PLANT BIOTECHNOLOGY

The United Nations predicted that 9.2 billion are likely to occupy our planet by 2050 and 11.2 billion in 2100 (Fig. 1 and 2). Global warming and resultant climate change poses another threat to the population. The food production must increase by 50% under the prevailing circumstances. The plant cell culture can offer continuous production systems for high-value food and health ingredients, independent of geographical or environmental constrains. Metabolic engineering using plant tissue culture systems has led to increase in yield potential of several metabolites and also helped in understanding the primary and secondary metabolisms. Induction of chloroplast development and carbon fixation studies in autotrophic callus cultures enabled basic understanding of "C4" cycle in C3 callus cultures of *Daucus carota* and *Arachis hypogaea* for the first time. Major efforts are currently underway to introduce carbon concentrating systems into C3 crops plants, such as wheat and rice, with special emphasis on converting C3 plants to C4 plants.

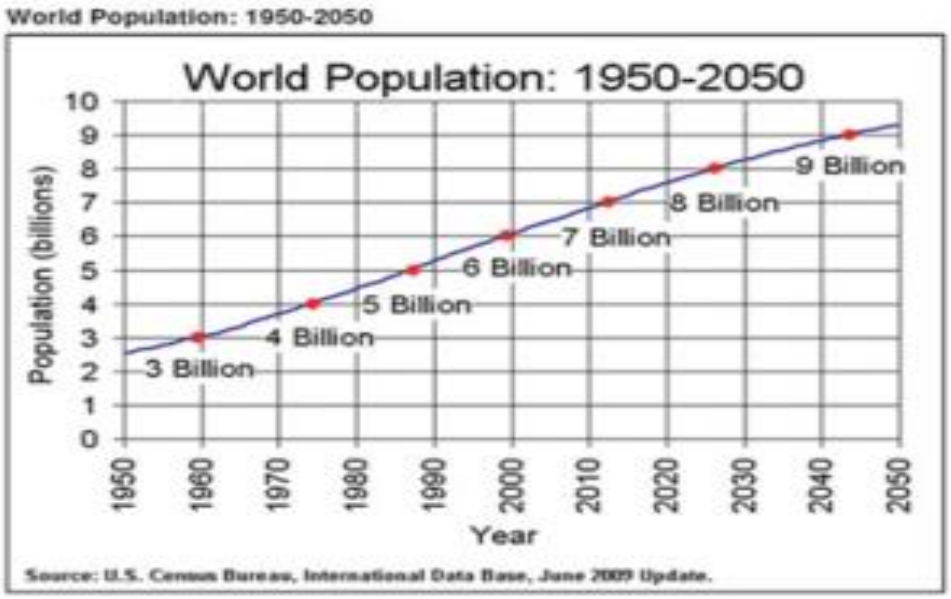

Figure 1. The rise of human population will outrum the food supply.

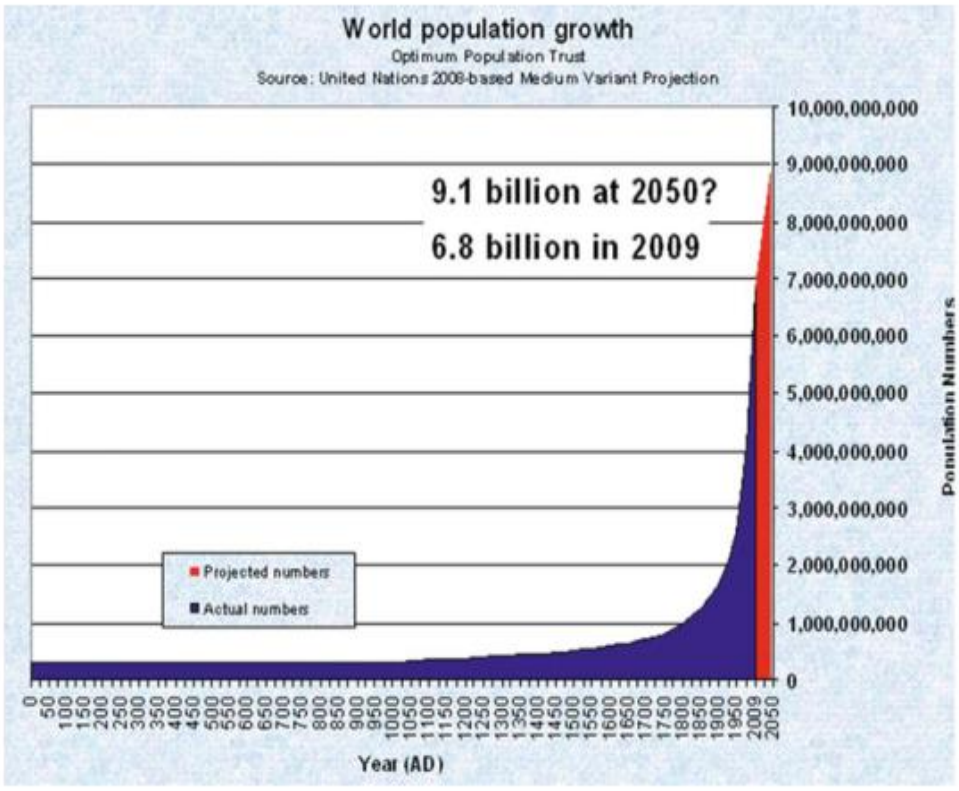

Figure 2. World population growth.

The increasing photosynthetic efficiency is of prime importance to increase plant productivity to meet the demands of a growing human population with respect to food, feed, fiber, and energy efficiency. It was suggested two major goals for plant metabolic engineering: (1) increasing the yield or quality of plant products or specialized metabolites which have health promoting properties and (2) increasing photosynthetic efficiency to optimize the amount of plant products that can be achieved with a given amount of fertilizer, water, and land. Plant cell culture technology can be used to obtain fundamental metabolic information, supply high-value products, propagate elite species, and promote somatic embryogenesis. This can only be achieved if conventional breeding is supported by biotechnological applications of plant tissue culture techniques (Fig. 3).

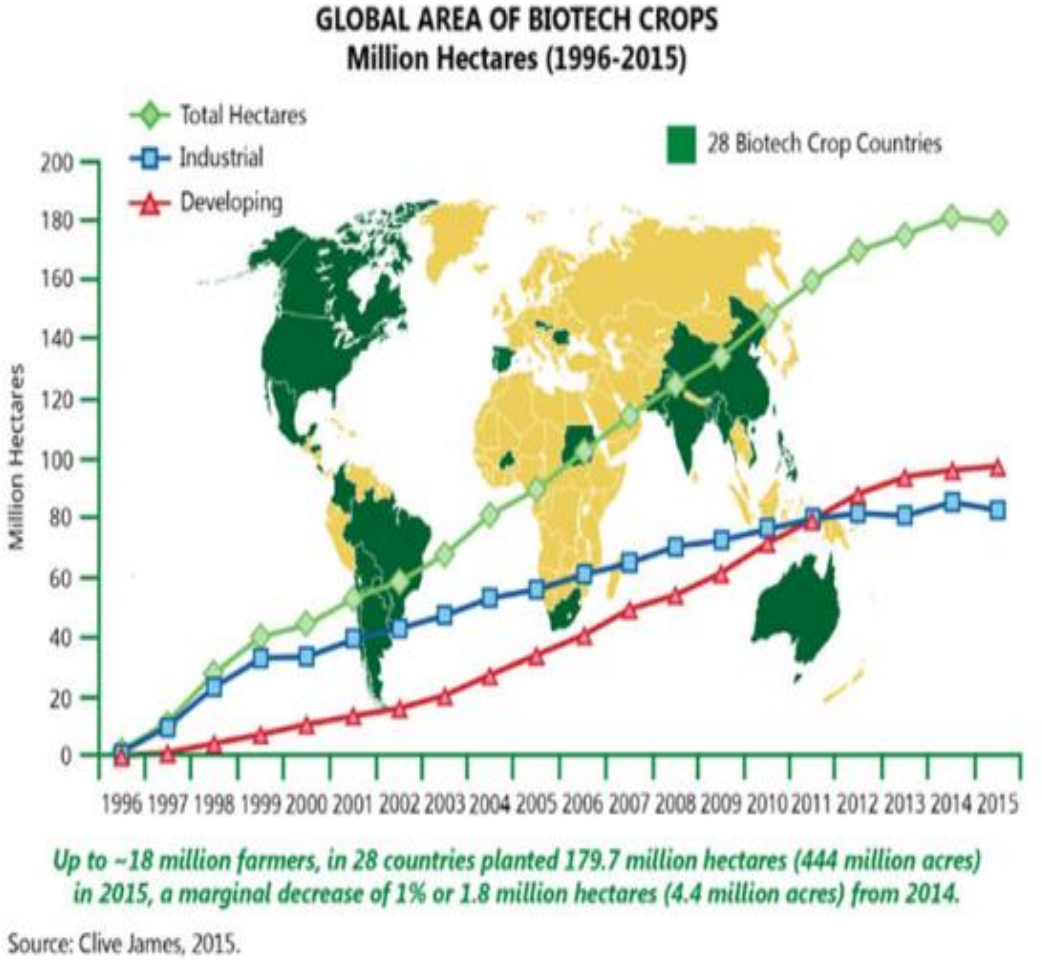

Figure 3. Gloal area under biotech crops.

Experimental systems based on plant cell and tissue culture are characterized by the use of isolated parts of plants, called explants, obtained from an intact plant body and kept on or in a suitable nutrient medium. This nutrient medium functions as replacement for the cells, tissue, or conductive elements originally neighboring the explant. Such experimental systems are usually maintained under aseptic conditions. Otherwise, due to the fast growth of contaminating microorganisms, the cultured cell material would quickly be overgrown, making a rational evaluation of experimental results impossible.

Some exceptions to this are experiments concerned with problems of phytopathology in which the influence of microorganisms on physiological or biochemical parameters of plant cells or tissue is to be investigated. Other examples are co-cultures of cell material of higher plants with Rhizobia to study symbiosis or to improve protection for micro-propagated plantlets to escape transient transplant stresses.

Using cell and tissue cultures, at least in basic studies, aims at a better understanding of biochemical, physiological, and anatomical reactions of selected cell material to specified factors under controlled conditions, with the hope of gaining insight into the life of the intact plant also in its natural environment.

Compared to the use of intact plants, the main advantage of these systems is a rather easy control of chemical and physical environmental factors to be kept constant at reasonable costs. Here, the growth and development of various explant can be studied without the influence of remote material in the intact plant body. In most cases, however, the original histology of the cultured material will undergo changes and eventually may be lost. In synthetic culture media available in many formulations nowadays, the reaction of a given cell material to selected factors or components can be investigated. As an example, cell and tissue cultures are used as model systems to determine the influences of nutrients or plant hormones on development and metabolism related to tissue growth. These were among the aims of the "fathers" of tissue cultures in the first half of the twentieth century.

The advantages of those systems are counterbalanced by some important disadvantages. For one, in heterotrophic and mixotrophic systems, high concentrations of organic ingredients are required in the nutrient medium (particularly sugar at 2% or more), associated with a high risk of microbial contamination. Other disadvantages are the difficulties and limitations of extrapolating results based on tissue or cell cultures to interpret phenomena occurring in an intact plant during its development. It has always to be kept in mind that tissue cultures are only model systems, with all positive and negative characteristics inherent of such experimental setups. To be realistic, a direct duplication of in situ conditions in tissue culture systems is still not possible even today in the twenty-first century and probably never will be. The organization of the genetic system and of basic cell structures is, however, essentially the same, and therefore tissue cultures of higher plants should be better suited as model systems than, e.g., cultures of algae, often employed as model systems in physiological or biochemical investigations.

The domain cell and tissue culture is rather broad and necessarily unspecific. In terms of practical aspects, basically five areas can be distinguished (Fig. 4), which here shall be briefly surveyed before being discussed later at length. These are callus cultures, cell suspensions, protoplast cultures, anther cultures, and organ or meristem cultures.

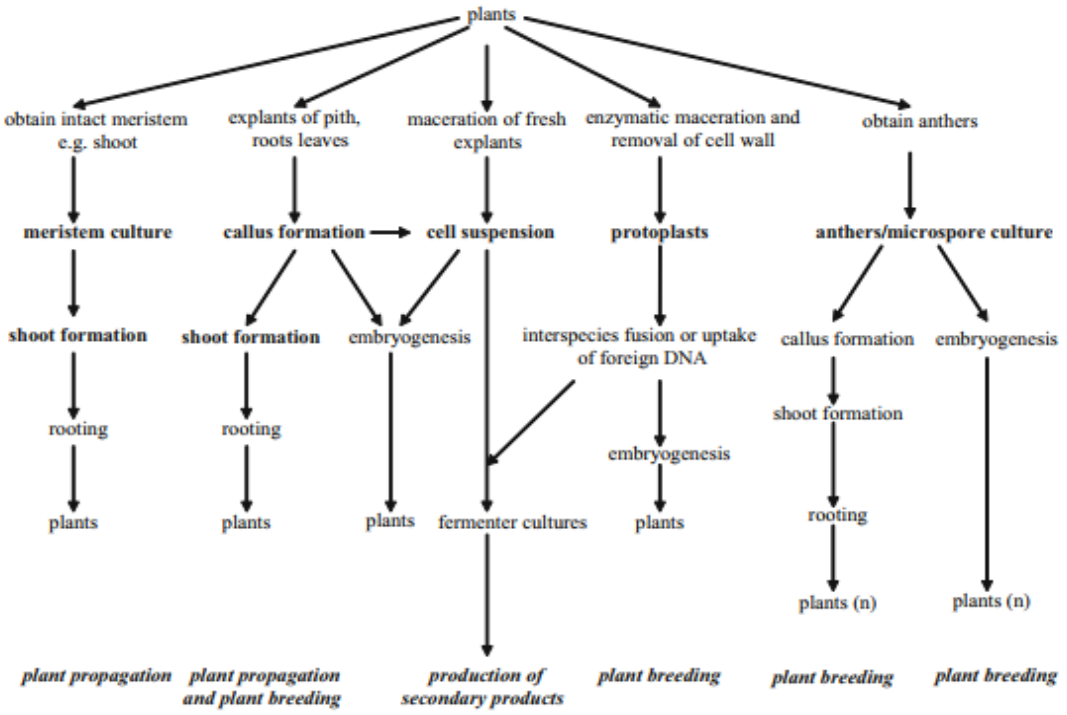

Figure 4. Schematic presentation of the major area of plant cell and tissue cultures and some fields of application.

Callus Cultures

In this approach, isolated pieces of a selected tissue, so-called explants (only some mg in weight), are obtained aseptically from a plant organ and cultured on or in a suitable nutrient medium. For a primary callus culture, most convenient are tissues with high contents of parenchyma or meristematic cells. In such explants, mostly only a limited number of cell types occur, and so a higher histological homogeneity exists than in the entire organ. However, growth induced after transfer of the explants to the nutrient medium usually results in an unorganized mass or clump of cells—a callus—consisting largely of cells different from those in the original explant.

Cell Suspensions

Whereas in a callus culture, there remain connections among adjacent cells via plasmodesmata; ideally in a cell suspension, all cells are isolated. Under practical conditions, however, also in these cell populations, there is usually a high percentage of cells occurring as multicellular aggregates. A supplement of enzymes able to break down the middle lamella connecting the cells in such clumps or a mechanical maceration will yield single cells. Often, cell suspensions are produced by mechanical sheering of callus material in a stirred liquid medium. These cell suspensions generally consist of a great variety of cell types (Fig. 5), and are less homogenous than callus cultures.

Protoplast Cultures in this approach, initially the cell wall of isolated cells is enzymatically removed, i.e., “naked” cells are obtained (Fig. 5), and the explant is transformed into a single-cell culture. To prevent cell lysis, this has to be done

under hypertonic conditions. This method has been used to study processes related to the regeneration of the cell wall and to better understand its structure. Also, protoplast cultures have served for investigations on nutrient transport through the plasmalemma but without the confounding influence of the cell wall. The main aim in using this approach in the past, however, has been interspecies hybridizations, not possible by sexual crossing. Nowadays, protoplasts are still essential in many protocols of gene technology. From such protoplast cultures, ideally plants can be regenerated through somatic embryogenesis to be used in breeding programs.

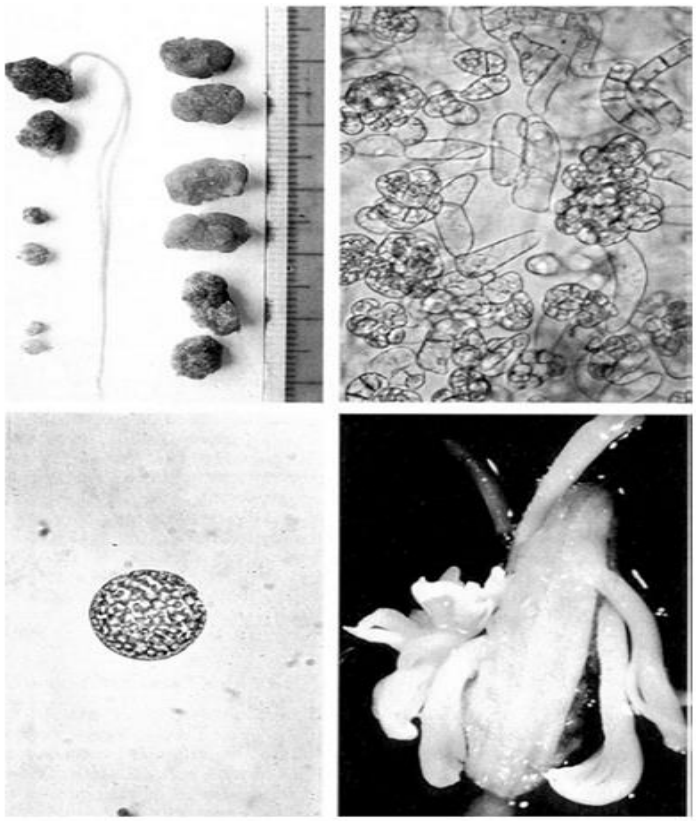

Figure 5. Various techniques of plant cell and tissue cultures, some examples: (top left) callus culture (top right) cell suspension. (bottom left) protoplast culture, (bottom right) anther.

Haploid Techniques

Culturing anthers (Fig. 5), or isolated microspores from anthers under suitable conditions, haploid plants can be obtained through somatic embryogenesis. Treating such plant material with, e.g., colchicines, it is possible to produce dihaploids, and if everything works out, within 1 year (this depends on the plant species) a fertile homozygous dihaploid plant can be produced from a heterozygous mother plant. This method is advantageous for hybrid breeding, by substantially reducing the time required to establish inbred lines.

Often, however, initially a callus is produced from microspores, with separate formation of roots and shoots that subsequently join, and in due time haploid plants can be isolated. Here, the production of "ploidy chimeras" may be a problem. Another aim in using anther or microspore cultures is to provoke the expression of

recessive genes in haploids to be selected for plant breeding or gene transfer purposes.

Plant Propagation, Meristem Culture, Somatic Embryogenesis in this approach, mostly isolated primary or secondary shoot meristems (shoot apex, axillary buds) are induced to shoot development under aseptic conditions. Generally, this occurs without an interfering callus phase, and after rooting, the plantlets can be isolated and transplanted into soil. Thereby, highly valuable single plants—e.g., a hybrid—can be propagated. The main application, however, is in horticulture for mass propagation of clones for the commercial market, another being the production of virus-free plants. Thus, this technique has received a broad interest in horticulture and also in silviculture as a major means of propagation.

Some Endogenous and Exogenous Factors in Cell Culture Systems Growth and differentiation of excised tissues are regulated by an interplay of endogenous and exogenous factors in coordination with genetic influences. It deals with growth regulators, nutrient uptake, and role of physical factors in growth and differentiation of cultured plant tissues.

Primary Metabolism

Cultured plant cells have the capacity to develop chloroplasts. However, subsequently elaborate studies lead to not only elucidation of chloroplast development in isolated carrot root explants but also studies on carbon dioxide fixation by such chloroplasts in “sputniks”; the special type of flasks on a rotatory machine under illuminated conditions not only developed chloroplasts but also showed their function with regard to carbon fixation. Also developed autotrophic cultures of *Arachis hypogaea* which were able to grow without carbon supplement at Jaipur.

Secondary Metabolism

Plant tissue cultures and organ cultures were thought to be excellent source of production of secondary metabolites under controlled conditions. Leading scientists from Germany, the USA, France, and Japan believed that by regulating the cultural conditions and developing better fermenter systems, the dream of obtaining secondary metabolites from cultured tissues could be realized. Biotechnological innovations and metabolic engineering further supported the production of secondary metabolites and other biologically active compounds. Metabolic engineering of plant cells and production of heterologous expression systems have been optimized for production of Secondary metabolites.

Rapid development of metabolic engineering and synthetic biology of microorganisms shows many advantages to replace the current extraction of these useful high-price chemicals from plants. Attempts to obtain benzylisoquinoline alkaloids (BIAs) which include many pharmaceutical compounds, such as berberine (antidiarrheal), sanguinarine (antibacterial), morphine (analgesic), and codeine (antitussive) have been reported successfully in microorganisms. presented total biosynthesis of opiates by stepwise fermentation using engineered

Escherichia coli. Application of biotechnology for production of biofuels, bioethanol and biodiesel, has gained attention.

Genetic Problems and Gene Technology

Plant tissue culture and its application in biotechnology have attracted industries to this field. Plant tissue culture and biotechnology some of the topic elaborated include somaclonal variations, ploidy stability, evaluation of genetic fidelity during longterm conservation, molecular markers, DNA fingerprinting and characterization of germplasm, and estimation of genetic diversity, gene technology, modes of gene modification, and wild crossing have been covered in addition to mutagenesis, genetic transformations techniques and applications, and gene silencing. The first CRISPR-edited crops presented to the US regulatory system can be cultivated and sold without oversight by the US Department of Agriculture (USDA). This opens new possibilities of achieving goal of feeding increasing world population and meeting their environmental needs with green technologies without causing further pollution. Plant tissue culture and its biotechnological options open new vistas. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been utilized to allow for site-specific gene mutation, replacement, or integration through non-homologous end joining or homologous recombination. In contrast to ZFNs and TALENs, the Cas9 protein is by nature a sequence-specific nuclease. Newer genetic engineering (GE) techniques that don't involve plant pests are quickly supplanting the old ones, and the USDA appears to be saying it does not have the authority to regulate the products of these techniques. The agency ruled similarly on plants transformed with other gene editing techniques, such as zinc finger nuclease and transcription activator-like effector nuclease systems. Invention of new genome engineering strategies, such as TALENs and CRISPR/Cas9 systems, would greatly facilitate researchers to develop more advanced strategies within shorter periods. Recently synthetic biology is perceived as an interdisciplinary field. It acts at the interface between recombinant DNA technology and biotechnology and enables reprograming of living cells to perform novel and improved functions. Cell engineering in synthetic biology is based on the concept that biological components from different organisms can be reassembled into genetic networks that operate either in parallel or together with natural biological systems to improve, restore, or add essential functions to cells. However, synthetic biology is beyond the scope of this publication hence would not be dealt in detail in this edition.

It has been added to provide practical aspect of plant tissue culture, and its application in biotechnology based on our practical experiences and lab exercises carried out on day to day basis. It has been added based on practical being conducted in the Institute a guide is prepared for all those interested in undertaking plant tissue culture in lab, in industry, and in research labs. Exact details of the procedures are given in simple language so that the guide can be followed easily by beginners and experts alike.

Summary of Some Physiological Aspects in the Development of Plant Cell and Tissue Culture.

Attempts have been made to elaborate on recent applications of plant cell and tissue culture with some examples and recent developments in focus. Millions of plants are commercially propagated annually via micropropagation which is labor-intensive and associated with developmental abnormalities; there have been many efforts to develop cost-effective and simple bioreactors with the aim of automating micropropagation. However, designing reactors for plant tissue culture must reconcile environmental factors (shear stress, aeration, RH, nutrient supply) with healthy plant development, cost, and simplicity of use. Numerous studies have applied bioreactors in plant cell and organ culture to obtain specific metabolites. In addition, a considerable number of researchers have cultured plant propagules in bioreactors to produce high-quality seedling.

STERILIZATION AND CONTAMINATION

The plant tissue culture laboratory must be maintained clean and sterilized, especially the cultivation and growth rooms because pollution means complete corruption of the crops, loss of time and effort, and loss of the chemicals that were worked on.

Sources of plant tissue culture laboratory contamination

1. Contamination of the chemicals involved in the composition of the medium and the tools used in handling them. Therefore, specific tools must be allocated for each chemical to prevent the transfer of contamination from one chemical to another.
2. Lack of cleanliness and sterilization of used tools, lack of cleanliness and sterilization of floors, failure to quickly remove waste from the laboratory, and workers' lack of interest in personal hygiene.
3. Unsealed windows and doors that allow dust to enter the laboratory are essential sources of pollution.

The laboratory must have precision in the engineering design to prevent dust from penetrating inside it.

Sterilization of the cultivation room

1. Clean the cabin table with 70% alcohol, then dry the cabin with a piece of good cloth that does not leave any residue.
2. Operating the cab for 30 minutes before starting work is preferable to ensure its safety.
3. Ultraviolet (UV) bulbs are sometimes lit all night to sterilize the cultivation room and its contents before using them the next day. Lighting them no less than 30 minutes before starting the laboratory may be preferable, ensuring that the doors are tightly closed and must not be looked at while in operation. To protect the eye from damage.

Laminar flow hood table

In this cabin, explant are separated and grown in laboratory cultivation containers.

It must be completely sterilized during the transplantation procedure. This cabin is distinguished by its ability to purify and sterilize the air passing inside it. The atmospheric air passes at a constant pressure through filters less than a micron to prevent dust particles and the microorganisms they carry. Then, the air passes into the cabin after it becomes highly pure and sterilised. The filter must be changed with a new one annually. Fluorescent lamps are installed in the cabin ceiling as a lighting source. The cabin has a gas source used as a flame to sterilize tools used in agriculture.

Cleaning and sterilization of laboratory instruments

Cleaning glassware

New glassware that has not been used before should be cleaned in a container containing a mixture of sulfuric acid or an industrial detergent. Then, they are washed under running water for no less than 5 minutes, followed by washing with sterile distilled water twice in a row, and then all of them are kept away from dust. It should be washed twice with distilled water before use if stored for long. If a floor is contaminated with a mixture of the two mentioned acids, it should be cleaned using a high-pressure water source. Therefore, the laboratory floor must be resistant to acids. Acid-resistant gloves must be used. Used glassware is also cleaned quickly before the agar dries and sticks to the wall of the vessel. The pipes are cleaned by removing the plugs and then placing them in hot water. He uses forceps and a suitable brush to remove the agar and plant residues in the tubes. It is preferable to use a dishwasher to wash glassware, rinse with ion-free distilled water, dry well in a drying oven after ensuring its cleanliness, and store it as quickly as possible to reduce the chance of contamination.

Sterilization of laboratory instruments

Laboratory instruments include all glass and plastic containers used in agriculture and other tools such as knives, scalpels, tweezers, scissors, etc. Sterilization uses gamma rays and steam pressure using an autoclave or dry heat. Do not use low-quality plastic containers once because they cannot withstand the high heat of sterilization, and they have a shelf life after which they become unusable. Radiation is successfully used to sterilize metal tools, glass and plastic utensils, regardless of their quality. Using an autoclave or dry heat ovens to sterilize glassware is preferable. Plastic containers that can withstand heat sterilization are usually expensive, sterilize like glassware, and have some problems, such as the release of ethylene. This compound is harmful to the environment and toxic to plants. Sterilize glassware by dry heat in an oven at 160°C for four hours, provided that it is made of heat-resistant glass such as Pyrex and Borosilicate, which are expensive, and the cheap types cannot withstand high temperatures, and toxic cations such as sodium, lead, and mercury are released into the food medium. Pyrex glass is used in particular for the cultivation of protoplasts, individual cells and meristematic tissues, and the following must be taken into account:

1. Laboratory tools, such as knives, scalpels, tweezers, scissors, and others, are wrapped in aluminium foil before being sterilized in the oven. They remain

wrapped after sterilization to maintain them sterile until the time of use. The pipettes and cultures are placed inside their boxes, and then each box is covered with aluminium foil to preserve them after sterilization.

2. During laboratory cultivation, the tip of the instrument used is dipped in 99% ethyl alcohol, then exposed to flame and then left to cool. Any instrument must not be placed in alcohol after it is directly exposed to a heat source so as not to cause an explosion or infection of the alcohol, which may harm the person performing the laboratory culture.
3. Laboratory tools must not touch the surface of the nutrient medium when transporting and cultivating the explant or tissue.
4. Using sterile pipettes or syringes is preferable to ensure that the food medium is not contaminated. A new, sterile one is allocated for each type of solution used.
5. The hair must be covered entirely, a mask must be placed over the nose and mouth, and the hands and any other exposed part of the arm must be disinfected with alcohol so that it is not a source of contamination of food media and explant.

Sterilization of nutritional medium

The purity and sterilization of the compounds included in the composition of the medium must be ensured, and their contamination prevented. Preparing it immediately before use is preferable, and its storage period should not exceed 14 days.

Moist heat sterilization

Most food media are sterilized in the autoclave, a steam sterilization device under pressure that is fast and easy to operate. The stabilizers vary in shape and size, some of which are horizontal, loaded from the front, and vertical, loaded from the top. The horizontal type is easier and more expensive, and the sterilization temperature reaches 115-135°C. Therefore, the autoclave is capable of destroying all microorganisms and viruses. Sterilisation efficiency depends on the duration of operation, the steam pressure used, the temperature, and the size of the materials to be sterilized. Sterilization of large volumes of food media in one package requires a more extended period to ensure uniform distribution of sterilization heat (Tab. 1). It also needs a more extended period after sterilization to lose its high temperature. Prolonging the sterilization period or the post-sterilization period may lead to damage to the medium or the burning of some of its components, such as the sugar used in preparing the medium. Therefore, it is preferable to distribute the medium into tubes or flasks at a rate of 20-100 ml each to ensure complete and homogeneous sterilization and to protect its components from alteration. Sterilization occurs after 15-20 min at 121°C and a steam pressure of one kg/cm^3.

Test tubes or glass flasks containing a food medium are closed before placing them in the freezer with a cotton plug surrounded by gauze to protect them from contamination. Special lids may be used for glass tubes and flasks, provided they

are not tightly closed to help expand the air inside them. They are preventing its explosion inside the autoclave. Covers that contain internal protrusions that touch the outside of the glass tube and allow gas exchange may be used. Suitable square pieces of 0.25 mm thick aluminium foil are also used. A piece of aluminium foil is placed over the mouth of the tube or flask. Then, its ends are gently folded around the circumference of the neck. Tightly sealing the containers isolates their contents from the outside environment and creates anaerobic conditions inside them. The aluminium cap can be removed, re-sterilized, and used to add a chemical or explant. After sterilisation, a polythene film can be used in 70% ethanol instead of aluminium foil. Devices are available in the market to prepare and sterilize quantities between 0.5-16 lit, which are sterilized under steam pressure. The medium is mixed and stirred during sterilization to ensure good mixing and dissolution of all its components, rapid heating, and uniform temperature in the medium. After sterilization, the medium is cooled quickly under a stream of running water. Then, add the heat-tolerant ingredients first, stirring. The medium then becomes sterile and suitable for distribution in cultivation pots.

Table 1. Duration of sterilization in the autoclave at a temperature of 121˚C.

Sterilization time (min)	Nutrient medium (ml)
15	25-20
20	50-25
25	500-50
30	1000-500
35	1500
40	2000

Equivalent to autoclaving sterilization

a. Hydrolysis of sucrose into fructose, glucose, sugars, and sugar acids occurs. Higher sterilization temperature than necessary may lead to complete decomposition.
b. There is a change in the acidity of the food medium (pH) by 0.5-0.3 units, which causes the separation of some of its components, the occurrence of some side reactions between them, and a decrease in their effectiveness.
c. Destruction of some compounds included in the formation of the medium, such as colchicine, zeatin, gibberellin, vitamin B, vitamin C, antibiotics, ethrel, and ethylene.

Dry heat sterilization

This method sterilises glassware and metal utensils made of stainless iron and other materials not damaged by high temperatures. Regular drying ovens that operate on gas or electricity are used for this purpose. Sterilization is carried out at a temperature of 160°C for four hours, and the tools and containers to be desired must be wrapped. Sterilize them with aluminium foil and then place them in the sterilization oven. After sterilization, the tools and containers wrapped in

aluminium foil are transported to the transport room where they are used. This method cannot sterilise tools or containers made of paper, cotton, or plastic. It would help if you also avoided sterilizing medical blades and scalpels this way because high temperatures affect or reduce the sharpness of their edges.

Sterilization by filtration

Special filters, such as the Millipore MF filter with a porosity of 0.22 μM, are used for sterilization. Liquid and other solutions are passed through the filter membranes to eliminate all suspended particles and microorganisms. This method is used instead of steam pressure sterilization (autoclave). Sterilization by filtration leads to the medium retaining its nutritional components unchanged, and some compounds are adsorbed to the surface of the filter. Therefore, it is preferable to exclude the first amount of the filtrate because it may not conform to the required specifications. Also, some viruses may be small and can pass through the filter.

Sterilization of explant

The explant separated from plants growing in the field are usually contaminated with various microorganisms. Air, dust, irrigation water, pests, etc., are considered the most important sources of pollution, and planting contaminated explant fails agriculture, so sterilizing explant from the field is essential.

Sterilization steps

1. Wash the explant under a continuous stream of running water for 30 minutes to clean its outer surface well and get rid of the dust stuck on it.
2. The explant is immersed in the sterilization solution to eliminate any remaining organisms stuck on its surface. The choice of the sterilizing chemical and the appropriate period for sterilization depends on the sensitivity of the explant. Severe sterilization leads to the elimination of microorganisms spread on the external surface and may also lead to the destruction of some of the superficial tissues of the explant. Therefore, the sterilization material, appropriate period for sterilization, and concentration must be carefully chosen.
3. Wash the explant with sterile distilled water several times to remove the remaining traces of the sterilizing substance, removing all affected and damaged tissues from the effect of the sterilizing importance.
4. The explant is cut into sizes suitable for practical cultivation under sterile conditions, and clean tools are used. The explant are then grown under sterile conditions in cultivation containers, such as test tubes, conical flasks, and Petri dishes containing nutrient media. It should be considered that the containers and food media are well sterilized.

Sterilizing explant by chemicals

Surface sterilizes explant using sodium hypochlorite (NaOCl), calcium hypochlorite, bromine water, hydrogen peroxide, silver nitrate, mercuric chloride, ethanol alcohol, and ethylene oxide. It is preferable to add a spreading material, such as Teepol, Trigetol, or Tween-20, to the sterilization solution to facilitate its spread on the external surfaces of explant and increase its efficiency in killing microorganisms. Sodium hypochlorate at a concentration of 1% is used

successfully in the sterilization process due to its low side effects. Sodium hypochlorate decomposes into chlorine, which has a practical sterilization effect. Sodium reacts with water to form sodium hydroxide, an easy compound to dispose of with water. Sterilization requires 5 to 30 min in a 1% sodium hypochlorate solution. Prolonging the sterilization period may harm the explant. Covering the wounded surfaces on the explant with paraffin may be necessary to prevent the sterilization solution from penetrating the internal tissues and releasing cellular fluid. Bromine at a concentration of 1% is used to sterilize seeds immediately before planting them. Bromine causes some damage to the embryo if the seed coat is thin. Hydrogen peroxide is used in sterilization, and it is easy to decompose into oxygen and water, which are harmless and evaporate easily. This compound causes damage as the plant tissue loses some moisture or leads to dryness. A diluted mercuric chloride solution successfully sterilizes plant tissue, especially covered explant with a cutin layer or uneven surfaces. Removing its remaining traces on the explant with water is difficult. It can be removed with ethanol, washing powder, or both. Therefore, the sterilization period and concentration of the sterilizing agent used must be determined for each plant species (Tab. 2 and 3).

Table 2. Period (min) and appropriate concentration of sodium hypochlorite.

Plant	Explant	Duration of sterilization	NaOCl (%)
Anthurium andreanum	Leaves	30	1
Hyacianthus scale	Tissue	15	1
Rhododendron	Stems	20	1
Gerbera	Atrium	15	1
Freesia	Buds	20	1
Strelitzia	Leaves	45	1
Tulipa	Seeds	30	2
Phaseolus	Stems	15	1
Nephrolepis	Top of stems	5	1

Table 3. Some clarifications about the different materials used to sterilize explant.

Sterilization material	Concentration (%)	Duration of sterilization	effect	Removal
Calcium hypochlorite	10-9	30-5	V. good	+++
Sodium hypochlorite	2	30-5	V. good	+++
Bromine water	2-1	20-10	V. good	+++
Hydrogen peroxide	12-10	15-5	good	++++++
Silver nitrate	1	30-5	good	+
Mercury chloride	1-0.1	10-2	acceptable	+
Antibiotics	5-4 ml/l	60-30	good	++

* Gradient from difficult to remove (+) to easy to remove (++++++)

Specifications of sterilization materials

Sterilization materials must have certain qualities, including the ease of removing their traces by washing them in water. It must not interact with components of plant tissue so that compounds that harm new growth are not formed. It must be easily decomposed into less toxic components than the original substance and easy to dispose of with water. During sterilization, the explant may appear brown due to the formation of melanin pigment. This process can be prevented by dipping the explant in a 100 mg/L ascorbic acid solution and then dipping them in 150 mg/L citric acid.

Self contamination

Sometimes, growths of microorganisms appear in some containers containing crops. This may be due to poor sterilization. The source of contamination may be in the internal tissues of the explant itself, which are difficult for sterilization materials to reach. Microbial growths appear if wounded surfaces on the explant of the nutrient medium come into contact. Infected changes appear weak, pale, and die; these pots must be disposed of immediately. A rich nutritional environment helps increase the spread of microbial infection. Microbial mutations may occur that can grow on poor media. The growing tip of the stem may be a source of contamination because it is difficult for sterilizing materials to penetrate between the gathered leaves and adjacent to them. Therefore, the growing top is sterilized in successive stages, starting with washing it well with water, then sterilizing it outwardly, then removing some leaves and sterilizing it again. Sterilizing is repeated after each removal of some leaves. Sometimes, it is preferable to conduct a test to estimate sterilization efficiency by preparing a medium to which 2-3% Tryptone or Peptone is added. Parts of the apical tip of one of the branches are distributed on it after cutting it lengthwise, with the wounded surface in contact with the medium. After a few days, the growths of microorganisms, if any, are visible. The results of this method are not always encouraging, as there is no standard food medium that can be used as a good guide for all types of bacteria that grow inside plant tissue. The following procedures help in the emergence of self-contamination:

1. Not washing and sterilizing hands, not using mouth and nose masks, hair covers, sterile gloves, and not sterilizing the floor. Do not allow large numbers of visitors into the agriculture room. And poor sterilization of the cultivation cabin. Do not cover the shoes with a plastic cover or dip the shoes in a sterile solution when entering the cultivation room. Therefore, it is necessary to ensure the safety and sterilization of the cultivation room and cabin by blowing sterile air into the cabin, irradiating it with UV rays during the night, and renewing the front filters of the cabin annually.
2. Failure to sterilize the workbench with ethanol, failure to sterilize the floors, and poor sterilization of the tools used.
3. The outer surfaces of tubes and conical flasks may not be well sterilized, so it is preferable to sterilize and store them in sterile conditions, even if they contain

a nutrient medium.

4. some living organisms, such as spiders, can easily penetrate through cotton covers and plastic strips, carrying microorganisms into the food medium in tubes and flasks.

Prevention of self-contamination

1. Meristemic tip cultivation: Using the meristematic tip surrounded by one or two leaf stems is preferable because it is free of the virus. It may be due to the incomplete formation of the transmitting vessels in the meristematic tip. Meristem is preferred from sterilized growing shoots, but it may not be necessary to sterilize them if they are separated from laboratory plants or grown in a greenhouse.
2. Adding antibiotics to the food media: Sometimes, some antibiotics are added to the food media, such as Benolate or Benomyl, at a concentration of 10 mg/L to stop the activity of microorganisms, and the latter compound may cause some damage to the growth and development of explant. Therefore, some people do not prefer to add such compounds to the medium due to the possibility of them interacting with the components of the nutrient medium and forming compounds that are toxic to growth and may cause direct damage to ribosomes, leading to growth inhibition. Mutations in microorganisms may appear resistant to antibiotics, and antibiotics do not affect viruses. The compounds used include Gentamycin, Achromycin, Rifampicin, Streptomycin, Oxytetracycline, Tetracyclin, and 8-hydroxy-quino.

Quesation

- How did plant biotechnology develop?
- What is the relationship between biotechnology and plant tissue culture?
- What is the role of biotechnology in the development of crop production?