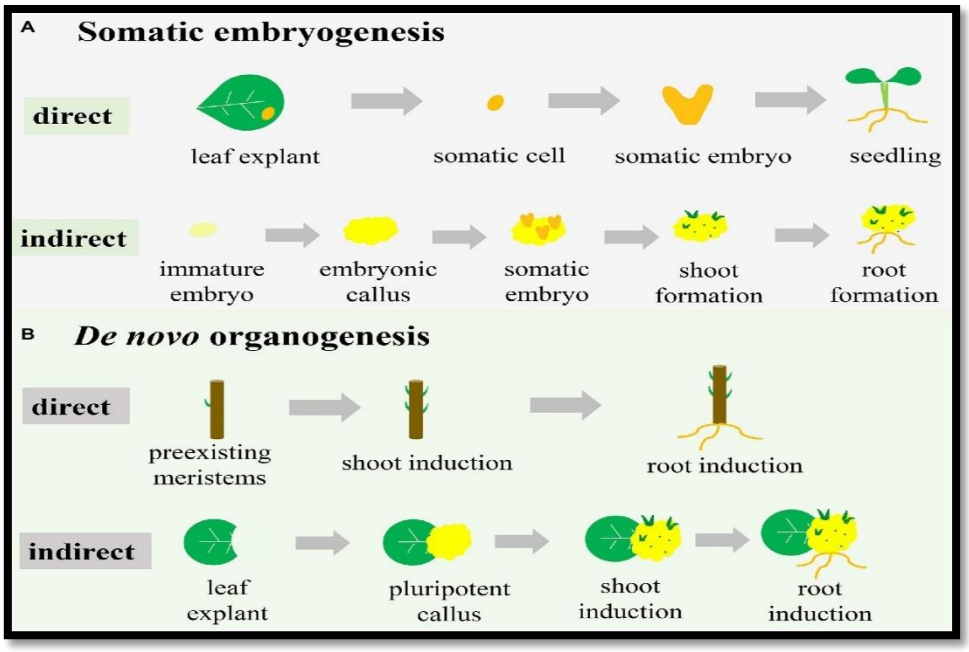

Figure 06: Somatic embryogenesis stages (Long et *al*., 2022)

3. Types

3.1. Immature embryo culture

This technique avoids the seed maturation phase and speeds up conventional selection procedures. The embryo is harvested a few days after fertilization, rather than when the seed matures, which means that several generations per year can be obtained. The embryos are cultured on a medium suitable for whole-plant regeneration (Shen et *al*., 2011).

3.2. Interspecific embryo rescue

Following interspecific crosses (sexual reproduction between two organisms belonging to two different species, generally genetically close), problems of incompatibility may arise between embryonic tissues and maternal tissues from the ovule; this technique is used to ensure embryo rescue (during these crosses, embryo development is rarely possible). To rescue the embryo, it is removed a few days after fertilization and cultured *in vitro*. If rescue is not assured, the embryo cannot develop degenerates and dies (Shen et *al*., 2011).

Some researchers have used this technique to transfer agronomic traits of interest between wild and cultivated species.

4. Advantages

The advantages of somatic embryogenesis are (Jain, 2007):

- Somatic embryogenesis, a clonal micropropagation technique, makes it possible to obtain genetically identical embryos.
- Somatic embryogenesis yields are high. In fact, this technique can rapidly produce several million of identical seedlings.
- It enables us to accumulate data on the biochemical and molecular events that take place during embryogenesis, embryo maturation and germination.
- Callus or embryogenic cultures can be cryopreserved in liquid nitrogen at -196°C, ensuring that the juvenility of the material is maintained indefinitely and that it is available over the long term. This also makes it possible to secure collections of plant material: to create cryobanks.

5. Disadvantages

The disadvantages of somatic embryogenesis are (Jain, 2007):

- Risk of genetic variation: obtaining plants that differ from the mother plant.
- It costs expensive.
- Young embryos are very difficult to retrieve, unlike more developed embryos, which are easier to retrieve.
- A very young embryo is likely to have a disturbed and slower growth.

II.2.3. Protoplast culture and Somatic hybridization

A. Definition

A protoplast is a plant, fungal or bacterial cell whose cell wall has been removed, obtained mechanically or enzymatically (Guédira, 2016).

Isolated protoplasts have been described as "naked" cells because the cell wall has been removed by either a mechanical or an enzymatic process.

Somatic fusion, also called **protoplast fusion**, is a type of tissue culture and genetic modification in plants by which two distinct species of plants are fused together to form a new hybrid plant with the characteristics of both : a **somatic hybrid**. Also it is used to overcome breeding barriers. Hybrids have been produced either between different varieties of the same species (e.g. between non-flowering potato plants and flowering potato plants) or between two different species (e.g. between wheat triticum and rye secale to produce Triticale).

Plant protoplasts have a wide range of applications from their use in the isolation of cell organelles such as chloroplasts and nuclei to developing new plant varieties as a result of the

fusion between cells of different plant species. This fusion, known as **somatic hybridization**, is the most promising use of protoplasts. This advantage makes it possible to induce fusion between protoplasts of plants that do not normally hybridize sexually, producing somatic hybrid plants that exhibit novel nuclear and cytoplasmic genetic combinations.

B. Technique description

1. Obtaining protoplasts

Protoplasts are obtained in the laboratory by (Fig.07) (Bhatia, 2015):

a. Mechanical Method

Mechanical method of protoplast isolation was first done by Klercher (1982).

Cut the tissue which is first plasmolysed with a sharp knife into small pieces. Then these pieces are deplasmolysed by using dilute solution to release the protoplasts. Generally protoplasts were isolated from highly vacuolated cells of storage tissues (onion bulbs, scales, radish root, beet root).

This method proved tricky and random, and was abandoned because of its disadvantages:

- It is not very efficient and cannot be reproduced.
- It requires elongated cells.
- Meristem cells are not suitable for this method.

In this method the protoplast released are very few in number.

b. Sequential Enzymatic Method

This method was first used Takebe and others in 1968 in two steps: the macerated tissue was first incubated in pectinase (degrade pectin cell wall) and then treated with cellulose (degrade cellulosic cell wall) for liberation of protoplasts.

The protoplast is isolated by degrading the cell wall.

c. Mixed Enzymatic Method

Simultaneous process.

This is one-step procedure in which both enzymes are used together to reduce time. Power and Cocking (1968) used this method for isolation of protoplasts.

Protolplasts can be isolated by treating cells, with a suitable mixture of cell wall degrading enzymes. The mixture of Pectinase (0.1-1.9%) and Cellulase (1-2%) is suitable for majority of plant parts.

The commercially available enzyme has enabled the isolation of protoplasts from practically every plant tissue. There pH value is adjusted between 4.7 to6 and is kept at temperature 25-30°C.

This method is used by most of the workers because it is less times consuming and reduces the chance of microbial contamination by excluding few steps.

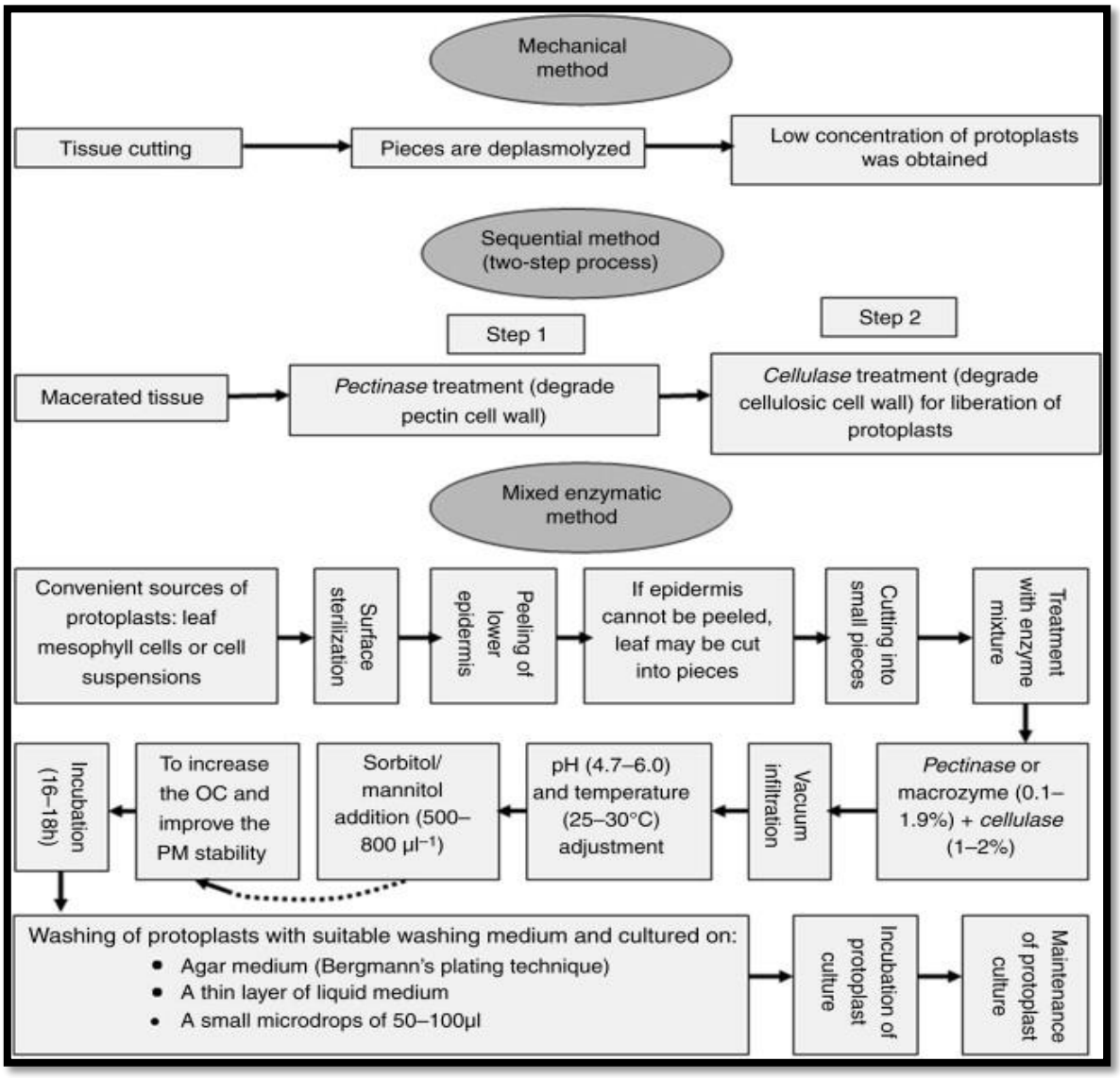

Figure 07: Methods for the isolation of the protoplasts (mechanical, sequential, mixed enzymatic methods) (Bhatia, 2015)

2. Testing the viability of isolated Protoplasts

The isolated protoplasts should be healthy and viable in order to undergo proper division and regeneration. This can be done by microscopic observation of untreated cells or after staining the cells with suitable chemicals to indicate active metabolism in the protoplasts. It is done by several methods (Tomar and Dantu, 2010).

a. Phase Contrast Microscopy: Cytoplasmic streaming movement and the presence of clear, healthy nucleus indicate that the cells are in viable state.

b. Tetrazolium Reduction: In this test respiratory efficiency of cells is measured by reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) to the red dye formazon. The formazon formed can be extracted and measured spectrophotometrically.

c. Fluorescein Diacetate Method: The 0.5% fluoresces in diacetate (FDA) in acetone is prepared and stored at 0°C. This was added at 0.01% of final concentration to protoplasts suspension with osmotic stabilizer. After 5 min incubation, the cells are observed under microscope with suitable filter.

d. Evan's Blue Staining: The 0.025% of Evan's Blue stain solution was used for staining the protoplasts. The stain gives color to the dead protoplasts by becoming permeable to dead ones, whereas viable protoplasts remain colorless due to impermeability of plasma membrane.

3. Culturing Protoplasts

a. Protoplast purification: at the end of enzymatic digestion, a mixture of isolated cell protoplasts and undigested plant debris is generally obtained. To achieve this, a combination of operations must be carried out: filtration - centrifugation - washing. Sucrose, mannitol and $CaCl_2$ can be used at different concentrations to purify protoplasts (Tomar and Dantu, 2010).

b. Culture: purified protoplasts with a minimum density (10^4 protoplasts/mL for successful culture) are transformed onto a suitable liquid or semi-solid culture medium. These protoplasts can not only fuse together, but also regenerate entire plants.

Protoplast fusion enables cross-breeding between two distantly related species when embryo rescue is not sufficient (chromosomal instability of the embryo).

4. Protoplasts Fusion

Several techniques enable the fusion of two protoplasts of different varieties (intraspecific fusions) or different species (interspecific fusions). Protoplast fusion can be broadly classified into two categories: Spontaneous fusion (fuse through their plasmodesmata) and Induced fusion (needs a fusion inducing chemicals) (Verma et *al*., 2008).

a. Spontaneous fusion

Can occur spontaneously during enzymatic digestion; fusion frequency increases with rising temperature (Verma et *al*., 2008).

b. Chemical fusion

Based on the use of high-molecular-weight chemicals to bring protoplasts closer together and subsequently fuse them (Verma et *al*., 2008).

✓ ***Fusion by sodium nitrate NaNo3:*** added to the enzyme solution at a low level of 0.5% (due to toxicity concerns).

✓ ***Fusion by Ca2+ ions:*** a high concentration of $Ca2^{+}$ ions is added at a high pH of 10.

✓ **Fusion by PEG (Polyethylene Glucole):** this is the most widely used and effective method for a large number of plants, giving a high fusion rate. Dilution of this substance induces molecular disruption, leading to protoplast fusion (Fig.08) (Bhatia, 2015).

c. Electrical fusion: Electrofusion

The protoplasts are subjected to an electric field induced by a current created between two non-symmetrical (different) electrodes; this causes them to align into chains that can be observed under an inverted microscope. Once the protoplasts have joined together, fusion is achieved by sending pulses of direct current for a few milliseconds (Verma et *al*., 2008).

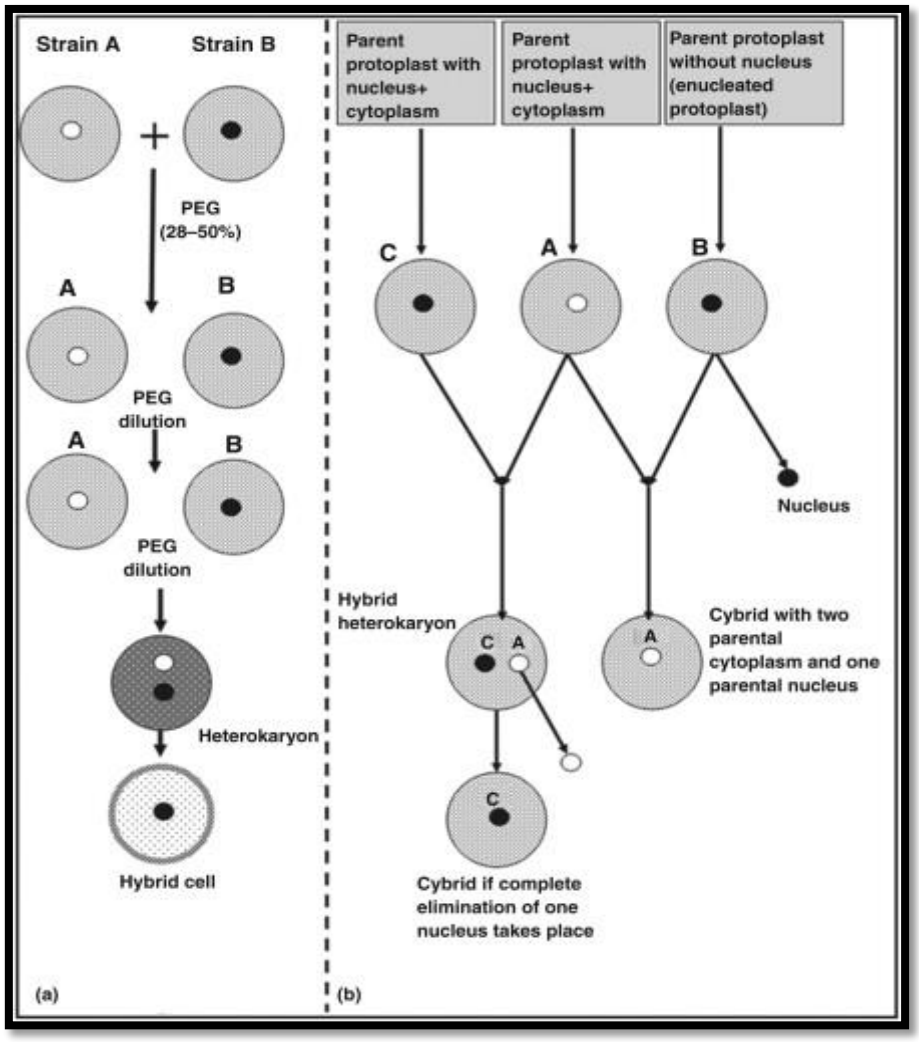

Figure 08: Protocol for the polyethylene glycol induced protoplast fusion (a) and production of cybrids through protoplast fusion (b) (Bhatia, 2015).

5. Fusion products diversity

a. Nuclear genome

- A single parental nucleus, which will be conserved (Cybrids: Plants or cells witch containing nucleus of one species but cytoplasm from boththe parental species.).
- Two nuclei fuse, in which case all chromosomes are retained.
- Partial elimination of one of the two genomes may occur in most cases.

b. Chloroplastic genome

- Regenerated plants have one or other type of chloroplast.

c. Mitochondrial genome

- Either a single parental population is conserved, or mitochondrial DNA recombination occurs, which is the most frequent case.

6. Factors affecting protoplast yield

Several factors can influence the yield and viability of protoplasts isolated from plants (Zhang et *al*., 2011).

1. Plant source, genotype, age, and growth conditions;
2. Tissue pre-treatment (osmotic treatment with mannitol, removal of epidermis to improve yield) and enzyme concentration used;
3. Growth conditions: (temperature, light, and cell density);
4. Culture medium (especially, plant growth regulators, osmotic stabilizers, and gelling agents);
5. Purification method.

7. Importance of protoplast isolation and culture

The protoplast isolation, culture and fusion are one of the most fascinating fields of research. The techniques are important for the following reasons (Lambardi et *al*., 2008; Hussain et *al*., 2012):

- To develop novel hybrid plant through protoplast fusion, genetic engineering would continued to be an exciting area of research in modern plant biotechnology. This technology holds great promises to synthesis a plant of desired characteristics.
- This helps in crop improvement by somatic hybridization and cell modification.
- The protoplast in culture can be regenerated into an entire plant.
- It provides a tool for isolating protoplasts and exploring the possibilities of genetic engineering.

- The technique in future will be one of the most frequently used research tools for tissue culturists, physiologists, pathologists molecular biologists, cytogenetics and biotechnologists.

8. Major limitations of protoplast culture

In practice, although protoplast culture is frequently used, you may encounter some challenges while performing it (Pasternak et *al*., 2020):

1. Protoplast culture is a process that demands specialized tissue culture expertise, requires complex manipulation, and can be time-consuming.
2. Protoplasts are very fragile and there is genetic instability associated with their culture.
3. Current methods for protoplast regeneration are very genotype-specific reducing its application and success.

II.3. Micropropagation stages

The plant micropropagation process aims to produce clones (true copies of a plant in large numbers). The following distinct stages are recognized for the micropropagation of most plants (Iliev et *al*., 2010; Sharma et *al*., 2015, Lal et *al*., 2021). The different stages of micropropagation are shown in figure 09.

Stage 0: Pre-propagation Stage

The pre-propagation stage requires proper maintenance of the mother plants in the greenhouse under disease and insect free conditions with minimal dust. Clean enclosed areas, glasshouses, plastic tunnels and net covered tunnels, provide high quality explant source plants with minimal infection.

Collection of explants for clonal propagation should be done after appropriate pre-treatment of the mother plants with fungicides and pesticides to minimize contamination in the *in vitro* cultures. This improves growth and multiplication rates of *in vitro* cultures. The control of contamination begins with the pretreatment of the donor plants.

The choice of explant depends on the methods of shoot multiplication to be followed. All plant organs viz. nodal segment, inter-nodal segments, shoot tip, root tip. For axillary bud induction, callus culture, somatic embryogenesis explants nodal segments, internodes and leaves are collected.

Stage 1: Initiation of Aseptic Culture:

In this stage sterilization of explants and establishment of explants were done. The plant organ used to initiate a culture is called explant. The choice of explant depends on the method of shoot multiplication to be followed:

- For micropropagation work the explant of choice is nodes
- For callus culture work the explant of choice is internodes and leaves.
- For somatic embryogenesis the explant is internodes and leaves.

Explant: The nature of explant to be used for *in vitro* propagation is governed by the method of shoot multiplication. For enhanced axillary branching, only the explants, which carry a pre-formed vegetative bud, are suitable. When the objective is to produce virus-free plants from an infected individual it becomes necessary to start with sub-millimeter shoot tips. If the stock is virus-tested or virus eradication is not necessary, then the most suitable explant is nodal cuttings. Small shoot-tip explants have a low survival rate and show slow initial growth. Meristem tip culture may also result in the loss of certain horticultural characteristics which are controlled by the presence of virus, such as the clear-vein character of the Geranium cv. Crocodile. Generally, the clear vein character is transmitted in petiole-segment culture but not in shoot-tip culture.

2. Sterilization: Special precautions need to be taken when explants are derived from field-grown materials, which is often necessary in cloning an elite tree. In such cases an ideal approach would be to take cuttings from the selected plant and grow them in greenhouse. Discarding the surface tissues from plant materials while preparing the explants also minimizes the loss of cultures due to microbial contamination.

3. Browning of medium: A serious problem with the culture of some plant species is the oxidation of phenolic compounds leached out from the cut surface of the explant. It turns the medium dark brown and is often toxic to the tissues. This problem is common with the adult tissues from woody species.

Stage 2: Multiplication of Culture:

This is the most important stage and the rate of multiplication determines the largely success of micropropagation system this can be achieved by:

- **Enhanced axillary branching:** The axillary bud present in the axil of each leaf either develops into a single shoot or form a cluster of shoots in the presence of cytokinins (BAP 1.0mg/l) in the medium.

- **Adventitious Bud Formation**: Buds arising from any part other than the leaf axils or shoot apex are called adventitious buds. It is a standard horticulture practice.
- **Through Callusing**: Plant cells are totipotent. In tissue culture, the mass of differentiated cells commonly known as callus. This either gives rise to shoot bud or bipolar structure resembling embryo (somatic embryo). This method is used when aim is to induce variability especially in self-pollinating species with narrow genetic base.

Stage 3: *In Vitro* Rooting of Shoots

In-vitro grown shoots lack root system. For induction of roots they were transferred to rooting medium. For rooting half strength MS medium supplemented with 1.0mg/l auxin was used.

Somatic embryos carry a pre-formed radical and may develop directly into plantlet. However, these embryos often show very poor conversion into plantlets, especially under *in vitro* conditions. They require an additional step of maturation to acquire the capability for normal germination. Adventitious and axillary shoots developed in cultures in the presence of a cytokinin generally lack roots. To obtain full plants the shoots must be transferred to a rooting medium which is different from the shoot multiplication medium, particularly in its hormonal and salt compositions. For rooting, individual shoots measuring 2 cm in length are excised and transferred to the rooting medium.

Stage 4: Hardening and Acclimatization of Tissue Culture Plantlets

This is the final stage and requires careful handling of plants. The transplantation from completely controlled conditions should be gradual.

This process of gradually preparing the plants to survive in the field conditions is called acclimatization. The plants produced in tissue culture, although green in color; do not prepare sufficient food for their own survival.

Also, inside the culture vessels, humidity is very high and thus the natural protective covering of cuticle is not fully developed. Therefore, immediately after transfer plants were maintained under high humidity. Optimum conditions were provided to plants in green house.

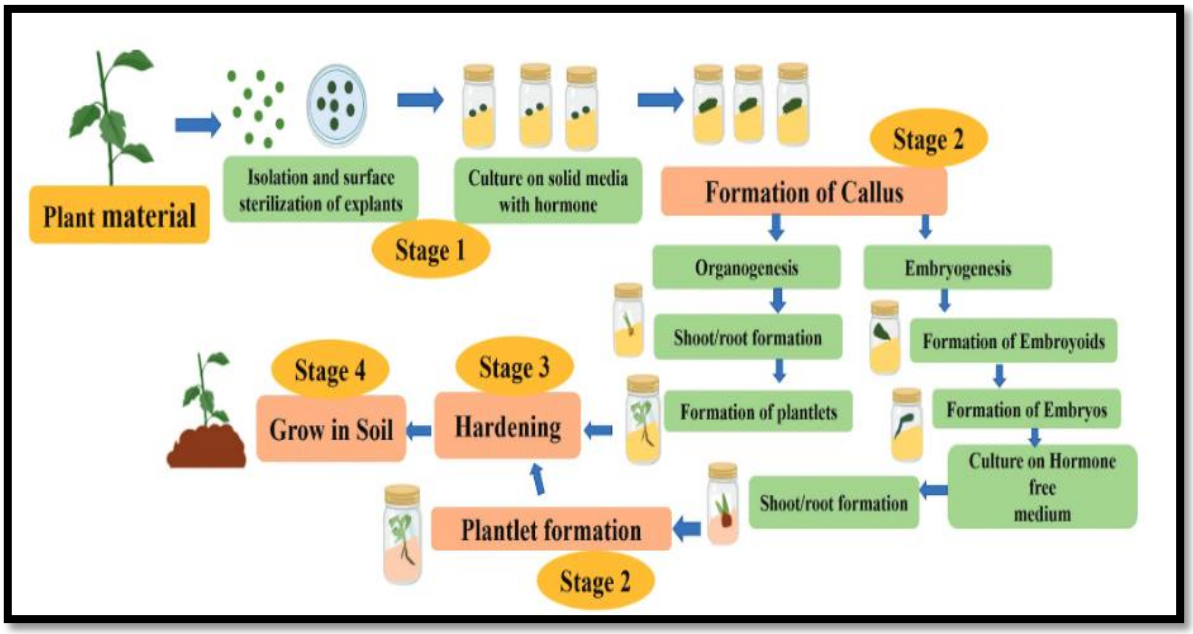

Figure 09: General methodology followed for tissue culture (Lal et *al*., 2021).

II.4. Somaclonals variations

Plant tissue culture techniques can sometimes produce vitroplants that differ from the mother plant (Guédira, 2016); these variations can be genotypic or phenotypic, which in the latter case can be either **genetic** or **epigenetic** in origin (Datta, 2019).

II.4.1. Epigenetic variations (Non-heritable Variations)

a. Definition

Epigenetic variations describe phenomena in which genetically identical cells or organisms express their genomes differently, causing phenotypic differences. These variations are generated during plant tissue culture, caused by temporary phenotypic changes and occur at low frequency (Datta, 2019).

b. Causes

Epigenetic variations are due to the physicochemical environment of the culture (Guédira, 2016), i.e.:

- Composition of the environment, which has become non-adapted as the growing period lengthens: depletion of certain elements, acidity and changes in osmotic pressure.
- Competition between seedlings in a space that has become too small.
- Confinement.

This leads to an evolution in the plant's gene regulation, resulting in non-conformities in the plant's subsequent development. Epigenetic variations generally disappear after transplanting or acclimatization, and even if they persist, they are not passed on to offspring.

II.4.2. Genetic variations (Heritable Variations)

a. Definition

Genetic changes that arise in vitro between clonal regenerants and their corresponding donor plants. These variations are: pre-existing variations in the somatic cells of explant, caused by mutations and other DNA changes and occur at high frequency (Datta, 2019).

b. Causes

The causes of somaclonal variations can be (Datta, 2019):

1. Physiological Cause

- Exposure of culture to plant growth regulators.
- Culture conditions

2. Genetic Cause

Change in chromosome number

- Euploidy: Changes chromosome Sets
- Aneuploidy: Changes in parts of chromosome Sets
- Polyploidy: Organisms with more than two chromosome sets
- Monoploidy: Organism with one chromasomes set

Change in chromosome structure

- Deletion
- Inversion
- Duplication
- Translocation

Gene Mutation

- Tansition
- Transversion
- Insertion
- Deletion

DNA sequence

- Change in DNA
- Detection of altered fragment size by using Restriction enzyme

- Change in Protein or Alteration in level of specific protein
- Methylation of DNA

3. Biochemical Cause

- Lack of photosynthetic ability due to alteration in carbon metabolism
- Biosynthesis of starch via carotenoid pathway
- Nitrogen metabolism
- Antibiotic resistance.

II.4.3. Somaclonal Variants Detection and Isolation

Somaclonal variations can be detected by several methods (Datta, 2019):

1. Analysis of morphological characters

- Qualitative characters: Plant height, maturity date, flowering date and leaf size.
- Quantitative characters: yield of flower, seeds and wax contents in different plant parts.

2. Variant detection by cytological Studies

- Staining of meristematic tissues like root tip, leaf tip with feulgen and acetocarmine provide the number and morphology of chromosomes.

3. Variant detection by DNA contents

- Cytophotometer detection of feulgen stained nuclei can be used to measure the DNA contents.

4. Variant detection by gel electrophoresis

- Change in concentration of enzymes, proteins and hemical products like pigments, alkaloids and amino acids can be detected by their electrophoretic pattern.

5. Detection of disease resistance variant

- Pathogen or toxin responsible for disease resistance can be used as selection agent during culture.

6. Detection of herbicide resistance variant

- Plantlets generated by the addition of herbicide to the cell culture system can be used as herbicide resistance plant.

7. Detection of environmental stress tolerant variant

- Selection of high salt tolerant cell lines in tobacco
- Selection of water-logging and drought resistance cell lines in tomato
- Selection of temperature stress tolerant in cell lines in pear.

- Selection of mineral toxicities tolerant in sorghum plant (mainly for aluminium toxicity)

II.4.4. Types of somaclonal variations

Based on the tissue from which variation originates, somaclonal variation can be divided into the following types (Fig.10) (Wijerathna-Yapa et *al*., 2022):

1. **Gametoclonal variation:** variation observed among the plants regenerated from gametic cultures.
2. **Androclonal variation**: observed among the plants regenerated from the anther (or) pollen culture.
3. **Gynoclonal variation:** from ovule (or) ovary culture.
4. **Protoclonal variation:** variation observed among the plants regenerated from protoplast cultures.
5. **Calliclonal variation:** variation observed among the plants regenerated from callus cultures.

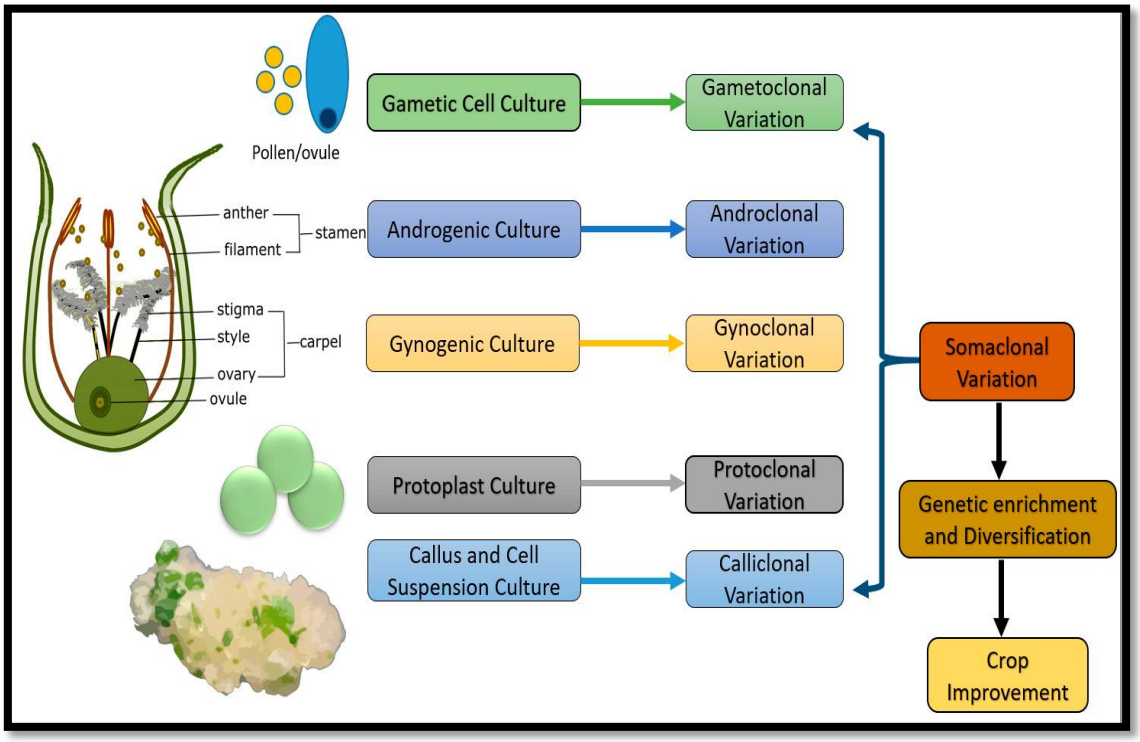

Figure 10 : Somaclonal Variations Types (Wijerathna-Yapa et *al*.,

II.4.5. Advantages of Somaclonal Variations

The benefits of somaclonal variations are (Datta, 2019):

- Help in crop improvement.
- Creation of additional genetic varitions.

- Increased and improved production of secondary metabolites.
- Selection of plants resistant to various toxins, herbicides, high salt concentration and mineral toxicity (abiotic stress).
- Suitable for breeding of tree species.

II.4.6. Disadvantages of Somaclonal Variations

The disadvantages of somaclonal variations are (Datta, 2019):

- A serious disadvantage occurs in operations which require clonal uniformity, as in the horticulture and forestry industries where tissue culture is employed for rapid propagation of elite genotypes.
- Sometime leads to undesirable results.
- Selected variants are random and genetically unstable.
- Require extensive and extended field trials.
- Not suitable for complex agronomic traits like yield, quality etc.
- May develop variants with pleiotropic effects, which are not true.

II.4.7. Ways of reducing somaclonal variation

According to (Datta, 2019) somaclonal variation can be avoided by:

- Increasing numbers of subculture, so the number of subcultures in micropropagation protocols should be kept to a minimum.
- Avoid 2,4-D in the culture medium, as this hormone is known to introduce variation.

Chapter III. *In vitro* culture techniques using reproductive cells: Haplomethodes

Introduction

Haplomethods are techniques for regenerating plants from male or female reproductive cells. The resulting cells are haploid with n chromosomes, and subsequently become diploid with 2n chromosomes, either naturally or artificially, so that they become fertile.

Although, the significance of haploids in genetics and plant breeding has been recognized for long time, production of haploids become an important component of biotechnology programs in different countries.

III.1. Haploid production methods

III.1.1. Spontaneous haploids

The different cases of **Spontaneous** haploid production are:

a. Polyembryony

Formation of two viable embryos from an embryo sac cell other than the oosphere; a synergid or antipodal cell, which develops alongside the fertilized oosphere. Two twin plants are obtained, one diploid and the other sometimes haploid (Kishore, 2014) (Fig11).

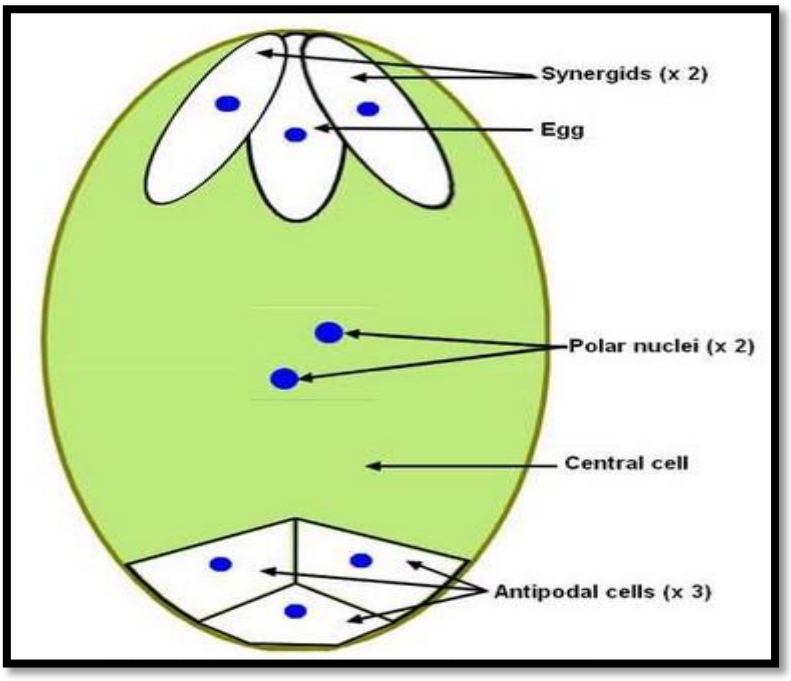

Figure 11: Embryo sac.

b. Parthenogenesis

This is a single-parent mode of reproduction, in which a cell from the mother fertilizes the ovum to form an embryo. Reproduction is carried out exclusively by females, without the intervention of a male gamete and with the development of an ovule without fertilization (Vijverberg et *al*., 2019). The embryo develops from an unfertilized female gamete, and the resulting plants are sometimes haploid.

III.1.2. Haploids induction *in vitro*

a. Androgenesis (motherless plants)

This is a technique for obtaining haploid plants from a culture of male cells: isolated anthers or microspores (Germanà, 2011).

1. Anther culture: this is a technically easier method, based on breaking the links between the mother plant and the anthers. It can be direct (embryos formed directly from explants) or indirect (embryos formed via callus).

It consists in removing intact anthers and placing them on an appropriate culture medium to form an embryo and obtain haploid plants.

2. Microspore cultivation: two cultivation techniques are possible:

2.1. Microspore culture with anther pre-culture: consists in culturing anthers while waiting for, or inducing, the release of microspores into the culture medium.

2.2. Isolated microspore culture without anther pre-culture: involves direct isolation of microspores after grinding the anthers, followed by filtration and centrifugation (for purification).

Factors affecting the success of androgenesis

Haploid induction is influenced by genotype, culture conditions, media components, and growth regulators (Shalaby, 2007; Bohanec, 2009).

1. Effect of the genotype: genetic factors are linked to the processes involved in androgenesis. The success of androgenesis is strongly linked to the plant family tested. Some genotypes have a high genetic variability to produce haploids; this ability is a trait that can be transmitted by crossing and self-fertilization, and its heritability is high (probability of transmission of the trait).

2. Effect of the physiological state of the donor plant: the growing conditions of anther and microspore donor plants appear to play a major role in the success of androgenesis.

These conditions: temperature, light and nutrition - must be precisely adjusted to ensure the success of the technique.

3. Anther collection stage: the stage at which the young, uninucleated microspore enters mitosis is the optimum for successful androgenesis.

b. Gynogenesis (fatherless plants)

The term gynogenesis is used for all haploid induction methods in which a female gametophyte is used as the origin of the haploid cells, regardless of whether it is a pseudofertilization process or not. *in vitro* gynogenesis is a technique used for obtaining haploids by unfertilized ovaries or ovules culture (Dong et *al*., 2016).

All attempts to regenerate plants using *in vitro* culture of an unfertilized ovum had failed, and it was not until 1976 that the first results were obtained by cultivating unpollinated barley ovaries.

Procedure

In the case of self-pollinated species, haploid are regenerated via *in vitro* culture of unfertilized female gametes by culturing flower buds prior to anthesis. However, in male-sterile or self-incompatible plants it is performed at any stage of ovule development, as they show a favorable response to gynogenic induction (Asif, 2013).

Explant pre-treatment:

Androgenesis and gynogenesis can be achieved by various physical or chemical pretreatments such as chilling, high temperature, high humidity, water stress, anaerobic treatment, centrifugation, sucrose and nitrogen starvation, ethanol, c-irradiation, microtubuli disruptive agents, electrostimulation, high medium pH and heavy metal treatment (Shariatpanahi et *al*., 2006).

1. Cold:

- Stimulates even microspore division.
- Stimulates embryo formation.
- Preservative action (maintains explant survival).

2. High temperatures:

- ✓ Some species require high temperatures to stimulate androgenesis and gynogenesis.

3. Mannitol:

- ✓ Stimulates androgenesis.

These pretreatments can also slow down the differentiation of microspores into pollen grains.

III.1.3. Haploids induction *in situ*

a. Interspecific crosses

The ovule of one species is fertilized by pollen from a distant species. The incompatible chromosomes of the pollinating parent are naturally rejected (Niu et *al*., 2014).

The haploid embryo develops, and an embryo rescue phase is necessary (Wei et *al*., 2018), otherwise the embryo degenerates and dies. The embryo is cultured, and the tissues differentiate to produce a haploid plant.

b. Crosses with irradiated (denatured) pollen

Pollen irradiation with UV, gamma rays, and X rays is the most commonly used technique to induce *in situ* parthenogenetic haploid plants production.

In some species, it has become imperative to induce gynogenesis by culturing ovaries pollinated with gamma-irradiated pollen (Chahal and Gosal, 2002). The aim of pollen irradiation is to disrupt sperm differentiation in male gametes, inhibiting fertilization. The oosphere can then divide without fertilization, giving rise to haploid embryo, and embryo rescue is necessary.

III.2. Stages of haplodiploidization

The haplodiploidization steps are (Adly, 2013):

1. Obtaining mother plants

- By crossing two parents with interesting and complementary characteristics.
- This cross will produce the F1 generation, the mother plants for haploid production.

2. Obtaining the haploid phase

- This involves recovering the cells that have undergone meiosis before fertilization, and this is where the laboratory work begins.
- Haploid plants can be obtained in *vitro* or *in situ*.

3. Return to the diploid fertile state

- To use plants regenerated by one of the haplomethods in breeding, diploid plants must be available.
- As the haploid state is unstable, the regenerated individual can be diploid: spontaneous doubling.
- If not, chromosomes can be artificially doubled using the chemical agent colchicine.
- The resulting plants are homozygous diploids: they have two identical copies of each of their chromosomes, and therefore carry identical pairs of genes.

Treatment with colchicine: can be carried out at the seedling stage, by soaking the roots or injecting the meristems.

4. Line selection

- The material thus fixed is delivered to the breeder, who will sort the plants according to the agronomic and technological criteria required.
- These plants are propagated by self-fertilization: all offspring are identical copies of their parents.

III.3. Advantages and uses of haploids

The advantages and the importance of haploid production are (Forster et *al*., 2007; Rajcan et *al*., 2011).

- ✓ Production of homozygous genotypes in a single generation.
- ✓ Rapid fixation of genetic material, facilitating the selection process.
- ✓ Expression of recessive genes.
- ✓ Microspore culture is a single-cell culture: all cells have n chromosomes (no mixing).
- ✓ Production of homozygous varieties in self pollinated crops.
- ✓ In cross-pollinated crops, the derivation from heterozygous material of pure lines for use as parents of the intended single cross or double cross hybrids.
- ✓ The obvious advantage of haploids is that they display mutations with successive effects in single dose.
- ✓ Effective fixation by chromosome doubling on transformation.
- ✓ Double haploid plants are also used in mutagenesis, biochemical, and physiological studies.
- ✓ Development of pure lines and disease resistant lines for mildew and yellow mosaic-barley.
- ✓ Parthenogenetic haploids in maize.
- ✓ Recovery of sexual inter specific hybrids between wild and domestic species – tomato.
- ✓ Development of pure lines and 100% male plants in asparagus.
- ✓ Complex hybrids for disease resistance in coffee.

III.4. Limits of haplomethods

III.4.1. Case of species recalcitrant to androgenesis

The success of androgenesis is partial or poor, or even non-existent.

Example: durum wheat: the induction and embryo production phases are generally similar to those of non-recalcitrant species, but problems arise above all during the regeneration

phase, when the embryos of most genotypes regenerate only albino plants or roots (Touraev et *al*., 2009).

III.4.2. Problem of albinism

Albinism is a common problem encountered in interspecific crosses and *in vitro* plants tissue culture experiments. It is characterized by partial or complete loss of chlorophyll pigments and incomplete differentiation of chloroplast membranes (Kumari et *al*., 2009).

1. Characteristics of albino plants

a. Phenotype: the leaf apparatus of albino plants is identifiable by a white or yellow color, and stems and leaves are devoid of chlorophyll pigments.

Albino plants are unable to produce functional chloroplasts, and therefore unable to carry out photosynthesis.

b. Morphology: Morphological disturbances of the chloroplasts.

c. Biochemistry: absence of chlorophyll a and b.

d. Genetics: reduction and alteration of chloroplast DNA copies.

2. Causes of albinism: albinism is due to:

- ✓ Incomplete differentiation of proplasts into chloroplasts.
- ✓ Rapid cell divisions that inhibit the synthesis of sufficient quantities of the specific structural proteins needed to form chloroplasts.

3. Results on plants: we can observe:

- ✓ A deficiency of cytoplasmic organelles: chlorophyll deficiency.
- ✓ Albinism may be initiated during the regeneration phase, or it may set in early during the androgenesis process.

4. Solution:

- ✓ The *in vitro* androgenesis reaction is hampered by the problem of albinism. Several pretreatments have been developed to reduce the risk of albinism: heat shock, osmotic shock and chemical pretreatments.

Example: a research project carried out by French researchers at INRA: protoplast fusion manipulation in rapeseed for the creation of hybrid varieties.

In the first stage, carried out by successful interspecific sexual hybridization with Radish, a male sterile Rapeseed was obtained; but this sterility was accompanied by a serious chlorophyll deficiency: yellow plants.

The anomaly was due to the transfer of Radish cytoplasm into the rapeseed cell: the mitochondria transmitted male sterility, the desired trait, but the chloroplasts transferred at the same time malfunctioned.

By fusing the protoplasts and regenerating them, it was possible to substitute the chloroplasts of the Turnip plant, one of Rapeseed's ancestors, for those of Radish.

Navette chloroplasts were also used to transfer herbicide resistance to rapeseed.

The resulting rapeseed therefore has the normal rapeseed nucleus, chloroplasts from the Turnip and mitochondria from the Radish.

III.4.3. Gametoclonal variations

The variation observed among the plants regenerated from *in vitro* culture of gametic cells is called gametoclonal variation. Such variations result from genetic recombination and are the core interest of plant breeders because of their simultaneous expression of dominant and recessive mutations. Gametoclonal variation can be obtained through haploid production androgenic gynogenic *in vitro* techniques (Shahzad et *al*., 2017).

Haploids can be developed either through the male gametophyte (anther and microspore) or female gametophyte (ovules and ovary) via *in vitro* culture approaches known as androgenesis and gynogenesis, respectively. However, these androgenic and gynogenic haploids are highly variable and susceptible to changes in ploidy level, gene shuffling, and mutation. Moreover, haploids are desirable and captivated by a plant breeder because of a single allelic gene present in a chromosome, which leads to the expression of traits governed by a recessive gene. Therefore, genetic manipulation and manifestation become easy and are able to induce mutation and enhance the genetic background of crops (Chadipiralla et *al*., 2020).

The variations observed in regenerated plants can be explained by (Datta, 2019):

1. Changes in chromosome number or structure (deletion, insertion, substitution).
2. Cytoplasmic mutations.
3. Changes in DNA quantity.

Gametoclonal variations may offer advantages in the selection of salinity-resistant plants.

Chapter IV: Applications, Advantages and Disadvantages of Plant *In vitro* Cultures

Introduction

Agronomic crops i.e., cereals, fruits, vegetables, ornamental plants and forest trees are currently being used for *in vitro* propagation.

The *in vitro* plant propagation offers potential applications as it guarantees a sustainable agricultural development providing solutions to major food security issues.

Plant tissue culture coupled with biotechnological approaches is applicable to the development of genetically modified plants as well as embryo rescue procedures (Sadiku et *al*., 2018). It plays a pivotal role for the production of transgenic plants with improved traits (Hasnain et *al*., 2020). Various crops with superior traits have been developed using this technology with enhanced nutritional value and biotic/abiotic stress resistance that leads to increased crop yield (Ragavendran and Natarajan, 2017). Different transcription factors, which regulate nutrient assimilation pathways, have been over expressed in crops that may improve crop yield (Hasnain et *al*., 2020).

IV.1 Application of *in vitro* culture in agriculture

According to Lambardi et *al*. (2008), Hussain et *al*. (2012) and Hasnain et *al*. (2022), Plant tissues cultures with different explants are used for:

IV.1.1. Creating new plant varieties

- ✓ Obtain plants resistant to biotic and abiotic stresses.
- ✓ Improvement of the technological qualities of agricultural products: modification of the physico-chemical composition of products (lipids - carbohydrates - etc.).
- ✓ Introduction of new cultivars eg. Dutch iris. Get 5 daughter bulbs annually. Takes 10 years for commercial quantities of new cultivars to be built up. Can get 100-1000 bulbs per stem section.
- ✓ Pathology - Eliminate viruses, bacteria, fungi etc. Use heat treatment and meristem culture. Used routinely for potatoes, carnation, mum, geranium, garlic, gypsophila

IV.1.2. Plant propagation and selection

- ✓ Clonal mass propagation. The important point here is that extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting, one can obtain upwards of 1,000,000 plants per year from one initial explant.
- ✓ Difficult or slow to propagate plants. Micropropagation enables growers to increase the production of plants that normally propagate very slowly such as narcissus and other bulbous crops.

- ✓ Vegetative propagation of sterile hybrids used as parent plants for seed production. Eg. cabbage.
- ✓ Time is a limiting factor in the breeder's work to produce new varieties. Reducing the length of selection cycles and the time needed to fix and multiply interesting genotypes is one of the breeder's main concerns.
- ✓ The haplodiploidization techniques used in breeding programs enable pure lines to be fixed very quickly. As a result, *in vitro* multiplication techniques can considerably increase the rate of multiplication per unit of time, and thus reduce production costs.
- ✓ The transfer of the male sterility gene makes it possible to obtain sterile plants, which are highly sought-after for their high, stable yield and high resistance.

IV.2. Advantages of *in vitro* culture

Micropropagation has a number of advantages over traditional plant propagation techniques (Sinclair et *al*., 2004; Rajput et *al*., 2023):

- o The main advantage of micropropagation is the production of many plants that are clones of each other. Many genetically identical plants can be created from one parent plant. Because plants are clones, the uniformity assures quality.
- o Used to produce disease-free plants (Healthier plants).
- o Allows many plants to growing in a small place in a short time
- o It can have an extraordinarily high fecundity rate, producing thousands of propagules while conventional techniques might only produce a fraction of this number.
- o Regenerating genetically modified cells or cells after protoplast fusion.
- o It is useful in multiplying plants which produce seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed cannot be stored (see recalcitrant seeds).
- o Some plants with very small seeds, including most orchids, are most reliably grown from seed in sterile culture.
- o A greater number of plants can be produced per square meter and the propagules can be stored longer and in a smaller area.

IV.3. Disadvantages of *in vitro* culture

Micropropagation is not always the perfect means of multiplying plants. Conditions that limits its use include (Sharma et *al*., 2015; Sharma et *al*., 2021):

- o It is very expensive, and can have a labour cost of more than 70%.
- o An infected plant sample can produce infected progeny.

- Not all plants can be successfully tissue cultured, often because the proper medium for growth is not known or the plants produce secondary metabolic chemicals that kill the explant.
- Some plants are very difficult to disinfect of fungal organism.

IV.4. Limits of *in vitro* culture

IV.4.1. Tissue vitrification problems

Tissue vitrification is an accident that cannot be foreseen at the outset, and can occur during *in vitro* cultivation, in the same way as malformations. Tissue vitrification is a major handicap to successful *in vitro* organogenesis propagation of certain plants **(**Bougeriaoui and Zaid, 1993).

a. Initiation phase: vitrified explants have spongy tissues that eventually become necrotic during successive transplanting.

b. In the multiplication phase: young shoots often have thick, turgid leaves, sometimes with a chlorophyll deficiency; their growth is very weak and accompanied by a low rate of multiplication.

Factors inducing this phenomenon: certain ions ($NH4^+$), certain growth regulators, temperature, humidity of the culture medium, ethylene.

IV.4.2. Loss of interesting characteristics

- Repeated production of a large number of uniform plants (clones) can lead to loss of the necessary genes, so it is necessary to keep the mother plants and go back to sexual reproduction.

IV.4.3. Technical problems

1. **Asepsis of the explant:** viruses, bacteria and fungi are not totally eliminated.
2. **Acclimatization:** somaclonal and gametoclonal variations.
3. **Difficulty in setting up *in vitro* cultures**: **a.** For some plants, *in vitro* installation requires in-depth research: sterilization - multiplication - ...etc. **b.** Skilled labor required.

Conclusion

Plant tissue culture (PTC) refers to the cultivation of undifferentiated mass of plant cells, tissues or organs on artificial media under aseptic and controlled environmental conditions. Any plant organ like leaf, apical meristem, embryo, cotyledon, hypocotyl, etc., can be used as an explant and whole plants can be regenerated *in vitro.*

Plant tissue culture media used for *in vitro* cultures is mainly composed of inorganic and organic supplements, carbon source, plant growth hormones, vitamins, gelling agents, antibiotics, etc.

Tissue culture can be categorized as organ culture, explant culture, callus culture, cell suspension culture, protoplast culture or single cell culture.

Plant tissue culture is routinely used for several applications in plant science, such as in micropropagation, synthetic seed formation, protoplast culture, haploid or triploid culture, virus free plants production, secondary metabolites production, etc.

Plant growth hormones play a vital role in plant tissue culture especially, different ratios of auxin and cytokinin are employed for either root or shoot regeneration depending upon the need or objective.

Somatic hybridization in plant tissue culture can be exploited to produce distantly related plants as well. Cultured cells or tissues may accumulate a higher concentration of secondary metabolites than its parents, under optimum environmental and nutritional conditions.

Several compounds of industrial importance have been successfully produced in tissue culture, like taxol, azadirachtin, shikonin.

Exercises

Exercise 01: Answer the following questions

1. What is plant tissue culture?

2. Describe the various components of plant tissue culture media.

3. What are the general steps of plant tissue culture?

4. Describe various applications of plant tissue culture.

5. How are somatic hybrids developed?

6. What are somaclonal variations?

7. Define explant and list five most commonly used explants for plant tissue culture.

8. Describe somatic embryogenesis and their application for the development of synthetic seeds.

9. Describe briefly the role of pH in nutrient media.

10. Describe the method of somatic hybridization and its advantages.

11. What are somaclonal variations and discuss their role for improving crops.

Exercise 02: Multiple Choice Questions

A. Which of the following tissues can be used as explant for regenerating complete plant through tissue culture?

1. Shoot apical meristem
2. Embryo
3. Leaf segments
4. All of the above

B. Which of the following explants are suitable for the production of virus free plants?

1. Leaf segments
2. Seeds
3. Apical meristem
4. Stem cuttings

C. The process of combining the nuclear genomes of one parent with the cytoplasmic genome of the other parent is called as:

1. Cybridization
2. Micropropagation
3. Regeneration

4. None of them

D. Which of the following components is not essential for Murashige and Skoog media?

1. Inorganic nutrients
2. Carbon source
3. Antibiotics
4. Organic Nutrients

E. Decrease in the pH of the media may result in:

1. Increase in hardiness of the solidified medium.
2. May interfere with the solubility of media salts.
3. Interfere with solidification of the medium and results in poor solidification.
4. All of the above.

F. Somatic clonal variation can be present in which of the following plants?

1. Plants regenerated through tissue culture
2. Plant generated through seeds
3. Plant generated through sexual reproduction which includes fertilisation of egg with pollen nuclei.
4. None of the above.

G. *In vitro* tissue culture can be used for the generation of:

1. Virus free plants
2. Somatic hybrid plants
3. Synthetic seeds
4. None of the above.

H. **Assertion:** Somatic seeds are encapsulated by a layer called seed coat.

Reason: Seed coat is the protective layer which prevents water desiccation.

1. Both assertion and reason are true and the reason is the correct explanation of the assertion.
2. Both assertion and reason are true but the reason is not the correct explanation of the assertion.
3. Assertion is true but reason is false.
4. Both assertion and reason are false.

I. **Assertion**: Virus free plants can be produced by growing apical/axillary meristem of virus-infected plants.

Reason: Apical/axillary meristems lack vascular bundle, which is required by the virus to replicate.

1. Both assertion and reason are true and the reason is the correct explanation of the assertion.
2. Both assertion and reason are true but the reason is not the correct explanation of the assertion.
3. Assertion is true but reason is false.
4. Both assertion and reason are false.

Exercise 03: Choice Questions

1. The most important methods currently utilized for haploid production include …….
   a) Anther or pollen culture
   b) Ovule culture
   c) Bulbosum technique
   d) All the above
2. Bulbosum technique is ……..
   a) Chromosome elimination
   b) Chromosome elimination following interspecific hybridization
   c) Chromosome elimination following intraspecific hybridization
   d) None of the above
3. Bulbosum technique is used for ……..
   a) Haploid production
   b) Triploid production
   c) Tetraploid production
   d) None of the above
4. The development of numerous pollen plantlets in anther culture of *Datura innoxia* was first reported by ……
   a. Guha
   b. Maheswari
   c. Both a & b
   d. None of the above

5. The process of parthenogenesis is ……
    a. Embryo development from fertilized egg
    b. Embryo development from unfertilized egg
    c. Embryo development
    d. None of the above
6. The capacity for haploid production ……
    a. Declines with age of donor plants
    b. Increases with age of donor plants
    c. Unaffected with age of donor plants
    d. None of the above
7. In pollen culture, isolation of pollen grains from the cultured anthers is by ……
    a. Mechanical method
    b. Float culture method
    c. Both a & b
    d. None of the above
8. Pollen dimorphism is exhibited by ……
    a. Tobacco
    b. Wheat
    c. Barley
    d. All the above
9. *In vitro* androgenesis is promoted by pretreatment of cultured anthers/pollen grains viz.
    a. Temperature shock
    b. Centrifugation
    c. γ irradiation
    d. All the above
10. Addition of..... found to increase the success rate of androgenesis.
    a. Ethrel
    b. Sucrose
    c. Nutrients specific to certain genotype
    d. All the above

- Important Terminology -

Aseptic – The state of being free of contaminating organisms (bacteria, fungi, algae and all micro-organisms except viruses : Free from microorganisms)

Callus – A mass of thin-walled, undifferentiated plant cells, meristematic (high regeneration capacity) in nature. It developed as the result of culture on nutrient media.

Clone –genetically identical plants developed by process of in-vitro tissue culture, vegetative propagation or, without involving sexual process.

De-differentiation – The term is used to denote the process of formation of unorganized tissues from the highly organised tissues.

Differentiation – A process in which unspecialized cells develop structures and functions characteristic of a particular type of cell. Development from one cell to many cells, accompanied by a modification of the new cells for the performance of particular functions. In tissue culture, the term is used to describe the formation of different cell types. The term differentiation is used in many different senses in biology. In broad sense, it is defined as the process by which meristematic cells are converted into two or more types of cells, tissues or organs which are qualitatively different from each other.

Excision – Cutting out and preparing a tissue, organ, etc., for culture.

Explant – Part of an organism used in "*in vitro*" culture. The excised piece of differentiated tissue or the organ which is used for culture is called as explant (Donar plant) e.g., embryos, young leaf, bud, etc Tissue aseptically obtained and prepared from the donor plant for culture

Hardening off – Adapting plants to outdoor conditions by gradually withholding water, lowering the temperature, increasing light intensity, or reducing the nutrient supply. The hardening-off process conditions plants for survival when transplanted outdoors. The term is also used for gradual acclimatization to in vivo conditions of plants grown *in vitro*, e.g., gradual decrease in humidity. cf acclimatization; free-living conditions.

IAA Indoleacetic acid – a plant hormone increasing cell elongation and, under certain circumstances, implicated in stimulating cell division and root formation. IAA moves in a polar manner in plants forming an IAA gradient in tissues. Orientation of plant organs, then,

influence callus formation and morphogenesis. "*in vitro*" "In glass"; as in tissue culture methods

In vitro – Test tube culture, outside the natural environment, in an artificial environment, typically in glass vessels in which cultured cells, tissues, or whole plants may reside.

In vivo – The natural conditions in which living organism or cell live.

Inoculum – A small piece of tissue cut from callus, or an explant from a tissue transferred into fresh medium for continued growth of the culture.

Inositol – C6H6(OH)6, A water-soluble nutrient frequently referred to as a "vitamin" in plant tissue culture.

Meristem – Undifferentiated tissue, the cells of which are capable of active cell division and differentiation into specialized and permanent tissue such as shoots and roots.

Morphogenesis – Attainment of biological organization or form is termed as morphogenesis. Under *in vitro* conditions this can be achieved by two routes: *de novo* origin of organs, either shoots or roots from the cultured tissues precisely termed as organogenesis and *de novo* origin of embryos with distinct root and shoot poles on opposite ends from the somatic cells or cells cultured *in vitro*, otherwise called as somatic embryogenesis. The historical background, achievements and the causes for the two routes are discussed below.

Organ Culture – This term is used for in-vitro culturing of organs like embryo, root or shoot apices.

Organogenesis – In plant tissue culture, organogenesis means genesis of organs like shoots, roots, leaves, flowers, etc.

Re-differentiation – The process of differentiation occurring in an undifferentiated tissue.

Regeneration – It is defined as the structuring of any part, which has been removed or physiologically isolated from the organism. In other words, genesis of an entire plant from cultured explants directly or *via* callus indirectly is called regeneration.

Root hairs – Epidermal cell extensions of young root that increase absorptive surface area.

Scarification – The chemical or physical treatment given to some seeds (where the seed coats are very hard or contain germination inhibitors) in order to break or weaken the seed coat sufficiently to permit germination.

Suspension Culture – Defined as the culture of cell and cell aggregates suspended in a liquid medium.

Tissue Culture – The in-vitro culture of the tissue e.g. Callus culture

Totipotency – The establishment of missing plant organs or parts; formation of a whole plant from a few cells or small portion of a plant.

Totipotency---A cell characteristic in which it has potential for forming all the cell types and develop in the entire organism.

Viability – The capability to live and develop normally.

Viable – Capable of germinating, living, growing and developing.

References

Adly M. (2013). Genetical studies on haploid production in some ornamental plants. Thesis: Department of Agriculture Botany Faculty of Agriculture, Cairo Al-Azhar University. 187p.

Asif M. (2013). Progress and Opportunities of Doubled Haploid Production; Springer International Publishing: New York, NY, USA, p. 75, ISBN 978-3-319-00732-8.

Bentahar S., Abada R. and Ykhlef N. (2023). Biotechnology: Definitions, types and main applications. YMER Digit., 22, 563–575.

Bhatia S. (2015). Plant Tissue Culture. In Modern Applications of Plant Biotechnology in Pharmaceutical Sciences. Pages 31-107. https://doi.org/10.1016/B978-0-12-802221-4.00002-9.

Bohanec B. (2009). Doubled haploids *via* gynogenesis. In: Touraev A., Forster B.P. and Jain S.M. (Eds.), Advances in Haploid Production in Higher Plants. Springer, pp. 35–46. DOI: 10.1007/978-1-4020-8854-4_2.

Bougeriaoui M. and Zaid, A. (1993). Effect of the ammonium nitrate content of the culture medium on the vitrification of date palm (*Phoenix dactylifera* L.) tissues cultivated *in vitro*. AL AWAMIA, 82: 177-196. (In French).

Chadipiralla K., Gayathri P., Rajani V. and Reddy P.V.B. (2020). Plant Tissue Culture and Crop Improvement. In Sustainable Agriculture. In the Era of Climate Change; Roychowdhury, R., Choudhury, S., Hasanuzzaman, M., Srivastava, S., Eds.; Springer International Publishing: Cham, Switzerland, pp. 391–412; ISBN 978-3-030-45669-6.

Chahal G.S. and Gosal S.S. (2002). Principles and procedures of plant breeding. Alpha Science, Oxford.

Datta S. (2019). Somaclonal Variation. Class Presentation; B.Tech, Plant Biotechnology; BT-603. 11p. DOI: 10.13140/RG.2.2.30417.17760

Diallo M.S., Ndiaye A., Sagna M. and Gassama-Dia Y.K. (2008). Plants regeneration from African cowpea variety (*Vigna unguiculata* L. Walp.). Af. J. Biotech. 7, 2828-2833.

Dong Y.Q., Zhao W.X., Li X.H., Liu X.C., Gao N.N., Huang J.H., Wang W.Y., Xu X.L. and Tang Z.H. (2016). Androgenesis, gynogenesis, and parthenogenesis haploids in cucurbit species. Plant Cell Rep., 35, 1991–2019.

Forster B.P., Heberle-Bors E., Kasha K.J. and Touraev A. (2007). The resurgence of haploids in higher plants. Trends Plant Sci. 12: 368–375. https://doi.org/10.1016/j.tplants.2007.06.007.

Germanà M.A. (2011). Gametic embryogenesis and haploid technology as valuable support to plant breeding. Plant Cell Rep. 30, 839–857.

Godoy S., Tapia E., Seit P., Andrade D., Sánchez E., Andrade P. *et al*. (2017). Temporary immersion systems for the mass propagation of sweet cherry cultivars and cherry rootstocks : development of a micropropagation procedure and effect of culture conditions on plant quality. In vitro Cell Dev Biol Plant; 53:494–504; doi: 10.1007/ s11627-017-9856-z

Guédira A. (2016). Plant biotechnology course in vitro culture. Rabat University Faculty of Natural and Life Sciences. 294p (In French).

Hasnain A., Irfan M., Bashir A., Maqbool A. and Malik K.A. (2020). Transcription factor TaDof1 improves nitrogen and carbon assimilation under low-nitrogen conditions in wheat. Plant Mol. Biol. Rep. 38, 441–451. doi: 10.1007/s11105-020-01208-z

Hasnain A., Naqvi S.A.H., Ayesha S.I., Khalid F., Ellahi M., Iqbal S., Hassan M.Z., Abbas A., Adamski R., Markowska D., Baazeem A., Mustafa G., Moustafa M., Hasan M.E. and Abdelhamid M.M.A. (2022). Plants in vitro propagation with its applications in food, pharmaceuticals and cosmetic industries; current scenario and future approaches. Front. Plant Sci. 13:1009395. doi: 10.3389/fpls.2022.1009395

Hussain A., Qarshi, I.A., Nazir H. and Ullah I. (2012). Plant tissue culture: current status and opportunities. Recent Adv. Plant Vitro culture., 1–28. doi: 10.5772/505668

Iliev I., Gajdoˇsova A., Libiaková G. and Jain S.M. (2010). "Plant micropropagation," in Plant Cell Culture: Essential Methods, M.R. Davey and P. Anthony, Eds. United Kingdom: John Wiley and Sons, pp. 1-23.

Jain S.M. (2007). Biotechnology and mutagenesis in genetic improvement of cassava (*Manihot esculenta*). Gene Conserve 6 (23): 329-343.

Kaya Y. and Huyop F.Z. (2020). An easy and reliable method for establishment and maintenance of tissue cultures of *nicotiana tabacum* cv TAPM 26. Int. J. Sci. Lett. 2 (2), 62–71. doi: 10.38058/ijsl.764947

Kaya Y. and Karakütük, S. (2018). Effects of different growth regulators on regeneration of Turkish upland rice. Anadolu. Tarım. Bilimleri. Dergisi. 33 (3), 226–231. doi: 10.7161/omuanajas.407998.

Kishore K. (2014). Polyembryony: In book: Reproductive Biology of Plants. In Reproductive Biology of Plants. Eds. Ramawat K., Merillon J.M. and Shivanna K. (Boca Raton: CRC Press), 133–146. doi: 10.1201/b16535-16

Kumar N. and Reddy M.P. (2011). *In vitro* Plant Propagation: A Review. Jpurnal of Forest and Environmental Science 27 (2): 61-72.

Kumari M., Clarke H.J., Small I. and Siddique K.H. (2009). Albinismin plants: a major bottleneck in wide hybridization, androgenesis and doubled haploid culture. Critical Reviews in Plant Sci. 28 (6): 393-409.

Lal S., Kumari A., Guleria I. , Dhatwalia J., Thakur S., Kumari S. and Sharma S. (2021). An insight on micro propagation of Myrica species for improvement in cultivation practices of nutraceutically important fruits. Journal of Applied Biology & Biotechnology Vol. 9 (06), pp. 34-40. DOI: 10.7324/JABB.2021.9604.

Lambardi M., Ozudogru E.A. and Benelli C. (2008). Cryopreservation of embryogenic cultures. In Plant cryopreservation: Pract. guide (Springer, New York, NY) pp, 177–210).

Long Y., Yang Y., Pan G. and Shen Y. (2022). New Insights Into Tissue Culture Plant-Regeneration Mechanisms. Front. Plant Sci., 13: 1-15. https://doi.org/10.3389/fpls.2022.926752

Murashige T and Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum.15:473-497.

Niu Z., Jiang A., Abu Hammad W., Oladzadabbasabadi A, Xu S.S., Mergoum M. and Elias, E.M. (2014). Review of doubled haploid production in durum and common wheat through wheat_maize hybridization. Plant Breed. 133, 313–320.

Pasternak T., Lystvan K., Betekhtin A. and Hasterok R. (2020). From single cell to plants: Mesophyll protoplasts as a versatile system for investigating plant cell reprogramming. Int. J. Mol. Sci., 21, 4195.

Ragavendran C. and Natarajan D. (2017). Role of plant tissue culture for improving the food security in India: A review update. In A. Dhanarajan (Ed.), Sustainable agriculture towards food security (pp. 231–262). https://doi.org/10.1007/978-981-10-6647-4_13

Rajcan I., Boersma J.G. and Shaw E.J. (2011). Plant genetic techniques: Plant breeder's toolbox: In Comprehensive Biotechnology; Moo-Young M. Ed.; Elsevier: Amsterdam, The Netherlands, 2011; pp. 133–147, ISBN 978-0-08-088504-9.

Sadiku M., Ashaolu T.J. and Musa, S. (2018). Biotechnology: A primer. Int. J. Eng. Res. Advanced. Technol. 4, 87–91. doi: 10.31695/IJERAT.2018.3325

Sawant M.S. (2021). Project report on Plant Tissue Culture. Regional Institute of co-operative Management. At/Post-Harali Kh., Tal- Gadhinglaj, Dist-Kolhapur,State-Maharashtra ,416502

Shahzad A., Parveen S., Sharma S., Shaheen A., Saeed T., Yadav V., Akhtar R., Ahmad Z., Upadhyay A. (2017). Plant Tissue Culture: Applications in Plant Improvement and Conservation. In Plant Biotechnology: Principles and Applications; Abdin, M.Z., Kiran, U., Kamaluddin, A.A., Eds.; Springer: Singapore, pp. 37–72; ISBN 978-981-10-2961-5.

Shalaby T.A. (2007). Factors affecting haploid induction through *in vitro* gynogenesis in summer squash (*Cucurbita pepo* L.). Scientia Horticulturae 115: 1–6. DOI: 10.1016/j.scienta.2007.07.008.

Shariatpanahi M.E., Bal U., Heberle-Bors E. and Touraev A. (2006). Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. Physiol Plant 127:519–534

Sharma A.K., Sharma V., Agarwal V.P. and Sharma N.K. (2021). Compendium on advances in plant tissue culture and its applications in agriculture. Published under:

National Higher Education Project. Swami Keshwanand Rajasthan Agricultural University. Beechwal. Bikaner – 334006.

Sharma G., Jagetiya S., Dashora R. (2015). General Techniques of Plant Tissue Culture; Lulu Press, Inc.: Morrisville, NC, USA.

Shen X., Gmitter F.G. and Grosser J.W. (2011). Immature embryo rescue and culture. Methods Mol Biol., 710: 75-92. doi: 10.1007/978-1-61737-988-8_7. PMID: 21207263.

Sinclair T.R., Purcell L.C. and Sneller C.H. (2004). Crop transformation and the challenge to increase yield potential. Trends Plant Sci. 9 (2), 70–75. doi: 10.1016/j.tplants.2003.12.008

Tomar U.K. and Dantu P.K. (2010). Protoplast Culture and Somatic Hybridization, in Cellular and Biochemical Sciences, Tripathi G, editor. I.K. International House Pvt Ltd New Delhi: 877-890.

Touraev A., Forster B.P. and Jain S.M. (2009). Advances in Haploid Production in Higher Plants; Springer: Berlin/Heidelberg, Germany.

Verma N., Bansal M. and Kumar V. (2008). Protoplast fusion technology and its biotechnological applications. Chem. Eng. Trans. 14: 113–120.

Vidyagina E.O., Kharchenko N.N. and Shestibratov K.A. (2021). Efficient cryopreservation of populustremula by *in vitro*-grown axillary buds and genetic stability of recovered plants. Plants (Basel). 2-10 (1), 77. doi: 10.3390/ plants10010077.

Vijverberg K., OziasAkins P. and Schranz M.E. (2019). Identifying and Engineering Genes for Parthenogenesis in Plants. Front. Plant Sci. 10: 128

Wang M.R., Cui Z.H., Li J.Y., Hao X.Y., Zhao L. and Wang Q.C. (2018). *In vitro* thermotherapy-based methods for plant virus eradication. Plant Methods. 14:87, 1-18. https://doi.org/10.1186/s13007-018-0355-y

Wei Y., Zhu M., Qiao H., Li F., Zhang S., Zhang S., Zhang H. and Sun R. (2018). Characterization of interspecific hybrids between flowering Chinese cabbage and broccoli. Sci. Hortic. 240, 552–557.

Wijerathna-Yapa A, Ramtekey V., Ranawaka B. and Basnet B.R. (2022). Applications of *In Vitro* Tissue Culture Technologies in Breeding and Genetic Improvement of Wheat. Plants11, 2273. https://doi.org/10.3390/plants11172273

Zhang J., Shen W., Yan P., Li X. and Zhou P. (2011). Factors that influence the yield and viability of protoplasts from *Carica papaya* L. African Journal of Biotechnology Vol. 10 (26), pp. 5137-5142. DOI: 10.5897/AJB10.1156