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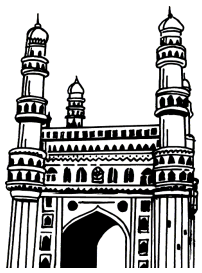

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## 1.1 Tissue Culture

### 1.1.1 Introduction

**Q1. Write an essay on Plant Tissue Culture?**

*Ans :*

**Plant Tissue Culture**

Plant tissue culture is the practice of cultivating and maintaining plant cells, tissues, and organs in sterile, nutritionally, and ecologically favorable conditions (in vitro). Micro propagation refers to the process of regenerating complete plants from cell cultures grown from meristematic cell explants.

Tissue culture is the in vitro cultivation of isolated plant cells, tissues, or organs in an aseptic environment. Plant tissue culture is a collection of experimental procedures for cultivating large numbers of isolated cells (or) tissues under controlled conditions. It is not a separate area of plant science like taxonomy, cytology, genetics, or others.

Tissue Culture Significance

Plant tissue culture plays an important role in plant biotechnology, particularly in crop enhancement programmes. The process of in-vitro culture of explants (pieces of living differentiated tissues) in nutritional medium under aseptic conditions is known as tissue culture. Tissue culture, on the other hand, encompasses not just tissue culture but also cell culture, organ culture, and suspension culture. Plant tissue culture is essential to almost all elements of plant biotechnology. Plant biotechnology is now widely recognized as one of the most useful of all sciences. Plant biotechnology products are quickly making their way from the lab to the fields. Plant tissue culture has also piqued the curiosity of molecular biologists, plant breeders, and even scientists.

Plant tissue culture is essential to almost all elements of plant biotechnology. Plant biotechnology is now widely recognized as one of the most useful of all sciences. Plant biotechnology products are quickly making their way from the lab to the fields.

Plant tissue culture has also piqued the interest of molecular biologists, plant breeders, and even industrialists, as it aids in the improvement of economically important plants. Aside from that, tissue culture is extremely useful in determining the patterns and components that control plant growth, metabolism, morphogenesis, and differentiation.

**History of Plant Tissue Culture And Research**

In 1902, German scientist G. Haberlandt cultivated completely developed plant cells obtained from various plants. This was the initial step in the plant cell and tissue culture process. The Cell Doctrine, which acknowledged that a cell is capable of demonstrating totipotency, made additional contributions. The identification of a number of substances such as cytokinin, auxin, other hormones, vitamins, and others, as well as their role in altering cell division and differentiation, led to the correct development of plant tissue culture systems. Gautheret, White, and Nobecourt were three other scientists who made significant contributions to the development of plant tissue culture techniques. Braun regenerated the first plant from a mature plant cell in 1959. The discovery of a million times increase in the multiplication of Cymbidium (an orchid) by G.M.Morel in 1960 set the groundwork for commercial plant tissue culture.

The University of Delhi pioneered tissue culture research in India in the 1950s. This initiation is credited to Shri Panchanan Maheshwari, who was working there at the time. Shri S.C. Maheshwari and Sipra Guha made significant contributions to India's plant tissue culture progress. Later, advances in nutritional media composition and genetic engineering laid the groundwork for even greater success in plant tissue culture techniques.

Gottleib Heberlandt, known as the "Father of Tissue Culture," was the first to attempt plant tissue cultivation. He established the notion of in-vitro culture of plant cells. Following that, there was some advancement in tissue culture techniques.

Some of the early classical important contributions in the field tissue culture are tabulated below:

| Year | Scientist | Contribution |
|---|---|---|
| 1902 | Haberlandt | First attempt of in-vitro culture of plant cell |
| 1922 | Robbins | In-Vitro culturing of roots |
| 1934 | White | Culture of Roots of Tomato |
| 1955 | Miller | Hormone Kinetin Discovered |
| 1957 | Skoog Miller | Discovered that Auxin-Cytokin in regulates the organ formation |
| 1970 | Maheswari& Guha | Successful Anther Culture |
| 1974 | Reinhard | Biotransformation in Plant Tissue Culture |

**Technical Procedure Of Plant Tissue Culture**

Plant tissue culture in vitro includes the following successive steps.

1. Culture Medium
2. Aseptic Conditions
3. Transfer of Explants
4. Incubation of the culture
5. Aeration

## 1. Culture medium

Plant tissues and organs that have been removed are unable to produce their own sustenance. As a result, nutrients must be provided exogenously via the culture media. During their cultural experiments, different workers utilized various sorts of media. However, no single medium can support the optimal growth of all plant tissues. As a result, the best medium for a specific tissue must be found by trial and error.

The media are frequently given names based on the names of the discoverers who created them. M.S. medium, B.5 medium, White's medium, S.H. medium, and so on... Murashige and Skoog developed the M.S medium in 1962, and Gamborg et al developed the B-5 medium in 1968. Schenk and Hilde brant developed the S. H medium in 1972.

In plant cell and tissue culture, the M.S medium and the S. H medium are commonly employed.

**M.S. medium composition (for a one-liter solution):**

**(a) Macro nutrients:** NH,NO, 1650mg; KNO, 1900mg; CaCl, .2H, O - 440mg; MgSo, 7H, O - 370mg and KH, PO, - 170mg.

**(b) Micronutrients:** H,BO, 6-2 mg; KI-0-83 mg; MnSo4, 4H, O-22-3 mg: ZnSo, 7H, O-8-6 mg; CuSo4, 5H, O-0-025 mg; CoCl2, 6H2O – 0.025 mg MnSo4, FeSo, 7H2O - 27-25mg; NaMoO4.H, 0-0.025mg and Na2, EDTA 37.25mg.

**(c) Carbon Source:** Sucrose - 30gms

**(d) Vitamins:** Nicotinic acid- 0.5 mg: Pyridoxine, HCI-0-5mg; Thiamine - HCI-0.1 mg

**(e) Amino acid:** Clycine-2mg.

Inorganic salts, an iron source, vitamins, amino acids, growth hormones, and a carbohydrate supply are all present in most culture mediums. Macronutrients and micronutrients are the two types of inorganic salts available. Macronutrients are salts that are required in greater levels. Nitrogen, phosphorus, sulphur, magnesium, calcium, and potassium are among them. Micronutrients are inorganic elements that are required in small amounts. Fe, Mn, Zn, B, Cu, and Mo are the micronutrients.

Some tissues grow on simple media containing sucrose and inorganic salts. Such a medium is called minimal (or) basal medium. But for most others it is essential to supplement the medium with Vitamins, amino acids and growth regulators. Such a medium is referred to as a synthetic medium. The most commonly used amino acid is glycine. The addition of growth hormones like auxins and cytokinins in different proportions promotes the initiation of organs (organogenesis). The auxins that are commonly used in culture medium are IAA,NAA,IBA, 2,4-D and BTOA. Auxins induce cell division in the cultured tissue. The most commonly used cytokinins are 6-BAP (6-Benzylamine Purine), Zeatin and 2IPA (2, isopeptenyl adenine). Gibberellins and Abscissic acid are rarely used in culture medium.

In terms of morphogenesis, the ratio of auxin and cytokinins in the culture media is critical. The required ratio of auxins to cytokinins for embryogenesis, callus initiation, and root initiation is high, while the reserve leads to shoot proliferation. Some plant tissues thrive in the presence of natural plant products such as coconut milk, casein hydrolysis, yeast extract, watermelon juice, malt extract, ripe tomato extract, orange juice, and so on. Diphenyl Urea, a growth factor present in coconut milk, causes cytokinin-like reactions. Coconut milk, at a concentration of 10-15% v/v, is added to the medium as a source of cytokinin. Yeast extract is a good source of vitamins and organic nitrogen. All of the common amino acids are present in casein hydrolysis. Fruit juices include a variety of critical nutrients and antioxidants.

**Preparation of nutrient medium:**

Various medium components are dissolved in distilled water. The medium's pH is adjusted to a range of 5-8. (The uptake of ions by the cells is affected by the pH of the medium.) A higher pH results in a hard medium, whereas a lower pH results in agar solidification that is unacceptable). With the addition of agar, the medium can be turned semisolid (5-8 percent). Liquid medium is a medium that does not contain agar and remains liquid. In general, callus culture is done using semisolid medium, while cell suspension culture is done with liquid medium.

**2. Aseptic conditions**

Microorganisms such as bacteria, fungus, and others will flourish in the culture media, especially if it contains sucrose. As a result, if they come into touch with the medium in cellular (or) spore form, the microorganisms grow quicker than the higher plant tissues, eventually killing them. As a result, maintaining a totally aseptic environment inside the culture vial is vitally essential.

The medium is contaminated from three key sources:

(a) Medium:

(b) Glass vials and

(c) Plant tissue.

**(a) Sterilization of medium:** Microorganisms may have been present in the media from the start. The culture vials containing the media must be sanitized to kill such microorganisms. It's possible to perform it with an autoclave (or) filter sterilization. The medium is sterilized by

auto calving for 15 minutes at a steam pressure of 15 lb/m2 and a temperature of 120° C. High pressure causes carbohydrate and other medium components to decompose, hence the pressure should not exceed 20lb/in. Thermo labile substances include vitamins, amino acids, plant extracts, and hormones. During autoclaving, they decompose. As a result, filter sterilization is required for these chemicals' solutions. The solutions are filtered using bacterial filters throughout this process.

**(b) Sterilization of culture vessels and instruments:** Brown paper is used to wrap glass culture vials and metal tools. They are then disinfected in a hot-air oven for 2-4 hours by being exposed to dry air (160-180° C). Metal equipment, such as forceps, scalpels, needles, and spatulas, are sterilized by soaking them in 95 percent ethanol and then burning and cooling them. Flame sterilization is the name for this method.

**(c) Proper plugging:** Microorganisms are prevented from entering the medium vials by plugging them with sterile cotton.

**(d) Sterilization of plant material:** Microorganisms, as well as the tissue being cultivated, may find their way into the media. The plant material from which the tissue is to be removed is surface sterilized to prevent this. The plant material is first carefully washed with a liquid detergent solution such as tee friend (5 percent v/v). It is properly rinsed with distilled water after 15 minutes. The surface is then disinfected with disinfectants such as chlorine water (or) sodium hypochlorite solution. The material is thoroughly rinsed with distilled water after disinfection to remove any traces of chlorine. Shoot apices, pollen, and buds, for example, should be sanitized by rinsing in ethyl alcohol (or) isopropyl alcohol for a few seconds.

### 3. Transfer of explants (inoculation)

Finally, at the time of inoculation, precautions must be taken to prevent the admission of any organism. Inoculation is the process of transferring surface sterilized explants to the nutritional medium. The tissue should only be inoculated within the inoculation chamber for this. The chamber used for inoculation purposes is sterilized using UV light (or) ethyl alcohol swabbing. Laminar air flow cabinets are now used in the majority of laboratories. For aseptic operations, this is a very practical and trustworthy device. It enables you to work for extended periods of time. A number of small blower motors are used in laminar air flow. They blast bacteria-free air through the working area at a rate of around 27:13 liters per minute. All the contaminants are blown away by the ultra clean air and there by an aseptic environment is maintained over the working area.

### 4. Incubation of the culture

The colonies are cultured in a culture room under controlled temperature, light, and humidity after being inoculated with tissue culture medium. With the help of air coolers, the temperature is kept at roughly 25:2ºC. Cultures can be grown in both light and darkness. Cool-light florescent bulbs suspended 18 inches above the culture offer illumination. For 16 hours, this bulb emits a light of 4-10x103 lux. The culture room's relative humidity is maintained at or above 50%.

### 5. Aeration

Aeration, or the correct supply of oxygen to plant tissues, is required. There is no requirement for an outside supply of oxygen if the tissue is developed on the surface of a semi-solid medium. The tissue, on the other hand, will be submerged if the medium is liquid. A particular device for aeration of the tissue must be used in this case.

Shaking the flasks (or tubes) on an automatic shaker can give aeration. The shaker's platform is equipped with clips for carrying conical flasks. The media is agitated, which not only provides aeration but also causes tissue fragmentation into single cells and tiny clumps. Single cells are extremely important in physiological and genetic research.

Aeration can also be provided by arranging a 'filter paper bridge'. The two legs of the bridge remain dipped in the medium, and the horizontal part carries the tissue.

### Tissue Culture – Procedure

To learn about the basic technique of culturing a tissue in vitro, a general method of tissue culture procedure is explained below.

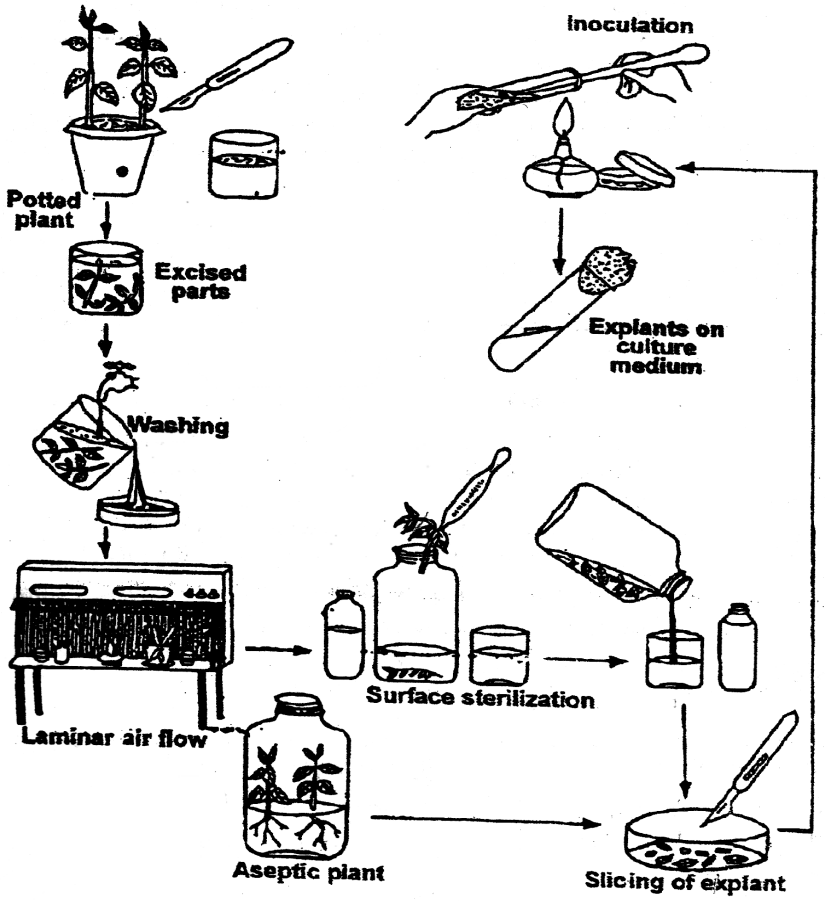



**Fig.: Tissue culture method – Diagrammatic View**

To begin, a tissue is a type of organ that may be cultivated and harvested from a healthy plant. This tissue or organ is sterilized by dipping it for a few seconds in an alcohol or hypochlorite solution. After that, the sterilized tissue is cultured in an aseptic nutritional medium. During incubation, cells divide and regicide to produce a huge tissue colony with no shape. CALLUS is the name of such a colony.

A callus is a clump of cells that is structured but undifferentiated in shape. There is no organ differentiation in the callus. To induce organ differentiation, phytohormones such as Auxins and Cytokinins are given to the callus culture medium in the appropriate ratios. When auxin is added to the callus medium, root growth is induced, and cytokines are added to the callus medium, shoot growth is induced. On the callus, one or more embroyoids (Non Zygotic embryos) form.

The embryoids above are collected and placed in separate jars with nutritional media. Normally, each embryonic grows into a plantlet, which is a miniature plant. Plantlets are generally fragile, with no cuticle layers covering their roots and stems.

High temperatures and dry circumstances are unsuitable for these plantlets. These are first permitted to grow in high-humidity environments. Then it gradually moved to drier circumstances. It may take 4 to 6 weeks for these plantlets to establish themselves as typical plants. Callus and embryoids are seen in organ cultures (such as ovary, ovule, and embryo).

## 1.2 Plant Tissue Culture

### 1.2.1 Sterilization Procedure

**Q2. Write in detail about the sterilization procedures employed in Tissue cultures.**

*Ans :*

**Introduction**

Sterilization is a technique for getting rid of microorganisms. For a successful Tissue Culture technique, aseptic or sterile conditions must be maintained. Because of the need for "ASEPSIS," all culture vessels, media, and devices used in tissue handling, as well as explants themselves, must be disinfected. All operations are carried out in sterile cabinets with laminar air flow.

**Procedure:** Generally sterilization procedure mainly focuses on three aspects.

(1) Preparation of sterile media, containers and instruments.

(2) Maintenance of aseptic condition.

(3) Preparation of sterile explants.

Sterilization is done for different materials by different methods. The methods of sterilization are categorized in to two types. They are:

(1) Physical Methods

(2) Chemical Methods

**1. Physical Methods**

They include (i) Sterilization by Heat;

(ii) Radiation; (iii) Filtration.

**(i) Sterilization by Heat:** It is the simplest and most reliable method. It is done in two ways

(a) Dry Heat

(b) Moist Heat

**(a) Dry Heat:** It is used for sterilizing glass were (Test tubes, petridishes etc.) Dry Heat is provided by hot air oven with Thermostat. High Temperature 160-170°C for one or two hours eliminates all forms of micro organisms. Dry heat also eliminates or can sterilize certain chemicals. Ex: Fats, Oils, Powders etc..

**(b) Moist Heat:** In this, Heat is in the form of steam under pressure. This moist heat kills micro organisms. Autoclave is employed in tissue cultures to provide moist heat, Moist heat is used to sterilize (a) Media Components (b) Dissolved chemicals and (c) Culture Tubes.

**(ii) Radiation:** Inoculation chambers and culture rooms employ these to eradicate germs. For sterilizing, UV light is commonly used. Prior to inoculation in the plant tissue culture method, inoculation chambers, culture media, and tools are disinfected by UV radiation. Filtration (iii): It is used to eliminate heat-sensitive microorganisms from a solution. Various filter types are employed. Special air filters called HEPA are utilized to sterilize Inoculation chambers and culture rooms. Membrane filters and Millipore filters are frequently employed for sterilization.

**2. Chemical Methods**

Different chemicals are utilized in vitro plant tissue culture processes, such as surface sterility, disinfectants, antibiotics, and so on. At normal temperatures, these substances must have particular characteristics such as antibacterial activity, lack of toxicity, penetrating capacity, and so on. Plants used in tissue culture, such as seeds, tissues, excised embryos, and so on, are disinfected with disinfectants such as H2O, ethyl alcohol, Mercuric Chloride, Sodium hypochlorite, and so on. It's known as 'Surface Sterilization.

Inoculation tools are autoclaved and surface sterilized after being dipped in alcohol and then flame sterilized. To keep or maintain aseptic conditions in culture rooms, potassium permanganate fumigants are used.

In place of the typical inoculation chamber, Laminar Flow Clean Air Benches have recently become popular. A "Laminar Air Flow Cabinet" is one in which air is drawn in from the outside and filtered via very small filters before reaching the inoculation table top.

## 1.3 EXPLANTS

**Q3. What do you mean by explants? Explain their method(s) of preparation and sterilization. Explain their methods of preparation and sterilization.**

*Ans :*

**Defnition**

An explant may be defined as any piece of plant part used for initiation of tissue culture.

Selection of Explants:

Explants are frequently taken from leaves, floral buds, seeds, roots, stems, flowers, or any other part of the flower. Explants with more vascular tissue, parenchyma, and cambium have a stronger ability to regenerate than smaller explants. Explants with massive undifferentiated cells have a strong cell division and proliferation capability structure, The plant components to be used as explants, on the other hand, are determined by the following factors. Explants are frequently taken from leaves, floral buds, seeds, roots, stems, flowers, or any other part of the flower. Explants with more vascular tissue, parenchyma, and cambium have a stronger ability to regenerate than smaller explants. Explants with massive undifferentiated cells have a strong cell division and proliferation capability. Nevertheless, the plant components that will be used as

**1. Plant age**

The physiological age of the plants is a significant factor in explant selection. Younger tissues are more sensitive in in vitro tests because they produce callus more easily and undergo the surface sterilising process more easily. As a result, the optimum explants are newly produced tissues.

**2. Season of Collection**

Spring season serves as the best season for collection of explants since in this season the shoots leaves, S are in a flush state of growth and hence are more to 3 min responsive than dormant shoots. As the season distilled progresses from spring, summer, rainfall and then to winter, contamination increases to a very high level and hence the responding ability of explants decreases.

**3. Plant Quality**

For best results, it is advised to obtain explants sterilizing from healthy plants rather than those exhibiting with sterile diseased symptoms or those under stresses.

**4. Size of Explant**

As a general rule, the larger the explants the higher is the content of their nutrient reserve and plant growth regulators. Hence it is easier to culture the larger explants than the smaller ones.

**5. Culture Goal**

Although any part of the plant can be used as an explant, besides the above mentioned factors, the choice of the explant also depends upon the ultimate goal of the tissue culture technique. For instance, if the ultimate goal is the clonal propagation, then the shoot A small part apex or lateral bud will serve as the best explant. For protoplast culture, leaf tissue and for callus culture seedling tissues serves as good explants. Moreover, 1.1.4 cu anther or pollen may be used to produce haploid plants or callus.

**Preparation and Sterilization of Explants**

Initially, the piece of the plant tissue or organ derived from the plant i.e., the explant is thoroughly for pla washed with tap water. Alcohol rinse or swabbing with alcohol-wetted cheese cloth may be done on explants with hairy or waxy surfaces.

The process of tissue culture is carried out in sterile conditions. Therefore, it is important for the explant to be sterile. The part of the plant from which depends upon the explant is excised should therefore be surface sterilized. Surface sterilization of explants may be carried out using the following agents.

1. Mercuric chloride (0.1-1%))
2. Silver nitrate (1%)
3. Sodium hypochlorite (1-2%)
4. Bromine water (1-2%)
5. Hydrogen peroxide (10-12%).

In addition, a suitable wetting agent (E.g: Tween) can also be included to increase the surface area of contact with the sterilizing agent. The technique used for surface sterilization depends on the part of plant from which the explant has been excised.

**1. Aerial Portions**

Aerial portions of the plants (buds, flowers, leaves, stems) are submerged in 70% ethanol for 2 to 3 minutes and rinsed twice or thrice with sterile distilled water.

**2. Seeds**

The seeds are immersed in 70% ethanol for about 2 minutes before being rinsed with sterile distilled water. They are then surface sterilised for a particular period of time using a suitable sterilising agent before being washed with sterile distilled water. However, the sterilant must be entirely removed by vigorous washing with distilled water, as the sterilant can be hazardous to the explant if it gets into the culture media. In an aseptic atmosphere, the seeds are then allowed to germinate. Pre-sterilized filter papers or cotton plugs wet with sterile distilled water can be used for this form of germination. The seeds to be germinated are placed on cotton plugs or double layers of filter papers and placed in a warm environment.

### 1.3.1. Culture Media - Composition and preparation

**Q4. Write in detail about Culture Media Types, composition and preparation?**

*Ans :*

**Culture Media**

A suitable, artificially prepared nutrient medium, on which excised p tissues and organs grow in vitro is called "Culture Medium". Several Scientists es Murashig, Skoog, White, Nitsch etc. proposed the composition of a nut Su medium. But no single medium shown the capacity to maintain optimum growth. Culture media are largely responsible for the in vitro growth morphogenesis of plant tissues.

Fundamentally, the composition of a medium is determined by the unique requirements of a certain culture system, which include the type of material employed (e.g., cells, tissues, etc.) and the type of plant species chosen for culture.

**Major types of Media**

The most popular and normally used Culture Media types are as follows:

**(a) White's Medium:** This is one of the earliest Plant Tissue Culture Media developed for root culture.

**(b) MS Medium:** Murashig and Skoog (MS formulated a medium to induce organogenesis and regeneration of plants in cultured tissues. MS Medium is presently in use for many types of culture systems.

**(c) B5 Medium:** Gamborg developed this type. It was designed for callus cultures and for cell suspension cultures. But at present by few modifications, it is now being used for protoplast culture.

**(d) N6 Medium:** Chu formulated Cereal anther culture. This medium and it is used specifically for Cereal anther culture

**(e) Nitsch Medium:** This was developed by Nitsch and Nitsch and mainly used few anther cultures.

Because of its performance with a variety of plant species and production systems, MS medium are regarded the most practical of the above-mentioned media.

Synthetic media are the ones mentioned above. For tissue culture, these varieties have almost completely superseded Natural Media for culture.

Culture Media – Expression of Concen trations: The concentrations of inorganic and organic components are normally expressed as mass value (mg/1 or ppm $1^{-1}$)

**Culture Media Components**

All culture media generally contain the following constituents.

(a) Inorganic Nutrients.

(b) Organic Supplements.

(c) Growth Regulators.

(d) Carbon and Energy Source.

(e) Solidifying Agents.

**(a) Inorganic Nutrients**

These nutrients are divided into two categories: micronutrients and macronutrients. N, P, K, Ca, Mg, and Sulphur have been identified as important Macronutrients for Tissue Cultures. Fe, Mn, Zn, B, Cu, and Molybdenum are the most common micronutrients required.

**(b) Organic Supplements**

Include vitamins, amino acids, activated charcoal organic extracts and antibiotics.

**(c) Growth Regulators**

Four phyto hormones namely Auxins, Cytokinin Gibberellins and ABA are the essential growth regulators employed in culture plant cells.

**(d) Carbon and Energy Source**

Sucrose and Glucose are considered as t vital compounds for Culture Media, which act as resources for carbon and energy.

**(e) Solidifying Agents**

These are required for the preparation of semisolid or solid culture media. The commonly used solidifying agents are Agar and Gelatin.

**(f) PH of the medium**

The optimal pH for most cultures is in the range of 5.0 – 6.0.

**Preparation of Media**

The general process for preparing a medium consists mostly of preparing STOCK solutions and storing them. They can be utilized whenever and wherever they are needed.

Plant tissue culture medium is now commercially manufactured and accessible as 'Dry Powders' on the market. By dissolving the powder in distilled water, the required medium can be created.

## 1.3.2 Nutrient and hormone requirements

**Q5. Write an essay on Nutrient and hormone requirements**

*Ans :*

Tissue culture of Meteor pea was obtained in July 1968 from the proliferation of an immature embryo. The tissue was isolated in the search for a system which might be useful in the study of gibberellin action at the level of unorganized cells. During the preliminary routine experiments aimed at finding the optimal culture medium, it was noted that the tissue was rather specific in its requirements for growth. The present paper reports the observations concerning the effect of mineral salts, carbohydrates, vitamins and hormones.

Callus tissues of different pea varieties have not been very often used in studies of nutrition and morphogenesis. Torrey and Shigemura (1957) obtained a callus tissue from Alaska pea roots using a modified Bonner mineral medium, with sucrose, auxin and vitamins. The root-forming capacity of several strains of that tissue was studied later on in relation to chromosomal constitution (Torrey, 1967). The production of cytokinins was investigated by using higher salt concentrations and a more complex mixture of vitamins and aminoacids (Short and Torrey, 1972). Hildebrandt etal . (1963) isolated a callus from the stem tissue, which was growing on W hite m ineral solution, sucrose, NAA, inositol and

casein hydrolysate. Bailey (1970) studied the production of pisatin by pea tissue cultivated on a similar medium, containing vitamins, glycine, 2,4-D and coconut milk. Ka11ak and Yarvekylg (1971) investigated the cytogenetic effect of 2,4-D in a pea callus, derived from cotyledons and grown on the Torrey medium. The influence of environmental conditions, nitrogen nutrition and hormones on shoot formation in a callus tissue obtained from excised field pea apices was studied by Gamborgetal. (1974). Apparently, pea callus tissue can satisfactorily be grown on various media and the precise nutritional requirements were not studied in most cases.

**Material and Methods:**

Green, not fully grown pea pods (Pisum sativum cv. Meteor) were opened and the seeds sterilized w ith 5°/o calcium hypochlorite and washed w ith sterile water. Embryos about 3 mm long were taken out of the seeds and placed on the agar medium. Most embryos proliferated in the root region. All tissues referred to in this paper originate from a single embryo. The isolated embryos were first grown on a medium used previously for pea callus tissue by Torrey and Shigemura (1957). Small proliferations were obtained from the axial tissue, which grew very slowly, so that only a few subcultures could be transplanted. Some of the explants were then transferred onto the medium prepared for wild carrot tissue (Ha1perin, 1964) and satisfactory growth was obtained. This medium was designated as basal medium (BM) and has been used since then for stock cultures. It contains mineral solution of Murashige and Skoog (1962), 1% agar, 3% sucrose and (in mg l -1): thiamine 3, nicotinic acid 5, adenine 2 and 2,4-D 0.1. After three more transfers, experiments were set up to determine which components of the medium were actually needed. Five mineral solutions were tried:

1. **T-medium:** was a mineral solution used for pea callus tissue by Torrey and Shigemura (1957).
2. **H-medium:** mineral solution of Heller (1953), which had been proved suitable for m any cultivated tissues.
3. **W-medium:** mineral solution by White (1943) for tomato roots, but also used widely for m any tissues.
4. **WB-medium:** was White's solution as modified by Wood and Braun (1961) for Vinca rosea tissue; it contains additional amounts of ammonium, nitrate, potassium and phosphate salts.
5. **MS-medium:** is a revised medium of Murashige and Skoog (1962) for tissue culture of tobacco.

Organic constituents tested were sucrose, glucose and fructose, vitamins and growth substances. The concentrations are indicated in the text or tables. If necessary, the pH of the media was adjusted with NaOH at 5.8 before autoclaving. Media were autoclaved at 115° C for 25 min. Experiments were carried out in 100ml Erlenm ayer flasks, containing 40 ml of nutrient agar, with three callus pieces in each. Each series comprised at least five flasks. Experiments were repeated three or more times. Cultures were kept in diffuse light from Sylvania "Gro-Lux" and warm white fluorescent tubes, in a day of 12 hours. Initial weight of the callus pieces at transfers was 20—30 mg. Growth was evaluated as fresh weight after 6—8 weeks.

**Results**

The callus tissue obtained was a slow growing tissue with average increase in w eight of 250—350 mg in the culture period. The tissue was rather friable, composed of small, firm masses, sometimes greenish in colour. By the completion of the experiments described here, the tissue had been subcultured 23 times.

**Mineral salts:**

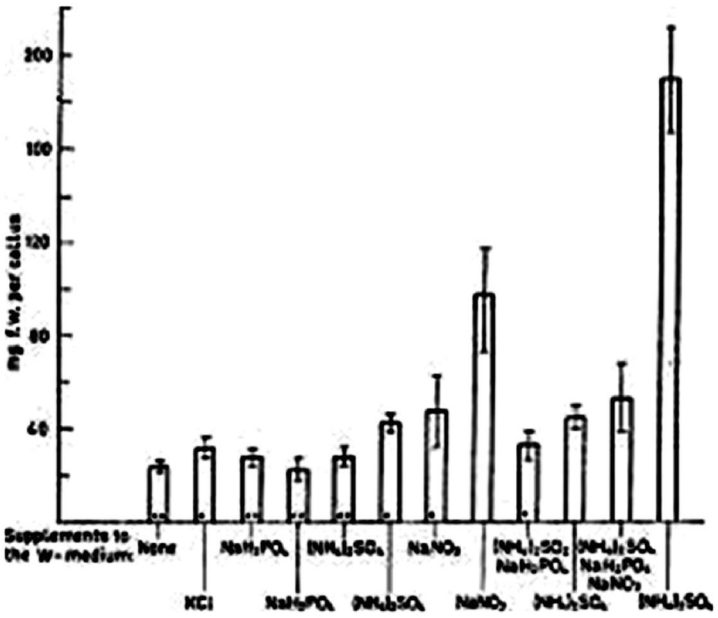
The requirements for mineral salts were studied by using the five solutions mentioned above. They were supplemented w ith sucrose and other organic constituents as in the BM. It was found that only two of 44 them could support the growth of the tissue. These were MS and WB media, the former being much superior.

Growth of Meteor pea callus tissue on different mineral solutions. Results of five separate experiments, each comprising five flasks with three callus pieces.

Figures indicate fresh weight in mg per callus, after six weeks of culture.

**Mineral solutions**

| T | H | W | WB | MS |
|---|---|---|---|---|
| 16.7 | 15.8 | 21.4 | 48.5 | 231.0 |
| 18.3 | 29.2 | 17.5 | 122.4 | 305.9 |
| 14.4 | 26.0 | 27.1 | 101.7 | 246.8 |
| 15.5 | 14.7 | 19.6 | 80.2 | 245.0 |
| 15.6 | 20.0 | 21.6 | 171.0 | 208.7 |




The growth of Meteor pea callus tissue on White's mineral medium, supplemented as indicated. Amounts of particular salts added same as in the complete WB medium. ** = tissues necrotic * = tissues partly necrotic 45. The media used differ greatly from one another in the presence and concentration of almost all salts. As the WB and MS media contain p art of their nitrogen in the form of an ammonium salt, it was thought in the beginning that the Meteor callus might be unable to reduce nitrate. However, when in MS medium all the nitrogen was given as nitrate, the tissue was still growing, although at a slower rate. Further experiments were set up to see whether the tissue required a higher concentration of all salts in general, or the stimulation of MS and WB media were due to some particular element. Therefore, the W medium was supplemented with (NH4)2S 0 4, N aN 03, KC1 and NaH2P 0 4 separately or combined, so as to obtain the concentration of these salts in the WB medium. The results are shown in Fig. 1. As can be seen, no compound alone could support grow th of the tissue. However, when a higher amount of nitrogen, either as ammonium or as nitrate, was added together with KC1, the tissue did not die and some increase in growth was achieved. Yet for the optimal growth on that medium all four salts must be present. It was shown before (Street, 1969) that tomato roots grown in W medium produce an increase in pH, causing iron to become unavailable. Therefore we tried to substitute the inorganic iron of the W medium by Na2Fe-EDTA as in the MS medium, but no growth was induced. When all trace elements of the MS medium were added to the W medium, the tissue did not grow either. Added to the WB medium, the same trace elements caused some increase in weight of the tissue.

Carbohydrates Sucrose, glucose and fructose were added to the BM in three different concentrations. It was rath er surprising th at sucrose was the only sugar tested which could support the grow th of the tissue.

The effect of sucrose, glucose and fructose on the growth of the tissue

| Sugar | conc | |
|---|---|---|
| Sucrose | 2 | 268.3 ± 59.1 |
| | 3 | 417.0 ± 168.4 |
| | 4 | 284.6 ± 58.7 |
| Glucose | 3 | 42.9 ± 10.2 |
| | | 29.9 ± 4.81 |
| | | 18.4 ± 4.4 |
| Fructose | 3 | 40.2 ± 5.1 5 |
| | | 31.4 ± 17.2 |
| Glucose + Fructose | 22 | 37.7 ± 20.2 |

**Vitamins**

The basal medium contains thiamine, nicotinic acid and adenine. In order to test whether these vitam ins were really necessary, the tissue was grown on media lacking one of them for several subsequent transfers.

As can be seen, tissues deprived of thiamine died in the third transfer, while without nicotinic acid their weight was very much decreased in the fifth transfer. Apparently, adenine was not needed, as the tissues could grow in its absence for five passages, with no sign of deficiency. An experiment was run through three transfers with 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10.0 mg l-1 of thiamine. Best growth was obtained w ith 1.0 and 3.0 mg V *, but all the other concentrations gave rather close values.

The effect of thiamine, nicotinic acid and adenine on the growth of the tissue (in mg f. w. per callus ± S. E.)

| Vitamin omitted from the BM | 1st | 2nd | 3rd | 4th | 5th |
|---|---|---|---|---|---|
| None | 230.8±59.6 | 237.6±78.2 | 302.4±38.4 | 300.4± 74.6 | 295.6±58.8 |
| Thiamine | 196.0±45.6 | 72.1 ±9.6 | 22.4 ± 5.1 | --- | --- |
| Nicotinic acid | 285.5±74.0 | 189.1±84.5 | 102.6±22.9 | 79.2 ±44.7 | 87.4 ± 46.9 |
| Adenine | 308.4±76.5 | 206.9±59.0 | 156.3±29.8 | 285.± 89.6 | 463.4±102.8 |

### 1.3.3 Micropropagation.

**Q6. What is Micro propagation? Write about its Technical procedure, Applications and disadvantages.**

*Ans :*

**Micropropagation**

Methods of plant propagation in nature may be either Asexual multiplication of Vegetative parts) or sexual (thro' seeds in higher plants).

Multiplication of genetically identical copies of desirable plants by reproduction is called CLONAL propagation. Clonal propagation of plants in vivo is difficult, expensive and unsuccessful.

Tissue culture methods offer an alternative means of plant vegetation propagation.

Clonal propagation through Tissue Culture is popularly called "Micro Propagation".

It is possible to produce plants in large members in short time and space Micro Propagation'.

**Micropropagation - Procedure:** The technique of micro propagation includes 5 stages (0, I, II, III, IV).

**Table - Showing Activity, Stage wise**

| Stage | Main Activity |
|---|---|
| 0 | Selection of Mother Plant and Maintenance |
| 1 | Initiation and establishment of Culture |
| 11 | Multiplication of Shoots/ Explants |
| 111 | In Vitro, Rooting of Shoots |
| 1V | Transfer of Plantlets to sterilized soil, Under Green House Environment |

**Stages**

**Stage-0**

It is the first step in micro propagation. It involves the selection and growth of stock plants under controlled conditions.

**Stage-I**

Culture, in a suitable medium is initiated in this stage. Suitable explants (eg. Shoot tips, Axiliary Buds) are selected. The chosen explant is surface sterilized and washed.

**Stage-II**

Multiplication of explants (taken in Stage-1) in a correct culture medium is the main activity in this stage.

**Stage-III**

The explants of Stage II are transferred to a medium for the development of shoot tips into rooting shoots (Plantlets).

**Stage-IV**

It entails the germination of plantlets in the soil. It is accomplished by first transporting the plantlets from the laboratory to the Greenhouse for acclimation, and then planting them in the solid of choice.

Other regeneration procedures, like as organogenesis and somatic embryogenesis in tissue cultures, are also classified as Micro propagation types since they produce the same results as those often used (e.g. Shoot Tips).

Factors affecting Micro Propagation Method (in vitro): Few main factors which control micro propagation are as follows:

(a) Genotype of the plant selected.

(b) Physiological status of explants.

(c) Culture Media type.

(d) Culture environmental status (Light, Temperature etc).

### Applications of Micropropagation

Micro propagation is now considered as a suitable alternative to conventional methods of vegetative propagation of plants.

### Advantages

1. A large number of plants can be grown from a small piece of tissue within a short period.
2. It can be carried out thro' out the year (irrespective of seasons).
3. Both storage (for many years) and transport of micro propagation source materials (Plant Propagates etc.,) are practically useful and easy.
4. Production of Disease free plants (by Meristem tip cultures) and particularly the production of virus-free plants can be successfully achieved in short time.
5. Sterile useful hybrids can be multiplied in short time.
6. Low production cost is another advantage.

### Disadvantages

1. Contamination of cultures.
2. Accumulation of growth inhibitory substances in the medium.
3. Low Genetic variability.
4. Hyper hydration of the cultures and explants/ leading to verification/ resulting in the death of shoots.

## 1.4 ORGAN CULTURE

### 1.4.1 Totipotency, Vegetative Organs- Root, Shoot, Leaf culture

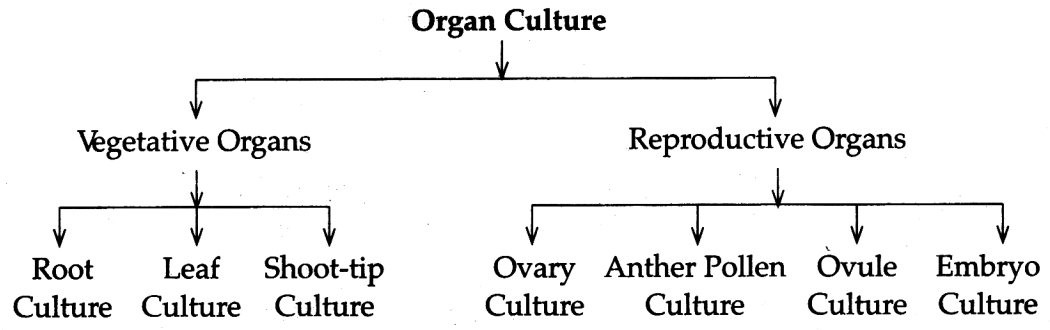
**Q7. Write in detail about Organ Culture?**

*Ans.*

### Introduction

Culturing of plant organs on nutrient medium is called organ culture, Organ culture provides an excellent opportunity to define the nutrients and growth factors normally received by the organ.

On the basis of the origin of the explants, organ culture can be categorized into different types, which are given below.



### Root Culture

Root culture is the process of growing the excised radical tips of aseptically germinated seeds in a nutritional media.

In 1922, Kotte (Germany) and Robbins (USA) developed root tip cultivation at the same time. Kotte cultivated pea and maize root tips that had been excised in a range of nutritional solutions. After two weeks, the growth slowed and the cultures were eventually lost. White later made a significant contribution to the continuous growth of root tip cultures (1934). He created a medium with three B vitamins, pyridoxine, thiamine, and nicotinic acid, in place of yeast extract. This medium, also known as white's synthetic medium, went on to become one of the most common media used in invitro cultures.

### Importance of Root culture

(a) Root tip cultures increase our knowledge of carbohydrate metabolism, role. of mineral ions, vitamins etc, in the growth of roots.

(b) These cultures proved the dependence of roots on shoots for growth hormones.

(c) Leguminous plant root cultures are an ideal medium for studying the process of nodule formation by nitrogen-fixing bacteria (Rhizobium sp). Raggio and his colleagues created nodules in Phaseolus vulgaris root cultures that had been removed.

(d) The roots of various plant species produce medicinally useful alkaloids. The production of such substances can be tracked down using root cultures. This method is also used to boost the production of these chemicals in cultivated roots. Hyoscyamina is commercially produced from Datura stramonium root cultures.

### Leaf Culture

Leaf culture is the culture of excised young leaf primordial (or) immature leaves in a chemically defined medium under controlled conditions.

T.A. Steeves and L.M. Sussex (1966) first realized that culture of excised leaf primordial would provide an experimental system to study the development of leaves under controlled conditions. They successfully cultured the leaf primordia of ferns, particularly Osmunda.

For leaf culture, leaf primordial (or) very young leaves are excised. They are surface sterilized and inoculated on an agar solidified nutrient medium. Mature leaves are not suitable for in vitro growth.

### Importance of leaf culture

1. It is useful to study the effects of various nutrients, growth environmental conditions on leaf growth.

2. In the case of ferns, leaf cultures are useful to study the development stages of sporangia.

### Shoot Tip Culture

The culturing of terminal (0.1-1.0 mm) portion of a young stem comprising the meristems is called shoot-tip (or) meristem culture.

For the first time, LOO (1945) described the culture of 5 mm shoot tips dodder and asparagus. Ball (1946) developed an indigenous method for identifying the exact section of the shoot system that gives rise to a full plant the following year. Virus infection in potato and dahlia can be removed by stem tip culture, according to Movel (1952-55).

The removed stem tips and meristem can be cultivated in a basic nutritional mixture that has been aseptically solidified. The meristem produces a single leafy shoot (or several shoots) right away. The most often utilised auxin and cytokin are NAA and BAP, respectively. The growth of shoot apices can also be aided by coconut milk and gibberellic acid.

**Importance of Shoot tip culture**

1. Generally the meristematic tissue is free from virus due to their fast mitotic activity. Therefore shoot tip culture of a plant is ideal for producing Virus free stock.
2. **Micropropagation:** Asexual (or) vegetative propagation of whole plants using tissue culture technique is reffered to as micropropagation. Shoot tip (or) meristem culture of many plant species can successfully be used for micropropagation.
3. Many plants produce seeds that are highly heterozygous in nature. Such seeds are not accepted for storing genetic resources. So, the meristemfor such plants can be stored in vitro.
4. **Propagation of haploid plants**: Haploid plants derived from anther/pollen culture always remain sterile until and unless they are made homozygous diploids. Meristem (or) shoot tip culture of haploid plants can be used for the propagation of such haploid plants.

### 1.4.2 Reproductive Organs Anther, Ovule, Embryo culture

**Q8. Write an essay on Anther and Pollen Culture?**

*Ans :*

Anther culture is a method of cultivating developing anthers on a nutritional medium. The microspores in the cultured anthers grow into calluses (or embryoids). Organogenesis (or) embryogenesis are the processes by which embryoids produce haploid plantlets.

The creation of haploid plants is the core premise of anther and pollen cultivation. It is based on microspore totipotency and the presence of a single chromosomal set in microspores.

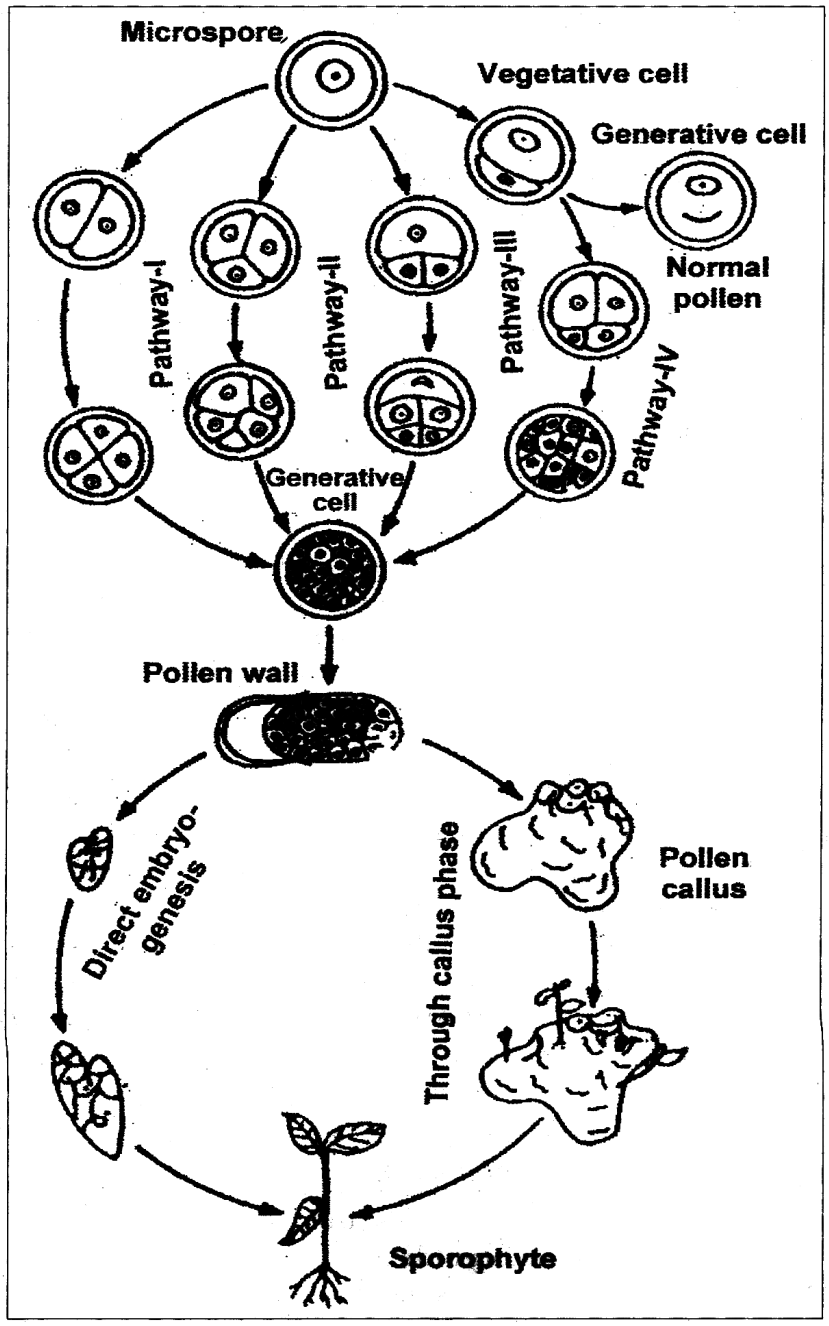
Shimakura (1934), a Japanese cytologist, was the first to use anther culture to better understand the physiology of meiosis. Later, Guha and Maheswari (1964) detected the formation of embryo-like structures within the cultivated anthers by mistake. Bourgin and Nitsch (1967) were the first to obtain haploid plants from isolated Nicotiana anthers. During the last decade much progress has been made in anther culture of rice, wheat, maize, potato, Atropa, Brassica and other crop species.

**Technique of anther culture**

The complete anther is usually cultured while growing haploids from microspores (pollen grains). The mature anthers are picked out of the flower buds and removed. Surface sterilisation is done using ethyl alcohol (or) chlorine water. The anthers are placed on the nutritional medium aseptically. At 28° C, the cultures are kept in alternate light (12-18h) and dark periods (2-6h). The wall tissues of responding anthers turn brown over time. They burst open after 3-8 weeks, exposing the multicellular tissue. The tissue in certain species progressively organises into a spherical embryo. This embryo develops into a single haploid plant called a haploid plantlet. Only one haploid plant is produced from a pollen grain (or) anther using this procedure. The complete anther is usually cultured while growing haploids from microspores (pollen grains).

e.g. Atropa, Datura and Nicotiana.

In the majority of plants, however, the tissue divides repeatedly to generate callus. Several embryoids are differentiated from this callus. On isolation, each embryoid develops into a plantlet. Due to endopolyploidy, the plants derived from the callus are frequently mixo ploids. The somatic cells of the anther wall can divide and produce unwanted diploid callus (or) plantlets in anther culture. Not only that, but numerous pollen grains are involved in the production of calluses. Instead of intact anthers, isolated pollen grains should be cultured to overcome these issues.Shart et al (1972) succeeded in raising haploid tissue clones from isolated tomato pollen. Nitsch (1974) developed a synthetic medium for raising haploid plants from isolated pollen grains of Datura and tobacco.




**Cytology of Androgenesis**

The invitro development of haploid plants originating from totipotent pollen grain is called Androgenesis. A problem of fundamental interest in pollen culture is the mode of origin of sporophyte (plant) from pollen grains.

Through detailed cytological investigations, four pathways have been 17 identified leading to invitro androgenesis.

**Pathway-1**

Microspores divide equally and both the daughter cells contribute to the further development of sporophyte. Vegetative and generative cells are not differentiated.

**Eg.** Datura innoxia

**Pathway-II**

Microspores divide by an unequal division to form vegetative cell and generative cell . Sporophyte arises from the vegetative cell alone.

**Eg.** Hordeum vulgare, Nicotiana tabacum

**Pathway-III**

Microspores undergo unequal division as in pathway II. But the sporophyte arises from the generative cell only.

**Eg.** Hyoscyamus niger

**Pathway-IV**

Microspores divide unequally and both the cells contribute to the formation of sporophyte. eg. Datura innoxia, Datura metel.

**Factors affecting Anther culture**

The following factors significantly affect haploid production through anther culture: (a) Temperature; (b) Culture medium (c) Stage of pollen at culture and (d) Physiological status of the donor plant.

**(a) Temperature**

High temperature (20° C or more) had a promotory effect on pollen embryogenesis, in tobacco and Datura. In Datura the optimum temperature is 25° C. A cold treatment to the flower buds before excising the anthers increase the output of embryoids. NITSCH (1974) reported that in Nicotiana tabacum upto 58% of anthers yielded embryos from the buds which were kept at 5° C for 72 hrs. In contrast only 21 percent anthers yielded embryos if the buds were maintained at 22° C for the same period.

**(b) Physiology of the donor plant**

Androgenesis is influenced by the age of the donor plant as well as the environmental conditions in which it is produced. Anthers from young plants are usually more sensitive short-day plants had a better response than those from long-day plants.

**(c) Culture medium**

The dietary needs of removed anthers are substantially more straightforward. Anthers can be grown on a base medium with coconut milk (or) kinetin added to it. Iron (40 mol) is one of the most significant mineral salts for the induction of haploids. Sucrose is most commonly used at a concentration of 2%. In cultures, adding activated charcoal (0-5-2%) to the media increases androgenesis.

**(d) Selection of anthers**

The anthers having uninucleate pollen grains (microspores) are the most convenient for anther culture. However, the optimal stage of microspore formation for maximal pollen generation differs by plant species. If pollen is grown at the first mitotic stage in Datura and Tobacco, anthers produce the best response. Anthers are more prolific when cultivated at the uninucleate microspore stage in most other cases. The microspore mother cells in meiosis have been discovered to be the optimal stage in Lycopersicon - 1. Brassica sp. anthers bearing ripe pollen, on the other hand, are acceptable for cultivation.

**Diploidization of Haploids**

Up to the flowering stage, haploid plants grown from anther (or) pollen culture thrive normally. However, due to the lack of homologous chromosomes, these plants are unable to create viable gametes. As a result, the plants are sterile and no seeds are produced. By duplicating the chromosomal complement, haploids may be sustained. By treating the haploids with 0.4 percent colchicines for 24-48 hours, they can be produced homozygous diploids.

**Importance of Haploids**

1. Homozygous diploid plants can be used as pure lines in breeding programme.
2. Haploid plants are useful in cytogenetic studies.
3. Recessive mutations can be easily detected in haploids, because they contain only one set of chromosomes.
4. Haploid plants become a good source for biochemical and for physiological studies, just like microorganisms.
5. Homozygous diploids (Isogenic lines) are beneficial, when the plants are self – in compatable. Eg. Rye
6. In genetic engineering, haploids can be successfully used for gene transfer. Haploid tissue of Lycopersicon has been used for the transfer and expression of three genes from E.Coli.

**Q9. Write an essay on Embryo culture and Ovuleculture.**

*Ans :*

**Embryo culture**

Like any other plant organ, sexually produced embryos (zygotic embryos) can be used as explant and cultured aseptically in a test tube containing nutrient medium. Zygotic embryo culture was first carried out by Hanning (1904) in two crucifers, *Cochleria* and *Raphanus.* He successfully raised transplantable seedlings from the embryos cultured on a semi-solid medium containing mineral salts and sugar. Subsequently, many workers obtained plants by culturing embryos especially obtained from abortive seeds.

There are two types of embryo culture.

**(a) Culture of immature embryos** : This type of culture is mainly used to grow immature embryos originating from unripe (or) hybrid seed which failed to germinate. Excising such embryos is very difficult. A complex nutrient medium is required to raise them to produce plants. The objective of this technique is to understand the factors that are regulating the development of embryos under natural conditions.

**(b) Culture of mature embryos** : Mature embryos are excised from ripe seeds and are cultured in nutrient medium. These embryo; require a simple nutrient medium containing mineral salts, sugar anc agar. This type of culture is precised mainly to avoid dormancy o: the seed before germination.

Dietrich (1924) studied the physiological behaviour of im-mature embryo's under invitro conditions. He noticed that excises immature embryos on a nutrient medium tend to bypass certair developmental stages. These embryos grew directly into wea- seedlings. This phenomenon of seedling formation withov completing normal embryonic development is called precocious germination.

**Importance of embryo culture**

1. Embryo culture helps to study the metabolic and chemical aspects of dormancy and seed germination.
2. Long periods of dormancy in seeds delay breeding work, expecially in horticultural and crop plants.

Using embryo culture technique, the life cycle can be shortened in these plants. For example, the life cycle of *Iris* was reduced from 2-3 years to less than one year.

3. The most useful and popular application of embryo culture is to raise rare hybrids by rescuing embryos of incompatible crosses. Such rare hybrides are successfully produced in case of RICE (interspecific) and also intergeneric crosses between Barley X wheat, Barley X Rye.
4. Germination of excised embryos is the more reliable test for rapid testing of viability in seeds, especially duringjhe dormancy period.
5. Seeds and embryos of several obligate parasites can be grown under aseptic conditions to study their dependence on the host. Johri and Bajaj (1962) sucessfully cultured the young embryo of Cuscuta reflexa, a stem parasite in the absence of host tissue.
6. Propagation of rare plants. It is achieved in Musa and Colocasia plants.

**Ovuleculture**

Ovule culture is an elegant experimental system by which ovules are aseptically isolated from the ovary and are grown aseptically on chemically defined nutrient medium.

Ovule culture is useful to study the behaviour of zygote (or) very young embryos which are difficult to culture.

Ovule culture was carried for the first time by White (1932) in Antirrhinum majus. However, the technique of ovule culture was perfected by Maheswari (1958)

**Importance of ovule culture**

1. The most important achievement of ovule culture is the development of technique of test tube pollination and fertilization. By this technique, it is possible to germinate pollen in the same culture along with the excised ovule and to induce invitro fertilization. Using this technique, it has been possible to fertilize the ovules of Argemone, Papaver, Nicotiana and Datura.
2. It is a boon for the plant breeders in obtaining seedlings from crosses which are normally unsuccessful because of abortive embryos.
3. In vitro ovule culture helps to understand the factors that regulate the development of a zygote.
4. In nature, the seeds of orchids germinate only in association with a proper fungus. As a result numerous seeds are lost due to unavailability of proper fungus. This problem can be overcome by culturing fertilized ovules.
5. In obligate root parasites such as Striga (or) Orobanche, the formation of seedlings is dependent on some stimulus from the host root. On the medium enriched with growth regulators, the seeds germinate and develop into shoots.

## 1.5 Callus Culture and Isolation and Fusion of Protoplast Culture

**Q10. Write an essay on callus culture.**

*Ans :*

**Introduction**

Callus is a term used to describe an undifferentiated, unorganized proliferating mass. Callus or callus-like components can form in various regions of an undamaged plant as a result of a wound or disease.

Experiments with cells from explants yield callus, which is vital in plant tissue culture. Explants are a term used to denote any minor excised area of any living healthy plant. On a nutritional medium containing specific phytohormones, the explants are cultivated aseptically in vitro under controlled conditions. In culture, these explants grow into huge, unorganized, and differentiated entities with a vast number of cells. Callus is a term used to describe an undifferentiated region of the body.

**Callus Culture:** Explants (small segments of plant parts) are cultured aseptically on solid or liquid nutrient medium under controlled conditions to produce the callus.

**History**

Several attempts were made in the past by many scientists to produce Callus tro. Few of those studies are summarized and depicted in the form of a table given below.

| Scientist | Year | Contribution |
|---|---|---|
| R.J. Gautheret | 1934-37 | First developed the callus from the excised cambial cells of Salix in a simple nutrient medium. |
| J. Van Over Beck | 1941 | First reported the importance of coconut milk in callus culture. |
| F. Skoog | 1954-55 | First produced the callus from the stem pieces of Tobacco in nutrient medium containing auxin. |
| Skoog and Miller | 1957 | First proposed and reported the hormonal control of organ formation from callus. |

**Callus Culture Method**

The initiation of callus culture from the explants requires three major steps. The three steps are as follows:

**Step I :**

Aseptic preparation of plant material.

**Step II :**

Selection of suitable nutrient medium supplemented with phytohormones like auxins and cytokinins.

**Step III:**

Incubation of culture under controlled conditions

**Procedure**

Explants are plant components that have been excised and washed in liquid detergents (5 percent v/v-Teepol). The explants are next surface sterilized for 10-15 minutes with chemicals such as Hg Cl or sodium hypochlorite, and then rinsed with autoclaved distilled water. The sterile explants material is split into small parts aseptically. These segments are placed aseptically on a nutrition media that has been solidified with Agar. For the induction of callus tissue, this solidified/semisolid Agar-nutrient media (which is sterilized by autoclave at 151bs pressure for 15mts) is utilized.

Finally, the previously incubated Agar culture medium is moved to the incubation culture rooms. Incubation should take place in a controlled environment. Temperatures of 25o2oC and relative humidity of 55 to 60 percent are considered excellent for callus growth.

After the callus has formed, sections of the callus tissue are excised and placed immediately on fresh nutritional medium to continue growing. Serial subcultures can keep these callus cultures alive.

Various plant tissues from a variety of plant species can be employed to induce callus development. Carrot roots, on the other hand, are quite particular to callus cultures. The technique of callus culture is primarily explained "Thro" the carrot root cultures, which is used as an example.

### Importance of Callus Culture

Callus Culture plays little role compared to other culture methods. But it is pass in many other experimental holds which help many areas of biology, to achieve the respective objectives of goals.

Callus culture shows its importance in certain areas like the following

1. Certain manipulations in the nutrients and hormones of callus culture medium help two phenomena like plant regeneration [PR] and somatic Embryogenesis GE). It is possible to regenerate whole plant in large number from callus tissue (PR) Embryos can be produced directly from the somatic cells of callus tissue (SE).
2. Cell suspension culture can be initiated from callus culture.
3. Callus tissue is considered as a good source of Genetic variability. Plants with genetic variations can be regenerated from such modified callus cells.
4. Secondary metabolites of commercial value can be obtained from callus cultures. Direct extraction of a secondary metabolite or a drug from the callus tissue (ie used as a source for such materials) is possible.

**Q11. Write an essay on protoplast culture.**

*Ans :*

### Introduction

Protoplast culture is a new technique in the realm of culture technology that involves culturing protoplasts in vitro under aspectic settings. Plasmalemma and everything enclosed inside it make up the protoplast of a plant cell.

Walled cells do not allow for experimental manipulation. For all practical reasons, naked protoplast without a cell wall is the most acceptable material in plant tissue culture systems when compared to others.

Protoplast culture is currently an important aspect of plant cell genetics, especially when it comes to cell fusions and gene transfer (Genetic Engineering). PPC also aids in the investigation of biochemical and metabolic pathways in plant systems.

The basic premise of protoplast culture is to isolate large numbers of intact living protoplasts aseptically, remove their cell walls, and culture them on a suitable nutrient medium for growth and development. In cultures, isolated protoplasts are nothing more than a naked plant cell surrounded by plasma lemma that is capable of cell wall renewal, cell division, growth, and plant regeneration.

### History

Various studies that were made in the past regarding the protoplast culture can be summarized and depicted in the form a table as below.

| Scientist | Year | Contribution |
|---|---|---|
| J. Kler cker | 1892 | First isolation of protoplast by mechanical method |
| E. K. U ster | 1927 | First adapted physiological method for protoplast isolation. |
| E. C. Cocking | 1960 | First isolation of protoplast by Enzymatic method. |
| J.B. Power & Cocking | 1968 | First employed cellulose and Macerase mixture for the isolation of protoplast. |
| G. Melchers etal | 1971 | First reported the plant regeneration from isolated protoplast in Nicotiana tabacum. |

**Protoplast Culture – Procedure**

Protoplast culture mainly includes two major steps like

(A) Isolation of the protoplast;

(B) Culturing the isolated protoplast;

**(A) Isolation of the protoplast**

Plant protoplasts can be extracted from a variety of tissues. Mesophylls from leaves and cells from liquid suspension cultures (CSC) are both convenient and acceptable materials. Cultures are more dependable and produce higher-quality protoplasts than other sources, which necessitate numerous precautions during isolation.

The removal of the cell wall without injuring the cell or protoplasts is a crucial step in protoplast isolation. Before removing the cell wall, the cell or tissue must be placed in a hypertonic solution (13 percent Mannitol). The cell is plasmolysed, and the hypertonic solution functions as an osmotic stabilizer. It aids in the removal of the cell wall without causing any damage to the cell.

After osmotic stabilization, the protoplasts can be separated from their call wa by a variety of methods, the most common of which are mechanical and enzymatic.

**(i) Mechanical Isolation:** In mechanical isolation, the protoplast is liberated by splitting apart each cell compartment. Cutting the cell walls is performed under a microscope with a micro scalpel or a sharp razer. From the severed ends of the cells, protoplasts are discharged. To avoid harm, the isolated protoplasts are subsequently moved to an isotonic liquid media. This approach has some drawbacks, such as the ones listed below.

(a) Tedious and time consuming.

(b) Only small number of protoplasts is isolated.

(c) Unfit for mature and meristematic tissues.

(d) Not suitable for large scale attempts.

**(ii) Enzymatic Method :** Enzymes like cellulose, hemicelluloses, pectinase or macroenzyme are employed to digest the cell walls in this method. It helps in the liberation of protoplast. These enzymes are available commercially; They are isolated mostly from fungi. This method helps to isolate the protoplast from any part of the plant body. But cells of mesophyll pollen mother cells (PMC) or pollen tetrads (PT) appear to be best materials, for this method.

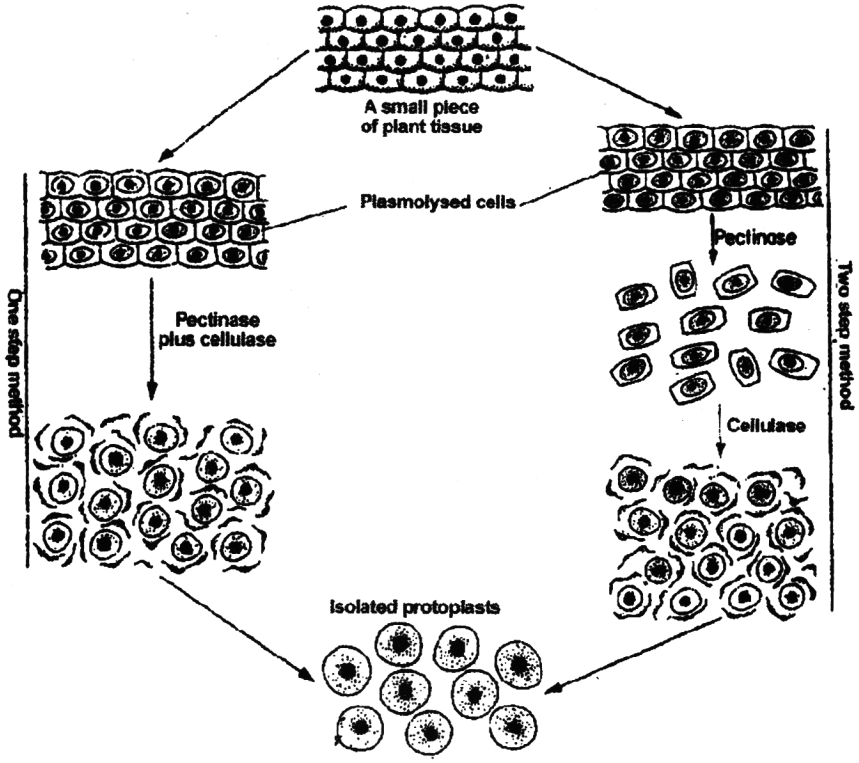



**Fig.: 3-4 : Method of Enzymatic isolation**

This method has certain advantages compared to mechanical Method. They are

(a) Injury to cells is very low or eliminated totally

(b) Large quantity of protoplast can be isolated.

(c) Side effects like osmotic shrinkage etc are limited.

Pectinase and cellulase enzymes are used separately (Two step method) session or as a mixture (one step method), at a time to dissolve cell wall parts.

**Isolation Technique :** The technique of isolation of protoplast is mesophyll cells includes several steps.

**Procedure :** It can be summarized as follows

**Steps:**

1. Surface sterilization of leaves (in 70% Ethyl alcohol etc)
2. Washing of leaves with sterilized distilled water.
3. Peeling of leaf pieces from the lower epidermis of the leaves.
4. Placing the leaf pieces in a chemical mixture (13% Mannital etc) plasmolysis and shaken in a water bath.
5. Placing the above treated leaf pieces in wall degrading enzyme mixture (Pectinase / Cellulose) and incubated (for 24 Hrs. at 25°C).

6. Filtering the above material and the filtrate is Centrifuged (100/min)
7. Separation and washing (Sorbitol) the protoplast settled at the bottom of the centrifuge tube, then placed in 20% sucrose solution.
8. The above solution is centrifuged (200g/min) and protoplasts are removes with a pipette and placed finally in a suitable culture medium.

**Isolation of Protoplast from Cell Suspension Culture (CSC)**

CSCs are considered as the most suitable material for protoplast isolation. More number of protoplasts can be obtained only after sub culturing the new cell suspensions. Fresh cell suspension preparations avoid all the obstacles and end in successful isolation.

**Procedure**

Several steps are involved in the isolation method. The main steps of this technique are given below in a sequence.

1. Filtering the harvested cell suspensions thro' a nylon sieve. Filtrate contains single cells and cell clumps.
2. Separation of the medium and single cells.
3. Transferring the single cells to a flask.
4. Addition of plasmolyticum (i.e., CPW 13M solution). to the cells in the (CPW = Cell and Protoplast Washing Medium) flask 13% Mannitol (13M) Cells are subjected to plasmolysis in this step.
5. Separation of plasmolyticum and cells.
6. Addition of Enzyme solution to the cells in the flask.
7. Rotation of the flask with a rotator shaker for 4-6 Hrs.
8. Separation of enzyme solution is cells by filtration and transferring the filtrate to centrifuge tubes.
9. Collection of the sediment containing protoplast pellets, after the removal of supernatant enzyme medium.
10. Resuspension of protoplast pellets in CPW 21% sucrose solution and subjected to spinning.
11. Collection of viable and pure protoplasts at the surface and washed with CPW 13M with repeated centrifugations.
12. Suspension of the protoplasts in measured volume of liquid culture medium in the final step.

## 1.6 ORGANOGENESIS

**Q12. Write an essay on Organogenesis?**

*Ans :*

The process of initiation and development of an organ is called organogenesis. In plant tissue culture, inducing organogenesis is an important way to regenerate plants from the culture. Plant cells are grown in culture which allows them to be manipulated and then induced to develop into whole plants.

**Importance of Organogenesis**

Organogenesis, in embryology, the series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo. The cells of an organ-forming region undergo differential development and movement to form an organ primordium, or anlage.

The process of initiation and development of an organ is called organogenesis. In plant tissue culture, inducing organogenesis is an important way to regenerate plants from the culture. Plant cells are grown in culture which allows them to be manipulated and then induced to develop into whole plants.

**Factor Affecting the Organogenesis**

- Size of Explant
- Source of Explant
- Age of the Explant
- Seasonal Variation
- Oxygen Gradient
- Quality and Intensity of Light
- Temperature
- Culture Medium

- PH of the Medium
- Polyploidy Level
- Age of Culture

In vitro organogenesis is controlled by a number of factors other than phytohomes such factors are discussed below:

**1. Size of Explant**

Organogenesis is generally dependent upon size of explant. The large explant consisting parenchyma, vascular tissues and cambium have greater regenerative abilitythan the smaller explant. Small group of homogenous tissues taken from epidermal or subepidermal layer could directly give rise to complex organs like flower or bud or roots.

**2. Source of Explant**

The most suitable part of the plant for starting culture will depend on species. Leaves and leaf fragment of many plant species like Begonia, Solanum, Nicotina, Crepis , etc have shown capacity to regenerate shoot buds. Bulb scale of Hillium, sps, regenerate adventitious bulbelts, flower stem explant of Tulip asps. Regenerate shoot bud , inflorescence axis of Haorthia sp. Also forms shoots and root section of Convolvulus sp. Produce shoot bud in culture.

**3. Age of the Explant**

Physiological age of explant is important for in vitro organogenesis. In Nicotiana species, regeneration of adventitious shoot is only noted if the leaf explant is collected from vegetative stage i.e. before flowering. Leaf explants of Echeveria sp. That are collected from young leaves only produce roots, whereas older leaves initiate only shoot buds and leaves of medium age produce both shoots and roots.

**4. Seasonal Variation**

Bulb scales of Lilium speciosum regenerate bulblets freely in vitro when explant is taken during spring and autuma period of growth but same explant collected from summer or winter season does not produce any bulblets.

**5. Oxygen Gradient**

In some cultures, shoot bud formation takes place when the gradient of available oxygen inside the culture vessel is reduce. But rooting requires a high oxygen gradient.

**6. Quality and Intensity of Light**

The blue region of spectrum promotes shoot formation and red light induce rooting. The treatment of blue light followed by treatment of red light also stimulates the organogenesis phenomenon. In some cultures artificial fluorescence light favours rooting and inhibits in others. In case of Pisum sativum shoot bud initiation takes place in dark followed by sudden treatment of lights.

Normally, organogeneses in culture take place with an illumination of 2000- 3000 lux. However, the callus tissue of Nicotina tabacum also produces shoot bud or embryo when tissue is exposed to high intensity of light of 1000-15000 lux.

**7. Temperature**

Most tissue culture are grown successfully at twp. Around 25 0C. In number of bulbous species optimum temperature may be much lower of about 15-18 0C. Increase in temp upto upto 330C may be associated with rise in growth of tobacco callus but for shoot bud initiation a lower temp of about 18 0C may be optimum.

**8. Culture Medium**

Medium solidified with agar favours bud formation although there are some reports about the development of leaf shoot buds on culture grown in a liquid medium.

**9. PH of the Medium**

The PH of the culture medium is generally adjusted between 5.6 and 5.8 before sterilization. The pH may have a determining role in organogenesis.

**10. Polyploidy Level**

Variation in chromosome number i.e anuploidy, polyploidy, etc of plant cell in culture has been well documented. With the increase in chromosome instability there is a general decline in morphogenetic potentiality of callus tissue. So the most important factor in maintaining organogenic potential of callus tissue is the maintenance of chromosome

stability. Frequency of subculture can affect the chromosome stability of cell culture. So in order to maintain chromosome stability, cultures are subcultured frequently and regularly.

**11. Age of Culture**

A young culture frequently produces organs. But the organogenic potential may decrease and ultimately disappear in old culture. In certain cultures of some plants, the plant regeneration capacity may retain indefinitely for many years.

**Organogenes is PPT**

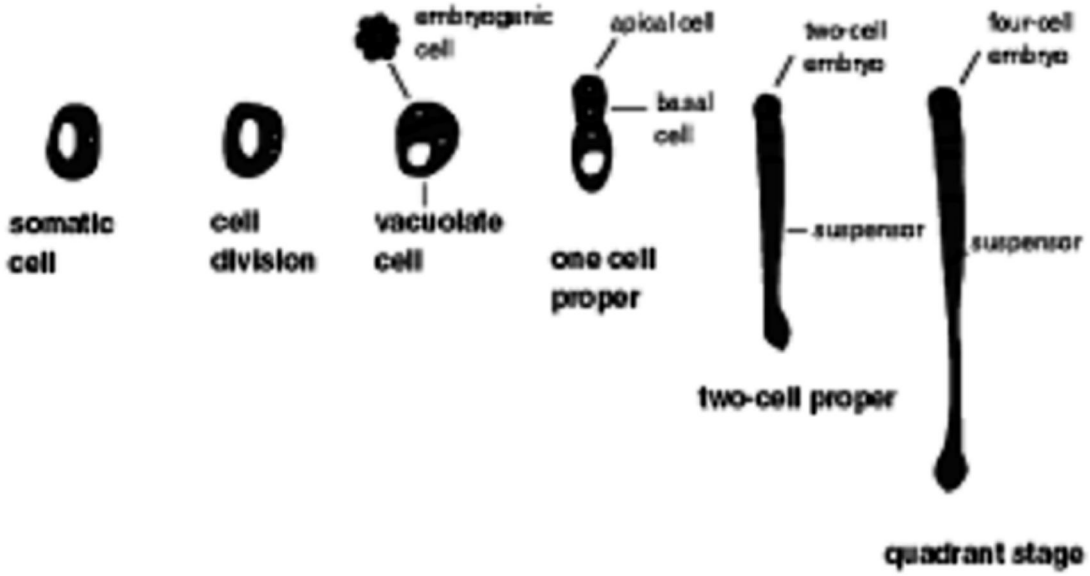
Organogenesis is the process by which the ectoderm, endoderm, and mesoderm develop into the internal organs of the organism. Cells of each germ layer proliferate, migrate, reaggregate and differentiate into various tissues that form the organs (organogenesis).

## 1.6.1 Embryogenesis(somatic and zygotic)

**Q13 What is embryogenesis in plant tissue culture?**

*Ans :*

The process of formation of an embryo is called embryogenesis. Somatic embryogenesis is another important way to regenerate new plants in plant tissue culture. Embryo development occurs through an exceptionally organized sequence of cell division, enlargement and differentiation.




**Types of Embryogenesis:**

Primary somatic embryogenesis: In this type, the embryogenesis can only be induced by using the explants.

Secondary somatic embryogenesis: In this phenomenon, the development of the embryo is induced through existing somatic embryos.

**Applications of somatic embryogenesis**

Applications. Applications of this process include clonal propagation of genetically uniform plant material; elimination of viruses; provision of source tissue for genetic transformation; generation of whole plants from single cells called protoplasts; development of synthetic seed technology.

**mportance of embryogenesis**

In woody plants, somatic embryogenesis plays a critical role in clonal propagation and is a powerful

tool for synthetic seed production, germplasm conservation, and cryopreservation. A key step in somatic embryogenesis is the transition of cell fate from a somatic cell to embryo cell.

**Characteristics of embryogenesis**

Embryogenesis is defined by a sequential series of dynamic processes that include cell division and growth, and the elaboration of differentiation programs leading to cell fate specification.

**Zygotic Embryogenesis**

In zygotic embryogenesis, plant development initiates within the embryo sac (female gametophyte) where the egg cells fuse with male gametes (which move into embryo sac by siphonogamy to fuse with egg cells) to produce zygote. The formed zygote carries three major events namely assymmetrical division, embryo patterning and transition respectively to produce mature embryo. The following three events occur as follows.

**Asymmetrical Division**

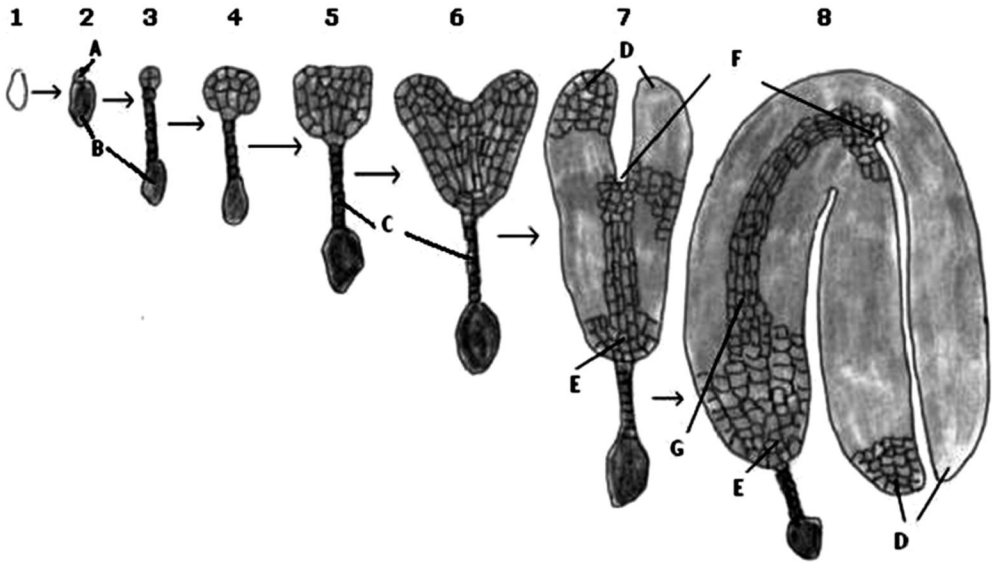
Firstly, the formed zygote divides assymmetrically into two cells i, small apical cell, which later develops into embryo and large basal cell (also calle d as suspensor) which provides nutrients to the embryo from endosperm for further development. Assymmetrical division plays a crucial role in the development of embryo because failure in the formation of basal cell turns out to be lethal for rygotic embryos

**Embryo Patterning**

The two-celled embryo proliferates to form octant (eight-celled embryo) which further divides to reach globular stage where significant embryonic pattern can be observed. In addition to this, the zygotic embryo and somatic embryo morphologi-cally resemble each other from globular stage.

**Transition**

Embryo at globular stage experiences transition through radial patterning by undergoing series of cell divisions leading to heart shaped embryo followed by torpedo stage then finally reaches cotyledonary stage. At this stage, embryogenesis reaches the verge of completion. Finally, the embryo elongates itselm and is enlarged to complete embryogenesis. Later on, the meristematic activity begins at physiologica level where growth process, accumulation and maturation of seed is initiated. However, in certaim plants like Arabidopsis, after the cotyledonary stage, the embryo undergoes other physiological changes like dessication, quiescence and dormancy along with accumulation and maturation to complete the seed formation process (which takes place only in zygotic embryogenesis). Whereas, in somati embryogenesis, the embryo directly develop into fully differentiated plantlet.



# Short Question & Answers

**1 Write short notes on the sterilization procedure.**

*Ans :*

Sterilization is a technique for getting rid of microorganisms. For a successful Tissue Culture technique, aseptic or sterile conditions must be maintained. Because of the need for "ASEPSIS," all culture vessels, media, and devices used in tissue handling, as well as explants themselves, must be disinfected. All operations are carried out in sterile cabinets with laminar air flow.

**Procedure**

Generally sterilization procedure mainly focuses on three aspects.

1. Preparation of sterile media, containers and instruments.
2. Maintenance of aseptic condition.
3. Preparation of sterile explants.

Sterilization is done for different materials by different methods. The methods of sterilization are categorized in to two types. They are:

(i) Physical Methods

(ii) Chemical Methods

**2. Major types of media.**

*Ans :*

The most popular and normally used Culture Media types are as follows:

**(a) White's Medium:** This is one of the earliest Plant Tissue Culture Media developed for root culture.

**(b) MS Medium:** Murashig and Skoog (MS formulated a medium to induce organogenesis and regeneration of plants in cultured tissues. MS Medium is presently in use for many types of culture systems.

**(c) B5 Medium:** Gamborg developed this type. It was designed for callus cultures and for cell suspension cultures. But at present by few modifications, it is now being used for protoplast culture.

**(d) N6 Medium:** Chu formulated Cereal anther culture. This medium and it is used specifically for Cereal anther culture

**(e) Nitsch Medium:** This was developed by Nitsch and Nitsch and mainly used few anther cultures.

Because of its performance with a variety of plant species and production systems, MS medium are regarded the most practical of the above-mentioned media.

**3. Importance of Root culture.**

*Ans :*

(a) Root tip cultures increase our knowledge of carbohydrate metabolism, role. of mineral ions, vitamins etc, in the growth of roots.

(b) These cultures proved the dependence of roots on shoots for growth hormones.

(c) Leguminous plant root cultures are an ideal medium for studying the process of nodule formation by nitrogen-fixing bacteria (Rhizobium sp). Raggio and his colleagues created nodules in Phaseolus vulgaris root cultures that had been removed.

(d) The roots of various plant species produce medicinally useful alkaloids. The production of such substances can be tracked down using root cultures. This method is also used to boost the production of these chemicals in cultivated roots. Hyoscyamina is commercially produced from Datura stramonium root cultures.

**4. Shoot Tip Culture**

***Ans :***

The culturing of terminal (0.1-1.0 mm) portion of a young stem comprising the meristems is called shoot-tip (or) meristem culture.

For the first time, LOO (1945) described the culture of 5 mm shoot tips dodder and asparagus. Ball (1946) developed an indigenous method for identifying the exact section of the shoot system that

gives rise to a full plant the following year. Virus infection in potato and dahlia can be removed by stem tip culture, according to Movel (1952-55).

The removed stem tips and meristem can be cultivated in a basic nutritional mixture that has been aseptically solidified. The meristem produces a single leafy shoot (or several shoots) right away. The most often utilised auxin and cytokin are NAA and BAP, respectively. The growth of shoot apices can also be aided by coconut milk and gibberellic acid.

**5. Ovuleculture.**

*Ans :*

Ovule culture is an elegant experimental system by which ovules are aseptically isolated from the ovary and are grown aseptically on chemically defined nutrient medium.

Ovule culture is useful to study the behaviour of zygote (or) very young embryos which are difficult to culture.

Ovule culture was carried for the first time by White (1932) in Antirrhinum majus. However, the technique of ovule culture was perfected by Maheswari (1958)

**6. Embryo culture.**

*Ans :*

Like any other plant organ, sexually produced embryos (zygotic embryos) can be used as explant and cultured aseptically in a test tube containing nutrient medium. Zygotic embryo culture was first carried out by Hanning (1904) in two crucifers, *Cochleria* and *Raphanus.* He successfully raised transplantable seedlings from the embryos cultured on a semi-solid medium containing mineral salts and sugar. Subsequently, many workers obtained plants by culturing embryos especially obtained from abortive seeds.

**7. Short notes oraganogenesis/**

*Ans :*

The process of initiation and development of an organ is called organogenesis. In plant tissue culture, inducing organogenesis is an important way to regenerate plants from the culture. Plant cells are grown in culture which allows them to be manipulated and then induced to develop into whole plants.

**8. What is embrogenesis?**

*Ans :*

The process of formation of an embryo is called embryogenesis. Somatic embryogenesis is another important way to regenerate new plants in plant tissue culture. Embryo development occurs through an exceptionally organized sequence of cell division, enlargement and differentiation.

**9. Zygotic Embryogenesis.**

*Ans :*

In zygotic embryogenesis, plant development initiates within the embryo sac (female gametophyte) where the egg cells fuse with male gametes (which move into embryo sac by siphonogamy to fuse with egg cells) to produce zygote. The formed zygote carries three major events namely assymmetrical division, embryo patterning and transition respectively to produce mature embryo. The following three events occur as follows.

**10. Characteristics of embryogenesis.**

*Ans :*

Embryogenesis is defined by a sequential series of dynamic processes that include cell division and growth, and the elaboration of differentiation programs leading to cell fate specification.

# Choose the Correct Answer

1. How many stages are there in Micropropagation [ d ]
   (a) 5 (b) 6
   (c) 8 (d) 5
2. How many major types of cultural media are present? [ b ]
   (a) 6 (b) 5
   (c) 7 (d) 9
3. How many pathways have been identified for leading to invitro androgenesis [ c ]
   (a) 7 (b) 2
   (c) 4 (d) 6
4. How many culture methods are there for isolation? [ d ]
   (a) 4 (b) 9
   (c) 5 (d) 5
5. In which year did J. Kler Cker make his contribution for Protoplast Culture? [ b ]
   (a) 1926 (b) 1892
   (c) 1960 (d) 1896
6. Who first reported the plant regeneration from isolated protoplast in nicotiana tabacum [ b ]
   (a) E.C. Cocking (b) G. Melchers et al
   (c) E.K. U Ster (d) J.kler cker
7. Which of the following is not a factor affecting organogenesis? [ c ]
   (a) Temperature (b) Culture method
   (c) Surroundings
8. The method defined by a sequential series of dynamic processes that include cell division and growth, and the elaboration of differentiation programs leading to cell fate specification, is ? [ c ]
   (a) Organogenesis (b) Protoplast Culture
   (c) Embryogenesis (d) Seasional variation
9. Who reported the importance of coconut milk in callus culture. [ b ]
   (a) F.Skoog (b) J. Van Over Beck
   (c) R.J.Gautheret (d) Skoog and Miller
10. L. Bergmann made his contribution to Single Cell Culture in the year [ c ]
   (a) 1909 (b) 1960
   (c) 1968 (d) 1970

# Fill in the blanks

1. Tissue culture may also be defined as _____
2. The MS. Medium was formulated by _____ in 1962
3. The most commonly used amino acid is _____
4. _____ is called as culturing of plant organs on nutrient medium
5. _____ is commercially produced from the root cultures of *Datura stramonium*
6. Ovary culture was developed by _____
7. Placing the isolated single cell on the wet filter paper raft along with growing _____
8. _____ occurs through an exceptionally organized sequence of cell division, enlargement and differentiation.
9. Somatic embryogenesis plays a critical role in _____
10. _____ is the process by which the ectoderm, endoderm, and mesoderm develop into the internal organs of the organism.

## ANSWERS

1. Culture of isolated plant cells
2. Murashige and Skoog
3. Glycine
4. Organ culture
5. Hyoscymina
6. Nitsch
7. Nurse Tissue
8. Embryo development
9. Clonal propagation
10. Organogenesis

# One Mark Answers

**1. What is Androgenesis ?**

*Ans :*

The invitro development of haploid plants originating from totipotent pollen grain is called Androgenesis.

**2. Give some examples of anther and pollen culture.**

*Ans :*

Atropa, Datura, Nicotiana.

**3. Mention the steps involved in Callus Culture Method.**

*Ans :*

The initiation of callus culture from the explants requires three major steps. The three steps are as follows:

Step I : Aseptic preparation of plant material.

Step II : Selection of suitable nutrient medium supplemented with phytohormones like auxins and cytokinins.

Step III: Incubation of culture under controlled conditions:

**4. What is nurse tissue means?**

*Ans :*

Placing the isolated single cell on the wet filter paper raft along with growing callus part is called nurse tissue.

**5. What is the procedure for protoplast culture?**

*Ans :*

Protoplast culture mainly includes two major steps like

(A) Isolation of the protoplast;

(B) Culturing the isolated protoplast;

Isolation of the protoplast:

**6. Write very briefly about plant regeneration.**

*Ans :*

The capacity of single cell to give rise to whole plant is called plant regeneration

**7. What is organogenesis ?**

*Ans :*

Organogenesis Organogenesis is the process by which the ectoderm, endoderm, and mesoderm develop into the internal organs of the organism.

**8. What is embryogenesis?**

*Ans :*

The process of formation of an embryo is called Embryogenesis.

**9. What is the Sterilization procedure for plant tissue culture?**

*Ans :*

Sterilization is a technique for getting rid of microorganisms. For a successful Tissue Culture technique, aseptic or sterile conditions must be maintained. Because of the need for "ASEPSIS," all culture vessels, media, and devices used in tissue handling, as well as explants themselves, must be disinfected.

**10. Name the culture media components.**

*Ans :*

Culture media components are.

(a) Inorganic Nutrients.

(b) Organic Supplements.

(c) Growth Regulators.

(d) Carbon and Energy Source.

(e) Solidifying Agents.

UNIT II

5. **Applications of tissue culture:** Production of pathogen free plants and stress resistant plants, somaclonal variants and synthetic seeds.
6. Induction of hairy roots and its applications in production of secondary metabolites.
7. Haploidy and triploids, Cryopreservation and Germplasm Conservation.
8. Somatic Hybrids and Cybrids

## 2.1 APPLICATIONS OF TISSUE CULTURE

### 2.1.1 Production of pathogen free plants and stress resistant plants

**Q1. Write in detail about the various applications of Tissue culture technology?**

*Ans :*

Today, tissue culture is playing a vital role in various fields like Agriculture Medicine, Plant Breeding, and Genetics etc. As now, Biotechnology is also utilizing Tissue culture methods particularly cell suspension cultures in its studies.

The refinement of methodology of Tissue culture is greatly helping the concerned to make suitable manipulations to improve or modify plants to the benefit of mankind.

The current applications of tissue culture are very wide and ovaries. Some of the important ones are as follows:

**Pathogen free plants**

Using tissue culture techniques, it is feasible to grow healthy plants from the shoot tips of virus-infected plants. It was discovered that, even in infected plants, virus-free cells exist in the shoot tips. To obtain virus-free plants, such shoot tips are cultivated in an appropriate culture medium. This approach aids in the complete eradication of virus in plants. This approach is frequently employed. In Horticulture, this approach is commonly used to generate virus-free ornamental plants.

It is possible to produce virus free plants thro tissue culture at commercial level. For which, specific cultured tissues are employed. Such cultured Tissues are obtained from

(a) Virus free plants

(b) Meristems - which are generally free of infection.

(c) Meristems treated with Heat shock (34-36°C) to inactivate the virus.

(d) Media-treated with chemicals.

Among the several culture techniques, meristem-tip culture is the most reliable method for virus and other pathogen elimination.

### 2.1.2 Somaclonal variants and synthetic seeds

**Q2. Write essay on Somaclonal variants and synthetic seeds**

*Ans :*

**Production of Somaclonal Variants**

Few of the achievements, relating to the production of useful somaclonal variants, are described below. Somaclonal variants with Somaclonal variants are genetic variations identified in grown cells in vitro. SOMACLONES (LARKIN etal) are plant variations generated through tissue culture of somatic tissue. When compared to their original plants, such variant plants (Somaclonal variations) may exhibit several important characteristics such as disease resistance, herbicide resistance, stress tolerance, and so on. Plant Breeders are now making extensive use of these somaclonal differences in their plant breeding methods. For crop development, only plants with stable, heritable, and beneficial somaclonal variants are chosen and used.

(a) Herbicide Resistance e.g. Tobacco resistant to Sulfonyl-UREA.

(b) Abiotic stress Resistance e.g. Salt tolerance in tobacco, Maize.

(c) Improved seed quality (with less neurotoxin) e.g. A new variety of Lathyru sativa (Bio.L. 212).

(d) Improved oil content eg. Cirtonella java Bio-13 variety - a medicinal plant with more oil and more citronellon.

(e) Improved fruit Quality e.g. super tomatoes with high solid component (obtained by screening somaclones).

(f) Disease resistance e.g Rice, Wheat, Apple, Tomato have been success fully me produced and developed in recent times.

**Production of Stress Resistant Plants**

Tissue Culture technique has emerged as an achievable and cost effective alternative tool for developing stress resistant / tolerant plants in recent years. Biotic and A-biotic stresses impose a major threat to Agriculture and limiting the productivity of commercial crops mainly by the major a-biotic stresses like Salinity, Drought heat etc. Tissue Culture Technique has the potential for selection of s resistant plants. Production of salt and drought resistant or tolerant plants Tissue Culture (in vitro selection) has been reported in many plant species cereals, vegetables, fruits, commercial crops etc.

**Achievements of Tissue Culture :** (a) Salt or NaCl stress resistant / tolerant plants.

**Examples:**

(i) Brassica Oleraceae / B. juncea

(ii) Chrysanthemum morifolium

(iii) Citrus aurantium (orange)

(iv) Citrus limon (LEMON)

(v) Nicotiana tabacum (Tobacco)

(vi) Lycopersicum esculentum (Tomato). -) Drought Stress Resistant / Tolerant plants.

**Examples :** Maize, Potato, Barley, Rice and woody plants (Mango, Grapes, etc.)

Biotic Stress Resistant / Tolerant Plant. Certain Bacteria, Fungi, Viruses etc., mostly act as pathogens and create biotic stress. In vitro selection is an alternative approach for development of biotic resistant/ tolerant strains / lines. It has been achieved in recent year's plants thro' Tissue Culture as described below:

| Plant Species | Plant | Resistant to the Pathogen |
|---|---|---|
| (1) Nicotiana tabacum | Tobacco | Pseudomonas syringae |
| (2) Zea maya | Maize | Helmintho sporium maydis |
| (3) Solanum tuberosum | Potato | Phytopthera infestans |
| (4) Oryza Sativa | Rice | Helminthosporium oryzae |
| (5) Hordeum vulgare | Barley | Fusarium sp. |

**Production of Secondary Metabolites:**

The plant cells when cultured can produce several different secondary metabolites. These include alkaloids, glycosides, terpenoids, latex, tannins etc.

The, invitro production of secondary metabolites is generally high from differentiated tissues. This, in vitro production of secondary metabolites, from cell cultures, includes several advantages like the following.

(a) Recovery of the secondary metabolite is easy.

(b) Production is not affected by external factors and their variations.

(c) Production time is less and cost effective.

(d) Production of high quality product is possible.

Cell-suspension culture in liquid medium helps in the large scale production of natural secondary metabolites.

Many of the secondary metabolites, particularly alkaloids, are of immense use in medicines. The one major achievement in this field is the production of shikonine, a dye, from cell cultures of Lithospermum erythrorhizon, on commercial scale. Vincristine, Digoxin, Pyrethrins are some other secondary metabolites produced thro' cell cultures like shikonine.

Some secondary metabolites, plant source and their uses are given in the following table.

| | Plant source | Product | Uses |
|---|---|---|---|
| 1. | Digitalis lanata | Digoxin | Cardiac tonic |
| 2. | Chrysanthemum ciner ariae folium | Pyrethrin | Insecticide |
| 3. | Papaver somniferum | Morphine | Sedative |
| 4. | Cinchona officinalis | Quinone | Anti malarial |
| 5. | Catharanthus roseus | Vincristine | Anti carcinogenic |

**Production of Synthetic seeds (Artificial seeds)**

Embryoids or Somatic embryos are structures that resemble embryos that are created during tissue/ cell/organ/culture. (Types that aren't Zygotic.) A synthetic seed is a man-made seed containing a somatic embryo encased in a protective gel-like material.

Somatic Embryos are encapsulated in a suitable substance (e.g. sodium alginate) combined with insecticides, herbicides, fungicides, and other chemicals in synthetic seeds. These artificial seeds can be used to mass-produce desired plant species and hybrid variations quickly and efficiently. Synthetic seeds provide many benefits like the following.

(a) Can be directly sown in the soil.

(b) Can be stored up to a year.

(c) Can be handled and delivered in a comfortable way.

All these characters of synthetic seeds help the plant Breeder, in a great way in field conditions.

**Crop Improvement**

The utilization or replacement of conventional breeding methods by tissue culture techniques such as anther, pollen or ovary cultures eliminates the need t wait for six to seven generations of selfing and crossing- over to obtain a pureline The same can be achieved with in two generations by tissue culture method. The haploids that are obtained thro' anther or pollen culture can be easily converted into homozygous diploids by chromosome doubling.

### Micropropagation

The regeneration of whole plant thro' tissue culture is called Micro propagation. It is possible to raise hundreds or thousands of plants in large scale from small amounts of tissue thro' micropropagation. Using this method, millions of genetically identical plants can be obtained from a single bud. This method is being utilized by the industries for the commercial production of orchids and fruit trees Shoot tip propagation is exploited intensively in horticulture and nurseries. This method is now being considered as an alternative to vegetative propagation.

### Benefits of Micropropagation

1. Rapid multiplication of superior clone thro' out the year.
2. Multiplication of Disease free plants.
3. Multiplication of sterile Hybrids of sexual origin.

### Genetic Transformation

Tissue culture, in combination with genetic engineering is very useful in gent transfers. The transfer of a useful or desirable bacterial gene, for example crygene (Crystal protein) from Bacillus thuringiensis, into a plant cell, followed by the regeneration of whole plants enables the integration and expression of the desirable gene in the expected fashion. Such plants are described as Transgenic plants.

### Storage of Germ plasm

Germplasm can be stored in the form of a tissue. It helps in the elimination of diseases and rapid multiplication of clones. Preservation of plant germplasm can be achieved by plant tissue culture. Usually plant tissues/cells/ meristems a stored at low temperature of liquid nitrogen (-196°C) for a desirable period. The technique is known as Cryopreservation. It helps to maintain the genetic stability there by it allows the preservation of germplasm for a desirable period.

## 2.2 Induction of Hairy Roots and its Applications in Production of Secondary Metabolites

**Q3. Write an essay on hairy roots and their production of Secondary metabolities.**

*Ans :*

### Introduction

The term "hairy Root" was first coined by Steward et al (1900). Ricker fi named the Hiary root causing organism as Phytomonas rhizogenes, which was latte renamed as Agrobacterium rhizogenes. It is a rod shaped, gram negative bacteri closely related to Rhizobium.

Virulent strains of A. rhizogenes contains a large mega plasmid (more than 200 kb), which plays a key role in hairy root formation. This plasmid is called " Plasmid". It alone carries the genes involved in hairy root formation. A. rhizogenes causes Hairy Root disease by entering in to a plant thro' wound and causing a proliferation of secondary or Hairy Roots. Ri Plasmid (Root inducing Plasmid) - Genome Structure: This plasmid carries three genetic components. They are designated as (a) t-DNA (b) Vir and

(c) Border sequences. t-DNA is a mobile element. This t-DNA enters into the nucleus of infected plant cells, then in to host genome and then promotes Transcription process. These effects lead to the formation of multi branched adventitious roots at the site of infection, resulting in Hairy Root Disease.

**(a) t-DNA :** It is approximately 10-30 Kbp in size.

**(b) Vir :** It represents or called virulence-area of Ri Plasmid. It contains several vir genes which do not enter the host plant cell but help the transfer of t-DNA c) Border Sequences: These reside in the chromosome of Agro bacterium itself and they contain about 25 bps only.

### Ri Plasmids - Types

Hairy Roots synthesize 'OPINES' as food to Agro erium. The Ri-Plasmids are identified as Two

Types or Strains depending on Opine Chemical nature". They are described as Agropine strains and Mann e strains. Agropine - Types or strains are highly virulent than Mann opine 5. So Agrapine strains are frequently used in Hairy Root Cultures.

**Hairy Root Culture (HRC) - Establishment**

Hairy Root culture, also called med root culture, is a type of plant Tissue Culture.

It is mainly used -

(a) to study plant metabolic pathways.

(b) to produce valuable Secondary Metabolites. o produce recombinant proteins etc.

(c) to produce recombinant proteins etc.

Success of Hairy Root Culture system establishment depends on ons. These conditions include

(i) Selection of the correct bacterial strain of A. rhizogenes. several

(ii) An appropriate explant.

(iii) A proper antibiotic.

(iv) A suitable culture medium.

Agropine strains are the most used strains in HRCs. The common explants of Care Shoot Tips, leaf, cotyledon, protoplast, storage root, tubers, petioles etc. The level of tissue differentiation determines the ability to give rise to transformed after A. rhizogenes inoculation. Successful infection is achieved by the addition of Acetosyingone Chemical. Vancomycin, Ampicillin sodium, Streptomycin Sulphate etc. (in 100-500 ug/ml entrations) are used to kill or eliminate bacteria.

Selection of Appropriate hairy root lines is done following or after sub culture, in all HRCS.

**Applications Of Hairy Root Culture**

The main applications of HRCs are as follows:

(a) Production of Secondary Metabolities.

(b) Understanding Metabolisms in Plants.

(c) Production of New compounds.

(d) Regeneration of whole plants.

**Production on of Secondary Metabolites :**

This is considered as the most common activity of Hairy Root Cultur g to the rest of the applications mentioned above, because of certain advantages and very few limitations.

(i) Hairy roots are acting like a special Biological system, playing a vital role in the production of valuable phyto-chemicals that are useful as pharmaceutical and food additions.

(ii) Hairy Root Cultures are also in use as an alternative to Agricultural methods for producing valuable secondary metabolities.

(iii) Special Alkaloid produced from plants like Atropa bellado s tricophyllus and Datura Candida, thro HRCs is now considere jor events in secondary metabolite production.

(iv) Special antitumour alkaloids like Campothecin, Taxol etc., are now b h HRC technique.

(v) A special, ribosome inactivating protein, by name 'trichosanthin' produced from the plant Trichosanthes "kirilowii', thro' HRC. It is proved that trichoanthin inhibits multiplication of HIV (AIDS Virus) and there by checks AIDS disease.

**Advantages Of Hairy Root Culture**

Few of them are as follows

(i) Secondary Metabolites produced thro HRCs are stable and in higher quantities compared to traditional methods.

(ii) Hairy Roots can be maintained as organ cultures for a long time and al help subsequent shoot regenerations without any side eff

(iii) Plantlet regeneration frequency in HRC is very high.

(iv) Plant derived Biopharmaceuticals, thro' HRC are safe medically, cheap cost wise and easy for storage and mass production,

(v) Suitable, modified Bioreactor systems are now being employed for the exploitation of hairy roots in commercial scale on account of their production capacity of Secondary metabolites.

In spite of several advantages as explained above, HRC system has not been utilized Globally in commercial scale.

HRC still requires a special focus and further research to make HRC, an effective TOOL' in culture systems in future.

## 2.3 HAPLOIDY AND TRIPLOIDS

### Q4. What is meant by Haploidy and Triploidy?

*Ans :*

Triploidy is a rare chromosomal abnormality. Triploidy is the presence of an additional set of chromosomes in the cell for a total of 69 chromosomes rather than the normal 46 chromosomes per cell. The extra set of chromosomes originates either from the father or the mother during fertilization.

**Triploidy**

**Introduction:**

Anytime the quantity or quality of the blueprint is not preserved, abnormal cell function, organ function (e.g., developmental delay), fetal development (e.g., birth defects), and miscarriage can occur. Triploidy is one of the most common genetic causes of fetal loss (approximately 20%). Estimates are that triploidy occurs in about 2% to 3% of conceptions. Triploidy, which can result in a partial mole, can derive through several distinct mechanisms, some of which originate as errors in meiosis (Fig.). Dispermy is the most common cause and leads to a diandrogenic conception. Another cause is abnormal chromosome segre-gation (meiosis I or meiosis II) of all chromosomes involving either sperm (diandrogenic) or egg (digynic). Fertilization of the primary oocyte and failure to extrude either polar body are additional digynic mechanical.

Triploidy is the term referring the presence of three sets of haploid (single) chromosomes in an organism or cell line and is termed 3n, where 1n is the haploid chromosome number for the species concerned. Triploids are both euploid and polyploid in that they contain a completely balanced extra set of chromosomes to the normal diploid (2n) state. Triploids are rarely found in a viable state in wild animal populations but can occur in plant communities. Triploids are widely used in both commercial fruit, fish, and mollusk production as they can have beneficial commercial traits such as improved growth, pathogen resistance, and infertility conferring protection of elite genetics.

**Other Organisms**

Triploidy is encountered occasionally in natural populations of flowering plants containing diploid (2n) and tetraploid (4n) plants. It is presumed that such triploids arise by natural crosses between diploid and tetraploid plants in the same population. However, there are many examples of triploid strains of cultivated plants that have been induced artifically by crossing diploid and tetraploid parental strains. Unlike human triploids, such triploid plants appear to be morphologically normal, but are characterized by being completely infertile and can only be propagated vegetatively. Their infertility arises during gamete formation. Typically during meiosis the three homologs of each chromosome join and cross-over to produce a trivalent at the first meiotic division. The resulting chromosome segregation from each trivalent is completely random and it is extremely unlikely that a sufficiently large number of genetically balanced gametes can be produced to provide fertility. This phenomenon of triploid sterility was widely studied in the 1930s and 1940s in various plant species with notable contributions from Darlington and Mather in triploid Hyacinthus (hyacinth) and Dermen in Petunia by studying chromosome segregation in pollen.

One of the most famous and ancient examples of a triploid plant species is the cultivated banana characterized by its widely used and fleshy seedless fruit. The cultivated banana is believed to have been derived from a cross between a diploid

species Musa acuminata and the tetraploid species M. balbisiana, both of which produce seeded fruit, some 1000 years ago in southeast Asia. This gave rise to a sterile triploid plant with large seedless fruit and enormous food-producing properties. Propagation of the cultivated banana occurs by dividing its root system. There are now more than 600 varieties of cultivated banana, including the plantain, which have been introduced into the majority of tropical countries. Although the original seeded wild species are still available, they are considered to be so inferior that they are only eaten in times of famine when the cultivated banana crop fails.

**Principles:**

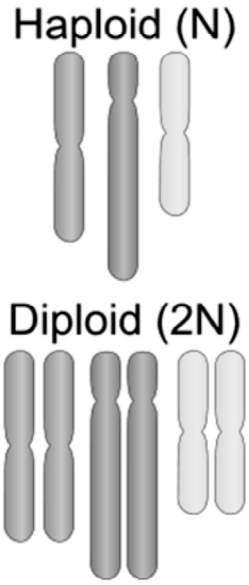
**Triploidy and Tetraploidy**

In addition to the diploid (2n) number characteristic of normal somatic cells, two other euploid chromosome complements,triploid (3n) and tetraploid (4n), are occasionally observed in clinical material. Both triploidy and tetraploidy have been seen in fetuses. Triploidy is observed in 1% to 3% of recognized conceptions; triploid infants can be liveborn, although they do not survive long. Among the few that survive at least to the end of the first trimester of pregnancy, most result from fertilization of an egg by two sperm (dispermy). Other cases result from failure of one of the meiotic divisions in either sex, resulting in a diploid egg or sperm. The phenotypic manifestation of a triploid karyotype depends on the source of the extra chromosome set; triploids with an extra set of maternal chromosomes are typically aborted spontaneously early in pregnancy, whereas those with an extra set of paternal chromosomes typically have an abnormal degenerative placenta (resulting in a so-called partial hydatidiform mole), with a small fetus. Tetraploids are always 92, XXXX or 92, XXYY and likely result from failure of completion of an early cleavage division of the zygote.

Triploidy most frequently results from fertilization of an oocyte by two sperms(dispermy). Failure of one of the meiotic divisions, resulting in adiploid oocyte or sperm, may account for some cases.Triploid fetuses account for approximately 20% of chromosomally abnormal spontaneous abortions.

**Haploidy**

The word haploid describes a condition, a cell, or an organism that contains half of the set of homologous chromosomes present in the somatic cell. Homologous chromosomes are two chromosomes that pair up by having the same gene sequence, loci, chromosomal length, and centromere location. Half of the homologous pairs are maternal (coming from the mother) whereas the other half, paternal (coming from the father). Thus, in other words, a haploid is when a cell, for instance, contains half of the total homologous chromosomes, i.e. a single set of chromosomes that are unpaired.




Examples of Haploid

**Haploid cell in humans**

Sex cells (gametes) are examples of haploid cells. In humans (as well as other higher forms of living things), the somatic cell contains two copies of genes. As such, they are referred to as diploids. They produce haploid gametes through gameto-genesis, a process that makes use of meiosis that halves the chromosomal set. This is essential so that during the union of gametes the resulting zygote is diploid. This preserves the integrity of chromosomal number throughout generations.

In particular, the human sex cells (a sperm or an egg cell) will have 23 chromosomes. This means

that the sperm cell and the egg cell contain only half the diploid number of a human somatic cell (which is 46). At fertilization, the two cells unite forming a zygote with now two sets of chromosomes. The human zygote, then, grows by going through mitosis. As a result, the archetypal chromosomal number of 46 in humans per somatic cell in the human body is maintained.

### 2.3.1 Cryopreservation and Germplasm Conservation

**Q5. What is Germplasm Conservation?**

*Ans :*

**Germplasm Conservation**

Germplasm broadly refers to the hereditary material (total content of genes) transmitted to the offspring through germ cells.

Germplasm provides the raw material for the breeder to develop various crops. Thus, conservation of germplasm assumes significance in all breeding programmes.

As the primitive man learnt about the utility of plants for food and shelter, he cultivated the habit of saving selected seeds or vegetative propagules from one season to the next one. In other words, this may be regarded as primitive but conventional germplasm preservation and management, which is highly valuable in breeding programmes.

The very objective of germplasm conservation (or storage) is to preserve the genetic diversity of a particular plant or genetic stock for its use at any time in future. In recent years, many new plant species with desired and improved characteristics have started replacing the primitive and conventionally used agricultural plants. It is important to conserve the endangered plants or else some of the valuable genetic traits present in the primitive plants may be lost.

A global body namely International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation. Its main objective is to provide necessary support for collection, conservation and utilization of plant genetic resources throughout the world.

**There are two approaches for germplasm conservation of plant genetic materials:**

1. In-situ conservation
2. Ex-situ conservation

**1. In-Situ Conservation**

The conservation of germplasm in their natural environment by establishing biosphere reserves (or national parks/gene sanctuaries) is regarded as in-situ conservation. This approach is particularly useful for preservation of land plants in a near natural habitat along with several wild relatives with genetic diversity. The in-situ conservation is considered as a high priority germplasm preservation programme.

**The major limitations of in-situ conservation are listed below:**

(i) The risk of losing germplasm due to environmental hazards.

(ii) The cost of maintenance of a large number of genotypes is very high.

**2. Ex-Situ Conservation**

Ex-situ conservation is the chief method for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic materials in the form of seeds or from in vitro cultures (plant cells, tissues or organs) can be preserved as gene banks for long term storage under suitable conditions. For successful establishment of gene banks, adequate knowledge of genetic structure of plant populations, and the techniques involved in sampling, regeneration, maintenance of gene pools etc. are essential.

**Germplasm conservation in the form of seeds**

Usually, seeds are the most common and convenient materials to conserve plant germplasm. This is because many plants are propagated through seeds, and seeds occupy relatively small space. Further, seeds can be easily transported to various places.

**There are however, certain limitations in the conservation of seeds**

(i) Viability of seeds is reduced or lost with passage of time.

(ii) Seeds are susceptible to insect or pathogen attack, often leading to their destruction.

(iii) This approach is exclusively confined to seed propagating plants, and therefore it is of no use for vegetatively propagated plants e.g. potato, Ipomoea, Dioscorea.

(iv) It is difficult to maintain clones through seed conservation.

Certain seeds are heterogeneous and therefore, are not suitable for true genotype maintenance.

**In vitro methods for germplasm conservation**

In vitro methods employing shoots, meristems and embryos are ideally suited for the conservation of germplasm of vegetatively propagated plants. The plants with recalcitrant seeds and genetically engineered materials can also be preserved by this in vitro approach.

**There are several advantages associated with in vitro germplasm conservation**

(i) Large quantities of materials can be preserved in small space.

(ii) The germplasm preserved can be maintained in an environment, free from pathogens.

(iii) It can be protected against the nature's hazards.

(iv) From the germplasm stock, large number of plants can be obtained whenever needed.

(v) Obstacles for their transport through national and international borders are minimal (since the germplasm is maintained under aspectic conditions).

**There are mainly three approaches for the in vitro conservation of germplasm:**

1. Cryopreservation (freeze-preservation)
2. Cold storage
3. Low-pressure and low-oxygen storage

**Q6. What is Cryopreservation.**

*Ans :*

**Cryopreservation**

Cryopreservation (Greek, krayos-frost) literally means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants.

**Cryopreservation broadly means the storage of germplasm at very low temperatures:**

(i) Over solid carbon dioxide (at -79°C)

(ii) Low temperature deep freezers (at -80°C)

(iii) In vapour phase nitrogen (at -150°C)

(iv) In liquid nitrogen (at -196°C)

Among these, the most commonly used cryopreservation is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), the cells stay in a completely inactive state and thus can be conserved for long periods.

In fact, cryopreservation has been successfully applied for germplasm conservation of a wide range of plant species e.g. rice, wheat, peanut, cassava, sugarcane, strawberry, coconut. Several plants can be regenerated from cells, meristems and embryos stored in cryopreservation.

**Mechanism of Cryopreservation**

The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state. Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze (even up to -68°C) compared to the freezing point

of pure water (around 0°C). When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.

**Precautions/Limitations for Successful Cry- opreservation**

Good technical and theoretical knowledge of living plant cells and as well as cryopreservation technique are essential.

**Other precautions (the limitations that should be overcome) for successful cryopreservation are listed below:**

(i) Formation ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell.

(ii) High intracellular concentration of solutes may also damage cells.

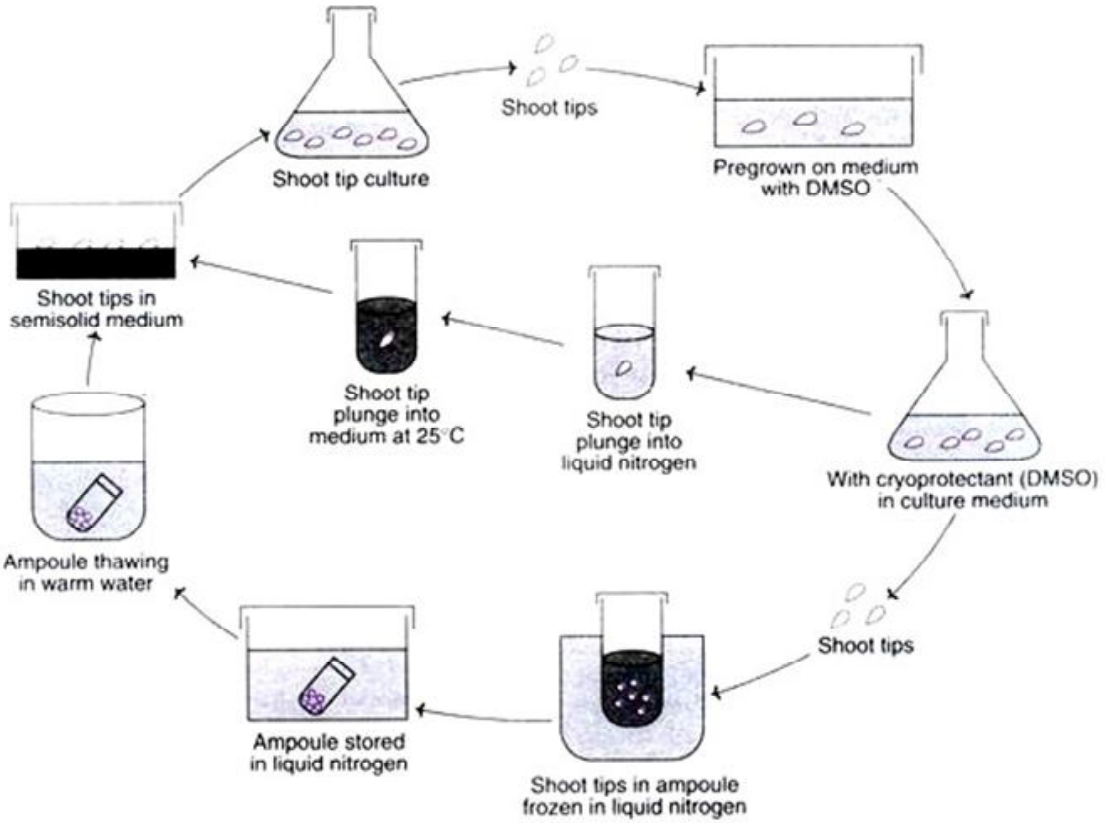
(iii) Sometimes, certain solutes from the cell may leak out during freezing.

(iv) Cryoprotectants also affect the viability of cells.

(v) The physiological status of the plant material is also important.

**Technique of Cryopreservation**

An outline of the protocol for cryopreservation of shoot tip is depicted in Fig. 48.1. The cryopreservation of plant cell culture followed by the regeneration of plants broadly involves the following stages



1. Development of sterile tissue cultures
2. Addition of cryoprotectants and pretreatment
3. Freezing
4. Storage
5. Thawing
6. Re-culture
7. Measurement of survival/viability
8. Plant regeneration.

The salient features of the above stages are briefly described.

**1. Development of sterile tissue culture**

The selection of plant species and the tissues with particular reference to the morphological and physiological characters largely influence the ability of the explant to survive in cryopreservation. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Among these, meristematic cells and suspension cell cultures, in the late lag phase or log phase are most suitable.

**2. Addition of cryoprotectants and pretreat- ment**

Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing. The freezing point and super-cooling point of water are reduced by the presence of cryoprotectants. As a result, the ice crystal formation is retarded during the process of cryopreservation.

There are several cryoprotectants which include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline and acetamide. Among these, DMSO, sucrose and glycerol are most widely used. Generally, a mixture of cryoprotectants instead of a single one is used for more effective cryopreservation without damage to cells/tissues.

**3. Freezing**

The sensitivity of the cells to low temperature is variable and largely depends on the plant species.

**Four different types of freezing methods are used:**

**(i) Slow-freezing method**

The tissue or the requisite plant material is slowly frozen at a slow cooling rates of 0.5-5°C/min from 0°C to -100°C, and then transferred to liquid nitrogen. The advantage of slow-freezing method is that some amount of water flows from the cells to the outside. This promotes extracellular ice formation rather than intracellular freezing. As a result of this, the plant cells are partially dehydrated and survive better. The slow-freezing procedure is successfully used for the cryopreservation of suspension cultures.

**(ii) Rapid freezing method**

This technique is quite simple and involves plunging of the vial containing plant material into liquid nitrogen. During rapid freezing, a decrease in temperature -300° to -1000°C/min occurs. The freezing process is carried out so quickly that small ice crystals are formed within the cells. Further, the growth of intracellular ice crystals is also minimal. Rapid freezing technique is used for the cryopreservation of shoot tips and somatic embryos.

**(iii) Stepwise freezing method:**

This is a combination of slow and rapid freezing procedures (with the advantages of both), and is carried out in a stepwise manner. The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes and then rapidly cooled by plunging it into liquid nitrogen. Stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds.

**(iv) Dry freezing method:**

Some workers have reported that the non-germinated dry seeds can survive freezing at very low temperature in contrast to water-imbibing seeds which are susceptible to cryogenic injuries. In a similar fashion, dehydrated cells are found to have a better survival rate after cryopreservation.

## 4. Storage

Maintenance of the frozen cultures at the specific temperature is as important as freezing. In general, the frozen cells/tissues are kept for storage at temperatures in the range of -70 to -196°C. However, with temperatures above -130°C, ice crystal growth may occur inside the cells which reduces viability of cells. Storage is ideally done in liquid nitrogen refrigerator — at 1 50°C in the vapour phase, or at -196°C in the liquid phase.

The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability. For long term storage, temperature at -196°C in liquid nitrogen is ideal. A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is essential. It is necessary to check the viability of the germplasm periodically in some samples. Proper documentation of the germplasm storage has to be done.

**The documented information must be comprehensive with the following particulars:**

(i) Taxonomic classification of the material

(ii) History of culture

(iii) Morphogenic potential

(iv) Genetic manipulations done

(v) Somaclonal variations

(vi) Culture medium

(vii) Growth kinetics

### Thawing

Thawing is usually carried out by plunging the frozen samples in ampoules into a warm water (temperature 37-45°C) bath with vigorous swirling. By this approach, rapid thawing (at the rate of 500-750°C $min^{-1}$) occurs, and this protects the cells from the damaging effects ice crystal formation.

As the thawing occurs (ice completely melts) the ampoules are quickly transferred to a water bath at temperature 20-25°C. This transfer is necessary since the cells get damaged if left for long in warm (37-45°C) water bath. For the cryopreserved material (cells/tissues) where the water content has been reduced to an optimal level before freezing, the process of thowing becomes less critical.

### Re-culture

In general, thawed germplasm is washed several times to remove cryoprotectants. This material is then re-cultured in a fresh medium following standard procedures. Some workers prefer to directly culture the thawed material without washing. This is because certain vital substances, released from the cells during freezing, are believed to promote in vitro cultures.

### Measurement of survival/viability

The viability/survival of the frozen cells can be measured at any stage of cryopreservation or after thawing or re-culture.

The techniques employed to determine viability of cryopreserved cells are the same as used for cell cultures .Staining techniques using triphenyl tetrazolium chloride (TTC), Evan's blue and fluorescein diacetate (FDA) are commonly used.

The best indicator to measure the viability of cryopreserved cells is their entry into cell division and regrowth in culture. This can be evaluated by the following expression.

$$\frac{\text{No. of cells / organs growing}}{\text{No. of cells /organs thawed}} \times 100$$

### Plant Regeneration

The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant. For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed, and grown. Addition of certain growth promoting substances, besides maintenance of appropriate environmental conditions is often necessary for successful plant regeneration.

A selected list of plants (in various forms) that have been successfully used for cryopreservation is given in Table

| Plant material | Plant species |
|---|---|
| Cell suspensions | Oryza sativa |
| | Glycine max |
| | Zea mays |
| | Nicotiana tabacum |
| Callus | Oryza sative |
| | Capsicum annum |
| | Saccharum sp |
| Protoplast | Zea mays |
| | Nicotlana tabacum |
| Meristems | Solanum tuberosum |
| | Cicer arietinum |
| Zygotic embryos | Zea mays |
| | Hordeum vulgate |
| | Manihot esculenta |
| Somatic embryos | Citrus sinensis |
| | Daucus carota |
| | Coltea arabica |
| Pollen embryos | Nicotiana tabacum |
| | Citrus sp |
| | Atropa belladona |

**Fig.: A selected list of plants in various forms that are successfully cryopreserved**

## 2.4 Somatic Hybrids and Cybrids

**Q7. Define somatic hybridization? Describe different steps involved in it and mention its uses.**

*Ans :*

### Introduction

Protoplasts are the entire plant cells which lack their cellulosic cell walls. Protoplasts can be isolated either directly from the different parts of whole plant or indirectly from an in vitro culture tichnique. Takebe et al (1968) first employed the use of cellulase and macerozyme for the isolation of mesophyll protoplast of tobacco.

The isolated protoplasts show the tendency to fuse with one another. As a result of this fusion, hybrid protoplasts are formed. These hybrid protoplasts are called somatic hybrids and the phenomenon is called somatic hybridization or parasexual hybridization. The somatic hybrids contain heteroploic cytoplasm and two fused parent nuclei (Heterokaryon).

Carlson and his associates in 1975 produced first somatic hybrids by using protoplasts from different species of tobacco plant (Nicotiana glauca X N.langsdorfi). Later Melchers, produced a somatic hybrid Pomatoes' by fusion of mesophyll protoplasts of tomato and potato.

Sometimes, the interaction of plastome (cytoplasmic characters) and genome leads to the formation of cybrids (cytoplasmic hybrids), Cybrids, in contrast to conventional hybrids, possess a nuclear genome from one parent and cytoplasmic genes from both parents. The process of protoplast fusion resulting in the development of cybrids is known as cybridization.

### Somatic Hybridization

Protoplast fusion and somatic hybridization in plants involve the following basic steps.

**I. Protoplast isolation**

It is the first step in somatic hybridization. The procedure and technique of isolation of protoplast is described in detail before under protoplast culture.

## II. Protoplast fusion

Protoplast fusion is a physcial phenomenon. During fusion, two or more protoplasts come together either spontaneously or in presence of fusion inducing chemicals. After adhesion, membranes of protoplasts dissolve in some localized areas and eventually the cytoplasm and nuclei of the two protoplasts intermingle with each other. As a result of this fusion, somatic hybrids are formed. The somatic hybrid will have the genomes and organelles of both the parent protoplasts.

Protoplast fusion can be classified into two types. They are as follows:

### (A) Spontaneous fusion of protoplasts

During enzymatic isolation protoplasts often fuse spontaneously and the phenomenon is called spontaneou fusion. As the cell walls are enzymatically degradaed, the plasmodesmatal connections between adjacent cells enlarge. This enlargement of plasmodesma allows the entry of organelles into neighbouring cells. As a result, hybn protoplasts are formed. The spontaneous fusion is strictly intraspecific and give re to homokaryons.

### (B) Induced fusion of protoplasts

Normally isolated protoplasts do not fo with each other, because the surface of them carries negative charge (10mV-30m out side the plasma mem-brane. So the fusion of isolated protoplasts from differe sources need fusion inducing chemicals (fusogens) or mechanical pressure.

#### (i) Mechanical Method

In this method, the protoplast suspension is sucked into a micropipette whose mouth is partially blocked. Later the suspension is forced outside through the partially sealed mouth of the micropipette. Due to th compression, the adjacent protoplasts are induced to adhere and fuse together.

#### (ii) Chemical method

Here, the isolated protoplasts are induced to fuse with each other, with the help of a range of chemical substances. These chemicals are called fusogens. Sodium nitrate, polyethylene glycol (PEG), calcium ions (Ca**). lysosyme, artificial ater, antibodies, dextran are the most commonly used fusogens. In animals, inactivated Sendia Virus is needed to induce fusion.

Mild electrical stimulation is also employed to fuse protoplasts. This technique is known as electrofusion. Zimmermann and Scheurich (1981) improved this method for large scale fusion of plant protoplasts.

## III. Identification of Somatic Hybrids

After fusion treatments, the protoplast mixture contains a number of hybrid protoplasts (somatic hybrids) and unfused parent protoplasts. The hybrid protoplast shows heterogeneity in its genome content and are usefull for crop improvement In some non viable protoplasts, the chromosomes and the cell inclusions of one parent protoplast are gradually eliminated. As a result, the hybrid protoplast contains the nucleus of one parent protoplast and the cytoplasm of both protoplasts. Such somatic hybrids are called cybrids. Thus, the somatic hybrid and cybrid differ by the presence of two nuclei and by the presence of a single nuclei respectively. In cybrids, heterozygosity for extrachromosal material can be obtained, which has a direct application in plant breeding experiments.

The preliminary identification of fusion product is done under a microscope. The identification is based on the visible characters of the protoplasts viz, colour of the protoplast, presence of chloroplasts and other pigments. In most of the hybridization experiments, green coloured and colourless protoplasts are used. This facilitates easy identification of fused protoplasts from unfused parental protoplasts. If both types of parental protoplasts look alike i.e, either colourless or pigmented, then the fusion product can be distingushed using nuclear staining technique. Patnaik (1982) used the dye fluorescein diacetate to detect the hybrid protoplasts.

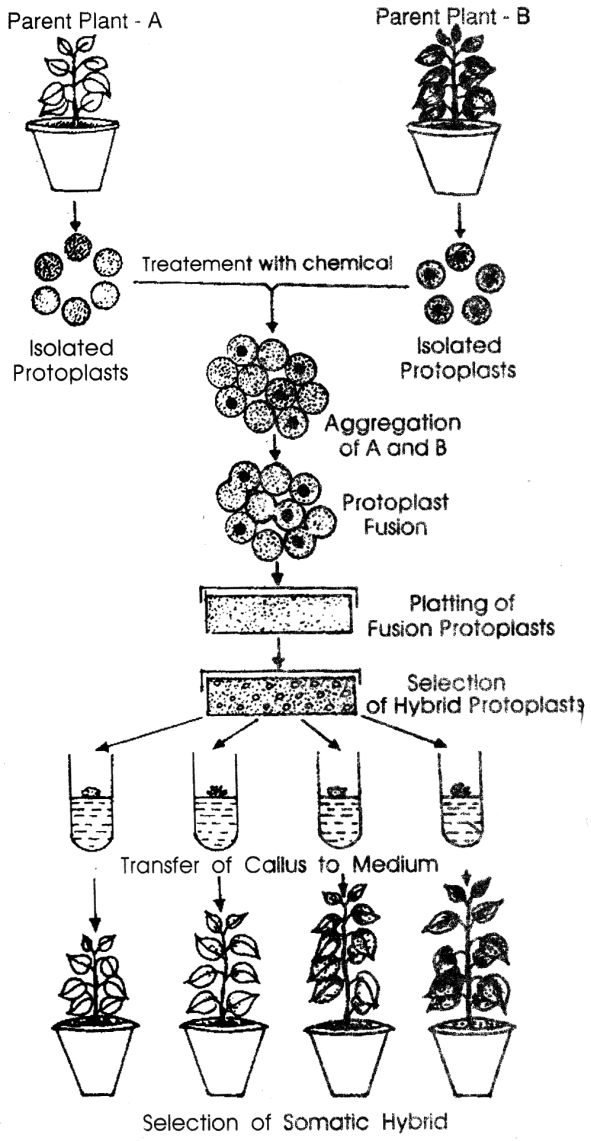



**Fig. : Diagramatic Illustration of Somatic Hybridization**

### (IV) Post fusion events

The selected hybrid (or) cybrid protoplasts are transferred to a fresh culture medium without agitation. The nutrient medium generally contains nutrients similar to those required for callus and suspension cultures. The viable hybrid protoplasts in the culture medum regenerates a wall around them and enter into mitotic cycle. Subsequent division resutls in the formation of a callus tissue. Complete hybrid or cybrid plants can be regenerated from such callus tissue. For this, the callus is transferred to a nutrient medium containing 6- Benzyl amino purine (6-BA) to induce shoots on it. The well-shooted callus is then transferred to another medium to induce root formation. After the proper development of roots, the plantlets so formed are planted in pots in a green house for proper acclimatization.

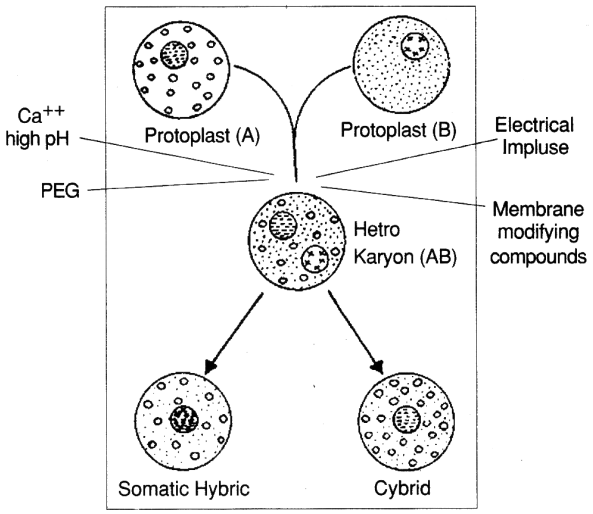



**Fig. : Fusion products of the different protoplasts**

### Importance of somatic hybridization

1. Somatic hybridization is an effective method to breed sexually incompible plants, where the conventional breeding programmes fail. But the experimental reutls are not very encouraging. This method is quite successful in few ces only So far the production of somatic hybrids of agronomically importint plants is not achieved.
2. The formation of cybrids has some application in plant improvement programme. By this, the cytoplasmic male sterility can be transferred to a hybrid protoplast.
3. Studies of fusion product can give information about compatibility or incompatibility of the nuclei or cytoplasm.
4. By protoplast fusion, it is possible to trasfer some useful genes such as discase resistance, nitrogen fixation, protein quality etc., from one species to another.

**Examples:**

1. Normal sexual reproduction is not possible between Datura inoxia x D. discolour and D. inoxia x D. stromonium. However, tetraploid amphidiploids were produced by fusing the diploid protoplast collected from mesophyll cells. These somatic hybrids contain 20 30% more scopalmine (alkaloid) than the normal diploid plants. This alkaloid is used in medicines.
2. A somatic hybrid Oryzochloa is produced through somatic hybridization between Oryza sativa and Eichinochloa oryzicola. This hybrid shows drought resistance.
3. A virus resistant hybrid is produced bewteen Solanum tuberosum and S. brevidens.
4. Mesophyll protoplasts of tomato (Lycopersicum esculentum) and mesophyll protoplasts of potato (Solanum tuberosum) were fused to produce hybrids called pomatoes. These plants are cytoplasmic male sterile lines and have great importance in crop breeding experiments.

**Cytoplasmic Hybrids Or Cybrids**

In somatic hybrids, the cytoplasm and nuclei are derived from both the parents. However in most cases, the two nuclei coming from different protoplasts, do not fuse and genetic information from one of the two nuclei is lost. Such hybrids are known as cytoplasmic hybrids or cybrids. A cybrid may be defined as a somatic hybrid where the cytoplasm is derived from both parents and nucleus is derived from one parent only.

Cybrids may be obtained by any one of the following methods:

(a) Fusion of normal protoplasts from one parent with enucleated protoplasts from the other parent.

(b) Fusion of normal protoplasts from one parent and protoplast containing non viable nucleus from the other.

(c) Selective elimination of chromosomes of one parent after fusion of the nuclei and

(d) Selective elimination of one of the nuclei from the heterokaryon.

**Importance of Cybrids**

1. Cytoplasmic male sterility has been transferred between Nicotiana line and Brassica species.
2. Streptomycin resistance has been transferred from Nicotiana tabacum other three species of tobacco.

# Short Questions and Answers

**1. Define Somaclonal variants and synthetic seeds**

*Ans :*

Few of the achievements, relating to the production of useful somaclonal variants, are described below. Somaclonal variants with Somaclonal variants are genetic variations identified in grown cells in vitro. SOMACLONES (LARKIN etal) are plant variations generated through tissue culture of somatic tissue. When compared to their original plants, such variant plants (Somaclonal variations) may exhibit several important characteristics such as disease resistance, herbicide resistance, stress tolerance, and so on. Plant Breeders are now making extensive use of these somaclonal differences in their plant breeding methods. For crop development, only plants with stable, heritable, and beneficial somaclonal variants are chosen and used.

**2. Write short notes Synthetic seeds.**

*Ans :*

Embryoids or Somatic embryos are structures that resemble embryos that are created during tissue/ cell/organ/culture. (Types that aren't Zygotic.) A synthetic seed is a man-made seed containing a somatic embryo encased in a protective gel-like material.

Somatic Embryos are encapsulated in a suitable substance (e.g. sodium alginate) combined with insecticides, herbicides, fungicides, and other chemicals in synthetic seeds. These artificial seeds can be used to mass-produce desired plant species and hybrid variations quickly and efficiently. Synthetic seeds provide many benefits like the following.

(a) Can be directly sown in the soil.

(b) Can be stored up to a year.

(c) Can be handled and delivered in a comfortable way.

**3. Micropropagation**

*Ans :*

The regeneration of whole plant thro' tissue culture is called Micro propagation. It is possible to raise hundreds or thousands of plants in large scale from small amounts of tissue thro' micropropagation. Using this method, millions of genetically identical plants can be obtained from a single bud. This method is being utilized by the industries for the commercial production of orchids and fruit trees Shoot tip propagation is exploited intensively in horticulture and nurseries. This method is now being considered as an alternative to vegetative propagation.

**4. Applications of Hairy Root Culture.**

*Ans :*

The main applications of HRCs are as follows:

(a) Production of Secondary Metabolities.

(b) Understanding Metabolisms in Plants.

(c) Production of New compounds.

(d) Regeneration of whole plants.

**5. What are the advantages of Hairy Root Culture?**

*Ans :*

Few of them are as follows

(i) Secondary Metabolites produced thro HRCs are stable and in higher quantities compared to traditional methods.

(ii) Hairy Roots can be maintained as organ cultures for a long time and al help subsequent shoot regenerations without any side eff

(iii) Plantlet regeneration frequency in HRC is very high.

(iv) Plant derived Biopharmaceuticals, thro' HRC are safe medically, cheap cost wise and easy for storage and mass production,

(v) Suitable, modified Bioreactor systems are now being employed for the exploitation of hairy roots in commercial scale on account of their production capacity of Secondary metabolites.

In spite of several advantages as explained above, HRC system has not been utilized Globally in commercial scale.

HRC still requires a special focus and further research to make HRC, an effective TOOL' in culture systems in future.

**6. What is meant by Haploidy and Triploidy?**

*Ans :*

**Haploidy**

The word haploid describes a condition, a cell, or an organism that contains half of the set of homologous chromosomes present in the somatic cell. Homologous chromosomes are two chromosomes that pair up by having the same gene sequence, loci, chromosomal length, and centromere location. Half of the homologous pairs are maternal (coming from the mother) whereas the other half, paternal (coming from the father). Thus, in other words, a haploid is when a cell, for instance, contains half of the total homologous chromosomes, i.e. a single set of chromosomes that are unpaired.

**Triploidy**

Triploidy is a rare chromosomal abnormality. Triploidy is the presence of an additional set of chromosomes in the cell for a total of 69 chromosomes rather than the normal 46 chromosomes per cell. The extra set of chromosomes originates either from the father or the mother during fertilization.

**7. What is Germplasm Conservation?**

*Ans :*

Germplasm broadly refers to the hereditary material (total content of genes) transmitted to the offspring through germ cells.

Germplasm provides the raw material for the breeder to develop various crops. Thus, conservation of germplasm assumes significance in all breeding programmes.

As the primitive man learnt about the utility of plants for food and shelter, he cultivated the habit of saving selected seeds or vegetative propagules from one season to the next one. In other words, this may be regarded as primitive but conventional germplasm preservation and management, which is highly valuable in breeding programmes.

**8. What is Cryopreservation.**

*Ans :*

Cryopreservation (Greek, krayos-frost) literally means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants.

**9. Somatic hybridization.**

*Ans :*

(i) Somatic hybridization is an effective method to breed sexually incompible plants, where the conventional breeding programmes fail. But the experimental reutls are not very encouraging. This method is quite successful in few ces only So far the production of somatic hybrids of agronomically importint plants is not achieved.

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### Importance of Cybrids

(i) Cytoplasmic male sterility has been transferred between Nicotiana line and Brassica species.

(ii) Streptomycin resistance has been transferred from Nicotiana tabacum other three species of tobacco.

# Choose the Correct Answers

1. Somaclonal variations are of how many types ? [ d ]
   (a) 4 (b) 6
   (c) 8 (d) 6
2. How many Achievements of Tissue Culture are there ? [ a ]
   (a) 3 (b) 5
   (c) 7 (d) 9
3. Benefits of Micropropagation are of how many ? [ b ]
   (a) 7 (b) 3
   (c) 6 (d 9
4. Usually Plant tissues/ meristems are stored at which temperature [ a ]
   (a) 190ºC (b) 150ºC
   (c) 120ºC (d) 170ºC
5. Ri-Plasmids are identified as _____ types. [ c ]
   (a) 4 (b) 6
   (c) 2 (d) 8
6. How many Triploidy is observed in recognized conceptions [ b ]
   (a) 4% - 7% (b) 1% - 3%
   (c) 2% - 5% (d) 6% - 9%
7. One of the most famous and ancient examples of a triploid plant species [ a ]
   (a) Cultivated banana (b) Cultivated guava
   (c) Cultivated apple (d) Cultivated mango
8. Protoplast fusion is classified into how many types [ c ]
   (a) 3 (b) 7
   (c) 2 (d) 8
9. What is the percentage of somatic hybrids in scopolamine. [ b ]
   (a) 10 – 20% (b) 20 – 30%
   (c) 30 – 40% (d) 52 – 60%
10. How many methods are there to obtain Cybrids [ d ]
    (a) 2 (b) 5
    (c) 6 (d) 4

# Fill in the blanks

1. _____ generally are free of infection .
2. The genetic variations found in vitro in clustered cells are called _____ variations.
3. _____ is the Resistant to the Pathogen.
4. Secondary metabolities, alkaloids, are of immense use in _____ .
5. Pollen culture can be converted into_____ diploids .
6. The term "hairy Root" was first coined by _____ .
7. Hairy root culture also called as _____ .
8. _____ is a rare chromosomal abnormality.
9. _____ is when a cell, for instance, contains half of the total homologous chromosomes, i.e. a single set of chromosomes that are *unpaired.*
10. During enzymatic isolation, protoplasts often fuse spontaneously and the phenomenon is called _____.

## ANSWERS

1) Meristem
2) Somaclonal
3) Pseudomonas syringe
4) Medicine
5) Homozygous
6) Steward et al (1900)
7) Transformed root culture
8) Triploidy
9) Haploid
10) Spontaneous fusion.

# One Mark Answers

**1. Give the example of improved Oil Content?**

*Ans :*

Eg. Cirtonella java Bio-13 variety – a medical plant with more oil and more citronellon.

**2. Give some examples of Drought Stress Resistant/Tolerant plants.**

*Ans :*

Maize, Potato, Barely, Rice and Woody Plants.

**3. What are the uses of Digitalis lantana**

*Ans :*

Uses of Digitalis lantana is Cardiac tonic

**4. What is the special role in hairy roots?**

*Ans :*

Hairy roots are acting like a special Biological system, playing a vital role in the production of valuable phyto-chemicals that are useful as pharmaceuticals, cosmetics and food additions.

**5. What is Germplasm Conservation?**

*Ans :*

Germplasm broadly refers to the hereditary material (total content of genes) transmitted to the offspring through germ cells.

**7. What is Cryopreservation?**

*Ans :*

Cryopreservation (Greek, krayos-frost) literally means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants.

**8. What is In-situ method of germplasm conservation?**

*Ans :*

The conservation of germplasm in their natural environment by establishing biosphere reserves (or national parks/gene sanctuaries) is regarded as in-situ conservation. This approach is particularly useful for preservation of land plants in a near natural habitat along with several wild relatives with genetic diversity. The in-situ conservation is considered as a high priority germplasm preservation programme.

**9. What is Ex-situ method of germplasm conservation?**

*Ans :*

Ex-situ conservation is the chief method for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic materials in the form of seeds or from in vitro cultures (plant cells, tissues or organs) can be preserved as gene banks for long term storage under suitable conditions.

**10. What is the Mechanism of cryopreservation**

*Ans :*

The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state. Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze (even up to -68°C) compared to the freezing point of pure water (around 0°C). When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.

UNIT III

9. Biotechnology Introduction history, scope and application.
10. rDNA technology: Basic aspect of gene cloning, Enzymes used in gene cloning-Restriction enzymes, Ligases, Polymerases.
11. Gene cloning: Recombinant DNA, Bacterial Transformation and selection of recombinant clones, vectors-cloning vehicles (Plasmid, Cosmids, Bacteriophages & Plasmids;Eukaryotic Vectors (YAC) Gene Construct; Applications of rDNA technology.

## 3.1 BIOTECHNOLOGY

**Q1. Write an essay on biotechnology and its applications in various fields.**

*Ans :*

The term biotechnology is composed of two words - bio (Greek - bios - means life) and technology (Greek technologic - means systematic treatment). It is a branch of applied biology. Biotechnology is a broad term for a group of technologies that are based on applying biological processes. It involves the use of living things or their derivatives to solve problems and make products.

Biotechnology has been defined by many in different ways and there is a considerable diversity regarding the exact definition of biotechnology.

The applications of biotechnology are so broad, and the advantages so compelling, that virtually every industry is using this technology. Developments are underway in areas like pharmaceuticals, diagnostics, textiles, aquaculture, forestry, chemicals, household products, environmental cleanup, food processing and forensics to name a few. Biotechnology is enabling these industries to make new or better products, often with greater speed, efficiency and flexibility. Biotechnology holds significant promise to the future but certain amount of risk is associated with any area. Biotechnology must continue to be carefully regulated so that the maximum benefits are received with the least risk.

The current applications of biotechnology are predominantly practiced in the fields of agriculture and medicine. Modern techniques allow for the production of new and improved foods. Virus resistant crop plants and animals have been developed and advances in insect resistance have been made. Biotechnology applications in the field of medicine have resulted in new antibiotics, vaccines for malaria, and improved ways of producing insulin. Diagnostic tests for detecting serious diseases such as hereditary cancers have been developed as well as ways of detecting and treating AIDS.

Recent developments, both in microbiology and genetic enginereing have opend up new areas where micro organisms can be exploited profitably for the production of industrially important biochemicals. The important biotechnological products include alcoholic beverages, cheese, antibiotics, fructose syrups, aminoacids, steriod hormones, vitamins, enzymes, albumins, pesticides etc.

### 3.1.1 Introduction history

**Q2. Write an account on Introduction history.**

*Ans :*

**History**

The History of Biotechnology begins with ZYMO-Technology which ommenced with a focus on Brewing Techniques. By world war 1 however motechnology would expand to tackle larger industrial uses and the potential of dustrial Fermentations gave rise to Biotechnology.

The initiation of Biotechnological processes can be traced back to pre historic es. The primitive people were well acquainted with alcoholic fermentation and gar formation from sugary substrates.

A Babylonian tablet of about 6000 B.C unearthed in 1881 explained the preparation of Beer. Sumerians and Babylonians prepared about 20 different types of beer in 3rd millenium. About 4000 BC, Egyptians employed yeast to make leavened bread.

Biotechnology was first used in 500 B.C., mold. The Greeks practiced crop rotation to increase soil fertility in 250 BC.. A that in 100B.C. chinese used powdered chrysanthemum as an insecticide.

In 14th century, first vinegar manufacturing industry was established France, Artificial cultivation of mushrooms was initiated in 1650 in France. In A.V. Leeuven hoek first observed yeast cells with his new microscope.

In addition to the above historical data, other developments of Biotechno in pre- 20th century and 20th century are summarized and described in a sequence order (year wise) in the following table.

## Historical Events Of Biotechnology

**Bio technology in Pre – 20th Century**

| Year | Development |
|---|---|
| 1590 | Jansen discovers Microscope. |
| 1677 | Anthony Van Leeuven Hook first observed yeast cells. |
| 1797 | Edward Jenner inoculates a child with a viral vaccine. |
| 1818 | Discovery of fermentation properties of yeasts. |
| 1857 | Louis Pasteur describes lacticacid Fermentation. |
| 1869 | F. Miescher isolates Nucleic acid. |
| 1878 | J. Lister reports lacticacid fermentation. |
| 1881 | Production of Lacticacid from micro-organisms. |
| 1883 | Rabin vaccine is developed. |
| 1888 | Waldeyer discovers chromosomes. |
| 1897 | E. Buchner describes alcoholic fermentation without yeast cells. |

**Biotechnology in First part of 20th century**

| Year | Development |
|---|---|
| 1902 | The term 'immunology' is first used. |
| 1915 | Discovery of Bacteriophages. German process for the manufacture Bakery yeast. |
| 1919 | Karl Ereky, a Hungarian Engineer, first proposes the term"Biotechnology |
| 1927 | Muller discovers that X-rays cause mutations. |
| 1928 | Alexander Flemming discovers penicillin. |
| 1941 | The term 'Genetic Engineering' is first used. |
| 1942 | Characterization of Bacteriophages by using Electron microscope. |
| 1944 | Large scale production of Penicillin. Discovery of streptomycin WAKSMAN. DNA is shown to be the building block of the Gene. |
| 1946 | Genetic Recombination in Bacteriophage. |
| 1949 | Pauling Proves that Sickle cell Anaemia is a molecular disease caused mutation. |
| 1953 | Watson and Crick describe the structure of DNA Cell culturing techniques. |
| 1954 | Cell culturing techniques. |
| 1955 | Isolation of an enzyme involved in the production of Nucleic acid. |
| 1956 | The fermentation process is perfected. |
| 1961 | The Genetic code is under stood. |
| 1962 | Use of microbes in the mining of URANIUM. |

**Biotechnology in 1970s and beyond**

| | |
|---|---|
| 1973 | Stanley Cohen and Herbert Boyer discover recombinant DNA technology considered to be the birth of modern biotechnology. |
| 1977 | First use of Genetically Engineered Bacteria |
| 1978 | Genetic engineering techniques used to produce human insulin in E. colit first biotech company on NY stock exchange; first successful transplantation of mammalian gene, discoverers of restriction enzymes receive Nobel Prize in medicine. |
| 1979 | Genentech, inc. produce human growth hormone and two kinds interferon DNA from malignant cells transformed a strain of cultured mouse gella new tool for analyzing ancer genes. The first monoclonal antibodies are synthesized. |
| 1980 | U.S. Superme Court decided that man-made microbes could be patened. |
| 1981 | Chinese scientists become first to clone a fish - a golden carp. The first genetically Engineered plant is reported. |
| 1982 | The first genetically engineered product, human insulin, (HUMULIN) approved for sale in USA. |
| 1983 | Genetech, inc. licensed Eli Lily to make insulin, first transfer of foreign gene in plants. |
| 1984 | The DNA Finger printing technique is developed. The first G.E. vaccine is produced. |
| 1985 | Patent Right is extended to PLANTS. |
| 1986 | First field trials of DNA recombinant plants resistant to insects, viruses. bacteria. The first Biotech inter feron drugs for cancer are synthe-sized. |
| 1988 | Congress funds the Human Genome project. |
| 1989 | Exxon Valdez oil spill off the coast of Alaska cleaned up with the help of bacteria. |
| 1990 | The first food products modified by biotechnology, an enzyme for cheese production and a yeast for baking, approved in USA and UK, respectively. |
| 1992 | FDA decides GM foods will be regulated as conventional foods. |
| 1993 | The first animal hormone produced with biotechnology, Bovine somatotropin, approved for use in USA. |
| 1994 | FlavrSavi Tomato becomes the first GM food approved for sale. The first Breast cancer gene is discovered. |
| 1995 | Three human biopharmaceuticial products receive approval of AIDS and cancer. |
| 1996 | Commercial production of biotechnology crops beginscorn, potato. |
| 1997 | Birth of Dolly, the sheep, announced as the firsy successful mammal cloned from an adult cell. |
| 1999 | The Genetic code of the Human Chromosome is deciphered |
| 2000 | Cotton, soybeans, sugar beet as well as insect or virus- resistant corn, cotton, papaya, potato, squash, tomato approved in the USA. Completion of rough Draft of human genome |
| 2001 | "Golden rice", which may help prevent millions of cases of blindness and death caused by Vitamin A and iron deficiencies, undergoes continued |
| 2001 | The sequence of the Human Genome is published in Science and Nature. |
| 2002 | Scientists complete the sequence of the pathogen of RICE a fungus. |
| 2003 | Dolly, the cloned sheep from 1997, is euthanized. |

### 3.1.2 Scope and Applications

**Q3. Write an essay on applications of biotechnology indifferent fields.**

*Ans :*

The applications of biotechnology are so broad, and the advantages o compelling, that virtually every industry is using this technology. Developments are underway in areas like pharmaceuticals, diagnostics, textiles, aquaculture, forestry, chemicals, household products, environmental cleanup, food processing and forensics to name a few. Biotechnology is enabling these industries to make new or better

products, often with greater speed, efficiency and flexibility. Biotechnology holds significant promise to the future but certain amount of risk is associated with any area. Biotechnology must continue to be carefully regulated so that the maximum benefits are received with the least risk.

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Recent developments, both in microbiology and genetic enginereing have opend up new areas where micro organisms can be exploited profitably for the production of industrially important biochemicals. The important biotechnological products include alcoholic beverages, cheese, antibiotics, fructose syrups, aminoacids, steriod. hormones, vitamins, enzymes, albumins, pesticides etc.

## Agricultural Biotechnology

Agricultural biotechnology aims at genetic improvement of crop plants to produce maximum yield and also to resist adverse climatic factors, diseases and herbicides. It also explains the method of culturing micro organisms which can be used as biofertilizers and as bioinsecticides in agriculture.

### 1. Biofertilizers

The word biofertilizer refer to the microbial population that is capable of enriching the soil fertility by their activities. They are used either by mixing them with seeds or by spreading them over field during agricultural operations.

Micro organisms such as nitrogen fixing organisms, cellulolytic microbes, and phosphate-solubilizing microbes are commonly used as biofertilizers.

**(a) Nitrogen-fixing organisms:** Nitrogen is one of the most important macronutrient required for plant growth. Though the atmosphere contains 78% of nitrogen, it is not readily available to plants. Only a small group of microorganisms called nitrogen - fixing organisms are capable of converting the molecular nitrogen into organic nitrogen. The nitrogen fixers are of two types namely symbiotic and non symbiotic. The symbiotic nitrogen fixers get associated with the roots of higher plants and fix the atmospheric nitrogen in the soil. Eg. Rhizobium, Anabaena etc. The non-symbiotic nitrogen fixers live freely in the soil and fix the nitrogen in the soil. Eg. Azotobacter, Azotococcus, Klebsiellla etc.

Recombination DNA technology helped in the cloning of nif- genes to non nitrogen fixing microorganisms. Thus E.coli, Salmonella typhimurium, Pseudomonas, Serratia marcesceus, Proteus mirabilis, Erwinia herbicola, Saccharomyces Cerevisine have been genetically transformed to fix atmospheric nitrogen in the soil. Cloning of the nif gens was first achieved by plasmid (PWK 120) mediated conjugation. Efforts are now being made to change the genetic systems of cereals so that they may have nif genes and fix atmospheric nitrogen.

**(b) Phosphate solubilizing microorganisms:** Phosphate is also one of the most important macronutrient required for plant growth. Plants absorb phosphate only in the form of soluble phosphate ions. In the soil, phosphates are present in combined form with calcium, iron, aluminum etc. They are insoluble in water but they can be converted into soluble phosphates by activity of a group of microbes. These include Penicillium digitatum, Bacillus species, Aspergillus species, Fusarium oxysporum, Xanthomonas, Pseudomonas, Flavor bacterium, Escherichia intermedia etc. So, their use reduces the necessity of phosphate fertilizers in agriculture.

**(c) Sea weeds :** Sea weeds are the gaint sized marine algal members. The important sea weeds include Macrocystis, Sargassum, Lessonia, Laminaria, Gracillaria, Enteromor-pha etc. They are used as green manure in the cultivation of wheat, potato, citrus, palm trees etc. They increase the quality and yield of plants due to the presence of nitrogen and potassium in their cells. They also contain trace elements like cobalt, manganese, boron, iodine etc., which enrich the soil.

**(d) Vesicular-Arbuscular Mycorrhiza:** VAM is a group of fungi which form symbiotic association with the root system of higher plants. They may form ecto. or endomycorrhizal associations in the roots of angiosperms, gymnosperms and pteridophytes. They infect a number of agricultural crops like rice, cotton, tobacco citrus, sugarcane etc. They also infect the plantation crops like rubber, coffee, tea papaya and produce mycorrhiza in their roots. They play an important role in plan growth. The VAM fungi increase the absorption of more phosphate, zinc, water, sulphur etc. by agricultural crops. Thus VAM infected plants show increased rate of growth. Fungal strains of Endogone, Glomus, Azotobacter Aspergillus are used for inoculation of seedlings.

## 2. Bio insecticides

A number of microorganisms are known to be pathogenic on insects. These microbes are called entomopathogens. They include viruses, bacteria, fungi and protozoa. Biotechnology helps in the production of these microbes in large number

**Table: Commercially-available microbial insecticides**

| S.No | Organisms | Target | Production process |
|---|---|---|---|
| 1. | Bacillus thuringiensis | Larvae of Lepidoptera and Diptera | Fermentation (WHO) |
| 2. | B sphaericus | Mosquito larvae | Fermentation |
| 3. | Beauveria spp | Many insects | Fermentation (USSR) |
| 4. | Hirsutella thompsonit | Citrus mites | Fermentation (Abbott) |
| 5. | Cotton bollworm nuclear polyhedrosis virus | Heliothis spp | In mass-reared larvae.(Sandoz) |

## 3 Tissue culture

Plant tissue culture plays an important role in agriculture. Some of the important applications are:

(i) Tissue culture technique helps in rapid production of more number of clones within a limited time and space. This is called micro propagation.

(ii) Apical meristem culture helps in eliminating pathogens like bacteria viruses and mycoplasmas. In potato, 136 virus - free cultivars have been raised by using this method.

(iii) Haploid individuals can be raised from anther/ pollen culture method.

(iv) Some times protoplasts are induced to fuse together in the medium. This gives rise to Somatic hybrids (or) cybrids.

(v) Sometimes genes are directly injected into the nucleus of plant cells through a micropipette.

(vi) Generally clones released through tissue culture methods show uniformity in their characters. Sometimes, variations are produced in the clones, which are not found in original parents. Such variations are called somaclonal variations. A number of somaclonal variations have been raised from plants like sugarcane. Potato, maize, oats, rice and other cereals.

## 4. Plant Protection

Biotechnology also helps in protecting the plants from several environmental stresses, diseases and pests. Biotechnological plant protection is mainly based on e two following factors;

1. Manipulation of genetic constitution of plants to improve resistance power of plants. This is carried out by using r-DNA technology.

2. By employing the microorganisms which are pathogenic to plant pathogens.

**(a) Resistance against Herbicides:** Resistance against herbicides in cultivated is created by using genetic engineering methods. The resistance genes are isolated either from micro-organisms or from resistant species of higher plants. The species genes so isolated are inserted into the cells of sensitive species of plant through some cloning vehicle. Agrobacterium tumefaction is the commonly used as cloning vehicle. By this method herbicide resistance is transferred to tobacco, tomato, cotton, sunflower, soybeans etc. Souza Machado (1982) suggested that c DNA (chloroplast DNA) of some species encodes for resistance to some herbicides. The transfer of these genes into sensitive plants leads to the generation of resistant plants.

**(b) Resistance against Diseases:** Sub-culturing of the callus tissue is one of the methods used for raising the resistant plants from sensitive strains. Fiji disease, one of the most serious diseases in sugar cane is caused by a Virus. Hussain and Hutchinson (1970) separate the Virus resistant clones from sensitive clones of sugarcane under cultural conditions. Similarly Reddi and Guinodi (1970) isolated plants with resistance to downy mildew from the sensitive strains.

**(c) Resistance against Stress:** A number of genes responsible for providing resistance against stresses such as heat, cold, salt, heavy metals have been identified. Efforts are now being made to produce transgenic plants resistant to a variety of stresses. Recently Murata et al(1992) succeeded in introducing a resistance gene against chilling into tobacco plants.

## Medical Biotechnology

Medical biotechnology deals with the production of immunologically active substances such as antibodies, interferon's, human growth hormones, vaccines, insulin etc., these compounds play an important role in diagnosis and treatment of some serious diseases.

### 1. Vaccines

Vaccine is a preparation containing attenuated or killed bacteria or viruses, which is injected into organisms to induce active immunity Vaccination is the intramuscular injection of vaccine to induce the formation of antibodies inside the organisms. This type of immunity is called acquired immunity.

Conventionally vaccines are prepared from animal material by isolating antigens. But this method of separation has the risk of contamination. Not only that, the collection of enough bacteria or viruses for vaccination is rather difficult. Therefore, biotechnology has been used to raise new organisms for the production of new vaccines and diagnostic agents. In such cases, a portion of DNA of disease causing organism is isolated and inserted into E. coli cells. The transformed E.coli cells receive the pathogenic property and can be used as vaccine for the disease concerned.

Hepatitis B virus causes serious diseases like fulminate chronic hepatitis, cirrhosis and primary liver cancer. Recently it has been possible to clone HBV-DNA obtained from infected patients into E.coli (Edman et al, 1981). The transformed E.coli cells were able to produce large quantity of antigen from the cloned HBV DNA. It made possible to produce hepatitis B vaccine.

Similarly, vaccines against the following pathogenic microbes have also been produced using r-DNA technology. They include -

(i) Salmonella causing typhoid,

(ii) Vibrio cholerae causing cholera

(iii) Plasmodium falciparus causing malaria

(iv) Feline leukaemia virus causing cancer

(v) Taenia solium causing cysticerosis

(vi) Rabies vaccine and

(vii) Aphthovirus causing Foot and mouth disease in cattle and other animals.

**2. Monoclonal Antibodies**

Antibodies are the globular proteins produced by animals against the antigens when they enter the body. The antigens include fungi, bacteria, Viruses etc. The reaction between antigen and antibody is very specific; one type of antibody reacts only with a particular antigen. Therefore, known antibodies can be used to detect diseases.

Antibodies are routinely obtained by injecting an antigen into an experimental animal and later collecting its serum. But the serum contains mixed types of antibodies, which are produced against different types of antigens. So the purification of a desired antibody from the serum becomes a problem. To overcome this difficulty, Kohler and Milstein (1975) came with a modern technology to produce identical, desired species of antibodies in a large amount. Such homologous antibodies are called monoclonal antibodies. This technology is called hybridoma technology.

Monoclonal antibodies (MAbs) play the following important roles in the field of medicine:

(i) Monoclonal antibodies are used as a diagnostic tool to determine the nature of infectious diseases. They include childhood diarrhea, malaria, cancer, tumors, leukemia and related diseases.

(ii) Monoclonal antibodies are used to treat several diseases.

(iii) MAbs are linked to the action chain of a biological toxin such as racing and diphtheria toxin. Such preparations are called immunotoxins. Immunotoxins act as two-edged swords against target cells, the two edges being specificity and cytotoxicity.

(iv) In recent years, MAbs are also used as enzymes.

**3. Inerferons**

Interferons are protein molecules which are produced by virus infected cells of blood, fibroblasts and T-lymphocytes. Interferons protect the organism from secondary infection by the viruses. They were first discovered by Issacs and Lindermann (1957). Gilbert and Weissman (1980) first time successfully produced human interferons by using genetically engineered E.coli cells. These commercial preparations have clinical importance. Leucocytic interferon's are used in the treatment of breast cancer. A-interferon is useful in the treatment of renal carcinoma in human beings.

**4. Insulin**

Insulin is a hormone which is made of amino acids. It is produced by the pancreas of the liver. Insulin is useful for treatment of persons who suffer from diabetes. Till a few years ago insulin is mainly extracted from the pancreas of dogs, swine's, oxen etc. But now techniques are available for the large scale "manufacture of insulin in bacterial cells by transferring the corresponding genies from human or animal cells into phages (or) bacteria. This makes the production of insulin relatively cheaper. Indian born scientist, Dr. Saran Narang, now working in Ottawa, Canada is involved in the cloning of insulin gene, The Eli Lilley Company presently (USA) producing human insulin from genetically transformed E.coli cells and has been marketing it under the trade name, HUMULIN

**Industrial Biotechnology**

Industrial biotechnology applies the techniques of modern molecular biology. It helps to improve the efficiency and reduce the environmental impacts of industrial manufacturing For example, industrial biotechnology companies develop. Biocatalysts, such as enzymes, to synthesize chemicals. Enzymes are proteins produced by all organisms. Using biotechnology, the desired enzyme can be manufactured in commercial quantities.

Commodity chemicals (e.g. polymer - grade acryl amide) can be produced using biotech applications. Traditional chemical synthesis involves large amounts of energy and often - undesirable products, such as HCI. Using biocatalysts, the same chemicals can be produced more economically and more environmentally friendly. An example would be the substitution of protease in detergents for other cleaning compounds. Protease production results in a biomass that in turn yields a useful byproduct- an organic fertilizer. Biotechnology is also used in the textile industry for the finishing of fabrics and garments. Biotechnology also produces biotech- derived cotton that is warmer, stronger, which is wrinkle and shrink resistance.

Some agricultural crops, such as corn, can be used in place of petroleum to produce chemicals. The crop's sugar can be fermented to acid, i.e. used as an intermediate to produce other chemical feed stocks for various products. It has been demonstrated, at test scale, that biopulping reduces the electrical energy required for wood pulping process by 30%.

Another important facet of industrial Biotechnology is fermentation technology. It contributes various useful products and a working machinery to the field of Biotechnology.

**Fermentation Biotechnology:**

Fermentation technology deals with the industrial application of some micro organisms in the production of valuable chemicals. Fermentation is the anaerobic break down of complex organic substances by the action of cell free enzymes. The fermentation process result in the production of a wide variety of organic compounds, such as amino acids, enzymes, proteins, antibodies, organic acids, single-cell proteins etc.

**1. Production of amino acids**

Amino acids are the building blocks of proteins. They are used in various industries such as food industry, pharmacy, cosmetic industry etc.

Amino acids are used in food for imparting flavors and for increasing nutrition value. L-glutamate, L-alanine and L-glycine are used for flavoring. L histidine is used for relief from gastric ulcers, L-cysteine for releif from bronchitis and L-arginine is used for alleviation of liver disorders.

Kinoshita and his co-workers (1957) first time reported that micro organisms were able to produce amino acids under cultural conditions. Since then, a number of workers have taken interest in raising new strains of micro organisms in order to produce amino acids. Most of the commercial amino acids belong to L- form.

| S.No. | Amino acid | Enzyme | Source of enzyme |
|---|---|---|---|
| 1. | L-glutamine and L-Glutamic acid | Glutarate,dehydrogenase | Corynebacterium glutamicum, Brevibacterium flavum, Micro bacterium ammoniaphilum |
| 2. | Aspartate | Aspartase | E. coli |
| 3. | Alanine | Aspartate decarboxylase | Pseudomonas decunhae |
| 4. | Lysine | Caprolactum racemase | Achrobacter obae |
| 5. | Tryptophan | Tryptophanase | Proteus rettgeri. |

**2. Production of Alcohols**

A number of bacteria produce alcohols during their fermentation activity. The microbes produce ethanol, methanol and butane in the fermentation medium. These alcohols are now used as fuels in many industries. They are called biofuels. Biofuels do not release too much of carbon dioxide during their combustion. This helps in reducing air pollution.

Ethanol is used in the manufacture of synthetic fibers like rayon, polyester etc. It is also used in manufacture of synthetic rubber, perfumes and acetaldehyde. Methane is a constituent of Biogas. Biogas consists of methane and Co, in the ratio of 2:1. Biogas is a colorless mixture of gases and burns with a blue flame,)

### 3. Production of organic acids

Different groups of micro organisms produce organic acids in the culture medium during their growth. Some of the important organic acids are:

| S.No. | Organic acid | Process organism |
|---|---|---|
| 1. | Aconitic acid | Aspergillus itaconicus |
| 2. | Citric acid | A.niger;Penicillium citratum |
| 3. | Formic acid | Rhizopus; Mucor |
| 4. | Gentistic acid | Penicillium griseo-fulvum |
| 5. | Glycolic acid | A.niger |
| 6. | Lactic acid | Rhizopus, Mucor, Lactobacillus |
| 7. | Oxalic acid | A.niger |
| 8. | Succinic acid | Rhizopus, Fusarium |
| 9. | Propionic acid | Propionobacterium, Proteus |

### 4. Production of antibiotics

The genus Streptomyces, is the source of over one-half of the known antibiotics. The other half being derived from Micromonospora, Actinoplanes Actinomadura, Nocardia, certain Actinomycetes, Penicillium, Cephalosporium, Bacillus and others. About 100 types of antibiotics are produced commercially using aerobic fermentations. Some of them are listed below:

| S.No. | Antibiotic | Source organism |
|---|---|---|
| 1. | Amphotericin-B | Streptomyces nodosus |
| 2. | Bacitracin | Bacillus subtilis |
| 3. | Bleomycin | Streptomyces verticillus |
| 4. | Cephalosporin-C | Cephalosporium acremonium |
| 5. | Chloramphenical | Streptomyces venezuelae |
| 6. | Cycloheximide | S. griseus |
| 7. | Chloromycetin | S. venezuelae |
| 8. | Erythromycin | S. erythreus |
| 9. | Fungimycin | S. coelicolor |
| 10. | Gentamycin | Micromonospora purpurea |
| 11. | Griseofulvin | Penicillium griseofulvum |
| 12. | Tetracyclin | Streptomyces rimosus |

### 5. Production of alcoholic beverages

A large number of beverages like beer, brandy, whisky, vinegar, wine etc. are produced by fermentation. Some important process organisms are listed below:

| S.No. | Source | Process organism | Product |
|---|---|---|---|
| 1. | Ripe grape juice | S. ellipsoids | Wine |
| 2. | Marsh grain of corn, rye, malt | S.cerevisiae | Whisky |
| 3. | Grape juice, apple, orange, peach | S,cerevisiae | Brandy |
| 4. | Cane molasses (or) Beet molasses | Clostridium Saccharobutyricum | Rum |

## 3.2 rDNA TECHNOLOGY

**Q4 What is meant by rDNA? Explain the steps involved in rDNA technology.**

*Ans :*

**rDNA technology**

Recombinant DNA technology (DNA technology) ng of DNA of interest for various purposes like production of proteins for therapeutic us, diagnosis and abs of diseases at molecular level, gene replacement therapy, in forensic medicine and others. In other word genetic manipulation or alteration of gene structure and function involves cutting and joining of DNA Hameets using restriction enzymes and ligases. The terms recombinant DNA technology. DNA cloning or genetic engineering involves isolation and lecular cloning gene, manipulation, genetic modification, genetic engineering or gene cloning all refer to the same proce

**Steps Involved in rDNA Technology**

The basic steps involved in genetic engineering technology are

### 1. Isolation and Specific Cleavage of Target Gene from the Organism

One of the basic steps in the process of creating rDNA involves identification and isolation of gene of interest. For this purpose, different genes that can create specific proteins are chosen whose functions are enlisted in a complimentary DNA or genomic DNA library. Isolation of desired gene involves the process of removing the target DNA from the rest of the sequence using restriction enzymes The desired gene is isolated and it is ready for introduction into the vector

### 2. Ligation of DNA Fragment to a Cloning Vehicle

A brief overview of making recombinant DNA (DNA) is described below

A recombinant DNA is the DNA that has been produced artificially by linking two or more fragments from different sources and incorporating it into a single recombinant molecule The purpose of producing rDNA molecule is,

(a) produce many identical copies of the same recombinant molecule called cloning.

(b) To get large quantities of a specific protein produced by a particular gene DNA.

(c) To incorporate the gene into the chromosome of target organism.

In order to produce many identical copies of a particular recombinant molecule, the process of cloning is done in vitro by polymerase chain reaction (PCR), In vivo cloning is accomplished in unicellular microbes like E. coll unicellular eukaryotes like yeast and in mammalian cells grown in tissue culture. Cloning as such, is necessary to produce numerous copies of the DNA since the initial supply is inadequate to insert into host cells.

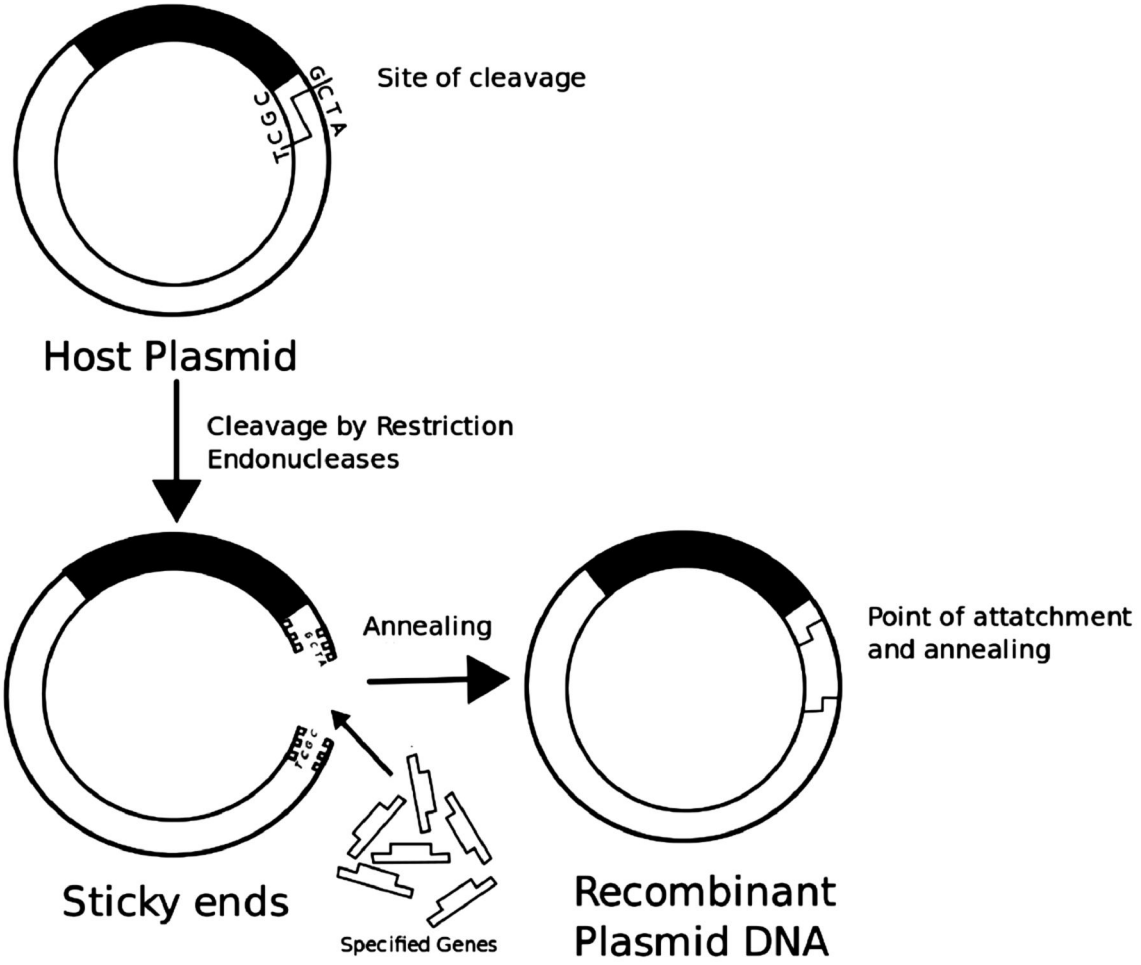



**Fig.: Recombinant DNA**

The recombinant DNA thus produced has to be taken up by the cell in the form in which it can be replicated and expressed. Vectors are used for this purpose.

### 3. Transformation and Selection

It is the process by which recipient bacterium takes up DNA from the surrounding environment. Only a small number of bacterial cells take up the plasmids and therefore there has to be a way to find out which bacteria contains the recombinant plasmids. This is called screening of clones.

4. Confirmation of the cloned gene by physical mapping and DNA sequencing.

5. Expression of cloned gene. Once the host cell gets the foreign vector, it starts synthesizing the foreign vector.

6. Recombinant proteins in excess amounts can be isolated from the host cells and purified.

### 3.2.1 Basic aspect of gene cloning

**Q5. What is Gene Cloning? Write in detail about Gene Cloning.**

*Ans :*

**Introduction**

Gene Cloning (DNA Cloning) is a molecular biology technique that makes up many identical copies of a DNA. The term gene cloning can be defined as the isolation and amplification of an individual gene sequence (or a desired gene of interest) by insertion of that gene of interest (GI) in to a bacterium where it can be replicated.

Cloning technology involves the construction or creation or production of novel DNA molecules in the form recombinant DNA types. This technique is also described as Gene manipulation or 'Genetic Engineering because of the potential for creating novel genetic combinations.

Mainly, Gene cloning refers to the Vivo production of multiple copies of a desired Gene.

Main steps involved in Gene Cloning: The procedure of Gene Cloning can be explained in a simple way thro' the following steps to know about Gene cloning methodology in a broader perspective.

**Gene Cloning - Steps**

**Step-1:**

**Isolation of DNA (Gene of Interest) Fragments to be cloned**

The operation of gene cloning primarily needs two basic things in their purified state - the gene of our interest (GI) and the vector. A GI is a fragment of gene whose product may be a protein, enzyme or a hormone.

The vector is a carrier molecule which can carry our GI into a host, replicate there along with the GI making its multiple copies. In this state the GI can also be expressed in the host cell producing the product of the gene which is needed. Desired DNA segments are obtained from Genomic library, C DNA library of produced by PCR method.

**Step-2:**

**Insertion of Isolated DNA into the Suitable Vector to Form the Recombinant DNA**

The next step is to cut both the vectors as well as the GI by using a special type of enzyme, called restriction endonuclease. A restriction endonuclease is an enzyme that cuts double-stranded or single-stranded DNA specific recognition nucleotide sequences known as restriction sites towards the inner region (hence endonuclease).

They are also regarded as molecular scissors as they cut open the DNA strands. The cutting step is followed by pasting. Here the GI is taken and pasted to the cut vector. This procedure also needs an enzyme, called DNA ligase. They are also considered as molecular glue.

The resulting DNA molecule is a hybrid of two DNA molecules - our GI and the vector. In the terminology of genetics this intermixing of different DNA strands is called recombination. Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and this technology is called recombinant DNA technology (RDT).

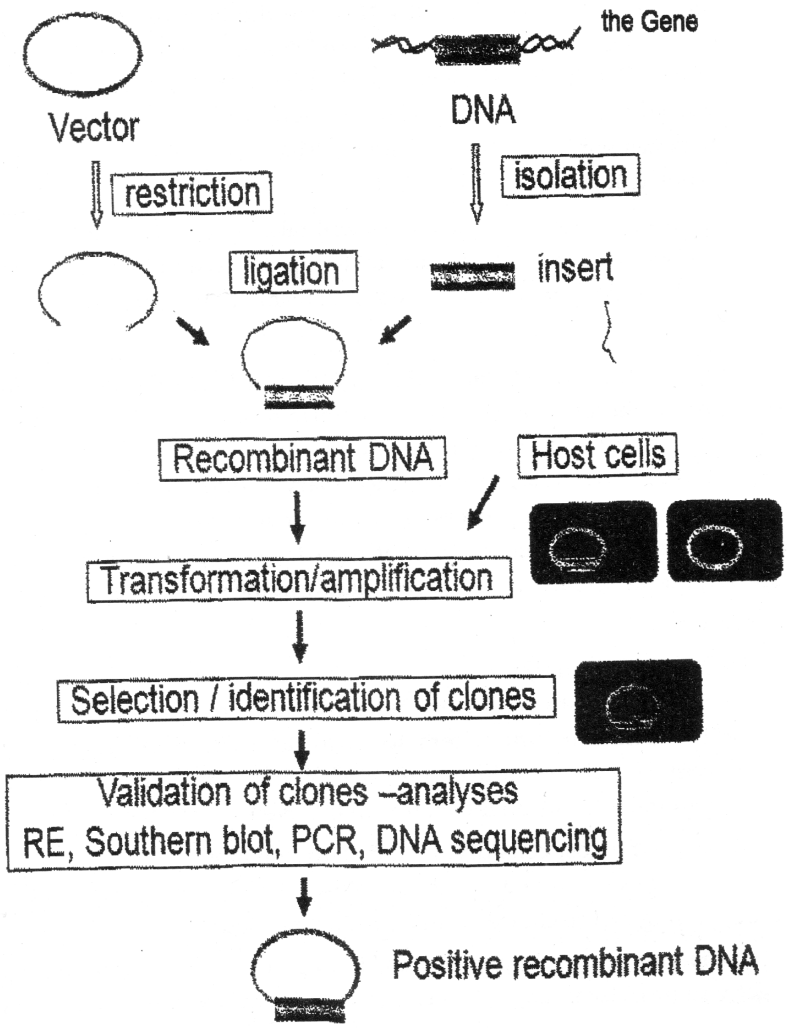



**Fig.: Basic Steps in gene cloning**

**Step 3:**

Introduction of the Recombinant DNA into a Suitable Organism known as Host

When the recombinant DNA molecule is ready, it is to be introduced into a living system known as host.

This is done either for one or both of the following reasons:

(a) To replicate the recombinant DNA molecule in order to get the multiple copies of our GI.

(b) To let our GI get express and produce the protein which is needed by us.

Introduction of the recombinant DNA into the host cell is done by various ways and strictly depends upon the size of the DNA molecule and the nature of GI. Some of the methods followed to carry out this step include electroporation, micro injection, lipofection etc. Generally CaCl, is added to E. Coli cultures. Host cell membrane becomes porous by this treatment. Then Heat shock is given. It helps or promotes the entry of rDNA in to host cell.

In this process some of the host cells will take up the recombinant DNA and some will not. The host cells which have taken up the recombinant DNA are called transformed cells and the process is called transformation.

**Step 4:**

Selection of the Transformed Host Cells and Identification of the Clone Containing the Gene of Interest

The transformation process generates a mixed population of transformed and non-trans-formed host cells. As we are interested only in transformed host cells it becomes necessary to filter them out. This is exactly what is done in the selection process. There are many existing selection strategies some of which include taking the help of reporter genes or colony hybridization technique, etc. Marker genes are employed mainly in the selection procedures.

**Step-5:**

Multiplication/Expression of the Introduced Gene in the Host: After obtaining purified transformed host cells by the screening process; they are provided with optimum parameters to grow and multiply. In this step the transformed host cells are introduced into fresh culture media which provide them rich nourishment followed by incubation in the oven at right temperature.

At this stage the host cells divide and re-divide along with the replication of the recombinant DNA carried by them. Now at this point, two methods are followed depending on the choice of final product needed.

(a) When the aim of the cloning process is to generate a gene library, then our target will be obtaining numerous copies of GI. So with this plan in our mind we will simply go for the replication of the recombinant DNA and not beyond that.

(b) If the aim of the cloning experiment is to obtain the product of GI, then we will go for a step ahead where we will provide favorable conditions to the host cells in which the GI sitting in the vector can express our product of interest (P1). Several multiplied colonies are ultimately produced.

The production of multiple colonies is all due to rapid division of E. Coli (Vector). This rapid rate (i.e., division of each 'E. Coli' at the end of every two minutes) of division produces E. Coli colonies in thousands. Each such colony contains about 100 to 200 Cells Genes with desired characters are available in millions in each colony of E. Coli.

**Step 6:**

**Screening**

It is done to identify and to isolate colonies having desired DNA inserts / Genes of Interest (GI).

The second stage of cloning comprises of identifying a colony of E. Coli, which contains the Gene of Interest (GI). For this, Blue-White screening method is adapted by employing new generation vectors like PUC 19, PGEM etc. The application of this method with special (pUC19) vectors, X-gal Assay, LB Agar Plate etc., after its completion, results in the formation of Blue and White colored colonies on the experimental screen.

White colonies contain desired DNA inserts and Blue ones are without any insert. This inference is based on the additional observations made by the Gene Cloning Experts.

The PUC vectors contain ampR (AMP Ampicillin Resistance) gene and a new gene called lacz, which was derived from the lac operon of E. Coli that codes for galactosidase enzyme. This enzyme can convert Lactose into Glucose and Galactose. This activity of -galactosidase of lac Z gene has been used as a tool for identification of clones. The activity of this enzyme can be measured in live cells by employing the chromogenic substrate X-gal (5 bromo-4-chloro-3-indolyl B-galactoside).

In normal E. Coli cells, breakdown of x-gal by lac-z gene produces a blue color, which can be seen as a Blue colony on Agar plates in X-gal Assay. E. Coli cells lacking lac.Z gene (lac operan mutants) appear as white colonies in X-gal assays. The results of the above application help in the identification of Non Transformed and Transformed Colonies of E. Coli cells in the screening procedure.

**Color Change and Associated Activity:**

**(a) Transformed E. Coli Types**

These are also called Recombinant Cells. They contain recombinant pUC plasmids, which are ampicillin resistant but can't synthesize ß-galactosidaze enzyme. Hence no breakdown of X-gal. The result is formation of only white colonies in the Media on the agar plates.

**(b) Non Transformed E. Coli Cells**

They contain normal pUC plasmids, which are also ampicillin resistant but can synthesize ß-galactosidaze enzyme, which causes X-gal breakdown. It leads to the formation of Blue Colored Colonies on the agar plates.

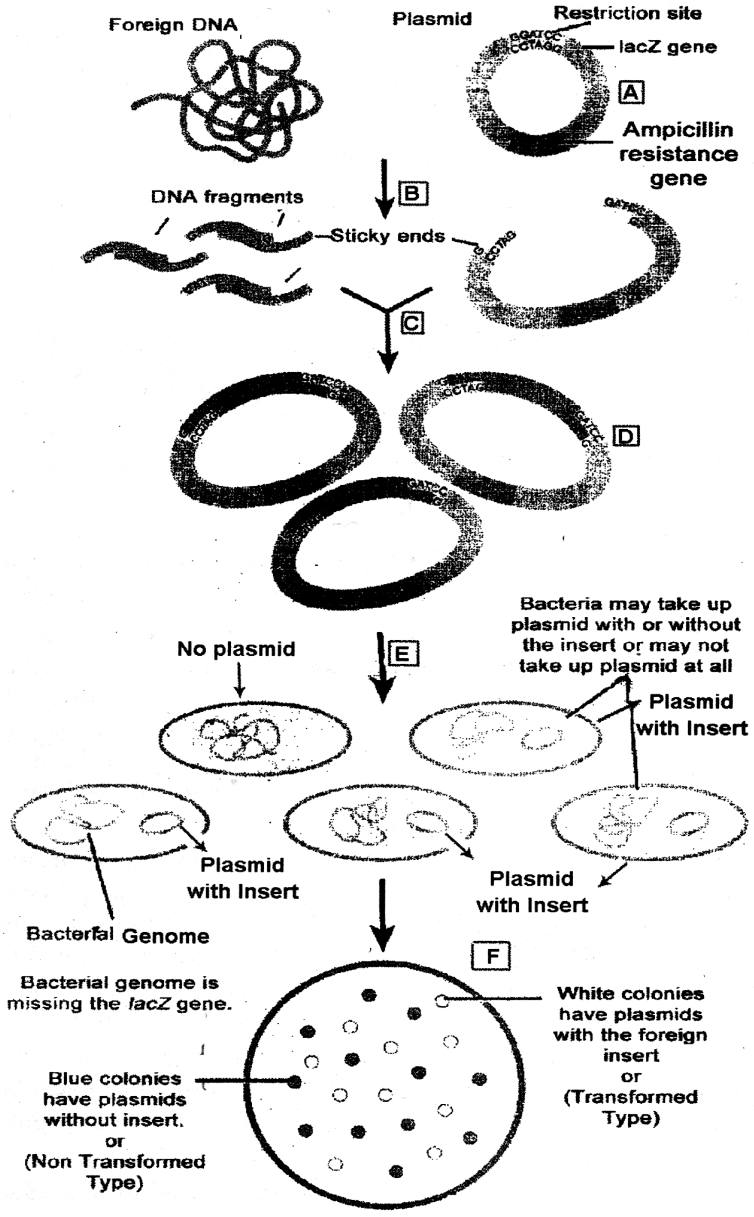



**Fig.: Diagramatic View And Explanation of Gene Cloning method**

A) Isolation of desired DNA and suitable vector.

B) Cutting desired DNA and vector with the same Restriction Enzyme.

C) Insertion of DNA fragments in to a suitable vector in to the restriction sites.

D) Formation of rDNA molecules.

E) Transformation, selection, Identification and Multiplication of clones containing the desired Gene. Formation of colonies in fresh cultures.

F) Screening for the identification and isolation of clones (having desired gene) in the colonies on the screening plate by the color at the colonies.

In Transformed Host Cells - No lac-Z gene- No chemical - Break downs - No color changes - Formation of white colonies. In Non-Transformed Host cells - Lac.Z gene is intact - Enzyme production - Chemical Break Down - Color change - Blue coloration Formation of Blue Colored colonies.

### 3.2.2 Enzymes used in gene cloning-Restriction enzymes

**Q6. Write about the enzymes used in Gene Cloning.**

*Ans :*

**Introduction**

**Refer to Q.No. 5**

**Enzymes Used In Gene Cloning**

Restriction Enzymes, Ligases and Polymerases are considered as key enzymest in Gene Cloning process and they play vital role in the Gene Cloning procedure.

**Restriction Endonucleases (REases)**

The nuclease enzyme that cuts the DNA at a unique sequence or at specific sites of DNA is called Restriction Endonuclease. They cut the DNA in a nonterminal region. Restriction Endonucleases are used to generate rejoinable DNA fragments.

REases are also known as Molecular Knives, Molecular Scissors etc. The sequence recognized by the RE, to cut the DNA is called restriction site or recognition site. This site-generally consists of 4-8 base pairs. Present Day Technology, specifically Gene Cloning is totally dependent on the ability to cut DNA molecules at specific sites with Restriction endonucleases Restriction Endonuclease or Restriction Enzyme consists of two enzymatic elements i.e., Nuclease and Methylase.

Nuclease helps restriction. Restriction means the identification of incoming DNA to the host cell (e.g. Bacteria) and its destruction by cleaving or cutting inte pieces, if it is recognized as foreign DNA.

Methylase helps the methylation activity of the Restriction Endonuclease i.e., modification. Modification is the protection of the cell's own DNA by methylation of certain bases so that host DNA is not cleaved. REs act as Vital Tools in Gene Cloning. have been isolated from a wide variety

**Nomenclature:** Many types of REases of Bacteria.

For easy identification of REases, scientists proposed a code system in their Nomenclature.

**First Type:** It is a three letter code type (Proposed by SMITH and NATHANS in 1973).

**Examples**

1. Hae for Haemophilus aegypticus.
2. Hin for Haemophilus influenzae.

**Second Type**

It is a four letter code type proposed later, which is now widely in use. It is named after the Bacterium (like 3 letter code) from which it was isolated, but includes Bacterial Genus, species, strain and order of its identification.

**Example for Four letter code:** EcoRI

| | |
|---|---|
| Letter 1 | E = Escherichia - Genus name |
| Letter 2 | co=coli - Species name |
| Letter 3 | R=RY 13 Strain |
| Letter 4 | I= First Identified - Order of Identification in the Bacterium |

**Types of REs**

Naturally occuring REs are categorized into FOUR groups (TYPE I, II, III and IV) based on their composition and enzyme cofactor requirements, the nature of their Target sequence and the position of their DNA cleavage site relative to the target sequence. But all types recognize specific short DNA sequences and carry out. the endo nucleolytic cleavage of DNA to give specific fragments with terminal 5' phosphates. They differ in their recognition sequence, sub unit composition cleavage position and cofactor requirements..

Type-II Restriction endonuclease is mainly used or employed in Gene cloning, in spite of the availability of several other types of REs, on account of the efficient nature and other properties of RE II (Ex: Eco RI).

**RE II- Useful Features in Gene Cloning**

(a) Ability to cut the DNA sequence within the specific recognition site.

(b) Ability to cut the DNA sequence in close proximity of their recognition site.

(c) Smaller and simple structure. (d) Ability to recognize palindromic sequences to cut the DNA.

### 3.2.3 ligases and Polymerases

**Q7. Discuss about ligases and Polymerases.**

*Ans :*

**1. Ligases**

Ligase is an enzyme that can catalyze the joining of two large molecules by forming a new chemical bond. Generally Ligase can joint two complementary fragments of nucleic acid and repair single stranded breaks that arise in double. stranded DNA during replication. Ligases are classified as EC 6 with Six sub classes.

But DNA Ligases are the principle types employed in Geng Cloning techniques.

**DNA Ligase:** It is the main enzyme in Gene Cloning. It catalyzes the formation of phosphodiester bond between 3-OH and 5-PO4 groups of nucleotides and facilitates the joining of DNA fragments. It is also known as a 'Molecular Stitcher. This ability enables the filling the gaps or the nicks of DNA and joining OKAZAKI fragments formed during DNA replication.

DNA ligases are mainly used in rDNA technology for the production of recombinant DNA molecules during Gene Cloning. DNA ligase joins DNA of interest (DI or GI) with vector DNA (Plasmids etc.) DNA ligase depends on a cofactor and Mg, + ions for its function. NAD+ acts as a cofactor for Bacterial DNA Ligases. While ATP acts as a cofactor for VIRUS and Eukaryotic DNA ligases.

**2. Polymerases**

A polymerase is an enzyme that synthesizes long chains of polymers or nucleic acids. DNA and RNA polymerase are the two common types.

**DNA Polymerase:** It is present in all living organisms. It helps in DNA synthesis and Genome replication. This ability of DNA polymerase is employed in Gene cloning. This enzyme catalyzes the synthesis of new DNA, complementary for the existing DNA.

DNA Polymerase I of E. Coli directs the synthesis of complementary nucleic acids using single stranded DNA as template. It converts single stranded DNA to the double stranded form. DNA polymerase synthesizes, DNA molecules from deoxyribonucleotides. DNA Polymerase adds nucleotides to the 3'OH (3' end) group of the primer sequence (Bases) and continues the strand formation to 5' direction i.e., elongation of the newly forming strand in a 5'-3' direction.

**Conclusion**

DNA ligase helps the joining DNA fragments by phosphodiester bonds, where as DNA polymerase helps the synthesis of new DNA. Though both enzymes are functionally different but they are required for DNA repairing, DNA replication and recombinant DNA technology activities. And these activities are essential for all Gene Cloning procedures in one way or other.

In PCR mediated Gene Cloning technique, a special Taq DNA polymerase is employed

## 3.3. GENE CLONING

**Q8. What is PCR Mediated Gene Cloning? Give differences between Gene Cloning and PCR Cloning.**

*Ans :*

**Pcr Mediated Gene Cloning**

**PCR (Polymerase Chain Reaction)**

The in vitro amplification of DNA by repeated cycles of strand separation and polymerization is called PCR. PCR was invented by Kary Mullis in 1985.

**PCR - Gene Cloning or PCR Cloning Method**

PCR Cloning differs from traditional cloning in that the DNA fragment of interest and even the vector can be amplified by the PCR and ligated together, without the use of restriction enzymes. PCR cloning is a rapid method for cloning genes and is often used for projects that require high out puts than traditional cloning methods can accommodate. It allows for the cloning of DNA fragments that are not available in large amounts.

PCR reaction is performed to amplify the sequence of interest (Desired Gene of Interest DI) and then DI is joined to the vector via a blunt or single base over hang ligation prior to transformation.

DNA polymerases with high fidelity (or trustful) are also now routinely used to amplify sequences with the PCR product containing no. 3 extensions. The blunt end, fragments are joined to a plasmid

vector thro' ligation reaction or by the action of an 'activated vector containing Topo-isomerese I, which facilitates the vector insert joining. Among all the PCR - mediated cloning methods (TA cloning, LIC (Liqase independent cloning etc) overlap extension PCR cloning is simple and reliable way. to create recombinant constructs.

**Differences Between Gene Cloning And Pcr Cloning**

| S.No. | Gene Cloning | PCR Cloning |
|---|---|---|
| 1. | r DNA is constructed in vitro and amplified in vivo inside a bacterium. | DNA is amplified in vitro and no need of r DNA construction. |
| 2. | Restriction Enzymes, DNA ligase, vector DNA and bacterial cells are required. | Taq DNA polymerase, RNA primers, DNA nucleotides and DNA segments to be amplified are required. |
| 3. | Restriction Enzyme is required for re-isolation of the amplified DNA for r DNA. | No need for reisolation or enzymes |
| 4. | Screening of the amplified DNA is necessary to obtain desired DNA / gene of interest. | No screening is needed. |
| 5. | Amplification requires minimum a microgram of DNA. | A nanogram of DNA is sufficient. |
| 6. | It requires 2-4 days time for completion. | It requires a maximum 4 hours of time. |
| 7. | Error possibility is more. | It is less. |
| 8. | Practical applications are limited. | Unlimited or wide. |

### 3.3.1 Recombinant DNA

### Q9. Describe the Significance of Recombinant DNA in Gene Cloning?

*Ans :*

**Recombinant DNA in Gene Cloning**

A single cell separated from tissues divides by repeated mitosis to form a group of homogenous cells is clone and the process of obtaining such a clone is called cloning. Thus, the cells in a clone are similar der genotype and karyotype.

**Significance of Cloning**

A pure sample of an individual gene of interest with a striking characteristic feature can be obtained, which enables the study of genetic disorders in a laboratory environment.

Cloning of an important organ can obliterate the need for organ transplantation, compatibility complications and the use of immunosuppressive drugs. Transgenic plants have been produced by cloning methods, by using genes coding for proteinase inhibitors. The plants so produced are resistant to herbicides, antibiotics, pest damage, infections and diseases.

Examples of such plants are wheat, rice, maize, soyabean, potato etc. Livestock breeders use cloning technology to make their operations more productive and more profitable.

Cloning helps to prepare advanced medicines for diagnosis and vaccines, for the treatment of heart diseases, cure from different kinds of cancer, kidney ailments, diabetes, hepatitis, multiple sclerosis, cystic fibrosis etc.

Cloning can be a promising conservative strategy for endangered species. The Cloning methods also help in identification of crime suspects.

### 3.3.2 Bacterial transformation and selection or Recombinant clones.

**Q10. Describe Bacterial transformation and selection or Recombinant clones.**

*Ans :*

**Introduction**

After the construction of rDNA (chimeric plasmid DNA) it is introduced in to a host cell. The most commonly used host in rDNA technology is E. coli. Under natural conditions the recipient cells are unable to uptake the chimeric plasmid DNAS. For this the bacterialRECOMBINANT DNAcell is treated with CaCl, which renders the cell membrane permeable to plasmid DNA. The transformed bacterial cell is then grown on nutrient agar plates under optimum conditions for the colonies to develop. As each colony of cells is the progeny of a single cell, all cells will have the same genetic make up. This is called a clone. After the entry of plasmid DNA into the bacterial cell, it should be protected by histone proteins of the recipient cells against the degrading activity of cellular endonucleases and exonucleases.

The rDNA gets integrated in to the genome of E. coli. The E. coli now gets the property of the desired gene. This process is called transformation. The transformation leads to the genetic manipulation of bacterial cells for synthesizing the product of the desired genes. For example, if the inserted gene is insulin gene, the E.coli begins to synthesize insulin which can be harvested from the medium.

**Selection of the cells containing the DNA**

The culture may contain transformed cells as well as the normal cells. The transformed cells are selected by two methods namely biochemical testing and colony hybridization. 1. Bio chemical selection: The plasmid DNA possess some sequences to confer resistance for the organism against certain drugs like tetracycline, ampicillin, chloramphenicol etc. These sequences are used as genetic markers to detect the transformed recipient cells.

For example PBR 322 plasmid has two sequences for giving resistance to the organism against drugs. One sequence gives resistance against tetracycline and the other sequence against ampicillin. If the gene is cloned at the tetracycline resistance sequence, the plasmid fails to give resistance to the organism against tetracycline. So these cells do not form colonies in an agar medium containing tetracycline.

Colony Hybridization: In this method, the radioactive probes containing complementary sequence of cloned gene are used to establish hybridization on the nitrocellulose filter paper. The doned gene forms complementary base pairing with the radio active probe. As a result hybrid DNAs are formed. The presence of hybrid DNAs indicate the clone containing the transformed bacterial cells.

### 3.3.3 Vectors-cloning vehicles (Plasmid, Cosmids, Bacteriophages & Plasmids)

**Q11. Write in detail about all Gene Cloning Vectors / Vehicles.**

*Ans :*

The DNA that carries the desired gene to the host cell is called Cloning Vector, It is also called Cloning Vehicle or DNA Carrier. Vector is considered as one of the most important elements in r DNA technology or in gene cloning cells.

There are different types of cloning vectors for use with different types of host Vectors - Features:

A Vector should have the following features:

1. It must contain a 'Replicon'.
2. It should have several marker genes.
3. It should have a unique cleavage site.
4. Vector DNA should contain suitable control meters, such as promoters, terminators and ribosome binding sites.

**Types Of Cloning Vectors**

Six main types of cloning vectors are identified which are used mostly in r DNA technology. They may belong to Pro or Eukaryote or a mixture of both types.

They are

(1) Plasmids

(2) Bateriophages

(3) Cosmids

(4) Phagemids

(5) Larger DNA fragments cloning vectors (YAC, PAC and BAC Types)

(6) ssDNA preparing vectors.

**1) Plasmids**

Plasmids are relatively small, circular DNA molecules that can exist independently of host chromosomes. They are found in many bacteria, some yeasts and fungi. They have their replication origins and are autonomously replicating and stably inherited. Plasmids possesses relatively a few genes, generally less than 25 to 30. There may be a single copy of plasmid (single copy plasmid) or more copies of the plasmid in a cell (multicopy plasmids). Plasmids often bear antibiotic resistance genes, which are used to select their bacterial hosts.

There are several plasmid cloning vectors such as PBR 322, PSC 102, COIEI, PUC, pRp4 pRk2, Ti- and Ri - DNA plasmids. The plasmid cloning vectors are designated by 'P', some abbreviations and a few numbers. For example, in PBR320, P means plasmid, BR refers to the researchers (F. Bolivar and R. Rodriguez who discovered the plasmid), and 322 is the numerical designation. Some of the plasmids and their characters are given below.

| Cloning Vector | Natural occurrence | Size (kb) | Selective marker* |
|---|---|---|---|
| pBR 322 | E. Coli | 4.36 | Amp ^ r, tet ^ r |
| pBR324 | E. Coli | 8.3 | Amp ^ r, tet ^ r, Elimon |
| pC194 | Staphylococcus aureus | 3.6 | Ery ^ r |
| pSA 0501 | S. aureus | 4.2 | St ^ r |
| pBS 161-1 | Bacillus subtilis | 3.65 | V |

(Resistance to ampicillin (Amp'), tetracycline (Tet), Erythromycine (Ery'), streptomycine (Str").)

The plasmids that occur naturally do not posses all the characteristes to be used as cloning vector. Therefore, they are restructured by inserting the genes of relaxed replication and genes for antibiotic resistance.

pBR322 is the first artificial cloning vector developed in 1977 by Boliver and Rodriguez from E. Coli plasmid Col. E. I. It is 4.362 kb long and most widely used cloning vector. It has genes for resistance against two antibiotics tetracycline (tet¹) and ampicillin (amp) and unique recognition sites for 20 restriction endonucleases. Six of these such as EcoR II, Bgl 1, Sal I, Bam HI, Hind III and Cla I are present within the gene coding for tetracycline resistance. Two sites. (Hind III and Cla I) are located within the promoter of the tetracycline resistance gene, and three sites (Hind II, PVU I, and Bgl I) are within the beta lactamase gene that provide resistance to ampicillin. If any foreign DNA is cloned into any of these 11 sites, insertional inactivation of any of the antibiotic resistance markers takes place. Foe example, if a foreign DNA is inserted at tet gene cluster, the property of tetracycline resistance will be lost.

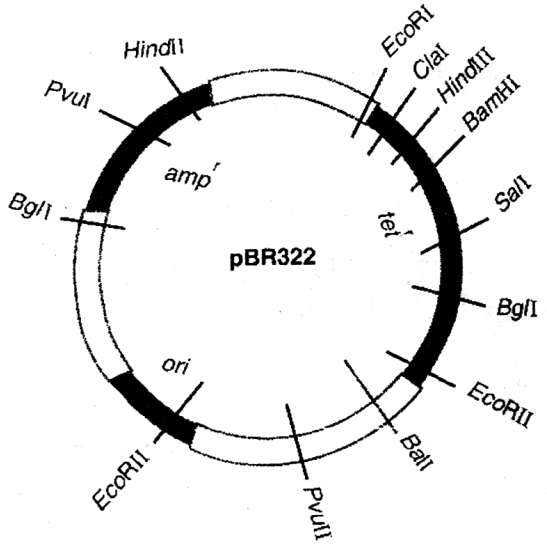



**Fig.: pBr322 plasmid**

### (i) pUC Vector Plasmids

These are another type of Plasmids. These genetically modified plasmids were first prepared in University of California and thus were named PUC. These plasmids possess several advantages as a vector for cloning like the following:

1. Small size but can carry relatively large DNA inserts.
2. PUC, like PUC18 plasmid in a host cell, can replicate to form 500 copies/cell, producing many clones of inserted DNA units.
3. Contain one ampicillin resistance gene and one replicon site.
4. Contain a lac Z gene with a Multiple Cloning Poly-Linker (MCPL) site.
5. PUC plasmids have a selection system.
6. PUC family plasmids are available in pairs.

### (ii) Phage Vectors

Bacteriophages are viruses which infect bacteria. These are commonly called phages. Both single and double stranded DNA phage vectors are being employed in gene cloning. Phage vectors are required for cloning of large DNA fragments. They are more complicated than plasmids. In addition to the normal replicating capability, phage DNA also contains genes coding for proteins that form the capsid of the virus. But phages lack the machinery necessary to actually make proteins, consequently they reproduce only in side living bacterial cells.

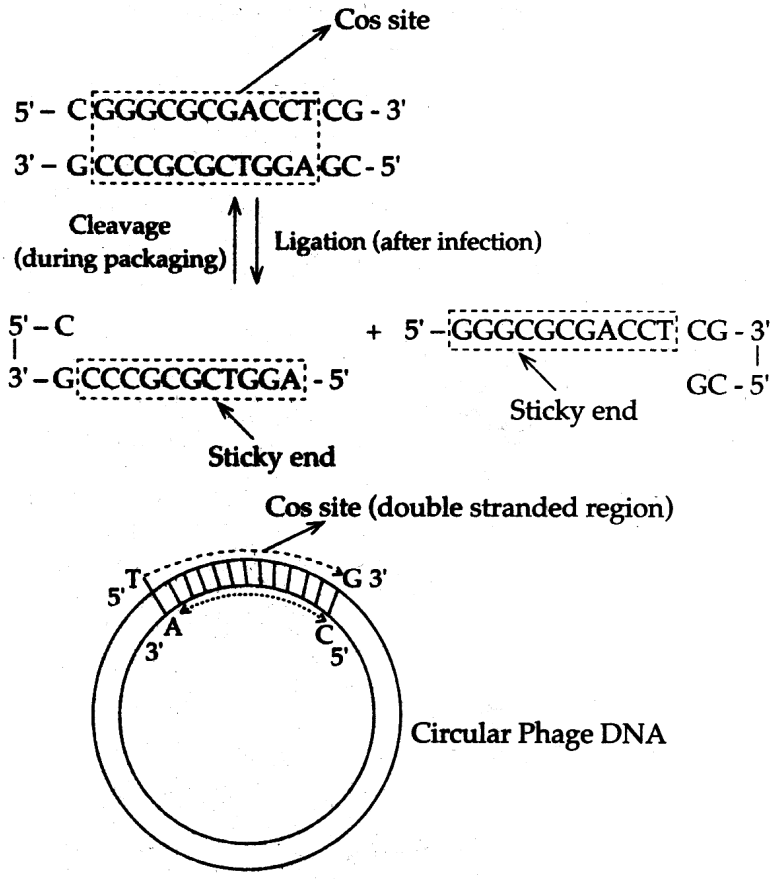



**Fig. : λ Phage DNA**

The two bacteriophages phase A(lambda) and M13 were modified and are commonly used as cloning vectors. Lambda phage commonly infects E.Coli. It has linear double stranded DNA molecule (50 genes, 48-514 kb) in the head region. This molecule has single stranded terminii of 12 nucleotides, commonly known as cos sites (cohesive sites). When this DNA is injected into bacteria, the two ends. are joined to form a circular DNA molecule. The double stranded region so formed is called a cos-site. The cos sites help in inserting large DNA fragments in the phase DNA molecule.

### 2) Cosmids

Cosmids are plasmids that contain lambda phage DNA containing cohesive ends (Cosmid = cos site + plasmid). For the first time it was developed by Collins and Hohn (1978).

Cosmids lack encoding viral proteins. Therefore, neither viral particles are formed nor cell lysis occurs. Cosmids like plasmids contain

(i) Origin of replication,

(ii) A marker gene coding for antibiotic resistance,

(iii) A special cleavage site for the insertion of foreign DNA and

(iv) The small size. They differ from plasmids in having are extra phase DNA, cos site, which is about 12 bases. It helps the whole genome in circularization and ligation.

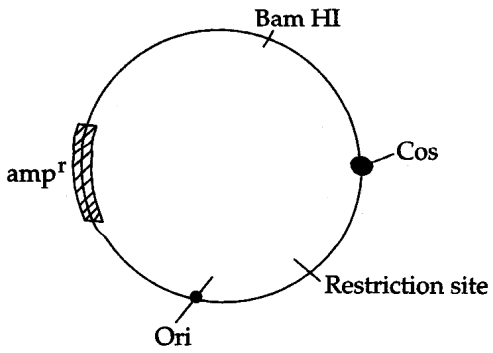



**Fig. : Structure of Col E 1 cosmid**

### 3) Phagemids /Bacteriophages

These are artificially prepared vectors like cosmids, by combing the features of phages with plasmids. Characters of Phagemids: These possess -

(a) A multiple cloning site.

(b) An inducible promoter.

(c) An origin of replication (Derived both from phage and plasmid) Example for this type is p Blue script 11 ks: It is derived from the plasmid PUC19 type and is 2961 bp long. The KS designates the orientation of polylinker.

### 4) Larger DNA fragments cloning vectors (Eukaryotic vectors)

These vectors are very useful for characterization and expression of large genes or gene complexes. A number of such vectors have been developed recently to utilize on large genomes of Humans and Mammalia.. Some of the popular examples for this type are YACS, PACs, BACs etc.

#### YAC (Yeast Artificial Chromosome)

YAC can clone very large DNA fragments upto 2 Mb. This character of YAC has made it a vital tool in creating physical maps of large genomes such as the human genome. Each YAC is made up of 3 regions, namely Telomere (TEL), Centromere (CEN) and a replicating sequence (RS). Hence YAC is described as a mini chromosome. It behaves as an additional chromosome.

#### Uses of YAC

1. It is used to form Genomic libraries of both Pro and Eukaryotes.
2. It is used in Human Genome Project (HGP) to construct Gene Map of Chromosome in Man.
3. It is used to clone large DNA fragments. PAC

It is called P, - Derived Artificial Chromosome. PACs are DNA constructs that are derived from the DNA of P, - bacteriophage. They can carry large amounts (100-300 Kbs) of other sequences for variety of Bio engineering purposes. It is one type of vector to clone DNA fragments in E. Coli cells.

This vector (PAC) originates from a phage (P, Type) instead of a plasmid. P, Phage is unique in nature and exists in two forms. In Lysogeny, it remains and acts like a plasmid and in Lysis, it destroys the Host Cell.

PAC contains both features of Plasmids and F factor (F= fertile plasmid like DNA sequence) i.e., P and F factor systems.

PAC can accommodate larger inserts of DNA than a plasmid or many other vectors.

PACS are constructed thro' a method called 'Electroporation'.

Uses or Applications:

1. PACs are used in the Genome Analysis and Map based cloning of Complex Plants and Animals.
2. PAC cloning is useful in phage therapy and helps the study of Antibiotic actions.

**6) ssDNA preparing Vectors**

These types can be synthesized by PCR-based methods. Besides, these can be obtained, using vectors, on certain bacteriophages, whose genomes assume a single stranded DNA in their life cycle. Examples for Type VI - M , Bacteriophages, M, Vectors.

### 3.3.4 Applications of rDNA technology.

**Q12. Describe the role of rDNA technology through its applications in the field of Medicine and other fields.**

*Ans :*

**Application Of Rdna Technology**

**Refer to Q.No. 7 Page No. 73**

Genetically engineered bacterial cells through recombinant DNA technology have many applications in the field of medicine and other branches.

1. E. coli containing rDNA made of human insulin gene, is used to produce human insulin.
2. E. coli containing r DNA can produce human growth hormone-Somatotropin.
3. Genetically engineered E. coli cells are used to produce rat insulin.
4. Genetically engineered colon bacillus cells are capable of producing beta endorphin which is used as antagonistic drug.
5. Gilbert and Weissmann (1980) raised a new strain of colon bacilli capable of producing human-a- interferon against viruses.
6. Genetically engineered microorganisms are used in the production of biogas in biogas plants.
7. Transformed bacterial cells are also used in the manufacture of steroid hormones.
8. They are also used in biology-based industries to produce alcholic beverages, organic acids, amino acids etc.
9. Some genetically engineered organisms are capable of degrading the complex herbicides and pesticides used in agriculture. Thus they reduce the pollution caused by these chemicals in our environment.
10. The Ti plasmid of Agrobacterium has been exploited as a means of transfering nif genes into a cereal plant. This transfer will help in reducing the dependence on nitrogenous fertilizers.
11. Recombinant DNA technology has been, exploited in synthesis of vaccines against hepatitis B Virus, malaria and foot and mouth disease in cattle.
12. r DNA studies have revealed the details of various infectious diseases, such as inborn errors of metabolism and cytogenetic abnormalities viz. Down's syndrome, coronary artery disease, orthosclerosis.
13. Recombinant DNA has also been used in animal husbandary through production of vaccines and transfer of commercially important gene traits to improve milk-yields.

**Q13. Write in detail about agricultural, medical and industrial biotechnology.**

*Ans :*

**Agricultural Biotechnology**

Agricultural biotechnology aims at genetic improvement of crop plants to produce maximum yield and also to resist adverse climatic factors, diseases and herbicides. It also explains the method of culturing micro organisms which can be used as biofertilizers and as bioinsecticides in agriculture.

**1. Biofertilizers**

The word biofertilizer refer to the microbial population that is capable of enriching the soil fertility by their activities. They are used either by mixing them with seeds or by spreading them over field during agricultural operations.

Micro organisms such as nitrogen fixing organisms, cellulolytic microbes, and phosphate-solubilizing microbes are commonly used as biofertilizers.

**(a) Nitrogen-fixing organisms**

Nitrogen is one of the most important macronutrient required for plant growth. Though the atmosphere contains 78% of nitrogen, it is not readily available to plants. Only a small group of microorganisms called nitrogen - fixing organisms are capable of converting the molecular nitrogen into organic nitrogen. The nitrogen fixers are of two types namely symbiotic and non symbiotic. The symbiotic nitrogen fixers get associated with the roots of higher plants and fix the atmospheric nitrogen in the soil. Eg. Rhizobium, Anabaena etc. The non-symbiotic nitrogen fixers live freely in the soil and fix the nitrogen in the soil. Eg. Azotobacter, Azotococcus, Klebsiellla etc.

Recombination DNA technology helped in the cloning of nif- genes to non nitrogen fixing microorganisms. Thus E.coli, Salmonella typhimurium, Pseudomonas, Serratia marcesceus, Proteus mirabilis, Erwinia herbicola, Saccharomyces Cerevisine have been genetically transformed to fix atmospheric nitrogen in the soil. Cloning of the nif gens was first achieved by plasmid (PWK 120) mediated conjugation. Efforts are now being made to change the genetic systems of cereals so that they may have nif genes and fix atmospheric nitrogen.

**(b) Phosphate solubilizing microorganisms**

Phosphate is also one of the most important macronutrient required for plant growth. Plants absorb phosphate only in the form of soluble phosphate ions. In the soil, phosphates are present in combined form with calcium, iron, aluminum etc. They are insoluble in water but they can be converted into soluble phosphates by activity of a group of microbes. These include Penicillium digitatum, Bacillus species, Aspergillus species, Fusarium oxysporum, Xanthomonas, Pseudo- monas, Flavor bacterium, Escherichia intermedia etc. So, their use reduces the necessity of phosphate fertilizers in agriculture.

**(c) Sea weeds**

Sea weeds are the gaint sized marine algal members. The important sea weeds include Macrocystis, Sargassum, Lessonia, Laminaria, Gracillaria, Enteromorpha etc. They are used as green manure in the cultivation of wheat, potato, citrus, palm trees etc. They increase the quality and yield of plants due to the presence of nitrogen and potassium in their cells. They also contain trace elements like cobalt, manganese, boron, iodine etc., which enrich the soil.

### (d) Vesicular-Arbuscular Mycorrhiza

VAM is a group of fungi which form symbiotic association with the root system of higher plants. They may form ecto. or endomycorrhizal associations in the roots of angiosperms, gymnosperms and pteridophytes. They infect a number of agricultural crops like rice, cotton, tobacco citrus, sugarcane etc. They also infect the plantation crops like rubber, coffee, tea papaya and produce mycorrhiza in their roots. They play an important role in plan growth. The VAM fungi increase the absorption of more phosphate, zinc, water, sulphur etc. by agricultural crops. Thus VAM infected plants show increased rate of growth. Fungal strains of Endogone, Glomus, Azotobacter Aspergillus are used for inoculation of seedlings.

## 2. Bio insecticides

A number of microorganisms are known to be pathogenic on insects. These microbes are called entomopathogens. They include viruses, bacteria, fungi and protozoa. Biotechnology helps in the production of these microbes in large number

**Table: Commercially-available microbial insecticides**

| | Organisms | Target | Production process |
|---|---|---|---|
| 1. | Bacillus thuringiensis | Larvae of Lepidoptera | Ferm entation (WHO) |
| 2. | B sphaericus | Mosquito larvae and Diptera | Fermentation |
| 3. | Beauveria spp | Many insects | Fermentation (USSR) |
| 4. | Hirsutella thompsonit | Citrus mites | Fermentation (Abbott) |
| 5. | Cotton bollworm nuclear Heliothis spp | In mass-reared | polyhedrosis virus larvae.(Sandoz) |

## 3. Tissue culture

Plant tissue culture plays an important role in agriculture. Some of the important applications are:

(i) Tissue culture technique helps in rapid production of more number of clones within a limited time and space. This is called micro propagation.

(ii) Apical meristem culture helps in eliminating pathogens like bacteria viruses and mycoplasmas. In potato, 136 virus - free cultivars have been raised by using this method.

(iii) Haploid individuals can be raised from anther/ pollen culture method.

(iv) Some times protoplasts are induced to fuse together in the medium. This gives rise to Somatic hybrids (or) cybrids.

(v) Sometimes genes are directly injected into the nucleus of plant cells through a micropipette.

(vi) Generally clones released through tissue culture methods show uniformity in their characters. Sometimes, variations are produced in the clones, which are not found in original parents. Such variations are called somaclonal variations. A number of somaclonal variations have been raised from plants like sugarcane. Potato, maize, oats, rice and other cereals.

## 4. Plant Protection

Biotechnology also helps in protecting the plants from several environmental stresses, diseases and pests. Biotechnological plant protection is mainly based on e two following factors;

(i) Manipulation of genetic constitution of plants to improve resistance power of plants. This is carried out by using r-DNA technology.

(ii) By employing the microorganisms which are pathogenic to plant pathogens.

**(a)** Resistance against Herbicides Resistance against herbicides in cultivated is created by using genetic engineering methods. The resistance genes are isolated either from micro-organisms or from resistant species of higher plants. The species genes so isolated are inserted into the cells of sensitive species of plant through some cloning vehicle. Agrobacterium tumefaction is the commonly used as cloning vehicle. By this method herbicide resistance is transferred to tobacco, tomato, cotton, sunflower, soybeans etc. Souza Machado (1982) suggested that c DNA (chloroplast DNA) of some species encodes for resistance to some herbicides. The transfer of these genes into sensitive plants leads to the generation of resistant plants.

**(b) Resistance against Diseases**

Sub-culturing of the callus tissue is one of the methods used for raising the resistant plants from sensitive strains. Fiji disease, one of the most serious diseases in sugar cane is caused by a Virus. Hussain and Hutchinson (1970) separate the Virus resistant clones from sensitive clones of sugarcane under cultural conditions. Similarly Reddi and Guinodi (1970) isolated plants with resistance to downy mildew from the sensitive strains.

**(c) Resistance against Stress**

A number of genes responsible for providing resistance against stresses such as heat, cold, salt, heavy metals have been identified. Efforts are now being made to produce transgenic plants resistant to a variety of stresses. Recently Murata et al(1992) succeeded in introducing a resistance gene against chilling into tobacco plants.

## Medical Biotechnology

Medical biotechnology deals with the production of immunologically active substances such as antibodies, interferon's, human growth hormones, vaccines, insulin etc., these compounds play an important role in diagnosis and treatment of some serious diseases.

### 1. Vaccines

Vaccine is a preparation containing attenuated or killed bacteria or viruses, which is injected into organisms to induce active immunity Vaccination is the intramuscular injection of vaccine to induce the formation of antibodies inside the organisms. This type of immunity is called acquired immunity.

Conventionally vaccines are prepared from animal material by isolating antigens. But this method of separation has the risk of contamination. Not only that, the collection of enough bacteria or viruses for vaccination is rather difficult. Therefore, biotechnology has been used to raise new organisms for the production of new vaccines and diagnostic agents. In such cases, a portion of DNA of disease causing organism is isolated and inserted into E. coli cells. The transformed E.coli cells receive the pathogenic property and can be used as vaccine for the disease concerned.

Hepatitis B virus causes serious diseases like fulminate chronic hepatitis, cirrhosis and primary liver cancer. Recently it has been possible to clone HBV-DNA obtained from infected patients into E.coli (Edman et al, 1981). The transformed E.coli cells were able to produce large quantity of antigen from the cloned HBV DNA. It made possible to produce hepatitis B vaccine.

Similarly, vaccines against the following pathogenic microbes have also been produced using r-DNA technology. They include -

(i) Salmonella causing typhoid

(ii) Vibrio cholerae causing cholera

(iii) Plasmodium falciparus causing malaria

(iv) Feline leukaemia virus causing cancer

(v) Taenia solium causing cysticerosis

(vi) Rabies vaccine and

(vii) Aphthovirus causing Foot and mouth disease in cattle and other animals.

**2. Monoclonal Antibodies**

Antibodies are the globular proteins produced by animals against the antigens when they enter the body. The antigens include fungi, bacteria, Viruses etc. The reaction between antigen and antibody is very specific; one type of antibody reacts only with a particular antigen. Therefore, known antibodies can be used to detect diseases.

Antibodies are routinely obtained by injecting an antigen into an experimental animal and later collecting its serum. But the serum contains mixed types of antibodies, which are produced against different types of antigens. So the purification of a desired antibody from the serum becomes a problem. To overcome this difficulty, Kohler and Milstein (1975) came with a modern technology to produce identical, desired species of antibodies in a large amount. Such homologous antibodies are called monoclonal antibodies. This technology is called hybridoma technology.

Monoclonal antibodies (MAbs) play the following important roles in the field of medicine:

(i) Monoclonal antibodies are used as a diagnostic tool to determine the nature of infectious diseases. They include childhood diarrhea, malaria, cancer, tumors, leukemia and related diseases.

(ii) Monoclonal antibodies are used to treat several diseases.

(iii) MAbs are linked to the action chain of a biological toxin such as racing and diphtheria toxin. Such preparations are called immunotoxins. Immunotoxins act as two-edged swords against target cells, the two edges being specificity and cytotoxicity.

(iv) In recent years, MAbs are also used as enzymes.

**3. Inerferons**

Interferons are protein molecules which are produced by virus infected cells of blood, fibroblasts and T-lymphocytes. Interferons protect the organism from secondary infection by the viruses. They were first discovered by Issacs and Lindermann (1957). Gilbert and Weissman (1980) first time successfully produced human interferons by using genetically engineered E.coli cells. These commercial preparations have clinical importance. Leucocytic interferon's are used in the treatment of breast cancer. A-interferon is useful in the treatment of renal carcinoma in human beings.

**4. Insulin**

Insulin is a hormone which is made of amino acids. It is produced by the pancreas of the liver. Insulin is useful for treatment of persons who suffer from diabetes. Till a few years ago insulin is mainly extracted from the pancreas of dogs, swine's, oxen etc. But now techniques are available for the large scale "manufacture of insulin in bacterial cells by transferring the corresponding genies from human or animal cells into phages (or) bacteria. This makes the production of insulin relatively cheaper. Indian born scientist, Dr. Saran Narang, now working in Ottawa, Canada is involved in the cloning of insulin gene, The Eli Lilley Company presently (USA) producing human insulin from genetically transformed E.coli cells and has been marketing it under the trade name, Humulin

**Industrial Biotechnology**

Industrial biotechnology applies the techniques of modern molecular biology. It helps

to improve the efficiency and reduce the environmental impacts of industrial manufacturing For example, industrial biotechnology companies develop. Biocatalysts, such as enzymes, to synthesize chemicals. Enzymes are proteins produced by all organisms. Using biotechnology, the desired enzyme can be manufactured in commercial quantities.

Commodity chemicals (e.g. polymer - grade acryl amide) can be produced using biotech applications. Traditional chemical synthesis involves large amounts of energy and often - undesirable products, such as HCI. Using biocatalysts, the same chemicals can be produced more economically and more environmentally friendly. An example would be the substitution of protease in detergents for other cleaning compounds. Protease production results in a biomass that in turn yields a useful byproduct- an organic fertilizer. Biotechnology is also used in the textile industry for the finishing of fabrics and garments. Biotechnology also produces biotech- derived cotton that is warmer, stronger, which is wrinkle- and shrink resistance.

Some agricultural crops, such as corn, can be used in place of petroleum to produce chemicals. The crop's sugar can be fermented to acid, i.e. used as an intermediate to produce other chemical feed stocks for various products. It has been demonstrated, at test scale, that biopulping reduces the electrical energy required for wood pulping process by 30%.

Another important facet of industrial Biotechnology is fermentation technology. It contributes various useful products and a working machinery to the field of Biotechnology.

**Fermentation Biotechnology**

Fermentation technology deals with the industrial application of some micro organisms in the production of valuable chemicals. Fermentation is the anaerobic break down of complex organic substances by the action of cell free enzymes. The fermentation process result in the production of a wide variety of organic compounds, such as amino acids, enzymes, proteins, antibodies, organic acids, single-cell proteins etc.

1. Production of amino acids Amino acids are the building blocks of proteins. They are used in various industries such as food industry, pharmacy, cosmetic industry etc.

   Amino acids are used in food for imparting flavors and for increasing nutrition value. L-glutamate, L-alanine and L-glycine are used for flavoring. L histidine is used for relief from gastric ulcers, L-cysteine for releif from bronchitis and L-arginine is used for alleviation of liver disorders.

   Kinoshita and his co-workers (1957) first time reported that micro organisms were able to produce amino acids under cultural conditions. Since then, a number of workers have taken interest in raising new strains of micro organisms in order to produce amino acids. Most of the commercial amino acids belong to L- form.

| S.No. | Amino acid | Enzyme | Source of enzyme |
|---|---|---|---|
| 1. | L-glutamine and L-Glutamic acid | Glutarate, dehydrogenase | Corynebacterium glutamicum, Brevi - bacterium flavum, Micro bacterium ammoniaphilum |
| 2. | Aspartate | Aspartase | E. coli |
| 3. | Alanine | Aspartate decarboxylase | Pseudomonas decunhae |
| 4. | Lysine | Caprolactum racemase | Achrobacter obae |
| 5. | Tryptophan | Tryptophanase | Proteus rettgeri. |

## 2. Production of Alcohols

A number of bacteria produce alcohols during their fermentation activity. The microbes produce ethanol, methanol and butane in the fermentation medium. These alcohols are now used as fuels in many industries. They are called biofuels. Biofuels do not release too much of carbon dioxide during their combustion. This helps in reducing air pollution.

Ethanol is used in the manufacture of synthetic fibers like rayon, polyester etc. It is also used in manufacture of synthetic rubber, perfumes and acetaldehyde. Methane is a constituent of Biogas. Biogas consists of methane and Co, in the ratio of 2:1. Biogas is a colorless mixture of gases and burns with a blue flame,)

## 3. Production of organic acids

Different groups of micro organisms produce organic acids in the culture medium during their growth. Some of the important organic acids are:

| | Organic acid | Process organism |
|---|---|---|
| 1. | Aconitic acid | Aspergillus itaconicus |
| 2. | Citric acid | A.niger;Penicillium citratum |
| 3. | Formic acid | Rhizopus; Mucor |
| 4. | Gentistic acid | Penicillium griseo-fulvum |
| 5. | Glycolic acid | A.niger |
| 6. | Lactic acid | Rhizopus, Mucor, Lactobacillus |
| 7. | Oxalic acid | A.niger |
| 8. | Succinic acid | Rhizopus, Fusarium |
| 9. | Propionic acid | Propionobacterium, Proteus |

## 4. Production of antibiotics

The genus Streptomyces, is the source of over one-half of the known antibiotics. The other half being derived from Micromonospora, Actinoplanes Actinomadura, Nocardia, certain Actinomycetes, Penicillium, Cephalosporium, Bacillus and others. About 100 types of antibiotics are produced commercially using aerobic fermentations. Some of them are listed below:

| | Antibiotic | Source organism |
|---|---|---|
| 1. | Amphotericin-B | Streptomyces nodosus |
| 2. | Bacitracin | Bacillus subtilis |
| 3. | Bleomycin | Streptomyces verticillus |
| 4. | Cephalosporin-C | Cephalosporium acremonium |
| 5. | Chloramphenical | Streptomyces venezuelae |
| 6. | Cycloheximide | S. griseus |

| | |
|---|---|
| 7. Chloromycetin | S. venezuelae |
| 8. Erythromycin | S. erythreus |
| 9. Fungimycin | S. coelicolor |
| 10. Gentamycin | Micromonospora purpurea |
| 11. Griseofulvin | Penicillium griseofulvum |
| 12. Tetracyclin | Streptomyces rimosus |

**5. Production of alcoholic beverages**

A large number of beverages like beer, brandy, whisky, vinegar, wine etc. are produced by fermentation. Some important process organisms are listed below:

| | Source | Process organism | Product |
|---|---|---|---|
| 1. | Ripe grape juice | S. ellipsoids | Wine |
| 2. | Marsh grain of corn, rye, malt | S.cerevisiae | Whisky |
| 3. | Grape juice, apple, orange, peach | S,cerevisiae | Brandy |
| 4. | Cane molasses (or) Beet molasses | Clostridium Saccharobutyricum | Rum |

Write an account of the history of Biotechnology.

**Biotechnology For The 21st Century**

Countries in Asia, Africa, and elsewhere are in a constant dilemma ie how to continue feeding a

# Short Questions and Answers

**1. Define Biotechnology.**

*Ans :*

The term biotechnology is composed of two words - bio (Greek - bios - means life) and technology (Greek technologic - means systematic treatment). It is a branch of applied biology. Biotechnology is a broad term for a group of technologies that are based on applying biological processes. It involves the use of living things or their derivatives to solve problems and make products.

**2. Write Short notes on Nitrogen-fixing organisms**

*Ans :*

Nitrogen is one of the most important macronutrient required for plant growth. Though the atmosphere contains 78% of nitrogen, it is not readily available to plants. Only a small group of microorganisms called nitrogen - fixing organisms are capable of converting the molecular nitrogen into organic nitrogen. The nitrogen fixers are of two types namely symbiotic and non symbiotic. The symbiotic nitrogen fixers get associated with the roots of higher plants and fix the atmospheric nitrogen in the soil. Eg. Rhizobium, Anabaena etc. The non-symbiotic nitrogen fixers live freely in the soil and fix the nitrogen in the soil. Eg. Azotobacter, Azotococcus, Klebsiellla etc.

**3. Medical Biotechnology**

*Ans :*

Medical biotechnology deals with the production of immunologically active substances such as antibodies, interferon's, human growth hormones, vaccines, insulin etc., these compounds play an important role in diagnosis and treatment of some serious diseases.

**4. Insulin**

*Ans :*

Insulin is a hormone which is made of amino acids. It is produced by the pancreas of the liver. Insulin is useful for treatment of persons who suffer from diabetes. Till a few years ago insulin is mainly extracted from the pancreas of dogs, swine's, oxen etc. But now techniques are available for the large scale "manufacture of insulin in bacterial cells by transferring the corresponding genies from human or animal cells into phages (or) bacteria. This makes the production of insulin relatively cheaper. Indian born scientist, Dr. Saran Narang, now working in Ottawa, Canada is involved in the cloning of insulin gene, The Eli Lilley Company presently (USA) producing human insulin from genetically transformed E.coli cells and has been marketing it under the trade name, Humulin

**5. Fermentation Biotechnology**

*Ans :*

Fermentation technology deals with the industrial application of some micro organisms in the production of valuable chemicals. Fermentation is the anaerobic break down of complex organic substances by the action of cell free enzymes. The fermentation process result in the production of a wide variety of organic compounds, such as amino acids, enzymes, proteins, antibodies, organic acids, single-cell proteins etc.

**6. Gene Cloning Steps.**

*Ans :*

**Step-1:**

**Isolation of DNA (Gene of Interest) Fragments to be cloned**

The operation of gene cloning primarily needs two basic things in their purified state - the gene of our interest (GI) and the vector. A GI is a fragment of gene whose product may be a protein, enzyme or a hormone.

The vector is a carrier molecule which can carry our GI into a host, replicate there along with the GI making its multiple copies. In this state the GI can also be expressed in the host cell producing the product of the gene which is needed. Desired DNA segments are obtained from Genomic library, C DNA library of produced by PCR method.

**Step-2**

**Insertion of Isolated DNA into the Suitable Vector to Form the Recombinant DNA**

The next step is to cut both the vectors as well as the GI by using a special type of enzyme, called restriction endonuclease. A restriction endonuclease is an enzyme that cuts double-stranded or single-stranded DNA specific recognition nucleotide sequences known as restriction sites towards the inner region (hence endonuclease).

They are also regarded as molecular scissors as they cut open the DNA strands. The cutting step is followed by pasting. Here the GI is taken and pasted to the cut vector. This procedure also needs an enzyme, called DNA ligase. They are also considered as molecular glue.

**7. Restriction Endonucleases (REases)**

*Ans :*

The nuclease enzyme that cuts the DNA at a unique sequence or at specific sites of DNA is called Restriction Endonuclease. They cut the DNA in a nonterminal region. Restriction Endonucleases are used to generate rejoinable DNA fragments.

REases are also known as Molecular Knives, Molecular Scissors etc. The sequence recognized by the RE, to cut the DNA is called restriction site or recognition site. This site-generally consists of 4-8 base pairs. Present Day Technology, specifically Gene Cloning is totally dependent on the ability to cut DNA molecules at specific sites with Restriction endonucleases Restriction Endonuclease or Restriction Enzyme consists of two enzymatic elements i.e., Nuclease and Methylase.

**8. ligases.**

*Ans :*

**Ligases**

Ligase is an enzyme that can catalyze the joining of two large molecules by forming a new chemical bond. Generally Ligase can joint two complementary fragments of nucleic acid and repair single stranded breaks that arise in double. stranded DNA during replication. Ligases are classified as EC 6 with Six sub classes.

But DNA Ligases are the principle types employed in Geng Cloning techniques.

**DNA Ligase:** It is the main enzyme in Gene Cloning. It catalyzes the formation of phosphodiester bond between 3-OH and 5-PO4 groups of nucleotides and facilitates the joining of DNA fragments. It is also known as a 'Molecular Stitcher. This ability enables the filling the gaps or the nicks of DNA and joining OKAZAKI fragments formed during DNA replication.

DNA ligases are mainly used in rDNA technology for the production of recombinant DNA molecules during Gene Cloning. DNA ligase joins DNA of interest (DI or GI) with vector DNA (Plasmids etc.) DNA ligase depends on a cofactor and Mg, + ions for its function. NAD+ acts as a cofactor for Bacterial DNA Ligases. While ATP acts as a cofactor for VIRUS and Eukaryotic DNA ligases.

**9. Polymerases.**

*Ans :*

**Polymerases**

A polymerase is an enzyme that synthesizes long chains of polymers or nucleic acids. DNA and RNA polymerase are the two common types.

**DNA Polymerase:** It is present in all living organisms. It helps in DNA synthesis and Genome replication. This ability of DNA polymerase is employed in Gene cloning. This enzyme catalyzes the synthesis of new DNA, complementary for the existing DNA.

DNA Polymerase I of E. Coli directs the synthesis of complementary nucleic acids using single stranded DNA as template. It converts single stranded DNA to the double stranded form. DNA polymerase synthesizes, DNA molecules from deoxyribonucleotides. DNA Polymerase adds nucleotides to the 3'OH (3' end) group of the primer sequence (Bases) and continues the strand formation to 5' direction i.e., elongation of the newly forming strand in a 5'-3' direction.

**10. Application Of Rdna Technology**

*Ans :*

Genetically engineered bacterial cells through recombinant DNA technology have many applications in the field of medicine and other branches.

1. E. coli containing rDNA made of human insulin gene, is used to produce human insulin.
2. E. coli containing r DNA can produce human growth hormone-Somatotropin.
3. Genetically engineered E. coli cells are used to produce rat insulin.
4. Genetically engineered colon bacillus cells are capable of producing beta endorphin which is used as antagonistic drug.
5. Gilbert and Weissmann (1980) raised a new strain of colon bacilli capable of producing human-a-interferon against viruses.

# Choose the Correct Answer

1. PCR was invented by Kary Mullis in [ c ]
   (a) 1986 (b) 1975
   (c) 1985 (d) 1990
2. REs are categorized into how many groups [ a ]
   (a) 4 groups (b) 6 groups
   (c) 8 groups (d) 10
3. Sumerians and Babylonians prepared about 20 different types of beer in [ d ]
   (a) $5^{th}$ millennium (b) $4^{th}$ millennium
   (c) $3^{rd}$ millennium (d) $6^{th}$ millennium
4. In $14^{th}$ Century, first Vinegar manufacturing industry was established in [ a ]
   (a) America (b) Italy
   (c) Germany (d) France
5. Plant tissue culture plays an important role in agriculture, in that how many important applications are there? [ c ]
   (a) 9 (b) 8
   (c) 6 (d) 5
6. How many types of antibiotics are produced in aerobic fermentations [ d ]
   (a) 200 types (b) 300 types
   (c) 400 types (d) 400
7. PCR was invented by Kary Mullis in [ c ]
   (a) 1986 (b) 1975
   (c) 1985 (d) 1984
8. REs are categorized into how many groups [ a ]
   (a) 4 groups (b) 6 groups
   (c) 8 groups (d) 10 groups
9. How many types of Clonal vectors are identified. [ b ]
   (a) 8 (b) 4
   (c) 10 (d) 6
10. What is the size of the Conal vector in pBR 322 [ a ]
    (a) 4.36 (b) 5.45
    (c) 6.65 (d) 7.40

# Fill in the blanks

1. Statistical Definition of Biotechnology is also described as _____
2. White biotechnology is also known as _____
3. Methane is a constituent of _____
4. Insertion of Isolated DNA into the Suitable Vector to Form the _____
5. The resulting DNA molecule is a hybrid of _____ molecules.
6. Transformed of E.Coli is called as _____ .
7. Ligases are classified as EC 6 with _____
8. DNA ligase is also known as _____
9. The plasmid cloning vectors are designated by _____
10. Bacteriophages are viruses which infec bacteria these are commonly called as _____

## ANSWERS

1. List based definition
2. Grey biotechnology
3. Biogas
4. Recombinant DNA
5. Two
6. Recombinant cells
7. Six sub classes
8. 'Molecular Stitcher'
9. 'P'
10. Phages.

# One Mark Answers

**1. What is biotechnology?**

*Ans :*

Biotechnology has been defined by many in different ways and there is a considerable diversity regarding the exact definition of biotechnology.

**2. What is entomopathogens?**

*Ans :*

A number of microorganisms are known to be pathogenic on insects. These microbes are called as entomopathogens.

**3. What is meant by Genetic therapy?**

*Ans :*

**Genetic therapy**

The treatment of a genetic disease by replacing or manipulating damaged genes with normal genes with normal genes or replacement of a defective gene in an organism suffering from a genetic disease by normal genes or use of a gene or cDNA to treat disease.

**4. What is meant by Genetic Engineering ?**

*Ans :*

Genetic engineering the manipulation of genes, composed of DNA to create heritable changes in biological organisms and products that are useful to people living things, or the environment. It is also called recombinant DNA technology.

**5. What is meant by Polymerase ?**

*Ans :*

A Polymerase is an enzyme that synthesizes long chains of Polymers or nucleic acids.

**6. What is Ligase.**

*Ans :*

Ligase is an enzyme that can catalyse the joining of two large molecules by forming a new chemical bond is called Ligase.

**7. What is the full form of PCR.**

*Ans :*

Polymerase Chain Reaction

**8. What is meant by Cloning Vechile?**

*Ans :*

The DNA that carries the desired gene to the host cell is called Cloning Vector/Cloning Vechile.

UNIT IV

12. **Gene Libraries:** construction penomic and cDNA libraries, colony hybridization; Probes oligonucleotide, Polymerase in Reaction (PCR) and its applications.
13. **Methods of gene transfer Agrobacterium-mediated:** Direct gene transfer by electroporation, Microinjection, Microprojectile bombardment, Selection of transgenics-selectable marker and reporter genes.
14. **Application of transgenics in improvement of crop productivity and quality traits:** Pest resistant transgenic crops (Bt-Cotton & Bt-Brinjal); herbicide resistant plants (Roundup Ready soybean);crops with quality traits (Flavr Savr tomato, Golden rice).

## 4.1 GENE LIBRARIES

**Q1. Write an essay on Genomic libraries.**

*Ans :*

### Genomic Libraries

### Introduction

A genomic library is a collection of the total genomic DNA clonal fragments from a single organism. The DNA is stored in a population of identical vectors, each containing a different insert of DNA. Generally, libraries made from organisms with larger genomes, require vectors having larger inserts. Genomic libraries are commonly used for sequencing applications. They have played an important role in the whole Genome sequencing of several organisms, including Human Genome. The first DNA -based Genome, fully sequenced was achieved by two times Noble Prize Winner, Frederick Sanger in 1977, created a library of the bacteriophage, Phi x 174 for use in DNA sequencing.

### Applications

After a library is created, the genome of an organism can be sequenced to elucidate how genes affect an organism or to compare similar organisms at the genome-level. The aforementioned genome-wide association studies can identify candidate genes stemming from many functional traits. Genes can be isolated through genomic libraries and used on human cell lines or animal models to further research. Furthermore, creating high-fidelity clones with accurate genome representation and no stability issues would contribute well as intermediates for shotgun sequencing or the study of complete genes in functional analysis.

### Hierarchical sequencing

One major use of genomic libraries is hierarchical shotgun sequencing, which is also called top-down, map-based or clone-by-clone sequencing. This strategy was developed in the 1980s for sequencing whole genomes before high throughput techniques for sequencing were available. Individual clones from genomic libraries can be sheared into smaller fragments, usually 500bp to 1000bp, which are more manageable for sequencing. Once a clone from a genomic library is sequenced, the sequence can be used to screen the library for other clones containing inserts which overlap with the sequenced clone. Any new overlapping clones can then be sequenced forming a contig. This technique, called chromosome walking, can be exploited to sequence entire chromosomes.

Whole genome shotgun sequencing is another method of genome sequencing that does not require a library of high-capacity vectors. Rather, it uses computer algorithms to assemble short sequence reads to cover the entire genome. Genomic libraries are often used in combination with whole genome shotgun sequencing for this reason. A high resolution map can be created by sequencing both ends of inserts from several clones in a genomic library. This map provides sequences of known distances apart, which can be used to help with the assembly of sequence reads acquired through shotgun sequencing. The human genome sequence, which was declared complete in 2003, was assembled using both a BAC library and shotgun sequencing.

**Genome-wide association studies**

Genome-wide association studies are general applications to find specific gene targets and polymorphisms within the human race. In fact, the International HapMap project was created through a partnership of scientists and agencies from several countries to catalog and utilize this data. The goal of this project is to compare genetic sequences of different individuals to elucidate similarities and differences within chromosomal regions. Scientists from all of the participating nations are cataloging these attributes with data from populations of African, Asian, and European ancestry. Such genome-wide assessments may lead to further diagnostic and drug therapies while also helping future teams focus on orchestrating therapeutics with genetic features in mind. These concepts are already being exploited in genetic engineering. For example, a research team has actually constructed a PAC shuttle vector that creates a library representing two-fold coverage of the human genome. This could serve as an incredible resource to identify genes, or sets of genes, causing disease. Moreover, these studies can serve as a powerful way to investigate transcriptional regulation as it has been seen in the study of baculoviruses. Overall, advances in genome library construction and DNA sequencing has allowed for efficient discovery of different molecular targets. Assimilation of these features through such efficient methods can hasten the employment of novel drug candidates.

### 4.1.1 Construction penomic and cDNA libraries

**Q2. Describe the method of construction of genomic and DNA libraries.**

*Ans :*

**Construction of genomic and DNA libraries - Procedure**

Construction of a genomic library involves mainly creating many recombinant molecules. Below are the steps for creating a Genomic Library from a large genome, Shotgun method is employed mostly in the construction procedure of Genomic Library.

**Steps:**

1. Isolation of genomic DNA from the organism. It includes extraction and purification of DNA.
2. Digestion of the DNA with a restriction enzyme. In this, DNA is partially cleaved into thousands of fragments (of 5-100 kb) by restriction endonuclease to get inserts of desired size range, compatible with the cloning vector (Plasmid, Phage Lambda, Cosmid, BAC, YAC etc.) used for library construction.
3. Insertion of the fragments of DNA into the cloning vector, which is also cut with the same restriction enzyme used for the cleavage of the isolated DNA in the first step.
4. Ligation of the DNA fragment with the cloning vector is done in this step by using the enzyme DNA ligase to seal the DNA fragment into the vector. This creates a recombinant DNA molecule or Hybrid DNA molecule. Generally a large pool our DNA molecules are created at experimental level.
5. Transformation is the main event in this step. The entry of r DNA molecules into a host-coll is described as Transformation. Host organism is commonly a population Bacterium (E. Coli) or Yeast Cells, to produce a library with each cell containing one vector molecule. Then transformed cells are subjected to cloning. Each cloning vector contains a different fragment of the genome. By this, all DNA in the genome is represented among the clones in the library. Both transformed host cell and cloning vector multiply producing a colony with host cells in millions, containing numerous copies of the cloning vectors or clones.
6. Selection of the clones with the desirable genes is done in this step, after cloning by special selection methods.
7. Construction of a Genomic library with the help of the selected clones is the main event in this step.

The number of clones required to include all the sequences in the genome depends on the size of the genome being investigated. The main activities in the final preparation of a Genomic library include sequencing, comparing, analyzing, characterizing, aligning of genomic DNA inserts by employing, if necessary, computer programs and other related scientific methods.

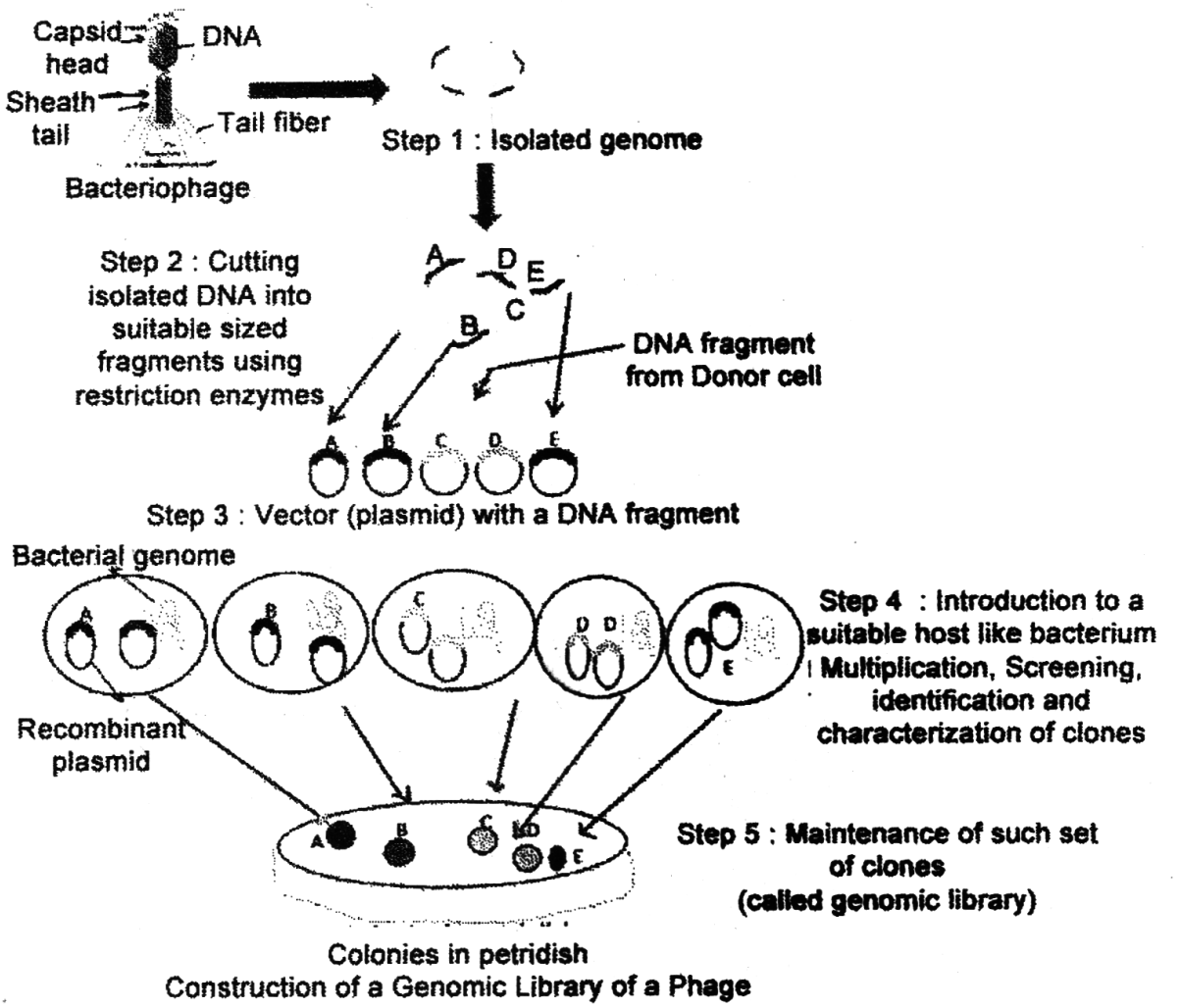



**Fig.: Genomic Library Construction – Diagrammatic Explanation**

### Determining titer of library

After a genomic library is constructed with a viral vector, such as lambda phage, the titer of the library can be determined. Calculating the titer allows researchers to approximate to many infections viral particles were successfully created in the library. To do this, dilutions of the library are used to transfect cultures of E. coli of known concentrations. The cultures are then plated on agar plates and incubated overnight. The number of viral plaques are counted and can be used to calculate the total number of infectious viral particles in the library. Most viral vectors also carry a marker that allows clones containing an insert to be distinguished from those that do not have an insert. This allows researchers to also determine the percentage of infectious viral particles actually carrying a fragment of the library.

A similar method can be used to titer genomic libraries made with non-viral vectors, such as plasmids and BACs. A test ligation of the library can be used to transform E.coli. The transformation is then spread on agar plates and incubated overnight. The titer of the transformation is determined by counting the number of colonies present on the plates. These vectors generally have a selectable marker allowing the differentiation of clones containing an insert from those that do not. By doing this test, researchers can also determine the efficiency of the ligation and make adjustments as needed to ensure they get the desired number of clones.

### 4.1.2 Colony hybridization

**Q3. Write briefly on screening of clones.**

*Ans :*

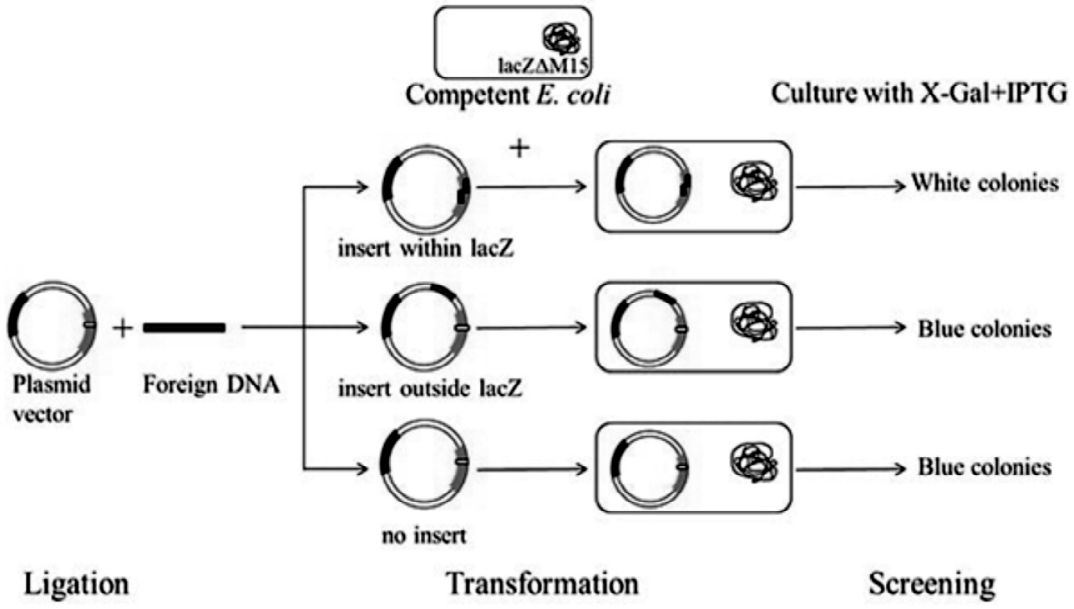
**Screening of Clones**

Recombinant DNA teshliggy involves DNA manipulation outside the cells DNA into the living cells by various methods Only a small number of bacterial cells take and therefore there has to be a way to find out which hacteria contain the roun carry genes that confers resistance to specific abnotic such as ampicilin. instance, if the gene for ampicillin resistance (Amp) is incorporated into the antibiotic ampicillin. This allows selection on the medium containing the actic ampicil only those bacteria that have been transformed and contain a plasmid will be able to grow the untransformed cells are killed by the antibiotic.

The plasmid vector, in addition to antibiotic resistance genes, contains the lac Zgene that producer enzyme B-galactosidase to metabolire the sugar X-gal (S-bromo-4-chloro-3-indolyt-B-D-galactopyranside.

When the cells are cultured in a medium called X-gal, the earyme B-galactosidase metabolizes the sugar X-gal to produce galactose and an indigo compound. The indigo turns the colonies blue.

If a foreign DNA is inserted into the plasmid where it inactivates the lac Z gene, the pre ps and the cell will be unable to metabolize X-gal. The reason for this is that, the foreign DNA dismuts the me of the lac Z gene. Therefore, functional lac Z protein, L.e., B-galactosidase is not produced. If such a e spread on the nutrient agar plate containing ampicillin and B-galactosidase substrate and cond, the ulting white colonies contain foreign DNA while the blue colonies do not comin foreign DNA.



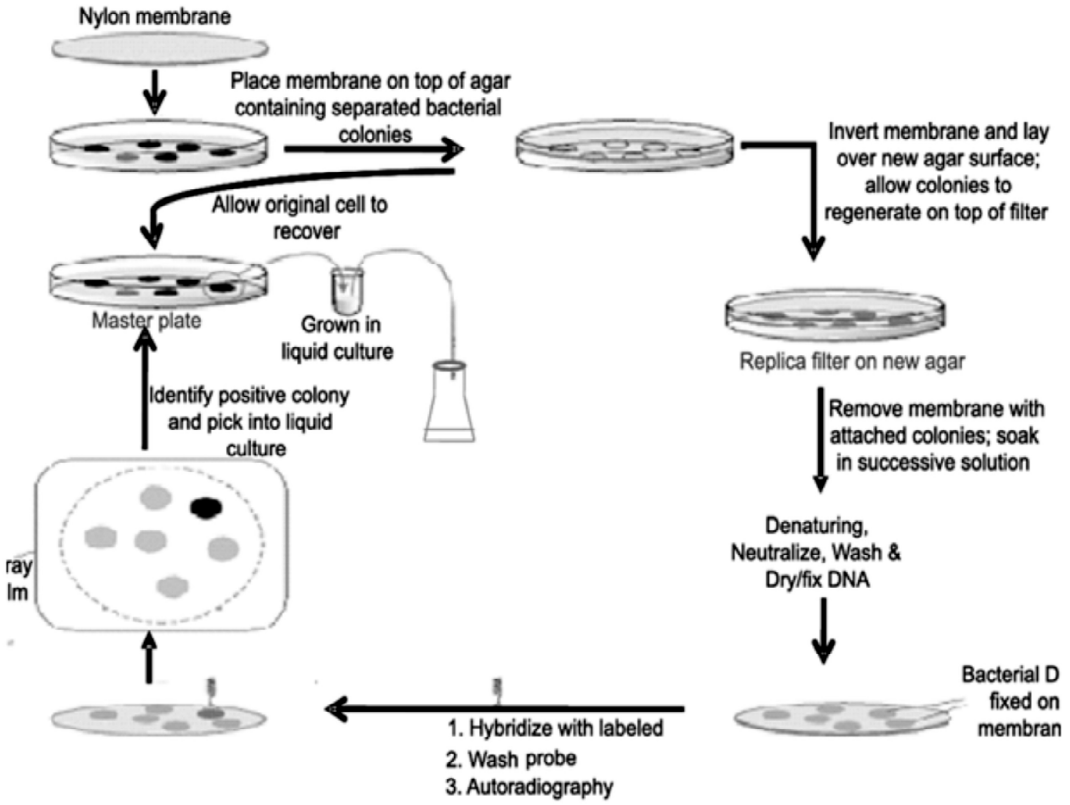
**Finding the Target Gene**

Colony hybridization is a common method of identifying us that carry target genes. When a dilute solution of culture is spread on an agar plase, each cell reproduces g millions of genetically identical cells. A challenging task is to idly which yo rest (the desired DNA base sequence). A gmatic probe is used for this purpose. Geti pe aded nocleic acid that has a complementary sequance of the game of the with dement P or a fluorescent dye for easy detection

**The steps in this process are,**

1. Anitrocellulose filter paper/nylon membrane is pressed against the master plate to make a replica/ copy of the colonies.
2. The filter paper is washed with detergent (SDS) to lyse the bacterial cells and the sodium hydroxide with to separate DNA into single stands.
3. A solution containing radioactive labelled probe is added. The probe hybridizes with the game of interest.
4. The filter is washed to remove the unbound probe and exposed to X-ray film (autoradiography).
5. The developed film is compared to the master plate to identify the colony containing the desired DNA.

The spots on the filter show where the probes have hybridized to the genes. These spots are compared to the original plate to select the colony containing the gene of interest. These colonies are removed from the plates and the plasmids are grown in bulk to produce large quantities of the desired gene.



### 4.1.3 Probes – Oligonucleotide

**Q4. What is a probe? Explain the radioactive and non radioactive labelling of probe**

*Ans :*

A probe is a machine sequence of 10 to 30 bases long used for detecting the DNA b complementary to its sequence. It can hybridize to target nucleic acid sequence through complement base pairing.

**Types of DNA Probes**

The main types of DNA probes include

**(i) c DNA Probe**

CDNA probe is produced from specific mRNA that codes for a specific protein mRNA template, which is catalyzed by the enzymes reverse transcriptase and DNA polymerase cDNA. This cDNA hybridizes with specific gene sequence

**(ii) Genomic DNA Probe**

Genomic DNA probes are single stranded DNA molecules used for detection of complementary acid sequences of a gene of interest

**(iii) Oligonucleotide Probe**

Oligonucleotide probes are usually 15 to 45 bases of single stranded nucleic acids that are chemically synthesized as a specified base sequence. The probe is allowed to base pair with the nucleic acids, i.e., hybridize. The probes are labeled with radioactive or fluorescent molecule for easy detection.

**Types of Nucleic Acid Hybridization Probe**

The nucleic acid hybridization probe can be of two types:

DNA Probes It is used to detect the presence of DNA or a gene present in a sample.

**RNA Probes**

RNA probes (also known as riboprobes or cRNA probes) are used to detect the presence of mRNA present in a biological sample

**Labelling of Probes**

Probes are marked with either of the following:

**Radioactive Labelling**

The different methods used for labelling of nucleic acids are as follows

**(i) Direct Labelling**

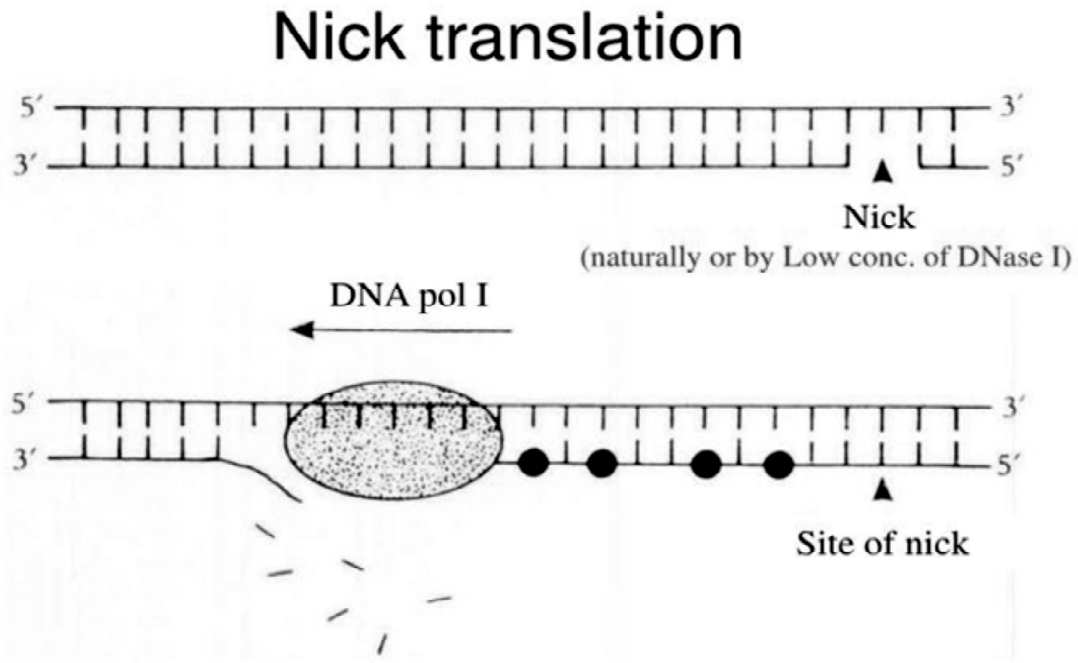
nucleotide labelled with $^{32}P$ or $^{3}58$ is added during the production of probe. The ad nucleotide gets covalently attached to the nucleic acid and thus gets incorporated in the p the alpha phosphate position.

**Nick Translation**

**(ii)** In this method, the DNA is treated with the enzyme DNase 1 for a short duration. The activity of the enzyme produces single-strand nicks (cuts) in DNA .

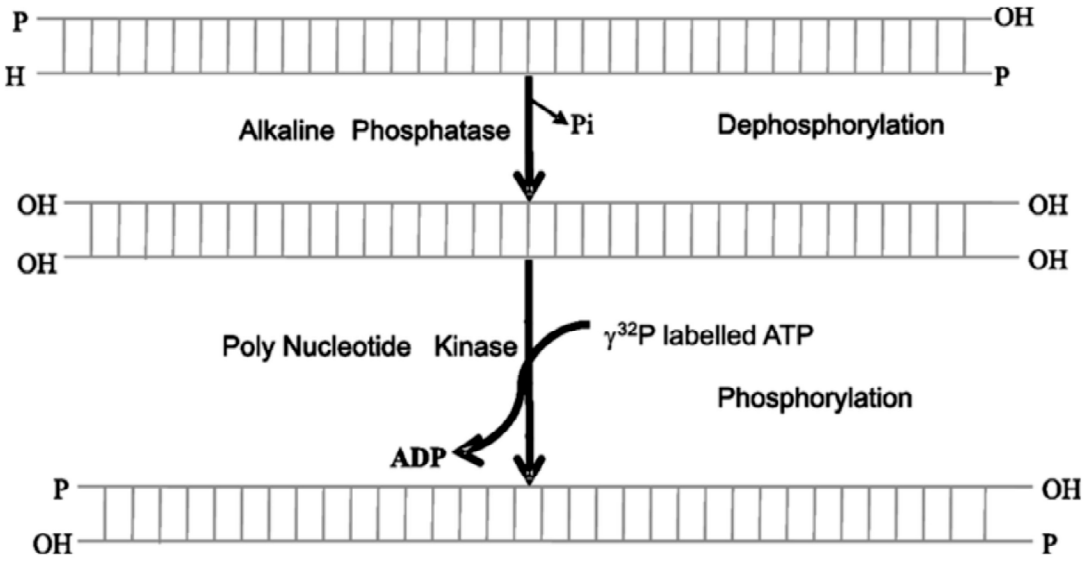
The nicked DNA sample is treated with E. coli DNA polymerase I and radio labelled nucleotides. The enzyme catalyzes the synthesis of DNA complementary to a DNA template.

The 5 ---- 3' exonuclease activity of the enzyme DNA polymerase I digests the existing homologous strand in the DNA duplex.




### (iii) End Labeling

End labeling of DNA probe can be performed at the 3'-end or 5'-end. The 5' end labeling of probe DNA is illustrated below.




The 3'end of DNA sequence can be labelled by the use of a polymerase without a 3''!'5 exonuclease activity. The steps involved are,

i) Incubation of DNA fragments with the enzyme terminal deoxynucleotide transferase (TDT) and radioactive $\alpha^{o}$ PATP.

ii) The enzyme TDT catalyses the incorporation of $\alpha^{3}2$ PATP at 3´ OH end of DNA fragment in template independent manner.

iii) Probes are ready with labeled nucleiotides at 3´OH.

iv) Primer Extension

In this method, a radiolabelled primer is allowed to anneal with the RNA. The enzyme reverse transcriptase is used to synthesize cDNA from RNA until it reaches the 5´ end of RNA.

**Advantages**

**1.** Radioactive probes can be rapidly prepared Nucleotides labeled with high energy-emitting radioisotopes (“P) are very sensitive. They can detect small amounts of target DNA

**Disadvantages**

(i) Short shelf life of radioactive labels.

(ii) Safety and regulatory issues.

**2. Non-radioactive Labelling**

In this method, the probes are labelled by one of the following methods

**(i) Direct Labelling**

This method involves direct incorporation of fluorescent tags or cross-linking enzyme molecules to the nuleic acid probe.

**(ii) Indirect Labelling**

This method involves the incorporation of nucleotides tagged with a happen (biotin or digoxigenin)

**During probe synthesis.**

Biotin, a water-soluble vitamin, (also known as vitamin H) is incorporated into nucleic acid by using dUTP-biotin, Such biotin-conjugated (biotin - conjugated) nucleotides are used for labeling the probes.

The dUTP of dUTP-biotin complex first binds with dATP of target DNA following the hybridization process, avidin / streptavidin is added that has horse radish peroxidase/alkaline phosphatase enzyme coupled to it. Avidin/ streptavidin binds specifically and irreversibly to biotin. A chromogenic substrate is added the changes the colour because of the action of peroxidase. The intensity of colour is measured by stand procedures.

Digoxigenin is a plant-derived steroid (Digitalis purpurea and Digitalis lunata) which is used t conjugating with nucleotides to produce digoxigenin labeled probe.

The bound digoxigenin is a highly antigenic compound.It can be visualized when the digoxigenin anti body conjugated with a reporter enzyme (horse radish peroxidase) or a fluorescent dye is added to it

**Advantages**

(i) Increased probe stability

(ii) Convinient handling

(iii) Superior safety profile

(iv) Multiple labels can be used in a single experiment for improved detection.

**Application of DNA Probes**

DNA probes are widely used in

(i) Screening gene libraries

(ii) Detecting nucleotide sequences with blotting methods

(iii) Nucleic acid and tissue microarrays.

### 4.1.4 Polymerase in Reaction (PCR) and its applications.

**Q5. What is PCR? Describe in detail about PCR and its applications.**

*Ans :*

**Polymerase chain reaction (PCR)**

The amplification of DNA fragments in vitro is described as the Polymerase Chain Reaction (PCR). The idea for PCR is credited to Kary Mullis and he was awarded the Noble prize in Chemistry in 1993 for his contribution to the development of PCR.

PCR helps in the selective "amplification of a chosen region of a DNA molecule.

Amplification of selected region from a complex of DNA mixture is carried out in vitro by the DNA polymerase-1 (Obtained from Thermus aquaticus, a bacterium that lives in hot springs). This DNA polymerase is called Taq Polymerase.

PCR can use the smallest sample of the DNA to be cloned and amplify it, to millions of copies, in just a few hours.

**Basic Principle of PCR**

The main activity of PCR includes the primer mediated enzymatic amplification of DNA. PCR employs the ability of DNA polymerase to synthesize new strand of DNA complementary to the selected template strand. For this a Primer is needed because DNA polymerase can add a nucleotide, only on to a preexisting 3'OH group to add the fist nucleotide. Then 3' end elongation is achieved by adding more nucleotides, by the catalytic action of DNA polymerase, to generate an extended region of double stranded DNA.

**Components of PCR**

The PCR requires the following components for its completion.

1. DNA template: The double stranded DNA of interest, separated from the sample.
2. Taq Polymerase: It is a thermally stable DNA polymerase which can function normally at higher temperatures without denaturation.
3. Primers: These are short-segments of nucleotides which are complementary to a section of the DNA, which is to be amplified in the PCR. Each Primer is a short strand of DNA (with 20-30 base pairs) complementary to the 3'ends of the sense and antisense strands of the target sequence.
4. Deoxy ribo nucleoside Triphosphates (dNTPs): These are single units of the bases A, T, G, C (d ATP, d TTP, d GTP, d CTP). These are energy providerfor polymerization and building blocks for DNA synthesis.
5. Buffer System: Magnesium and Potassium are included as ingredients in these systems. They provide suitable conditions for DNA denaturation and denaturation. They also control polymerase activity to continue in a suitable status.

**PCR Procedure**

All the PCR components are mixed together and placed in a PCR machine. PCR machine increases and decreases the temperature of the PCR mixture in automatic, programmed steps which generate copies of the target sequence very rapidly in large amounts.

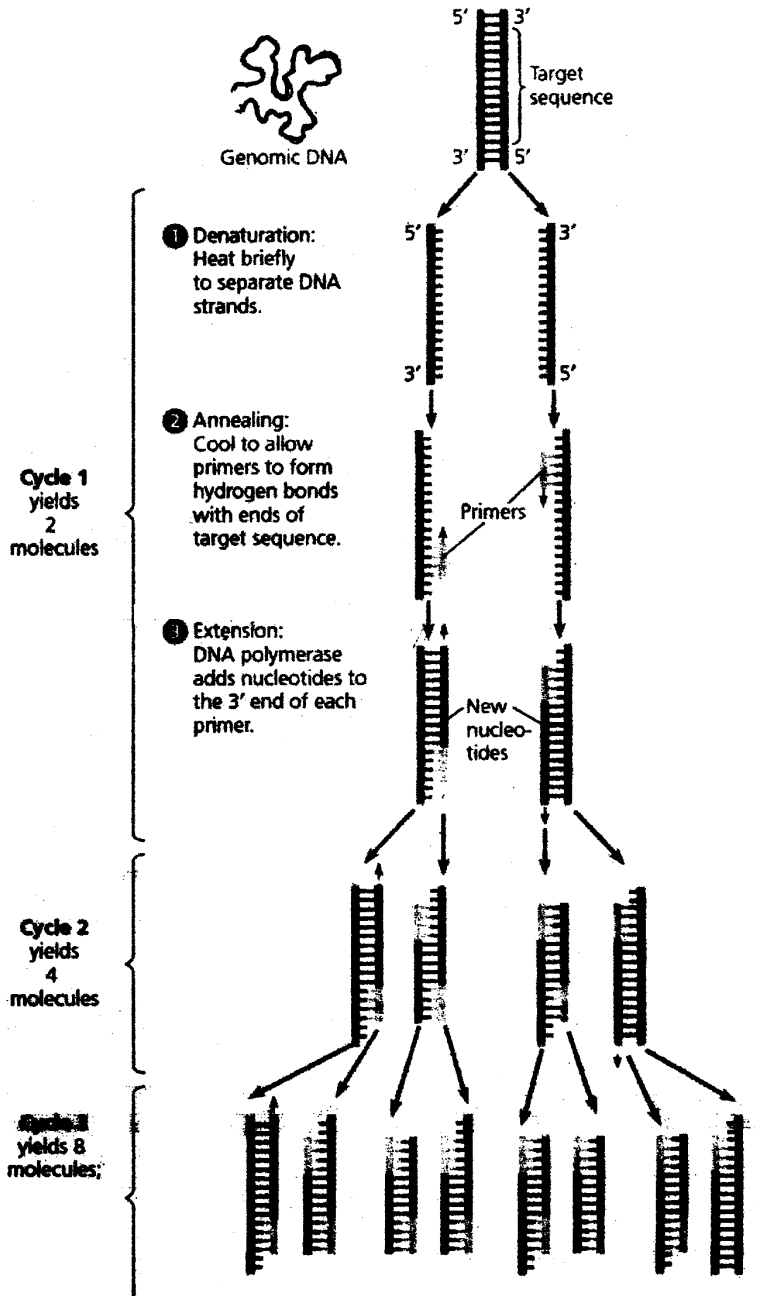



**Fig. : PCR Technique - Diagrammatic Explanation**

The PCR procedure includes three major successive steps. They are described as follows:

**Step-1:** Denaturation

**Step-2:** Annealing

**Step-3:** Elongation

**Step-1: Denaturation**

The separation of the two strands of DNA in to single strands is called denaturation. This step involves heating the PCR mixture to 94°C for 15-30 seconds. It results in the denaturation of double stranded DNA in the mixture and formation of single strands of DNA by breakage in weak H, bonds.

**Step-2: Annealing**

Binding of primers to the template DNA to their complementary sequence is described as Annealing.

In this step, reaction temperature is rapidly lowered to 54-60°C for 20-40 seconds. It allows annealing or binding of primers at the appropriate positions/sequences on the single separated strands of DNA formed in the first step.

**Step-3: Elongation**

It is also called 'Extension' type. The main activity in this step is the synthesis of new DNA. It starts at the annealed primer and usually occurs at 72-80°C. In this step Taq Polymerase sequentially adds bases to the 3' end primer, extending the DNA sequence in the 5 to 3' direction. Taq polymerase can add about 1000 bp/minute under optimum conditions.

The completion of the above three steps is treated as one CYCLE in which a single segment of dsDNA template is amplified into two separate pieces of double stranded DNA. These two pieces are ready for amplification in the next cycle. As the cycles are repeated more and more copies are generated.

**Applications of PCR**

PCR has been playing a vital role in various scientific fields like Molecular Biology, Medicine, Pathology, Forensic Science and others:

PCR is now widely in use as a Practical, Biochemical tool by extending its role in the following applications.

**PCR helping in the**

1. Diagnosis of Genetic disorders such as phenylketonuria, Hemophilia, Sickle cell Anemia etc.
2. Detection of Nucleic acid sequences of pathogenic organisms in clinical samples.
3. Genetic analysis of Forensic samples is done by the technique of Genetic Finger Printing. This technique is used by Forensic Scientists. And this, Genetic Finger Printing can also be achieved by PCR.
4. Forensic Scientists now a days employing PCR as a tool, in Genetic Finger Printing (GFP). GFP Technology can identify any one person from millions of others in case of CRIME Scene; rule out suspects by testing very small amounts of Sample Specimens (Stains of Blood, hair etc.)
5. Analysis of Homologous Genes in the evolutionary Biology (Phylogeny).
6. Molecular Biology activities like
   (a) Generation of Probes or cDNAs.
   (b) Generation of large cDNA libraries from small amounts of mRNA.
7. Detection of Genetic Mutations.
8. Monitoring of the Gene, thereby helping Gene Therapy.

9. Detection of HIV, TB, Hepatitis, Malaria etc., by clinical analysis of Specimens / Samples. 10. Mapping of all the Genes in Human beings through HGP (Human Genome Project).

10. Detection of antimicrobial resistance.

## 4.2 GENE TRANSFER

**Q6. What is Gene Transfer? Describe in detail about the Gene Transfer Methods in Plants.**

*Ans :*

### Introduction

Gene transfer is defined simply as a technique to efficiently introduce foreign genes into the Genome of targets. Gene transfer technologies were originally developed as a research tool for investigating gene expression and function. Currently, there are a number of gene transfer technologies available, which vary greatly in their efficiency of Gene Transfer.

Gene transfer technology has found its way into clinical applications: designed to treat hereditary diseases like Cancer, AIDS etc.

### Gene Transfer Methods In Plants

The Gene Transfer techniques in plant genetic transformation are broadly divided into two types or categories. They are:

1) Vector Mediated Gene Transfer

II) Vector less or Direct Gene Transfer

### 1. Vector Mediated Gene Transfer

It is also described as "indirect" Gene transfer. This type of Gene transfer is mostly carried out by Agrobacterium-mediated transformation.

Agrobacterium Mediated Gene Transfer Method: Agrobacterium tumefactions are a soil borne, Gram-negative bacterium. It is considered as one of the efficient vectors in gene transfer techniques. The Ti Plasmid of A. tumefactions has been widely used as a vector in plant transformation. These bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection.

Ti Plasmids are used as Gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti Plasmid in place of unwanted sequences.

Leaf discs (in case of Dicots) or Embryo genic Callus (in case of Monocots) are collected and infected with Agrobacterium carrying recombinant Ti Plasmid vector for Transformation activity.

The infected tissue is then cultured on shoot generation medium for 2 or 3 days, during which transfer of T-DNA takes place along with foreign genes. Then transformed tissues are transferred on to a selection cum plant regeneration 'MEDIUM', supplemented with an antibiotic of specific concentration to eliminate selectively, non-transformed, tissues.

After 3-5 weeks the regenerated shoots are transferred to root inducingMedium.

After another 3-4 weeks, complete plants are transferred to soil. Techniques like PCR etc., are used to detect the presence of foreign genes in transgenic plants.

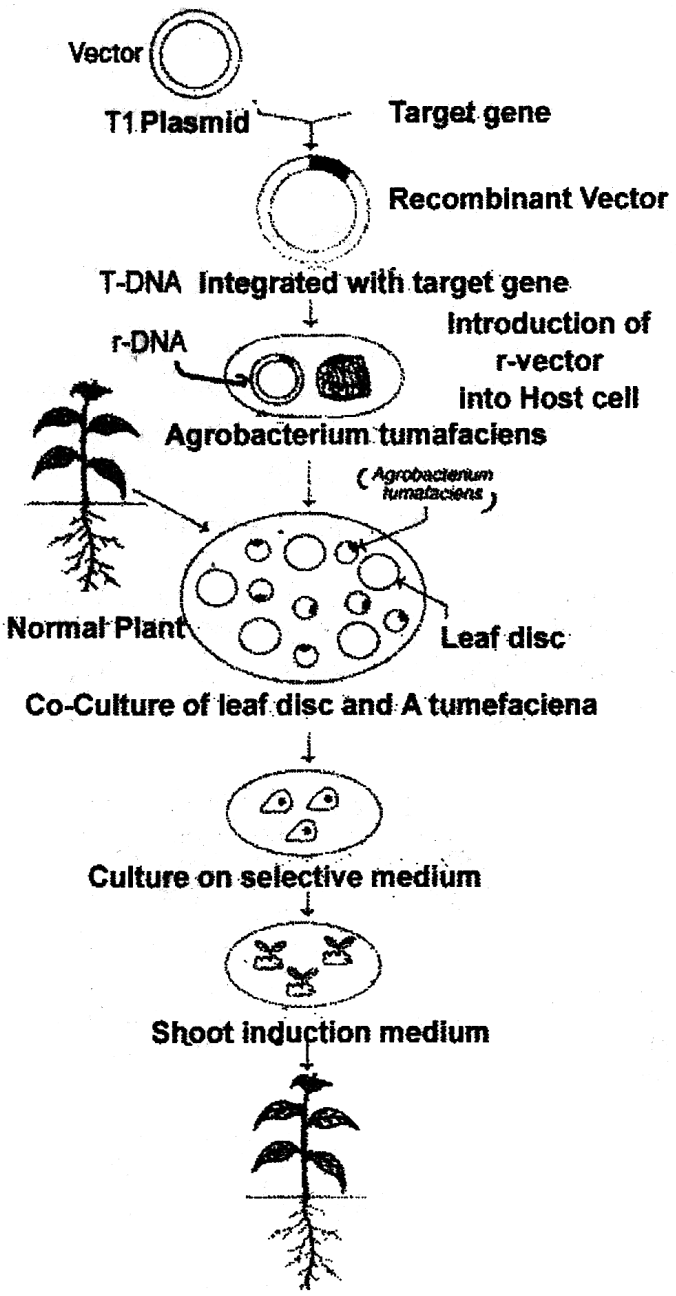



**Fig.: Agrobacterium Mediated Gene Transfer - Method - Diagrammatic View and Explanation**

### Advantages

1. It is a natural method of gene transfer.
2. Agrobacterium can conveniently infect any explants (cells / tissues / organs).
3. Even large fragments of DNA can be efficiently transferred.
4. Stability of transferred DNA is reasonably good. 5. Transformed plants can be regenerated effectively.

**Limitations**

1. There is a limitation of host plants for Agrobacterium, since many crop plants (monocotyledons e.g., cereals) are not infected by it. In recent years, virulent strains of Agrobacterium that can infect a wide range of plants have been developed.
2. The cells that regenerate more efficiently are often difficult to transform, e.g., embryonic cells lie in deep layers which are not easy targets for Agrobacterium.

**2. Direct (or) Vector-less DNA Transfer**

The term direct or vector less transfer of DNA is used when the foreign DNA is directly introduced into the plant genome. Direct DNA transfer rely on the delivery of naked DNA into the plant cells. This is in contrast to the Agrobacterium or vector-mediated DNA transfer which may be regarded as indirect method. Majority of the direct DNA transfer methods are simple and effective. And in fact, several transgenic plants have been developed by this approach.

**Limitations**

The major disadvantage of direct gene transfer is that the frequency of transgene rearrangements is high. This results in higher transgene copy number and high frequencies of gene silencing.

**Types**

The direct DNA transfer can be broadly divided into three categories.

1. Physical gene transfer methods electro-poration, Micro projectile, microinjection, liposome fusion, silicon carbide fibers.
2. Chemical gene transfer methods - Polyethylene glycol (PEG) mediated Diethyl amino ethyl (DEAE) dextran-mediated, calcium phosphate precipitation.
3. DNA imbibition by cells / tissues / organs.

**Q7. Write about the Gene Transfer-mediated by Agrobacterium, its advantages and limitations.**

*Ans :*

Agrobacterium Mediated Gene Transfer Method: Agrobacterium tumefactions are a soil borne, Gram-negative bacterium. It is considered as one of the efficient vectors in gene transfer techniques. The Ti Plasmid of A. tumefactions has been widely used as a vector in plant transformation. These bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection.

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**Limitations**

1. There is a limitation of host plants for Agrobacterium, since many crop plants (monocotyledons e.g., cereals) are not infected by it. In recent years, virulent strains of Agrobacterium that can infect a wide range of plants have been developed.

2. The cells that regenerate more efficiently are often difficult to transform, e.g., embryonic cells lie in deep layers which are not easy targets for Agrobacterium.

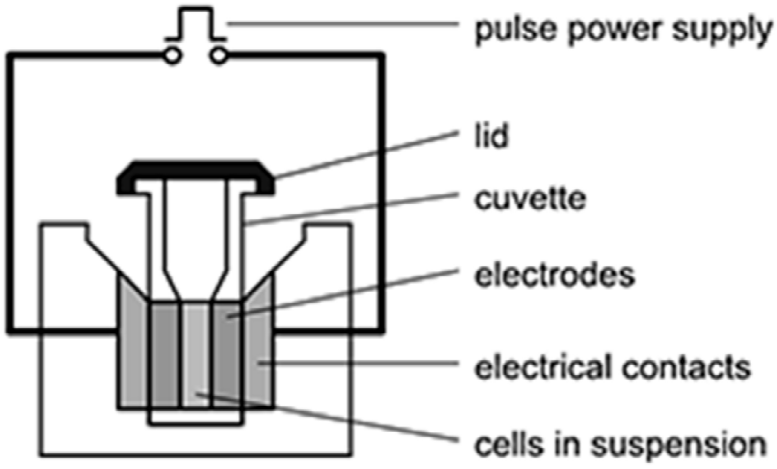
### 4.2.1 Direct gene transfer by electroporation, Microinjection, Microprojectile bombardment

**Q8. Write about the Micro projectile method of gene transfer.**

*Ans :*

**Electroporation (also known as electropermealization)**

Electroporation is a convenient, fast, simple and efficient method for DNA uptake by the cells. In this process, the cells are placed in a solution containing DNA. Electrical impulse of high voltage (1.5 KV) process for short duration and low voltage (350V) for long duration is applied to the protoplasts of cell induce the formation of transient hydrophilic pores in the lipid bilayer of cell membrane, for the uptake of exogenous DNA. The exogenous DNA migrates through the pores into the cytoplasm and in nucleus, followed by its integration into the host genome.




**Micro projectile Method (Particle Bombardment (Biolistics))**

Micro projectile is the most effective method for gene transfer and creation of transgenic plants. This method is versatile due to the fact that it can be successfully. Used for the DNA transfer in mammalian cells and microorganisms.

The micro projectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Biolistics is a combination of biological and ballistic. There are other names for this technique-particle gun, gene gun, bio blaster. A diagrammatic representation of micro projectile bombardment system for the transfer of genes in plants is depicted in Figure. A and briefly described below.

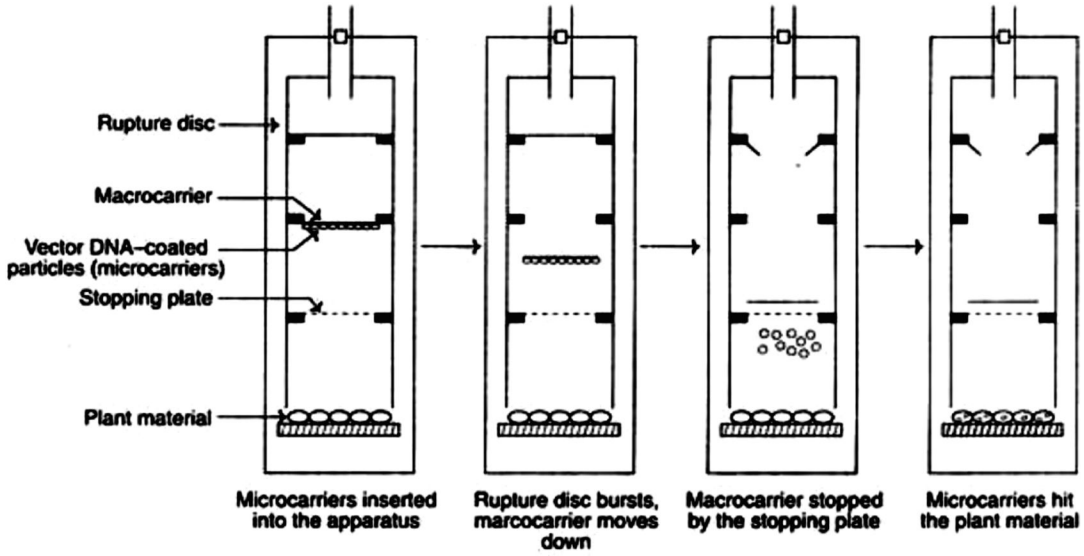



**Fig.: A diagrammatic representation of particle bombardment (biolistics) or Microprogectile system for gene transfer in plants**

Micro carriers (micro projectiles), the tungsten or gold particles coated with DNA, are carried by macro carriers (macro projectiles). These macro-carriers are inserted into the apparatus and pushed downward by rupturing the disc.

The stopping plate does not permit the movement of macro carrier while the micro carriers (with DNA) are propelled at a high speed into the plant material. Here the DNA segments are released which enter the plant cells and integrate with the genome.

### Plant material used in bombardment

Two types of plant tissue are commonly used for particle bombardment.

1. Primary explants which can be subjected to bombardment that is subsequently induced to become embryo genic and regenerate.
2. Proliferating embryonic tissues that can be bombarded in cultures and then allowed to proliferate and regenerate.

In order to protect plant tissues from being damaged by bombardment, cultures are maintained on high osmoticum media or subjected to limited plasmolysis.

### The success of bombardment

The particle bombardment technique was first introduced in 1987. It has been successfully used for the transformation of many cereals, e.g. rice, wheat, maize. In fact, the first commercial genetically modified (GM) crops such as maize containing Bt-toxin gene were developed by this approach.

A selected list of the transgenic plants (developed by biolistics) along with the sources of the plant materials used is given in Table A.

Table 'A' selected list of transgenic plants (along with cell sources) developed by microprojectile bombardment)

| | Plant | Cell source(s) |
|---|---|---|
| 1. | Rice | Embryonic callus, immature zygotic embryos |
| 2. | Wheat | Immature zygotic embryos |
| 3. | Sorghum | Immature zygotic embryos |
| 4. | Corn | Embryonic cell suspension, immature zygotic embryos. |
| 5. | Barley | Cell suspension, immature zygotic embryos. |

**Advantages of particle bombardment**

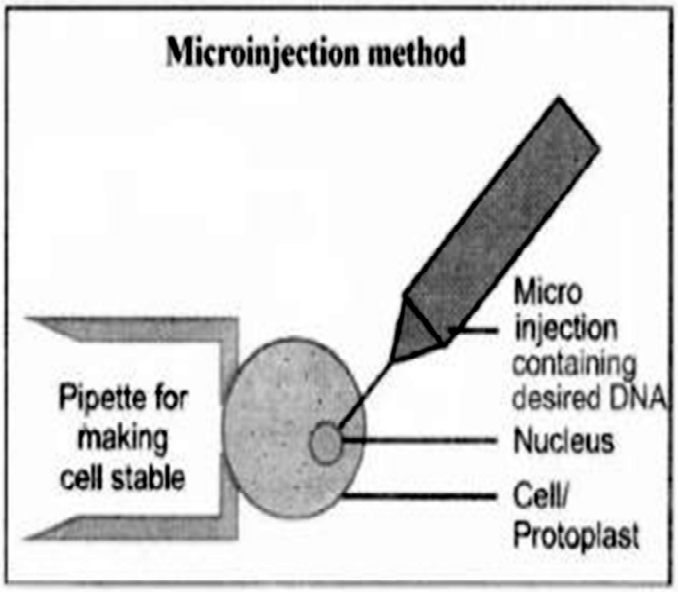
1. Gene transfer can be efficiency done in organized tissues.
2. Different species of plants can be used to develop transgenic plants.

**Limitations of particle bombardment**

1. The major complication is the production of high transgene copy number. This may result in instability of transgene expression due to gene silencing.
2. The target tissue may often get damaged due to lack of control of bombardment velocity. 3. Sometimes, undesirable chimeric plants may be regenerated.

**Microinjection**

In this method glass aucropipettes with fine capillary needles (0.5 pm - 10 um in diameter) are used to transfer DNA precisely into the cells. This method is efficient to transfer DNA into the nuclei of the target cell. The disadvantage in this method is that only one cell can be injected. This the specialized skill and instrumentations.



### 4.2.2. Selectable Marker

**Q9. Write a brief account on selectable marker used in transgenesis**

*Ans*

During the gene transfer experiments, selection system allows the preferential growth of transformed cells. Hence, marker genes are needed for the selection of transgenic cells/tissues. They are placed alongside the gene of interest for the selection of transformed cells/ tissues;

**Selectable Marker Genes**

The selectable marker genes form an important part in the generally present alongside the target gene in a vector.

The marker genes code for proteins that confer resistance when the transformed cells are grown on a medium containing the selection agent (antibiotic, herbicide, antimetabolite). The transformed cells survive whereas the non-transformed cells get killed.

**Types of Selectable Marker Genes**

The different types of selectable marker genes can be grouped into the following three categories (a)

**(a) Antibiotic Resistance Genes**

Some genetically modified plants possess antibiotic resistance genes that make the plant resista antibiotics. Thus, the antibiotic resistance genes serve as selectable markers.

**Example**

**(i) Neomycin Phosphotransferase II Gene (npt II Gene)**

The npr II gene encodes neomycin phosphotransferase which inactivates the antibiotics, karamyci and neomycin by phosphorylation. The transformed cells can be checked by applying the antibiot solution and the transformed cells can be selected.

**(ii) Bialaphos Gene (bar Gene)**

The bar gene encodes phosphinothricin acetyltransferase (PAT). The enzyme converts the herbicide phosphinothricin (PPT) into a nontoxic acetylated form and confers resistance to the cell. Thus, the growth of transformed cells in the presence of herbicide phosphinothricin is allowed.

**(b) Antimetabolite Marker Genes**

Some genetically modified plants possess antimetabolite marker genes that make the plant resistant antimetabolites.

**Example**

Dihydrofolate Reductase gene (dhfr gene) the dhfr gene encodes the enzyme dihydrofolate reductase which catalyzes the conversion of dihydrofolate to tetrahydrofolate and is involved in purines and thymidylate synthesis.

The antimetabolite methotrexate inhibits the enzyme dihydrofolate reductase, thus inhibiting the synthesis of purines and pyrimidines.

Scientists have identified mutant dhfr gene in mouse that codes for the enzyme with a low affinity methotrexate. Fusion of mutant mouse dhfr gene to the CaMV 35S promoter (Cauliflower Mosaic Vi 355) yield a methotrexate resistance marker that can be used for the selection of transformed pl species.

### Herbicide Resistance Markers

Some genetically modified plants possess herbicide resistant genes that code for a modified tam protein and make the plant resistant to herbicides.

### Example

Enolpyruvylshikimate phosphate synthase gene (epsps aro A genes).

Glyphosate, a broad-spectrum herbicide, is widely used by farmers and gardeners for weed commu his sprayed on the leaves of plants to kill both broadleaf plants and grasses. It inhibits the process of photosynthesis and prevents the plants from making proteins that are needed for plant growth.

The herbicide blocks the activity of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, a key enzyme involved in the shikimic acid pathway, which is involved in the synthesis of somatic amino acids namely tryptophan, tyrosine and phenylalanine, that are needed for protein synthesis. Researchers have identified a soil-borne, glyphosate-resistant bacterium, Agrobacterium sp and Penia The glyphosate resistant crops possessing transgenic EPSP enzyme have been developed that allow the herbicide on these crops without injuring the plants.

| Selectable Marker Gene | Abbreviation | Substrate used for Selection |
|---|---|---|
| Antibiotic Resistance | | |
| Neomycin phosphotransferase II | Npt 11 Kanamycin, | Geneticin |
| Neomycin phosphotransferase III | Npt 11I Kanamycin, | Geneticin |
| Hygromycin phosphotransferase | hpt/hyg Hygromycin | |
| Bleomycin ble | Bleomycin | |
| Aminoglycoside adenyltransferase aad A | Streptomycin, | Spectinomycin |
| Aatimetabolite Markers | | |
| Dyhyrofolate reductase | dhfr | Methotrexate |
| Dyhydropteroate synthase | dhps/sul | Sulfonamides |
| Herbicide Resistance | | |
| Phosphinothricin acetyltransferase | bar/pat Glufosinate, | L-phosphinothricin, Bialophos |
| Enolpyruvyl shikimate | phosphate | synthase |
| Aceolactase synthase | Als | Sulfonylurea |
| Glyphosate oxidoreductase | Gox | Glyphosate |
| Bromoxynil nitrilase | Bxn | Bromoxynil |

## 4.3 APPLICATION OF TRANSGENICS IN IMPROVEMENT OF CROP PRODUCTIVITY AND QUALITY TRAITS

**Q10. Write in detail about the production and applications of transgenic plants in crop improvement.**

*Ans :*

### Introduction

The term transgenic plants' refers to plants created in a laboratory using Recombinant DNA technology. A transgenic plant contains a foreign genre which has been artificially inserted. The inserted gene sequence (Known as "Transgene") may come from another unrelated plant or from a completely different species or kingdom.

Transgenic plants are produced by adding one or more foreign gesto plant's genome by a process (described before) called Transformation,

Transgenic plants, Containing Transgene, are identified as a class of Genetically Modified organisms. (GMO). But all transgenic plants have always and inserted DNA originated in a different species But contrary to this, some GMO contain no DNA from other species. From this it is concluded that all GMOs are no transgenic and they are described differently as CISGENIC types. So transmic plants, broadly, come under the category of GMOs, but as a distinct or special group.

### Types

There are three general types of transgenic plants.

1. Those with genes to improve the quality of the product.
2. Those with genes to show resistance to diseases or to the insects which killthem.
3. Those with genes to show resistance to specific herbicides.

### Role of Transgenic Plants

Generally, transgenic plants have been engineered to possess several desirable traits like resistance to pests, herbicides, improved nutritional value, improved product shelf life and tolerance to cold, heat and drought etc. Transgenic technology enables plant Breeders to bring together in one plant useful or desirable genes from wide range of living resources, not just from withinthe crop species or from closed related plants. This technology helps to identify, isolate the genes in one kind organism and transfer such genes to a totally different organism / plant where desirable characters are expressed in the expected fashion. This powerful to enables plant Breeders to generate more useful and productive crop varietiescontaining new combination of genes.

### Production of Transgenic Plants

The production of Transgenic Plants is the result of integrated application a NA technology, Gene Transfer Methods and Tissue Culture Techniques

Transgenic Plants are produced by adding one or more foreign genes to plant are genome by a process called Transformation. The overall process of transformation involves Introduction, Integration and Expression of foreigner) the recipient host plant. Transgenic Plants (or GMPts) are generated mostly by the Microprojectile Bombardment method or by Agrobacterium tumefaciens mediated transformation method.

### 1. Microprojectile Method

It is also called Biolistic or Particle Bombardment or Gene Gun Method. This method is most commonly used in the plants like Com and Rice. In this method, DNA (Foreign Gene) is bound to the tiny particles of Tungsten on Gold, which is subsequently shot into Plant Tissue or single plant cells, under high pressure using gun. The accelerated particles penetrate in to the cell wall and the membranes. The DNA separates from the coated metal and it integrates into the plant Genome inside the nucleus. This method has been used successfully and applied for many crops, especially monocots like Maize or Wheat. This above method is considered as direct, vector less, gene transfer method. Tungsten/Gold coated DNA particles in this method are called Micro-carriers.

**2. Agrobacterium Mediated Method**

This method is also used to produce or generate Transgenic Plants. Agrobacterium tumefaciens, a soil bacterium is used in this method. This bacterium has the ability to infect plant cells with a piece of its DNA. This piece of DNA that infects a plant is integrated into a plant chromosome through a tumor inducing plasmid (Ti Plasmid). This Ti Plasmid replicates independently and produces many copies of its own bacterial DNA.

This Ti plasmid contains regions of transfer DNA (t DNA/T DNA)), where Transgenic Scientist can insert a gene to form r Ti plasmid, this r Ti plasmid is transferred to a plant cell through a process known as 'Floral dip'.

A floral dip involves. Dipping flowering plants into a solution of Agrobacterium carrying the gene of interest, followed by the transgenic seeds. Being collected directly from the plant. The above method includes indirect or vector mediated Gene Transfer steps and considered as a natural acceptable technique for the production of transgenic crops

This method works especially well for the Dicot plants like Potato, Tomato and Tobacco.

## 4.3.1 Pest resistant transgenic crops (Bt-Cotton & Bt-Brinjal)

**Q11. Write an essay on Bt.Cotton and Bt-Brinjal.**

*Ans :*

**Introduction**

Genes establishing insect resistance in transgenic plants has been achieved through the use of insect control protein genes. These genes have beenobtained from micro-organisms

**Examples:**

1) Bt gene from Bacillus thuringiensis.

2) iPt gene from Agrobacterium tumefaciens.

**1) Bt-gene-Mode of Activity**

B. thuringiensis (Bt) is an entomocidal bacterium that produces an insect control protein. Bt genes code for the 'Bt TOXIN. Most of the Bt TOXINS are active against Lepidopteran larvae but some are specific for Dipteran and Colopteran insects. Bt Toxins accumulate as crystal proteins. These are designated as Cry 1, Cry 2 Cry 3 etc. types. These ultimately kill the infected insects by causing disruption in their cell membranes etc.

The Cry 1 Ac protein type is highly toxic to both Tobacco BUD WORM andCotton BOLLWORM larvae and is expressed in Bt cotton varieties.

**Bt-Cotton**

It is genetically modified (Transgenic) pest resistant cotton variety, which produces insecticide to Bollworm.

The gene coding for Bt Toxin has been inserted into cotton, as a Transgene causing it to produce insecticide in its tissues. Bt cotton protein kills larvae ofLepidopteran.

Bt cotton is created by the addition of Bt genes encoding toxin crystals in the Cry group of endotoxin. Cry 1 Ac is a specific toxin of Bt-Cotton, which is highly toxic to Cotton Bollworm and kills the infecting pathogen as explained before in Bt gene-Mode of Activity.

Applications of Bt-Cotton: Bt-cotton has several advantages over non-Bt cotton plants. The important applications / advantages of Bt-cotton are as follows:

1. The effective control of Boll-worms by Bt-cotton leads to the increase in yieldof cotton that is under cultivation.
2. It reduces the cost of cultivation of cotton crops.
3. Cultivation of Bt-cotton varieties indirectly reduces the use of insecticides.
4. Bt. Cotton varieties exhibit high resistance to different environmental factors compared to non-Bt-cotton varieties.

### Bt-Brinjal

Bt- brinjal is a Transgenic crop generated by the similar procedure as that of cotton. Like Bl-cotton, Bi-brinjal carries a specific Bi-gene that provides inbuill ect protection against Fruit Shoot Borer (FSB) by name Lucenodes orbionall ich is the most destructive and unmanageable pest of Brinjal (som Br-Types).

The development of Bt-Brinjal involves the introduction of Cry 1 Argen ressing insecticidal protein to confer resistance against POB

Mode of Action of Bt Brinjal to control FSB B Brinjal transgentes taining Cry 1 Ac gene, express BL-protein in all parts of the plant when po vae feed on Bt-Brinjal plants, they ingest Bt-protein, which to activated by the gut leases generating Toxins. These toxins finally cause the death of FSB.

### Applications of Bt-Brinjal

1. Bt-Brinjal is highly effective in controlling FSB pest
2. Consumption of Bt-Brinjal by human beings is proved safe.
3. Bt-Brinjal crops will produce more undamaged fruits than non Bt Brinjal variety. It leads to higher yields and higher income for farmers. The yield gain in Bt-hybrids was 37 3% over non-Bt-Types.
4. Bt-Brinjals require fewer insecticidal sprays. On account of this Farmers and Farmer workers, exposure to insecticides will be minimized.
5. The utilization of B1-Brinjal hybrids would benefit consumers also interms of reduction in price of Brinjal to 3 to 15%.
6. Cost of production of Bt-Brinjal is also reduced due to the reduction of the use of insecticides and labour.
7. Bt-Brinjal is one of the important components of IPM (Integrated Pest Management).

### Application of Transgenic Plants in Crop Improvement

1. Transgenic technology may ultimately replace the long, difficult traditional plant Breeding methods in future to produce new and desirable plants which help in Crop Improvement.
2. Transgenic plants have been used for bioremediation of contaminated soils. Transgenic plants, containing genes for bacterial enzymes, are used to remove pollutants like Mercury, Selenium, etc from the soils. Such soils are utilized an additional cultivable lands.
3. Several transgenic plants have been developed with desirable characters like
   (a) Longer shelf life eg, Flavr Savr Tomato.
   (b) Disease Resistance.
   (c) Herbicide Resistance eg. RR Soybean.
   (d) Pest Resistance eg. (GMO-SOY, GMO-Corn).
   (e) Stress Resistance eg. Tobacco (resistant to chilling)
   (f) High nutritional value (eg, Golden Rice)
   (g) Male sterility eg. Brassica napus.
4. Transgenic plants or crops are now being grown or cultivated through world, As of 2006 there were around 250 million acres of G.E. Crops being grown in 22 Countries. The USA has adopted this technology and cultivated the transgenic crops like soybeans, cotton, com etc. which showed resistance to herbicides and insects.
5. The introduction of Flavr Savr. Tomato (a crop), which is strictly an in door variety, helped the USA scientists to grow certain GMO crops such GMO-Soy and GMO-Corn in outdoors on large scale. 6. Several Transgenic Bacteria are used to produce insulin (for diabetes), clotting factors (for haemophilia) humilin hormone (for Dwar fism), pesticides (Toxic to pests).

7. A group of transgenic plants are now being designed to produce speciality chemicals and pharmaceuticals. Transgenic plants are used by Biotechnologists as Factories or Bioreactors. The levels of the produc produced are increased by using G.E techniques. This area is generally referred to as "MOLECULAR FARMING".

   **Example 1:** A transgenic tobacco, that promotes increased levels of Mannitol, is developed by transferring a gene for MDHase from E. coli to a normal tobacco plant.

   **Example 2:** A transgenic potato, which produces a, ß cyclodextrins in its tuben, is developed by the transfer of a bacterial gene (from klebsiella) for CGTase to a normal potato plant.

8. Transgenic plants with male sterility have been produced in certain plants like Brassica napus.

   A gene constructs having another promoter (from TA 29 gene of Tobacm and a barnase gene (from Bacillus amyloliquefaciens) are used for production of male sterile strains in B. naupus. These strains help to eliminate the normal manual emasculation usually carried on by plant breeders.

9. Transgenic plants, suitable for food processing, have been produced.

   **Example :** Bruise resistant tomatoes.

### 4.3.2 herbicide resistant plants (Roundup Ready soybean);crops with quality traits (Flavr Savr tomato, Golden rice).

**Q12. Tomatoes exhibiting delayed ripening.**

*Ans :*

The transgenes, in the above transgenic tomatoes, activate the enzymes like Poly Galacto Uronase (PGU) and Acc de aminase (ACC). PGU attacts pectin in the cellwalls of ripening fruit and there by softens the SKIN. ACC deaminase prevents ethylene production (by degrading the precursors of ethylene) and leads to delayed ripening.

**Risks and Concerns**

The transgenic technology with its achievements in Agriculture, Medicene, industry, has generated many doubts or questions about the possible negative consequences of using G.E crops, G. E foods etc. People with concerns are raising many objections. These objections are mainly concerned about :

1. Damage to Human health.
2. Damage to natural Environment or Ecological systems.
3. Disruption of traditional practices of Farming.

   A few of the specific concerns relating to transgenic plants, foods, crops etc, can be listed as follows

1. Getting Allergy by the use of G.E. foods.
2. Loss of the ability of Antibiotic drugs in due course of time.
3. General increase in the level of antibiotic resistance in the environment. It may be due to crop to soil Microorganism Gene flow.
4. Crop to crop Gene flow favoring transfer of unwanted genes to natural crops.
5. Crop to weed Gene flow producing herbicide resistant weeds. 6. Killing of Monarch Butterfly by Bt corn pollen.

All the above concerns, visualized by MAN, may not come true because several field trials proved that transgenic crops/foods etc are as safe as that of conventional types

**Q13. What are Herbicide resistant plants?**

*Ans :*

Herbicide resistance is the predominant trait of cultivated GM crops and will remain so in the near future. GM crops resistant to the broad-spectrum herbicides glyphosate and glufosinate have first been cultivated commercially in the 1990s, and GM crops with resistance to other herbicides are under development, or already on the market, with various HR traits increasingly combined in one crop. Another, more recent strategy is the development of plants that are resistant to high concentrations of glyphosate without exhibiting a yield drag.

Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway for biosynthesis of aromatic amino acids and phenolics in plants and microorganisms. This enzyme is not present in human or animal cells. Glufosinate ammonium is an equimolar, racemic mixture of the D- and L-isomers of phosphinothricin (PPT). The L-isomer inhibits plant glutamine synthetase, leading to the accumulation of lethal levels of ammonia.

To confer resistance to glyphosate, most glyphosate-resistant crops express a glyphosate-insensitive EPSPS derived from Agrobacterium spp., some also the glyphosate-degrading enzyme glyphosate oxidoreductase (GOX) and/or the enzyme glyphosate acetyltransferase (GAT) that modifies glyphosate. In addition, various crops have also been transformed with one of the two bacterial genes pat or bar from Streptomyces spp. conferring resistance to glufosinate-based herbicides. These genes encode the enzyme phosphinothricin acetyl transferase (PAT) which detoxifies L-PPT. Other transgenes contained in HR crops confer resistance to ALS inhibitors.

While many transgenic HR crop species have been tested in the field, only four are widely grown commercially since the late 1990s: soybean, maize, cotton, and canola. In 2013, of the 175.2 million ha global GM crop area, about 57% (99.4 million ha) were planted with HR varieties and another 27% (47 million ha) with stacked HR/IR crops. Hence, 84% of the GM crops carried HR genes (146.4 million ha). HR soybean is the dominant GM crop and grown mainly in North and South America, making up about 80% of the global soybean area and 46% of the total GM crop area. In GM maize and GM cotton, HR traits are often combined with IR genes. In the US, HR crops such as alfalfa, sugar beet, creeping bentgrass, and rice, are already deregulated and on the market or pending for deregulation.

## Yields of HR crops

Contrary to widespread assumptions, HR crops do not provide consistently better yields than conventional crops. Increased yield is not the main reason for farmers to adopt HR crops. If there are yield differences between HR and conventional crops, they may be due to various factors, such as scale and region of growing, site and size of farms, soil, climate, tillage system, weed abundance, genetic background/varieties, crop management, weed control practice, farmer skills, and the education of the farm operators. Reviewing data about the agronomic performance of GM crops, Areal et al. concluded that although GM crops, in general, perform better than conventional counterparts in agronomic and economic (gross margin) terms, results on the yield performance of HR crops vary. A consistent yield advantage for HR crops over conventional systems could not be demonstrated.

The actual yield reduction in RoundupReady soybean observed in some studies might be due to several causes:

(i) The present resistance gene in the first generation of RoundupReady line (40-3-2) and

(ii) Reduced nodular nitrogen fixation upon glyphosate application and/or

(iii) A weaker defence response.

Application of glyphosate seemed to affect nodule number and mass which have been correlated with nitrogen fixation and cause the symptom of “yellow flashing” which leads to a decrease in grain yield. The second generation RR2Y soybean (MON 89788) was introduced to provide better yields, but when tested in the greenhouse, different cultivars of RR2Y performed less well than RR 40-3-2.

## Eco-toxicological attributes of complementary herbicides

Impacts of HR crops on biodiversity are possible through the altered herbicide management option, that is, application of a broad-spectrum herbicide during crop growth and its impacts on

weed abundance and diversity. These impacts, also called indirect effects, are dealt with later in this text. Direct impacts relate to the toxicity of the herbicide, of residues, and breakdown products. First, an update of eco-toxicological attributes and direct effects of relevant complementary herbicides of HR crops is given.

**Q14. Write briefly on herbicide resistant plants (Flavr Savr Roundup Ready soybean & Golden Rice).**

*Ans :*

Weeds are undesirable plants that grow in places where they are unwanted. They cause many problems in different ways,

i) They compete with crop plants for water, nutrients, sunlight and space.

ii) They cause yield reduction of crop plants.

iii) Some weeds are injurious to livestock.

iv) They serve as host/ shelier Row pests.

v) They may increase the risk of fire hazard.

vi) They may interfere with the harvesting procedure of the crops

Farmers use chemical herbicides to control weeds. The herbicides are generally sprayed onto the foliage, applied to soil and applied to the aquatic system. They act by inhibiting cell division, impede the photosynthetic process or hamper the synthesis of amino acids The chemical herbicides are generally preferred because they are less expensive and very effective at killing the weeds.

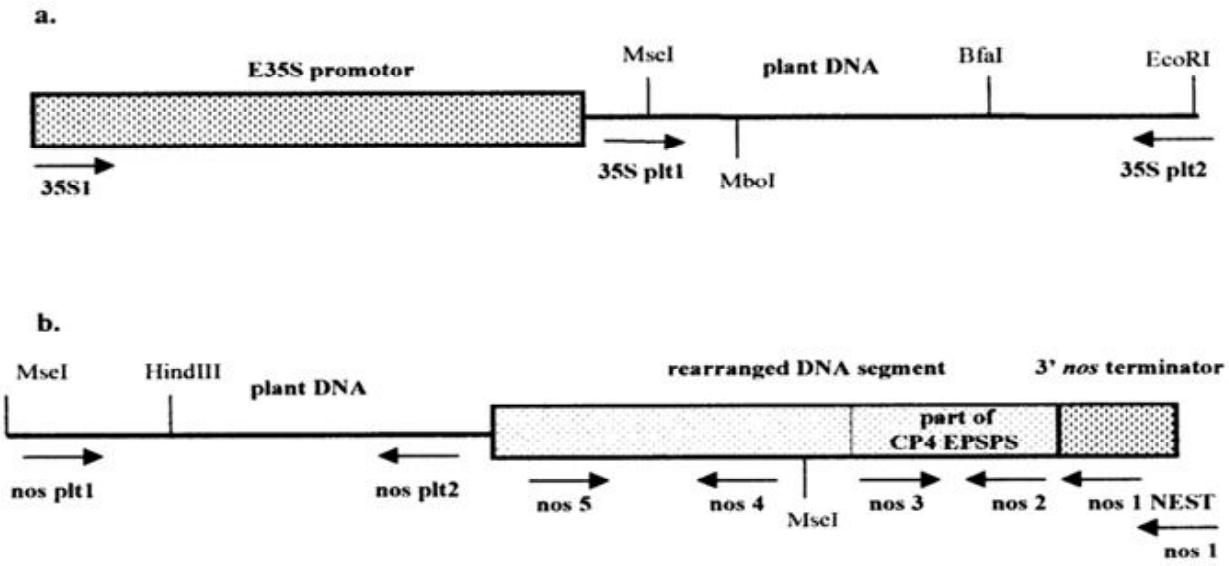
Glycophosate is a broad spectrum herbicide widely used to control weeds. It is the main ingredient in the herbicide Roundt/p It works by preventing the plants from synthesizing proteins needed for plant growth be able to a specific enzyme pathwas, namely the shikimic acid pathway. An effective herbicide should be able to kill the target weeds with no harm to the crop plants. Development of transgenic plants that are resistant to herbicides can solve the problems arising from the use of chemical herbicides, Crops such as soybean and corn have been genetically modified to be resistant to glyphosate and they were named “RoundUp Ready”.

The researchers at Monsanto Company (an American agrochemical and agricultural biotechnology corporation) identified the gene for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from a sed bacterium, that had a slightly altered amino acid sequence from that found in plants. Hence, the bacterium was found to be insensitive to treatment with glyphosate. The bacterial EPSPS gene was inserted into plants by the particle bombardment method to produce herbicide resistant plant.

The shikimic acid pathway is an important metabolic pathway for the synthesis of essential aromatic amino acids, namely tryptophan, phenylalanine and tyrosine, that are essential for plant growth.

In a normal plant, the EPSPS gene is transcribed to mRNA, which is then translated to protein in the cytoplasm. The EPSPS protein is transported to chloroplast where the shikimate pathway occurs.

A chloroplast transit peptide was attached to the 5´ end of the bacterial EPSRS gene to target the protein into the chloroplasts




Now the bacterial EPSPS protein can enter the chloroplast and catalyze the biosynthesis of aromatic aminoacids when glyphosate is sprayed.

Roundup ready soyabeans have been accepted commercially across the globe the most popular transgenic plant. Now glyphosate resistance has been introduced into a variety of crops.

**Q15. How is golden rice produced?**

*Ans.*

Rice (Oryza sativa) is commonly consumed parts of African and Asian countries. The paddy rice is the individual rice kernel containing the following parts.

**(i) Hull**

It is the fibrous, indigestible shell of the rice kernel.

**(ii) Bran**

It is the outer layers of the edible rice kernel containing the pericarp, aleurone and subaleurone layers. It is brown in colour, which gives the characteristic colour to brown rice. It is rich in edible fats, vitamins, minerals, antioxidants and protein.

**(iii) Endosperm**

It is the white interior part of the rice kernel and the most common edible portion of the grain. It contains starch, protein, vitamins and minerals. The outermost cell layer of endosperm is the aleurone.

**(iv) Germ**

The rice germ (embryo) is the reproductive part that can sprout into a new plant upon germination. It has the richest nutrient content including oils, vitamins, minerals, antioxidants and protein.

During the paddy milling operations, the hull, bran and germ are removed, and what remains is the bright y starchy endosperm which is widely consumed as the Rice": T white rice. Moreover, in hot and humid tropical and sufficier subtropical regions, the fat-rich aleurone layer needs to be removed

during milling, to avoid rancidity of reduce t the grain upon storage. It may be observed that several significa essential nutrients, including the carotenoids that exhibit provitamin A activity needed for maintenance G of health, are removed during the milling processes.

The developing countries encounter dietary vitamin deficiencies as white rice is a staple food. Vitamin A deficiency can lead to blindness in children, and increase the susceptibility to other illnesses such as measles, HIV/AIDS and malaria.

An effective approach to solving this problem body. was to provide provitamin A in the form of beta carotene in rice, which led to the development of modest a Golden Rice in 1999.

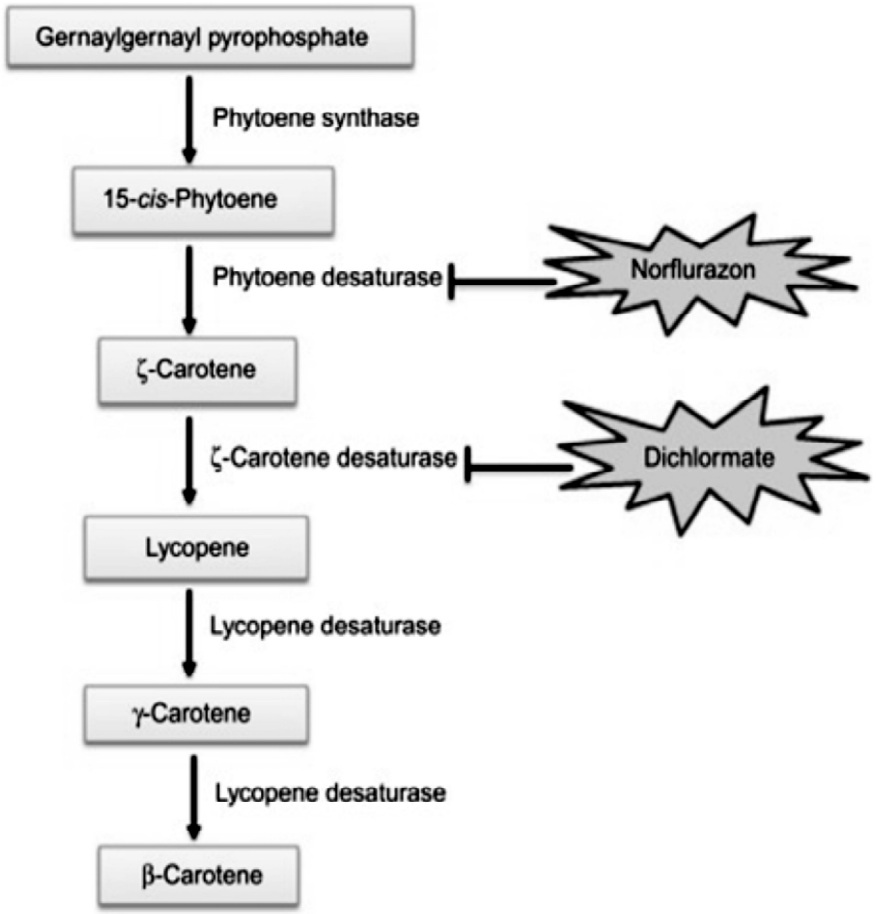
Golden rice was produced through genetic engineering by Ingo Potrykus, Proffesor Peter Beyer of the centre for Applied Biosciences University of Freiburg, Germany.

The researchers created transgenic rice plants via particle bombardment of two â-carotene biosynthesis genes, namely

(i) The gene for phytoene desaturase originated from the soil bacterium, Erwinia urdovora.

(ii) The gene for phytoene synthase came form daffodit (Narcissus pseudonar cissus).

The enzymes produced from inserted genes catalyzed the biochemical reactions in the synthesis of – β carotene



The transgenic rice plants produced grains of bright yellow colour, which led to the name “Golden Rice”. The carotene levels in the golden rice were sufficient to give yellow colour.

The consumption of Golden Rice could reduce the incidence of vitamin A deficiency diseases significantly. However, it could not provide optimal nutritional benefit.

Golden Rice 2 was developed by scientists at Syngentia as an improved version of transgenic rice that had higher levels of total carotenoids and â-carotene.

It had the phytoene synthase gene inserted from corn rather than daffodil along with phytoene desaturase from the original golden rice. The “Golden Rice 2” contained 23 times more provitamin A in the body.

Observations indicate that consumption of modest amounts of Golden Rice 2 provided enough beta-carotene to alleviate vitamin A deficiency.

# Short Question and Answers

**1. Write about notes on Genomic libraries.**

*Ans :*

A genomic library is a collection of the total genomic DNA clonal fragments from a single organism. The DNA is stored in a population of identical vectors, each containing a different insert of DNA. Generally, libraries made from organisms with larger genomes, require vectors having larger inserts. Genomic libraries are commonly used for sequencing applications. They have played an important role in the whole Genome sequencing of several organisms, including Human Genome. The first DNA -based Genome, fully sequenced was achieved by two times Noble Prize Winner, Frederick Sanger in 1977, created a library of the bacteriophage, Phi x 174 for use in DNA sequencing.

**2. Write 5 points about genomic libraries.**

*Ans :*

1. Isolation of genomic DNA from the organism. It includes extraction and purification of DNA.
2. Digestion of the DNA with a restriction enzyme. In this, DNA is partially cleaved into thousands of fragments (of 5-100 kb) by restriction endonuclease to get inserts of desired size range, compatible with the cloning vector (Plasmid, Phage Lambda, Cosmid, BAC, YAC etc.) used for library construction.
3. Insertion of the fragments of DNA into the cloning vector, which is also cut with the same restriction enzyme used for the cleavage of the isolated DNA in the first step.
4. Ligation of the DNA fragment with the cloning vector is done in this step by using the enzyme DNA ligase to seal the DNA fragment into the vector. This creates a recombinant DNA molecule or Hybrid DNA molecule. Generally a large pool our DNA molecules are created at experimental level.
5. Transformation is the main event in this step. The entry of r DNA molecules into a host-coll is described as Transformation. Host organism is commonly a population Bacterium (E. Coli) or Yeast Cells, to produce a library with each cell containing one vector molecule. Then transformed cells are subjected to cloning. Each cloning vector contains a different fragment of the genome. By this, all DNA in the genome is represented among the clones in the library. Both transformed host cell and cloning vector multiply producing a colony with host cells in millions, containing numerous copies of the cloning vectors or clones.

**3. Write different Types of DNA Probes**

*Ans :*

The main types of DNA probes include

**(i) c DNA Probe**

CDNA probe is produced from specific mRNA that codes for a specific protein mRNA template, which is catalyzed by the enzymes reverse transcriptase and DNA polymerase cDNA. This cDNA hybridizes with specific gene sequence

**(ii) Genomic DNA Probe**

Genomic DNA probes are single stranded DNA molecules used for detection of complementary acid sequences of a gene of interest

**(iii) Oligonucleotide Probe**

Oligonucleotide probes are usually 15 to 45 bases of single stranded nucleic acids that are chemically synthesized as a specified base sequence. The probe is allowed to base pair with the nucleic acids, i.e., hybridize. The probes are labeled with radioactive or fluorescent molecule for easy detection.

**4. What is Polymerase Chain Reaction.**

*Ans :*

The amplification of DNA fragments in vitro is described as the Polymerase Chain Reaction (PCR). The idea for PCR is credited to Kary Mullis and he was awarded the Noble prize in Chemistry in 1993 for his contribution to the development of PCR.

PCR helps in the selective "amplification of a chosen region of a DNA molecule.

Amplification of selected region from a complex of DNA mixture is carried out in vitro by the DNA polymerase-1 (Obtained from Thermus aquaticus, a bacterium that lives in hot springs). This DNA polymerase is called Taq Polymerase.

PCR can use the smallest sample of the DNA to be cloned and amplify it, to millions of copies, in just a few hours.

**5. Write the Applications of PCR**

*Ans :*

PCR has been playing a vital role in various scientific fields like Molecular Biology, Medicine, Pathology, Forensic Science and others:

PCR is now widely in use as a Practical, Biochemical tool by extending its role in the following applications.

**PCR helping in the**

1. Diagnosis of Genetic disorders such as phenylketonuria, Hemophilia, Sickle cell Anemia etc.
2. Detection of Nucleic acid sequences of pathogenic organisms in clinical samples.
3. Genetic analysis of Forensic samples is done by the technique of Genetic Finger Printing. This technique is used by Forensic Scientists. And this, Genetic Finger Printing can also be achieved by PCR.
4. Forensic Scientists now a days employing PCR as a tool, in Genetic Finger Printing (GFP). GFP Technology can identify any one person from millions of others in case of CRIME Scene; rule out suspects by testing very small amounts of Sample Specimens (Stains of Blood, hair etc.)
5. Analysis of Homologous Genes in the evolutionary Biology (Phylogeny).
6. Molecular Biology activities like
   (a) Generation of Probes or cDNAs.
   (b) Generation of large cDNA libraries from small amounts of mRNA.
7. Detection of Genetic Mutations.
8. Monitoring of the Gene, thereby helping Gene Therapy.
9. Detection of HIV, TB, Hepatitis, Malaria etc., by clinical analysis of Specimens / Samples. 10. Mapping of all the Genes in Human beings through HGP (Human Genome Project).
10. Detection of antimicrobial resistance.

**6. What are the Advantages of gene transfer.**

*Ans :*

1. It is a natural method of gene transfer.
2. Agrobacterium can conveniently infect any explants (cells / tissues / organs).
3. Even large fragments of DNA can be efficiently transferred.
4. Stability of transferred DNA is reasonably good. 5. Transformed plants can be regenerated effectively.

**7. What short notes on Electroporation.**

*Ans :*

Electroporation is a convenient, fast, simple and efficient method for DNA uptake by the cells. In this process, the cells are placed in a solution containing DNA. Electrical impulse of high voltage (1.5 KV) process for short duration and low voltage (350V) for long duration is applied to the protoplasts of cell induce the formation of transient hydrophilic pores in the lipid bilayer of cell membrane, for the uptake of exogenous DNA. The exogenous DNA migrates through the pores into the cytoplasm and in nucleus, followed by its integration into the host genome.

**8. What are the Advantages of particle bombardment**

*Ans :*

1. Gene transfer can be efficiency done in organized tissues.
2. Different species of plants can be used to develop transgenic plants.

**9. Bt-Cotton**

*Ans :*

It is genetically modified (Transgenic) pest resistant cotton variety, which produces insecticide to Bollworm.

The gene coding for Bt Toxin has been inserted into cotton, as a Transgene causing it to produce insecticide in its tissues. Bt cotton protein kills larvae ofLepidopteran.

Bt cotton is created by the addition of Bt genes encoding toxin crystals in the Cry group of endotoxin. Cry 1 Ac is a specific toxin of Bt-Cotton, which is highly toxic to Cotton Bollworm and kills the infecting pathogen as explained before in Bt gene-Mode of Activity.

Applications of Bt-Cotton: Bt-cotton has several advantages over non-Bt cotton plants. The important applications / advantages of Bt-cotton are as follows:

1. The effective control of Boll-worms by Bt-cotton leads to the increase in yieldof cotton that is under cultivation.
2. It reduces the cost of cultivation of cotton crops.
3. Cultivation of Bt-cotton varieties indirectly reduces the use of insecticides.
4. Bt. Cotton varieties exhibit high resistance to different environmental factors compared to non-Bt-cotton varieties.

**10. Applications of Bt-Brinjal.**

*Ans :*

1. Bt-Brinjal is highly effective in controlling FSB pest
2. Consumption of Bt-Brinjal by human beings is proved safe.
3. Bt-Brinjal crops will produce more undamaged fruits than non Bt Brinjal variety. It leads to higher yields and higher income for farmers. The yield gain in Bt-hybrids was 37 3% over non-Bt-Types.
4. Bt-Brinjals require fewer insecticidal sprays. On account of this Farmers and Farmer workers, exposure to insecticides will be minimized.
5. The utilization of B1-Brinjal hybrids would benefit consumers also interms of reduction in price of Brinjal to 3 to 15%.
6. Cost of production of Bt-Brinjal is also reduced due to the reduction of the use of insecticides and labour.
7. Bt-Brinjal is one of the important components of IPM (Integrated Pest Management).

# Choose the Correct Answers

1. Frederick Sanger in 1977, created a library of the bacteriophage, no is _____. [ a ]
   (a) Phi x 174 (b) Phi x 185
   (c) Phi x 190 (d) Phi × 195
2. Screening methods are of how many types [ d ]
   (a) 4 (b) 6
   (c) 6 (d) 5
3. What is the optimum temperature in Taq polymerase [ b ]
   (a) 2000 bp (b) 1000 bp
   (c) 3000 bp (d) 4000 bp
4. Gene transfer techniques in plant genetic transformation are broadly divided into how many types. [ a ]
   (a) 2 (b) 4
   (c) 6 (d) 8
5. Direct DNA transfer can be broadly divided into how many categories. [ b ]
   (a) 9 (b) 3
   (c) 6 (d) 7
6. The particle bombardment technique was first introduced in [ c ]
   (a) 1985 (b) 1986
   (c) 1987 (d) 1988
7. How many types of Trangenic plants are there? [ b ]
   (a) 5 (b) 3
   (c) both 4 (d) All
8. The yield gain in Bt-hybrids was [ d ]
   (a) 36.5% (b) 38.4%
   (c) 39.6% (d) 37.3%
9. In transgenic plants give the example of Stress Resistance [ a ]
   (a) Tobacco (b) Soyabean
   (c) Rice (d) Millet
10. Transgenic plants or crops are grown in how many countries [ c ]
   (a) 30 (b) 20
   (c) 22 (d) 50

# Fill in the Blanks

1. Genomic libraries are commonly used for _____ applications.
2. The entry of r DNA molecules into a host cell is described as _____.
3. Colony hybridization is also called _____.
4. Vector Mediated Gene Transfer is also known as_____.
5. The micro projectile bombardment method was initially named as _____.
6. Microinjection injection is used for the transfer of _____.
7. r Ti plasmid is transferred to a plant cell through a process known as _____.
8. The Cry1 Ac protein type is highly toxic to both Tobacco _____ & _____
9. Transgenic plants with male sterility have been produced in certain plants like _____.
10. Getting Allergy by the use of _____ foods.

## ANSWERS

1. Sequenced
2. Transformation
3. Replicating
4. "indirect" gene transfer
5. biolistics
6. cellular organelles
7. 'Floral dip'
8. BUD WORM & Cotton BOLLWORM
9. Brassica napus
10. G.E

# One Mark Answers

**1. What is Polymerase Chain Reaction?**

*Ans :*

The amplification of DNA fragments in vitro is described as Polymerase Chain Reaction

**2. What is gene transfer?**

*Ans :*

Gene transfer is defined simply as a technique to efficiently introduce foreign genes into the Genome of targets.

**3. What are the three main Physical Gene Transfer methods, name them?**

*Ans :*

i) Electroporation
ii) Microprojectile Bombardment
iii) Microinjection

**4. What is Agrobacterium Mediated Gene Transfer Method/ tumefaciens?**

*Ans :*

Agrobacterium tumefaciens is a soil borne, Gram-Negative bacterium.

**5. What is Transgenic Plants?**

*Ans :*

The term "Transgenic Plants" refers to plants created in a laboratory using Recombinant DNA technology.

**6. What is transformation?**

*Ans :*

Transgenic plants are produced by adding one or more foreign genes to a plants genome by a process is called Transformation.

**7. GMO means.**

*Ans :*

Genetically modified Organisms

**8. What is Microprojectile Method?**

*Ans :*

It is also called as Biolistic or Particle Bombardment or Gene Gun Method.

**9. What is Bt-gene-Mode of Activity**

*Ans :*

It is an entomocidal bacterium that produces an insect control protein.

**10. Give examples of Transgenic plants which is suitable for food processing**

*Ans :*

i) Bruise resistant tomatoes
ii) Tomatoes exhibiting delayed ripening

# FACULTY OF SCIENCE

## B.Sc. VI - Semester(CBCS) Examination

## Subject : Botany

# TISSUE CULTURE AND BIOTECHNOLOGY

## MODEL PAPER - I

Time : 3 Hours] [Max. Marks : 80

**Part - A (8 × 4 = 32 Marks)**

**ANSWERS**

**(I) Write short answer of the following**

1. Write short notes on the sterilization procedure. **(Unit-I, SQA - 1)**
2. Write short note on Shoot Tip Culture **(Unit-I, SQA - 4)**
3. Write short notes Synthetic seeds. **(Unit-II, SQA - 2)**
4. What is meant by Haploidy and Triploidy? **(Unit-II, SQA - 6)**
5. Write Short notes on Nitrogen-fixing organisms **(Unit-III, SQA - 2)**
6. Fermentation Biotechnology **(Unit-III, SQA - 5 )**
7. Write about notes on Genomic libraries **(Unit-IV, SQA -1)**
8. Write the Applications of PCR **(Unit-IV, SQA - 5)**
9. Importance of Root culture. **(Unit-I, SQA - 3)**
10. Define Somaclonal variants and synthetic seeds **(Unit-II, SQA - 1)**
11. Define Biotechnology. **(Unit-III, SQA - 1)**
12. Write different Types of DNA Probes **(Unit-IV, SQA -3)**

**Part - B (4 × 12 = 48 Marks)**

**(II) Essay Questions**

13. (a) Write an essay on Plant Tissue Culture? **(Unit-I, Q.No. 1)**

OR

(b) Write in detail about the sterilization procedures employed in Tissue cultures. **(Unit-I, Q.No.2)**

14. (a) Write essay on Somaclonal variants and synthetic seeds **(Unit-II, Q.No. 2)**

OR

(b) Write an essay on hairy roots and their production of Secondary metabolities. **(Unit-II, Q.No.3)**

15. (a) Write an essay on biotechnology and its applications in various fields. **(Unit-III, Q.No.1)**

OR

(b) Write an account on Introduction history. **(Unit-III, Q.No. 2)**

16. (a) Write an essay on Genomic libraries. **(Unit-IV, Q.No.1)**

OR

(b) Describe the method of construction of genomic and DNA libraries. **(Unit-IV, Q.No. 2)**

# FACULTY OF SCIENCE

## B.Sc. VI - Semester(CBCS) Examination

## Subject : Botany

# TISSUE CULTURE AND BIOTECHNOLOGY

## MODEL PAPER - II

Time : 3 Hours] [Max. Marks : 80

**Part - A (8 × 4 = 32 Marks)**

**ANSWERS**

**(I) Write short answer of the following**

1. Ovuleculture. **(Unit-I, SQA - 5)**
2. Embryo culture. **(Unit-I, SQA - 6)**
3. Applications of Hairy Root Culture. **(Unit-II, SQA - 4)**
4. What are the advantages of Hairy Root Culture? **(Unit-II, SQA - 5)**
5. Insulin **(Unit-III, SQA - 4)**
6. Gene Cloning Steps. **(Unit-III, SQA - 6)**
7. What are the Advantages of gene transfer. **(Unit-IV, SQA - 6)**
8. What short notes on Electroporation. **(Unit-IV, SQA -7)**
9. What is embrogenesis? **(Unit-I, SQA - 8)**
10. Micropropagation **(Unit-II, SQA - 3)**
11. Polymerases. **(Unit-III, SQA - 9)**
12. What is Polymerase Chain Reaction. **(Unit-IV, SQA -4)**

**Part - B (4 × 12 = 48 Marks)**

**(II) Essay Questions**

13. (a) Write an essay on Nutrient and hormone requirements **(Unit-I, Q.No.5 )**

OR

(b) What is Micro propagation? Write about its Technical procedure, Applications and disadvantages. **(Unit-I, Q.No.6)**

14. (a) What is Germplasm Conservation? **(Unit-II, Q.No. 5)**

OR

(b) What is Cryopreservation. **(Unit-II, Q.No.6)**

15. (a) What is meant by rDNA? Explain the steps involved in rDNA technology. **(Unit-III, Q.No.4)**

OR

(b) What is Gene Cloning? Write in detail about Gene Cloning. **(Unit-III, Q.No. 5)**

16. (a) What is PCR? Describe in detail about PCR and its applications. **(Unit-IV, Q.No.5)**

OR

(b) What is Gene Transfer? Describe in detail about the Gene Transfer Methods in Plants. **(Unit-IV, Q.No.6)**

# FACULTY OF SCIENCE

## B.Sc. VI - Semester(CBCS) Examination

## Subject : Botany

# TISSUE CULTURE AND BIOTECHNOLOGY

## MODEL PAPER - III

Time : 3 Hours] [Max. Marks : 80

**Part - A (8 × 4 = 32 Marks)**

**ANSWERS**

**(I) Write short answer of the following**

1. Short notes oraganogenesis. **(Unit-I, SQA - 7)**
2. Zygotic Embryogenesis. **(Unit-I, SQA - 9)**
3. What is Germplasm Conservation? **(Unit-II, SQA - 7)**
4. Cytoplasmic Hybrids Or Cybrids **(Unit-II, SQA - 10)**
5. Restriction Endonucleases (REases) **(Unit-III, SQA - 7)**
6. ligases. **(Unit-III, SQA - 8)**
7. What are the Advantages of particle bombardment **(Unit-IV, SQA -8 )**
8. Bt-Cotton **(Unit-IV, SQA - 9)**
9. Characteristics of embryogenesis. **(Unit-I, SQA - 10)**
10. What is Cryopreservation. **(Unit-II, SQA - 8)**
11. Application Of Rdna Technology **(Unit-III, SQA - 10)**
12. Applications of Bt-Brinjal. **(Unit-IV, SQA -10)**

**Part - B (4 × 12 = 48 Marks)**

**(II) Essay Questions**

13. (a) Write an essay on callus culture. **(Unit-I, Q.No.9)**

OR

(b) Write an essay on Organogenesis? **(Unit-I, Q.No.11)**

14. (a) What is meant by Haploidy and Triploidy? **(Unit-II, Q.No. 4)**

OR

(b) Define somatic hybridization? Describe different steps involved in it and mention its uses. **(Unit-II, Q.No.7)**

15. (a) What is PCR Mediated Gene Cloning? Give differences between Gene Cloning and PCR Cloning. **(Unit-III, Q.No.8)**

OR

(b) Describe Bacterial transformation and selection or Recombinant clones. **(Unit-III, Q.No.10)**

16. (a) Write in detail about the production and applications of transgenic plants in crop improvement. **(Unit-IV, Q.No.10)**

OR

(b) Tomatoes exhibiting delayed ripening. **(Unit-IV, Q.No.12)**

# FACULTY OF SCIENCE

## B.Sc. VI - Semester (CBCS) Examination

## May / June - 2019

## Subject : BOTANY

## TISSUE CULTURE AND BIOTECHNOLOGY

Time : 3 Hours] [Max. Marks : 60

**PART - A (5 × 3 = 15 Marks)**

**(Short Answer Type)**

**Note : Answer any Five of the following questions.**

**Answers**

1. Root tip culture **(Unit-I, Q.No. 7)**

2. Single cell culture - Advantages

*Ans :*

1) Callus cultures can be used for long term maintenance of cell lines.

2) It is useful for studying organogenesis and meristematic cultures.

3) Helpful in determining the nutritional requirements of plants, somaclonal variations and genetic transformations.

4) It is a useful source for studying the production and regulation of secondary metabolites in plants by using tracers.

3. Secondary metabolites **(Unit-I, Q.No. 3)**

4. DNA Polymerase **(Unit-III, SQA. 9)**

5. Cosmids

*Ans :*

Cosmids are plasmids that contain lambda phage DNA containing cohesive ends (Cosmid = cos site + plasmid). For the first time it was developed by Collins and Hohn (1978).

Cosmids lack encoding viral proteins. Therefore, neither viral particles are formed nor cell lysis occurs. Cosmids like plasmids contain

(i) Origin of replication,

(ii) A marker gene coding for antibiotic resistance,

(iii) A special cleavage site for the insertion of foreign DNA and

(iv) The small size. They differ from plasmids in having are extra phase DNA, cos site, which is about 12 bases. It helps the whole genome in circularization and ligation.

6. Restriction enzymes **(Unit-III, SQA. 7)**

7. Somaclonal variation **(Unit-II, SQA. 1)**

8. Micropropagation. **(Unit-II,Q.No. 6)**

**PART - B (3 × 15 = 45 Marks)**

**(Essay Answer Type)**

**Note : Answer All questions from the following**

9. (a) Write an essay on callus culture and its advantages. **(Unit-I,Q.No. 10)**

OR

(b) Write in detail about somatic hybrids and cybrids. **(Unit-II,Q.No. 7)**

10. (a) Write in detail about the advantages of tissue culture technique.

*Ans :*

**Advantages**

1. The biomass obtained is uniform at all times as the conditions required for the propagation are reproducible.
2. Those medicinal compounds which are impossible or very difficult to synthesize chemically can be produced using plant tissue culture.
3. The cultured cells can be maintained free from microbes and insects.
4. By adding labelled precursors to the tissue cultures, the particular metabolic pathway involved in the synthesis of secondary metabolites can be interpreted.

OR

(b) Write an essay on applications of biotechnology in different fields. **(Unit-III,Q.No. 3)**

11. (a) Describe methods of gene transfer in plants. **(Unit-IV,Q.No. 6)**

OR

(b) Write in detail about the scope and applications of transgenic plants. **(Unit-IV,Q.No. 11)**

# FACULTY OF SCIENCE

## B.Sc. VI - Semester(CBCS) Examination

## September / October - 2020

## Subject : BOTANY

## TISSUE CULTURE AND BIOTECHNOLOGY

Time : 2 Hours] [Max. Marks : 60

**PART - A (4 × 5 = 20 Marks)**

**Note : Answer any Four questions**

**Answers**

1. Sterilization **(Unit-I, SQA. 1)**
2. Explant **(Unit-I, Q.No. 3)**
3. Somaclonal variation **(Unit-II, SQA. 1)**
4. Synthetic seeds **(Unit-II, SQA. 2)**
5. Plasmids **(Refer Aug.-21, Q.No. 5)**
6. Bacteriophages **(Unit-II, Q.No. 11)**
7. Cybrids **(Unit-II, SQA. 10)**
8. Secondary metabolites. **(Unit-II, Q.No. 3)**

**PART - B (2 × 20 = 40 Marks)**

**Note : Answer any Two questions.**

9. Write an essay callus culture and its advantages. **(Unit-I, Q.No. 10)**
10. Define somatic hybridization. Write its procedure and its applications. **(Unit-II, Q.No. 7)**
11. Write in detail about the various applications of plant tissue culture technique. **(Unit-II, Q.No. 1)**
12. Write an essay on recombinant DNA (r-DNA) technology. **(Unit-III, Q.No. 4)**
13. Write in detail about methods of gene transfer in plants. **(Unit-IV, Q.No. 6)**
14. Write in detail about the production of transgenic plants and applications of transgenic in crop improvement. **(Unit-IV, Q.No. 10)**

# FACULTY OF SCIENCE

## B.Sc. VI - Semester (CBCS) Examination

## July / August - 2021

## Subject : BOTANY

## TISSUE CULTURE AND BIOTECHNOLOGY

Time : 2 Hours] [Max. Marks : 60

**PART - A (4 × 5 = 20 Marks)**

**Note : Answer any Four questions**

**Answers**

1. Explant sterilization. **(Unit-I, SQA. 1)**
2. Somatic hybrids. **(Unit-II, SQA. 9)**
3. Synthetic seeds. **(Unit-II, Q.No. 2)**
4. DNA ligases. **(Unit-III, Q.No. 8)**
5. Plasmid.

*Ans :*

Plasmids are relatively small, circular DNA molecules that can exist independently of host chromosomes. They are found in many bacteria, some yeasts and fungi. They have their replication origins and are autonomously replicating and stably inherited. Plasmids possesses relatively a few genes, generally less than 25 to 30. There may be a single copy of plasmid (single copy plasmid) or more copies of the plasmid in a cell (multicopy plasmids). Plasmids often bear antibiotic resistance genes, which are used to select their bacterial hosts.

6. Microprojectile bombardment. **(Unit-IV, Q.No. 8)**
7. Bt-brinjal. **(Unit-IV, Q.No. 11)**
8. Genomic libraries. **(Unit-IV, SQA. 1)**

**PART - B (2 × 20 = 40 Marks)**

**Note : Answer any Two questions.**

9. What is micro propgation? Add a note on its importance in forestry. **(Unit-I, Q.No. 6)**
10. Add a detailed note on protoplast and cell suspension culture. **(Unit-I, Q.No. 11)**
11. Write an essay on hairy roots and its application in production of secondary metabolites. **(Unit-II, Q.No. 3)**
12. Explain recombinant DNA technology with neat labelled diagram. **(Unit-III, Q.No. 4)**
13. Describe agrobacterium-mediated method of gene transfer in plants. **(Unit-IV, Q.No. 6)**
14. Explain role of transgenic plants in crop improvement. **(Unit-IV, Q.No. 10)**