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Indira Gandhi National
Open University
School of Sciences

BBYET-143
ECONOMIC BOTANY AND BIOTECHNOLOGY

VOLUME 2

BIOTECHNOLOGY

Blocks 3 and 4

Indira Gandhi
National Open University
School of Sciences

BBYET-143
ECONOMIC BOTANY AND PLANT BIOTECHNOLOGY

VOL

2

ECONOMIC BOTANY AND PLANT BIOTECHNOLOGY

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ECONOMY BOTANY AND PLANT BIOTECHNOLOGY

The second volume of the elective course Economic Botany and Plant Biotechnology comprises of two blocks- Third and Fourth block. These blocks are based on the concept of biotechnology and its applications. We have tried to give you an overview of the concept of biotechnology, its origin and its applications in the field of botany (plant biotechnology). The role of biotechnology in improvement of various areas such as medicine, agriculture and many more has also been discussed in detail. In depth knowledge about plant biotechnology, plant tissue culture technique and applications of biotechnology in development of improved plant varieties will make you appreciate the designing of the course and give you to an enthusiasm to learn more.

The Third block belongs to plant biotechnology. It has three units (10 to 12).

In Unit 10, you will be introduced to the concept of biotechnology, its origin and history. You will appreciate the concept of the classical and modern day biotechnology. You will be studying about various categories, techniques and applications of biotechnology. This will be followed by comprehensive information about various methods used in plant biotechnology and its applications.

The Unit 11 introduces you to the technique of plant tissue culture. A brief historical account given in this unit will make you aware about the various developments in the plant tissue culture technique. A detailed information about the plant tissue culture set up and requirements is discussed. In this unit you will also be studying about the concept of totipotency and techniques of tissue culture such as organogenesis, embryogenesis and protoplast fusion.

Unit 12 of this block describes in detail about various applications of plant tissue culture. A lucid account of various applications of tissue culture such as micropropagation, embryo culture, androgenesis, gynogenesis, production of secondary metabolites and germplasm conservation is given.

Fourth block deals with various techniques and applications of biotechnology. It also contains three units (13 to 15).

In Unit 13 deals with a detailed information on molecular techniques used in biotechnology. You will study about some of the well known techniques such as blotting (Northern, Southern and Western), DNA fingerprinting, DNA sequencing, PCR, ELISA and use of molecular markers in this unit.

Unit 14 of this Block will provide a detailed account of recombinant DNA technology. The role of vectors in gene cloning is discussed along with the concept of construction of genomic and

cDNA libraries. The last section of this unit gives you a detailed account related to techniques involved in plant genetic engineering. A crisp account of CRISPER technology has also been included in this unit.

The last Unit of this block i.e. Unit 15 gives an overview of the major applications of plant biotechnology. You will appreciate the detailed information related to role of plant biotechnology in improvement of plants. Use of plant biotechnology in various areas such as agriculture, horticulture, development of herbicide and pest resistant crops has been discussed in detail in this unit. A brief account of some of the genetically modified crops such as golden rice, Bt cotton, Flavr Savr tomato is also included.

Objectives

After going through this course, you will be able to :

- describe the concept of origin of biotechnology, various facets of plant biotechnology, and list out its various applications;
- enlist major techniques and applications of plant tissue culture used in plant biotechnology;
- explain techniques such as micropropagation, haploid production and germplasm conservation;
- appreciate the details about the technique of recombinant DNA technology;
- describe the molecular techniques commonly used in biotechnology;
- enlist major methods of plant genetic engineering; and explain the role of biotechnology in development of crops with high nutritional value, better tolerance capacity and long shelf life.

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BBYET-143
ECONOMIC BOTANY AND PLANT BIOTECHNOLOGY

Block

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Acknowledgements:

- Mr. Manoj Kumar for CRC Preparation.

May, 2022

ISBN

Further information on the Indira Gandhi National Open University courses may be obtained from the official website of IGNOU at www.ignou.ac.in.

Printed and published on behalf of Indira Gandhi National Open University, New Delhi by Director, SOS, IGNOU

BLOCK 3 : PLANT BIOTECHNOLOGY

Block 3 deals with plant biotechnology. Three units (units 10 to 12) of this block introduce you to the concept of biotechnology, plant biotechnology and the techniques involved in it. You will study about origin, development of technique of biotechnology, scope of plant biotechnology and its uses. Biotechnology originated long back when animals were domesticated, plants were cultivated and microbes were used in getting many products. Later on the concept of biotechnology was applied to various areas such as agriculture and medicine. You will be studying about plant biotechnology and main techniques used in it. A brief account about origin of Plant tissue culture and its applications will also be discussed.

Unit 10 of this block introduces you to the concept of Biotechnology, its origin and development with time. A brief account of major contributions of scientists is also given. You will appreciate the concept of the classical, modern day biotechnology, major events in the development of Biotechnology and methods involved used in it. Various methods used in Plant Biotechnology will also be discussed in this Unit.

Unit 11 of this block introduces you to the concept of plant tissue culture. You will appreciate a brief historical account related to the development of the plant tissue culture technique. Detailed information related to various equipments and methods of plant tissue culture is provided to you. The concepts such as totipotency, organogenesis, embryogenesis and protoplast culture is also discussed in detail in this unit.

Unit 12 is devoted exclusively to the applications of Plant Tissue Culture. In this unit, you will be studying in detail about techniques such as micropropagation, production of androgenic and gynogenic haploids, embryo culture and secondary metabolite production. The role of Plant tissue culture in germplasm conservation will also be discussed in the last part of the unit.

Objectives

After studying this block you will be able to :

- describe the concept of origin of biotechnology;
- list out the applications of biotechnology;
- appreciate the role of biotechnology in improvement of plants;
- enlist the major techniques and methods in plant biotechnology;
- describe the importance of plant tissue culture; and
- enumerate the major applications of plant tissue culture.

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UNIT 10

INTRODUCTION TO BIOTECHNOLOGY

Structure

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Crop Improvement

Genetic Transformation

Industrial

Pharmaceutical

10.9 Summary

10.10 Terminal questions

10.11 Answers

10.1 INTRODUCTION

Humans have been using biological processes to improve the quality of life for the last 10,000 years. The biological agents such as microorganisms have been used to get various products. Use of living organisms to develop technologies and products that help improve our lives and the health is referred as **Biotechnology**. The concept of biotechnology began to develop in the 19th century when plant and animal cells as well their constituents were cultured in vitro conditions to get desired products. Since then microorganisms have been used for large-scale production of biochemicals (such as alcohol, antibiotics), food processing and environmental remediation. The major applications of biotechnology include therapeutics, diagnostics, food processing, bioremediation, waste treatment, production of energy and genetically modified crops for improvement of agriculture.

The advent of Recombinant DNA technology has made development of microbes, plants and animals that possess superior and novel capabilities

possible. Genetically Modified Organisms (GMO) have been created by transferring one or more genes from one organism to another. Genetically modified plants have been developed for tolerance to high temperature, salinity stress and resistance to pathogen and pest attack. These approaches have proven to be very useful in increasing the yield of crop plants and reducing post-harvest losses. Development of crop plants with the improved nutritional value and reduced reliance on chemical pesticides (pest-resistant crops) has been made possible via technique of Biotechnology. The present Unit introduces you to the concept of biotechnology and provides you with information about various methods of plant biotechnology and its applications.

Objectives

After studying this unit, you would be able to:

- describe the concept of biotechnology;
- discuss the basics of plant biotechnology;
- enlist various methods in plant biotechnology; and
- enumerate various applications of plant biotechnology.

10.2 GENERAL ACCOUNT

Since ancient times, man has been using living things to improve his life. In the early times (about 8000-4000 B.C.) man has been using plants and animals for various services. As the time passed, various developments took place in the area of biotechnology.

Various stages in the development of Biotechnology

The development of biotechnology can be categorized as

- Ancient biotechnology (8000–4000 BC): Early history of biotechnology relates to domestication of animals and cultivation of plants.
- Classical biotechnology (2000 BC; 1800–1900 AD): This era of biotechnology was based on use of microbes in fermentation, production of food and medicine. In this period Genetics was developed (1900–1953) and DNA research was initiated (1953–1976).
- Modern biotechnology (1977): In this period, genetic information in organisms was manipulated via genetic engineering.

Ancient biotechnology

In the period before the year 1800, events such as domestication of animals to get milk or meat, cultivation of plants such as rice, barley and wheat as major source of food were categorized as biotechnological developments. Breeding of plants and animals has been done to improve them by introducing desirable characteristics.

Classical biotechnology

The production of cheese, yogurt and bread from micro-organisms, alcoholic drinks such as beer and wine via fermentation has been done since ancient times. The role of microorganisms (such as bacteria, yeast or molds) in hydrolysis sugars and hence fermentation was discovered. In 1796, Edward Jenner demonstrated role of vaccination in providing immunity against smallpox, hence is considered the founder of vaccinology. Scientific evidence for fermentation was first described by Louis Pasteur in the late 1800s. He proposed a theory known as germ theory stating microorganisms play a major role in the process of fermentation. Honey, soybean curds has been used as natural antibiotic and found effective in healing wounds since ancient times. Ukrainian farmers used moldy cheese to treat infected wounds. Later it was found that antibiotics present in molds killed bacteria and prevented the spread of infection. Enzymes and proteins have been discovered in 1830.

In 1919, Hungarian engineer **Karl Ereky** coined the term **"**Biotechnology". According to him, biotechnology is the use of living organisms to produce materials or products of importance. In other words, it is the technology that uses biological processes, organisms or systems to produce products that improve human lives.

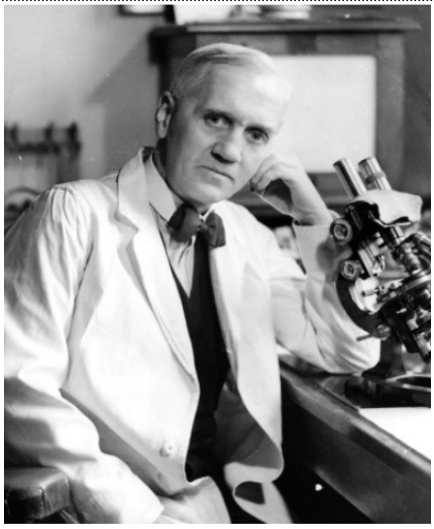
In 1920, **Chaim Weizman** tried to produce useful chemical compounds through biological techniques. He used *Clostridium acetobutylicum* that converted starch to butanol. Later, biotechnology such as fermentation was refined to develop paint solvents for the automobile industry and acetone from starch. These processes were promoted during World War I. During World War II, penicillin was discovered. In 1929, **Alexander Fleming** produced antibiotic penicillin from cultures of *Penicillium notatum*. The penicillin proved useful for the treatment of wounded soldiers during World War II (Fig. 10.1). The focus of biotechnology shifted to pharmaceuticals in 1930s. Also in1930s use of biotechnology shifted to agriculture sector.

Selective breeding of plants and animals has been done in the past. In this procedure organisms with desirable traits were allowed to mate to further enhance these traits in their offspring. It was found that selective breeding could improve yield as well as productivity. Mule is one of the most primitive examples of crossbreeding for the benefit of humans. Man started using mules for transportation, carrying loads and farming. Later on focus of biotechnology moved to pharmaceuticals. The microorganisms were used in preparation of antibiotics.

At the end of the eighteenth century and the commencement of nineteenth century, focus of biotechnology was shifted to crop rotation (including leguminous crops), development of vaccination and animal based studies. The complexity of biotechnology increased owing to the evolution of new technologies and better understanding of various principles of life science.

By the end of the nineteenth century, many microorganisms were discovered, Mendel's work on genetics was accomplished and microbial processes such as fermentation were explored. At the beginning of the twentieth century, industry and agriculture started using biotechnology methods.

(a)



(b)

Fig. 10.1: a) Karl Ereky; b) Alexander Fleming.

Modern biotechnology

Later in 1960s and 70s molecular, cellular technologies began to emerge. In the period between1953 to 1976, DNA research was initiated. Cells and biological molecules (DNA, RNA and proteins) were used to make useful products. The real biotechnology was initiated with the production of Insulin in 1970. The first restriction enzyme was also discovered in 1970. The discovery enabled the researchers to insert foreign genes in bacteria. This formed the basis of recombinant DNA technology and is considered the birth of modern-day biotechnology. The DNA of living organisms was manipulated and the products obtained from genetically modified organisms were used in the manipulation of molecules. In 1977, researchers in Scotland cloned a sheep named Dolly, later on, sheep named Polly was cloned using nuclear transfer technology as it had human gene encoding Factor IX, a protein involved in preventing haemophilia. Companies such as Genentech, Amgen, Biogen, Cetus, and Genex started were established in the mid-to late 1970s. They manufactured genetically engineered substances primarily for medical and environmental uses. In 1998, the importance of cloning Dolly was realized. Molecular biologists became interested in studying stem cells. In 1999, antibody analysis was found as another tool of use. A glance of the historical events in biotechnology has been provided in Table 10.1.

Table 10.1: A summary of historical events in Biotechnology.

Peiod	Time/Year	Event
Pre-1800	6000 BC	Yeast was used to prepare beer
	4000 BC	In Egypt, yeast was used to prepare bread.
	420 BC	Greek philosopher Socrates found that similar characteristics are found between parents and their offspring.
	320 BC	Greek philosopher Aristotle gave the concept that characters get inherited from father.
	1000 AD	Theory of abiogenesis, or spontaneous generation was proposed.

	1630	William Harvey discovered the circulation of blood in the body.
	1660–1675	Marcello Malpighi found blood circulation in capillaries using a microscope and found that the brain is connected to the spinal cord by bundles of fibers which form the nervous system.
	1673	Antonie van Leeuwenhoek identified micro-organisms such as protozoa and bacteria, and suggested that micro-organisms play an active role in fermentation.
	1701	Giacomo Pylarini found that the administration of smallpox prevents its occurrence later in life. The procedure was termed 'vaccination'.
1800–1900	1809	Nicolas Appert invent a technique using heat to sterilize food
	1856	Justus von Liebig, Pasteur (1822–1895) suggested that microbes are responsible for fermentation.
	1859	Charles Darwin (1809–1882) speculated 'natural selection'.
	1863	The method of pasteurization was discovered by Pasteur. Heinrich Anton de Bary found that fungus was responsible for potato blight.
	1865	Mendel suggested 'the laws of heredity'.
	1868	Johannes Friedrich Miescher separated nuclein (a compound made of nucleic acid) from pus cells.
	1870	Walther Flemming discovered mitosis.
	1871	Koch investigated anthrax and explored techniques to identify, culture and stain micro-organisms.
	1880	Louis Pasteur explored weakened (attenuated) strains of micro-organisms that might not be virulent.
	1881	Koch explained techniques for harvesting bacterial colonies on potato slices, gelatin and agar medium.
	1884	Gram described the differential staining technique for cellular peptidoglycan-containing bacteria now known as Gram staining.
1900–1953	1900	Mendel's work was revived by de Vries, von Tschermak and Correns.
	1902	Sutton found that chromosomes (paired) contain certain elements which are transferred from one generation to another.
	1905	Edmund Beecher Wilson and Nettie Stevens demonstrated that a single Y chromosome determines maleness, while two copies of the X chromosome decide femaleness.
	1905–1908	William Bateson and R C Punnett found that several genes alter or modify the action of other genes.

	1906	Paul Erlich also investigated atoxyl compounds and discovered the important features of Salvarsan (the first chemotherapeutic agent).
	1907	Thomas Hunt Morgan revealed that chromosomes have a defined role in heredity. He discovered mutation theory into fruit flies.
	1909	Wilhelm Johannsen used the word 'gene' for the carrier/transporter of heredity. He also coined the terms 'genotype' and 'phenotype'.
	1910	Morgan demonstrated that genes are present on chromosomes.
	1911	Morgan established the separation of certain inherited features that are generally linked to the separation/breaking of chromosomes during the process of cell division.
	1912	William Lawrence Bragg discovered the application of X-rays in the determination of the molecular structure of crystalline substances (Crystallography).
	1918	Herbert M Evans found that human genetic material is made up of 48 chromosomes.
	1926	Hermann Joseph Muller discovered that X-rays are responsible for genetic mutations in fruit flies.
	1928	Frederick Griffiths observed the 'transforming principle' by which a rough type of bacterium is transformed to a smooth type. Alexander Fleming studied an old culture of bacteria infected by fungus *Penicliium notatum* and discovered antibiotic penicillin
	1938	Proteins and DNA were studied by means of X-rays. The term 'molecular biology' was coined.
	1941	George Wells Beadle and Edward L Tatum proposed 'one gene, one enzyme' theory.
	1943	Canadian scientist Oswald Theordore Avery discovered that DNA is the carrier of genes.
	1943-1953	Cortisone, a steroid hormone was considered as the first biotech product.
	1944	Selman Abraham Waksman explored streptomycin, an antibiotic active against TB.
	1945-1950	Animal cell cultures were harvested for the first time in laboratories, giving birth to the field of animal tissue culture.
	1947	Barbara McClintock first demonstrated 'transposable elements' known as 'jumping genes' with the capability to move (or jump) from one site on the genome to another site.
	1950	Erwin Chargaff discovered that the same levels of adenine and thymine are present in DNA, as are the same levels of guanine and cytosine. These associations were later named 'Chargaff's rules'.

	1952	George Otto Gey created a cell line known as HeLa from a human cervical carcinoma.
1953–1976	1953	Watson and Crick's article based on unfolding the double-helix structure of DNA was published in the journal, *Nature*.
	1953–1976	The discovery of the structure of DNA revolutionized molecular biology and genetics.
	1957	Crick and Gamov studied 'central dogma',demonstrating how DNA functions to construct protein.
	1958	Arthur Kornberg created DNA in a test tube for the first time. The mechanical protein sequencer, the Moore–Stein amino acid analyzer, was developed.
	1959	François Jacob and Jacques Lucien Monod documented concept of genetic regulation. They explained the concept of 'repressor' and 'operon'.
	1960	A French researcher discovered messenger RNA (mRNA).
	1961	François Jacob and Jacques Monad gave the concept of Operon.
	1962	Watson and Crick were awarded the Nobel Prize in Physiology or Medicine with Maurice Wilkins.
	1963	Samuel Katz and John F Enders developed the first vaccine for measles.
	1964	Enzyme reverse transcriptase was discovered.
	1966	The genetic code was explored by several researchers. Marshall Warren Nirenberg, J Heinrich Matthaei and S Ochoa reported that a genetic sequence of three nucleotide bases (called codons) decides each of 20 amino acids.
	1967	Arthur Kornberg reported a study using single-stranded natural viral DNA.
	1969	An enzyme is synthesized *in vitro* conditions.
	1970	Virologists Peter H Duesberg and Peter K Vogt identified the first oncogene in a virus. Restriction enzymes were discovered.
	1967-1971	Maurice Hilleman made the first American vaccine for mumps. The first vaccine for rubella was developed.
	1972	Paul Berg, a biochemist, utilized a restriction enzyme to cut DNA into fragments. He employed a ligase enzyme to join two DNA strands concurrently to form a hybrid circular molecule.
	1973	Bruce Nathan Ames, a biochemist developed an investigation to distinguish chemicals that damage DNA. Later, the Ames test became extensively used to identify cancer-causing substances.

		Stanley Cohen and Herbert Boyer used bacterial genes to perform the first successful rDNA experiment. Sir Edwin Mellor Southern developed a blotting technique for DNA called the Southern blot.
	1974	The first vaccine for chicken pox was developed.
	1975	Colony hybridization and Southern blotting were explored for identifying specific DNA sequences. The first monoclonal antibodies were prepared.
	1976	J Michael Bishop and Harold Varmus established the presence of cancer-causing genes called oncogenes on animal chromosomes. The NIH published the first guidelines for rDNA research.
1977– (Modern biotechnology)	1977	With the advent of genetic engineering it was possible to produce human protein in bacteria for the first time. R Austrian and his coworkers developed the first vaccine for pneumonia.
	1978	Herbert W Boyer synthesized synthetic human insulin by introducing the insulin gene into the bacterium *Escherichia coli*. Louise Brown, the first test-tube baby, was born in the UK. The first vaccine for meningococcal meningitis was developed
	1980	Kary Mullis and coworkers established a tool for multiplying DNA sequences *in vitro* using the polymerase chain reaction (PCR).
	1981	Baruch Blumberg and Irving Millman developed the first vaccine for hepatitis B. The first transgenic animal (mice) was produced.
	1982	US Food and Drug Administration (FDA) allowed the first genetically engineered drug in the form of human insulin produced by bacteria. Michael Smith at the University of British Columbia, Vancouver, established a procedure for producing precise amino acid changes anywhere in a protein.
	1983	The first genetically modified (transgenic) plant is formed.
	1984	The DNA fingerprinting technique was discovered. The first genetically engineered vaccine was discovered for hepatitis B.
	1985	The NIH published guidelines for performing experiments in gene therapy on humans. Genetically engineered plants resistant to viruses, insects and bacteria were field-tested for the first time.

	1986	The NIH sanctioned guidelines for executing trials of gene therapy on humans. The automated DNA sequencer was discovered in California.
	1987	Calgene Inc. obtained a patent for the tomato polygalacturonase DNA sequence, which was later used to synthesize an antisense RNA sequence that can further extend the shelf life of fruit. Maynard Olson and colleagues at Washington University discovered yeast artificial chromosomes, which are expression vectors for large proteins.
	1989	A gene responsible for cystic fibrosis was explored.
	1990	Human Genome Project was launched.
	1993	Kary Mullis won the Nobel Prize in Chemistry for inventing the tool of PCR. FDA approved a recombinant protein to treat multiple sclerosis. A global research team, led by Daniel Cohen created a rough map of all 23 pairs of human chromosomes.
	1994	A number of genes, human and others were identified and their functions explained.
	1995	The first vaccine for hepatitis A was explored. Researchers at the Institute for Genomic Research completed the first full gene sequence of a living organism for the bacterium *Haemophilus influenzae.*
	1997	Researchers at the Roslin Institute in Scotland cloned a sheep called Dolly from the cell of an adult ewe. Dolly was the first mammal cloned by a technique called nuclear transfer technology. The first human artificial chromosome was discovered.
	1998	A group of researchers succeeded in culturing embryonic stem cells. A draft of the human genome map is created that locates more than 30,000 genes. Embryonic stem cells were employed to regenerate tissue and produce disorders mimicking diseases.
	2000	Sir Hargobind Khorana synthesized DNA in a test tube. He demonstrated the role of nucleotides in protein synthesis and helped in finding the genetic code**.**
	2001	The journals *Science* and *Nature* reported the human genome sequence.
	2002	A very rapid shotgun sequencing of major genomes was completed
	2003	Celera and the NIH successfully finished the sequencing of the human genome.

	2004	The FDA supported the first monoclonal antibody for cancer therapy
	2006	The FDA sanctioned a recombinant vaccine against human papillomavirus. Researchers established the three-dimensional (3D) structure of HIV.
	2008	Japanese chemists developed the first DNA molecule made nearly entirely of artificial parts.
	2009	Scientists identified three new genes connected with Alzheimer's disease, paving the way for possible new diagnostics and therapeutics.
	2013	Researchers established the three-dimensional (3D) structure of HIV, which causes AIDS.
	2010	Researchers from the J.Craig Ventere Institute create the first synthetic cell.

Modern biotechnology, thus has developed as a science with enormous potential for human welfare in areas ranging from food processing to human health and environmental protection. Biotechnology is now being used in numerous disciplines including bioremediation, energy production, agriculture and food processing. Biotechnology has vast scope in agriculture for the production of plants that are resistant to insects, weeds and plant diseases. Production of medicines (biopharmaceuticals) through cloning of vectors with genes of interest (GOIs) has been made possible via biotechnology. Modern biotechnology is applied in medicine and healthcare in therapeutics, mainly for the discovery, development and production of novel drugs and in diagnostics, for protein and nucleic acids based tests. In the area of environment, biotechnology has been primarily used to treat waste and prevent pollution. Thus modern biotechnology makes significant contribution in improving the lives of humans.

10.3 TYPES OF BIOTECHNOLOGY

In the recent years, the scope of biotechnology has expanded. It has been categorized into different types depending on the field in which it is used.

1. Medical Biotechnology

It refers to use of living cells and other materials to improve health of humans. It is used for finding cure for diseases or prevention of diseases, as a research tool for studying human health, understanding pathogens and human cell biology. The technique is used for producing pharmaceutical drugs as well as other chemicals to combat diseases. The field is used for development of new drugs and treatments. Examples- vaccines, an anti-lymphoma vaccine has been developed using genetically engineered tobacco plants that exhibit RNA (a similar chemical to DNA) from malignant (actively cancerous) B-cells.

2. Agricultural Biotechnology

Biotechnology has been used in developing genetically modified plants to increase crop yields or introduce characteristics that impart tolerance against

biotic, abiotic stress, and provide resistance against pest and pathogen attack. Examples- development of crops that express anti-pest characteristics. The genes of *Bacillus thuringiensis* have been transferred to crops. A protein (Bt) produced by Bacillus t*huringiensis*is found to be very effective against pests such as the European corn borer.

3. Industrial Biotechnology

Biotechnology also finds its applications in industrial sector. It includes use of microorganisms, or components of cells like enzymes, to generate products such as food and feed, chemicals, detergents, paper and pulp, textiles, biofuels, and biogas that are industrially useful. Examples- Biocatalysts have been developed via industrial biotechnology to synthesize chemicals.

4. Environmental Biotechnology

Biotechnology is used in treatment of waste and prevention of pollution. These methods efficiently clean up wastes and significantly reduce our dependence on methods for land-based disposal. Examples- in Bioremediation, potential of living organisms are exploited for removal/treatment of wastes. Enzyme bioreactors that pretreat industrial and food waste components and allow their efficient removal via sewage system has been developed.

Recently a new system of classifying Biotechnology has been adopted. In this type of classification, various colours have been given to various branches of biotechnology (Table 10.2, Fig. 10.2).

Table 10.2: Categorization of various branches of Biotechnology on the basis of colour.

Color designation	Description
Gold biotechnology or Bioinformatics or computational biology	Use of computational techniques for analysis of biological data.
Red Biotechnology (Biopharma)	relates to medicine and veterinary products. It includes development of new drugs, vaccines and antibiotics, regenerative therapies, molecular diagnostics and genetic engineering techniques to cure diseases applying genetic manipulation.
White Biotechnology	includes designing of energy-efficient, less polluting, and low resource-consuming processes and products.
Yellow Biotechnology	relates to the use of biotechnology in food production such as making wine, cheese, and beer by fermentation.
Grey Biotechnology	Includes environmental applications of biotechnology such as removal of pollutants or contaminants using microorganisms and plants.
Green Biotechnology	Use of biotechnology in agriculture for creating new plant varieties of agricultural interest, biopesticides,

	and biofertilizers. This area includes production of transgenics via genetic modification.
Blue Biotechnology	use of marine resources to create products and their application in various sectors.
Violet Biotechnology	deals with the law, ethical and philosophical issues related to biotechnology
Dark Biotechnology	Relates to bioterrorism, biological weapons and biowarfare using microorganisms, and toxins to cause diseases and death in humans, domestic animals, and crops.

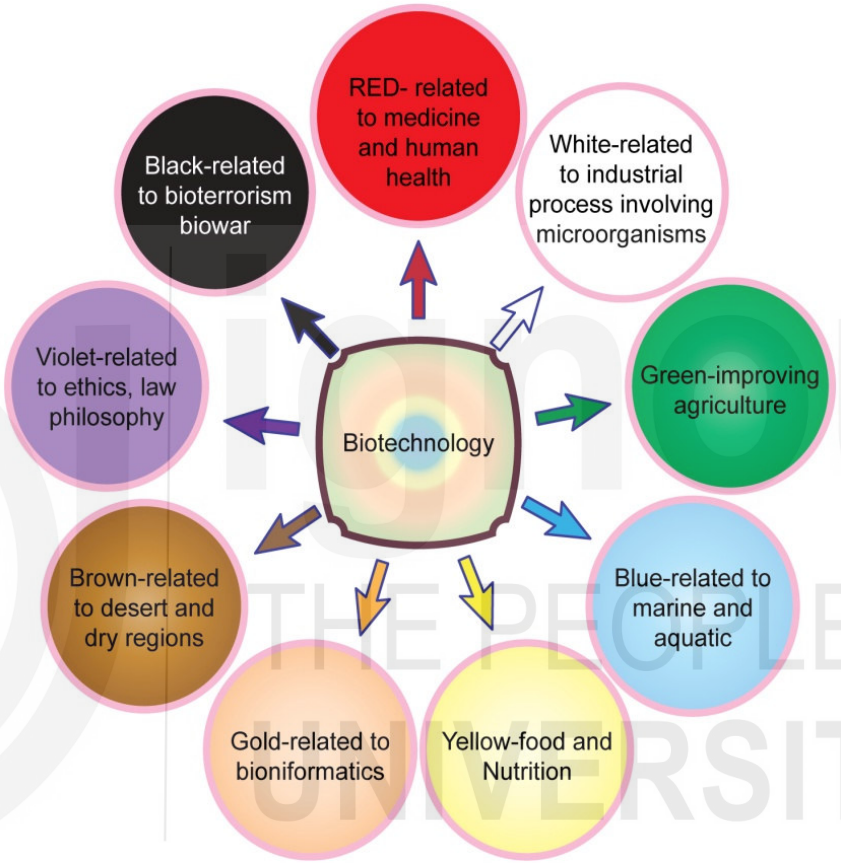


Fig. 10.2: Categorization of branches of Biotechnology on the basis of colour.

10.4 VARIOUS TECHNIQUES IN BIOTECHNOLOGY

Various techniques have been followed in biotechnology. Some of the major ones include:

- Genetic Engineering Technology

The technique involves the manipulation of the DNA of an organism by inserting foreign DNA.

- Protein Engineering Technology

The technique involves improvement in existing proteins or creating novel proteins to make useful products.

- Enzyme engineering

This technology involves improvement in efficiency of enzyme or formulation of an enzyme activity by altering its amino acid sequence. Genetic engineering techniques are widely used to improve enzyme efficiency.

- Cell and Tissue Culture Technology

In this technique, cells/tissues are grown under laboratory conditions to produce a organism or new products.

- Antisense or RNAi Technology

RNA interference (RNAi), is a mechanism that involves use of small RNAs (less than 30 bases long) in regulating the expression of genes in eukaryotic organisms (Fig. 10.3). An RNAi-based mechanism has found its applications in studies of gene function and therapeutics for the treatment of disease.

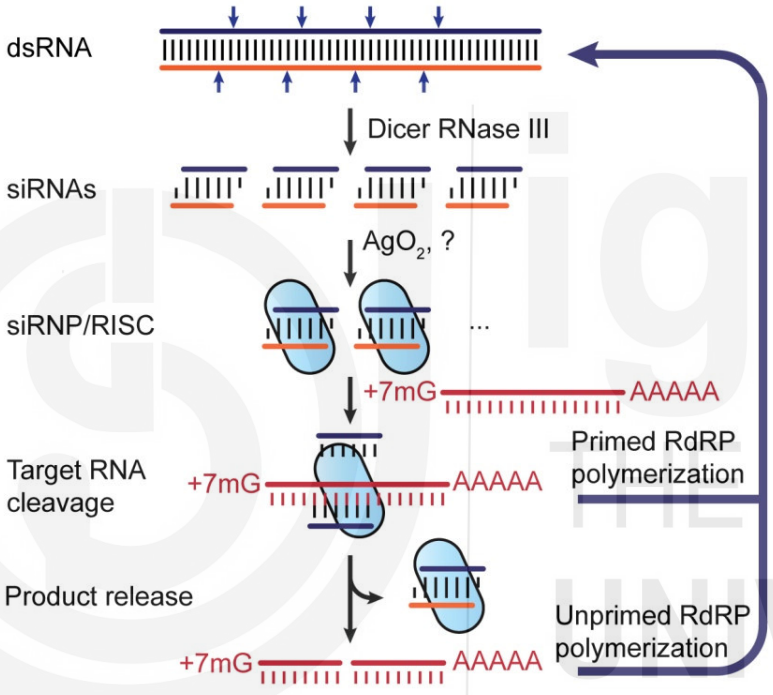


Fig. 10.3: An overview of RNAi technique.

- Bioinformatics Technology

Computational analysis of biological data, e.g., sequence analysis macromolecular structures, high-throughput profiling data analysis.

- Protein separation and identification techniques

Techniques such as electrophoresis, microarrays and blotting techniques that are helpful in separation and identification of desired DNA.

- Genomics

The genome approaches are used to determine the biological function of genes and their products. Transcriptomics (profiling of microarray expression), proteomics (structures/modifications/interactions of proteins), metabolomics (e.g. metabolite profiling, chemical fingerprinting, flux analysis) are some of its diversifications (Fig. 10.4).

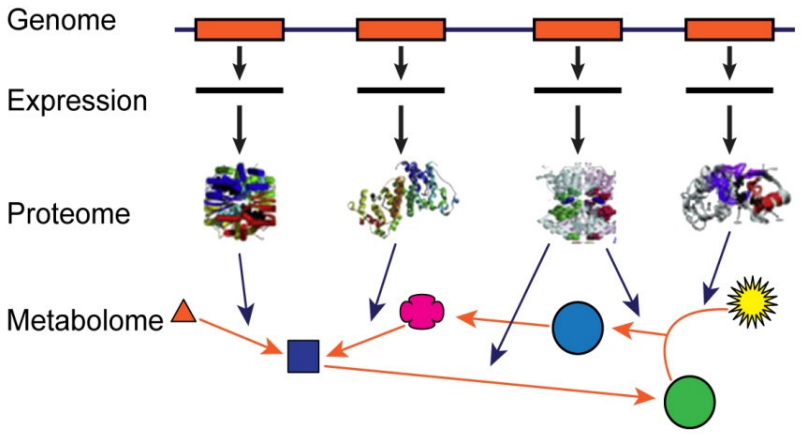


Fig. 10.4: Genome approaches used to determine the biological function of genes and their products.

10.5 APPLICATIONS OF BIOTECHNOLOGY

Biotechnology has been successfully applied in various areas such as the environment, medicine, agriculture, industries and many more.

1. Environment

Biotechnological techniques have been used in the diagnosis of environmental problems, their monitoring and abatement. This technology has been used in areas of waste management, bioremediation (use of living organisms to treat environmental pollutants), phytoremediation (use of plants to remove pollutants from the environment). Apart from these renewable resources, biodegradable products and alternative energy sources have also been developed using biotechnology. Biotechnology is used to engineer microbes that show immense potential for environmental clean-up by removing a wide range of pollutants from various sectors of the environment.

2. Medicine

Biotechnological techniques have been used in the diagnostics, therapeutics, vaccines, human Genome Research. The development of insulin and vaccines such as hepatitis B are some of the major application of biotechnology in medicine.

Recombinant DNA technology has made an immense impact in the area of healthcare by enabling the mass production of effective therapeutics. Transgenic animals are been used to understand how genes contribute to the development of human diseases such as cancer, cystic fibrosis, rheumatoid arthritis and Alzheimer's. Gene therapy had made the treatment of hereditary diseases possible *via* the insertion of genes into individual's cells and tissues.

Box 10.1: Role of biotechnology in abatement of Covid 19 pandemic.

Biotechnology has emerged as a leader in the fight against COVID -19. Vaccines for the treatment of corona virus infection have been developed using biotechnological tools. Pfizer-BioNTech and Moderna developed high efficacy mRNA vaccines against COVID-19. The mRNA vaccine developed by BioNTech/Pfizer was shown to be 90% effective in preventing COVID-19 infection. Other vaccines are also been developed based on other technologies such as an interference RNA that blocks the virus in the

form of nebulizers, recombinant ACE2 proteins that trick the virus into not binding to human cells, and antibodies that attack the virus.

Viral vector vaccines, AZD1222 and Sputnik V were developed by AstraZeneca. mRNA vaccines, mRNA-1273 and BNT162b2, were developed by Moderna (Cambridge, MA, USA) and Pfizer (New York, USA)

3. Agriculture

Biotechnological techniques have proved very useful in the area of developing crops with better nutrition content, high yield, and resistance to various abiotic and biotic stresses. Food, horticultural crops and tree species have been modified successfully using genetic engineering. For e.g. Oil seed can be modified to produce fatty acids for petrochemicals, fuels. Efforts have been made to develop vaccines from plants. Crops that show tolerance against a broad range of pests/insects have been developed.

4. Industries

Biotechnology has been applied for the production of cellular structures or production of biological elements for numerous uses such as development of new materials in the construction industry, and the manufacture of beer and wine, washing detergents, and personal care products.

Biotechnology is used in the textile industry for the finishing of fabrics and garments. It helps in production of warmer, stronger, wrinkle and shrink-resistant clothes along with other properties such as improved dye uptake and retention, enhanced absorbency. Biotechnology has been successfully applied in the food processing industry.

5. Biofuels

Biotechnology has been used in sector of energy production. Bioremediation waste can be converted to biofuel to run generators. Bacteria that degrade sulfur liquor, which is a waste product of the paper manufacturing industry, can help in production of methane.

6. Evolutionary studies

Genes associated with ecological traits and evolutionary diversification can easily be traced using biotechnological tools.

Other Uses

Microbes can be induced to produce enzymes required to turn plant and vegetable materials into building blocks for biodegradable plastics.

Table 10.3: Applications of biotechnology in different areas.

Area	Applications
Plant biotechnology	Transgenic plants, production of secondary metabolite, production of pathogen-free plants or crop improvement, production of herbicide-resistant crops, pest-resistant ('Bt concept' pest-resistant transgenic) plants, drought resistance, flood resistance, salt tolerance, high-yielding GM crops, nitrogen

	fixing ability, acidity and salinity tolerance, *in vitro* germplasm conservation, genetic variability, *in vitro* pollination, induction of haploidy, somatic hybridization, genetic transformation, molecular pharming, somatic embryogenesis, organogenesis, phytoremediation, *in vitro* plant germplasm conservation, mutant selection, somaclonal variation, plant genome analysis, hybrid seeds, artificial seeds.
Animal biotechnology	Biopharmaceuticals: Production of hormones, growth factors, interferons, enzymes, recombinant proteins, vaccines, blood components, oligonucleotides, transcription factor-based drugs, oligonucleotides.
	Antibiotics, Diagnostics: antibodies, biosensors, PCR, therapeutics, vaccines, medical research tools, human genome research, development of biosensors
	Gene therapy, Stem cell therapy, Animal tissue culture: Cell, tissue and organ culture, Gene cloning: rDNA technology, genetic engineering, transgenic animals, antibiotics, DNA markers, animal husbandry, xenotransplantation, medical biotechnology,
	Biopharmaceuticals (drug or vaccine developed through biotechnology), therapeutants, i.e. products used to maintain health or prevent disease, biopharming, i.e. production of pharmaceuticals in cultured organisms,
	Biopolymers, Designer drugs
Agricultural biotechnology	crop biotechnology, horticultural biotechnology, tree biotechnology, food processing, plant biotechnology (photosynthesis improvers, bio-fertilizers, stress-resistant crops and plants, bio-insecticides and biopesticides)
	Food: Increased milk production
	Pharmaceuticals: Animals engineered to produce human proteins for drugs, including insulin and vaccines
Environmental biotechnology	Environmental monitoring, Waste management, Pollution prevention.
Fuel and fodder	renewable fuels, Tissue culture technique for rapid afforestation of degraded forests and regeneration of green cover
Industrial biotechnology	Metabolite production (acetone, butanol, alcohol, antibiotics, enzymes, vitamins, organic acids), anaerobic digestion (for methane production), waste treatment (both organic and industrial), production of bio-control agents, fermentation of food products, bio-based fuel and energy, recovery of metals and minerals, bioethanol, pulp and paper, food, textiles and leather, pharmaceuticals, an enzymatic process for producing antibiotics.
Aquatic biotechnology	Aquaculture, restoring and protecting marine ecosystems, improving seafood quality, environmental remediation, marine byproducts for human health, biomaterial and bioprocessing, marine molecular biotechnology.

10.6 PLANT BIOTECHNOLOGY

Plant biotechnology has gained importance because of its role in manipulating plants for improved agronomic performance. Plant biotechnology involves processes or scientific techniques that are used to develop beneficial plants

and/or their products and development of new plant traits. The use of biotechnological tools helps in achieving crop improvement at a faster rate and facilitates the transfer of genes from unrelated species.

The two processes involved in plant biotechnology are tissue culture and genetic engineering. Plant tissue culture is the most popular technique of plant biotechnology. The plant tissue culture had its beginning in 1939 when Gautheret tried to cultivate isolated cells and root tips on an organized medium. The tissue culture techniques provide the platform for the rapid multiplication of plant species. Plant tissue culture technique has been used for getting regeneration of plant cells for endangered/extinct plant species, rapid production of elite varieties with superior genotypes on a large scale in a comparatively short time. The plants are genetically modified plants by transferring genes from one organism to plant to get the desired characteristics. Plants have been genetically modified to induce herbicide tolerance, salinity tolerance, drought tolerance, pest resistance, enhanced nitrogen-fixing ability, improved nutritional value and food quality.

Plant biotechnology also provided an option for germplasm conservation and production of disease-free varieties. Increased production of biochemical constituents (secondary metabolites) in plants has also been achieved using this technique. Production of artificial seeds, biopharmaceuticals, plant-made pharmaceuticals, therapeutic proteins and plant-made vaccines or antibodies (plantibodies) is the other area where plant biotechnology is being applied.

SAQ 1

a) Answer in one word only:

i) Who coined the term ‘biotechnology’?

ii) What is the ‘part’ of plant used for culture called?

iii) Name the scientist who tried to cultivate isolated cells and root tips on an organized medium.

iv) Name the antibiotic produced by Alexander Fleming.

v) Name of the sheep cloned by researchers in Scotland.

vi) Name the technique used for computational analysis of biological data.

b) Match the following:

	Column A		**Column B**
i)	Yellow biotechnology	1.	environmental applications of biotechnology
ii)	1984	2.	Arthur Kornberg created DNA in a test tube for the first time.
iii)	1990	3.	Har Gobind Khorana synthesized DNA in a test tube
iv)	Grey biotechnology	4.	Sheep cloned using nuclear transfer technology

v)	1958	5.	Human genome project was launched
vi)	Dolly	6.	The DNA fingerprinting technique was discovered.
vii)	2000	7.	use of biotechnology in food production

10.7 METHODS IN PLANT BIOTECHNOLOGY

Various methods in plant biotechnology include:

- Genetic engineering/ recombinant DNA technology
- Tissue culture
- Molecular Assisted Breeding

Genetic engineering

Alteration of genetic material (hereditary material) in an organism forms the basis of genetic engineering. Genetic engineering or recombinant DNA technology involves the removal of the gene(s) from one organism and transfer to another organism. Integration of new DNA into a plant's original DNA creates a transgenic plant or genetically modified organism (GMO). The basic requirements for successful genetic engineering are restriction enzymes, cloning vehicles (vectors) to carry the genes of interest and detection and selection of cloned genes.

Gene transfer techniques used in genetic engineering include:

- *Agrobacterium*-mediated gene transfer: The desired trait is isolated from the DNA of an organism, inserted into *Agrobacterium* and the target plant is infected. Cells that accept the DNA are grown into plants with the new trait.
- Gene gun: DNA that codes for the desired trait is coated onto tiny particles of tungsten and fired into a group of plant cells. Cells that accept the DNA show the desired trait.
- Chemicals such as polyethylene glycol (PEG) help in rapid uptake of DNA by plant protoplasts and integration into the plant's chromosomal DNA.
- Electroporation is another method in which short high-voltage electrical pulses induce the formation of transient micropores in cell membranes allowing DNA to enter the cell and then the nucleus.
- Apart from these lipofection, microinjection, calcium phosphate transfection are some other methods that are also followed to transfer DNA.

Agrobacterium vectors are commonly used for the transfer of transgenes into plants. The DNA sequences may be cleaved at several points and the resulting fragments inserted into different parts of the genome (Fig. 10.5). Transgenes inserted into the genome contain regulatory elements and a selectable marker, often an antibiotic- or herbicide-resistant gene.

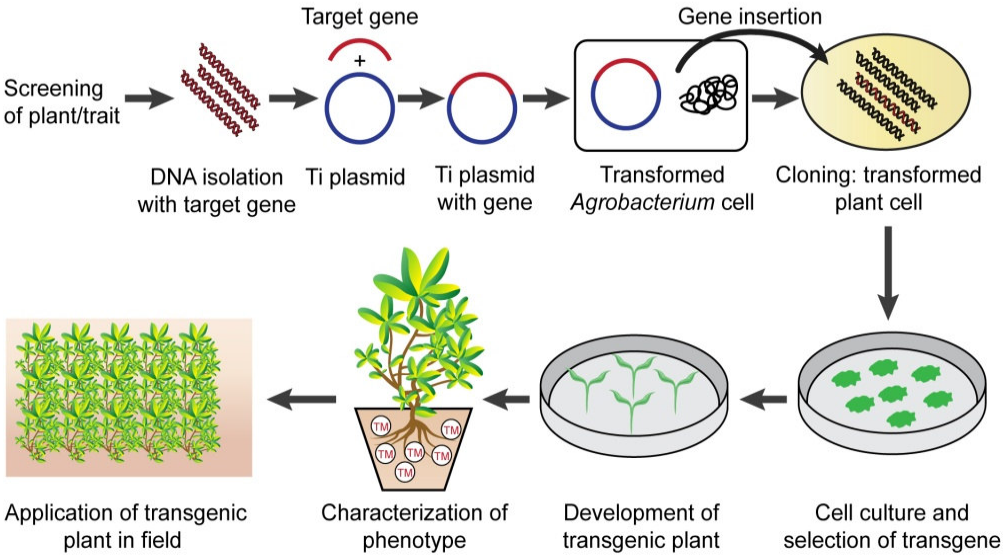


Fig. 10.5: An overview of plant genetic engineering.

Plant tissue culture

Cells, anthers, pollen grains, or other tissues are cultured under laboratory conditions to raise the plants. They are then multiplied in large numbers. Under cultural conditions, parts of plants (explants) or tissues form callus (undifferentiated mass of cells) develop directly into embryos (somatic embryos). The plant tissue culture allows breeders to introduce new cultivars at a much faster rate due to rapid multiplication of plants.

The tissue culture process involves placing of tissue on a nutrient medium, multiplication of cells to form callus or the embryos followed by their transfer to medium containing plant growth regulators and finally root formation (Fig. 10.6). The plantlets are transferred to greenhouses. A large variety of ornamental plants, crops and medicinal plants have been raised via plant tissue culture technique.

Cell culture helps in producing clones of a single cell, embryo-rescue, introgression of characters such as disease resistance into ~~elite~~ breeding lines and production of haploid plants for developing homozygous breeding lines. This technique is now widely used for the improvement of important crops including major cereals, legumes and trees.

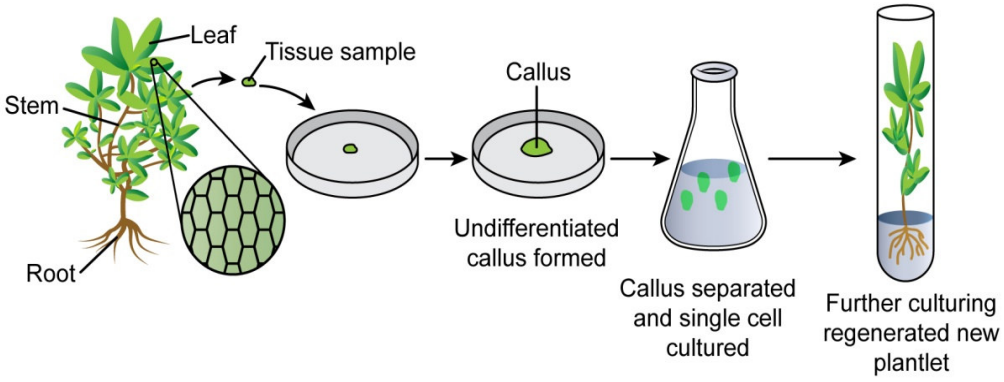


Fig. 10.6: Diagrammatic representation of the plant tissue culture technique.

Molecular Assisted Breeding (MAB)/Marker Assisted Selection (MAS)

Molecular breeding is the technique of using DNA markers that are tightly linked to phenotypic traits to assist in selection. Genetic markers such as

microsatellites, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphism (AFLP) are important components of molecular breeding. Molecular markers have been developed for many crops including trees. The information generated from molecular markers, transcriptome profiling, and genome sequencing facilitate genetic improvement in plants. The markers can be used to track the presence of valuable characters in large segregating populations as part of a crop-breeding program. For example, if a useful trait such as disease resistance or high oil yield is linked with a specific marker, many hundreds or even thousands of young plantlets can be screened for the likely presence of the trait without growing the plants to maturity. The use of molecular markers can decrease the time scale of crop-breeding programs by several years.

Genomic selection is emerging as a molecular breeding method used for crop improvement. It assists in the identification of superior genotypes with higher breeding values and helps in segregating breeding populations (Fig. 10.7). It is based on genome-wide marker profile data. MAS have proved as an efficient breeding method for drought and salt-tolerant oilseed crops such as *Brassica*. It has provided valuable contributions in the designing and development of oilseed crop ideotype(s).

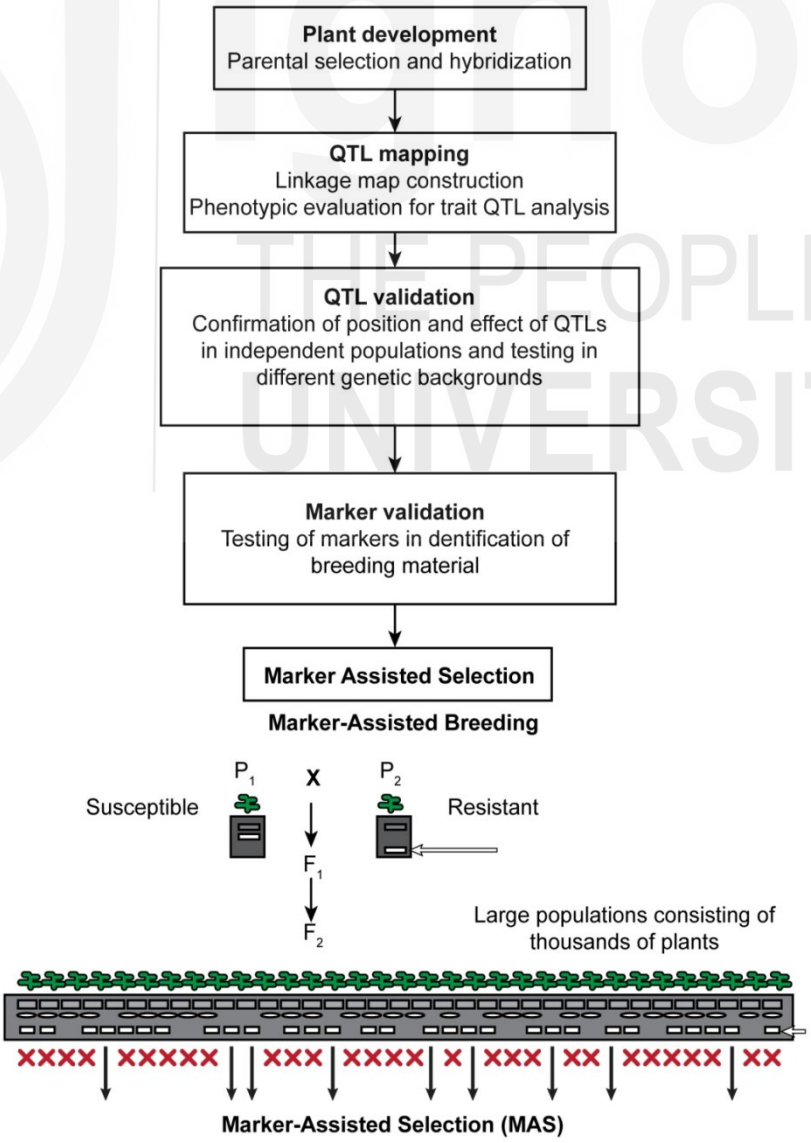


Fig. 10.7: Process of marker assisted breeding technique.

SAQ 2

Fill in the blanks with appropriate words:

a) Improvement of crops done *via* incorporation of desired agronomic traits is known as …… .

b) The plants formed after incorporation of genes for desired characters are called …….. .

c) Undifferentiated mass of cells produced via tissue culture is called …… ..

d) During …….. phase of the plant tissue culture, callus or the embryos are transferred to a medium containing plant growth regulators.

e) Polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) are examples of …….. .

10.8 APPLICATIONS OF PLANT BIOTECHNOLOGY

Plant biotechnology has broad applications in several areas. These include basic research and commercial applications. The basic application of plant biotechnology deals with understanding the concepts such as molecular pathways in plant cells. Plant biotechnology techniques such as tissue culture and genetic engineering have been applied to plants for modification and improvement. These include raising of pathogen-free plants, germplasm conservation, clonal propagation, secondary metabolite production and study of cell behaviour. Biotechnology has got its application in crop improvement programs.

Various areas of application of Plant Biotechnology are:

- Improvement of varieties for various agronomic traits such as:
  - Crop improvement (resistance to biotic stress: pests, viruses, pathogens, abiotic stress tolerance to drought, salinity)
  - Value addition: enrichment of crops with vitamins.
  - Increasing the shelf life to delay the fruit ripening
  - Food processing
  - Ornamental plants for characters such as size, color, smell
- Phytoremediation: removal of contaminants
- Biofuels: bioenergy crops
- Commercial products such as biopolymers, therapeutic proteins, biodegradable plastics, etc.
- protection of natural biodiversity

10.8.1 Crop Improvement

Plant tissue culture technique

Tissue culture helps in getting rapid clonal multiplication/propagation of plants. This is called micropropagation. The progeny obtained by vegetative propagation i.e. asexual reproduction of a single plant or individual is called a clone. Shoot buds produced on explants are separated and rooted to get plantlets. The technique proves useful in eliminating viruses and other pathogens.

Plant cell suspension culture and immobilized cells are being used to get large-scale production of useful chemical compounds. *C*ell and organ culture helps in the conservation of endangered genotypes and preservation of vegetative tissues. The plants that do not produce seeds (sterile plants) or have 'recalcitrant' seeds can be preserved *via in vitro* techniques. Cryopreservation involves the storage of cells or tissues in liquid nitrogen at ultralow temperature (–196°C) under *in vitro* conditions. This method helps in the long-term conservation of essential biological material and genetic resources. The embryonic tissues are generally cryopreserved for future use.

10.8.2 Genetic Transformation

In the genetic transformation technique, the genes with the desirable trait are transferred into host plants to get transgenic plants. In plants, genetic transformation is done by vector-mediated i.e. *Agrobacterium*-mediated genetic transformation, or vector less i.e. direct gene transfer method. The technique offers a great potential for genetic improvement in various crop plants. The technique involves integration of plant biotechnology and breeding programmes. Traits such as increased yield and better quality can be introduced into plants. For e.g.: Development of the transgenic 'golden rice'. Transgenic rice contains three genes encoding the enzymes responsible for the conversion of geranylgeranyl diphosphate to β-carotene (provitamin A). The transgenic rice produced is enrich in iron content. The plants are obtained by expressing ferritin or metallothionein transgenes, or making the existing iron more available by reducing levels of the iron-sequestering protein, phytase.

The use of transgenes can help in producing cells having a high capacity of the desired compounds that can have industrial uses. The modification of exogenous compounds by plant cells is called biotransformation. The bioconversion reactions are catalyzed by the enzymes present within the plant cell. The genetic engineering method has proven useful in developing plants that show resistance to or protection against various pests, diseases, viruses.

10.8.3 Industrial

Plants produce fuels, proteins, antibodies (plantibodies) and other products which are of commercial use. For e.g. avidin and β-glucuronidase (GUS) are proteins produced in transgenic maize. Avidin is used as a biochemical reagent for research and diagnostics, and also as a biopesticide. Plant oils are used as a feedstock for the production of oleochemicals. The soybean crop has been transformed to get high oleic acid content.

Non-toxic biodegradable polymers called polyhydroxyalkanoates (PHAs) are produced by plants and their production can be increased at the agricultural level. It is possible to obtain a high yield of the polymer from leaves or seeds. Attempts are been made to obtain transgenic plants so that production of these copolymers in the plant can be increased (including one in oil palm).

Starch produced by plants has also got uses in industry. Interest is also developed in manipulating complex carbohydrates in cereal crops such as rice, wheat, maize and barley. The starches are used for the development of packaging materials or even biodegradable plastics. Many of the enzymes involved in starch biosynthesis have been characterized and their genes cloned and attempts are been made to develop plants with enhanced capacity for the production of starch.

10.8.4 Pharmaceuticals

Plant-made pharmaceuticals, secondary metabolites, proteins, drugs and vaccines have been produced *via* classical and non-classical techniques such as plant cell culture and genetic engineering.

Apart from these, a new branch of biotechnology i.e. nanobiotechnology which involves the use of nanomaterials has proven useful in the development of crops mainly by manipulating the plant nutrient content, imparting disease and pesticide resistance in plants. Metallic nanoparticles exhibiting relatively superior anti-pathogenic, anti-fungal and anti-bacterial qualities are being used in developing nano pesticides. Nanoparticles of zinc oxide and iron helps in efficient uptake of nutrients by the plant, hence act as nanofertilizers. Their success depends on factors like plant species, susceptibility and the size, composition and chemical properties of nanomaterials.

SAQ 3

a) Define the following:

   i) Molecular breeding

   ii) Cryopreservation

   iii) Electroporation

   iv) Nanobiotechnology

b) Explain the term ‘transgenic’? How are they useful?.

10.9 SUMMARY

- Use of biological agents such as microorganisms, plants, cells of higher organisms and their constituents for generating desired products is called biotechnology.
- Biotechnology has been used since ancient times in as the animals were domesticated, crops were cultivated and micro-organisms were used to

get products such as cheese, yogurt, bread, beer and wine. Later antibiotics were discovered. Genetics laid the foundation for modern day biotechnology.

- Modern biotechnology has got its applications in areas such as medicine, healthcare, therapeutics, diagnostics for development and production of novel drugs, agriculture for development of improved crop varieties, environment for treatment of waste and prevents pollution.
- Plant biotechnology is referred to the use of plant cells, tissues, organs for the generation of useful products. The plant parts (explants) are cultured under *in vitro* conditions to get plantlets. Plant biotechnology plays a promising role in the field of agriculture, industry and pharmaceutical sciences.
- Plant biotechnology has many applications such as large scale production of biochemicals, rapid clonal multiplication, development of homozygous lines *via* production of haploids, conservation of germplasm etc.
- The main methods in plant biotechnology are plant tissue culture, genetic engineering and marker assisted breeding.
- Clonal multiplication, raising of virus-free plants can be done via plant tissue culture technique. The development of plants with tolerance against abiotic and biotic stresses, better nutritional quality, better storage can be achieved via manipulation of DNA by genetic engineering.
- The plant tissue culture technique can also be used for getting products of commercial value. These include bioplastics, biofuels, edible plant-based vaccines, bio-oils, etc

10.10 TERMINAL QUESTIONS

1. Discuss various techniques of Biotechnology.
2. Describe in brief various applications of Biotechnology.
3. How does Biotechnology be used for the improvement of crop plants?
4. Describe the methods of Plant Biotechnology.
5. Enumerate various applications of Plant Biotechnology.
6. How does Plant Biotechnology play a role in germplasm conservation?

10.11 ANSWERS

Self-Assessment Questions

1. a) i) Karl Ereky in 1919

   ii) explants

   iii) G. Haberlandt

iv) Penicillin

v) Dolly

vi) Bioinformatics

b) i) Use of biotechnology in food production

ii) The DNA fingerprinting technique was discovered

iii) Human genome project was launched

iv) environmental applications of biotechnology

v) Arthur Kornberg created DNA in a test tube for the first time.

vi) Sheep cloned using nuclear transfer technology

vii) Har Gobind Khorana synthesized DNA in a test tube

2. a) transformation

b) transgenic

c) callus

d) multiplication

e) genetic markers

3. a) i) Refer to Section 10.7.

ii) Refer to Subsection 10.8.1.

iii) Refer to Section 10.7.

iv) Refer to Subsection 10.8.4.

b) Refer to Subsection 10.8.2.

Terminal Questions

1. Refer to Section 10.4.
2. Refer to Section 10.5.
3. Refer to Section 10.8.
4. Refer to Section 10.7.
5. Refer to Section 10.8.
6. Refer to Section 10.8.

Acknowledgements

Fig. 10.1 : Source: https://www.google.co.in/imgres?imgurl=https%3A%2F%2Finstonchurchill.hillsdale.edu%2Fwp-content%2Fuploads%2F2018%2F09%2FFleming.jpg

UNIT 11

PLANT TISSUE CULTURE (I)

Structure

11.1 Introduction

Objectives

11.2 Historical Account

11.3 Equipment Required for Plant Tissue Culture

Basic Steps in Plant Tissue Culture

11.4 Composition of Media

11.5 Totipotency

11.6 Organogenesis

11.7 Embryogenesis

11.8 Protoplast Isolation, Culture and Fusion

11.9 Summary

11.10 Terminal Questions

11.11 Answers

11.1 INTRODUCTION

The process of raising plants under *in-vitro* aseptic conditions by culturing explants (parts of differentiated tissues) in a nutrient medium under conditions is termed as plant tissue culture. Cells, tissues, organs, or whole plant is cultured under *in vitro* conditions. Ideal conditions such as the proper supply of nutrients, pH and temperature provide a favorable environment for the growth and multiplication of plants under *in vitro* conditions. Plant tissue culture technique plays a significant role in the area of crop improvement. The plant tissue culture technique has been successful in getting large-scale production of plants in a short time duration. Many economically important plant species have been improved using the tissue culture technique. In addition, the technique has also contributed to increasing our understanding related to factors responsible for growth, metabolism, morphogenesis and differentiation in plants. Plant propagation, production of disease-free, nutritionally improved and highly tolerant (to conditions such as salinity drought, cold) plants has been achieved using plant tissue culture technique. Production of secondary metabolites of immense importance has been increased via this technique. Besides, many endangered, threatened and rare species can be successfully grown and conserved using this technique. Tissue culture techniques have been used for the improvement and propagation of various types of plants including cereals, grasses, legumes, vegetable crops, root and tuber crops, oilseeds, fruits, plantation crops, forest trees and ornamentals.

Before studying in detail about various practical applications of plant tissue culture technique, we need to understand the basic methods involved in culture and the requirements for the implementation of this technique. The first part of this unit provides you with an overview of various scientists that played an important role in developing the plant tissue culture technique, the various steps of the technique and the major requirements (equipments and chemicals). The second part of this unit describes the important aspects of concepts of totipotency, techniques such as Organogenesis, Embryogenesis and Protoplast culture.

Objectives

After studying this unit, you would be able to:

- list the contributions of various scientists in the plant tissue culture technique;
- explain the conditions and other requirements for raising plants through tissue culture technique;
- explain the concept of 'totipotency' and describe the processes of 'Organogenesis' and 'Embryogenesis'; and
- appreciate the techniques of protoplast isolation, culture and fusion.

11.2 HISTORICAL ACCOUNT

The German Botanist Guttlieb Haberlandt was first to propose the importance of plant tissue and cell culture in 1902. He grew the tissue of *Lamium purpureum* and *Eichhornia crassipes*, the epidermis of *Ornithogalium* and epidermal hairs of *Pulmonaria mollissima* on a salt solution with sucrose. He was the first to culture single palisade cells from leaves in Knop's salt solution enriched with sucrose. He observed growth in the cells. The cells grew in size, changed shape, accumulated starch and showed thickening of cell walls. The cells showed no division. The cells remained alive for up to 1 month. This happened because highly differentiated cells lack the growth hormones required for inducing division in mature cells. His experiments were unsuccessful but they laid down the foundation of tissue culture technology. He is regarded as the 'father of plant tissue culture'. He predicted that artificial embryos can be successfully cultivated from vegetative cells. He was the first one to propose the concept of **totipotency**.

The first true plant tissue cultures were obtained by Gautheret. He was able to raise cambial tissue from *Acer pseudoplatanus*. He was also able to culture explants of *Ulmus campestre, Robinia pseudoacacia*, and *Salix capraea* using agar-solidified medium of Knop's solution, glucose and cysteine hydrochloride. Later, he included indole acetic acid and vitamin B in the medium to get carrot root tissue cultures and a hybrid of *Nicotiana glauca* and *Nicotiana langsdorffii*. The tissues are grown in a culture medium get differentiated into roots and shoots.

Mature embryos of plants like *Raphanus sativus* were excised by Hanning (1940) and successfully grown on mineral salts and sugar solutions. Van Overbeck (1941) and co-workers demonstrated the stimulatory effect of coconut milk on embryo development and callus formation in *Datura*. These studies proved a turning point in the field of embryo culture as young embryos could be cultured successfully under *in vitro* conditions. Braun (1959) was able to generate a plant from a mature plant cell. The foundation of commercial plant tissue culture was laid down in 1960 by Morel. He was able to get multiplication of *Cymbidium* (an orchid) under culture conditions.

Later Raghavan and Torrey (1963), Norstog (1965) and his coworkers developed synthetic media for the culture of younger embryos. Laibach (1925, 1929) was able to show the practical application of embryo culture. He was successful to isolate embryos from seeds of a *Linum* plant and raise them to maturity on a nutrient medium. Robbins and Kotte (1922) successfully cultured isolated root tips of maize, pea, and cotton. In 1934, White reported success in cultures of tomato root tips. Extensive work on root culture was done by Street (1957). He cultured excised tomato roots to understand the role of inorganic ions, carbohydrates, amino acids, hormones and vitamins in plant growth. Gautheret (1934), White (1939) and Nobecourt successfully cultured cells of *Salix, Nicotiana* and carrot on synthetic media. They demonstrated that the addition of growth regulators and vitamins in media leads to the proliferation of cells forming a callus.

Skoog (1944), Tsui (1951), and Miller (1955) demonstrated induction of divisions in isolated, mature, and differentiated cells using syntheticmedia supplemented with natural compounds. The technique of growing single cells of *Tagetes erecta* and *Nicotiana tobacum* in a liquid medium was developed by Muir (1953). Vasil and Hildeprandt (1965) raised whole plants from single cells of tobacco. Skoog and Miller (1957) demonstrated that changes in the relative concentrations of the two substances (growth hormones) in the medium regulate the differentiation of organs. The formation of an embryo from carrot tissue was reported by Reinert and Steward in 1958-59. Whole plants of *Lupinus* and *Tropaeolum* could be raised by culturing shoot tips.

Ball (1946), Morel and Martin (1952) raised virus-free *Dahlia* plants by culturing shoots. Murashige used the plant tissue culture technique to multiply several plants species (ranging from ferns to foliage, flower and fruit plants) in large numbers. Guha and Maheshwari (1966) demonstrated the possibility of raising large numbers of haploid plants from pollen grains of *Datura.* The first somatic hybrid was produced via protoplast fusion by Carlson and coworkers in 1972. The use of cell wall degrading enzymes was done in protoplast culture technology in the 1970s. The totipotency of protoplasts was first demonstrated by Takebe and his coworkers in tobacco plants. Interspecific hybrid plants of tobacco were regenerated from mesophyll protoplasts.

With time, various developments were noted in the plant tissue culture techniques. The major developments have been summarized in Table 11.1.

Table 11.1: Developments in Plant tissue culture technique with time.

Year	Contribution
1902	Haberlandt proposed the concept of *in vitro* cell culture.
1904	Hannig cultured embryos from several cruciferous species.
1922	Kolte and Robbins successfully cultured root and stem tips respectively.
1926	Went discovered the first plant growth hormone i.e. Indole acetic acid.
1934	White introduced vitamin B as a growth supplement in tissue culture media for tomato root tip.
1939	Gautheret, White and Nobecourt established endless proliferation of callus cultures.
1941	Overbeek was first to add coconut milk for cell division in *Datura.*
1946	Ball raised whole plants of *Lupinus* by shoot tip culture.
1954	Muir was first to break callus tissues into single cells.
1955	Skoog and Miller discovered kinetin as cell division hormone.
1957	Skoog and Miller gave concept of hormonal control (auxin: cytokinin) of organ formation.
1959	Reinert and Steward regenerated embryos from callus clumps and cell suspension of carrot (*Daucus carota).*
1960	Cocking was first to isolate protoplast by enzymatic degradation of cell wall.
1960	Bergmann filtered cell suspension and isolated single cells by plating.
1960	Kanta and Maheshwari developed test-tube fertilization technique.
1962	Murashige and Skoog developed MS medium with a higher salt concentration.
1964	Guha and Maheshwari produced the first haploid plants from pollen grains of *Datura* (Anther culture).
1966	Steward demonstrated totipotency by regenerating carrot plants from single cells of tomato.
1970	Power and co-workers successfully achieved protoplast fusion.
1971	Takebe and co-workers regenerated the first plants from protoplasts
1972	Carlson produced the first interspecific hybrid of *Nicotiana tobacum* by protoplast fusion.
1974	Reinhard introduced biotransformation in plant tissue cultures.
1977	Chilton and co-workers successfully integrated Ti plasmid DNA from *Agrobacterium tumefaciens* in plants.
1978	Melchers and co-workers carried out somatic hybridization of tomato and potato to develop pomato.
1981	Larkin and Scowcroft introduced the term somaclonal variation.
1983	Pelletier and co-workers conducted intergeneric cytoplasmic hybridization in Radish and Grape.
1984	Horsh and co-workers developed transgenic tobacco by transformation with *Agrobacterium.*

1985	Gheysen and co workers developed a very efficientgene transfer system using *Agrobacterium tumefaciens.*
1986	Crossway and his coworkers developed a direct way of transferring cloned genes into nucleus of tobacco mesophyll protoplasts by microinjection of DNA.
1986	Kinsara and his coworkers produced somatic hybrids between *Lycopersicon esculentum* and *L. peruvianum*.
1987	Terada and his coworkers regenerated plantlets from somatic hybrid cells of *Oryza sativa*, and *Echinochloa oryzicola.*
1987	Klien and co-workers developed a biolistic gene transfer method for plant transformation.
1987	Neuhaus and his coworkers achieved gene transfer by microinjecting the DNA into the cells of microspore derived proembryos.
1988	Rhodes an his coworkers produced transgenic maize by electroporation.
1988	Toriyama and his coworkers produced transgenic rice by electroporation.
1989	Shimamoto and his coworkers produced fertile transgenic rice plants from transformed protoplasts.
1990	Milanova and his coworkers succeeded in overcoming hybrid incompatibility between Nicotiana africana and N. tabacum. They produced cytoplasmic male sterile plants by embryo culture.
1990	Iida and his coworkers delivered genes into cultured plant cells by DNA-coated gold particles accelerated by a pneumatic particle gun.
1991	Kyozuka and his coworkers succeeded in getting tissue specific expression of maize alcohol dehydrogenase I gene in transgenic rice plants and their progenies.
1991	Sautter and his coworkers developed a novel method (called micro targeting) for the acceleration of micro projectiles.

By the early 1980s techniques related to morphogenesis, somatic embryogenesis were developed. In this period, culture of cereals, grasses, legumes, and conifers that were previously considered to be recalcitrant groups was successfully done. Interspecific and intergeneric hybrids were produced using *in vitro* pollination. Plant tissue culture methods were developed for overcoming sexual self-incompatibility and induction of haploid plants. Embryo, ovary, anther and ovule cultures were successfully done for large number of plant species. Protoplasts cultures have been developed for more than more than 100 species of angiosperms.

Later in 1990s tissue culture techniques were developed for many types of plants including cereals and grasses, legumes, vegetable crops such as potato, root, tuber crops, oilseeds, temperate/ tropical fruits, plantation crops, forest trees and ornamentals. Cryopreservation technology for storage of germplasm was also developed. Plant tissue culture studies prove very useful in understanding the concepts of morphogenesis, cytodifferentiation, organogenesis and embryogenesis.

Genetic modification of plants by direct DNA transfer via vector-independent and vector-dependent means has been achieved during this period. Vector-independent methods such as electroporation, liposome fusion, microinjection and high-velocity microprojectile bombardment (biolistics) were developed. Advances in molecular biology proved useful in genetic engineering of plants via insertion of foreign genes from diverse biological systems. The development of shuttle vectors for harnessing the natural gene transfer capability of *Agrobacterium*, use of these vectors for direct transformation of regenerable explants and the development of selectable markers has been focused in the recent years. The current emphasis and importance of plant tissue culture is the improvement of plants. Over 100 species of plants have been genetically engineered including dicotyledonous crops, few monocotyledonous as well as some woody plants. Rice genome was sequenced under International Rice Genome Sequencing Project in 2005. In addition, technical improvements such as increased transformation efficiency, commercial germplasm storage and low production costs for transgenic plants have been done in the recent years.

Box 11.1 Contribution of Indian scientists in Plant tissue culture

In India, the plant tissue culture was initiated during 1950s at the University of Delhi. Panchanan Maheshwari showed the production of haploid plants under *in vitro* conditions. S.C. Maheshwari and Shipra Guha also made a remarkable contribution to plant tissue culture by developing haploid plants from cultured anthers of *Datura innoxia* that opened the new area of androgenesis. Techniques of *in vitro* culture of floral and seed parts were developed during this period. Endosperm cultures and plantlet regeneration via organogenesis were achieved later.

SAQ 1

Fill in the blanks with appropriate words.

a) The German Botanist …… first proposed the importance of plant tissue and cell culture.

b) …….. cultured embryos of plants like *Raphanus sativus*.

c) …… demonstrated the possibility of raising large numbers of plantlets from pollen.

d) …… first isolated protoplast by enzymatic degradation of the cell wall.

e) ……. regenerated plants from protoplasts.

11.3 EQUIPMENTS REQUIRED FOR PLANT TISSUE CULTURE

There are a few essential equipments that are required for the plant tissue culture technique. A plant tissue culture laboratory needs to have an aseptic chamber for culture and culture rooms or incubators.

Some of the basic equipments required for the tissue culture laboratory include:

Laminar airflow cabinets: The most important aspect to be taken care of in tissue culture is to maintain a contaminant-free environment within the lab. A laminar airflow cabinet provides a place free from dust particle/micro contaminants and helps in carrying out cell culture under an aseptic environment. These cabinets help in maintaining an aseptic environment.

Autoclave machine: An autoclave machine is designed for sterilization. The device will produce pressurized steam that helps in eradicating bacteria, fungi and viruses. Autoclaves can be used for dry sterilization, i.e. sterilization of scalpels and glass-wares such as Petri-dishes, pipettes etc. It is also used for sterilization of the culture medium.

Water distillation apparatus: This is used for producing distilled water or deionized water.

Culture rooms and/or Cabinets: The culture rooms/cabinets are equipped with adjustable photoperiods including the intensity of light along with temperature range so that controlled conditions can be maintained for the cultivation of the cells.

Shakers: A rotary shaker or a reciprocal shaker is required for the maintenance of suspension cultures.

A BOD incubator is required for maintaining a constant temperature to facilitate the culture of microorganisms and their subsequent maintenance. Fermentors or bioreactors are required to cultivate plant cells for the large-scale production of chemicals or compounds. A pH meter is used for adjusting the pH of the medium, a shaker to maintain cell suspension culture, a weighing balance to weigh various components of the nutrient medium. A freezer (used to store chemicals and stock solutions) is one of the basic equipment found in a tissue culture laboratory. A spirit burner or gas micro burner is needed for flame sterilization of instruments.

Incubation room or incubator provides the ideal environmental conditions required for the growth and differentiation of cultured tissues. A typical incubation chamber has light and temperature-controlled devices managed for 24 h period. AC or room heaters are required to maintain the desired temperature range. Light needs to be adjusted in the terms of photoperiod duration. Humidity in the range of 20-90% is required. Shelves are designed in a way so that the culture vessels can be placed without any hindrance in the light, temperature and humidity conditions.

Glassware is used to store cultures. Different kinds of culture vessels, large test tubes (25×150 mm), wide mouth conical flasks are required in large numbers for raising of cultures. In addition, graduated pipettes, measuring cylinders, beakers, filters, funnel, and petri dishes are also required. Scissors, scalpels and forceps are required for the preparation of explants from the excised plant.

(a)

(b)

(c)

(d)

Fig. 11.1: The basic equipments used in plant tissue culture. a) photograph of an autoclave; b) photograph of an incubator; c) photograph of a laminar air flow; d) a view of culture room.

11.3.1 Basic Steps in Plant Tissue Culture

Plant tissue culture is an *in-vitro* culture of plant cells, tissues, or organs to form a complete plant. *In-vitro* culturing of plants involves the following steps**:**

a) Selection and sterilization of explant:

A suitable explant is selected and is then excised from the donor plant. The explant is then sterilized using disinfectants. An explant is surface sterilized using mercuric chloride (0.11%), calcium hypochlorite (1-2 %) and alcohol (70%). The tissue is immersed in sterilizing agent for 10 s to 15 min, and thereafter washed with distilled water.

b) Preparation and sterilization of Culture Medium:

A suitable culture medium is prepared depending upon the type of explant to be cultured. Culture medium prepared is transferred into sterilized vessels and then sterilized in an autoclave.

c) Inoculation:

The sterilized explant is inoculated (transferred) on the culture medium under aseptic conditions.

d) Incubation:

Cultures are then incubated in the culture room under appropriate controlled conditions of light, temperature and humidity.

e) Subculturing:

Cultured cells are transferred to a fresh nutrient medium to get plantlets.

f) Transfer of Plantlets:

After the hardening process (i.e., acclimatization of plantlets to the environment), the plantlets are transferred to greenhouse or pots.

11.4 COMPOSITION OF MEDIA

Various media have been tried for raising different cultures using different explants. A suitable culture medium is required for culturing cells, tissues, protoplasts, embryos, anthers, root tips, from the plant. The explant is a part of the plant like root, stem, leaf, or meristematic tissue like cambium, floral parts like anthers, stamens, etc. Growth and morphogenesis of the plant tissues in *in vitro* conditions is governed by the composition of culture media. The principal components of plant tissue culture media include:

- macro and micronutrients,
- carbon source,
- organic supplements such as vitamins, amino acids
- growth regulators and
- a gelling agent.

A complex nutritive medium composed of chemically defined compounds is called a **synthetic medium**.

Although Knop's solution was used. Later on, Murashige and Skoog (MS) (1965) was developed as a new medium to induce organogenesis and regeneration of plants. MS media consisted of macro-and micro-nutrients, a carbon source, reduced N, B vitamins, and growth regulators. Another medium was developed by Linsmaier and Skoog in 1965. The medium has a similar component as Murashige and Skoog with some modifications of vitamins. It was used for inducing organogenesis, callus culture, cell suspension, and micropropagation. In 1968 O. L. Gamborg (Gamborg/B5) developed a

medium. This medium is a blend of nutrients like inorganic salts, vitamins, and carbohydrates. The medium has a higher concentration of nitrate and potassium and a lower concentration of ammonia. The media was used for callus, cell suspension culture and protoplast culture.

Nitsch and Nitsch (NN) medium was developed by J. P. Nitsch in 1969 for the establishment of in *vitro* anther culture of *Nicotiana*. It contains a high concentration of thiamine, biotin, and folic acid. White's medium was developed by P. R. White in 1963. This culture media was developed for root culture. It has a lower salt concentration and a higher concentration of $MgSO_4$. Another medium named N6 (Chu) was developed by Chu and co-workers in 1975 to culture anthers of rice plants. The medium promotes the initiation, growth and differentiation of callus. The medium is a blend of inorganic salts, vitamins, amino acids and carbohydrates. Media named EI was developed specifically to culture soybean, red clover and other legumes. Haberlandt's media is used to produce whole plants (such as tobacco) from a single cell.

Various components of media help in the growth of explants. The concentration of components of tissue culture media is expressed as mg/L or ppm. According to the recommendations of the International Association for Plant Physiology, the nutrients whose concentration should be greater than 0.5 g/L are **macronutrients**, while those nutrients whose concentration is less than 0.5 g/L are called **micronutrients**. Hormones, vitamins and other organic constituents are also generally added in concentrations less than 0.5 g/L.

Inorganic nutrients

These include macro and micronutrients. Macronutrients include elements namely nitrogen, phosphorus, potassium, calcium, magnesium and sulphur which are essential for plant cell and tissue growth. Micronutrients include iron, manganese, zinc, boron, copper and molybdenum. The stock solutions of macronutrients prepared are generally 10 times the strength of the working medium, while stock solutions of micronutrients are made up at 100 times the concentration of the working medium. Stock solutions are stored in a refrigerator at 2- 4º C.

Carbon source

Sucrose is the most preferred external source of carbon used in the media. Sucrose levels range from 2 to 6%.

Organic supplements

Thiamine (B1), nicotinic acid (B3) pyridoxine (B6), calcium pantothenate (B5) and myoinositol are vitamins commonly used in tissue culture. Vitamins such as thiamine, biotin, folic acid, riboflavin are also used. Vitamins are prepared as either 100 or 1000 times concentrated stock solutions. Amino acids are added to the media to stimulate the growth of cells, protoplast culture and establish cell lines. Casein hydrolysate, L-glutamine, L-glycine, L-arginine and L-cysteine are common sources of organic nitrogen used in the culture media. Organic supplements such as protein hydrolysates, coconut milk, yeast extract, malt extract, potato extract and tomato juice are also added to the media.

In some media activated charcoal is also added. It also helps in reducing toxic compounds produced during the culture and promotes the unhindered growth of cells. Sometimes antibiotics such as Streptomycin and Kanamycin are added in the culture media in very low concentrations to control systemic infection.

Growth regulators

One or more hormones are added to culture media to promote organogenesis and differentiation of tissues. Auxin and cytokinins play a role in cell division and differentiation. Culture media is generally supplemented with auxins such as indole acetic acid (IAA), naphthalene acetic acid (NAA), indole butyric acid (IBA), 2, 4 diphenoxyacetic acid (2,4 D). GA_3 is the most commonly used gibberellin. It promotes the growth of cell cultures, enhances callus growth and induces shoot elongation. The most commonly used cytokinins are 6 benzyl amino purine (BAP), 6-benzyl adenine (BA), purine (zeatin). Balance of auxin and kinetin in the medium influences morphogenesis. High levels of auxin favour rooting while low levels help in the formation of the shoot and intermediate levels to the proliferation of callus or wound parenchyma tissue. Stock solutions of growth regulators are usually prepared at 100-1000 times the final desired concentration and stored in a freezer (-20^{o}C) for 2-3 months.

Gelling agent

A solidifying or gelling agent is added for preparing semisolid or solid media. Agar, a compound obtained from seaweeds is generally used as a gelling agent. Generally 0.5 to 1% of agar is added to the medium.

Iron source such as Iron EDTA is also added to the media. The stock solutions of Fe are 100 times concentrated than the final working medium.

The pH of the media is also important as it affects the growth of plants and the activity of plant growth regulators. It is adjusted to the value between 5.4 - 5.8. Both the solid and liquid mediums can be used for culturing.

Full, half, or quarter strength media is prepared and this is based on the explants, type of culture and many other conditions. The composition of some commonly used media used in tissue culture has been given in Table 11.2.

Table 11.2: Composition of various Plant Tissue Culture Media.

Contents	Type of media					
	MS	**White**	**B5**	**Nitsch**	**Heller**	**NT**
Inorganic						
NH_4NO_3	1650	-	-	720	-	825
KNO_3	1900	80	2528	950	-	950
$CaCl_2.2H_2O$	440	-	150	-	75	220
$CaCl_2$	-	-	-	166	-	-
$MgSO_4.7H_2O$	370	750	247	185	250	1233
KH_2PO_4	170	-	-	68	--	680
$(NH_4)_2SO_4$	-	-	134	-	-	-
$Ca(NO_3)_2.$ $4H_2O$	-	300	-	-	-	-

$NaNO_3$	-	-	-	-	600	-
Na_2SO_4	-	200	-	-	-	-
$NaH_2PO_2.H_2O$	-	19	150	-	125	-
KCl	-	65	-	-	750	-
KI	0.83	0.75	0.75	-	0.01	0.83
H_3BO_3	6.2	5	10	3	1	6.2
$MnSO_4.4H_2O$	22.3	5	-	25	0.1	22.3
$MnSO_4.H_2O$	-	-	10	-	-	-
$ZnSO_4.7H_2O$	8.6	3	2	10	1	-
MoO_3	-	0.001	-	-	-	-
$CuSO_4.5H_2O$	0.03	0.01	0.03	0.25	0.03	0.25
$CoCl_2.6H_2O$	0.03	-	0.03	-	-	-
$CoSO_4.7H_2O$	-	-	-	-	-	0.03
$AlCl_3$	-	-	-	-	0.03	-
$NiCl_2.6H_2O$	-	-	-	-	0.03	-
$FeCl_3.6H_2O$	-	-	-	-	1	-
$Fe_2(SO_4)_3$	-	2.5	-	-	-	-
$FeSO_4.7H_2O$	27.8	-	-	27.8	-	27.8
$Na_2EDTA.2H_2O$	37.3	-	-	37.3	-	37.3
Sequestrene 330 Fe	-	-	28	-	-	-
Organic						
Inositol	100	-	100	100	-	100
Nicotinic acid	0.5	0.05	1	5	-	-
Pyridoxine.HCl	0.5	0.01	1	0.5	-	-
Thiamine HCl	0.1	0.01	10	0.5	-	-
Glycine	2	3	-	2	-	-
Folic acid	-	-	-	0.5	-	-
Biotin	-	-	-	0.05	-	-
Sucrose	3%	2%	2%	2%	-	-
D Mannitol	-	-	-	-	-	12.70%

The constituents are given in the unit mg/L.

SAQ 2

Answer in one word

a) The part of the plant used in tissue culture.

b) Chemical used for the surface sterilization of the explants.

c) The primary source of carbon used in the media.

d) Exogenous balance of these hormones in the medium influences the morphogenic fate of callus.

e) A relatively high level of this plant hormone favors rooting.

f) The medium containing a blend of nutrients like inorganic salts, vitamins, and carbohydrates. The medium has a higher concentration of nitrate and potassium and a lower concentration of ammonia**.**

g) Nutrients required in concentrations less than 0.5 g/L.

11.5 TOTIPOTENCY

In plant tissue culture technique, an explant is taken that is cultured on a nutrient medium under the controlled conditions and develops into a whole new plant. The potential of a plant cell to grow and develop into a whole new multicellular plant is described as cellular **totipotency**. It depends upon the inherent capacities of plant cells for differentiation. In other words, the capacity of a cell to get differentiated into other cell types is called totipotency. The term totipotency was coined in 1901 by Morgan. Totipotency of cells was first hypothesized in the mid-19th century based on the regeneration capacity of plants. In 1953, Muir was able to regenerate plants from isolated cells, demonstrating the theory of cellular totipotency.

The potential of totipotency depends upon the differentiation potential of cells. The cell undergoes dedifferentiation and re-differentiation (two inherent processes). Differentiation (cyto-differentiation) is the phenomenon that involves reverting of mature cells to dividing state. The ability of a dedifferentiated cell to form a whole plant or plant organs is known as redifferentiation. Differentiation of one organ directly from another organ is known as trans-differentiation. To express totipotency, a differentiated cell first undergoes dedifferentiation followed by re-differentiation.

Different plant parts and plants show different totipotent abilities. For e.g. mostly somatic cells in the plant body are totipotent.

Various applications of cellular totipotency are:

- Crop improvement
- Micro-propagation of commercially important plants
- Production of artificial or synthetic seeds
- Conservation of germplasm (genetic resources)
- Production of haploids
- Production of somatic hybrids and cybrids
- Cultivation of plants whose seeds are very minute and difficult to germinate
- High scale and efficient production of secondary metabolites
- Genotypic modifications

SAQ 3

Match the following:

	Column A		Column B
a)	Totipotency	1.	Murashige and Skoog medium
b)	Unorganized mass of cells	2.	Muir
c)	Demonstrated the theory of cellular totipotency	3.	Morgan
d)	Most extensively used medium in tissue culture	4.	Callus
e)	Complex nutritive medium	5.	Synthetic medium

11.6 ORGANOGENESIS

The development of organs like roots, shoots, and flowers directly from an explant or from the callus is called organogenesis. In other words, it is the development or regeneration of a complete organized structure i.e. whole plant from the cultured cells/tissues. Organogenesis occurs due to stimulus produced by the components of culture medium, substances present in the explants and compounds produced during culturing.

The hypothesis of organogenesis was given by Torrey (1966). He suggested that organogenesis starts with callus formation via the development of a group of meristematic cells i.e. meristemoids. The meristemoids initiate the formation of primordium which forms either shoot or embryoid. Shoot bud differentiation was first demonstrated by White (1939). Organogenesis may occur either through callus formation (indirect) or through the formation of adventitious organs (like adventitious shoot) (direct) (Fig. 11.2). The direct mode of organogenesis does not involve the formation of callus. Organogenesis has been obtained from the culture of various explants such as cotyledons, hypocotyl, stem leaf, shoot apex, root, young inflorescence, flower petals, petiole and embryos. Hence the process of organogenesis involves two steps: dedifferentiation and redifferentiation. Dedifferentiation results in the formation of callus from the explant tissue whereas redifferentiation causes the development of primordia from a group of callus cells.

The two main steps of Organogenesis are caulogenesis and rhizogenesis**.** The initiation of root formation (adventitious roots) is termed **rhizogenesis** while the initiation of shoot formation is called **caulogenesis**. Caulogenesis starts with the induction of adventitious shoot buds. Caulogenesis is induced by the optimal concentration of cytokinin present in the media. Rhizogenesis is induced only after caulogenesis.

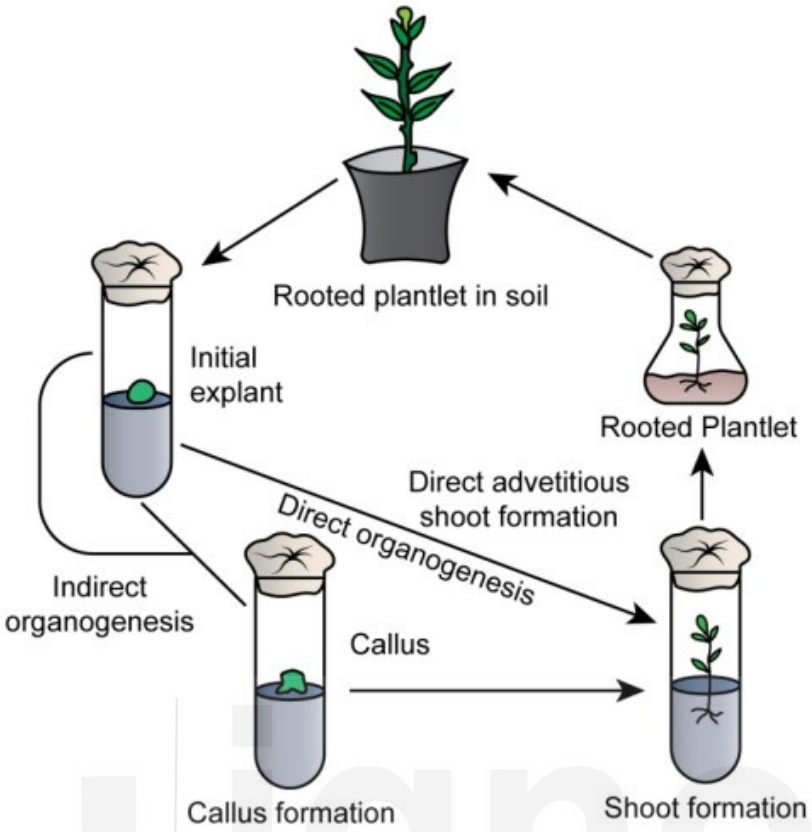


Fig. 11.2: Diagrammatic representation of various steps involved in organogenesis.

Various culture media are used for organogenesis include MS (Murashige and Skoog (1962), B5 (Gamborg (1969), white's medium (White, 1963) and SH (Schenk and Hildebrandt, 1972).

The process of organogenesis is regulated by various chemical and physical factors such as physiological state and size of explants, medium and environmental conditions such as temperature, quality and quantity of light. High light intensity has been shown to inhibit shoot-bud formation. Blue light promotes shoot-bud differentiation while red light stimulates rooting. Temperature up to 33° C supports growth of callus but lower temperature (18° C) stimulates differentiation of shoot-bud.

The concentration of growth regulator is critical for morphogenesis. Skoog and Miller (1957) were the first to demonstrate the role of growth regulators in morphogenesis. Skoog indicated that organogenesis is chemically controlled i.e. chemical supplements in media regulate the process of organogenesis. The formation of root and shoot is affected by the proportion of auxin and cytokinins. A moderate to a high concentration of auxin such as 2, 4-D induces callus formation by promoting the proliferation of cells. A relatively high concentration of auxin favours cell proliferation and root differentiation while high levels of cytokinin promote shoot bud differentiation.

Organogenesis proves very useful in understanding the mechanism of the action of hormones (plant growth regulators), differentiation of cells and tissues, development of plant organs, and other molecular mechanisms involved in the plant.

SAQ 4

Differentiate between the following:

a) Dedifferentiation and Redifferentiation.

b) Rhizogenesis and Caulogenesis.

c) Direct and Indirect organogenesis.

11.7 EMBRYOGENESIS OR EMBRYOGENIC DIFFERENTIATION

When embryos regenerate from somatic cells of plant organs, the process is called somatic embryogenesis or embryogenic differentiation or embryogenesis. Embryos formed from the somatic cells of the embryo sac (or cells surrounding it) without gametic fusion under *in-vitro* conditions are called somatic embryos or embryoids.

Induction of embryogenesis is achieved by two methods: direct and indirect method. Direct embryogenesis involves the development of embryos directly from the cells of explants or immature embryos. In indirect embryogenesis, an intermediate step of callus growth is involved in the process. Explants are cultured in a media containing growth regulators to produce embryogenic callus which is later transferred to the proliferation medium.

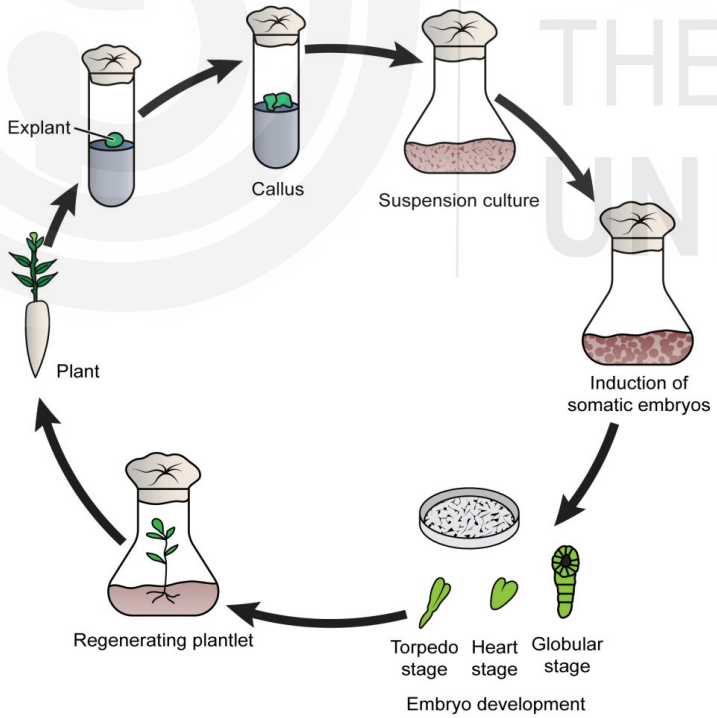


Fig. 11.3: Diagrammatic representation of various steps involved in the formation of plants from somatic embryos.

Somatic embryogenesis was first observed by Steward and his coworkers (1958) in carrot (*Daucus carota*). Thereafter, somatic embryoids were obtained from many plants namely Citrus, Coffea, *Zea mays*, etc. Embryoids can be produced in specialized nutrient media. The process of formation of somatic

embryos has three main steps which include the induction of embryogenesis, embryo development, and embryo maturation (Fig.11.3).

Embryogenesis is affected by physiological conditions, genotype of the plant and type of explants. Auxin plays an essential role in the first step of embryogenesis.

Importance

Somatic embryos (SEs) act as a valuable source for the large-scale propagation of germplasm resources. The process of somatic embryogenesis doesn't involve the steps of fertilization (sexual fusion of gametes) and thus, facilitates the rapid large-scale propagation of the plants. It also acts as a powerful tool for cryo-storage of the embryo and valuable germplasm.

SAQ 5

Differentiate between direct and indirect embryogenesis.

11.8 PROTOPLAST CULTURE

A plasma membrane-bound vesicle formed as a result of removal of the cell wall is called a protoplast. *In vitro* fusion of plant protoplasts derived either from a somatic cell of the same plant or from two genetically different plants is called somatic hybridization. The protoplasts are generally isolated from leaf and mesophyll cells. The fused protoplast (parasexual hybrid protoplast) is grown *in vitro* to produce a plant. The protoplast can be cultured and regenerated into a whole plant. Takebe and his co-workers obtained tobacco plants from mesophyll protoplasts.

J. Klercker (1892) first mechanically isolated protoplast from the plasmolyzed cell of water warrior (*Stratiotes aloides*). E. Kiister (1927) isolated protoplasts from fruits of several plants like *Solatium nigrum, Lycopersicon esculetum,* etc. Kuster used a physiological method that involved hydrolysis of the cell wall for isolating protoplasts. E. C. Cocking (I960) first reported the enzymatic method for isolation of protoplast in a large number from root tip cells of *Lycopersicon esculentum.* He used a concentrated solution of cellulase enzyme, prepared from cultures of the fungus *Myrothecium verrucaria* to degrade the cell wall. I. Takebe, Y. Otsuki and S. Aoki (1968) used cellulase and macerozyme sequentially (in two steps) for the isolation of mesophyll protoplast of tobacco. J. B. Power and E. C. Cocking (1968) demonstrated for the first time that a mixture of two enzymes (cellulase + macerozyme) can be used simultaneously (one-step method) for getting isolation of protoplasts. I. Takebe, G. Labib, G. Melchers (1971**)** reported the plant regeneration from isolated protoplast in *Nicotiana tabacum*.

The protoplasts are isolated from the plants utilizing chemical or enzymatic procedure. The cell wall is removed by mechanical or enzymatic methods. Isolated protoplast is placed in a hypertonic solution of a metabolic sugar such as mannitol (13%) to prevent plasmolysis (Fig 11.4). The isolated protoplasts are purified and then tested for their viability. The viable protoplasts are cultured under *in-vitro* conditions using a suitable nutrient medium (either a liquid medium or a semisolid agar medium).

Fusion of isolated protoplasts can be done mechanically by bringing the isolated protoplasts in intimate physical contact mechanically under microscope using micromanipulator and perfusion micropipette. The fusion of protoplasts can also be induced by some chemical agents called fusogens. Some commonly used fusogens are Poly Ethylene Glycol (PEG), high concentration of Ca^{2+}, Dextran, Poly Vinyl Alcohol, and synthetic phospholipids. The polymer binds with the lipid membrane of cells and thus induces fusion. The protoplasts carry negative charges and repel one another. A fusogen reduces electronegativity and induces fusion. Fusion of protoplast can also be induced by an electric current of low strength which is followed by an electric impulse of high intensity (100 KV/m) applied for microseconds.

Protoplast fusion or somatic cell hybridization produces a hybrid cell that contains two nuclei of two different parental protoplasts. The presence of two different parental nuclei in the hybrid cell, i.e. heterodikaryotic condition can be distinguished using the Carbol fuchsin staining technique or conventional aceto-orcein or aceto-carmine staining. Non-toxic fluorochromes (fluorescein isothiocyanate or FITC) or (rhodamine isothiocyanate or RITC) can also be used for this. Heterokaryon exhibits apple-green fluorescence when FITC is used and red fluorescence when rhodamine isothiocyanate is used.

Protoplast viability is influenced by the growing conditions such as light, photoperiod, humidity, temperature, nutrition, and water, age of the plant and leaf.

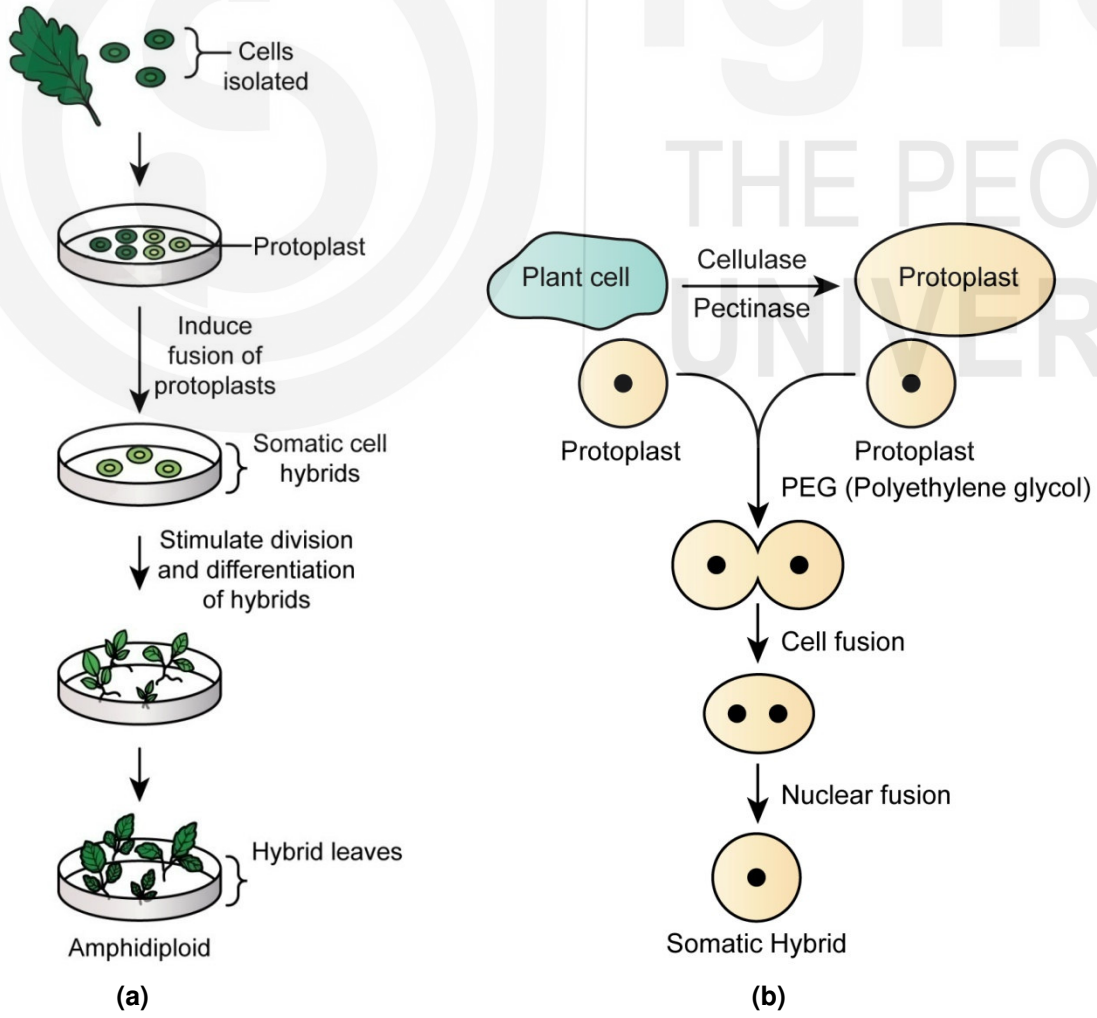


Fig. 11.4: a) Various steps in somatic hybridization; b) Flow diagram depicting process of formation of somatic hybrid.

Importance of Protoplast Culture

- Protoplast culture has immense potentialities for the improvement of different plant species and varieties having high economic values. The practical applications of protoplast culture include:
- Somatic hybrids can be developed between two sexually incompatible species. Thus it proves the best method with the potential of overcoming the sexual incompatibility barrier between plants.
- Cybrids (hybrids) which contain the nuclear and cytoplasmic genome of one parent and only the cytoplasmic genome of the second parent are produced by protoplast fusion. Cytoplasmic male sterile (CMS) lines may be developed through protoplast fusion.
- Isolated plant protoplasts act as suitable material for genetic and cytoplasmic modification experiments.
- Protoplasts are the ideal target for direct gene transfer. Direct gene transfer can be done by using isolated protoplasts. Transfer of genes for disease resistance, N_2 fixation, rapid growth rate, protein quality, frost hardiness and drought resistance has been successfully done through protoplast culture.
- In case of vegetatively propagated plants, genetic variation can be induced through the protoplast fusion of two species, varieties, or two different genera.
- Production of plants with greater hybrid vigour has been obtained using somatic hybrids. Success has been achieved in apomictic development of somatic hybrids through protoplast fusion in cases of orange (*Citrus* spp.) and mango (*Mangifera indica*).
- Regeneration of plants can be done using protoplast culture in several forest tree taxa. For e.g. *Eucalyptus*, *Populus alba*, *Picea*, *Abies*, Larix and *Pinus taeda*.
- Protoplast fusion technology is used for the fusion of plant cells and yeast cells and is important for the characterization of yeast artificial chromosomes (YAC), a potential vehicle for gene transfer.
- Protoplast culture has been used for the improvement of cereal crops like wheat and maize. Successful production of tetraploids and triploids of orange (*Citrus* spp.) has been achieved through protoplast fusion. Intergeneric hybrids have been developed through protoplast culture in *Brassica*.

SAQ 6

Fill in the blanks with appropriated words;

a) A membrane-bound vesicle which consists of a naked cell formed as a result of removal of the cell wall is called …….. .

b) …… is the first reported enzymatic method for isolation of protoplast from root tip cells of *Lycopersicon esculentum*.

c) An essential step in the isolation of protoplast is the removal of the …… from the cell.

d) Fusion induced by some chemical agents called fusogens is called …… .

e) Polyethylene glycol (PEG) has been identified as a potent chemical used for …… .

11.9 SUMMARY

- In the plant tissue culture technique, the cell, organs are grown under *in-vitro* aseptic conditions by culturing explants in a nutrient medium.
- Plant tissue culture has a great significance in crop improvement programs. It helps in getting large-scale production of plants in short time duration and improving the growth of plants of economic importance.
- The principal components of most tissue culture media include inorganic nutrients (macro and micronutrients), carbon sources, organic supplements, growth regulators and a gelling agent. Besides these vitamins, amino acids are also supplemented in the media.
- The potential of a plant cell to grow and develop into a whole new multicellular plant is called cellular totipotency. It depends upon the inherent capacities of plant cells for differentiation.
- Organogenesis is defined as the development of organs like roots, shoots, and flowers directly from an explant or the callus. The process of organogenesis involves two steps: dedifferentiation and redifferentiation.
- The somatic cells of plant organs develop into embryos which are called somatic embryos. The process is called somatic embryogenesis or embryogenesis. Somatic embryos are obtained indirectly (with the formation of callus) or directly from the explant.
- The protoplast can be cultured and regenerated into a whole plant. The first demonstration of the totipotency of protoplasts was by Takebe and co-workers.

11.10 TERMINAL QUESTIONS

1. Describe in brief the various components of the tissue culture medium.
2. What is the role of plant growth hormones in tissue culture medium?
3. What is totipotency? Enumerate its applications.
4. Organogenesis is chemically controlled. Justify the statement giving a suitable explanation.

5. Enumerate the various steps of protoplast culture.
6. Discuss the importance of the technique of protoplast fusion in tissue culture.

11.11 ANSWERS

Self Assessment Questions

1. a) Guttlieb Haberlandt
   b) Hanning
   c) Guha and Maheshwari
   d) Cocking
   e) Takebe and co-workers
2. a) explants
   b) mercuric chloride
   c) Sucrose
   d) auxin and kinetin
   e) auxin
   f) Gamborg (B5) medium
   g) micronutrients
3. a) Morgan
   b) Callus
   c) Muir
   d) Murashige and Skoog medium
   e) Synthetic medium
4. a) Refer to Section 11.5.
   b) Refer to Section 11.5.
   c) Refer to Section 11.5
5. Refer to Section 11.6
6. a) protoplast.
   b) E. C. Cocking
   c) cell wall
   d) Chemofusion
   e) fusogen

Terminal Questions

1. Refer to Section 11.3.
2. Refer to Section 11.3.
3. Refer to Section 11.4.
4. Refer to Section 11.6.
5. Refer to Section 11.7.
6. Refer to Section 11.7.

Acknowledgements

Fig. 11.1 : a) https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fwww.narang.com%2Fimages%2Fthumbnails%2Fautoclaves-pressure-steam-sterilizers.jpg&imgrefurl=https%3A%2F%2Fwww.narang.com%2Fautoclave-sterilizers%

b) https://www.google.co.in/imgres?imgurl=https%3A%2F%2F5.imimg.com%2Fdata5%2FTA%2FGF%2FKO%2FSELLER-3125581%2Flaboratory-incubators-500x500.jpg&imgrefurl

c) https://www.google.co.in/imgres?imgurl=http%3A%2F%2Fwww.pureairsystemindia.com%2Finnovtouch%2Fuploads%2F2021%2F07%2F2.png&imgrefurl=http%3A%2F%2Fwww.pureairsystemindia.com%2Fproducts%2Flaminar-airflow%2F&tbnid

d) https://www.google.co.in/imgres?imgurl=https%253A%252F%252Fthumbs.dreamstime.com%252Fz%252Fplant-tissue-culture-collection-shelves-tissue-culture-room-science-laboratory-techniques-used-to-maintain-grow-plant-

UNIT 12

PLANT TISSUE CULTURE (II)

Structure

12.1 Introduction

Objectives

12.2 Applications of Plant Tissue Culture

12.3 Micropropagation

12.4 Haploid Production through Androgenesis and Gynogenesis

Applications of Haploids

12.5 Embryo Culture

Culture of Immature Embryos/Embryo Rescue

Applications of Embryo Culture

12.6 Secondary Metabolite Production

12.7 Germplasm Conservation

Methods of Germplasm Conservation

Applications of Germplasm Storage

12.8 Summary

12.9 Terminal Questions

12.10 Answers

12.1 INTRODUCTION

In the previous units, we have discussed various techniques of tissue culture and their uses. Tissue culture is a term used for methods that help in maintaining and propagating plant cells and/or organs *in vitro* under aseptic conditions. In tissue culture technique, the property of cellular totipotency helps in getting differentiation of tissues, organs and the regeneration of the entire plant. Clonal propagation of plants in a laboratory is done *via* micropropagation. Explants obtained from a donor plant are cultured in sterile conditions in tissue culture media. Explants develop into a complete plantlet through somatic embryogenesis or organogenesis through callus formation. This technique is used in genetic engineering to raise transgenic plants, germplasm conservation, forestry, horticulture and many more.

In this unit, various applications of plant tissue culture such as conservation of plant genetic resources, haploid production through androgenesis,

gynogenesis and production of secondary metabolites have been included. *In vitro* methods have proved helpful in overcoming limitations found in conventional methods. All the above-mentioned methods have helped in the conservation of fast vanishing biodiversity.

Objectives

After studying this unit, you would be able to:

- enlist different ways in which plant genetic resources can be conserved;
- have a complete understanding of pollen haploids and their applications in agriculture and horticulture;
- appreciate embryo culture and its applications;
- differentiate between primary and secondary metabolites of plants;
- describe the use of plant tissue culture for the production of secondary metabolites;
- describe the role of plant tissue culture in germplasm conservation; and
- explain the technique of cryopreservation.

12.2 APPLICATIONS OF PLANT TISSUE CULTURE

The tissue culture technique has been extensively used for vegetative multiplication of plant species, getting virus-free plants, production of somatic hybrids, genetic transformation and germplasm preservation. The process involves growing/culturing plants cells in an agar medium to form the undifferentiated mass of cells in the form of callus or as suspension culture mass of free cells in a liquid medium (suspension culture). Some of the major applications of plant cell and tissue culture are enumerated below:

1. Clonal propagation

The most widely used application of tissue culture technology is the vegetative propagation of plants. Mass clonal propagation of plants can be achieved using this technique. Clonal propagation refers to the multiplication of genetically identical copies of individual plants (clones) through the process of asexual reproduction. Clonal propagation involves the culture of apical shoots, axillary buds and meristems on a suitable nutrient medium. Shoot meristems can be regenerated in large numbers and thus many plantlets can be produced (Fig. 12.1).

Clonal propagation is the rapid and large-scale production of plants from the same genetic stock in a very short time. The technique of clonal propagation is very useful commercially as several identical copies of plants can be obtained. This technique is widely used in horticulture and silviculture. The plant material can be provided in large quantities throughout the year irrespective of seasonal variation. A large population of plant species with desirable traits can be obtained using clonal propagation. This technique helps in providing

disease-resistant plants, multiplication of sexually derived sterile hybrids, overcoming long seed dormancy of tree species. This technique also facilitates the long-term storage of valuable germplasm. Clonal propagation has been successfully done in plant species with dormant seeds, tree species, orchids and many fruit plants. Apple, pears, strawberry, cardamom and many ornamentals like orchids have been successfully propagated.

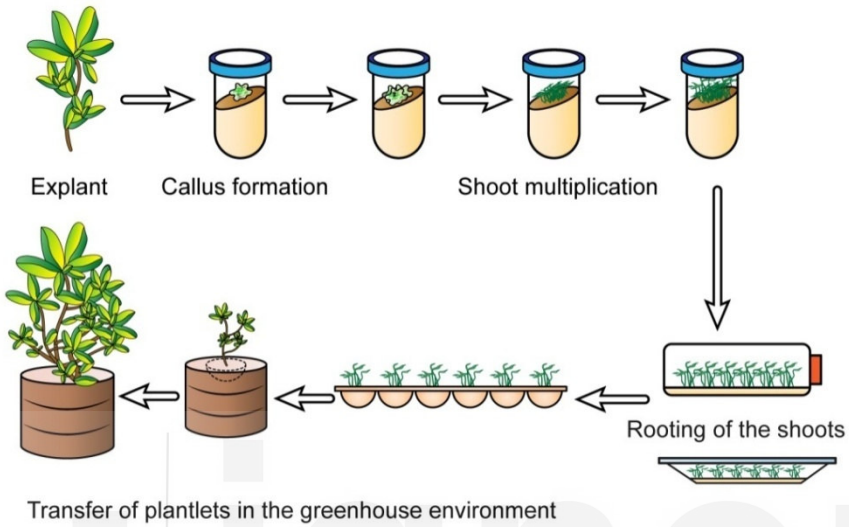


Fig. 12.1: Production of clones *via* clonal propagation.

2. Production of virus-free plants

Synthetic seeds or artificial seeds include encapsulated somatic embryos, shoot buds, cell aggregates, or any other meristematic tissue. These seeds have the ability to form a whole plant. The nutrients present inside the encapsulation ensure and promote development of the plants.

Tissue culture has been used to produce virus-free plants for many crops and ornamental plants at a commercial level. The meristems of *in vitro* raised plants are generally free of infection, therefore meristem-tip culture is done to obtain virus and pathogen-free plants. Virus-free plants of several economically important plant species have been raised using the meristem culture. For example: Potato virus X from potato, and mosaic virus from cassava have been removed using the same plant tissue culture technique.

In vitro propagation of forest trees on a commercial scale has been done using the technique of micropropagation. It has been successfully done for several economically important tree species like *Acacia nilotica*, *Albizia lebbeck, Azadirachta indica*, *Butea monosperma*, *Dendrocalamus strictus*, *Shorea robusta*, *Tectona grandis*, *Cedrus deodara*, *Cryptomeria japonica*, *Picea smithiana* and *Pinus sylvestris*.

3. Production of synthetic seeds

Somatic embryos produced by tissue culture are encapsulated in a suitable matrix (e.g. sodium alginate), along with substances like mycorrhizae, insecticides, fungicides and herbicides to form “artificial seeds”. These seeds can be utilized for rapid and mass propagation of desired plant species and hybrid varieties. The synthetic seeds have several advantages such as :

i) easy to handle

ii) act as useful units of delivery

iii) can be easily stored for a long time without loss of viability

iv) directly sown in the soil like natural seeds without any acclimatization in the green house.

4. Breaking Dormancy

The embryo (zygotic) culture technique helps in reducing the period of seed dormancy. The breeding cycle can be shortened in many plants. Two generations of flowering can be obtained in many plant species. For e.g. *Rosa* sp., *Malus* sp, *Ilex* sp., *Telia americana* etc. The life cycle of *Iris* was reduced from 2-3 years to less than one year using this technique.

5. Haploid Plants

Haploid plants can be obtained through anther or pollen culture (androgenesis) or ovaries or ovule culture (gynogenesis). The anther culture and haploid plant production has been done in many of the crop plants. Haploids are of immense importance for the production of homozygous diploid or polyploid lines without going into a series of backcrossing in a very short period. This technique is of immense utility to a plant breeder as the time of breeding gets reduced.

Haploids are sterile and possess a single set of chromosomes. They can be converted into homozygous diploids by spontaneous or induced chromosome doubling. The doubling of chromosomes helps in restoring the fertility of plants. The double haploids become pure breeding new cultivars. The production of haploids helps in overcoming the constraints of seed dormancy and the non-viability of the embryo. Haploid plants show resistance to various biotic and abiotic stresses and other desired traits.

6. Embryo rescue

In some plants, seeds take a long time to germinate and sometimes the seeds do not germinate at all. Embryo culture can rescue this situation by embryo rescue technique. The seeds are surface sterilized and split open in aseptic condition and the tiny embryo is excised and planted in a nutrient medium and then grows to a complete plant. Immature embryos can be recovered or regenerated by tissue culture technique.

7. Production of Secondary Metabolites

Secondary metabolites are the cell constituents that are not essential for the survival of plants but act as a good source of various compounds that are useful at the commercial level. Plants act as the source of various biochemicals which can be primary or secondary metabolites. These compounds find their application in pharmacy, medicine and industry. These metabolites possess antimicrobial, antibiotic, insecticidal, hormonal and pharmacological properties. These include alkaloids, glycosides (steroids and phenolics), terpenoids, latex, and tannins, etc. Secondary metabolites are the most important chemicals produced by using cell culture. As the cells undergo morphological differentiation and maturation during plant growth, some of the cells specialize to produce secondary metabolites. Differentiated tissues produce a high quantity of secondary metabolites under *in vitro* conditions. The cultured cells produce biochemical compounds in high amounts under appropriate culture conditions. The change in the physiological and biochemical conditions enhances the production of these biochemicals in cells.

Enhanced productivity of secondary metabolites can be achieved *via* tissue culture technique. Elicitors are the chemical compounds or molecules that stimulate the production of metabolites. Elicitors can be of plant or microbial origin. The elicitors of plant origin include polysaccharides derived from cell walls for example – pectin, cellulose.

8. Genetic Variability/Somaclonal Variations

Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits. Such a variation is known as somaclonal variation. This genetic variability is because of various ploidy levels of cells and the genetic constitution of the explant. Different cultural conditions can also induce such variations. Somaclonal variation may be utilized in the improvement of crops since this reduces the time required for the release of the new variety.

Such variations also show some useful characteristics such as resistance to a particular disease, herbicide resistance, stress tolerance, etc. and some agronomical traits like tiller number, panicle size, flowering time, plant height, lodging resistance, yield, nutrient content, and different kinds of morphological variations in leaf.

9. Somatic Hybrids

Somatic cell hybridization/ parasexual hybridization or protoplast fusion acts as a good method for obtaining hybrids between species or genera with desirable traits that cannot be produced *via* the conventional method of sexual hybridization. Regeneration of plants by culturing protoplast *in vitro* has opened a new avenue in various fields of plant breeding and plant biotechnology. Products of fusion between two protoplasts (heterokaryon) are cultured to regenerate a new somatic hybrid plant of the desired genotype. Cybrid obtained by fusion of two protoplasts helps in the production of male sterile line. Genes providing resistance to disease resistance such as Tobacco mosaic virus, potato virus X, club rot disease can be successfully transferred to species of interest.

10. Transgenic Plants

Transgenic plants are genetically modified (GM) plants in which a foreign gene has been incorporated by the biotechnological procedure. Several transgenic plants have been produced by incorporating genes for different traits such as insect resistance, herbicide tolerance, delayed ripening, increased amino acid, vitamin content and improved oil quality.

Various methods have been used to introduce foreign genes *via* plant tissue culture. These include direct (electroporation, microinjection, or particle bombardment) or *Agrobacterium*-mediated indirect processes. Important crops can be improved by genetic engineering by isolating a specific gene and then transferring it to selected crop plants.

11. Plant Germplasm Conservation

Germplasm refers to the sum of all the genes present in a crop and its related species. The conservation of germplasm involves the preservation of the genetic diversity of a particular plant or genetic stock for its use at any time in

the future. It is important to conserve the endangered plants or valuable genetic traits present in the existing and primitive plants otherwise they will be lost. The germplasm is preserved in the following two ways:

a) ***In-situ* conservation -** The germplasm is conserved in natural conditions by establishing biosphere reserves such as national parks, sanctuaries. This is used in the preservation of land plants in their natural habitat along with several wild types.

b) ***Ex-situ* conservation -** This method is used for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic material in the form of seeds or *in vitro* cultures is preserved and stored as gene banks for long-term use.

12. Biomass production

The plant tissue culture has proved useful in improving the capacity of the plant to produce more biomass by providing appropriate conditions such as suitable culture media. Optimum culture conditions and nutrient medium increase production of biomass by several folds. The enhancement in the biomass production depends upon plant species, explants used and composition of the medium. Various applications have been summarized in Fig 12.2.

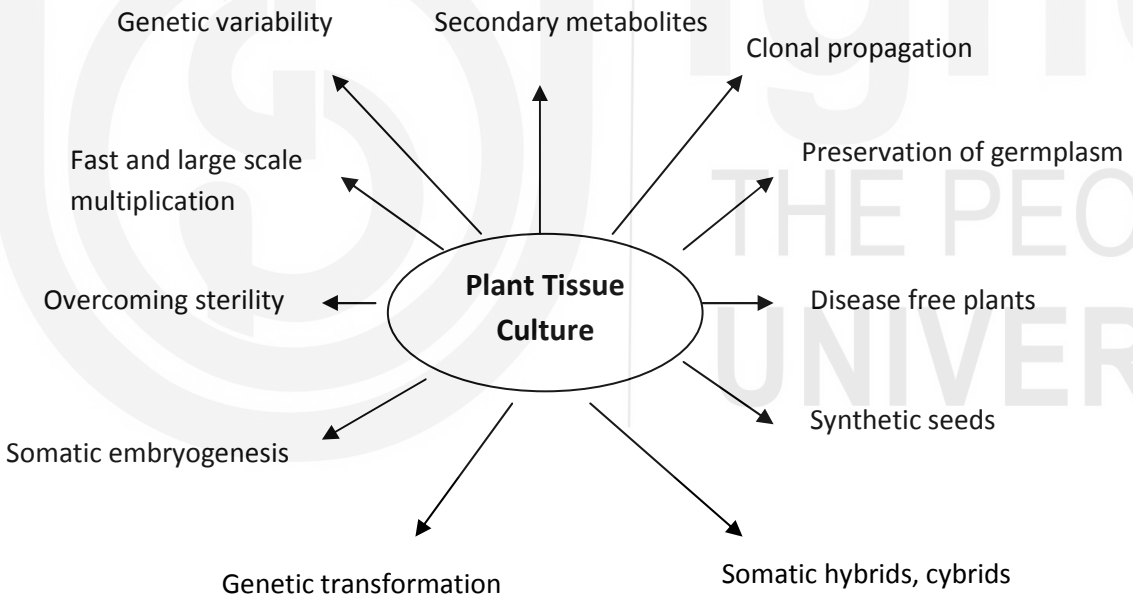


Fig. 12.2: An overview of various applications of plant tissue culture.

You will be made familiar with various techniques of germplasm conservation in the subsequent sections of this unit.

SAQ 1

State whether the statements given below are True (T) or False (F):

a) The vegetative propagation of plants is a labour-intensive seasonal process that is low in productivity.

b) The methods involved in the *in vitro* conservation of germplasm include cryopreservation, low pressure and low oxygen storage, and cold storage.

c) Virus-free plants in tissue culture are produced by nodal culture.

d) Somaclonal variation arises because of chromosome structural changes like deletions, duplication, and gene mutations.

e) The major benefits of synthetic seeds include ease to store without viability loss, rapid and mass propagation.

12.3 MICROPROPAGATION

Multiplication of genetically identical copies of a plant species by asexual reproduction is called **clonal propagation**. In nature, clonal propagation occurs by apomixis (seed development without meiosis and fertilization) and/or vegetative propagation (regeneration of new plants from vegetative parts). Tissue culture has become a popular method for the vegetative propagation of plants. Clonal propagation under *in vitro* conditions is called **Micropropagation**. A large number of the same types of plants can be obtained in a relatively short time from a single individual. Micropropagation is the only commercially viable method of clonal propagation for many horticultural crops such as Orchids.

Micropropagation starts with the selection of plant tissues (explants or any part of the plant such as leaf, apical meristem, bud and root) from a healthy, vigorous parent plant. The whole process can be summarized into the following stages as shown in Fig. 12.3.

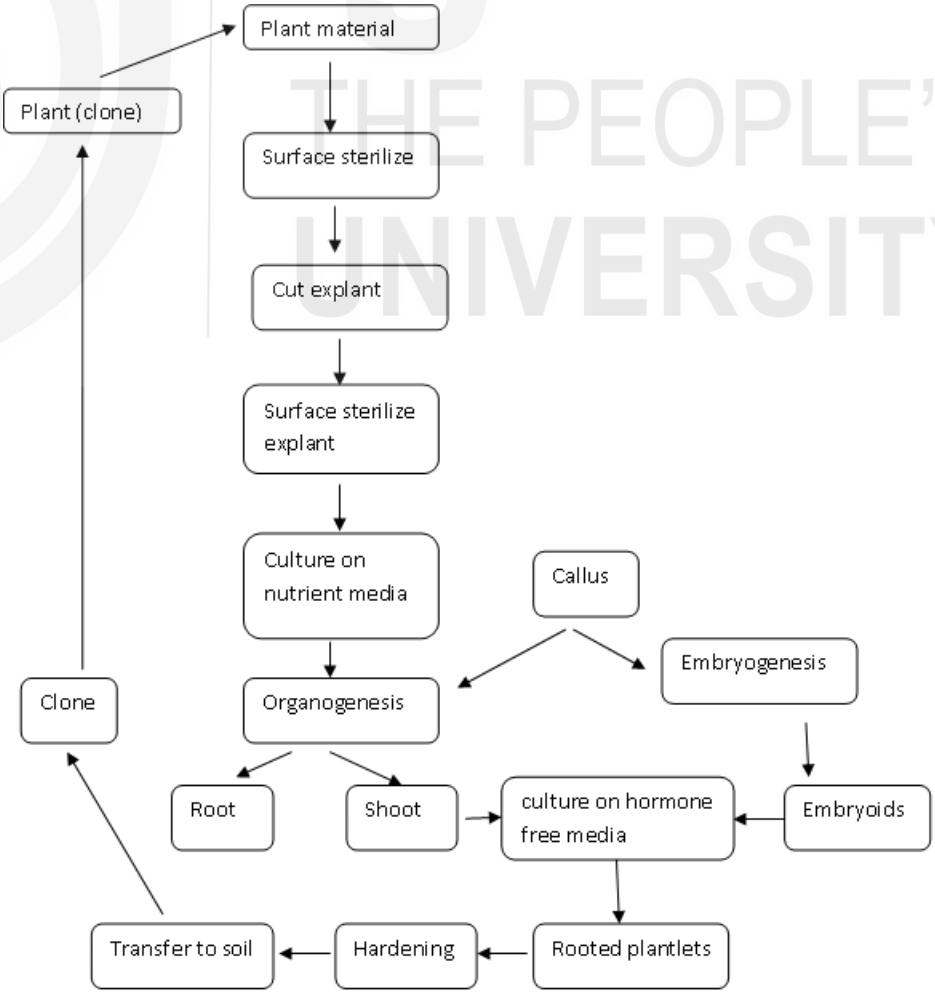


Fig.12.3: A summary flow-chart of various steps involved in micropropagation.

Explants used in micropropagation

Different kinds of explants have been used in micropropagation. In orchids, shoot tip (*Anacamptis pyramidalis, Aranthera, Calanthe, Dendrobium*, *Cymbidium, Odontioda*), axillary bud (*Aranda, Brassocattleya, Cattleya, Laelia*), inflorescence segment (*Aranda, Ascofinetia, Neostylis, Vascostylis*), lateral bud (*Cattleya, Rhynocostylis gigantean*), leaf base (*Cattleya*), leaf tip (*Cattleya, Epidendrum*), nodal segment (*Dendrobium*), flower stalk segment (*Dendrobium, Phalaenopsis*) and root tips (*Neottia, Vanilla*) are being used in micropropagation.

Various steps involved in the process of micropropagation have been listed below:

Stage 0: Preparation of donor plant

Suitable plant tissue or explant is taken from the plant growing under natural conditions and cultured under *in vitro* conditions.

Stage I: Initiation stage

In this stage, initiation and establishment of a culture in a suitable medium is done. The explant is surface sterilized and transferred into a nutrient medium. The surface sterilization removes contaminants with minimal damage to plant cells. The most commonly used disinfectants are sodium hypochlorite, calcium hypochlorite, ethanol and mercuric chloride. Thereafter, the cultures are incubated in a growth chamber either under light or dark conditions.

Stage II: Multiplication stage

This stage mainly involves the multiplication of shoots or formation of embryo from the explant. The cultures are maintained in growth chamber at of 20–24° C and light intensity of 2000- to 4000-lux. During this stage, the number of propagules is increased. This is done by repeated subcultures until the desired number of plantlets are obtained.

Stage III: Rooting stage

This stage involves the transfer of shoots to a medium that help in the formation of roots. The rooting is induced by addition of plant growth regulators such as auxins in the media.

Stage IV: Acclimatization Stage

The plants raised under *in vitro* conditions are weaned and hardened. Hardening is done by transferring the plant from conditions of high to low humidity and from low light intensity to high light intensity. In this stage plantlets are established in the soil. The plantlets of stage III from the laboratory are transferred to the greenhouse. In case of some plant species, shoots are directly planted in pots or suitable compost mix.

Advantages of micropropagation

i) Mass propagation of similar plants (clones) can be done through this method. Instead of getting 10000 plants per year from an initial cutting in vegetative propagation, we can get more than 1,000,000 plants per year from one explant through micropropagation.

ii) Culture is initialized from small parts of plants and not much space is required. From a small space of about 1 m^2 in the culture room, about 20000 - 100000 plantlets can be produced per year.

iii) Disease and virus-free plantlets can be obtained.

iv) Micropropagation enables to increase production of plants that normally propagate very slowly. Example bulbous crops and *Narcissus*.

v) Vegetative propagation of sterile hybrids can be done to get seed production. For e.g. cabbage

Disadvantages of micropropagation

i) Expensive laboratory equipments are required.

ii) Plants are not autotrophic during culture conditions.

iii) The plants are poorly adapted to field conditions and need to be acclimatized before transferring to field.

iv) There is a risk of genetic changes.

v) Mass propagation is not possible for all crops. For e.g. plants such as cowpea is highly recalcitrant, and attempts to regenerate it using *in vitro*-cultured explants have not been very successful.

vi) Adult woody plants cannot be easily regenerated *via* this technique.

vii) The induction of roots is not easily *ac*hieved in many species.

viii) Each explant has different *in vitro* growth rates and maturation, hence uniform growth of plants cannot be obtained from tissue culture in the case of many flowering plants.

SAQ 2

Fill in the blanks with appropriate words/terms:

a) …… is the crucial stage in tissue culture.

b) For producing virus-free plants, …… is considered as the most suitable explant.

c) Stage I in tissue culture is marked by……...

d) Rooting occurs in ------stage of micropropagation.

e) The *in vitro* plants are weaned and need to be hardened in the ……stage.

12.4 HAPLOID PRODUCTION THROUGH ANDROGENESIS AND GYNOGENESIS

The higher plants are normally diploid, with two sets of chromosomes in their somatic cells. Haploids (with one set of chromosomes) arise in nature by

parthenogenesis due to malfunction in the normal sexual process. Haploids are sterile having only a single set of chromosomes and can be converted into homozygous diploids by spontaneous or induced chromosome doubling. Haploid plants are of great significance as they produce homozygous lines (homozygous plants).

The production of haploids was first discovered in 1921 by **Bergner** in *Datura stramonium*. The development of haploid embryos and plantlets from microspores of *Datura innoxia* by the cultures of excised anthers was first reported by **Guha and Maheshwari**. To date androgenic haploids of over 200 species including many major crop plants (cereals, mustards, potato, and tomato) have been developed.

For the culture, anthers at the late uninucleate stage of microspore development are excised from surface-sterilized buds and cultured on a nutrient medium. A low temperature (4-5° C) treatment is given for initial 2-3 days to enhance the androgenic response. However, in some cases like *Brassica,* a higher temperature (30-35° C) is required. The microspores undergo repeated divisions to form multicellular structures. Such structures directly develop into an embryo or form a callus from which plants are regenerated *via* organogenesis or embryogenesis.

A summary of the standard protocol/procedure employed in raising the androgenic haploids is outlined in Fig 12.4.

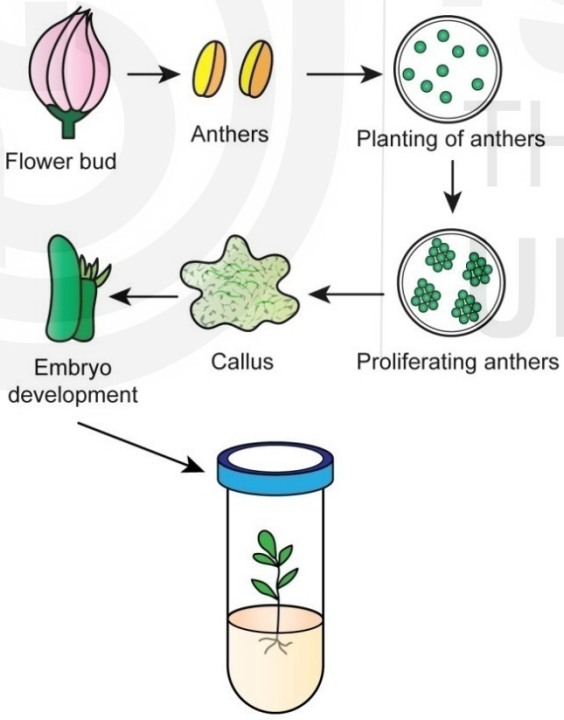


Fig 12.4: Diagrammatic representation of the production of plants by anther and pollen culture.

In contrast to androgenesis which involves the development of an egg cell containing a male nucleus into a haploid, a technique of gynogenesis is also employed for *in vitro* production of haploids. It involves the culture of unfertilized ovules whether egg (parthenogenesis) or any other haploid cells of the embryo sac (apogamy) on media to produce an embryo bypassing the process of fertilization. The gynobasic haploids were first developed from the

ovary cultures of *Hordeum vulgare* (Barley) by **San Noem** in1976. Haploids through gynogenesis have been obtained in cereals like wheat, rice, and maize and sugar beet, tobacco, sunflower.

The haploid plants obtained either by androgenesis or gynogenesis do not possess a complete set of homologous chromosomes, they remain sterile and do not form seeds. To obtain fertile plants, the haploids need to be made diploid which results in the formation of fertile homozygous diploid plants.

12.4.1 Applications of Haploids

i) Using this technique, homozygous plants can be obtained in a relatively short time as compared to the conventional breeding methods. The doubling of chromosomes is achieved by colchicines treatment which restores the fertility of plants. The double haploids show the potential to become pure breeding new cultivars.

ii) Haploids help to detect recessive mutations which are normally not expressed in heterozygous diploids.

iii) This technique also saves the plant breeders from the laborious and lengthy procedure of inbreeding.

iv) *In vitro* androgenesis allows the breeders to detect gametoclonal variation (variations occur due to recombination and segregation during meiosis).

v) Anther culture has also contributed to the production of super males (male populations with desirable features associated with male plants).

vi) Since the haploids show chromosomal instability, they can be used for the introduction of alien chromosomes or genes in breeding programs. Genetically transformed haploids have shown promising results by exhibiting resistance to various biotic and abiotic stresses.

Box 12.1: Bulbosum technique

A technique popularly called “bulbosum technique” has been successfully used to produce haploids in barley. The zygotic embryo culture of the tetraploid barley is used to achieve this. A tetraploid *Hordeum vulgare* is first fertilized by a diploid *H. bulbosum*. Zygotes of such hybrids are dissected out and the chromosomes of *H. bulbosum* are eliminated, the remaining embryonal tissue is cultured on a nutritive medium to produce dihaploid barley. Further crosses of this dihaploid produce the progeny comprising haploids. Two varieties of barley, *viz.*, ‘Gwylan” and “Mingo” have been commercially produced by the bulbosum technique.

SAQ 3

a) Match the statements/terms given in Column A with those of Column B:

	Column A		Column B
i)	Guha and Maheshwari	1.	Androgenic haploids in *Datura*
ii)	Parthenogenesis	2.	Embryo development from an unfertilized egg

iii)	Bulbosum technique	3.	Chromosome elimination following interspecific hybridization
iv)	*Hordeum vulgare*	4.	gynobasic haploids
v)	Colchicine	5.	Double haploids

b) State whether the statements are True (T) or False (F).

i) Microspores and unfertilized eggs are known to form haploid plants in culture.

ii) Haploids are important in genetic studies as they help to detect recessive mutants.

iii) Haploids production by tissue culture is of academic interest only as such plants are abnormal and cannot be integrated into conventional breeding programs.

iv) Micropropagation allows the production of a large number of propagules in a relatively short time throughout the year under aseptic conditions.

12.5 EMBRYO CULTURE

Embryo culture involves the culture of an excised immature or mature embryo from an ovule on a nutrient medium. The plant develops directly from the embryo or indirectly through the formation of callus and then the subsequent formation of shoots and roots. The technique has been very useful in:

- breaking seed dormancy,
- testing the vitality of seeds,
- production of rare species, haploid plants, and
- Shortening the breeding cycle of plants.

There are two types of embryo culture — mature embryo culture and immature embryo culture (embryo rescue). Mature embryos are isolated from ripe seeds and cultured *in vitro* when the embryos remain dormant for long periods or there is low survival of embryos *in vivo*. This technique helps in overcoming seed dormancy since the problem of dormancy due to inhibitors can be overcome and sterile seeds can be converted into viable seeds. Embryo culture is a relatively easy culture technique as embryos can be grown on a simple inorganic medium supplemented with an energy source (usually sucrose). This is because the mature embryos excised from the developing seeds are autotrophic.

12.5.1 Culture of Immature Embryos/ Embryo Rescue

Embryo rescue involves the culture of immature embryos to rescue them from becoming a part of unripe or hybrid seeds which fail to germinate (Fig. 12.5).

This approach is very useful to avoid embryo abortion and produce a viable plant. Wild hybridization involving the crossing of two different species of plants from the same genus or different genera often results in such a type of failure. This is mainly because the normal development of zygote and seed is hindered due to genetic barriers. Consequently, hybrid endosperm fails to develop leading to the abortion of hybrid embryos. The endosperm may also produce toxins that ultimately kill the embryo. Embryo abortions are due to failure in endosperm development. Embryo abortion can be avoided by isolating and culturing hybrid embryos. The most important application of embryo rescue is the production of interspecific and inter-generic hybrids from wild plant species.

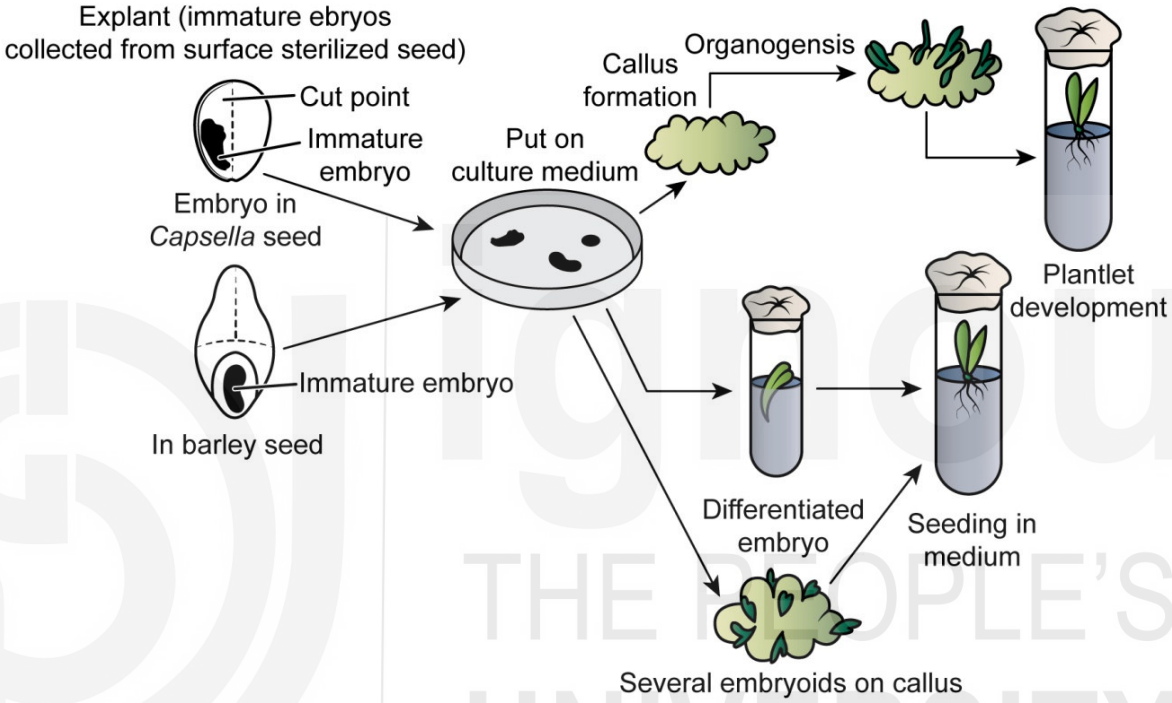


Fig 12.5: Diagrammatic representation of embryo culture.

12.5.2 Applications of Embryo Culture

i) Incompatibility barriers in interspecific and inter-generic hybridization programs leading to embryo abortion can be successfully overcome by embryo rescue.

ii) Many distant hybrids have been obtained through embryo rescue technique.

iii) Seed dormancy is caused by several factors endogenous inhibitors, embryo immaturity, and physical factors like specific light and temperature requirements, and dry storage requirements. Many plants have a long natural period of seed dormancy. Embryo culture is successfully applied to overcome seed dormancy and to produce viable seedlings in such plant species.

iv) Some of the plants have long breeding cycles in their natural state. This is mostly due to seed dormancy due to seed coat and/or endosperm. The embryos can be excised and cultured *in vitro* to develop into plants within a short period and can shorten the germination period.

v) Embryo culture has been successfully used to produce haploid (or monoploid) plants e.g. the Bulbosum technique in barley.

vi) Certain plant species produce sterile seeds that do not germinate. Seed sterility is mostly associated with incomplete embryo development which leads to the death of the germinating embryo. Using embryo cultures, it is possible to raise seedlings from sterile seeds of early-ripening fruits. For e.g., apricot, plum and cherry.

vii) Embryos are ideally suited for *in vitro* clonal propagation. Since the embryos are juvenile with high regenerative potential, it is possible to induce organogenesis and somatic embryogenesis from embryonic tissues.

SAQ 4

a) State whether the following statements are True (T) or False (F).

i) Ovary culture was first reported by San Noem.

ii) Bulbosum technique is used to overcome seed dormancy.

iii) Embryo culture is a relatively easy culture technique as the mature embryos excised from the developing seeds are autotrophic.

iv) The most important application of embryo rescue is the production of interspecific and inter-generic hybrids from wild plant species.

b) Give the technical term for the following statement:

i) *In vitro* production of plant from pollen grains.

ii) *In vitro* production of plants from unfertilized egg cell.

iii) *In vitro* propagation of plants.

iv) Variation among the plants raised from pollen grains.

12.6 SECONDARY METABOLITE PRODUCTION

In 1873, Sach found that metabolism gives rise to many by-products in plants. These compounds called secondary products have economical importance. These metabolites are synthesized by plants in low amounts (<1% of dry weight). The production of such compounds varies with the development stage and the physiological status of the plant.

Considering the medical importance of many of these secondary metabolites, *in-vitro* studies have been focused on the production of commercially valuable chemicals. Plants are the main source of about 25% of pharmaceutical compounds. More than 11% of the basic and essential drugs listed by WHO are exclusively derived from flowering plants. Tissue culture techniques have proven very useful as they increase the production of these secondary metabolites by many folds.

A commercial-scale production of secondary metabolites can be done *via* tissue culture technique. The problems like variation in quality, pricing, seasonal variation and can also be avoided/overcome by this technique. Plant tissue cultures also provide a viable alternative to protect valuable biodiversity. Compounds like Vincristine and Vinblastine, have been obtained from *Catharanthus roseus.* In addition, the anti-cancerous drug paclitaxel (Taxol), podophyllotoxin, camptothecin, morphine, diosgenin have also been successfully obtained from cultured plants.

The advantages of plant cell cultures in metabolite production are manifold that are listed below:

- shortening of maturity time of plants.
- The small amount of plant material is needed to get large-scale production of phytochemicals.
- a short time period is required to produce large amount of compounds
- Preservation of valuable natural resources.
- Suspension cultures to obtain a higher yield of the desired products/s.
- Potential for the production of hitherto unknown novel compounds by recovering new routes of biosynthesis from mutant and deviant cell lines by incorporation of latest techniques of metabolic engineering, metabolite mapping and use of recombinant DNA technology.
- Possibility of conversion of specific substrates to more valuable products by single or multiple-step enzymes activity.

Some of the notable examples of early tissue culture-based secondary metabolite production are:

- Increased levels of nicotine production in *Nicotiana tabacum.*
- Anthocyanin in *Euphorbia milii.*
- Anthraquinones in *Morinda citrifolia, Crocus sativa, Asperula, GaliumSherardia.*
- Shikonins in *Lithospermum erythrorhizon.*
- Phenolic compounds in *Acer pseudoplatinus.*
- *L-hydroxyquiphenylalanine (L-Dopa)* in *Stizolobium hassjoo.*
- Ferruginol in *Salvia miltorrhiza.*
- Sanguinarine in *Papaver somniferum.*
- Ajmalcine and serpentine in *Catharanthus roseus.*
- Rosmarinic acid in *Coleus blumei.*
- Diosgenin in *Dioscorea deltoids.*
- Ginseng in *Panax ginseng.*

Tissue culture production is used to get large-scale production of secondary metabolites and chemicals useful at the industrial level. Bioreactor systems have been developed that are capable of producing various chemicals and getting biological reactions under controlled conditions. Bioreactors provide a biologically active environment and chemical process can be carried out using microorganisms or biochemically active substances derived from such organisms.

Some of the secondary metabolites produced by tissue culture are listed in Table 12.1.

Table 12.1: List of some important secondary metabolites produced commercially using tissue culture.

Plant name	Secondary metabolite
Adhatoda vasica	Vasine
Artemisia annua	Artemisnin
Azadirachta indica	Azadirachtin
Capsicum annum	Capsiacin
Panax notoginseng	Ginseng saponin
Podophyllum hexandrun	Podophyllotoxin
Taxus chinensis	Taxane, Palitaxel
Lithospermum erythrorhizon	Shikonin
Coscinium fenustratum	Berberin
Cassia senna	Sennosides
Brucea javanica	Cathin
Catharanthus roseus	Ajmalicine, Vincristine

SAQ 5

Match the plant names given in Section A with secondary metabolites given in Section B**.**

	Section A		Section B
a)	*Bruncea annum*	1.	Vincristine
b)	*Papaver somniferum*	*2.*	Rosmarinic acid
c)	*Lithospermum erythrorhizon*	3.	Shikonin
d)	*Coleus blumei*	4.	Cathine
e)	*Catharanthus roseus*	5.	Sangunarine

12.7 GERMPLASM CONSERVATION

Indiscriminate denudation of forests, clearing of agricultural lands for other purposes, uncontrolled exploitation of wild plants for their economic benefit

has destroyed many natural habitats. As a result, there has been a continuous depletion of biodiversity and erosion of the gene pool. The number of wild species is decreasing and many of them are either endangered or vulnerable. Some wild species have already gone extinct. Efforts have been made at the international level to control and minimize the depletion of naturally occurring genetic resources.

The practice of saving and selecting seeds or vegetative propagules for the next sowing season has been a very ancient practice, which was also a form of germplasm conservation and management. Populations in ancient China and India exercised forest conservation practices as early as 700 B.C. Since the genetic diversity once lost can never be regained, alternative methods of conservation of the endangered genotypes, *viz.*, tissue culture (*in vitro* cell and organ culture) and germplasm conservation offer high promise to safeguard the genetic wealth.

Germplasm broadly refers to the hereditary material (total content of genes) transmitted from one generation to the next. Germplasm provides the raw material to the breeder. Thus, the conservation of germplasm has significance in all breeding programs. This further necessitates the need to conserve the genetic diversity of a particular species for future use.

Two global bodies, namely the International Board of Plant Genetic Resources (IBPGR) and subsequently International Plant Genetic Resources Institute (IPGRI) have been established for germplasm conservation. The main objective of these organizations is to provide the necessary support for the collection, conservation, and utilization of plant genetic resources for the benefit of present and future generations.

12.7.1 Methods of Germplasm Conservation

Two approaches are generally followed for germplasm conservation:

1. *In situ* conservation implies the conservation of plant biodiversity within its natural habitat along with its ecosystem. This approach is useful for the preservation of land plants in their natural habitat along with wild relatives. This approach has received attention because the plant breeders cannot simulate this system in the laboratory. The conservation of wild and cultivated species is done by retaining them in the National Parks, Sanctuaries, Biosphere Reserves.

This approach has advantages as it permits species-pathogen interactions and their coevolution. It is also a dynamic conservation method as it allows the conservation of lesser diversity in a single site. In addition, *in situ* conservation permits the preservation of many recalcitrant species (recalcitrant seeds are the seeds that will not survive during drying and freezing in *ex situ* conservation).

Some major drawbacks of *in situ* conservation include:

a) The risk of losing germplasm due to environmental hazards, natural/man-made disasters,

b) High cost of maintenance of many genotypes, and

c) Non-readily availability of germplasm for use.

2. *Ex-situ* conservation - *Ex-situ* conservation is the chief method for the preservation of germplasm obtained from cultivated and wild plant materials which are rare or endangered. It is mainly used for crop plants. 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. Adequate knowledge of the genetic structure of plant populations, sampling techniques, regeneration methods, and maintenance of varietal gene pools, especially in cross-pollinated species is essential for the successful establishment of gene banks.

Methods of *ex-situ* germplasm conservation include:

a) Seed Banks

b) Botanical Gardens

c) Arboreta

d) *In vitro* methods

e) Cryopreservation

During the onset of the Green Revolution, high-yielding varieties of rice and wheat were being grown everywhere as these varieties had a uniform genetic constitution and their agronomic yields were high. Cultivation of locally adapted varieties declined drastically causing a concern arose amongst scientists that this may lead to loss of genetic diversity of these crops. This led to the establishment of various agricultural institutions that aimed at the conservation of germplasm. In 1974, International Board for Plant Genetic Resources (IBPGR, now called Bioversity International) was established to coordinate global efforts to maintain a system of collection and conservation of plant biodiversity, throughout the world. Earlier, the focus for *ex situ* conservation was only for food crops and their wild relatives but since the last few years, importance has been given to rare and threatened wild species as well.

One of the most common and convenient methods of germplasm conservation is in the form of seeds. Seeds occupy less space, allow easy transportation and are economical to store. Seeds of plants behave differently when stored. Their response to low moisture tolerance, desiccation and low temperature influences their longevity and viability. Based on their storage behaviour, seeds can be classified as **orthodox, recalcitrant,** or **intermediate**.

Orthodox seeds have low moisture content (20% or less on a wet basis) and can further be dried (5% or less moisture content) without losing their viability. These seeds are desiccation-tolerant and can remain viable for several years. Storage of these seeds at low temperature or cryopreservation enhances their viability. Orthodox seeds include most grains and legumes, Cashew (*Anacardium occidentale*), *Citrus aurantifolia*, *Lantana camara*, guava (*Psidium guajava*), *Capsicum annum* and *Hamelia patens*.

Recalcitrant (unorthodox/desiccation sensitive) seeds are short-lived and very sensitive to desiccation. These cannot be dried below a critical moisture level

and cannot tolerate freezing. They do not undergo maturation drying before being shed like most other species. Since they have high moisture content, they encourage microbial growth and rapid deterioration. They cannot be freeze-stored like normal seeds as the ice crystals produced cause freeze injury. Recalcitrant seed-producing plants are mostly propagated vegetatively. Some notable examples are avocado, cacao, coconut, jackfruit, lychee, mango, rubber, tea, and some horticultural trees.

Intermediate seeds are tolerant to desiccation but at the same time are sensitive to low temperature. The seeds of coffee (*Coffea arabica*), papaya (*Carica papaya*), Citrus, Florida royal palm (*Roystonea regia*), and oil palm (*Elaies guineensis*) are examples of intermediate type.

There are certain drawbacks in conservation by seeds:

i) Recalcitrant and intermediate seeds lose their viability with time.

ii) Seeds are susceptible to pathogen or insect infection.

iii) Distinct clones cannot be maintained, and

iv) Seed conservation is applied only to those plants that are propagated by seeds and those that are propagated vegetatively.

To circumvent the above-mentioned shortcomings, *in vitro* methods for germplasm conservation are employed which are very useful for vegetatively propagated plants, species with recalcitrant seeds and genetically engineered materials. This approach has several advantages:

i) Small space is required for the maintenance of even large quantities of material.

ii) The germplasm can be maintained in a pathogen-free environment.

iii) The conserved material can be protected against natural hazards.

iv) A Large number of plants can be obtained from the germplasm stock.

v) Since the germplasm is maintained under aseptic conditions, the material can be easily transported across national and international borders.

Despite having so many advantages, the *ex-situ* methods suffer from a serious drawback. By this method, we are freezing the evolutionary development in plants that could have taken place in nature.

Various materials can be stored by an *in-vitro* method. These include isolated protoplasts, cells from suspension or callus cultures, propagules, seeds, or meristem tips. Each material shows different responses to repeated cultures. Cell or callus cultures which are in an undifferentiated phase are more susceptible to genetic changes like additions and deletions during subculturing. During culture, somaclonal or gametoclonal variations can lead to genetic heterogeneity. To minimize the frequency of subculturing, it is preferred to maintain the material as plantlets or store them as somatic embryos.

Three basic approaches are followed for the *in vitro* conservation of germplasm. These are: cold-Storage, cryopreservation, and storage under low-pressure and low-oxygen conditions. The selection of these methods depends upon two broad parameters i) slow-growth systems and ii) systems at zero-metabolism rate.

1. Cold Storage: It involves conservation of germplasm at a low and non-freezing temperature (1-9° C). This temperature treatment slows down the growth of plant. This is a cost-effective method for germplasm storage and has been employed successfully for storage of *in vitro* developed grapes (*Vitis vinifera*), strawberry (*Fragaria* sp), rye grass (*Lolium*), Lotus (*Nelumbo nucifera*), alfalfa (*Medicago sativa*), raspberry, blueberry (*Rubus* sp.), white and red clover (*Trifolium*sp) and apple (*Malus domestica*) plants. Since the plant material is not completely frozen therefore there is no cryogenic injury. The plants have been preserved for several years by this method.

2. Cryopreservation: The word *cryo* is derived from the Greek word *krúos*, = icy cold, chill, frost. It implies the storage of germplasm in sub-zero temperatures. It is a safe and cost-effective method for long-term conservation of structurally intact living cells and tissues. The plant cell and tissue cultures are brought to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants.

Sub-zero temperatures can be achieved by subjecting the tissue to any four of the following: i) solid carbon dioxide (-79° C), ii) low-temperature deep freezers (-80° C), iii) vapour phase nitrogen (-150° C), and iv) liquid nitrogen (-196° C). Liquid nitrogen is the most preferred cryopreservative as the cells remain in a completely inactive state and thus can be conserved for long periods. The cells do not divide and remain genetically stable. The formation of ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell. In addition, cells may also be damaged by a high intracellular solute concentration. Leaking out of certain solutes during freezing may also pose problems. The viability of cells may also get altered by exposure to cryoprotectants.

Successful cryopreservation of the material is based on the survival rate of cell and tissue and their ability to re-grow or regenerate into complete plants or form new colonies. The genetic integrity of recovered germplasm should be assessed to determine whether it is 'true-to-type' following cryopreservation. **Cryobionomics** is the study of genetic stability in the cryopreserved plant materials, where the fidelity of recovered plants can be assessed at phenotypic, histological, cytological, biochemical, and molecular levels.

The process of cryopreservation involves the following steps:

i) Selection of material.

ii) Pre-treatments (Cryoprotective treatments, dehydration, vitrification, encapsulation).

iii) Freezing (rapid, slow or stepwise).

iv) Storage

v) Thawing

vi) Reculture

vii) Measurement of survival/viability

viii) Plant regeneration.

i) The ability of the explant to survive in cryopreservation is greatly influenced by the morphological and physiological characters. Although the meristematic cells and suspension cell cultures, in the late lag phase (phase in which little to no cell division occurs) or log phase (phase that show cell doubling) are most suitable, other tissues like meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses can also be used.

ii) Damage to cells by freezing or thawing can be prevented by using certain compounds called cryoprotectants. Ice crystal formation is prevented or reduced as these chemicals lower the freezing point and supercooling point of water. Dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline and acetamide are some of the commonly used cryoprotectants. Generally, a mixture of cryoprotectants is more effective than using one for more effective cryopreservation without causing damage to cells/tissues.

iii) Since cells respond differently to low temperature, specific freezing methods are employed for cryopreservation.

a) **Slow-Freezing Method:** This procedure is employed to cryopreserve cells with suspension cultures. The tissue is slowly frozen at a slow cooling rate of 0.5-4℃/min from 0℃ to -100℃, and then transferred to liquid nitrogen. The advantage of the slow-freezing method is that some amount of water flows from the cells to the outside, which promotes extracellular ice formation. Intracellular freezing is prevented and as a result, the plant cells get partially dehydrated and survive better.

b) **Rapid freezing method:** This technique involves treating the plant material with liquid nitrogen. During the process, the temperature decreases at the rate of -300° to -1000℃/min. Since the freezing is rapid, the intracellular ice crystals are not formed within the cells. Shoot tips and somatic embryos respond best to the rapid freezing technique as they are small and have low water content. Other tissues may not survive rapid freezing, and therefore, would require stepwise freezing.

c) **Stepwise freezing method:** This method is a combination of slow and rapid freezing procedures and combines the advantages of both. It is carried out in a stepwise manner. The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes. This is followed by plunging the material into liquid nitrogen for rapid cooling. During initial freezing, ice is formed only outside the cells while the cell protoplasm does not freeze. This unfrozen protoplasm subsequently loses water to the outside due to vapour pressure deficit.The stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds as these tissues show a better survival rate than when subjected to rapid freezing.

d) **Dry freezing method:** It has been observed that the non-germinated dry seeds can survive freezing at very low temperatures in contrast to water-imbibing seeds which are susceptible to cryogenic injuries. Cells frozen after dehydration in a vacuum are found to have a better survival rate. The dry freezing technique requires variable dehydration optimum for different species.

e) **Storage:** In germplasm conservation maintenance of the frozen cultures at a specific temperature is important. 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 the viability of cells.

f) **Thawing:** Thawing is usually carried out by plunging ampules containing the frozen samples into warm water bath (at 37-45°C) with vigorous swirling. Rapid thawing occurs at the rate of 500- 750°C min^{-1}. This protects the cells from the damaging effects of ice crystal formation. After thawing (ice completely melts), the ampoules are quickly transferred to a water bath at 20-25°C. This transfer is necessary to protect cells from getting damaged if left for long in a warm (37-45°C) water bath.

g) **Reculture:** Thawed germplasm is washed several times to remove cryoprotectants. The material is then re-cultured in fresh medium following standard procedures. Many a time, directly culturing the thawed material without washing yields better results as certain vital substances get released from the cells during freezing.

h) **Measurement of survival/viability:** The viability of the cryopreserved material is checked using stains like FDA (fluorescein diacetate), Evan's blue and TTC (2,3,5-triphenyl tetrazolium chloride). The entire cryopreserved material may not always regenerate into complete plants. The proportion of material that survives and divides to regenerate complete plants can be quantified by the following formula:

$$\text{Survival (\%)} = \frac{\text{Number of cells/organs growing}}{\text{Number of cells/organs thawed}} \times 100$$

i) **Plant Regeneration:** The cryopreserved cells/tissues are carefully nursed and grown by adding certain growth-promoting substances. Appropriate environmental conditions are maintained to ensure successful plant regeneration. A list of selected plants whose various forms have been successfully cryopreserved is given in Table 12.2.

Table 12.2: List of some successfully cryopreserved plants.

Plant species	Plant material
Oryza sativa	Cell suspension
Glycine max	
Zea mays	
Nicotiana tabacum	
Capsicum annum	

Oryza sativa *Capsicum annum*	Callus
Zea mays *Nicotiana tabacum*	Protoplast
Solanum tubersosum *Cicer arietinum*	Meristems
Zea mays *Hordeum vulgare* *Manihot esculentum*	Zygotic embryos
Citrus sinensis *Daucus carota* *Coffea arabica*	Somatic embryos
Nicotiana tabacum *Citrus sp.* *Atropa belladona*	Pollen embryos

Low-Pressure and Low-Oxygen Storage: These techniques are developed as an alternative to cryopreservation and cold storage. These techniques are useful for both short-term and long-term storage. Low-Pressure storage involves reducing the pressure of gases in contact with the plant material by reducing the atmospheric pressure itself, Low-Oxygen Storage involves a reduction in oxygen by combining it with an inert gas like nitrogen. Low-Pressure Storage reduces the activity of pathogenic organisms and inhibits spore germination in the plant culture systems. These techniques have also been useful for extending the shelf life of fruits, cut flowers, potted plants, and cuttings.

Synthetic Seed Technology: One of the modern approaches for short-term germplasm conservation is the production of synseeds (synthetic seeds/artificial seeds), which are produced by encapsulating somatic embryos in a protective coating. You have studied this technique of short-term non-cryogenic preservation in Section 12.2.

12.7.2 Applications of Germplasm Storage

Some of the important applications of cryopreservation germplasm storage are:

1. Maintenance of stock cultures does not require sub-culturing. No need to extend viability.
2. An ideal method for long-term conservation of cell cultures that produce secondary metabolites (e.g., medicines, biologically active compounds).
3. Storage of disease (pathogen)-free plant materials and their propagation as per requirement.
4. Long-term maintenance of recalcitrant seeds.
5. Conservation of somaclonal and gametoclonal variations in cultures.

6. Conservation of plant materials from endangered species.
7. Conservation of pollen for enhancing longevity.
8. Storage of rare germplasms developed through somatic hybridization and other genetic manipulations.
9. Reliable method for the selection of cold-resistant mutant cell lines for developing frost-resistant plants, and
10. Establishment of germplasm banks for the exchange of information at the national and international levels.

One of the major limitations of germplasm storage is that the process requires costly equipment and well-trained personnel.

SAQ 6

Answer in one word.

a) The hereditary material (total content of genes) transmitted from one generation to the next.

b) The conservation of plant biodiversity within its natural habitat along with its ecosystem.

c) Seeds that are short-lived and sensitive to desiccation.

d) The storage of germplasm in sub-zero temperatures.

e) Compounds that prevent damage to cells by freezing or thawing.

f) The technique in which the plant material is treated with liquid nitrogen.

12.8 SUMMARY

- Tissue culture is a term used to collectively refer to different methods which are used to maintain and propagate plant cells and/or organs *in vitro* under aseptic conditions.
- Tissue culture techniques have many important applications for various industrial uses as well as for the protection of plant biodiversity.
- The property of totipotency helps to achieve differentiation of various tissues and organs and the regeneration of the entire plant in tissue culture.
- The technique of micropropagation allows clonal propagation of plants in a laboratory and involves various steps starting from procurement of explants from a donor plant and its culture in sterile conditions in tissue culture media culminating in its development into a complete plantlet through somatic embryogenesis or organogenesis.

- Micropropagation is now routinely used in genetic engineering to raise transgenic plants and in other fields like germplasm conservation, forestry, and horticulture. These techniques can also be used to raise virus-free plants through callus culture of meristem tips.
- Another important application is to produce secondary metabolites which have many industrial uses, mainly in the pharmaceutical industry. Plants produce many of these secondary metabolites and manipulations during culture can augment the production.
- Conservation of plant genetic resources is needed to protect and/or store germplasm of crop species, a wild relative of crops as well as wild species which are rare or endangered. Various *in situ* or *ex-situ* methods are employed for germplasm conservation. *In vitro* conservation is an important method for *ex-situ* type of conservation. Cryopreservation involves keeping the material at very low temperatures, which suppresses all the metabolic activity while retaining viability.

12.9 TERMINAL QUESTIONS

1. Enumerate various applications of plant tissue culture.
2. Write a short note on artificial seeds.
3. What is micropropagation? What are the advantages of micropropagation over the conventional methods of clonal propagation of plants?
4. Give a brief account of embryo culture.
5. How can cell suspension cultures be used for the production of secondary metabolites?
6. Discuss *in vitro* germplasm conservation. Compare the advantages and disadvantages of *in-situ* and *ex-situ* germplasm conservation.
7. Describe the steps involved in the cryopreservation of germplasm.
8. Define the following terms:
   i) explants
   ii) callus
   iii) androgenesis
   iv) embryoids
   v) cryoprotectants
   vi) somaclonal variation
   vii) germplasm
   viii) secondary metabolites

12.10 ANSWERS

Self-Assessment Questions

1. a) True; b) True; c) False; d) True; e) True
2. a) Multiplication
   b) shoot tip
   c) Initiation of culture
   d) III
   e) acclimatization.
3. a) i) gynobasic haploids
      ii) Double haploids
      iii) Androgenic haploids in *Datura*
      iv) Embryo development from an unfertilized egg
      v) Chromosome elimination following interspecific hybridization
   b) i) True; ii) True; iii) False; iv) True
4. a) i) True; ii) False; iii) True; iv) False
   b) i) androgenesis
      ii) parthenogenesis/gynogenesis
      iii) micropropagation
      iv) gametoclonal variation
5. a) cathin
   b) sanguinarine
   c) shikonin
   d) Rosmarinic acid;
   e) vincristine
6. a) Germplasm
   b) *In situ* conservation
   c) Recalcitrant
   d) Cryopreservation
   e) Cryoprotectants
   f) Rapid freezing method

Terminal Questions

1. Refer to Section 12.2.
2. Refer to Section 12.2.
3. Refer to Section 12.3.
4. Refer to section 12.5.
5. Refer to Section 12.6.
6. Refer to Section 12.7.
7. Refer to Section 12.7.
8. a) Any part of a plant that is used for culturing is known as explant.

   b) An unorganized mass of cells capable of differentiating and developing into a whole plantlet is known as a callus.

   c) Production of haploid plants *via* a process of anther culture is known as androgenesis.

   d) Embryoids are embryo-like structures formed *in vitro* cultures and have the potential to develop into full-fledged plants.

   e) The compounds that prevent damage to cells by freezing or thawing are called cryoprotectants.

   f) Heritable variations for both qualitative and quantitative traits found in cultured cells are known as somaclonal variations.

   g) Germplasm- It is the hereditary material (total content of genes) transmitted from one generation to the next.

   h) The chemical compounds that are produced by the plant cell but are not directly involved in primary metabolic processes of a plant.