COURSE GUIDE

CRP 511
INTRODUCTION TO CROP BIOTECHNOLOGY

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INTRODUCTION

CRP 511 is a two (2)-credit unit course on introduction to crop Biotechnology. The course is broken into 34 units in all in six (6) modules. These units will teach and explainBiotechnology study as a tool and systematic process for ensuring that biotechnologyconsiderations are taken into account in all proposed activities. This course guide defines what the course is all about as well as the course material that you will need to consult to ensure that the course is simple and within your reach. It suggests some general guidelines for the amount of time you are likely to spend studying each unit in order to complete it successfully. It also gives you some guidance on your tutor-marked assignments. The course will deal with the basic principles and selected applications of biotechnology for agricultural crops, emphasizing the need for a different type of agriculture, in order to support the increasing needs for food (quantity and quality) facing climatic changes as well as increased abiotic and biotic stress conditions. The basic aspects of the major biotechnological tools and solutions will be evaluated.

WHAT YOU WILL LEARN IN THIS COURSE

On successful completion of this module, you will be able to:

1. To describe the major basic biotechnologies related to agricultural production.
2. To explain the major practical biotechnologies aimed at solving agriculture and food production problems.
3. To evaluate the achieved progress, the possible risks and future needs of agricultural biotechnologies.
4. To examine the ecological, sociological and ethical issues associated with genetically-modified (GM) plants and their products.

COURSE AIMS

The Course aims and objectives are follows:

1. To expose the students to the basic scientific evidence and technical aspects of the different disciplines of agricultural biotechnologies (mainly for plants and crops).
2. To clarify the major scientific, ecological and sociological aspects of biotechnology in agriculture and food production.
3. To discuss the general issues and interrelationships of science, agriculture and human well-being.

COURSE OBJECTIVES

For the aims to be achieved, there are set objectives. Each unit of this course also has its specific objectives that are found at the beginning of each unit. You will need to understand these objectives before you start working on each unit. You are encouraged to refer to them periodically to check on your progress in learning and assimilating the content. On completion of a unit, you may re-examine the objectives to ensure that you fully learn what is required. By so doing you can be sure that you have achieved what the unit expects you to acquire. By meeting these objectives, the aims of the course as a whole would have been achieved. These objectives include:

1) Define and explain the meaning of biotechnology
2) Understand the Background to general biotechnology:
3) Explain Agricultural biotechnologies and breeding - global perspectives and trends.
4) Describe the Applications of plant Biotechnology in crop improvement.
5) Understand the concept of Micro techniques
6) Explain Plant Genetic Engineering and Production of Transgenic Plants.
7) State the importance of Applications of Genetic Engineering
8) Describe the Application of Cell Culture Systems in Metabolic Engineering.
9) State the aims and scope of Molecular Farming and Applications.

WORKING THROUGH THIS COURSE

To complete this course, you are required to read the study units carefully and read other recommended materials. You will be required to answer some questions based on what you have read in the content to reaffirm the key points. At the end of each unit there is some Tutor-Marked Assignments (TMA) which you are expected to submit for Marking. The TMA forms part of your continuous assignments. At the end of the course is a final examination. The course should take you 12to 13 weeks to complete. The component of the course is given to you to know what to do and how you should allocate your time to each unit in order to complete the course successfully on time.

COURSE MATERIALS

The major components of this course are:

1. Course Guide
2. Study Units/Course Materials
3. Tutor Mark Assessment (TMA)
4. References and Further Reading

COURSE GUIDE

The material you are reading now is called the course guide which introduced you to this course.

STUDY UNITS

Module 1 Background to General Biotechnology

Unit 1 Introductory Remarks
Unit 2 Agricultural Biotechnologies and Breeding - Global Perspectives and Trends
Unit 3 Improved Nutritional Content

Module 2 Applications of Plant Biotechnology in Crop Improvement

Unit 1 Protoplast Isolation and Regeneration
Unit 2 Introduction to Plant Tissue Culture
Unit 3 Introduction to Micropropagation in Biotechnology
Unit 4 Doubled Haploid Production
Unit 5 Lab Facilities and Operations
Unit 6 Tissue Culture Media
Unit 7 Adventitious Shoot Proliferation
Unit 8 Axillary Shoot Proliferation
Unit 9 Cryopreservation

Module 3 Micro Techniques

Unit 1 Cytological and Various Staining Procedures for Ploidy Analysis
Unit 2 Microscopy
Unit 3 Electron Microscopy
Unit 4 Flow Cytometry and Cell Sorting
Unit 5 Plant Histological Techniques

Module 4 Introduction to Plant Genetic Engineering

Unit 1 Genetic Material of Plant Cells
Unit 2 Mode of Gene Delivery in Plant
Unit 3 Selection and Screening of Transformations
Unit 4 Gene Silencing
Unit 5 Applications of Genetic Engineering

Module 5 Application of Cell Culture Systems in Metabolic Engineering

Unit 1 Hairy Root Cultures
Unit 2 Screening of High Yielding Cell Lines and Extraction of High Value Industrial Products
Unit 3 Fractionation and Bioassays of Plant Extract
Unit 4: Growth and Production Kinetics of Cell Cultures in Shake Flasks
Unit 5 Bioreactors for Plant Engineering
Unit 6 Manipulation in Production Profile by Abiotic and Biotic Elicitation
Unit 7 Biotransformation
Unit 8 Advantages of Plant Cell, Tissue and Organ Culture as Source of Secondary Metabolites

Module 6 Molecular Farming and Applications

Unit 1 Aims Scope and Bottlenecks of Molecular Farming
Unit 2 Production of Industrial Enzymes and Biodegradable Plastics
Unit 3 Production of Antibodies
Unit 4 Metabolic Engineering for Production of Fatty Acids, Industrial Oils, Terpenoids and Flavonoids

TUTOR-MARKED ASSIGNMENT (TMA)

There are Tutor Marked assignments and self assignment in each unit. You would have to do the TMA as a revision of each unit. And there are four Tutor Marked Assignments you are required to do and submit as your assignment for the course. This would help you to have broad view and better understanding of subject. Your tutorial facilitator would inform you about the particular TMA you are to submit to him for marking and recording. Make sure your assignment reaches your tutor before the deadline given in the presentation schedule and assignment file. If, for any reason, you cannot complete your work on schedule, contact your tutor before the assignment is due to discuss the possibility of an extension. Extensions will not be granted after the due date unless

there are exceptional circumstances. You will be able to complete your assignment questions from the Contents contained in this course material and References/Further reading; however, it is desirable to search other References/Further reading, which will give you a broader view point and a deeper understanding of the subject.

REFERENCES AND FURTHER READING

Altman, A. & Hasegawa, P.M. (2012). Introduction to plant biotechnology 2011: Basic aspects and agricultural implications. In: A. Altman & P.M. Hasegawa, (Eds). 2012. *Plant Biotechnology and Agriculture: Prospects for the 21st Century.* Elsevier and Academic Press.Amsterdam. ISBN 978-0-12-381466-1.pp. xxix-xxxviii.

Moshelion, M. & Altman, A. (2015). Current challenges and future perspectives of plant and agricultural biotechnology. *Trends in Biotechnology* 33: 1-6 (Invited Opinion Review).

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Crop Biotech Update (weekly electronic update)- selected

Selected chapters and/or paragraphs from the above book (1- an electronic page 3 / 5 version is available in the library).

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Altman, A. & Hasegawa, P.M. (Eds.). *Plant Biotechnology and Agriculture: Prospects for the 21st Century*. Elsevier and Academic Press, San Diego; ISBN : 978-0-12-381466-1. This book is available in an electronic version in the library. Please consult Nurit (or the library) how to access it.

Slater, A., Scott, N.W., Fowler, & M.R., (Eds). (2003). *Plant Biotechnology,* Oxford University Press, UK.

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MAIN COURSE

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MODULE 1 BACKGROUND TO GENERAL BIOTECHNOLOGY

Unit 1 Introductory Remarks; Meaning of Biotechnology
Unit 2 Agricultural Biotechnologies and Breeding - Global Perspectives and Trends;
Unit 3 Improved Nutritional Content

UNIT 1 INTRODUCTORY REMARKS; MEANING OF CROP BIOTECHNOLOGY

CONTENTS

1.0 INTRODUCTION

This module provides a brief description of current and emerging uses of biotechnology in crops and forestry with a view to understanding the technologies themselves and the ways they complement and extend other approaches. It should be emphasized that the tools of biotechnology are just that: tools, not ends in themselves. As with any tool, they must be assessed within the context in which they are being used. Biotechnology is the broad area of biology involving living systems and organisms to develop or make products, or "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use" (UN Convention on Biological Diversity, Art. 2).Depending on the tools and applications, it often overlaps with the (related) fields of molecular biology, bio- ngineering, biomedical engineering, biomanufacturing, molecular engineering, etc.

For thousands of years, humankind has used biotechnology in agriculture, food production, and medicine.The term is largely believed to have been coined in 1919 by

Hungarian engineer KárolyEreky. In the late 20th and early 21st centuries, biotechnology has expanded to include new and diverse sciences such as genomics, recombinant gene techniques, applied immunology, and development of pharmaceutical therapies and diagnostic tests.

The wide concept of "biotech" or "biotechnology" encompasses a wide range of procedures for modifying living organisms according to human purposes, going back to domestication of animals, cultivation of the plants, and "improvements" to these through breeding programs that employ artificial selection and hybridization. Modern usage also includes genetic engineering as well as cell and tissue culture technologies.

By contrast, bioengineering is generally thought of as a related field that more heavily emphasises higher systems approaches (not necessarily the altering or using of biological materials directly) for interfacing with and utilising living things. Bioengineering is the application of the principles of engineering and natural sciences to tissues, cells and molecules. This can be considered as the use of knowledge from working with and manipulating biology to achieve a result that can improve functions in plants and animals. Relatedly, biomedical engineering is an overlapping field that often draws upon and applies biotechnology (by various definitions), especially in certain sub-fields of biomedical or chemical engineering such as tissue engineering, biopharmaceutical engineering, and genetic engineering.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- define biotechnology
- state the importance of biotechnology
- list and explain the forms of biotechnology.

3.0 MAIN CONTENT

3.1 Definition

The American Chemical Society defines biotechnology as the application of biological organisms, systems, or processes by various industries to learning about the science of life and the improvement of the value of materials and organisms such as pharmaceuticals, crops, and livestock. Per the European Federation of Biotechnology, biotechnology is the integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services.

Biotechnology is based on the basicbiological sciences (e.g. molecular biology, biochemistry, cell iology, embryology, genetics, microbiology) and conversely provides methods to support and perform basic research in biology.

Biotechnology is the research and development inthe laboratory using bioinformatics for exploration, extraction, exploitation and production from any living organisms and any source of biomass by means of biochemical engineering where high value-added products could be planned (reproduced by biosynthesis, for example), forecasted, formulated, developed, manufactured, and marketed for the purpose of sustainable operations (for the return from bottomless initial investment on R & D) and gaining durable patents rights (for exclusives rights for sales, and prior to this to receive national and international approval from the results on animal experiment and human experiment, especially on the pharmaceutical branch of biotechnology to prevent any undetected side-effects or safety concerns by using the products). The utilisation of biological processes, organisms or systems to produce products that are anticipated to improve human lives is termed biotechnology.

Broadly speaking, biotechnology is any technique that uses living organisms or substances from these organisms to make or modify a product for a practical purpose . Biotechnology can be applied to all classes of organism - from viruses and bacteria to plants and animals - and it is becoming a major feature of modern medicine, agriculture and industry. Modern agricultural biotechnology includes a range of tools that scientists employ to understand and manipulate the genetic make-up of organisms for use in the production or processing of agricultural products.

3.2 Importance of Biotechnology

Biotechnology is being used to address problems in all areas of agricultural production and processing.

This includes:

- plant breeding to raise and stabilise yields;
- to improve resistance to pests, diseases and abiotic stresses such as drought and cold;
- to enhance the nutritional content of foods.
- Biotechnology is being used to develop low-cost disease-free planting materials for crops such as cassava, banana and potato and is creating new tools for the diagnosis and treatment of plant

and animal diseases and for the measurement and conservation of genetic resources.

- Biotechnology is being used to speed up breeding programmes for plants, livestock and fish and to extend the range of traits that can be addressed.
- Animal feeds and feeding practices are being changed by biotechnology to improve animal nutrition and to reduce environmental waste.
- Biotechnology is used in disease diagnostics and for the production of vaccines against animal diseases.
- Some applications of biotechnology, such as fermentation and brewing, have been used for millennia.
- Other applications are newer but also well established. For example, micro-organisms have been used for decades as living factories for the production of life-saving antibiotics including penicillin, from the fungus Penicillium, and streptomycin from the bacterium Streptomyces.
- Modern detergents rely on enzymes produced via biotechnology, hard cheese production largely relies on rennet produced by biotech yeast and human insulin for diabetics is now produced using biotechnology.

3.3 Forms of Biotechnology

1. Agricultural biotechnology

Agricultural biotechnology, also known as agritech, is an area of agricultural science involving the use of scientific tools and techniques, including genetic engineering, molecular markers, molecular diagnostics, vaccines, and tissue culture, to modify living organisms: plants, animals, and microorganisms. Crop biotechnology is one aspect of agricultural biotechnology which has been greatly developed upon in recent times. Desired trait is exported from a particular species of Crop to an entirely different species. These transgene crops possess desirable characteristics in terms of flavor, color of flowers, growth rate, size of harvested products and resistance to diseases and pests.

2. Medical Biotechnology

Medical biotechnology is the use of living cells and other cell materials for the purpose of bettering the health of humans. Essentially, it is used for finding cures as well as getting rid of and preventing diseases. The science involved includes the use of these tools for the purpose of research to find different or more efficient ways of maintaining human health, understanding pathogen, and understanding the human cell biology.

Here, the technique is used to produce pharmaceutical drugs as well as other chemicals to combat diseases. It involves the study of bacteria, plant and animal cells to first understand the way they function at a fundamental level.

3. Industrial Biotechnology

The industrial applications of biotechnology range from the production of cellular structures to the production of biological elements for numerous uses. Examples include the creation of new materials in the construction industry, and the manufacture of beer and wine, washing detergents, and personal care products.

4.0 CONCLUSION

This module provides a brief description of current and emerging uses of biotechnology in crops and forestry with a view to understanding the technologies themselves and the ways they complement and extend other approaches. It should be emphasized that the tools of biotechnology are just that: tools, not ends in themselves. As with any tool, they must be assessed within the context in which they are being used.

5.0 SUMMARY

Broadly speaking, biotechnology is any technique that uses living organisms or substances from these organisms to make or modify a product for a practical purpose. Biotechnology is being used to address problems in all areas of agricultural production and processing. This includes;

- plant breeding to raise and stabilise yields;
- to improve resistance to pests, diseases and abiotic stresses such as drought and cold;
- to enhance the nutritional content of foods.
- Biotechnology is being used to develop low-cost disease-free planting materials for crops such as cassava, banana and potato and is creating new tools for the diagnosis and treatment of plant and animal diseases and for the measurement and conservation of genetic resources.
- Biotechnology is being used to speed up breeding programmes for plants, livestock and fish and to extend the range of traits that can be addressed.
- Animal feeds and feeding practices are being changed by biotechnology to improve animal nutrition and to reduce environmental waste.

- Biotechnology is used in disease diagnostics and for the production of vaccines against animal diseases.
  While the forms of biotechnology are agricultural biotechnology,medical biotechnology and industrial biotechnology

6.0 TUTOR-MARKED ASSIGNMENT

1. Give three definitions of crop biotechnology.
2. State four importance of crop biotechnology.
3. State one type of biotechnology and discuss it.

7.0 REFERENCES/FURTHER READING

Altman, A. & Hasegawa, P.M. (2012). Introduction to plant biotechnology 2011: Basic aspects and agricultural implications. In: A. Altman and P.M. Hasegawa, eds. 2012. *Plant Biotechnology and Agriculture: Prospects for the 21st Century.* Elsevier and Academic Press.Amsterdam. ISBN 978-0-12-381466-1. pp. xxix-xxxviii.

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UNIT 2 AGRICULTURAL BIOTECHNOLOGIES AND BREEDING - GLOBAL PERSPECTIVES AND TRENDS

CONTENTS

1.0 INTRODUCTION

Agricultural biotechnology, is an area of agricultural science involving the use of scientific tools and techniques, including genetic engineering, molecular markers, molecular diagnostics, vaccines, and tissue culture, to modify living organisms: plants, animals, and microorganisms.Crop biotechnology is one aspect of agricultural biotechnology which has been greatly developed upon in recent times. Desired trait is exported from a particular species of Crop to an entirely different species. These transgene crops possess desirable characteristics in terms of flavor, color of flowers, growth rate, size of harvested products and resistance to diseases and pests.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand more about agricultural biotechnologies and breeding
- global perspectives and trends
- state and explain crop modification techniques.

3.0 MAIN CONTENT

3.1 History

Farmers have manipulated plants and animals through selective breeding for tens of thousands of years in order to create desired traits. In the 20th century, a surge in technology resulted in an increase in agricultural biotechnology through the selection of traits like increased yield, pest resistance, drought resistance, and herbicide resistance. The first food product produced through biotechnology was sold in 1990, and by 2003, 7 million farmers were utilizing biotech crops. More than 85% of these farmers were located in developing countries.

3.2 Crop Modification Techniques

3.2.1 Traditional Breeding

Traditional crossbreeding has been used for centuries to improve crop quality and quantity. Crossbreeding mates two sexually compatible species to create a new variety with the desired traits of the parents. For example, the honeycrisp apple exhibits a specific texture and flavor due to the crossbreeding of its parents. In traditional practices, pollen from one plant is placed on the female part of another, which leads to a hybrid that contains genetic information from both parent plants. Plant breeders select the plants with the traits they're looking to pass on and continue to breed those plants. Note that crossbreeding can only be utilized within the same or closely related species.

3.2.2 Mutagenesis

Mutations (the changing of the structure of a gene) can occur randomly in the DNA of any organism. In order to create variety within crops, scientists can randomly induce mutations within plants. Mutagenesis uses radioactivity to induce random mutations in the hopes of stumbling upon the desired trait. Scientists can use mutating chemicals such as ethyl methanesulfonate, or radioactivity to create random mutations within the DNA. Atomic gardens are used to mutate crops. A radioactive core is located in the center of a circular garden and raised out of the ground to radiate the surrounding crops, generating mutations within a certain radius. Mutagenesis through radiation was the process used to produce ruby red grapefruits.

3.2.3 Polyploidy

Polyploidy can be induced to modify the number of chromosomes in a crop in order to influence its fertility or size. Usually, organisms have

two sets of chromosomes, otherwise known as a diploidy. However, either naturally or through the use of chemicals, that number of chromosomes can change, resulting in fertility changes or size modification within the crop. Seedless watermelons are created in this manner; a 4-set chromosome watermelon is crossed with a 2-set chromosome watermelon to create a sterile (seedless) watermelonwith three sets of chromosomes.

3.2.4 Protoplast Fusion

Protoplast fusion
Protoplast fusion is the joining of cells or cell components to transfer traits between species. For example, the trait of male sterility is transferred from radishes to red cabbages by protoplast fusion. This male sterility helps plant breeders make hybrid crops.

3.2.5 RNA Interference

RNA interference (RNAIi) is the process in which a cell's RNA to protein mechanism is turned down or off in order to suppress genes. This method of genetic modification works by interfering with messenger RNA to stop the synthesis of proteins, effectively silencing a gene.

3.2.6 Transgenics

Transgenics involves the insertion of one piece of DNA into another organism's DNA in order to introduce a new gene(s) into the original organism. This addition of genes into an organism's genetic material creates a new variety with desired traits. The DNA must be prepared and packaged in a test tube and then inserted into the new organism. New genetic information can be inserted with biolistics. An example of transgenics is the rainbow papaya, which is modified with a gene that gives it resistance to the papaya ringspot virus.

3.2.7 Genome Editing

Genome editing is the use of an enzyme system to modify the DNA directly within the cell. Genome editing was used to develop herbicide resistant canola to help farmers control weeds.

4.0 CONCLUSION

Traditional crossbreeding has been used for centuries to improve crop quality and quantity. Crossbreeding mates two sexually compatible species to create a new variety with the desired traits of the parents.

History of agricultural biotechnologies were highlighted, Crop modification techniques were discussed and list as; Traditional breeding,Mutagenesis,Polyploidy,Protoplastfusion,RNAinterference,Transgenics and Genome editing.

5.0 SUMMARY

Crop biotechnology is one aspect of agricultural biotechnology which has been greatly developed upon in recent times. Desired trait is exported from a particular species of Crop to an entirely different species. These transgene crops possess desirable characteristics in terms of flavor, color of flowers, growth rate, size of harvested products and resistance to diseases and pests.Some of the Crop modification techniques are as follows: Traditional breeding,Mutagenesis,Polyploidy,Protoplast fusion,RNA interference,Transgenics and Genome editing.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is agricultural biotechnology?
2. Define crop modification techniques.
3. List all the crop modification techniques given the module and briefly explain any three.

7.0 REFERENCES/FURTHER READING

Altman A. & P.M. Hasegawa (2012). Introduction to plant biotechnology 2011: Basic aspects and agricultural implications. In: A. Altman and P.M. Hasegawa, (Eds). *Plant Biotechnology and Agriculture: Prospects for the 21st Century.* Elsevier and Academic Press.Amsterdam. ISBN 978-0-12-381466-1. pp. xxix-xxxviii.

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UNIT 3 IMPROVED NUTRITIONAL

CONTENTS

1.0 INTRODUCTION

Agricultural biotechnology has been used to improve the nutritional content of a variety of crops in an effort to meet the needs of an increasing population. Genetic engineering can produce crops with a higher concentration of vitamins. For example, golden rice contains three genes that allow plants to produce compounds that are converted to vitamin A in the human body. This nutritionally improved rice is designed to combat the world's leading cause of blindness—vitamin A deficiency. Similarly, the Banana 21 species developed in Uganda has worked to improve the nutrition in bananas to combat micronutrient deficiencies. By genetically modifying bananas to contain vitamin A and iron, Banana 21 has helped foster a solution to micronutrient deficiencies through the vessel of a staple food and major starch source in Africa. Additionally, crops can be engineered to reduce toxicity or to produce varieties with removed allergens.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- meaning of improved nutritional content
- understand the Genes and traits of interest for crops
- Agronomic traits and quality traits
- Common GMO crops.

3.0 MAIN CONTENT

3.1 Meaning of Improved Nutritional Content

Agricultural innovation has always involved new, science-based products and processes that have contributed reliable methods for increasing productivity and sustainability. Biotechnology has introduced a new dimension to such innovation, offering efficient and cost-effective means to produce a diverse array of novel, value-added products and tools. Developing plants with these improved traits involves overcoming a variety of technical, regulatory, and indeed perception challenges inherent in the perceived and real challenges of complex modifications. Both traditional plant breeding and biotechnology-based techniques are needed to produce plants with the desired quality traits. Continuing improvements in molecular and genomic technologies are contributing to the acceleration of product development.

Plants are the main source of calories for humans, yet only a handful of species are used for human consumption. Rice, wheat, corn, beans, cassava, potato, sweet potato, sugar beets, and others, are the dietary base for the world's population and represent important food staples for low-income communities. Besides being energy-dense and carbohydrate-rich, these crops are also an important entry point into the human diet for micronutrients, vitamins and trace elements (both essential and toxic). Thus, breeding efforts aimed at improving crop nutrition and/or avoiding accumulation of hazardous elements have direct impacts on human health.

3.2 Genes and Traits of Interest for Crops

3.2.1 Agronomic Traits

1. Insect resistance

One highly sought-after trait is insect resistance. This trait increases a crop's resistance to bugs and allows for a higher yield. These genetically engineered crops can now produce their own Bt (Bacillus thuringiensis), which contains toxin-producing proteins that are non-harmful to humans. Bt corn and cotton are now commonplace, and cowpeas, sunflower, soybeans, tomatoes, tobacco, walnut, sugar cane, and rice are all being studied in relation to Bt.

2. Herbicide tolerance

Weeds have proven to be an issue for farmers for thousands of years; they compete for soil nutrients, water, and sunlight and prove deadly to crops. Biotechnology has offered a solution in the form of herbicide tolerance. Chemical herbicides are sprayed directly on plants in order to

kill weeds and therefore competition, and herbicide resistant crops have to the opportunity to flourish.

3. Disease resistance

Often, crops are afflicted by disease spread through insects (like aphids). Spreading disease among crop plants is incredibly difficult to control and was previously only managed by completely removing the affected crop. The field of agricultural biotechnology offers a solution through genetically engineering virus resistance. Developing GE disease-resistant crops now include cassava, maize, and sweet potato.

4. Temperature tolerance

Agricultural biotechnology can also provide a solution for plants in extreme temperature conditions. In order to maximize yield and prevent crop death, genes can be engineered that help to regulate cold and heat tolerance. For example, papaya trees have been genetically modified in order to be more tolerant of hot and cold conditions.[6] Other traits include water use efficiency, nitrogen use efficiency and salt tolerance.

3.2.2 Quality Traits

Quality traits include increased nutritional or dietary value, improved food processing and storage, or the elimination of toxins and allergens in crop plants.

3.3 Common GMO Crops

Currently, only a small number of genetically modified crops are available for purchase and consumption in the United States. The USDA has approved soybeans, corn, canola, sugar beets, papaya, squash, alfalfa, cotton, apples, and potatoes. GMO apples (arctic apples) are non-browning apples and eliminate the need for anti-browning treatments, reduce food waste, and bring out flavor. The production of Bt cotton has skyrocketed in India, with 10 million hectares planted for the first time in 2011, resulting in a 50% insecticide application reduction. In 2014, Indian and Chinese farmers planted more than 15 million hectares of Bt cotton.

4.0 CONCLUSION

Agricultural biotechnology has been used to improve the nutritional content of a variety of crops in an effort to meet the needs of an increasing population. Genetic engineering can produce crops with a higher concentration of vitamins.

It has been almost two decades since the first papers on biofortification were published, and flagship programs such as HarvestPlus were launched. Using a variety of plant modification strategies, including conventional breeding and genetic engineering, more nutritious crop varieties with higher amounts of vitamin A, iron (Fe) and zinc (Zn), the three micronutrients identified by the WHO as most lacking in human diets globally, have been generated. Through this work many genes and pathways important for nutrient accumulation in the edible parts of crop plants have been described and their potential uses for biotechnological application have been demonstrated. However, to date only a few biofortified crops are grown for human consumption, highlighting the fact that transfer of basic research into final products is still necessary.

5.0 SUMMARY

From a consumer perspective, the focus on value-added traits, especially improved nutrition, is of greatest interest.
Developing plants with these improved traits involves overcoming a variety of technical, regulatory, and indeed perception challenges inherent in the perceived and real challenges of complex modifications. Both traditional plant breeding and biotechnology-based techniques are needed to produce plants with the desired quality traits. Continuing improvements in molecular and genomic technologies are contributing to the acceleration of product development

6.0 TUTOR-MARKED ASSIGNMENT

1. What is nutritional content improvement?
2. What are the traits in crop biotechnology?
3. Give three examples each of the mentioned traits.

7.0 REFERENCES/FURTHER READING

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MODULE 2 APPLICATIONS OF PLANT BIOTECHNOLOGY IN CROP IMPROVEMENT

UNIT 1 PROTOPLAST ISOLATION AND REGENERATION

CONTENTS

1.0 INTRODUCTION

The term protoplast was introduced by Hanstein in 1880. It refers to the cellular content excluding cell wall or can also be called as naked plant cell. It is described as living matter enclosed by a plant cell membrane. Protoplast isolation for the first time was carried out by Klercker in 1892 using mechanical method on the plasmolysed cells. The application of protoplast technology for the improvement of plants offers fascinating option to complement conventional breeding programs. The ability of isolated protoplasts to undergo fusion and take up macromolecules and cell organelles offers many possibilities in genetic engineering and crop improvement (Bhojwani et al . 1977). The experiments involving protoplasts consist of three stages:

i. protoplast isolation
ii. protoplast fusion (leading to gene uptake)
iii. development of regenerated fertile plants from the fusion product (Hybrid).

Depending upon the species and culture conditions, the protoplasts may have the potential to:

i. regenerate a cell wall
ii. differentiate to form callus
iii. divide mitotically and proliferate clonally
iv redifferentiate into shoots, roots or embryos and produce a complete plantlet.

However, to fully explore the potentials for protoplast-technology, efficient and reproducible methods for protoplast isolation and purification must first be established. Since leaf tissue is a readily accessible source of genetically uniform cells, it is often desirable to use mesophyll protoplasts in somatic hybridisation studies, but, leaf tissues, in general, do not yield large number of protoplasts owing to the difficulty in removing the lower epidermis (Chaturvedi 2003). An alternative, therefore, is the cultured cell material where protoplasts can show greater potential to divide (Bhojwani and Razdan 1996).

2.0 OBJECTIVES

By the end of this unit, you will be able:

i. protoplast isolation
ii. understand protoplast fusion (leading to gene uptake)
iii. development of regenerated fertile plants from the fusion product (Hybrid).

3.0 MAIN CONTENT

3.1 Protoplast Isolation

Protoplast isolation may be carried out by Mechanical disruption method or enzymatic method. Out of these two methods, enzymatic method is preferred as it provides better protoplast yield with low tissue damage while mechanical method causes maximum tissue chopping with lower protoplast yields.

3.1.1. Factors Affecting Yield and Viability of Protoplasts

i. **Source of material:** Leaves were the most convenient source of the plant protoplasts because it allows the isolation of a large number of relatively uniform cells without killing the plants. Moreover, the mesophyll cells are loosely arranged, the enzymes have an easy access to the cell wall. The parent plant age and the conditions in which it is growing have profound effect on the yield of protoplast. Due to the difficulty in isolating culturable protoplast from leaf cells of cereals and some other species their cultured cells can be used as a source material. The yield of protoplasts depends upon the growth rate and growth phase of the cells. Generally embryogenic suspension cultures are used to obtain totipotent protoplasts.

ii. **Pre-enzyme treatments:** To facilitate the penetration of enzyme solution into the intercellular spaces of leaf, which is essential for effective digestion, various methods are followed. The most commonly used method is to peel the lower epidermis and float the stripped pieces of leaf on the enzyme solution in a manner that the peeled surface is in a contact with the solution. Most of the time it is not convenient to peel the epidermis, in such cases cutting the leaf or tissue into small strips (1- 2 mm wide) has been found useful. When combined with vacuum infiltration the latter approach has proved very effective. Brushing of leaves with a soft brush or with the cutting edge of a scalpel may also improve the enzymatic action. Large calli are chopped into pieces and can be transferred to enzyme mixture. Agitation of incubation mixture during enzyme treatment improves protoplast yield from cultured cells.

iii. **Enzyme treatment :** The release of protoplast is very much dependent on the nature and concentration of the enzymes used. The two major enzymes required for the isolation of protoplast are cellulase and pectinase. The cellulase is required to digest the cellulosic cell walls and the pectinase mainly degrades the middle lamella. Some of the tissues may require other enzymes like, hemicellulase, driselase, macerozyme and pectolyase. The activity of enzyme is pH dependent. The pH of the enzyme solution is adjusted somewhere between 4.7 to 6.0.

3.1.2 Choice of Plasmolyticum

The two most commonly used compounds are the sugar alcohols - mannitol and sorbitol. Of these, mannitol is the most preferred since it is not metabolised by the plant cells. Once the protoplast divides and regenerates the cell wall, no more osmoticum is required. It is, therefore, should be removed gradually from the medium otherwise cell division

stops. To slowly remove the osmoticum from the medium, the protoplast can be isolated in a high osmoticum mixture consisting of both mannitol and sucrose, the sucrose will be metabolised by the dividing protoplasts and thus, will reduce the osmolarity of the medium. Normally, mannitol is used at concentration range of 11-13%. A solution into which the osmoticum is often, but not always, added is called CPW salts mix or CPW for short. This has been observed much more beneficial than using distilled water as a solvent in obtaining high yields of viable protoplasts:

Table 1.1: Salt mix of protoplast washing media solution (Cocking, Peberdy and White – CPW)

KH_2PO_4	27.2 mg
KNO_3	101 mg
$CaCl_2.2H_2O$	1480 mg
$MgSO_4.7H_2O$	246 mg
KI	0.16 mg
$CuSO_4.5H_2O$	0.025 mg
Made to 1 litre with water : pH 5.8	

Although CPW is most widely used solution into which osmoticum or enzymes are added, sometimes culture medium used to grow cells or plants can also be utilised for protoplast isolation at one tenth concentration. Low concentration of culture medium is much more advantageous when compared with CPW.

3.1.3 Protoplast Purification

Enzyme treatment results in suspension of protoplast, undigested tissues and cellular debris. This suspension is passed through a metal sieve or a nylon mesh (50-100 µm) in order to remove undigested cellular clumps. The filtered protoplast-enzyme solution is mixed with a suitable volume of osmoticum, solution is centrifuged to pellet the protoplasts, pellet of protoplast is resuspended in osmoticum of similar concentration as used in enzyme mixture. The protoplast band is sucked in Pasteur pipette and is put into other centrifuge and finally suspended in culture medium at particular density; this is explained by the Figure 12. 2.

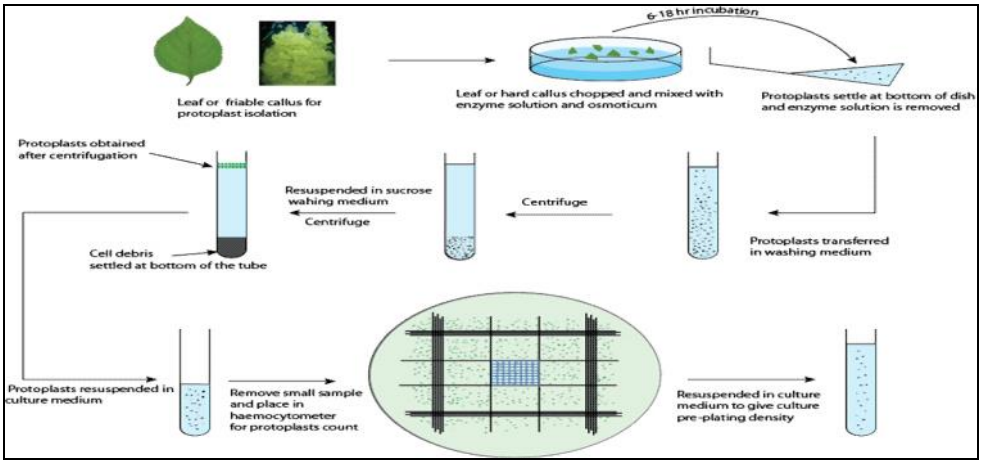

Figure 1.1: schematic diagram of protoplast purification

3.1.4 Protoplast Viability

The isolated protoplast must have a spherical shape when observed by a light microscope, protoplast can be stained using following stain:

- *Fluorescein diacetate staining method:* FDA accumulates inside the plasmalemma of viable protoplasts. Live protoplasts contain esterases which cleave FDA to release fluorescein which fluoresces yellowish-green using fluorescence microscopy within 5 min. FDA dissociates from membrane after about 15 min. It is used at a concentration of 0.01% dissolved in acetone.
- *Calcofluor White staining:* This staining method assures protoplast viability by detecting onset of cell wall formation. Calcofluor binds to beta linked glucosides in newly synthesized cell wall which can be observed as a fluorescent ring around the membrane. Optimum staining is achieved when 0.1 ml of protoplast is mixed with 5.0 µl of 0.1% w/v solution of CFW.
- *Protoplast viability* can also be detected by monitoring oxygen uptake of cells by oxygen electrode, which shows respiration.
- *Variation of protoplast size* with changing osmotic concentration also enables viability of protoplast.

3.2 Protoplast Culture

3.2.1 Protoplasts Culture Techniques

The culture requirements and the culture methods are same for both protoplasts and single cells. The main difference is the requirement of suitable osmoticum for protoplasts until they regenerate a strong wall. Isolated protoplasts are either cultured in liquid or semisolid agar or agarose media plates, sometimes the protoplast is first grown in liquid

media and then transferred into the agar media plates. The following techniques have been adopted in order to maintain number of protoplast population between minimum and maximum effective densities after plating up:

i. *Liquid method* **:** This method is preferred in earlier stages of culture as it provides (a) easier dilution and transfer, (b) the osmotic pressure of liquid media can be effectively reduced after a few days of culture (c) the cell density can be reduced or cells of special interest can be isolated easily. In Liquid medium, the protoplast suspension is plated as a thin layer in petriplates, incubated as static culture in flasks or distributed in 50-100 µl drops in petriplates and stored in a humidifier chamber.

ii. *Embedded in Agar/ Agarose* : Agarose is a preferred choice in place of agar and this has improved the culture response. This method of agar culture keeps protoplast in fixed position, thus, prevents it from forming clumps. Immobilised protoplasts give rise to clones which can then be transferred to other media. In practice, the protoplasts suspended in molten (40°C) agarose medium (1.2% w/v agarose) are dispensed (4ml) into small (3.5-5cm diameter) plates and allowed to solidify. The agarose layer is then cut into 4 equal sized blocks and transferred to larger dishes (9 cm diameter) containing liquid medium of otherwise the same composition. Alternatively, protoplasts in molten agarose medium are dispensed as droplets (50-100 µl) on the bottom of petri plates and after solidification the droplets are submerged in the same liquid medium.

iii. *Feeder layer* : In order to culture protoplast at low density, a feeder layer technique is adopted. A feeder layer of X-ray irradiated non-dividing but metabolically active protoplasts after washing are plated in soft agar medium. Non-irradiated protoplasts of low density are plated over this feeder layer. The protoplasts of the same species or different species can be used as a feeder layer.

iv. *Co-culturing:* This method involves co-culture of protoplasts from two different species to promote their growth or that of the hybrid cells. Metabolically active and dividing protoplasts of two types - slow and fast growing are cultured together, the fast growing protoplast provide other species with diffusible chemicals and growth factors which helps in cell wall formation and cell division. The co-culture methods are generally used where calli arising from two types of protoplasts can be morphologically distinguished. For example, protoplasts isolated from albino plants and green plants are easily distinguishable based on color where albino protoplast will develop non green colonies.

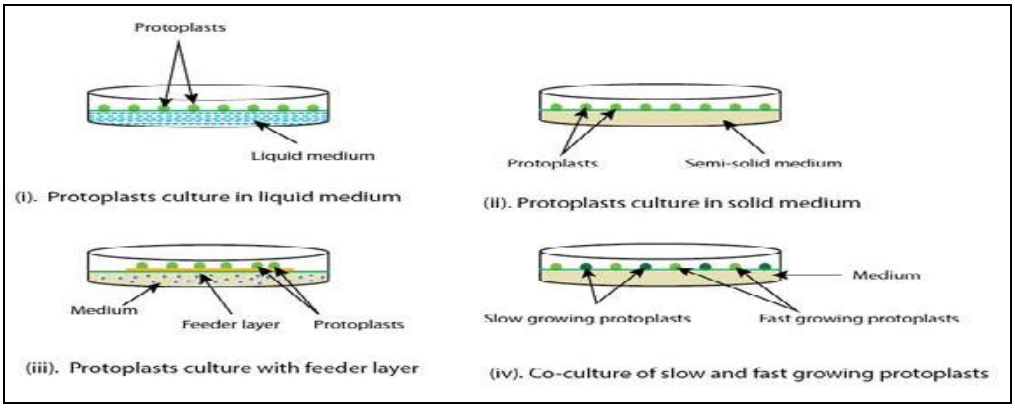

Figure 1.2: protoplast culture techniques

3.2.2 Culture Medium

The nutritional requirement of protoplast is almost similar to that of the cultured plant cells. Mostly the salts of MS (Murashige and Skoog, 1962) and B_5 (Gamborg et al. 1968) media and their modifications have been used. Ammonium salts have been found detrimental to protoplasts survival of many species, and media have been devised that either have a reduced concentration of ammonium or lack it. Concentration of zinc is reduced while the concentration of calcium is increased as it enhances the membrane stability. Osmolarity is maintained by addition of sorbitol, mannitol, glucose or sucrose and mannitol being widely used osmoticum as it is not used by the dividing cells, thus, maintains the osmolarity of the medium.

Glucose is preferred carbon source as sucrose do not satisfy protoplast culture. One or two amino acids are added at low concentration. Growth regulators are required essentially in protoplast culture generally high concentration of auxins (NAA, 2,4-D) along with lower concentration of cytokinins (BAP, Zeatin) is used. Environmental conditions: High light intensity inhibits growth of protoplast hence initially protoplast is grown in dim light for few days and then transferred to light of about 2000-5000 lux. However, better results are obtained when cultured in darkness.

3.2.3 Plating Density

Like cell cultures, the initial plating density of protoplasts has profound effect on plating efficiency. Protoplasts are cultured at a density of 1 x 10^4 to 1 x 10^5 protoplasts ml^{-1} of the medium. At high density the cell colonies arising from individual protoplasts tend to grow into each other resulting into chimera tissue if the protoplast population is genetically heterogeneous. Cloning of individuals cells, which is desirable in

somatic hybridisation and mutagenic studies, can be achieved if protoplasts or cells derived from them can be cultured at a low density.

3.3 Protoplast Development and Regeneration

Protoplast starts to regenerate a cell wall within few days (2-4 days) of culture and during this process; protoplasts lose their characteristic spherical shape which has been taken as an indication of new wall regeneration. Cell wall regeneration can be confirmed by Calcofluor White staining method. There is direct relationship between wall formation and cell division. Protoplasts which are not able to regenerate a proper wall fail to undergo normal mitosis. Protoplasts with a poorly developed wall often show budding and may enlarge several times their original volume. They may become multinucleate because karyokinesis is not accompanied by cytokinesis. Among other reasons, inadequate washing of the protoplasts prior to culture leads to these abnormalities.
And completes process when provided with suitable condition of light, pH and temperature newly synthesised protoplast can be visualized by staining. Once the cell wall formation is completed, cells undergo division resulting in increased size of cells. After an interval of 3 weeks, small cell colonies appear, these colonies are transferred to an osmotic-free callus induction medium. This is followed by introduction into organogenic or embryogenic medium leading to plantlet development.

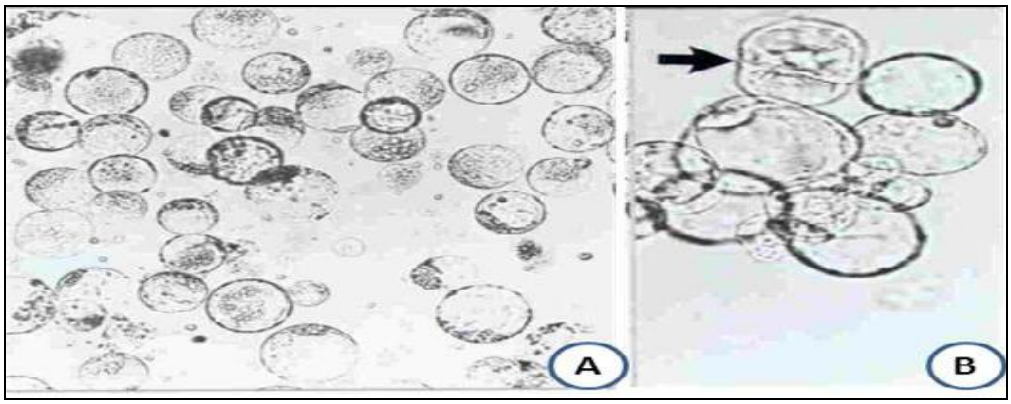

Figure 1.3: Protoplast isolation and cell wall regeneration. **A.** Isolated protoplast showing spherical structure; **B.** Wall is regenerated around the protoplast and one of the protoplasts showing cell division (arrow marked)

4.0 CONCLUSION

However, to fully explore the potentials for protoplast-technology, efficient and reproducible methods for protoplast isolation and purification must first be established. Since leaf tissue is a readily accessible source of genetically uniform cells, it is often desirable to use mesophyll protoplasts in somatic hybridisation studies, but, leaf tissues, in general, do not yield large number of protoplasts owing to the difficulty in removing the lower epidermis (Chaturvedi 2003). An alternative, therefore, is the cultured cell material where protoplasts can show greater potential to divide (Bhojwani and Razdan 1996).

5.0 SUMMARY

The term protoplast was introduced by Hanstein in 1880. It refers to the cellular content excluding cell wall or can also be called as naked plant cell. It is described as living matter enclosed by a plant cell membrane. Protoplast isolation for the first time was carried out by Klercker in 1892 using mechanical method on the plasmolysed cells. The application of protoplast technology for the improvement of plants offers fascinating option to complement conventional breeding programs. The ability of isolated protoplasts to undergo fusion and take up macromolecules and cell organelles offers many possibilities in genetic engineering and crop improvement (Bhojwani et al . 1977). The experiments involving protoplasts consist of three stages –

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6.0 TUTOR-MARKED ASSIGNMENT

1. Define protoplasts and why the protoplast can be used as a starting material for genetic manipulation.
2. What is the role of cell wall degrading enzymes?
3. Name the best source used for protoplast isolation.

4. Which chemical is considered to be a most important media component for protoplast isolation and culture?
5. How do you count protoplasts and why it is essential?
6. List out different protoplast culture techniques. Why do you think a sequential reduction in osmoticum is necessary during culture?

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UNIT 2 INTRODUCTION TO PLANT TISSUE CULTURE

CONTENTS

1.0 INTRODUCTION

Plant tissue culture has become popular among horticulturists, plant breeders and industrialists because of its varied practical applications. It is also being applied to study basic aspects of plant growth and development. The discovery of the first cytokinin (kinetin) is based on plant tissue culture research. Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation, including:

- The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.
- To quickly produce mature plants.
- The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
- The regeneration of whole plants from plant cells that have been genetically modified.
- The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e.: orchids and Nepenthes.
- To clear particular plants of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.

- Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stems or roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know the meaning of plant tissue culture
- explain the various plant tissue techniques
- discuss regeneration pathways of plant tissue
- outline the choice of explant
- state the applications of plant tissue.

3.1 Plant Tisssue Culture Techniques

Preparation of plant tissue for tissue culture is performed under aseptic conditions under HEPA filtered air provided by a laminar flow cabinet. Thereafter, the tissue is grown in sterile containers, such as petri dishes or flasks in a growth room with controlled temperature and light intensity. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so their surfaces are sterilised in chemical solutions (usually alcohol and sodium or calcium hypochlorite) before suitable samples (known as explants) are taken. The sterile explants are then usually placed on the surface of a sterile solid culture medium, but are sometimes placed directly into a sterile liquid medium, particularly when cell suspension cultures are desired. Solid and liquid media are generally composed of inorganic salts plus a few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, usually purified agar.

Figure 2.1:In vitro tissue culture of potato explants

The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the morphology of the tissues that grow from the initial explant. For example, an excess of auxin will often result in a

proliferation of roots, while an excess of cytokinin may yield shoots. A balance of both auxin and cytokinin will often produce an unorganised growth of cells, or callus, but the morphology of the outgrowth will depend on the plant species as well as the medium composition. As cultures grow, pieces are typically sliced off and subcultured onto new media to allow for growth or to alter the morphology of the culture. The skill and experience of the tissue culturist are important in judging which pieces to culture and which to discard. As shoots emerge from a culture, they may be sliced off and rooted with auxin to produce plantlets which, when mature, can be transferred to potting soil for further growth in the greenhouse as normal plants.

3.2 Regeneration Pathways of Plant Tissue Culture

The specific differences in the regeneration potential of different organs and explants have various explanations. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators, and the metabolic capabilities of the cells. The most commonly used tissue explants are the meristematic ends of the plants like the stem tip, axillary bud tip and root tip. These tissues have high rates of cell division and either concentrate or produce required growth regulating substances including auxins and cytokinins.

Figure 2.2: Plant tissue cultures being grown at a seed bank.

Shoot regeneration efficiency in tissue culture is usually a quantitative trait that often varies between plant species and within a plant species among subspecies, varieties, cultivars, or ecotypes. Therefore, tissue culture regeneration can become complicated especially when many regeneration procedures have to be developed for different genotypes within the same species. The three common pathways of plant tissue culture regeneration are propagation from preexisting meristems (shoot culture or nodal culture), organogenesis and non-zygotic embryogenesis.

The propagation of shoots or nodal segments is usually performed in four stages for mass production of plantlets through in vitro vegetative

multiplication but organogenesis is a common method of micropropagation that involves tissue regeneration of adventitious organs or axillary buds directly or indirectly from the explants. Non-zygotic embryogenesis is a noteworthy developmental pathway that is highly comparable to that of zygotic embryos and it is an important pathway for producing somaclonal variants, developing artificial seeds, and synthesising metabolites. Due to the single cell origin of non-zygotic embryos, they are preferred in several regeneration systems for micropropagation, ploidy manipulation, gene transfer, and synthetic seed production. Nonetheless, tissue regeneration via organogenesis has also proved to be advantageous for studying regulatory mechanisms of plant development.

3.3 Choice of Explant

The tissue obtained from a plant to be cultured is called an explant.
Explants can be taken from many different parts of a plant, including portions of shoots, leaves, stems, flowers, roots, single undifferentiated cells and from many types of mature cells provided are they still contain living cytoplasm and nuclei and are able de-differentiate and resume cell division. This has given rise to the concept of to tipotentency of plant cells. However this is not true for all cells or for all plants. In many species explants of various organs vary in their rates of growth and regeneration, while some do not grow at all. The choice of explant material also determines if the plantlets developed via tissue culture are haploid or diploid. Also the risk of microbial contamination is increased with inappropriate explants.

The first method involving the meristems and induction of multiple shoots is the preferred method for the micropropagation industry since the risks of somaclonal variation (genetic variation induced in tissue culture) are minimal when compared to the other two methods. Somatic embryogenesis is a method that has the potential to be several times higher in multiplication rates and is amenable to handling in liquid culture systems like bioreactors.

Some explants, like the root tip, are hard to isolate and are contaminated with soil microflora that become problematic during the tissue culture process. Certain soil microflora can form tight associations with the root systems, or even grow within the root. Soil particles bound to roots are difficult to remove without injury to the roots that then allows microbial attack. These associated microflora will generally overgrow the tissue culture medium before there is significant growth of plant tissue.
Some cultured tissues are slow in their growth. For them there would be two options:

(i) Optimising the culture medium;
(ii) Culturing highly responsive tissues or varieties. Necrosis can spoil cultured tissues. Generally, plant varieties differ in susceptibility to tissue culture necrosis. Thus, by culturing highly responsive varieties (or tissues) it can be managed.

Aerial (above soil) explants are also rich in undesirable microflora. However, they are more easily removed from the explant by gentle rinsing, and the remainder usually can be killed by surface sterilisation. Most of the surface microflora do not form tight associations with the plant tissue. Such associations can usually be found by visual inspection as a mosaic, de-colorisation or localised necrosis on the surface of the explant.

An alternative for obtaining uncontaminated explants is to take explants from seedlings which are aseptically grown from surface-sterilized seeds. The hard surface of the seed is less permeable to penetration of harsh surface sterilising agents, such as hypochlorite, so the acceptable conditions of sterilisation used for seeds can be much more stringent than for vegetative tissues. Tissue cultured plants are clones. If the original mother plant used to produce the first explants is susceptible to a pathogen or environmental condition, the entire crop would be susceptible to the same problem. Conversely, any positive traits would remain within the line also.

3.4 Applications of Plant Tissue Culture

Plant tissue culture is used widely in the plant sciences, forestry, and in horticulture. Applications include:

- The commercial production of plants used as potting, landscape, and florist subjects, which uses meristem and shoot culture to produce large numbers of identical individuals.
- To conserve rare or endangered plant species.
- A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance.
- Large-scale growth of plant cells in liquid culture in bioreactors for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals.
- To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.
- To rapidly study the molecular basis for physiological, biochemical, and reproductive mechanisms in plants, for example in vitro selection for stress tolerant plants.

- To cross-pollinate distantly related species and then tissue culture the resulting embryo which would otherwise normally die (Embryo Rescue). For chromosome doubling and induction of polyploidy, for example doubled haploids, tetraploids, and other forms of polyploids. This is usually achieved by application of antimitotic agents such as colchicine or oryzalin. As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants. Certain techniques such as meristem tip culture can be used to produce clean plant material from virused stock, such as sugarcane, potatoes and many species of soft fruit. Production of identical sterile hybrid species can be obtained. Large scale production of artificial seeds through somatic embryogenesis

1.5 Advantages of Plant Tissue Culture Over Conventional Agricultural Production

The most important advantage of *in vitro* grown plants is that it is independent of geographical variations, seasonal variations and also environmental factors. It offers a defined production system, continuous supply of products with uniform quality and yield. Novel compounds which are not generally found in the parent plants can be produced in the in vitro grown plants through plant tissue culture. In addition, stereo- and region- specific biotransformation of the plant cells can be performed for the production of bioactive compounds from economical precursors. It is also independent of any political interference. Efficient downstream recovery of products and rapidity of production are its added advantages (Figure 5.1).

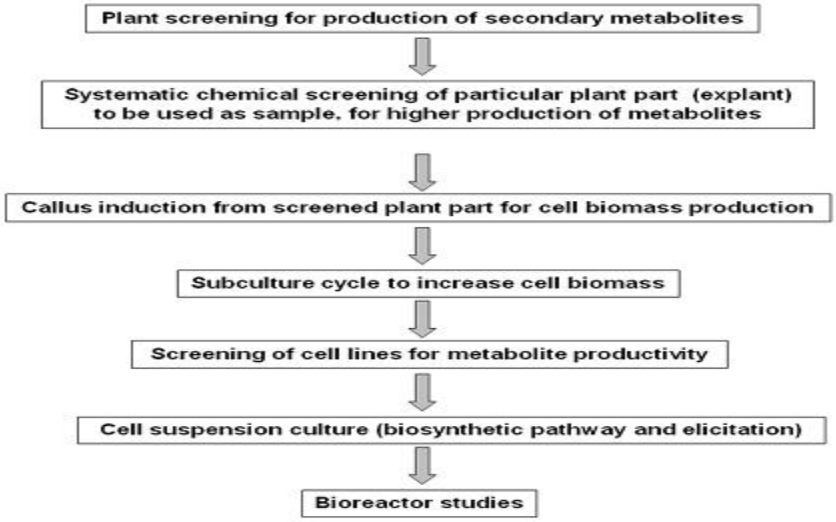

Figure 2.3: Steps involved in the production of secondary metabolites from plant cell

4.0 CONCLUSION

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as micropropagation.

5.0 SUMMARY

Preparation of plant tissue for tissue culture is performed under aseptic conditions under HEPA filtered air provided by a laminar flow cabinet. Thereafter, the tissue is grown in sterile containers, such as petri dishes or flasks in a growth room with controlled temperature and light intensity. The specific differences in the regeneration potential of different organs and explants have various explanations. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators, and the metabolic capabilities of the cells. The tissue obtained from a plant to be cultured is called an explant. Explants can be taken from many different parts of a plant, including portions of shoots, leaves, stems, flowers, roots, single undifferentiated cells and from many types of mature cells provided are they still contain living cytoplasm and nuclei and are able de-differentiate and resume cell division. The tissue obtained from a plant to be cultured is called an explant.

Explants can be taken from many different parts of a plant, including portions of shoots, leaves, stems, flowers, roots, single undifferentiated cells and from many types of mature cells provided are they still contain living cytoplasm and nuclei and are able de-differentiate and resume cell division. Plant tissue culture is used widely in the plant sciences, forestry, and in horticulture. Most common Applications include: The commercial production of plants used as potting, landscape, and florist subjects, which uses meristem and shoot culture to produce large numbers of identical individuals.However The most important advantage of *in vitro* grown plants is that it is independent of geographical variations, seasonal variations and also environmental factors.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is the meaning of plant tissue culture?
2. Explain plant tissue techniques.
3. Discuss regeneration pathways of plant tissue.
4. Outline the choice of explant.
5. State the applications of plant tissue.

6. Highlight the advantage of plant tissue culture over conventional agricultural production.

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UNIT 3 INTRODUCTION TO MICROPROPAGATION IN BIOTECHNOLOGY

CONTENTS

1.0 INTRODUCTION

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micropropagation is used to multiply plants such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain the stages of micropropagation
- discuss the methods of micropropagation
- list the advantages of micropropagation
- list the disadvantages of micropropagation

3.0 MAIN CONTENT

3.1 Stages of Micropropagation

In short, steps of micropropagation can be divided into four stages.

1. Establishment (Selection of mother plant)
2. Multiplication

3. Pretransplant Rooting and acclimatising
4. Transfer from culture Transfer new plant to soil

3.1.1 Establishment

Micropropagation begins with the selection of plant material to be propagated. The plant tissues are removed from an intact plant in a sterile condition. Clean stock materials that are free of viruses and fungi are important in the production of the healthiest plants. Once the plant material is chosen for culture, the collection of explant(s) begins and is dependent on the type of tissue to be used; including stem tips, anthers, petals, pollen and other plant tissues. The explant material is then surface sterilised, usually in multiple courses of bleach and alcohol washes, and finally rinsed in sterilised water. This small portion of plant tissue, sometimes only a single cell, is placed on a growth medium, typically containing sucrose as an energy source and one or more plant growth regulators (plant hormones). Usually the medium is thickened with agar to create a gel which supports the explant during growth. Some plants are easily grown on simple media,(figure 3.1) but others require more complicated media for successful growth; the plant tissue grows and differentiates into new tissues depending on the medium. For example, media containing cytokinin are used to create branched shoots from plant buds.

Figure 3.1: In vitro culture of plants in a controlled, sterile environment

3.1.2 Multiplication

Multiplication is the taking of tissue samples produced during the first stage and increasing their number. Following the successful introduction and growth of plant tissue, the establishment stage is followed by multiplication. Through repeated cycles of this process, a single explant sample may be increased from one to hundreds and thousands of plants.

Depending on the type of tissue grown, multiplication can involve different methods and media. If the plant material grown is callus tissue, it can be placed in a blender and cut into smaller pieces and recultured on the same type of culture medium to grow more callus tissue. If the tissue is grown as small plants called plantlets, hormones are often added that cause the plantlets to produce many small offshoots. After the formation of multiple shoots, these shoots are transferred to rooting medium with a high auxin\cytokinin ratio. After the development of roots, plantlets can be used for hardening.

3.1.3 Pretransplant

This stage involves treating the plantlets/shoots produced to encourage root growth and "hardening." It is performed in vitro, or in a sterile "test tube" environment. Banana plantlets transferred to soil (with vermicompost) from plant media. This process is done for acclimatisation of plantlets to the soil as they were previously grown in plant media. After growing for some days the plantlets are transferred to the field.

"Hardening" refers to the preparation of the plants for a natural growth environment. Until this stage, the plantlets have been grown in "ideal" conditions, designed to encourage rapid growth. Due to the controlled nature of their maturation, the plantlets often do not have fully functional dermal coverings. This causes them to be highly susceptible to disease and inefficient in their use of water and energy. In vitro conditions are high in humidity, and plants grown under these conditions often do not form a working cuticle and stomata that keep the plant from drying out.

Figure 3.2: Banana plantlets transferred to soil (with vermicompost) from plant media

When taken out of culture, the plantlets need time to adjust to more natural environmental conditions. Hardening typically involves slowly weaning the plantlets from a high-humidity; low light, warm environment to what would be considered a normal growth environment for the species in question.

3.1.3 Transfer from Culture

In the final stage of plant micropropagation, the plantlets are removed from the plant media and transferred to soil or (more commonly) potting compost for continued growth by conventional methods. This stage is often combined with the "pretransplant" stage.

Figure 3.3: Plant tissue cultures being grown at a seed bank

3.2 Methods of Micropropagation

3.2.1 Meristem Culture

In Meristem culture the Meristem and a few subtending leaf primordial are placed into a suitable growing media. An elongated rooted plantlet is produced after some weeks, and is transferred to the soil when it has attained a considerable height. A disease free plant can be produced by this method. Experimental result also suggests that this technique can be successfully utilised for rapid multiplication of various plant materials, e.g. Sugarcane, strawberry.

3.2.2 Callus Culture

A callus is mass of undifferentiated parenchymatous cells. When a living plant tissue is placed in an artificial growing medium with other conditions favorable, callus is formed. The growth of callus varies with the homogenous levels of auxin and Cytokinin and can be manipulated by endogenous supply of these growth regulators in the culture medium. The callus growth and its organogenesis or embryogenesis can be referred into three different stages.

Stage I: Rapid production of callus after placing the explants in culture medium

Stage II: The callus is transferred to other medium containing growth regulators for the induction of adventitious organs.

Stage III: The new plantlet is then exposed gradually to the environmental condition.

3.2.3 Suspension Culture

A cell suspension culture refers to cells and or groups of cells dispersed and growing in an aerated liquid culture medium (Street, 1997, Thorpe1981) is placed in a liquid medium and shaken vigorously and balanced dose of hormones. Suezawa et al. (1988) reported Cyotkinin induced adventitious buds in kiwi fruit in a suspension culture sub-culture for about a week.

3.2.4 Embryo Culture

In embryo culture, the embryo is excised and placed into a culture medium with proper nutrient in aseptic condition. To obtain a quick and optimum growth into plantlets, it is transferred to soil. It is particularly important for the production of interspecific and intergeneric hybrids and to overcome the embryo.

3.2.5 Protoplast Culture

In protoplast culture, the plant cell can be isolated with the help of wall degrading enzymes and growth in a suitable culture medium in a controlled condition for regeneration of plantlets. Under suitable conditions the protoplast develops a cell wall followed by an increase in cell division and differentiation and grows into a new plant. The protoplast is first cultured in liquid medium at 25 to 28 C with a light intensity of 100 to 500 lux or in dark and after undergoing substantial cell division, they are transferred into solid medium congenial or morphogenesis in many horticultural crops respond well to protoplast culture.

3.3 Advantages of Micropropagation

Micropropagation has a number of advantages over traditional plant propagation techniques:

a. The main advantage of micropropagation is the production of many plants that are clones of each other.
b. Micropropagation can be used to produce disease-free plants.
c. It can have an extraordinarily high fecundity rate, producing thousands of propagules while conventional techniques might only produce a fraction of this number.
d. It is the only viable method of regenerating genetically modified cells or cells after protoplast fusion.

e. It is useful in multiplying plants which produce seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed cannot be stored (see recalcitrant seeds).
f. Micropropagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods - like seeds or cuttings.
g. Some plants with very small seeds, including most orchids, are most reliably grown from seed in sterile culture.
h. A greater number of plants can be produced per square meter and the propagules can be stored longer and in a smaller area.

3.4 Disadvantages of Micropropagation

Micropropagation is not always the perfect means of multiplying plants. Conditions that limit its use include:

a. It is very expensive, and can have a labour cost of more than 70%.[clarification needed]
b. A monoculture is produced after micropropagation, leading to a lack of overall disease resilience, as all progeny plants may be vulnerable to the same infections.
c. An infected plant sample can produce infected progeny. This is uncommon as the stock plants are carefully screened and vetted to prevent culturing plants infected with virus or fungus.
d. Not all plants can be successfully tissue cultured, often because the proper medium for growth is not known or the plants produce secondary metabolic chemicals that stunt or kill the explant.
e. Sometimes plants or cultivars do not come true to type after being tissue cultured. This is often dependent on the type of explant material utilised during the initiation phase or the result of the age of the cell or propagule line.
f. Some plants are very difficult to disinfect of fungal organism
g. The major limitation in the use of micropropagation for many plants is the cost of production; for many plants the use of seeds, which are normally disease free and produced in good numbers, readily produce plants (see orthodox seed) in good numbers at a lower cost. For this reason, many plant breeders do not utilize micropropagation because the cost is prohibitive. Other breeders use it to produce stock plants that are then used for seed multiplication.
h. Mechanisation of the process could reduce labour costs, but has proven difficult to achieve, despite active attempts to develop technological solutions.

4.0 CONCLUSION

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micropropagation is used to multiply plants such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

5.0 SUMMARY

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. The stages of micropropagation are establishment (selection of mother plant), multiplication, pretransplant rooting and acclimatising and transfer from culture transfer new plant to soil. While the methods of Micropropagation are summarized as; Meristem culture, Callus culture, Suspension culture, Embryo culture and Protoplast culture. Micropropagation has a number of advantages over traditional plant propagation techniques and the main advantage of micropropagation is the production of many plants that are clones of each other. While the disadvantages of micropropagation are discussed as used to multiply plants such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

6.0 TUTOR-MARKED ASSIGNMENT

1. Define micropropagation.
2. Explain the stages of Micropropagation.
3. Discuss the methods of Micropropagation.
4. List the advantages of Micropropagation.
5. List the disadvantages of Micropropagation.

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UNIT 4 DOUBLED HAPLOID PRODUCTION

CONTENTS

1.0 INTRODUCTION

Haploids are sexually sterile and, therefore, doubling of the chromosomes is required to produce fertile plants which are called doubled haploids or homozygous diploids. Haploids have attracted great interest of plant physiologists, embryologists, geneticists and breeders. Spontaneous production of haploids usually occurs through the process of parthenogenesis (embryo development from an unfertilised egg). However, occasionally, they bear the characters of male parent only, suggesting their origin through ‘ovule androgenesis' (embryo development inside the ovule by the activity of the male nucleus alone where elimination or inactivation of egg nucleus occurs before fertilisation).

Haploid and diploid lines play a vital role in genomics and have been used for the purpose of physical mapping, genetic mapping and also for integration of genetic and physical maps. Additionally, haploid and doubled haploid plants are adapted for mutagenesis and genetic transformation experiments, presenting the advantage of immediate production of homozygous lines. It is also expected that, in the near future, haploid and doubled haploid plants will play an increasingly important role in whole genome sequencing projects, where homozygosity is of particular advantage. The technique has special significance for genetic improvement of the tree species where breeding is made difficult due to long generation intervals and highly heterozygous nature of these plants as a result of cross pollination.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand what androgenes is
- know what gynogenes is
- state factors affecting haploid androgenesis
- know the meaning of diploidisation of haploids
- list applications of haploid production.

3.0 MAIN CONTENT

3.1 Androgenesis

In androgenesis, the male gametophyte (microspore or immature pollen) produces haploid plants. The basic principle is to stop the development of pollen into a gamete (sex cell) and force it to develop into a haploid plant or sporophyte. The remarkable discovery that haploid embryos and plants can be produced by *in vitro* culture of anthers of *Datura* (Guha and Maheshwari 1964,1966) brought renewed interest to haploidy. This method of androgenic haploid production was quickly attempted in many species to hasten the breeding programme in several economically important plants. Haploid production through anther/microspore culture scores higher over other methods due to the fact that anthers harbour large numbers of haploid microspores per anther and is a potentially efficient means to generate homozygous true-breeding progeny lines in plant breeding programs.

3.1.1 Methodologies

In androgenesis, immature pollen grains are induced to follow the sporophytic mode of development by the application of various physical and chemical stimuli. There are two methods for *in vitro* production of androgenic haploids –
(A) Anther culture, and (B) Isolated pollen (microspore) culture.

3.1.3 Comparison between Anther and Pollen Culture

Anther culture is an easy, quick and practicable approach. Anther walls act as conditioning factors and promote culture growth. Thus, anther cultures are reasonably efficient for haploid production. The major limitation is that the plants not only originate from pollen but also from other somatic parts of the anther. This results in the development of plants at different ploidy levels viz., haploids, diploids, aneuploids, as a result of which the final tissue, derived, may not be of purely

gametophytic origin. Moreover, the plants arising from an anther would constitute a heterogenous population. It has been observed in some species that anther cultures show asynchronous pollen development, the older grains may suppress the androgenic potential of younger grains by releasing toxic substances. The disadvantages associated with Anther culture can be overcome by pollen culture as it offers the following advantages:

- Undesirable effects of anther wall and associated tissues can be avoided.
- Androgenesis, starting from a single cell, can be better regulated.
- Isolated microspores (pollen) are ideal for various genetic manipulations like transformation, mutagenesis etc.
- The yield of haploid plants is relatively higher.

3.1.4 Pathways of Development

The early divisions in responding pollen grains may occur in any one of the following four pathways (Figure 9.3):

i) Pathway I - The uninucleate pollen grain may divide symmetrically to yield two equal daughter cells both of which undergo further divisions e.g. *Datura innoxia* .

ii) Pathway II - In some other cases e.g. *N.tabacum*, barley, wheat etc., the uninucleate pollen divides unequally. The generative cell degenerates, callus/embryo originates due to successive divisions of the vegetative cell.

iii) Pathway III - But in few species, the pollen embryos originate from the generative cell alone; the vegetative cell either does not divide or divides only to a limited extent forming a suspensor like structure.

iv) Pathway IV - Finally in few other species e.g. *Datura innoxia*, the uninucleate pollen grains divide unequally, producing generative and vegetative cells, but both these cells divide repeatedly to contribute to the developing embryo/callus.

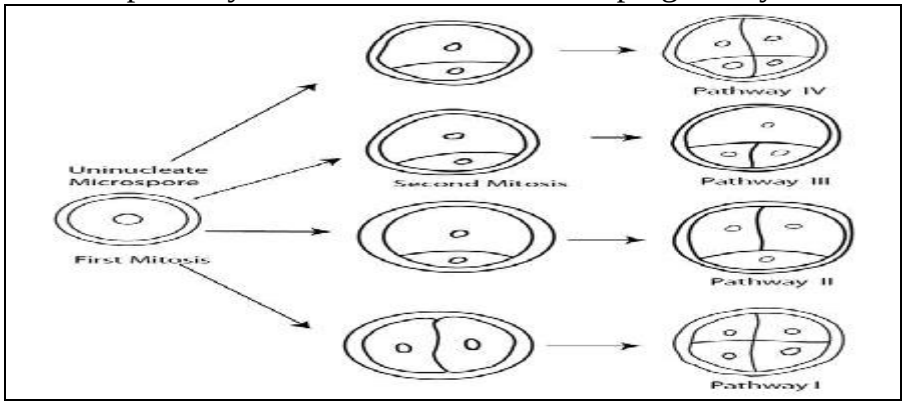

Figure 4.1: Pathways of development of microspores

3.2 Gynogenesis

Gynogenic development of plants from unfertilized cells of female gametophyte (embryo-sac) in ovary/ovule/young flower cultures is one of the available alternatives for haploid production. It was first reported in barley San Noeum (1976). This method of haploid production is more tedious than androgenesis. The reasons for this being the indefinite numbers of microspores (male gametes) within the anther wall for androgenesis as against single egg cell (female gamete) per flower for gynogenic haploid production, which too, is deep seated within the embryo-sac (female gametophyte), thus making the entire process very cumbersome. The technique is very useful where anther culture has been unsuccessful, plants are male sterile or androgenesis is confronted with the problem of albino or non-haploid formation. The following techniques are generally used for production of haploids via *in vitro* gynogenesis either through direct embryogenesis or via callusing.

1. In situ parthenogenesis induced by irradiated pollen followed by in vitro embryo culture
2 Ovary slice culture
3. Ovule culture

3.3 Factors Affecting Haploid Androgenesis

There are numerous endogeneous and exogeneous factors that affect *in vitro* haploid production. These factors can be genetic, physiological, physical and chemical may also interact amongst each other to divert the microspores/egg cell to enter into a new developmental pathway. Some of the crucial factors affecting haploid production in plants have been discussed below:

3.3.1 Genotype of the Donor Plant

The genotype of the donor plants, i.e. genetic factor, has a great influence on the anther, ovary and ovule culture response. In earlier studies, significant differences in callus formation using varieties or crosses were observed. In some species only a few genotypes have responded of many tested. In fact genetic factors contribute in a major way to the differences in the number of haploid plants produced (Custódio et al, 2005).

3.3.2 Physiological Status of the Donor Plant

The physiological conditions of the donor plant, *i.e* the environmental conditions and age of the donor plant, directly affects both *in vitro* androgenesis and *in vitro* gynogenesis in almost all plant species.

A correlation between plant age and anther response has also been demonstrated. Similar is the case with ovary culture. The frequency of androgenesis is usually higher in anthers harvested at the beginning of the flowering period and showed a gradual decline in relation to plant age (Bhojwani and Razdan 1996). Varying temperature and light conditions during the growth of donor plants also affect Anther response. In Anther culture of grape, the induction frequency of embryoids derived from spring flowers was higher than that derived from summer flowers (Zhou and Li 1981). The microscopical observations showed that some varieties of rubber tree often have a lot of degenerated and sterile microspores in their anthers in early spring or hot summer due to the influence of unfavourable climatic conditions (Chen et al, 1982). As a result no pollen embryoids were obtained from such anthers but only the somatic calli.

3.3.3 Stage of Explants Material at the Time of Inoculation

3.3.3.1 Stage of Microspores

The stage of microspores at the time of inoculation is one of the most critical factors in the induction of androgenesis. Detailed cytological studies conducted on poplars, rubber (Chen 1986) and apple (Zhang et al, 1990) have shown that androgenic callus and embryos were mainly induced through a deviation of the first pollen mitosis to produce two undifferentiated nuclei. Besides affecting the overall response, the microspore stage at culture also has a direct bearing on the nature of plants produced in Anther culture (Sunderland and Dunwell 1977). About 80% of the embryos obtained from binucleate microspores of *Datura innoxia*, a highly androgenic species, were non-haploids (Sunderland et al, 1974). In a vast majority of species where success has been achieved, anthers were cultured when microspores were at the uninucleate stage of microsporogenesis (Chaturvedi et al, 2003; Pedroso and Pais 1994; Sopory and Munshi 1996).

3.3.3.2 Stage of the Embryo Sac

It has been reported that the effect of ovule development on gynogenesis is profound as it harbours the embryo sac comprising of the egg cell. The stage of embryo sac is an important determining factor for *in vitro* gynogenesis in various plant species. However, it is difficult to know the stage of embryo sac at the time of inoculation. Several authors prefer to describe the inoculation stage according to the developmental stage of the flower bud or stage of pollen development. However, this could not be possible in several species, where male and female gametophytes do not mature simultaneously, a phenomenon known as protandry, the maturation of anthers before carpels (e.g., onion, leek,

sunflower, sugar beet and carrot) and the opposite protogyny (e.g., pearl millet). In such cases, the stage of embryo-sac at culture can be determined by histological preparations of ovary/ovules that are at identical stage with that of cultured ovary/ovules.

Although a wide range of embryo sac stages are responsive to gynogenic development, but, in most cases nearly mature embryo-sac stage gave better results. This is quite contrary to Anther culture in which mature pollen is non responsive to androgenesis. In Barley and rice, unfertilised ovary cultures with late staged mature embryo sacs gave good results (San Noeum 1976, 1979; Wang and Kuang 1981) while others reported success with ovary cultures containing uninucleate to mature embryo sacs (Zhou and Yang, 1981b, 1982; Kuo, 1982; Huang et al., 1982).

3.3.4 Anther Wall Factor

One of the important research subjects in Anther culture of woody plants is to avoid the over proliferation of callus from anther wall tissues and to achieve a high yield of pollen embryoids and pollen calli. In Anther culture of most woody plants, both pollen calli (or embryoids) and somatic calli from anther wall tissues grew simultaneously. The development of callus from somatic tissues of anther can be avoided by culture of isolated microspores. However, microspore culture is not very successful in woody plants (Chaturvedi et al, 2003). Pelletier and Ilami (1972) had introduced the concept of “Wall Factor”, according to which the somatic tissues of anther play an important role in the induction of sporophytic divisions in pollen. In Anther culture of rubber, 47% of the microspores in close contact with the surrounding somatic cells could develop into multicellular masses as compared to only 5% of microspores away from the wall (Chen 1986). Anther wall callusing was regarded as a pre-requisite for the formation of androgenic haploids (Chaturvedi et al, 2003; Chen 1986; Chen et al, 1982).

3.3.5 Chemical Factors

The constituents of the basal medium and combination/s of growth regulators serve as important factors in eliciting successful androgenesis or gynogenesis. However, it is difficult to suggest one culture medium or one growth regulator for all the systems. The requirement of growth regulators and culture medium in terms of kind and concentration may vary with the system. Generally, there is an agreement that the source and amount of total nitrogen as well as a combination of a cytokinin and an auxin is necessary for pollen embryogenesis and pollen callusing in several woody plants (Chaturvedi et al, 2003; Chen 1986; Nair et al, 1983). Sucrose has generally been used as the major carbohydrate

source in the culture medium. The effect of sucrose on Anther culture has been investigated in a number of species. Generally, sucrose is supplied at 2-3% concentration. However, increase in its concentration can lead to beneficial morphogenic potential by suppressing the proliferation of somatic tissues.

3.3.6 Effect of Female Flower Position

Position of female flowers on the plant stem affected induction of embryos from ovule cultures of *Cucurbita pepo* L (Shalaby, 2007). One of the possible explanations for enhancing responses of tissue culture could be attributed to indigenous hormonal level (Johansson, 1986).

3.4 Diploidisation of Haploids

The haploids may grow normally up to flowering stage but in the absence of homologous chromosomes the meiosis is abnormal and consequently, viable gametes are not formed. To obtain fertile homozygous diploids, the chromosome complement of the haploids must be duplicated. For long colchicine has been used for the purpose and is more effective. Another method to diploidise the haploids is by utilizing the tendency of *in vitro* growing callus cells to undergo endomitosis. Segments from vegetative parts of haploid plants are grown on a suitable culture medium and made to proliferate into calli. After sometimes many of the callus cells become diploid due to endomitosis. By transferring such calli to an appropriate medium fertile diploids shoot can be obtained.

3.5 Applications of Haploid Production

3.5.1 Development of Pure Homozygous Lines

Homozygous, true breeding cultivars are highly important for screening of high yielding lines and to produce hybrid vigour as a method of crop improvement. Obtaining homozygous diploid plants by conventional methods is difficult in perennials. From several decades to over a hundred years are required to obtain a pure line by means of successive inbreeding throughout many generations. The seed set by inbreeding in many trees is very low, usually only a few of ten thousandth or sometimes no seed can be obtained at all; therefore, it is impractical to obtain pure lines by inbreeding (Chen, 1986). Moreover, conventional method of haploid production by inbreeding is impossible if the plant is strictly cross-pollinating in nature. On the other hand, homozygous diploid plants can be achieved in a single generation by diploidisation of *in vitro* raised haploids by colchicine treatment.

3.5.2 Genetic Studies

Because of the lack of accurate materials in research work, the progress in the study of genetics in trees is much slower than that in annual herbaceous plants. The genetics of a lot of important traits in economically important plant species has not been clearly demonstrated as yet. As a result, it is still unknown whether the desirable characters of the parents will appear in their progenies. Only when crossing between different homozygous diploid plants is carried out, we can gain a clear idea of dominance of genes controlling various characters and that these characters are either monogenic or polygenic (Chen 1986). Furthermore, if we can use the haploid plants as samples of gametes, then we can obtain directly the recombination value between genes. Moreover, we can also use the haploid plants to study chromosome homology within genome or between genomes.

3.5.3 Gametoclonal Variation

The "gametoclonal variation" arises among plants regenerated from cultured gametic cells consisting of differences in morphological and biochemical characteristics as well as in chromosome number and structures that are observed. Besides yielding haploids, *in vitro* androgenesis helps in the screening of gametophytic variation at plant level. Pollen grains within an anther form a highly heterogeneous population because they are the product of meiosis which involves recombination and segregation. Therefore, each pollen plant is genetically different from the other. The gametoclonal variants being hemizygous in nature expresses also the recessive characters in the R_0plants (Bhojwani and Razdan, 1996). Different sources of variation can explain gametoclonal variation such as new genetic variation induced by cell culture procedures, from segregation and independant assortment, chromosome doubling procedures etc (Morrison and Evans, 1987; Huang, 1996).

3.5.4 Induction of Mutations

In general, majority of induced mutations are recessive and, therefore, are not expressed in diploid cells due to the presence of dominant allele. Since, haploid plants have only one set of chromosomes, their dominant and recessive characters can be seen simultaneously on separate plants. It is extremely advantageous to provide a convenient system for the induction of mutations and selection of mutants with desirable traits in the absence of their dominant counterparts (Bhojwani and Razdan, 1996)

3.5.5 Obtaining New Genotypes with Alien Chromosomes

The technique of interspecific and intergeneric hybridisation can be combined with Anther culture techniques (Thomas et al., 2003) for obtaining new genotypes with alien chromosomes. Thus, new genotypes with various reconstructed chromosome complements can be obtained after their successful chromosome doubling.

3.5.6 Genetic Manipulation

As microspore culture is a single cell system, it makes selection at the single cell level possible and, furthermore, offers new prospects for genetic manipulation like mutagenesis and transformation. Direct gene transfer by microinjection offers the possibility of transgenic plant formation by using isolated pollen culture having high regeneration efficiency (Kasha and Maluszynski, 2003). Moreover, if transgenes can be incorporated into the haploid microspore genome, prior to DNA synthesis and chromosome doubling, the doubled haploids may also be homozygous for the transgenes. Thus, isolated microspores not only provide a good target for bombardment but, also are readily amenable to transgene *in vitro* selection. Jahne et al. (1994) were the first to achieve plants homozygous for the transgenes using biolistic bombardment of barley microspores.

3.5.7 Genomics

Doubled haploids play a vital role in genomics, especially, in the integration of genetic and physical maps, thereby, providing precision in targeting candidate genes (Kunzel et al., 2000; Wang et al., 2001). Doubled haploids combined with marker assisted selection provides a short cut in backcross conversion, a plant breeding method for improving an elite line defective in a particular trait (Toojinda et al., 1998). Expressed sequence tags may help in identification of genes that determine any trait of interest.

4.0 CONCLUSION

The life cycle of angiosperms (higher plants) is characterized by alternating generations of sporophytes and gametophytes. The gametophytic phase arises when the diploid cells undergo meiosis (reduction division) to form male and female gametes. This phase is short lived as fertilisation of the egg re-establishes the diploid sporophytic phase. The sporophytic phase characterised by chromosome number (2n) is the product of fertilisation of male and female gametes, containing the haploid (n) set of chromosomes from each parent (Forster et al., 2007). Therefore, haploid is a generalised term for plants that

contain the gametic chromosome number (n). This is in contrast to diploid plants, which contain two sets (2n) of chromosomes. Haploids are sexually sterile and, therefore, doubling of the chromosomes is required to produce fertile plants which are called doubled haploids or homozygous diploids.

5.0 SUMMARY

Haploids have attracted great interest of plant physiologists, embryologists, geneticists and breeders. Spontaneous production of haploids usually occurs through the process of parthenogenesis (embryo development from an unfertilised egg). However, occasionally, they bear the characters of male parent only, suggesting their origin through 'ovule androgenesis' (embryo development inside the ovule by the activity of the male nucleus alone where elimination or inactivation of egg nucleus occurs before fertilisation). Although *in vivo* occurrence of haploids has been reported in several species but at low and variable frequencies and was regarded as a special biological phenomenon (Figure 4.1). The low frequency of spontaneously arising to these haploid plants severely limited the utilisation of haploids for crop improvement and genetic studies.

6.0 TUTOR-MARKED ASSIGNMENT

1. Why do an isolated microspore culture is advantageous over complete anther culture?
2. Of what importance are haploid plants for the crop improvements?
3. With the help of a flow chart briefly describe the early patterns of cleavage in cultured pollen grains and the different modes of subsequent development of the proembryogenic mass so obtained.
4. Explain briefly, the three crucial factors, which influence the induction of androgenesis?

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UNIT 5 LAB FACILITIES AND OPERATIONS

CONTENTS

1.0 INTRODUCTION

Nearly any room can be converted to a biotechnology lab training facility. It's important to ensure that you have plenty of table/counter tops, electricity and computers. Water and sinks should be on one side of the wall along with gas lines. Additionally, you'll need enough room for the storage of glass, plastics, chemicals and equipment.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- understand laboratory design and development for a tissue culture.

3.0 MAIN CONTENT

3.1 Laboratory Design and Development

The size of tissue culture lab and the amount and type of equipment used depend upon the nature of the work to be undertaken and the funds available. A standard tissue culture laboratory should provide facilities for:

- washing and storage of glassware, plasticware
- preparation, sterilisation and storage of nutrient media
- aseptic manipulation of plant material
- maintenance of cultures under controlled temperature, light and humidity
- observation of cultures, data collection and photographic facility
- acclimatisation of in vitro developed plants. The overall design must focus on maintaining aseptic conditions.

At least three separate rooms should be available one for washing up, storage and media preparation (the media preparation room); a second room, containing laminar-air-flow or clean air cabinets for dissection of plant tissues and subculturing (dissection room or sterilisation room); and the third room to incubate cultures (culture room). This culture room should contain a culture observation table provided with binoculars or stereozoom microscope and an adequate light source. Additionally, a green house facility is required for hardening-off in vitro plantlets.

3.1.1 Media Preparation Room

The washing area in the media room should be provided with brushes of various sizes and shapes, a large sink, preferably lead-lined to resist acids and alkalis, and running hot and cold water. It should also have large plastic buckets to soak the labware to be washed in detergent, hot-air oven to dry washed labware and a dust-proof cupboard to store them. If the preparation of the medium and washing of the labware are done in the same room, a temporary partition can be constructed between the two areas to guard any interference in the two activities. A continuous supply of water is essential for media preparation and washing of labware. A water distillation unit of around 2 litre/h, Milli-Q water purification systems needs to be installed.

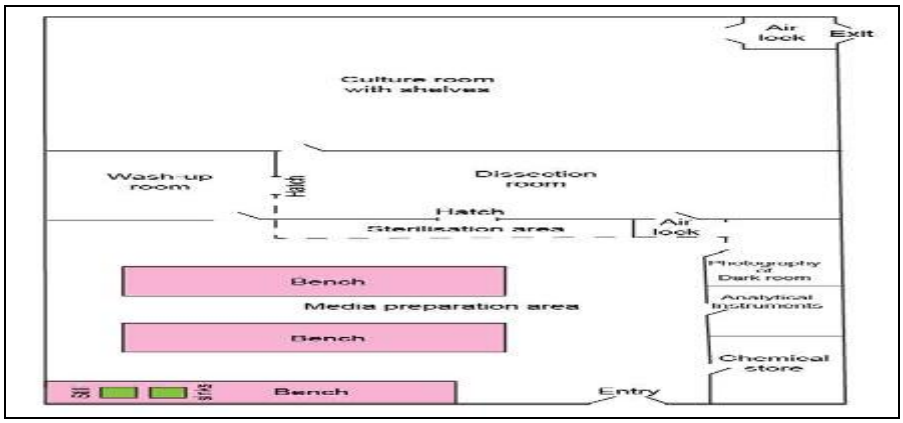

Figure 5.1: A floor plan for plant tissue culture laboratory

3.1.2 Culture Room

The room for maintaining cultures should be maintained at temperature 25 ±2°C, controlled by air conditioners and heaters attached to a temperature controller are used. For higher or lower temperature treatments, special incubators with built-in fluorescent light can be used outside the culture room. Cultures are generally grown in diffuse light from cool, white, fluorescent tubes. Lights can be controlled with automatic time clocks. Generally, a 16-hour day and 8-hour nights are

used. The culture room requires specially designed shelving to store cultures. Some laboratories have shelves along the walls; others have them fitted onto angle-iron frames placed in a convenient position. Shelves can be made of rigid wire mesh, wood or any building material that can be kept clean and dust-free. Insulation between the shelf lights and the shelf above will ensure an even temperature around the cultures. While flasks, jars and petridishes can be placed directly on the shelf or trays of suitable sizes, culture tubes require some sort of support. Metallic wire racks or polypropylene racks, each with a holding capacity of 18-24 tubes, are suitable for the purpose.

Figure 5.2: Culture room facility with humidifier, timer, wall cabinets, illuminated trolleys and test-tube racks

3.1.3 Dissection Room or Sterilisation Room

This area should have restricted entry, which is needed to ensure the sterile conditions required for the transfer operations. For sterile transfer operations, the laminar-air-flow cabinets are used. Temperature control is essential in this room as the heat is produced continuously from the flames of burners in the hoods. The room should be constructed in a way to minimise the dust particles and for easy cleaning. Several precautions can be taken including the removal of shoes before entering the area.

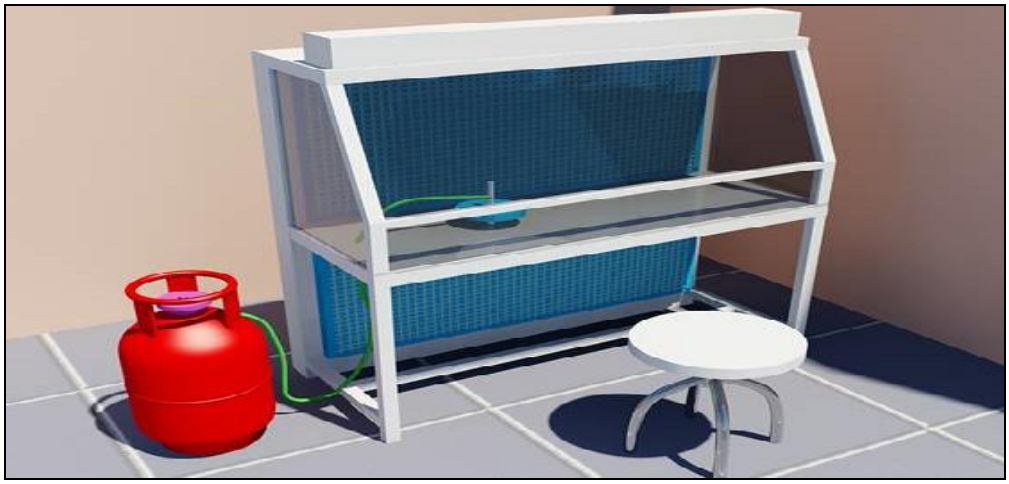

Figure 5.3: Laminar-air-hood with coarse filter, HEPA filter, gas cock, gas cylinder and electrical outlets

3.1.4. Greenhouse

The greenhouse facility is required to grow parent pants and to acclimatise in vitro raised plantlets. The size and facility inside the green house vary with the requirement and depends on the funds available with the laboratory. However, minimum facilities for maintaining humidity by fogging, misting or a fan and pad system, reduced light, cooling system for summers and heating system for winters must be provided. It would be desirable to have a potting room adjacent to this facility.

3.1.5 Equipments and Apparatus

3.1.5.1 Media Preparation Area

- benches at a height suitable to work while standing.
- pH meter is used to determine the pH of various media used for tissue culture. pH indicator paper can also be used for the purpose but it is less accurate. The standard media pH is maintained at 5.8.
- hot-plate-cum-magnetic stirrer for dissolving chemicals and during media preparation.
- an autoclave or domestic pressure cooker is crucial instrument for a tissue culture laboratory. High pressure heat is needed to sterilise media, water, labware, forceps, needles etc. Certain spores from fungi and bacteria can only be killed at a temperature of 121°C and 15 pounds per square inch (psi) for 15-20 min. A caution should be taken while opening the door of autoclave and it should be open when the pressure drops to zero. Opening the door immediately can lead to a rapid change in the temperature, resulting in breakage of glassware and steam burning of operator.

- plastic carboys for storing distilled water required for media preparation and final washing of labware.
- balances near dry corner of the media room. High quality microbalance are required to weigh smallest of the quantities. Additionally a top pan balance is required for less sensitive quantities.
- hot-air oven to keep autoclaved medium warm before pouring into vessels. It is also used for the dry heat sterilization of clean glassware like, Petridishes, culture tubes, pipettes etc. Typical sterilising conditions are 160-170 °C/1hr.
- Dishwasher for cleaning glass pipettes in running water

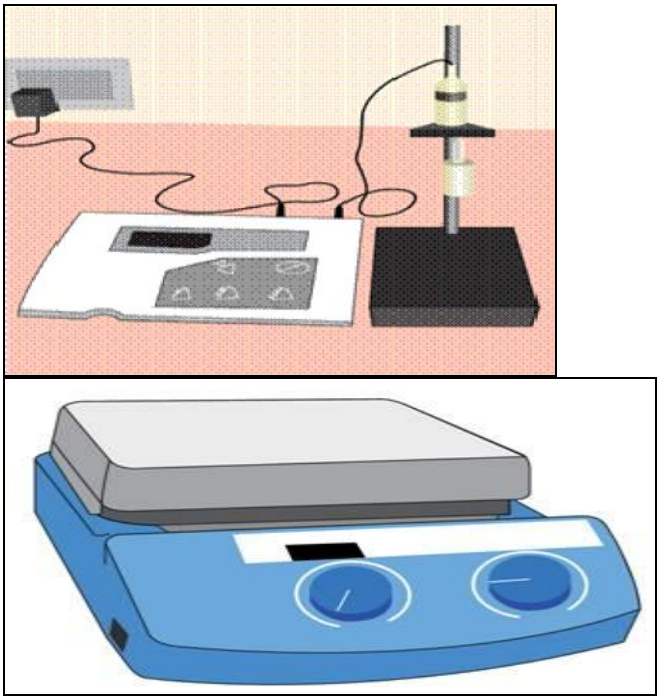

Figure 5.4: pH Meter and Magnetic stirrer-cum-heater

Figure 5.5: Autoclave with accessories and High Precision Weighing Balance

3.1.5.2. Storage Area

- A deep freezer (-20°C to -80°C) / refrigerator for storage of enzyme solutions, stock solutions plant materials and all temperature-sensitive chemicals.
- microwave oven to melt agar solidified media.
- Upright and inverted light microscope with camera attachment for recording the morphogenic responses from various explants, calli, cells and protoplasts. Inverted microscope gives the clear views of cultures settled at the bottom of Petridishes.

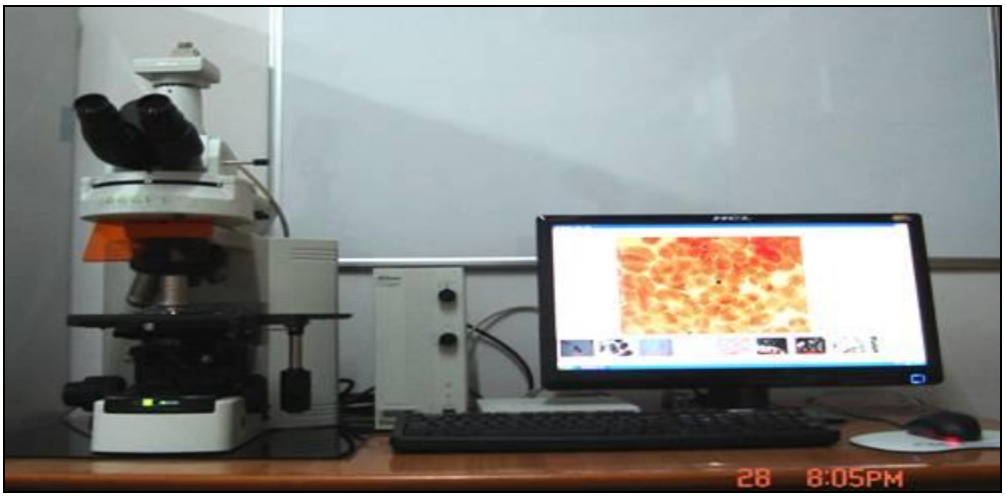

Figure 5.6: Upright light microscope with CCD camera attachment

3.1.5.3. Dissection Room

- laminar-air-flow cabinet within which tissue culture work can be carried out under sterilised environment
- glass bead steriliser where temperature of beads is raised to 250°C in 15-20 min with 15 s cut off. Here the sterilisation of instruments is effecting by pushing them into the beads for 5-7 s. This is much safer compare to the Bunsen burner heating of instruments like, forceps, needles, scalpels etc.
- binocular microscope to observe surface details and morphogenic responses of cultures and their possible contamination.
- low speed table-top centrifuge to sediment cells or protoplasts

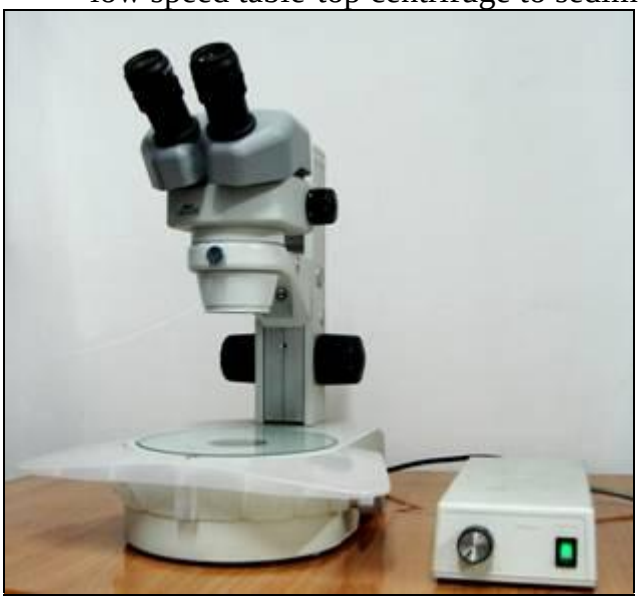

Figure 5.7: Binocular stereozoom microscope (left side) and Centrifuge (right figure)

3.1.4.4 Culture Room

- air (or heating / cooling system) to maintain 25±2 °C temperature.
- racks for holding test-tubes.
- lights to provide diffuse light and to maintain photoperiod.
- shakers with various sized clamps for different sized flasks to grow cells in liquid medium.
- thermostat and time clock for lights.
- wall cabinets for dark incubation of cultures.

Figure 5.8: Incubator shaker and Test-tube racks

3.1.5.5 Other Apparatus

- beakers (100 mL, 250 mL, 1 L, 5 L).
- measuring cylinders (5 mL, 10 mL, 25 mL, 50 mL, 100 mL, 500 mL, 1L, 2 L, 5 L).
- graduated pipettes and teats.
- reagent bottles for storing liquid chemicals and stock solutions (glass or plastic).
- culture tubes and flasks (glass or polypropylene or disposable)
- plastic baskets.
- filter membrane, preferably nylon, of sizes 0.22 µm and 0.45 µm, holders and hypodermic syringes (for solutions requiring filter sterilisation).
- large forceps (blunt and fine points) and scalpels for dissecting and subculturing plant material.
- Scalpel handles (no. 3) and blades (no. 11).
- Chemicals and reagents for preparing culture media.
- Disposable gloves and masks.
- Micropipettes of maximum volume size 5000 µL, 1000 µL, 500 µL, 250 µL, 100 µL

Figure5.9:(A) Syringe with filter assembly fitted on conical flask,(B) Disassembled filter assembly

Figure5.10: a) Forceps and scalpels for dissection and
b) Micropipettes.

4.0 CONCLUSION

The first step in the design process is to understand the purpose of the laboratory. A laboratory designed for typical commer-cial analysis of samples can be far different from a laboratory designed for meeting the needs of a process quality control lab, or an analytical research laboratory. All conduct analysis and may use some of the same instrumentation but it is at this very basic point that they begin to differ in purpose and, therefore, also need to differ in design.

5.0 SUMMARY

The cells or tissues are obtained from any part of the plant like stem, root, leaf etc. which are encouraged to produce more cells in culture and to express their totipotency (i.e. their genetic ability to produce more plants). Cells or tissues are grown in different types of glass vials containing a medium with mineral nutrients, vitamins and phytohormones. Therefore, to carry out the experiments using tissue culture techniques, a well-equipped laboratory is first required. An ideal tissue culture laboratory should have at least two big rooms and a small room. One big room is for general laboratory work such as preparation of media, autoclaving, distillation of water etc. The other big room is for keeping cultures under controlled light, temperature and humidity. The small room is for aseptic work and for keeping autoclaved articles.

6.0 TUTOR-MARKED ASSIGNMENT

1. Write down the list of ways by which the sterilisation might be achieved.
2. What do you think are the possible dangers of using flames in a laminar-air-flow cabinet?
3. Apart from providing a source of energy, what is the other important effect of light on intact plants?

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UNIT 6 TISSUE CULTURE MEDIA

CONTENTS

1.0 INTRODUCTION

Culture media are largely responsible for the in vitro growth and morphogenesis of plant tissues. The success of the plant tissue culture depends on the choice of the nutrient medium. In fact, the cells of most plant cells can be grown in culture media. Basically, the plant tissue culture media should contain the same nutrients as required by the whole plant. It may be noted that plants in nature can synthesise their own food material. However, plants growing in vitro are mainly heterotrophic i.e. they cannot synthesise their own food.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain the meaning of culture media
- know what media is
- list the different types of media
- describe the constituents of media as well as its composition.

3.0 MAIN CONTENT

Composition of media

The composition of the culture media is primarily dependent on two parameters:

1. The particular species of the plant.
2. The type of material used for culture i.e. cells, tissues, organs, protoplasts.

Thus, the composition of a medium is formulated considering the specific requirements of a given culture system. The media used may be

solid (solid medium) or liquid (liquid medium) in nature. The selection of solid or liquid medium is dependent on the better response of a culture.

Major types of media
The composition of the most commonly used tissue culture media is given and briefly described below.

White's medium
This is one of the earliest plant tissue culture media developed for root culture.

MS medium
Murashige and Skoog (MS) originally formulated a medium to induce organogenesis, and regeneration of plants in cultured tissues. These days, MS medium is widely used for many types of culture systems.

B5 medium
Developed by Gamborg, B5 medium was originally designed for cell suspension and callus cultures. At present with certain modifications, this medium is used for protoplast culture.

N6 medium
Chu formulated this medium and it is used for cereal anther culture, besides other tissue cultures.

Nitsch's medium
This medium was developed by Nitsch and Nitsch and frequently used for anther cultures. Among the media referred above, MS medium is most frequently used in plant tissue culture work due to its success with several plant species and culture systems.

Synthetic and natural media
When a medium is composed of chemically defined components, it is referred to as a synthetic medium. On the other hand, if a medium contains chemically undefined compounds (e.g., vegetable extract, fruit juice, plant extract), it is regarded as a natural medium. Synthetic media have almost replaced the natural media for tissue culture.

Expression of concentrations in media
The concentrations of inorganic and organic constituents in culture media are usually expressed as mass values (mg/l or ppm or mg I^{-1}). However, as per the recommendations of the International Association of Plant Physiology, the concentrations of macronutrients should be expressed as mmol/l⁻ and micronutrients as µmol/l⁻.

Constituents of media

Many elements are needed for plant nutrition and their physiological functions. Thus, these elements have to be supplied in the culture medium to support adequate growth of cultures in vitro.

3.1 Preparation of Media

The general methodology for a medium preparation involves preparation of stock solutions (in the range of 10x to 10Ox concentrations) using high purity chemicals and demineralised water. The stock solutions can be stored (in glass or plastic containers) frozen and used as and when required. Most of the growth regulators are not soluble in water. They have to be dissolved in NaOH or alcohol.

3.2 Dry Powders in Media Preparation

The conventional procedure for media preparation is tedious and time consuming. Nowadays, plant tissue culture media are commercially prepared, and are available in the market as dry powders. The requisite medium can be prepared by dissolving the powder in a glass distilled or demineralised water. Sugar, organic supplements and agar (melted) are added, pH adjusted and the medium diluted to a final volume (usually 1 litre).

3.3 Sterilisation of Media

The culture medium is usually sterilised in an autoclave at 121°C and 15 psi for 20 minutes. Hormones and other heat sensitive organic compounds are filter-sterilised, and added to the autoclaved medium.

3.4 Selection of a Suitable Medium

In order to select a suitable medium for a particular plant culture system, it is customary to start with a known medium (e.g. MS medium, B5 medium) and then develop a new medium with the desired characteristics. Among the constituents of a medium, growth regulators (auxins, cytokinins) are highly variable depending on the culture system. In practice, 3-5 different concentrations of growth regulators in different combinations are used and the best among them are selected. For the selection of appropriate concentrations of minerals and organic constituents in the medium, similar approach referred above, can be employed.

3.5 Composition of Culture Media

The culture media usually contain the following constituents:

1. Inorganic nutrients
2. Carbon and energy sources
3. Organic supplements
4. Growth regulators
5. Solidifying agents
6. pH of medium.

3.5.1 Inorganic Nutrients

The inorganic nutrients consist of macronutrients (concentration >0.5 mmol/l^{-}) and micronutrients (concentration <0.5 mmol/l^{-}). A wide range of mineral salts (elements) supply the macro- and micronutrients. The inorganic salts in water undergo dissociation and ionisation. Consequently, one type of ion may be contributed by more than one salt. For instance, in MS medium, K^+ ions are contributed by KNO_3 and KH_2PO_4 while NO_3^- ions come from KNO_3 and NH_4NO_3.

3.5.1.1 Macronutrient Elements

The six elements namely nitrogen, phosphorus, potassium, calcium, magnesium and sulfur are the essential macronutrients for tissue culture. The ideal concentration of nitrogen and potassium is around 25 mmol I^{-1} while for calcium, phosphorus, sulfur and magnesium; it is in the range of 1-3 mmol I^{-}. For the supply of nitrogen in the medium, nitrates and ammonium salts are together used.

3.5.1.2 Micronutrients

Although their requirement is in minute quantities, micronutrients are essential for plant cells and tissues. These include iron, manganese, zinc, boron, copper and molybdenum. Among the microelements, iron requirement is very critical. Chelated forms of iron and copper are commonly used in culture media.

3.5.2 Carbon and Energy Sources

Plant cells and tissues in the culture medium are heterotrophic and therefore, are dependent on the external carbon for energy. Among the energy sources, sucrose is the most preferred. During the course of sterilisation (by autoclaving) of the medium, sucrose gets hydrolysed to glucose and fructose. The plant cells in culture first utilise glucose and then fructose. In fact, glucose or fructose can be directly used in the

culture media. It may be noted that for energy supply, glucose is as efficient as sucrose while fructose is less efficient.

It is a common observation that cultures grow better on a medium with autoclaved sucrose than on a medium with filter-sterilised sucrose. This clearly indicates that the hydrolysed products of sucrose (particularly glucose) are efficient sources of energy. Direct use of fructose in the medium subjected to autoclaving, is found to be detrimental to the growth of plant cells. Besides sucrose and glucose, other carbohydrates such as lactose, maltose, galactose, raffinose, trehalose and cellobiose have been used in culture media but with a very limited success.

3.5.3 Organic Supplements

The organic supplements include vitamins, amino acids, organic acids, organic extracts, activated charcoal and antibiotics.

3.5.3.1 Vitamins

Plant cells and tissues in culture (like the natural plants) are capable of synthesising vitamins but in suboptimal quantities, inadequate to support growth. Therefore, the medium should be supplemented with vitamins to achieve good growth of cells. The vitamins added to the media include thiamine, riboflavin, niacin, pyridoxine, folic acid, pantothenic acid, biotin, ascorbic acid, myoinositol, Para amino benzoic acid and vitamin E.

3.5.3.2 Amino Acids

Although the cultured plant cells can synthesize amino acids to a certain extent, media supplemented with amino acids stimulate cell growth and help in establishment of cells lines. Further, organic nitrogen (in the form of amino acids such as L-glutamine, L-asparagine, L- arginine, L-cysteine) is more readily taken up than inorganic nitrogen by the plant cells.

3.5.3.3 Organic Acids

Addition of Krebs cycle intermediates such as citrate, malate, succinate or fumarate allow the growth of plant cells. Pyruvate also enhances the growth of cultured cells.

3.5.3.4 Organic Extracts

It has been a practice to supplement culture media with organic extracts such as yeast, casein hydrolysate, coconut milk, orange juice, tomato

juice and potato extract. It is however, preferable to avoid the use of natural extracts due to high variations in the quality and quantity of growth promoting factors in them. In recent years, natural extracts have been replaced by specific organic compounds e.g., replacement of yeast extract by L-asparagine; replacement of fruit extracts by L-glutamine.

3.5.3.5 Activated Charcoal

Supplementation of the medium with activated charcoal stimulates the growth and differentiation of certain plant cells (carrot, tomato, orchids). Some toxic/inhibitory compounds (e.g. phenols) produced by cultured plants are removed (by adsorption) by activated charcoal and this facilitates efficient cell growth in cultures. Addition of activated charcoal to certain cultures (tobacco, soybean) is found to be inhibitory, probably due to adsorption of growth stimulants such as phytohormones.

3.5.3.6 Antibiotics

It is sometimes necessary to add antibiotics to the medium to prevent the growth of microorganisms. For this purpose, low concentrations of streptomycin or kanamycin are used. As far as possible, addition of antibiotics to the medium is avoided as they have an inhibitory influence on the cell growth.

3.5.4 Growth Regulators

Plant hormones or phytohormones are a group of natural organic compounds that promote growth, development and differentiation of plants. Four broad classes of growth regulators or hormones are used for culture of plant cells-auxins, cytokinins, gibberellins (Fig. 43.1) and abscisic acid. They promote growth, differentiation and organogenesis of plant tissues in cultures.

3.5.4.1 Auxins

Auxins induce cell division, cell elongation, and formation of callus in cultures. At a low concentration, auxins promote root formation while at a high concentration callus formation occurs. A selected list of auxins used in tissue cultures is given. Among the auxins, 2, 4-dichlorophenoxy acetic acid is most effective and is widely used in culture media.

3.5.4.2 Cytokinins

Chemically, cytokinins are derivatives of a purine namely adenine. These adenine derivatives are involved in cell division, shoot differentiation and somatic embryo formation. Cytokinins promote RNA

synthesis and thus stimulate protein and enzyme activities in tissues. The most commonly used cytokinins are given in Table 43.3. Among the cytokinins, kinetin and benzyl-amino purine are frequently used in culture media.

3.5.4.3 Ratio of Auxins and Cytokinins

The relative concentrations of the growth factors namely auxins and cytokinins are crucial for the morphogenesis of culture systems. When the ratio of auxins to cytokinins is high, embryogenesis, callus initiation and root initiation occur. On the other hand, for axillary and shoot proliferation, the ratio of auxins to cytokinins is low. For all practical purposes, it is considered that the formation and maintenance of callus cultures require both auxin and cytokinin, while auxin is needed for root culture and cytokinin for shoot culture. The actual concentrations of the growth regulators in culture media are variable depending on the type of tissue explant and the plant species.

3.5.4.4 Gibberellins

About 20 different gibberellins have been identified as growth regulators. Of these, gibberellin A_3 (GA_3) is the most commonly used for tissue culture. GA_3 promotes growth of cultured cells, enhances callus growth and induces dwarf plantlets to elongate. Gibberellins are capable of promoting or inhibiting tissue cultures, depending on the plant species. They usually inhibit adventitious root and shoot formation.

3.5.4.5 Abscisic Acid (ABA)

The callus growth of cultures may be stimulated or inhibited by ABA. This largely depends on the nature of the plant species. Abscisic acid is an important growth regulation for induction of embryogenesis.

3.5.5 Solidifying Agents

For the preparation of semisolid or solid tissue culture media, solidifying or gelling agents are required. In fact, solidifying agents extend support to tissues growing in the static conditions.

3.5.5.1 Agar

Agar, a polysaccharide obtained from seaweeds, is most commonly used as a gelling agent for the following reasons

1. It does not react with media constituents.

2. It is not digested by plant enzymes and is stable at culture temperature.

Agar at a concentration of 0.5 to 1% in the medium can form a gel.

3.5.5.2 Gelatin

It is used at a high concentration (10%) with a limited success. This is mainly because gelatin melts at low temperature (25°C), and consequently the gelling property is lost.

3.5.5.3 Other Gelling Agents

Bio-gel (polyacrylamide pellets), phytagel, gelrite and purified agarose are other solidifying agents, although less frequently used. It is in fact advantageous to use synthetic gelling compounds, since they can form gels at a relatively low concentration (1.0 to 2.5 g l^{-1}).

3.5.6 pH of Medium

The optimal pH for most tissue cultures is in the range of 5.0-6.0. The pH generally falls by 0.3-0.5 units after autoclaving. Before sterilization, pH can be adjusted to the required optimal level while preparing the medium. It is usually not necessary to use buffers for the pH maintenance of culture media. At a pH higher than 7.0 and lower than 4.5, the plant cells stop growing in cultures. If the pH falls during the plant tissue culture, then fresh medium should be prepared. In general, pH above 6.0 gives the medium hard appearance, while pH below 5.0 does not allow gelling of the medium.

Medium-utmost important for culture
For tissue culture techniques, it is absolutely essential that the medium preparation and composition are carefully followed. Any mistake in the preparation of the medium is likely to do a great harm to the culture system as a whole.

4.0 CONCLUSION

Culture media are largely responsible for the in vitro growth and morphogenesis of plant tissues. The success of the plant tissue culture depends on the choice of the nutrient medium. In fact, the cells of most plant cells can be grown in culture media.

5.0 SUMMARY

Basically, the plant tissue culture media should contain the same nutrients as required by the whole plant. It may be noted that plants in nature can synthesise their own food material. However, plants growing in vitro are mainly heterotrophic i.e. they cannot synthesise their own food.

6.0 TUTOR-MARKED ASSIGNMENT

1) Explain the meaning of tissue culture media. And list its composition.

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UNIT 7 ADVENTITIOUS SHOOT PROLIFERATION

CONTENTS

1.0 INTRODUCTION

Adventitious shoot proliferation in plant cell and tissue culture, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, randomly, all over the tissue other than the pre-existing meristem. It is a multistep process and a series of intracellular events, collectively called induction that occurs before the appearance of morphologically recognisable organs. Micropropagation via adventitious shoot regeneration may occur directly or indirectly via an intervening callus phase (Figure 7.1A, B). Indirect regeneration often results in somaclonal variations, making this strategy less desirable for large-scale clonal multiplication. Therefore, regeneration of shoots directly from the explants is regarded as the most reliable method for clonal propagation. Various explants like leaf, cotyledon, embryo and root have been tried with different media combinations by the scientists to obtain adventitious shoot proliferation.

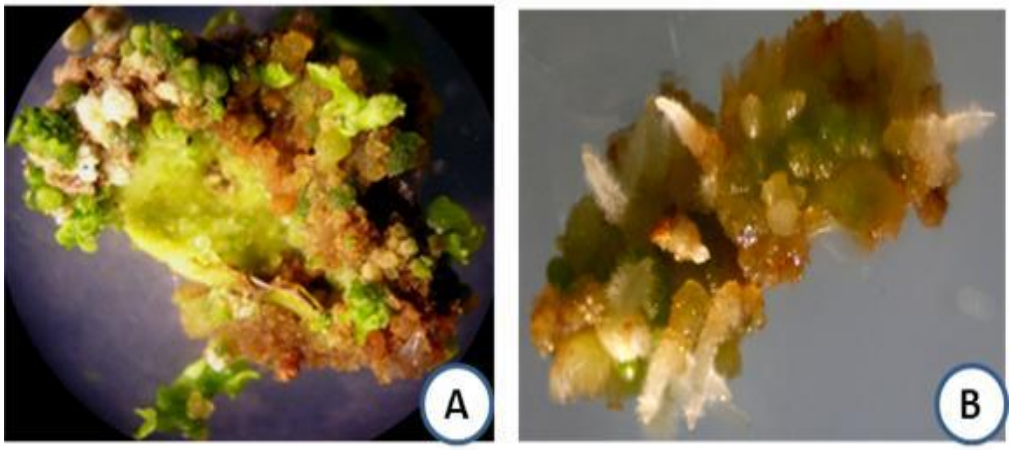

Figure7.1.: Organogenesis from leaf explants indirectly via callusing **A.** Shoot differentiation **B.** Root differentiation

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain organogenic differentiation
- discuss induction of organogenic differentiation
- state what is ontogeny of shoot buds
- list the factors affecting shoot-bud differentiation.

3.0 MAIN CONTENT

3.1 Organogenic Differentiation

Regeneration of plant from the cultured explant may occur either through differentiation of shoot-buds or somatic embryogenesis (Figure 7.2 A-E, and Figure 7.3).

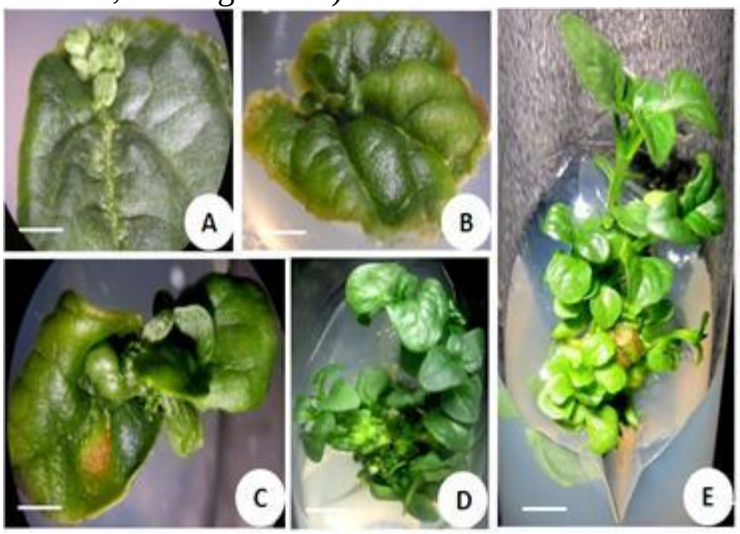

Figure 7.2: Direct shoot proliferation from leaf-disc culture

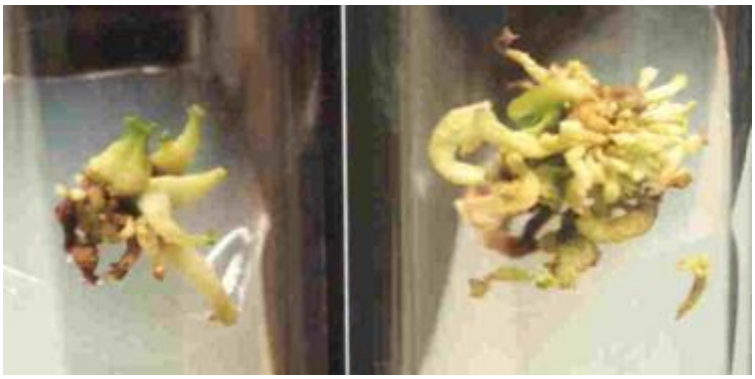

Figure 7.3: Direct differentiation of somatic embryos from hypocotyl explants

The shoot-bud and embryo formation can be distinguished by the distinct morphological features. The shoot-bud is a monopolar structure. It develops from the procambial strands which establish a connection with the pre-existing vascular tissue dispersed within the callus or the cultured explants (Figure 7.4 A-D).

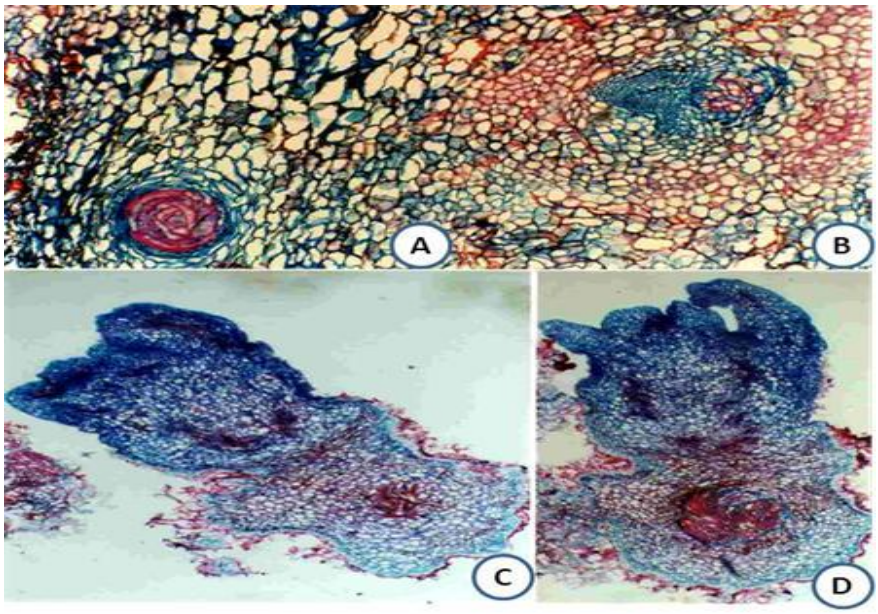

Figure 7.4: Shoot differentiation from callus tissue. **A-B**, Development of vascular nodules randomly in the callus, note a small shoot-bud originated from vascular tissue in **figure B**. **C-D,** Shoot-buds establish a connection with pre-existing vascular tissue developed from the callus

Plant regeneration from isolated cells, protoplasts or unorganised mass of cells (callus) is generally more difficult than that obtained from the intact explants such as, cotyledons, hypocotyl segments and immature embryos. The regeneration obtained through de novo differentiation of shoot buds or somatic embryogenesis directly from explants may also exhibit genetic variability.

3.2 Induction of Organogenic Differentiation

Induction is a multistep process. A schematic representation of adventitious shoot proliferation from leaf-disc culture is presented in (Figure 7.5). A series of intracellular events, collectively called induction, occur before the emergence of morphologically recognisable organs.

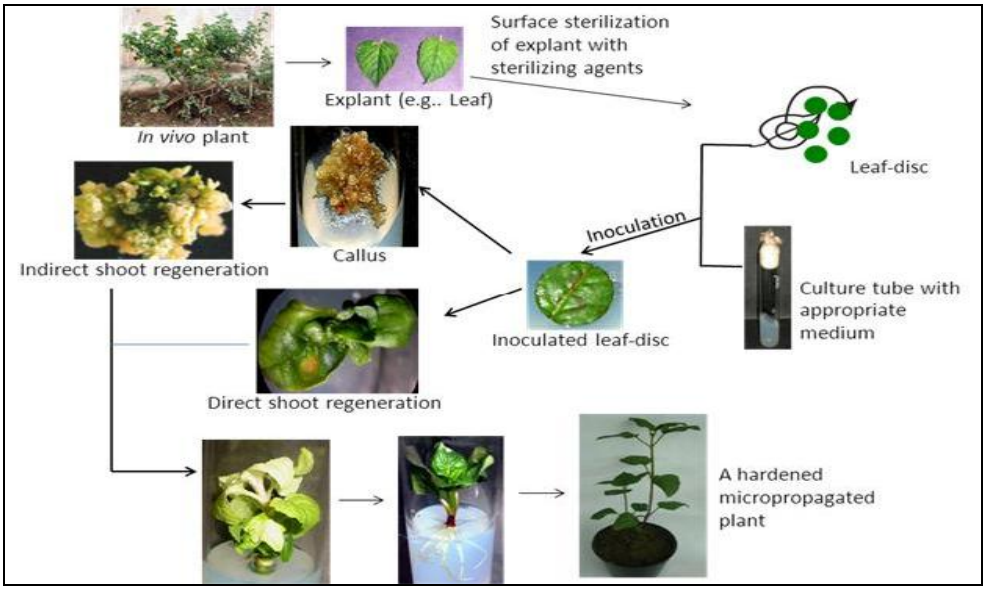

Figure 7.5: A schematic representation of *in vitro* adventitious shoot proliferation from leaf-disc culture

Under the optimal growth regulator combinations, the cells induced to form a specific organ and would continue to develop into that organ even if the inductive growth regulators are removed. Hence, induction favours the irreversible commitment of cells to follow a particular developmental pathway. For example, *Brassica juncea*, undergoes the induction of organogenic differentiation where a cytokinin, BAP induces shoot-bud differentiation at the cut end of the cotyledon petiole. In the absence of BAP (basal medium) only roots are formed at the same site. The cotyledons transferred to basal medium after 11 days of incubation on BAP leads to the development of only shoots and no roots. Similarly, the cotyledons lose the potential to form shoots on BAP medium if they are pre-cultured on BAP free medium for more than seven days.

3.3 Ontogeny of Shoot Buds

Under the optimal conditions, meristems formed from the callus are random and scattered. On transferring to the medium supporting organised growth promotes first the appearance of localised clusters of cambium like cells (Figure 7.4 A). These meristemoids (or nodules or growing centers), which may become vascularised due to the appearance of tracheidal cells in the centre, are the site for organ formation in the callus, as seen in above figure 7.4 B-C. Initially, the meristemoids exhibits plasticity and can form shoots and roots.

3.4 Factors Affecting Shoot-Bud Differentiation

The genotype and plant growth regulators are well known to affect regeneration frequency. Plant growth regulators play a major role in the regeneration which mainly depends upon the concentration and type of growth regulators used. For *in vitro* differentiation genotype plays equally, if not more critical role as the growth regulator. Besides, there are certain other factors which play a critical role in regeneration are:

3.4.1 Explant

Regenerability of an explant is influenced by several factors such as, the organ from which it is derived, the physiological state of the explant like age of the explant, young vs. mature, position of the explant on the plant and the explant size. Orientation of the explant on the medium and the inoculation density may also affect shoot-bud differentiation. There may be a decline in the number of shoots per culture and the percent cultures showing regeneration with increasing age of the seedlings.

3.4.2 Preparation of Explant

Sharma et al (1990) studied that in cotyledon cultures of *Brassica juncea* , shoot buds or roots are formed at the cut end of the petiole, depending on the culture medium. Lamina lacks this potential. However, the presence of laminar tissue is essential for the petiolar cells to exhibit totipotency. Therefore, the ideal explant to achieve regeneration is the lamina together with a short (1mm) petiole.

3.4.3 Orientation of The Explant

Orientation of explant is proved to be critical for organogenic differentiation in cotyledon cultures of *B. juncea.* Inoculating the cotyledons with their abaxial surface (lower surface away from the stem) in contact with the medium and the petiolar cut end embedded in the medium gave best response. The explants in which due to expansion and curling of the lamina, the petiole lost contact with the medium within 3-5 days after culture, failed to form roots or shoots. Generally, the explants inoculated horizontally on the medium produced three times more shoots than those planted vertically.

3.4.4 Physical Factors

i. Explants grown on liquid or semi-solid medium give different degree of organogenesis. In few species, like tobacco, the medium with 1% agar showed only flower formation. With lowering the agar concentration, the frequency of flower

formation dropped and vegetative bud differentiation occurred. In liquid medium, the tissue exhibited callusing and vegetative bud formation.

ii. The quality of light also influences organogenic differentiation. Alternating light and dark period (diffused light, 15-16 hrs) proved best. Callus maintained under continuous light remained whitish and may not exhibit organogenesis. Blue light promotes shoot-bud differentiation whereas red light stimulated rooting in tobacco. Calli of *Brassica oleracea* grown in dark for 20 days formed shoot-buds 12 days after transfer to light while those shifted to light after 12 days of growth in dark differentiated shoots within nine days.

iii. Skoog (1944) studied the effect of a range of temperature on tobacco callus growth and differentiation. Growth of callus increased with rise in temperature up to 33°C, but for shoot-bud differentiation 18°C was optimum.

4.0 CONCLUSION

Adventitious shoot proliferation in plant cell and tissue culture, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, randomly, all over the tissue other than the pre-existing meristem. It is a multistep process and a series of intracellular events, collectively called induction that occurs before the appearance of morphologically recognisable organs. Micropropagation via adventitious shoot regeneration may occur directly or indirectly via an intervening callus phase.

5.0 SUMMARY

Indirect regeneration often results in somaclonal variations, making this strategy less desirable for large-scale clonal multiplication (Marcotrigiano and Jagannathan 1988; Thorpe et al. 1991). Therefore, regeneration of shoots directly from the explants is regarded as the most reliable method for clonal propagation. Various explants like leaf, cotyledon, embryo and root have been tried with different media combinations by the scientists to obtain adventitious shoot proliferation.

6.0 TUTOR-MARKED ASSIGNMENT

1. Give a brief note on induction of organogenic differentiation.
2. Does induction leads to irreversible commitment of cells to follow a particular developmental pathway? Comment on the statement with an example.

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UNIT 8 AXILLARY SHOOT PROLIFERATION

CONTENTS

1.0 INTRODUCTION

Axillary buds are usually present in the axil of each leaf and every bud has the potential to develop into a shoot. In nature these buds remain dormant for various periods. The species with strong apical dominance show the growth of axillary buds into shoot only if the terminal bud is removed or injured. The phenomenon of apical dominance is regulated by the interplay of growth regulators. The application of cytokinin to the axillary buds can overcome the apical dominance effect and stimulate the lateral buds to grow rapidly in the presence of terminal buds. If the exogenous growth regulator diminishes the lateral shoot stop growing.

2.0 OBJECTIVE

By the end of this unit, you will be able to:

- understand rate of shoot multiplication
- state the factors affecting axillary shoot proliferation
- differentiate axillary shoot proliferation and adventitious shoot proliferation
- differentiate axillary shoot proliferation and conventional method of propagation.

3.0 MAIN CONTENT

3.1 Rate of Shoot Multiplication

In plant tissue culture, the rate of shoot multiplication can be determined by enhanced axillary branching. Due to continuous availability of cytokinin, the shoots formed by the bud, a priori present on the explant (nodal segment or shoot-tip cutting), develops axillary buds which may grow directly into shoots. This process may be repeated several times and the initial explant transformed into a mass of branches. There is a limit to which shoot multiplication can be achieved in a single passage, after which further axillary branching stops. At this stage, if shoots are excised and planted on a fresh medium of same composition, the shoot multiplication cycle can be repeated. This process can go on indefinitely, and can be maintained throughout the year independent of the season and the region.

In some plants, it may not be possible to break apical dominance by the application of growth regulator compositions, and the bud present a priori on the initial explant grows into an unbranched shoot. The rate of shoot multiplication in such cases would depend on the number of nodal cuttings that can be excised from the newly developed shoot at the end of each passage. With this alternative method of enhanced axillary branching, 6-7-fold shoot multiplication every 4-6 weeks could be achieved in the plants with strong apical dominance.

3.2 Factors Affecting Axillary Shoot Proliferation

i. Effects of season on culture establishment

The extent of contamination as well as bud-break is highly dependent on the season. The cultures initiated during spring season (January to April) shows best response not only in terms of the frequency of bud-break but also in the vigor of the shoots with least contamination rate. Since, summer (May-August) is the period that concurs with rainy season in certain regions like India, the cultures are prone to infection. By winter the shoots become old and it is difficult to break the dormant state of the buds.

ii. Effect of carbon source on shoot proliferation

In cultured plant tissues, a continuous supply of carbohydrate from the medium is essential which are needed for growth and organized development of the plant and are necessary as a source of energy and carbon skeletons for biosynthetic process. For shoot induction from axillary buds, three carbon sources, sucrose, glucose and maltose are

utilized in maximum plant tissue cultures at a fixed concentration of 30 g l^{-1} . Of these, sucrose is the most commonly used carbohydrate for plant tissue cultures and most culture media have it as the sole carbohydrate source. It favors higher growth of shoot, number of nodes per shoot and the rate of shoot multiplication compare to maltose and glucose. Sucrose is easily recognised and hydrolysed by cell wall bound invertase into more efficiently utilisable forms of sugars, glucose and fructose which are incorporated into the cells. Glucose, derived from sucrose hydrolysis, is more accessible to the cultured tissues than glucose derived by maltose hydrolysis, due to a rapid sucrose hydrolysis but a slow maltose hydrolysis in the media.

iii. Effect of growth regulators on shoot proliferation

In general, cytokinins favors shoot proliferation and auxins favors root formation. In *S. acmella,*nodal explants bearing two opposite axillary buds were when cultured on MS basal medium or basal medium supplemented with BAP, Kinetin or 2-iP at 3 µM concentration, the frequency of bud-break was appreciable in basal medium but incorporation of BAP to the basal medium has further improved the incidence of bud-break and promoted multiple shoot formation (2 shoots/explant). While the least bud-break was observed on Kinetin supplemented medium and 2-iP was noticed to be inhibitory for axillary bud proliferation. The addition of a low concentration of GA_3 to the BAP supplemented medium further promoted multiple shoot formation. On the other hand, single shoot with long internodes was developed from axillary buds in cultures when NAA was added to BAP containing medium. The frequency of bud-break varied with the concentration of the BAP and at its optimum level of 5 µM, 10-fold shoot multiplication occurred every five weeks.

3.3. Axillary Shoot Proliferation Vs Adventitious Shoot Proliferation

i. The axillary shoot proliferation is the most popular approach to clonal propagation of crop plants because the cells of the shoot apex are uniformly diploid and are least susceptible to genotypic changes under culture conditions.
ii. Chimeras, whose breakdown is common during adventitious bud proliferation, are perpetuated in shoot-bud culture and, thus, the cause for change in ploidy sometimes. While axillary shoot proliferation favours genetically uniform plant formation.
iii. Moreover adventitious bud formation and callusing methods require denovo differentiation of shoot-buds which is not always possible.

iv. Further, the axillary shoot proliferation is comparatively a quicker method of shoot multiplication as pre-existing meristem only proliferate into shoots, thus, reducing the time required to form de novo meristem formation.

3.4 Axillary Shoot Proliferation Vs Conventional Method of Propagation

i. The conventional method of vegetative propagation by stem cuttings utilises the ability of axillary buds to take over the function of main shoot in the absence of a terminal bud. However, the number of cuttings that can be taken from a selected plant in a year is extremely limited because in nature the vegetative growth is periodic. In *in vitro* conditions, axillary shoot proliferate irrespective of seasons and regions.
ii. A minimal size of cuttings required in conventional methods is around 24-30 cm in order to establish a plant from it. Thus, it may restrict the multiplication of plants if the stock of parent plant is limited or if the species is endangered.
iii. With axillary shoot proliferation, minimum cutting size required is <1cm, thus, it favours large scale multiplication even with the limited sample.
iv. With the axillary shoot proliferation method, juvenile nodal cuttings are made available throughout the year that helps to maintain faster rate of multiplication compare to conventional methods of vegetative propagation where juvenile phase is short lived and with mature cuttings it is difficult to establish propagation as the buds in the axil undergo dormancy.

4.0 CONCLUSION

Axillary buds are usually present in the axil of each leaf and every bud has the potential to develop into a shoot. In nature these buds remain dormant for various periods. The species with strong apical dominance shows the growth of axillary buds into shoot only if the terminal bud is removed or injured. The phenomenon of apical dominance is regulated by the interplay of growth regulators. The application of cytokinin to the axillary buds can overcome the apical dominance effect and stimulate the lateral buds to grow rapidly in the presence of terminal buds. If the exogenous growth regulator diminishes the lateral shoot stop growing.

5.0 SUMMARY

The phenomenon of apical dominance is regulated by the interplay of growth regulators. The application of cytokinin to the axillary buds can overcome the apical dominance effect and stimulate the lateral buds to

grow rapidly in the presence of terminal buds. If the exogenous growth regulator diminishes the lateral shoot stop growing.

6.0 TUTOR-MARKED ASSIGNMENT

1. Why is node culture considered the most reliable and rapid method for in vitro propagation of true-to-type plants?
2. How this method is considered superior over either adventitious shoot proliferation or the conventional method of vegetative propagation?

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UNIT 9 CRYOPRESERVATION

CONTENTS

1.0 INTRODUCTION

In recent years, with the enormous increase in the population, pressure on the forest and the land resources have increased. This results in depletion of population of medicinal and aromatic plant species. Even some of the plant species are at the verge of vanishing from the forest. The list of endangered species is growing day by day. The conventional methods of germplasm preservation are prone to possible catastrophic losses because of:

i. Attack by pathogen and pests
ii. Climatic disorders
iii. Natural disasters
iv. Political and economic causes.

In addition, the seeds of many important medicinal plants lose their viability in a short time under conventional storage system.
The conservation of germplasm can be done by two methods:

1. **In-situ preservation:** Preservation of the germplasm in their natural environment by establishing biospheres, national parks etc.
2. **Ex-situ preservation:** In the form of seeds or by *in vitro* cultures.

Seeds form the most common material to conserve plant germplasm; however, the method has the following disadvantages:

i. Discrete clones cannot be maintained in the form of seeds.
ii. Some plants do not produce fertile seeds.
iii. Loss of seed viability.
iv. Seed destruction by pests, etc.

v. Poor germination rate.
vi. This is useful for seed propagating plants and is not applicable to vegetative propagated crops, like potato, ginger etc.

In vitro preservation by tissue culture has several advantages over seed preservation

i. Large amount of materials can be stored in a small area.
ii. The material could serve as an excellent form of nucleus stock to propagate large number of plants rapidly, when required.
iii. Under special storage conditions the plants do not require frequent splitting and pruning.
iv. Being free from known viruses and pathogens, the clonal plant material could be sent from country to country, thus, minimizing the obstructions imposed by quarantine systems on the movement of live plants across national boundaries.
v. Protection from natural hazards.
vi. The plants are not exposed to the threat of changing government policies and urban development.

There are few disadvantages of in vitro system to be used for conservation of plant material

i. It is a costly process.
ii. In cultures, plants can be maintained by serial subcultures at frequent intervals for virtually unlimited periods. However, the storage of germplasm by serial subcultures risks the loss of plant material by microbial contamination due to human error and also, is uneconomical. Moreover, in long-term callus and suspension cultures, the regeneration potential, biosynthetic properties and genetic make-up of the cells suffer. The basic requirement of a plant tissue culture method is the preservation of genetic resources, therefore, is to reduce the frequency of subcultures to a bare minimum.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain cryopreservation (Greek, karyos - frost)
- state Steps involved in cryopreservation.

3.0 MAIN CONTENT

3.1 Cryopreservation (Greek, karyos - frost)

Cryopreservation means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to zero metabolism or non-dividing state by mean of storage of germplasm at a very low temperatures, (i) Over solid CO_2 (-79°C), (ii) Deep freezers (-80°C), (iii) in vapor phase nitrogen (-150°C), (iv) in liquid nitrogen (-196°C). Among these, the most commonly used non-lethal storage of biological material at ultra-low temperature is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods.

3.2 Steps Involved in Cryopreservation

The technique of cryopreservation involves the following steps:

1. Selection of plant material
2. Pre-culture
3. Cryoprotective treatment
4. Freezing and storage
5. Thawing
6. Reculture

3.2.1 Selection of Plant Material

The morphological and physiological conditions of the plant material, prior to freezing, considerably influence its ability to survive freezing at -196°C. Generally, small, richly cytoplasmic and meristematic cells survive better than the larger, highly vacuolated cells. Therefore, cell suspensions should be frequently subcultured and frozen in the late lag phase or exponential phase when the majority of the cells are in the preferred condition. While preservation of cell lines remains useful with respect to *in vitro* production of secondary metabolites, cultured cells are not the ideal system for germplasm storage. Instead, organized structures, such as shoot apices, embryos or young plantlets are preferred. The reasons to shift from cell cultures to organised cultures are as follows:

i. The genetic instability of cells in long term callus and cell suspension cultures is a very common phenomenon and there is no effective measure to control it so far. Moreover, most of the callus cultures are initiated from non-meristematic cells of the plant body which might exhibit polysomaty. Hence, the cultured cells may exhibit genetic heterogeneity from the very beginning.

In contrast, plants raised from shoot apices have generally proved to be true- to-type.

ii. Cultured cells of several important plants do not exhibit tot potency. Moreover, in few cases these cells initially form organs/ embryos and whole plants but this potentiality is often lost after some time in culture. Besides, shoot apices possess a high regeneration ability which is retained in prolonged cultures. Shoot apices are mostly preferred to develop a virus free plants and also for the rapid clonal multiplication.

iii. Haploidy, which is highly unstable in callus and suspension cultures can be maintained through shoot tip culture and axillary-bud proliferation.

iv. The cells of shoot-tip and young embryos are small and meristematic. They appear to be better suited than larger cells to survive liquid nitrogen (LN) freezing and thawing.

3.2.2 Pre-Culture

In several cases, a brief culture of shoot apices for at least 48h at 4°C before freezing has proved beneficial for consistently high frequency of survival of shoot apices after freezing in liquid nitrogen. The other treatments include the application of additives that known to enhance plant stress tolerance, for example ABA, proline, osmoticum (sucrose, mannitol), dimethylsulfoide (DMSO, 1-5%). Sugars acts as osmotically effective agents, although they do not penetrate inside the cells. Dehydration of cells/tissues occurs in the presence of sugars during the preculture, which prevents lethal ice crystal formation during freezing. Proline may act by reducing the level of latent injury to the cells or it may actively participate in recovery metabolism.

3.2.3 Cryoprotective Treatments

There are two potential sources of cell damage during cryopreservation (1) Formation of large ice crystals, inside the cells, leading to rupture of organelle and the cell itself, (2) intracellular concentration of solutes increases to toxic levels before or during freezing as a result of dehydration. Addition of cryoprotectants controls the appearance of ice crystals in cells and protects these cells from the toxic solution effect. Cryoprotectants are categorized as: (a) Penetrating, which exert their protective colligative action, (b) Non-penetrating, which affect through osmotic dehydration. A large number of heterogeneous groups of compounds have been shown to possess cryoprotective properties with different efficiencies, e.g. glycerol, DMSO etc. Cryoprotectant depresses both the freezing and super-cooling point of water, i.e. the temperature at which the homogeneous nucleation of ice occurs, thus, retarding the

growth of ice crystal formation in cells and protect cells from toxic effect. The cryoprotectants used in cryopreservation are:

a. **Alcohols:** Ethylene glycol, glycerol, propylene glycol, sorbitol, mannitol
b. **Sulphur containing compounds:** Amino acids, dimethyl sulphoxide (DMSO), sugar (glucose, saccharose)
c. **Polymers:** Hydroxyethyl amidon, polyethylene glycol, polyvinyl pyrrolidine

3.2.3.i Vitrification

At a sufficiently low temperature, highly concentrated aqueous solutions of cryoprotective agents become so viscous that they solidify into an amorphous "glassy" state, without ice crystal formation (crystallization) at practical cooling rates, this phenomenon is called vitrification. The significance of vitrification in cryopreservation of biological materials is that the cells applied with highly concentrated solution of osmotially active compounds, are protected from internal damage from ice crystal formation during freezing. This pretreatment also causes dehydration of cells. The commonly used cryoprotectants are employed for vitrification like DMSO.

3.2.3.ii Cryoprotective Dehydration

If cells are sufficiently dehydrated they may be able to withstand immersion in liquid nitrogen without further application of traditional cryoprotectant mixtures. Dehydration can be achieved by growing the cultures in the presence of high concentration of osmotically active compounds (sugars) and /or air desiccations in laminar-air-flow cabinet or over silica gel. Dehydration reduces the amount of water available for the ice formation.

3.2.3.iii Encapsulation and Dehydration

This involves the encapsulation of tissues in calcium alginate beads which are pre-grown in liquid culture media containing high concentrations of sucrose. The beads are transferred to sterile airflow in a laminar cabinet and desiccated further. After these treatments, the cells are able to withstand exposure to liquid nitrogen without application of chemical cryoprotectants.

3.2.4. Freezing and Storage

The type of crystal water within stored cells is very important for survival of the tissue. Different tissues have different sensitivities for

cooling rates. In general, there are three different types of freezing procedures:

3.2.4.i. Rapid Freezing

The plant material is placed in vials, liquid nitrogen is poured directly in the vial and dipping the vial into an open flask filled with liquid nitrogen. In this procedure, cooling between -10°C and -70°C occurred at the rate of >1000°C/min. The quicker the freezing is done, smaller the intracellular ice crystals are formed. In combination with desiccation or vitrification pre-treatments, ultra rapid cooling is proved to be the most attractive method for cryopreservation of plant materials. This method has been successfully used for the cryopreservation of shoot-tips, somatic embryos and embryonal axes from zygotic embryos of a number of plant species. The survival rate of cryopreserved tissues by this method is high and when the desiccation pretreatment is applied even the cryoprotectants are not required.

3.2.4. ii. Slow Freezing

The tissue is slowly frozen at a slow cooling rate of 0.5-4°C/min from 0 to -100°C, and then transferred to liquid nitrogen. Survival of cells frozen at slow freezing rates may involve some beneficial effects of dehydration, which minimises the amount of water that freezes intracellularly. Slow cooling permits the flow of water from the cells to the outside, thereby promoting extracellular ice formation instead of intracellular freezing. It is generally agreed that upon extracellular freezing the cytoplasm will be effectively concentrated and plant cells will survive better when adequately dehydrated. This has been successfully employed for cryopreservation of meristems of few plants and has proved especially successful with cells from suspension cultures.

3.2.4.iii Stepwise Freezing

Firstly, the material is cooled gradually (ca 1°C/min) or step-wise (5°C/min) to an optimum intermediate temperature (-30°C to -50°C) for about 30 min, and then rapidly cooled by dipping into liquid nitrogen. The method is highly favorable for freeze preservation of shoot apices and buds. It is equally successful to cells from suspension cultures.
The initial slow freezing reduces the amount of intracellular freezable water by dehydrating the cells. Early in the freezing process ice is formed first outside the cells, and the unfrozen protoplasm of cells loses water due to the vapor pressure deficit between the supercooled protoplasm and the external ice. This initial cooling, thus, acts as another pre-treatment for dehydration of the cells.

Storage

Maintaining the frozen material at the correct temperature is as important as proper freezing itself. Temperatures above -130°C may allow ice-crystal growth inside the cells and, as a result reduce their viability. Long-term storage of the material frozen at -196°C, therefore, requires a liquid nitrogen refrigerator. Generally, the frozen cells or tissues are immediately kept for storage at temperature ranging from -70°C to -196°C. The storage is ideally done in liquid nitrogen refrigerator at -150°C in the vapor phase or -196°C in the liquid phase. The temperature should be sufficiently low for long term storage of cells to arrest all metabolic activities and to prevent biochemical injury.

3.2.5 Thawing

Rapid thawing of the material frozen at -196°C is achieved by plunging it into water at 37 to 40°C which gives thawing rate of 500-750°C/min. After about 90s, the material is transferred to an ice bath and maintained there until recultured or its viability is tested. The transfer is necessary because the cells might get damage if it is left long in the water bath 37-45°C. Rapid thawing protects the cells from the damaging effects of ice crystal formation, which may occur during slow warming.

3.2.6 Re-Culturing

The material after thawing should be washed several times to remove the cryoprotectant which may otherwise be toxic to the cells. A gradual dilution of the cryoprotectant is desirable in-order to avoid any deplasmolysis injury to the cells. The plant material frozen at -196°C may need some special requirements for better survival when re-cultured. Shoot-tips from frozen seedlings of tomato directly developed into plantlets only if the medium was supplemented with GA_3. In its absence, apices callused, followed by the differentiation of adventitious shoots.

4.0 CONCLUSION

In recent years, with the enormous increase in the population, pressure on the forest and the land resources have increased. This results in depletion of population of medicinal and aromatic plant species. Even some of the plant species are at the verge of vanishing from the forest. The list of endangered species is growing day by day. The conventional methods of germplasm preservation are prone to possible catastrophic losses because of:

i. Attack by pathogen and pests.

ii. Climatic disorders
iii. Natural disasters
iv. Political and economic causes.

In addition, the seeds of many important medicinal plants lose their viability in a short time under conventional storage system.

5.0 SUMMARY

In vitro preservation by tissue culture has several advantages over seed preservation

i. Large amount of materials can be stored in a small area.
ii. The material could serve as an excellent form of nucleus stock to propagate large number of plants rapidly, when required.
iii. Under special storage conditions the plants do not require frequent splitting and pruning.
iv. Being free from known viruses and pathogens, the clonal plant material could be sent from country to country, thus, minimising the obstructions imposed by quarantine systems on the movement of live plants across national boundaries.
v. Protection from natural hazards.
vi. The plants are not exposed to the threat of changing government policies and urban development.

There are few disadvantages of in vitro system to be used for conservation of plant material

i. It is a costly process.
ii. In cultures, plants can be maintained by serial subcultures at frequent intervals for virtually unlimited periods. However, the storage of germplasm by serial subcultures risks the loss of plant material by microbial contamination due to human error and also, is uneconomical. Moreover, in long-term callus and suspension cultures, the regeneration potential, biosynthetic properties and genetic make-up of the cells suffer. The basic requirement of a plant tissue culture method is the preservation of genetic resources, therefore, is to reduce the frequency of subcultures to a bare minimum.

6.0 TUTOR-MARKED ASSIGNMENT

1. Define germplasm and briefly discuss its significance.
2. Briefly describe the various approaches for in vitro germplasm conservation, their advantages and limitations.
3. Write short notes on:
   a. Vitrification
   b. Cryoprotectants
   c. Stepwise freezing
4. What do you understand by cryoprotectant? Name any two most frequently used cryoprotectant.

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MODULE 3 MICRO TECHNIQUES

UNIT 1 CYTOLOGICAL AND VARIOUS STAINING PROCEDURES FOR PLOIDY ANALYSIS

CONTENTS

1.0 INTRODUCTION

Cytology is a scientific discipline that deals with the study of cells, their physiological and structural properties. Knowing the components of cells and how cells undergo divisions is the main aim of cytology. It helps to understand various chromosomal mutations and genetic rearrangements. The gross structural changes in chromosomes can be evaluated by cytological procedures, which involve staining the chromosomes and evaluating the morphology at metaphase stages of mitosis and meiosis. Cytological staining is particularly used to predict the total content of DNA or ploidy of an individual cell within the mixed population of cells. The determination of ploidy level is an essential technique in plant breeding, genetics and plant tissue culture. The ploidy analysis can be performed either by chromosome counting or by flow cytometry.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain chromosome counting
- state Procedure for sample preparation for chromosome counting
- explain flow cytometry.

3.0 MAIN CONTENT

3.1 Chromosome Counting

Counting of the chromosomes is the most definitive way to differentiate ploidy of an organism. In this technique, smears and squashes of the tissues are prepared which are then observed under the microscope for studies on chromosome counting, morphological differences and karyotyping. Although this technique retains the morphological information, it is tedious and requires longer time for analysing. The sample taken should be of small quantity for better analysis. The meiotic chromosome smears can be prepared using pollen mother cells isolated from immature anthers dissected from flower buds. Alternately, mitotic chromosomes can be counted from root-tip or axillary-bud or shoot-tip squash preparations. A schematic representation of squash preparation is shown in Scheme 13.1. Following stains are used to enhance the contrast of the microscopic images:

i. **Propidium iodide** is a fluorescent nucleic acid dye which binds only to double stranded nucleic acids. As a counter stain, propidium iodide stains the nucleus light to dark red in colour.
ii. **Hoechst stains** are a family of blue fluorescent dyes used to stain DNA. These dyes are excited by ultraviolet light at around 350nm, and emit blue / cyan fluorescent light around an emission maximum at 461nm.
iii. **Aceto-carmine** is a saturated solution of carmine (1-2%) prepared in 45% glacial acetic acid by boiling gently. It is used especially for the rapid staining of fresh unfixed chromosomes. Stained chromosomes are distinguished from other organelles, as it gives the chromosome a red colour.
iv. **Aceto-orcein** is extracted from two species of lichens, *Rocellatinctoria* and *Lecanoraparella*. Orcein is also available in synthetic form, but the natural form is preferred for chromosome analysis because it gives better contrast. Orcein is used in the form of 1% solution prepared in 45% glacial acetic acid by boiling gently.
v. **DAPI** (4',6-diamidino-2-phenylindole) is a cationic fluorescent dye, which specifically binds to adenine – thymine rich DNA. It

is used for cytofluorometric determination of the DNA base content in chromosomes.

vi. **Acridine orange** is a nucleic acid and also a fluorescent cationic dye. When bound to DNA it shows an excitation maximum at 502 nm and an emission maximum at 525 nm (green).

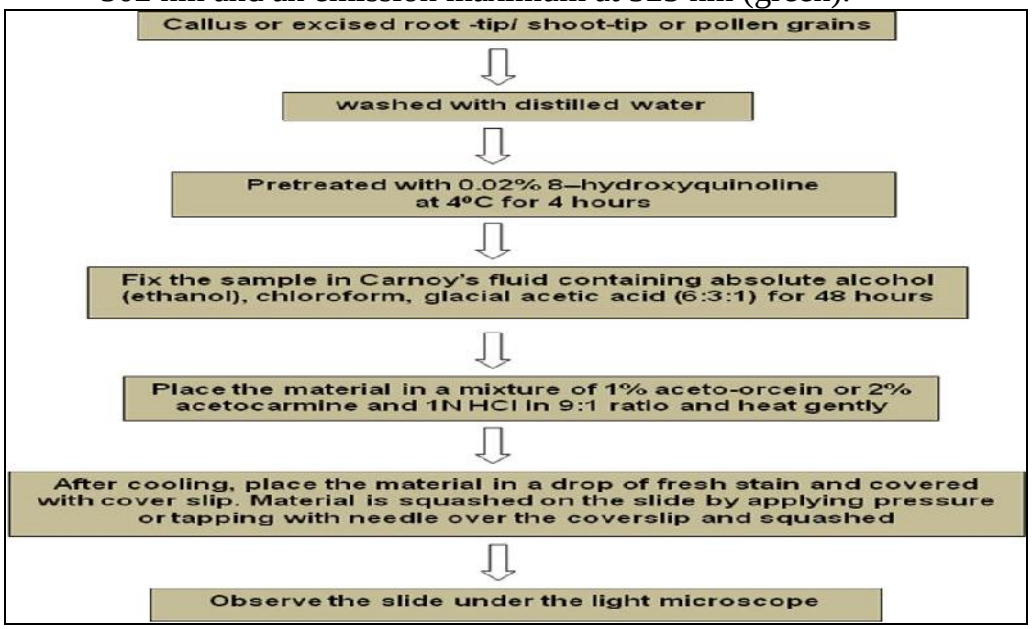

Figure 1.1: Schematic representation of sample preparation for plant ploidy analysis

3.2 Procedure for Sample Preparation for Chromosome Counting

3.2,1 Materials and Equipments

Meristematic region of root-tips is the most widely used plant material but when the root material is fine and shows only primary root region in such cases other regions such as shoot-tip and young flower buds can be used. Phase contrast microscope or high resolution microscope, waterbath, vials, staining dishes, rotar shaker, slide holder, glacial acetic acid, ethanol, colchicines, hydrochloric acid, activated charcoal, sorbitol, NaCl, basic fuchsin, Giemsa, orcein, carmine.

3.2.2. Fixative Preparation

Carnoy's fixative is used for fixation and the reagent consist of 60% ethanol, 30% chloroform and 10% glacial acetic acid . The pre-treated material is transferred to the freshly prepared cool, carnoy's fixative for about 2-4 hrs and finally stored in deep freezers until used. For longer storage, fixative can be replaced with 70% ethanol and stored at 4°C.

3.2.3. Preparation of Various Stains

Staining of plant chromosomes prior to squash preparation is usually done by using Feulgen stain, aceto-carmine or aceto-orecin.

3.2.3.1. Feulgen Stain Preparation

1 gm of basic Fuchsin is dissolved in 200ml of boiling distilled water followed by cooling at 50ºC and filtering. Add 20 ml of 1N HCL and 1 gm of potassium metabisulphate to the filtered fuchsin solution. Leave the solution in dark for about 12 hrs. Finally add 0.5 gm of activated charcoal, shake well and store in a dark bottle at 4°C.

Feulgen staining protocol

1. The plant material pre-fixed in fixative is rinsed properly with distilled water.
2. Hydrolyse the material in 5N HCl at 20ºC and leave for 40 mins.
3. Wash the material again with distilled water and transfer it to Feulgen stain for duration of 1-2 hrs.
4. Wash with distilled water.
5. Place the material under a phase contrast microscope, add a drop of 45% acetic acid, and cover with cover slip and squash.
   Root tips, Callus, Protoplast and suspension culture material can be very accurately visualised by using this staining technique, besides it is also suitable for study of somatic cell preparations of cereals, *Brassica* and *Medicago sativa* .

3.2.3.2. Aceto-Orcein and Aceto-Carmine Stain Preparation

Aceto-orcein : 2gm of orcein is dissolved in 100 ml hot 45% glacial acetic acid, shake well and filter the stock solution. Mix 9 parts of orcein with 1 part of 1N HCL. Aceto-carmine : 0.5% carmine is dissolved in 100 ml hot 45% glacial acetic acid, boil for half an hour and finally cool and filter.

Staining protocol

1. On a microscopic slide, the material fixed priorly with the fixative is placed and a drop of aceto-orcein or aceto-carmine is added.
2. Slide is warmed over a spirit lamp keeping in mind that the temperature should not increase beyond 60ºC.
3. Stain is removed with the help of a piece of filter paper, 45%acetic acid is added.
4. Material is covered with the cover slip and squash is prepared.

5. In order to avoid drying of chromosomes the edges of cover slip are sealed with a nail varnish or acid gelatin for temporary vision or keeping slide as a permanent one.

Immature stage of pollen anther and flower bud can be well visualised using Carmine staining method. Heating of the slide during this process causes cells to swell resulting in chromosome separation preventing their overlapping. Orcein works well for staining the microspores in *Brassica.*

3.2.3.3 Fluorescent Staining with DAPI (4'6-diamino-2 phenylindole)

DAPI solution preparation: 100 µg of DAPI is dissolved in 1ml of distilled water; reagent is stored in an aliquot for years at -20ºC. Working solution can be prepared from stock in McIlvaine buffer to a final concentration of 2-4µl/ml (w/v).

Mcilvaine buffer preparation.

Mix (0.1M citric acid (18 ml) with 0.2M Na_2HPO_4 x $2H_2O$ (82ml) and adjust the at pH at 7.0.

Staining protocol

1. 50µl of DAPI working solution is added to the slide at room temperature in dark for the duration of about half an hour.
2. Slide is washed with distill water and air dried.
3. Slide is then mount with antifade buffer Citifluor (AFI, Pelco).
4. DAPI stained chromosomes can be destained with methanol-acetic acid (3:1) for 6-12 hrs, rinsed with methanol and air dried.

3.2.3.4 Non Fluorescent Giemsa Staining

Stain preparation
Giemsa stain is prepared by adding 4% giemsa in söerensen phosphate buffer (0.05M Na_2HPO_4 and 0.05M KH_2PO_4)
Staining protocol.

1. To the slide with prefixed material Giemsa stain is added and kept for 20-30 mins.
2. Slide containing stained material is rinsed with distill water and air dried.
3. Stained chromosomes can be destained with 96% ethanol for 1-2 hrs.

Provides clear visualisation of well spread chromosomes preventing overlapping of chromosomes.

3.2.4. Squash Preparation

1. Small drop of stain is placed on a slide.
2. A root-tip is placed using a tweezer on the very slide with stain.
3. Intensely stained meristematic region of root is cut off using a scalpel; tissue is thereafter teased with the needle.
4. Major root region in root cap consisting of epidermal and sub epidermal region layers is removed. This provides flatter squash to be prepared.
5. The drying of slide should be prevented a cover slip of size which does not allow the stain to fall is used to cover the material.
6. Slide is gently heated over alcohol flame.
7. The excess of stain, if any, can be gently removed by pressing. Flatten the cells by applying pressure keeping in mind that majority of the cells are in same plane of focus.

3.2.5 Chromosome Count Analysis

The number and morphological appearance of chromosomes for particular species is almost fixed. Basic chromosome structure consists of two arms and a primary constriction known as centromere which attaches the two arms of a chromosome. The location of centromere may vary from median or centre known to be Metacentric compared with other having the centromere at very end of chromosome termed as Telocentric while those which have their centromere slightly shifted away from centre are known as submetacentric chromosomes. The prepared slide is viewed under a microscope and scanned for a chromosome spread. The chromosomes in the spread are examined and manually counted in order to obtain the ploidy of the sample. Place the slide under microscope and scan it properly so as to visualise separated, non-overlapping chromosomes. Count and record the number of chromosomes and draw the structure of the chromosome as under microscope on a piece of paper.

On the basis of these countings and structure, normal chromosome appearance and deviations from it, if any, which have been occurred due to overlapping of chromosome, deletion or translocation can be easily studied. C-banding Giemsa staining method is followed to study regenerated callus, protoplast and suspension studying the size position and no of bands on each chromosome. In *in-vitro* regenerated plants mostly from callus and protoplast, the chromosome number and the karyotype is most of the times stabilised. In such cases any variation, if observed, can be due to mechanical procedure of squash prepration.

3.3 Flow Cytometry

Flow cytometry analysis involves the estimation of total nuclear DNA content and not microscopic evaluation of chromosome number. Thus, the terms *Ploidy* and *DNA ploidy* should be used to distinguish between karyotype and DNA content analysis, respectively.

Advantages of flow cytometric assay are:

- Rapidity, precision and convenience (several hundred samples per working day).
- No need for pressing cells.
- Non-destructive (requires small amount of tissue).
- Analysis of large populations of cells (detection of subpopulations - mixoploidy).

4.0 CONCLUSION

Cytology is a scientific discipline that deals with the study of cells, their physiological and structural properties. Knowing the components of cells and how cells undergo divisions is the main aim of cytology. It helps to understand various chromosomal mutations and genetic rearrangements. The gross structural changes in chromosomes can be evaluated by cytological procedures, which involve staining the chromosomes and evaluating the morphology at metaphase stages of mitosis and meiosis. Cytological staining is particularly used to predict the total content of DNA or ploidy of an individual cell within the mixed population of cells.

5.0 SUMMARY

The determination of ploidy level is an essential technique in plant breeding, genetics and plant tissue culture. The ploidy analysis can be performed either by chromosome counting or by flow cytometry.
Advantages of flow cytometric assay are:

- Rapidity, precision and convenience (several hundred samples per working day).
- No need for pressing cells.
- Non-destructive (requires small amount of tissue).
- Analysis of large populations of cells (detection of subpopulations - mixoploidy).

6.0 TUTOR-MARKED ASSIGNMENT

1. Describe the technique for ploidy analysis?

2. How ploidy levels of plants can be analysed by chromosome counting?
3. Write the steps of sample preparation for plant ploidy analysis through chromosome counting.
4. What are the advantages of flow cytometry?
5. What materials can be analysed by chromosome counting and flow cytometry?

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UNIT 2 MICROSCOPY

CONTENTS

1.0 INTRODUCTION

A microscope is an optical instrument that produces an enlarged image of an object. A light source built into its base illuminates a specimen through a set of lenses to produce a magnified image that is projected onto the retina of the eye or onto an imaging device (Figures 2.1 and 2.2). The objective lens and the eyepiece (or ocular lens) work together to produce the final magnification *M* of the image such that

$M_{final} = M_{objective} \times M_{ocular}$

Two important components are of critical importance in forming the image. These are:

i. The objective lens, which collects the light that is diffracted by the specimen and form a magnified real image at the real intermediate image plane near the oculars or eyepiece
ii. The condenser lens, which focuses light from the illuminator onto a small area of the specimen

The condenser lens gathers the diffuse rays from the light source and illuminates the specimen with a small cone of bright light that allow very small part of the specimen to be seen after magnification. The light rays focussed on the specimen by the condenser lens are then collected by the microscope's objective lens. The light rays are brought to focus by the objective lens to form a real, enlarged image of the object within the column of the microscope. The image formed by the objective lens is used as an object by a second lens system, the ocular lens, to form an enlarged and virtual image. A third lens system located in the front part of the eye uses the virtual image produced by the ocular lens as an object to produce a real image on the retina. For an imaging device, the intermediate image is recorded directly or projected as a real image onto a camera.

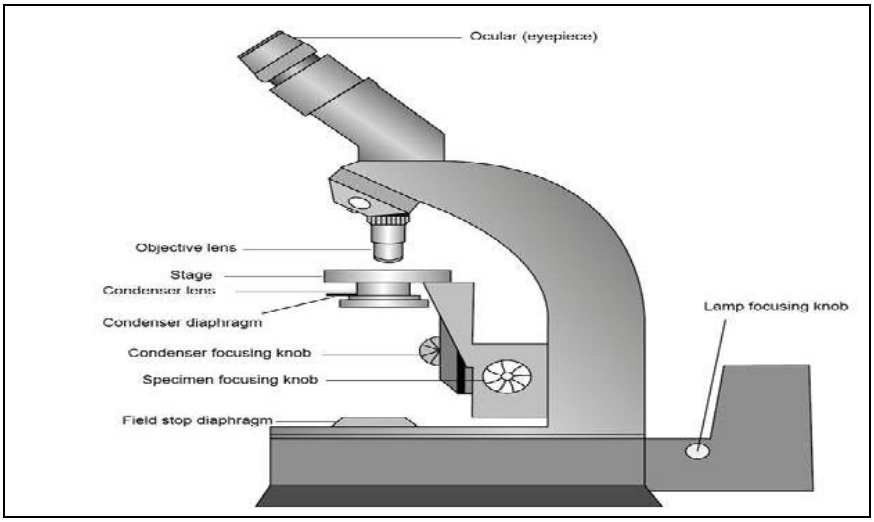

Figure 2.1: The light microscope

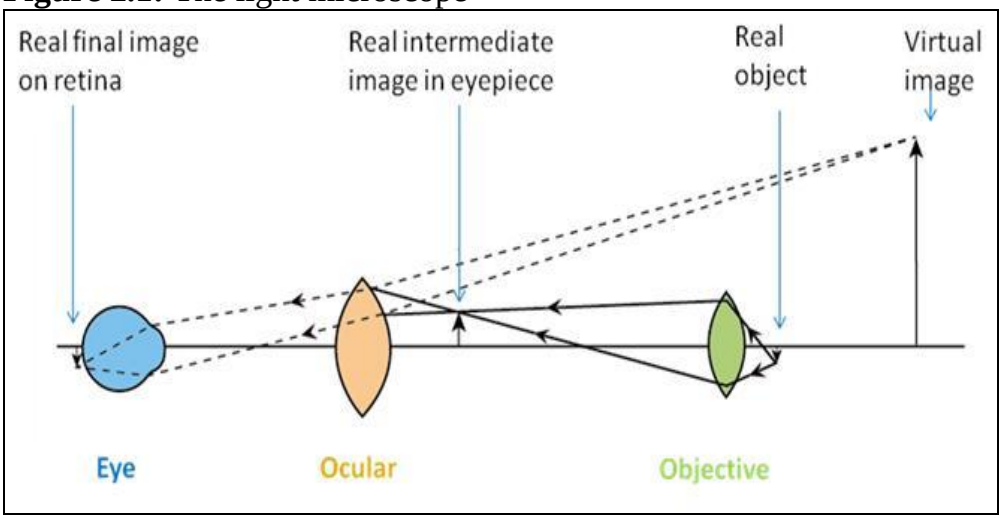

Figure 2.2: Perception of a magnified virtual image of a specimen in a microscope

The objective lens forms a magnified image of the object (called the real intermediate image) in or near the eye piece; the intermediate image is examined by the eyepiece and the eye, which forms a real image on the retina.

2.0 OBJECTIVE

By the end of this unit, you will be able to:

- analyse of the concept of microscope
- explain confocal microscopy.

3.0 MAIN CONTENT

3.1 Analysis of Microscope

3.1.1 Resolving Power of Microscope

Resolution or resolving power of a microscope can be defined as the smallest distance apart at which two points on a specimen can still be seen separately. Sometimes, blurred images may be seen through the lenses. This is because of the placing of two distinct points too close to each other, which results in overlapping of the images. Magnification can neither improve nor decrease the resolving power of the microscope. The resolution R is determined essentially by three parameters:

i. the wavelength λ of the illuminating light
ii. the numerical aperture (NA) of the objective lens (NA^{obj})
iii. the numerical aperture of the condenser (NA^{cond})
where,
$R = 1.22*\lambda / (NA^{obj} + NA^{cond})$ (i)

When the aperture of the condenser is adjusted to that of the objective, i.e. the aperture of the condenser is essentially same as the objective aperture, the equation (i) simplifies to:
$R = 0.61*\lambda/NA^{obj}$ (ii)

The resolution of an image is limited by the wavelength of radiation used to view the sample. This is because when objects in the specimen are much smaller than the wavelength of the radiation being used, they do not interrupt the waves, and so are not detected. The wavelength of light is much larger than the wavelength of electrons, so the resolution of the light microscope is a lot lower. Using a microscope with a more powerful magnification will not increase this resolution any further. It will increase the size of the image, but objects closer than 200nm will still only be seen as one point.

3.1.2. Numerical Aperture

The numerical aperture of a microscope objective is defined as a measure to gather light and, thus, resolve fine specimen detail at a fixed objective distance.

The numerical aperture is given by the following formula:

$NA = n*(\sin\mu)$
where,

- **n** refers to the refractive index of the medium between the specimen's cover glass and the front lens of the objective. It is, n = 1.00 for air and n = 1.5 for immersion oil.
  μ is one half the angular aperture (cone angle). The bigger the value of μ, the higher is the numerical aperture.

The numerical aperture of a lens depends upon two parameters, the angle of incidence of light onto the lens, and the refractive index (n) of the glass of which the lens is composed. The angle of incidence is also known as the cone angle and 1 / 2 of this value is designated by the symbol μ. The refractive properties of a lens are summed up in a measurement known as the refractive index (n). The refractive index is a function of the bending of light from air through glass and back again.

3.2 Confocal Microscopy

Confocal microscopy is an imaging technique which uses a spatial pinhole to increase micrograph contrast and/or to reconstruct three-dimensional images by eliminating out-of-focus light or flare in specimens that are thicker than the focal plane. The principle of confocal imaging was patented by Marvin Minsky in 1961. It is an integrated microscope system consisting of a fluorescence microscope, multiple laser light sources, a confocal box or scans head with optical and electronic equipment, a computer and monitor for display, and software for acquiring, processing, and analyzing images (Figure 14.3).

3.2.1 Principle

In this type of microscope, the specimen is illuminated by a finely focussed laser beam through a pin hole that rapidly scans across the specimen at a single depth, thus, illuminating only a thin plane or optical section within the specimen. Short wavelength incident light is absorbed by the specimen and remitted at longer wavelengths. Lights emitted from the specimen are brought to focus at a site within the microscope that contains a second pinhole aperture. The light emitted from the illuminated plane of the specimen passes back, up through the objective lens, through the dichroic mirror and emission filter, and through a second pin hole. Light rays that might emanate from above or below this plane are prevented from participating in image formation. As a result, out – of – focus points in the specimen becomes invisible. The emitted light is finally detected by a photomultiplier behind the second pin hole. The two pin holes – illuminating and detecting – are located in planes conjugate to the plane of focus of the image.

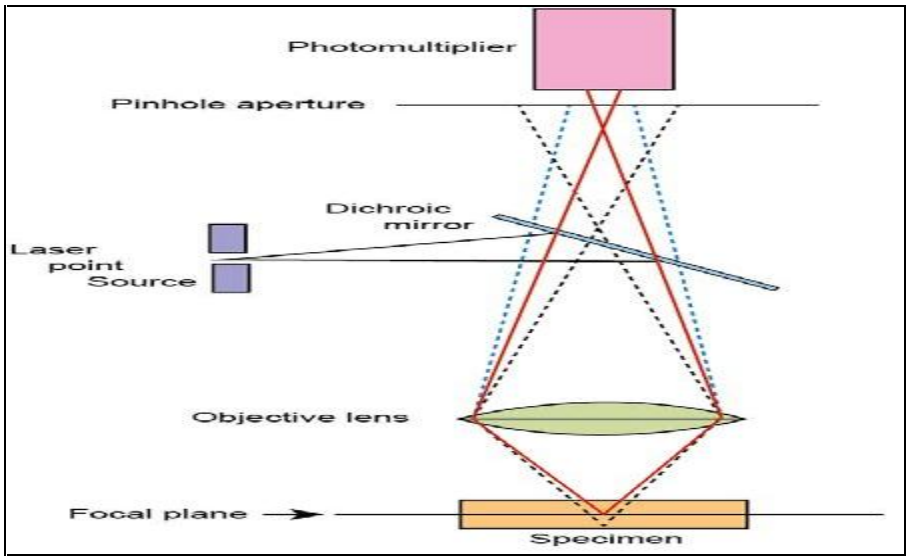

Figure 2.3: Confocal principle in epifluorescence laser scanning microscope

3.2.2 Practical Use of Confocal Microscopes

The above principle describes the confocal imaging of a single point on the specimen. For practical use of confocal microscopy, however, a method is needed to image successive points to build up an image. This can be done by either scanning the illuminated spot across the specimen in a raster fashion (scanned beam design) or the specimen can be moved through a stationary light path (stage scanning design). The stage scanning design has the advantage of a simple and accurate optical design, but suffers from lack of speed in scanning an image, particularly at low magnification. The first confocal microscopes built were of this stage scanning type.

However, all current biological confocal microscopes used the scanned beam design. An angular deflection of the light beam at a diffraction plane becomes transformed into a translation in the specimen / image planes. Thus, a system of two deflecting mirrors scanning back and forth about two axes positioned at or near a diffraction plane can be used to scan the light beam in a 2D raster across the specimen. The scan driving and measurement circuitry are interfaced together so that light intensity measurements are taken which cover the specimen area in a regular raster. These intensities are digitised into a computer to produce a digital image.

In order to increase the scanning to video frame rates, slit scanning design has been developed. Instead of a pin whole aperture, a narrow slit of light is scanned in a direction at right angles to its length across the specimen. The emitted light is then passed through a narrow detector

slit. This is of advantageous, since only one-dimensional scan is required; the scanning rate can be much faster than a point scanning system. Also, because a line of specimen is imaged at one time, the rate of light accumulation from the specimen is much higher. However, this design is not capable of producing clean optical sections as a point scanning system because a proportion of the out – of – focus light is also detected.

Confocal microscopes are also designed with an extended light source and an array of many pin holes on a disc called the Nipkow disc which is placed in a conjugate image plane. The full image is obtained by spinning the disc rapidly, so that pin holes that are usually arranged in a spiral pattern, scan across the whole image area. The major hurdle of this design is that the light source has to be spread out over the whole of the disc, and so is less bright than single hole / laser arrangement. Moreover, only a very small proportion of light passes back through the disc to be detected resulting in the difficulty in recording enough light for a satisfactory image.

3.2.3 Objective Lenses in Confocal Imaging

Although any objective can be used, objectives with higher numerical aperture are preferred as the brightness of the image is strongly dependent on the numerical aperture of the objectives.

3.2.4 Specimen Preparation for Confocal Imaging

Generally, confocal imaging (confocal fluorescence microscopy) simply requires a fluorescent specimen. However, since confocal microscopy are capable of producing clean optical sections from thick, three dimensionally well-preserved specimens, care should be taken to preserve the three-dimensional structure of the specimen. For living specimens, methods that keep the tissue or cells alive and active will almost certainly preserve the three-dimensional structure. For dead specimens, fixatives such as formaldehyde solutions with small percentage of glutaraldehyde which is consistent with the labelling method should be used. The fixed tissues also often need extra permeabilisation to allow penetration of probes. In case of plant material, the cell wall is partially digested with cellulose or other cell wall degrading enzymes. Producing good specimen depends on maintaining a balance between preservation of the structures of interest but at the same time disrupting them so as to allow probes in to visualise the structures.

3.2.5 Uses of Confocal Microscopy

Since confocal microscopy possesses the advantage of elimination of out - of - focus light, it is mainly used for observing specimens with substantial thickness.

4.0 CONCLUSION

A microscope is an optical instrument that produces an enlarged image of an object. A light source built into its base illuminates a specimen through a set of lenses to produce a magnified image that is projected onto the retina of the eye or onto an imaging device.

5.0 SUMMARY

Confocal microscopy is an imaging technique which uses a spatial pinhole to increase micrograph contrast and/or to reconstruct three-dimensional images by eliminating out-of-focus light or flare in specimens that are thicker than the focal plane. The principle of confocal imaging was patented by Marvin Minsky in 1961. It is an integrated microscope system consisting of a fluorescence microscope, multiple laser light sources, a confocal box or scans head with optical and electronic equipment, a computer and monitor for display, and software for acquiring, processing, and analysing images.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is the principle of light microscope?
2. What is magnification?
3. What do you mean by resolution?
4. Describe the principle of confocal microscopy?
5. Write short notes on:
   i. Numerical aperture
   ii. Specimen preparation for confocal imaging
   iii. Uses of confocal microscopy
   iv. Perception of a magnified virtual image of a specimen in a microscope.

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UNIT 3 ELECTRON MICROSCOPY

CONTENTS

1.0 INTRODUCTION

Electron microscopes are scientific devices that employ a beam of highly energetic electrons to study objects on a very fine scale. This inspection can yield information about the topography (surface features of an object), morphology (shape and size of the particles making up the object), composition (the elements and compounds that the object is composed of and the relative amounts of them) and crystallographic information (how the atoms are arranged in the object) of the sample. The wide spread use of electron microscopes are based on the fact that they allow the observation and characterisation of materials on a nanometer (nm) to micrometer (μm) scale. Electron microscopes were made due to the limitations of bright-field light microscopes which are limited by the physics of light to 500x or 1000x magnification and a resolution of 0.2 micrometers.

The Transmission Electron Microscope (TEM) was the first category of electron microscope that was developed by Max Knoll and Ernst Ruska in Germany in 1931. The first Scanning Electron Microscope (SEM) debuted in 1942 with the first commercial instruments around 1965. Electron microscopes work exactly like their optical counterparts except that they use a focused beam of electrons instead of light to form image of the specimen and gain information as to its structure and composition. The basic steps involved in all electron microscopes are:

a. A stream of electrons is formed in high vacuum by electron guns
b. This stream is accelerated towards the specimen with a positive electrical potential while is confined and focused using metal apertures and magnetic lenses into a thin, focused, monochromatic beam.

c. The sample is irradiated by the beam and interactions occur inside the irradiated sample, affecting the electron beam.
d. These interactions and effects are detected and transformed into an image.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain the concept of electron gun
- understand Scanning Electron Microscope (SEM)
- discuss Transmission Electron Microscope (TEM).

3.0 MAIN CONTENT

3.1 Concept of Electron Gun

The first and basic part of the microscope is the source of electrons. It is usually a V-shaped filament made up of tungsten that is wreathed with Wehnelt electrode (Wehnelt Cap). Owing to negative potential of the electrode, the electrons are emitted from a small area of the filament called a point source. A point source is important because it emits electrons with similar energy. The two common types of electron guns (Figure 3.1) are the conventional electron guns and the Field Emission Guns (FEG). In a conventional electron gun the positive electrical potential is applied to the anode and the filament (cathode) is heated until a stream of electrons is generated. The electrons gather speed by the positive potential down the column, and due to the negative potential of cap, all electrons are repelled toward the optic axis. A group of electrons occur in the space between the filament tip and cap, which is called a space charge. Those electrons at the base of the space charge (nearest to the anode) can exit the gun area through the small (<1 mm) hole in the cap and then travel down the column to be used in imaging.

A field emission gun comprises of sharply pointed tungsten tip held at several kilovolts negative potential relative to a nearby electrode, so that there exist a very high potential gradient at the surface of the tungsten tip. This results in the potential energy of an electron as a function of distance from the metal surface to have a sharp peak, and then drops off quickly (due to electron charge travelling through an electric field). Since electrons are quantum particles and have a probability distribution to their location, a certain number of electrons that are nominally at the metal surface will find themselves at some distance from the surface, such that they can reduce their energy by moving further away from the surface. This transport-via-delocalisation is called 'tunnelling', and is the basis for the field emission effect. FEGs produce much higher source

brightness than in conventional guns (electron current > 1000 times), better monochromaticity, but requires a very good vacuum (~10-7 Pa).

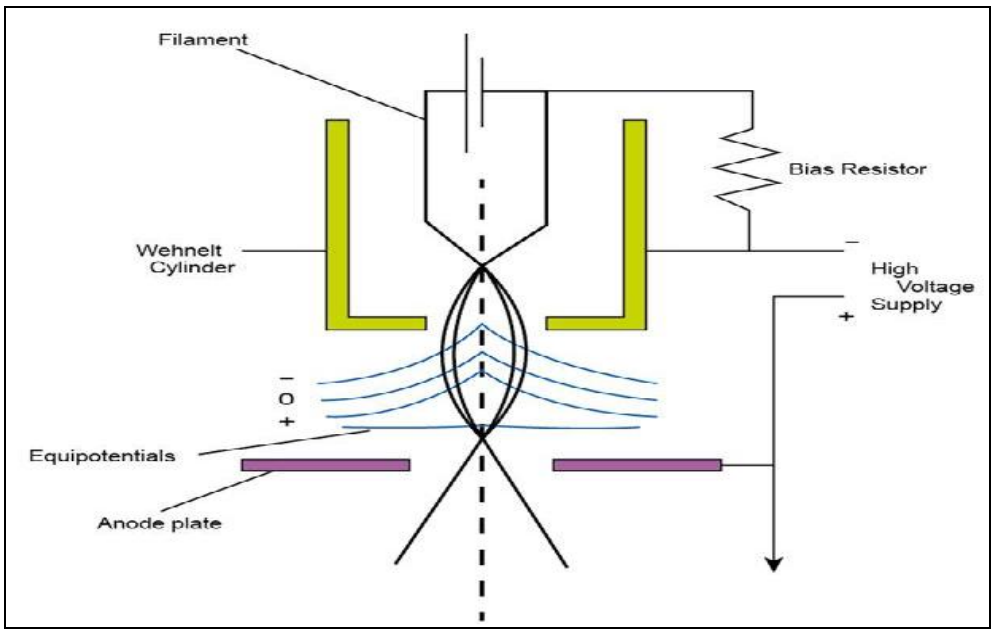


Figure 3.1: The electron gun

3.1.1 Electron–Specimen Interaction

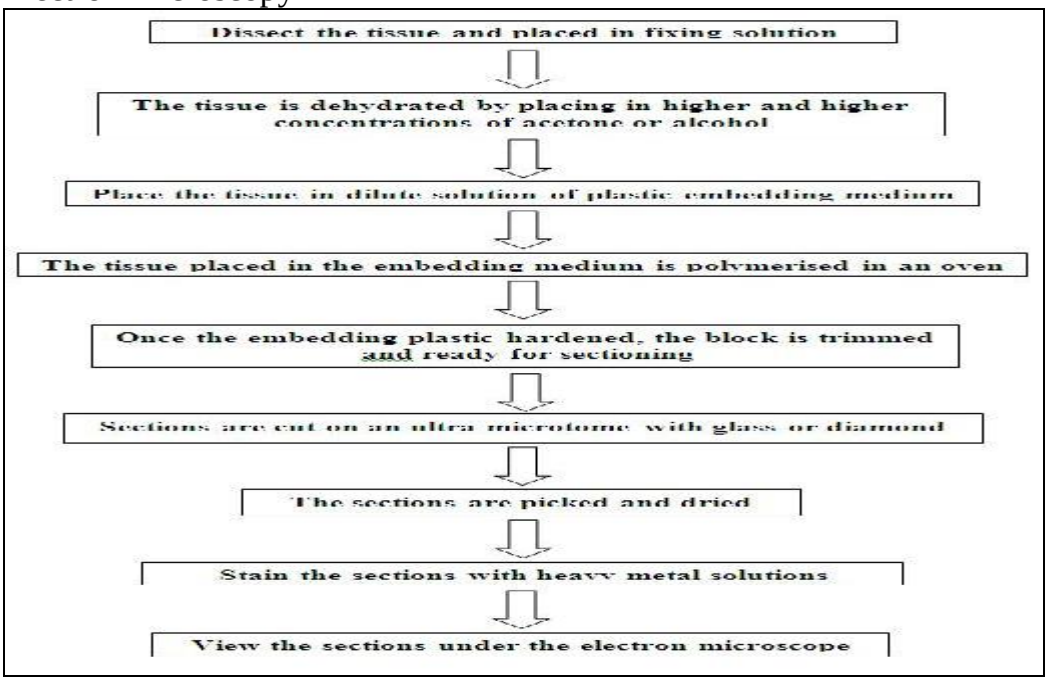
The individual incident electrons of an electron beam interact with the atoms in a sample, to undergo two types of scattering-elastic and inelastic. During elastic scattering, only the trajectory changes, and the kinetic energy and velocity remain constant. However, in inelastic scattering, some incident electrons will actually ram with and displace electrons from their orbits (shells) around nuclei of atoms comprising the sample. This interaction places the atom in an excited and unstable state. This specimen interaction is what makes electron microscopy possible.

3.1.2 Specimen Preparation for Electron Microscopy

Tissues to be examined in the electron microscope must be fixed, embedded and sectioned. Fixation of tissues for electron microscopy (Scheme 3.1) is much more critical than for light microscopy because the sections are subjected to a much greater scrutiny. A fixative must stop the life of a cell without significantly altering the structure of that cell and cell components. To obtain the most rapid fixation and the least cellular damage, very small pieces of tissues are fixed and embedded. Fixatives are chemicals that denature and precipitate cellular macromolecules. Once the tissue is fixed, the water is removed by dehydration in alcohol, and the tissue spaces are filled with a material

that supports tissue sectioning. Tissues to be sectioned for electron microscopy are usually embedded in epoxy resins, such as Epon or Araldite. Sections are cut by slowly bringing the plastic block down across an extremely sharp cutting edge made of cut glass or a finely polished diamond after which the sections are thoroughly dried and stained using heavy metal solutions, such as uranyl acetate or lead citrate. These heavy metals bind to the macromolecules and provide the atomic density required to scatter the electron beams.

Scheme 3.1: Schematic Representation of Specimen Preparation for Electron Microscopy



3.2 Scanning Electron Microscope (SEM)

A scanning electron microscope (SEM) is a type of electron microscope that forms images of a sample by scanning it with a high-energy beam of electrons. The electrons interact with the atoms that make up the sample resulting in signal production that contains information about the sample's surface topography, composition, and other properties, such as electrical conductivity. SEM is used primarily to examine the surfaces of objects (Figure 3.2).

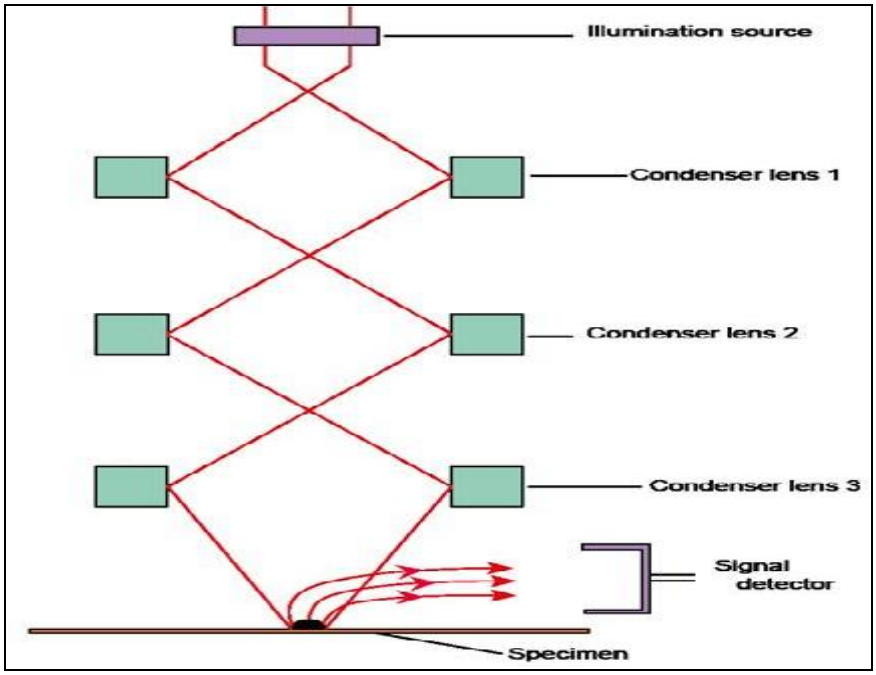


Figure 3.2: Configuration of scanning electron microscope

3.2.1 Operation of SEM

In SEM, a source of electrons is focussed in vacuum into a fine probe that is raster over the surface of the specimen. The electron beam passes through scan coils and objective lens that deflect horizontally and vertically so that the beam scans the surface of the sample. As the electrons penetrate the surface, numerous interactions occur that results in the emission of electrons or photons from or through the surface. A reasonable fraction of the electrons emitted can be gathered by suitable detectors, and the output can be used to modulate the brightness of a cathode ray tube (CRT) whose x- and y- inputs are driven in synchronism with the x-y voltages raster the electron beam. In this way an image is produced on the CRT. Every point that the beam strikes on the sample is mapped directly onto a corresponding point on the screen. As a result, the magnification system is simple and linear magnification is calculated by the equation:

$M=L/l$ (1)
Where L refers to the raster's length of the CRT monitor and l is the raster's length on the surface of the sample.

SEM functions on a voltage between 2 to 50kV and its beam diameter that scans the specimen is 5nm-2µm. The principle images produced in SEM are of three types: secondary electron images, backscattered electron images and elemental X-ray maps. Secondary and backscattered electrons are conventionally separated according to their energies. When the energy of the emitted electron is less than about

50eV, it is referred as a secondary electron and backscattered electrons are considered to be the electrons that exit the specimen with energy greater than 50eV. Detectors of each type of electrons are positioned in the microscope in proper locations to collect them.

3.2.2 Reactions Exploited in SEM

3.2.2.1 Secondary Electrons

When a sample is bombarded with electrons, the strongest region of the electron energy spectrum is as a result of secondary electrons. The secondary electron yield depends on many factors, and is by and large higher for high atomic number targets, and at higher angles of incidence. Secondary electrons are produced when an incident electron excites an electron in the sample and loses most of its energy in the process. The excited electron moves towards the surface of the sample experiencing elastic and inelastic collisions until it reaches the surface, where it can escape if it still has sufficient energy. The production of secondary electrons is very topography related. Due to their low energy (5eV) only secondary electrons that are very near the surface (<10 nm) can exit the sample and be examined. Any changes in topography in the sample that are larger than this sampling depth will change the yield of secondary electrons due to collection efficiencies. Collection of these electrons is assisted by using a "collector" in conjunction with the secondary electron detector.

3.2.2.2 Backscattered Electrons

Backscattered electrons constitute high-energy electrons originating in the electron beam that are reflected or backscattered out of the specimen interaction volume. The production of backscattered electrons differs directly with the specimen's atomic number. This inconsistent production rates causes elements of higher atomic number to appear brighter than lower atomic number elements. This interaction is exploited to differentiate parts of the specimen that have different average atomic number.

3.2.2.3 Relaxation of Excited Atoms

Inelastic scattering puts the atom in an excited (unstable) state. The atom “wants” to return to a ground or unexcited state. Therefore, at a later time the atoms will relax giving off the excess energy. Cathodoluminescence, X - Rays and Auger electrons are three types of relaxation. The relaxation energy is the fingerprint of each element.
When the sample is bombarded by the electron beam of the SEM, electrons are ejected from the atoms on the specimen's surface. A

resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted to balance the energy difference between the two electrons. The X-ray detector measures the number of emitted x-rays versus their energy. The energy of the x-ray is characteristic of the element from which the x-ray was emitted.

Cathodoluminescence is the emission of photons of characteristic wavelengths from a material that is under high-energy electron bombardment. The electron beam is typically produced in an electron microprobe or scanning electron microscope. Auger electrons are electrons expelled by radiation - less excitation of a target atom by the incident electron beam. Auger electrons are a feature of the fine structure of the atom and have energies between 280 eV (carbon) and 2.1 keV (sulphur).

3.2.3 Advantages and Disadvantages

The electrons in SEM penetrate into the sample within a small depth, and are, therefore, suitable for surface topology of every kind of samples (metals, ceramics, glass, dust, hair, teeth, bones, minerals, wood, paper, plastics, polymers, etc). It can also be employed for chemical composition of the sample's surface since the brightness of the image formed by backscattered electrons is increasing with the atomic number of the elements. This suggests that regions of the sample consisting of light elements (low atomic numbers) appear dark on the screen and heavy elements appear bright. Backscattered are used to form diffraction images that describe the crystallographic structure of the sample. Consequently, SEM is only employed for surface images, and both resolution and crystallographic information are limited (because they are only referred to the surface). Other limitations are firstly the samples must be conductive, so non-conductive materials are carbon-coated and secondly, the materials with atomic number smaller than the carbon are not detected with SEM.

3.3 Transmission Electron Microscope (TEM)

Transmission electron microscopy (TEM) is a microscopic technique in which a beam of electrons is transmitted through an ultra-thin specimen, resulting in interaction with the specimen as it passes through. An image is produced from the interaction of the electrons transmitted through the specimen, which is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or detected by a sensor. TEM has been exploited most widely in the examination of the internal surface of cells (Figure 3.3).

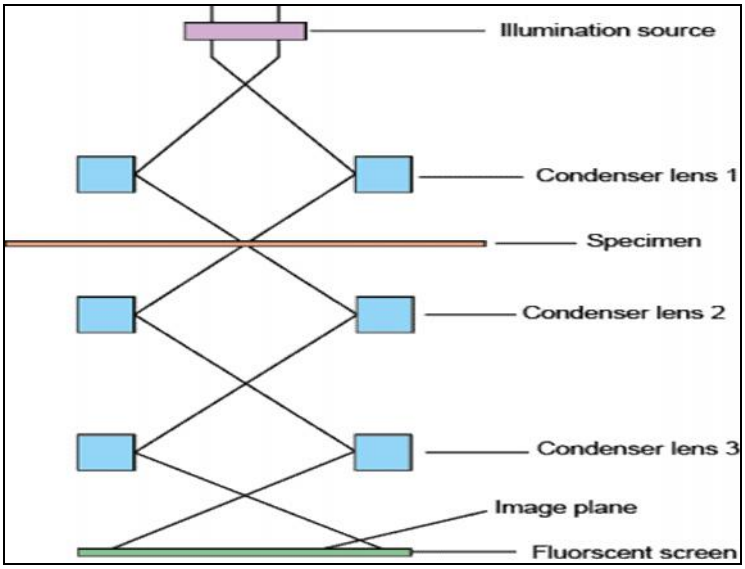


Figure 3.3: Organisation of Transmission Electron Microscope

3.3.1 Operation of TEM

To operate TEM, it requires an ultra-high vacuum and a high voltage. Through a sequence of adjustments of focus and brightness of the beam, the setting of the microscope is adjusted so that by shifting, the sample holder finds the thin area of the sample. Then tilting of the sample begins by rotating the holder. This is a way to view as much areas as we can, so we can obtain as much information. Various images are obtained in TEM by properly utilising the apertures and by using different types of electrons. Diffraction patterns are seen because of the scattered electrons. If the unscattered beam is selected, the bright field image is obtained. Dark field images are achieved if diffracted beams are selected by the objective aperture. In transmission microscopy, the specimen's structure and its atomic columns are observed, thus compositional and crystallographic information is attained. However, being a very expensive technique, expertise is needed and the sample preparation phase is too difficult in order to achieve very thin samples.

3.3.2 Sample Preparation

The first step is to decide whether the sample is useful to be observed and in which view, planar or cross-section. Due to the strong interaction between electrons and matter, the specimens have to be rather thin, less than 100nm. This is achieved with several methods, depending on the material. In general, mechanical thinning is used to thin and polish the sample. Then it is glued with epoxy glue on a really small and round holder. Whereas TEM data come from the edges of a hole in the centre of the specimen, in sample preparation, the hole is created by the method of ion thinning. Ion thinning is a method where a specimen is irradiated with beams of Ar ions and after a period of time a hole is

created. To minimise the damage created, the embedded sample can first be coated with a metal deposition layer. Consequently, sample preparation is a precise and a severe procedure, which may affects the results of the microscopic analysis and study.

3.3.3 Reactions Exploited in TEM

TEM exploits three different interactions of electron beam-specimen; Unscattered electrons (transmitted beam), elastically scattered electrons (diffracted beam) and inelastically scattered electrons. When incident electrons are transmitted through the thin specimen without any interaction occurring inside the specimen, then the beam of these electrons is called transmitted. The transmission of unscattered electrons is inversely proportional to the specimen thickness. The areas of the specimen that are thicker will have lesser unscattered electrons that are transmitted and so will appear darker, conversely the thinner areas will have more transmitted electrons and, thus, will appear lighter. Another component of the incident electrons are scattered (deflected from their original path) by atoms in the specimen in an elastic fashion (no loss of energy). These scattered electrons are then transmitted through the remaining portions of the specimen.

All electrons follow Bragg's Law and, thus, are scattered according to
$n\cdot\cdot \lambda = 2\cdot d\sin(\theta)$
Where
λ is the wavelength of the rays
θ is the angle between the incident rays and the surface of the crystal and d is the spacing between layers of atoms.

All incident electrons possess the same energy and wavelength and enter the specimen normal to its surface. All incident electrons that are scattered by the same atomic spacing will be scattered by the same angle. These scattered electrons can be collated using magnetic lenses to form a pattern of spots; each spot corresponding to a specific atomic spacing (a plane). This pattern can then yield information about the orientation, atomic arrangements and phases present in the area being examined. Finally, another way that incident electrons can interact with the specimen is in- elastically. Incident electrons that interact with specimen atoms in an inelastic fashion, loses energy during the interaction.

3.3.4 Limitation

There are numerous downsides to the TEM technique. Many materials need extensive sample preparation to generate a sample thin enough to be electron transparent, which makes TEM analysis a relatively time

consuming procedure with a low throughput of samples. The structure of the sample may also be altered during the preparation process. Also the field of view is relatively small, increasing the possibility that the region analysed may not be characteristic of the whole sample. There is potential that the sample may be destroyed by the electron beam, particularly in the case of biological materials.

4.0 CONCLUSION

Electron microscopes are scientific devices that employ a beam of highly energetic electrons to study objects on a very fine scale. This inspection can yield information about the topography (surface features of an object), morphology (shape and size of the particles making up the object), composition (the elements and compounds that the object is composed of and the relative amounts of them) and crystallographic information (how the atoms are arranged in the object) of the sample.

5.0 SUMMARY

There are numerous downsides to the TEM technique. Many materials need extensive sample preparation to generate a sample thin enough to be electron transparent, which makes TEM analysis a relatively time consuming procedure with a low throughput of samples. The structure of the sample may also be altered during the preparation process. Also the field of view is relatively small, increasing the possibility that the region analysed may not be characteristic of the whole sample. There is potential that the sample may be destroyed by the electron beam, particularly in the case of biological materials.

6.0 TUTOR-MARKED ASSIGNMENT

1. What are the basic steps involved in electron microscope?
2. What are the steps involved in preparation of specimen for observation in the electron microscope?
3. What is the principle for calculation of magnification in SEM?
4. Describe the reaction exploited in SEM and TEM both.
5. How sample prepared for TEM.
6. What are the limitations of TEM?
7. Write short notes on

i. Electron microscope
ii. Electron gun
iii. SEM.
iv Advantages and disadvantages of SEM and TEM

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UNIT 4 CELL SORTING

CONTENTS

1.0 INTRODUCTION

Because the flow cytometer display data from hundreds to thousands of cells that can highlights different population of cells, it has been exploited to separate ultrapure populations of cells from the original, heterogeneous sample and, thus, functions as a cell sorter.

2.0 OBJECTIVE

By the end of this unit you should be able to:

- define and explain cell sorting
- discuss high speed cell sorting
- state protocol for sample preparation
- interprete data analysis.

3.0 MAIN CONTENT

3.1 Cell Sorting

As the name implies, cell sorters have the added benefit of being able to select individual particles of interest and divert them from the fluid stream into a collection vessel. This is attained by introducing a slight vibration on the nozzle to create small waves on the surface of the jet as it emerges from the nozzle, causing it to break into regular droplets downstream of the point of laser intersection. If a given particle is desired, an electric charge is applied to those drops containing the particles of interest. Electrostatic charging occurs at a precise moment called the ‘break-off point', which describes the instant the droplet

containing the particle of interest separates from the stream. The particle-containing drops are deflected in an electric field and collected while the remaining uncharged drops are disposed of. The amount of charge applied will affect the degree of deflection of individual drops, and hence, multiple population of cell can be separated simultaneously by using charge of different polarity and intensity (Figure 19.3). The speed of cell sorting depends on several factors including particle size and the rate of droplet formation. A typical nozzle is between 50-70 μM in diameter, and depending on the jet velocity from it, can produce 30,000-100,000 droplets per second, which is ideal for accurate sorting.

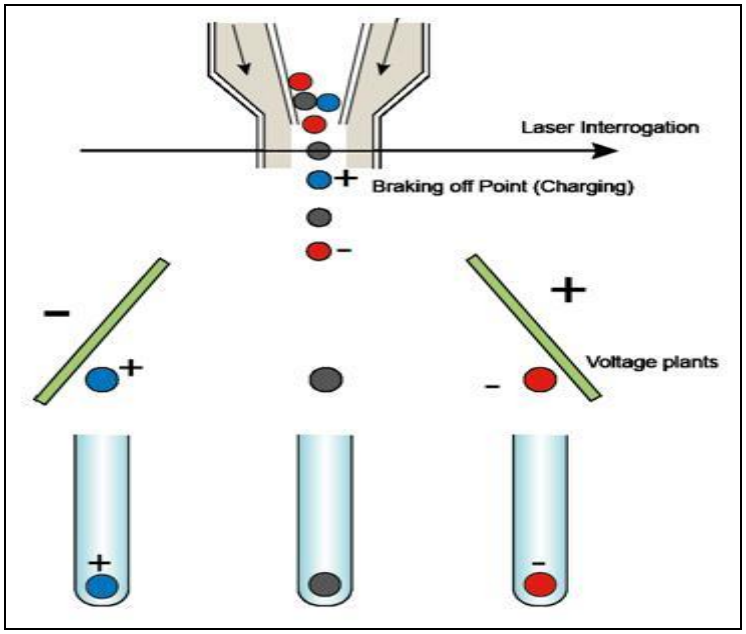

Figure 4.1: Electrostatic flow sorting

3.2 High Speed Cell Sorting

Because each and every particle is analysed individually, the performance of cell sorters is intrinsically low. However, advances in fluidics, optics, computers and the electronics of cell sorters have led to the development of instruments of high speed that can analyse and sort cells at very high rates. It is important not to compromise sort quality and yield when designing a flow cytometer capable of operating at high rates. As particles are illuminated by one or more laser beams, signals that are detected by the photo- detectors are sent to specialised circuits which perform analog to digital conversion, classify the events and issue sort commands for those cells that fall within the specified sort windows. Signals on the order of 1MHz can be easily processed by modern high level data acquisition system and analog to digital convertors and, therefore, do not limit the overall sort process. Modern high speed cytometers uses digital electronic circuitry which have little problem in quantifying and classifying measurement parameters in the

time it takes a cell to cross the laser interrogation point. However, it is unlikely to sort events at a higher rate than that at which sort drops are produced. Therefore, to attain the highest possible sort rate, the speed of drop generation should approximate the speed of electronics.

3.3 Protocol for Sample Preparation

Single cells must be suspended at a density of $10^5 - 10^7$ cells/ml to prevent the narrow bores of the flow cytometer and its tubing from clogging up. Phosphate buffered saline (PBS) is a common suspension buffer for flow cytometry of non-adherent cells. For analysis of cells from solid tissues, the solid material must be disaggregated. In case of woody plant samples, generally the woody plant buffer is used.

Preparation of plant tissue for flow cytometric analysis:

i. About 20 mg of fresh sample, explants like callus, leaves, seeds, were taken in a Petri dish.
ii. Into the Petri dish, 2 - 3 ml of the woody plant buffer was added and the sample was chopped into fine pieces. The quantity of the chopping buffer should be adjusted to the amount of plant material chopped.
iii. After chopping, the suspension was filtered through a 30µm membrane.
iv. Into the filtered sample, 50µl/ml of RNase was added and was mixed well with a Pasteur pipette to accelerate the release of nuclei.
v. About 50µl/ml of propidium iodide was also added and the sample was mixed.
vi. The above steps are to be carried out at 4°C.
vii. The sample prepared can then be introduced into the flow cytometer where it is analysed and evaluated accordingly.

3.4 Data Analysis

The ploidy level in the sample is determined by measuring the amount of nuclear DNA in the plant cells. The flow cytometer automatically, rapidly and accurately makes this determination. The term used to describe ploidy is C-values rather than chromosome number *n* values, and 1C value represents the amount of DNA corresponds to haploid complement (Figures 4.4, 4.5). The C-value information can be obtained from isolated and stained interphase nuclei. The best tissue to analyse the ploidy by flow cytometry is the young leaf tissue. In somatic diploid tissues, nuclei arrested in both G_1 and G_2 stages are usually found representing 2n and 4n nuclei, respectively. To determine the haploid status of plantlets, firstly, the known ploidy of diploid tissue is analysed

and gain values are set to appropriate channel. For example, if the first peak of the diploid tissue represents 2C nuclei (G_1 stage) and appears at 200 channel position, the second peak (G_2 stage) will be appeared at 400 channel position, assuming that a linear scale is used. The apparatus is thus, adjusted using diploid tissue of known origin as external standard. The unknown sample, like haploid tissue, can be analysed under the set conditions; in this case, the first peak would be expected at around channel position 100 and the second peak at around channel position 200.

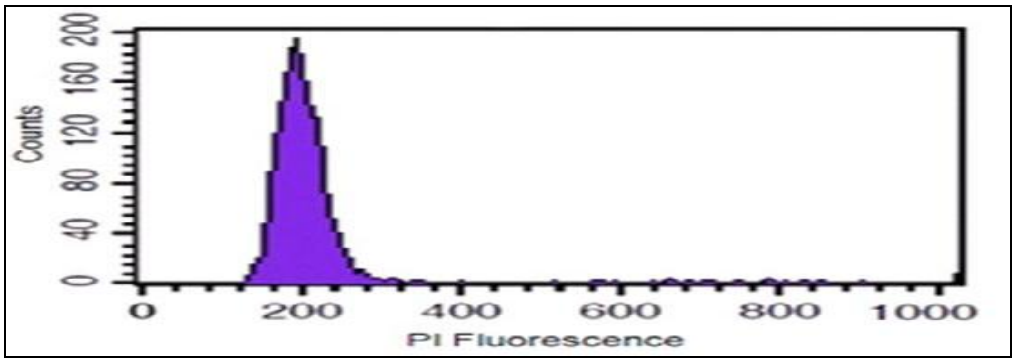

Figure 4.2: Pure diploid sample, showing a peak in channel position 200, as measured by the flow-cytometer

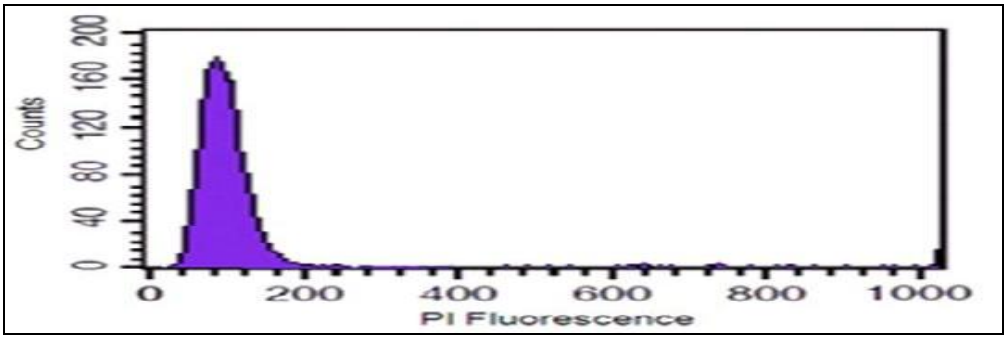

Figure 4.3: Sample showing haploid peak at channel position 100 as detected by the flow cytometer

4.0 CONCLUSION

Cell sorting is a well-established technology that will continue to see expansion of its uses in many areas of clinical and scientific research. Demands of the new biology requires machine to function at higher speed and efficiency and capable of increasing experimental complexity but at the same time should be robust and affordable.

5.0 SUMMARY

From a separation perspective, cell sorting as an indispensable technology, where heterogeneous cells suspensions can be purified into fractions containing a single cell type based upon virtually unlimited combinations of user-defined parameters will lead to a more precise understanding of biology and contribute to our increasingly detailed view of processes at the single cell level.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is the basic principle of flow cytometry?
2. Give some example of fluorophores which are used for labeling nucleic acids in flow cytometry.
3. Describe the hydrodynamic focusing in flow cytometry.
4. What is high speed cell sorting?
5. Describe the steps for plant sample preparation protocol for flow cytometry.
6. If a plant sample is tetraploid and peeks were coming at channel number 400 in flow cytometry. At what channel position peeks will occur for following ploidy levels of same plant.
   a) Haploid plant b) Diploid plant c) Hexaploid plant.

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UNIT 5 PLANT HISTOLOGICAL TECHNIQUES

CONTENTS

1.0 INTRODUCTION

Plant histology is the branch of biology concerned with the composition and structure of plant tissues in relation to their specialized functions. Its aim is to determine how tissues are organized at all structural levels, from cells and intercellular substances to organs. It provides a realistic interpretation of morphology, physiology, and phylogeny of the structure of cells and tissues. A variety of techniques are used for histological studies by using various fixatives, stains, the use of microtome for preparing thin sections, light microscopy, electron microscopy, and X-ray diffraction. To study tissues, the specimen is generally sliced into thin sections then contrast within tissues is induced using dyes, heavy metals, or fluorochromes. Specific staining is obtained by using a dye which has an affinity for a particular cell type or tissue element, or by the use of specific probes, such as labelled antibodies or labelled RNA or DNA probes. The basic requirements of histological studies are listed in Table below

Table 5.1 : Basic equipments and reagents required for histology

Basic Equipments	Basic Reagents
Glass vial	Ethanol
Rotator/shaker	Histoclear
Hotplate	Distilled water
Glass microscope slides	DPX permanent mounting medium
Cover slips	Aqueous mounting medium

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain tissue processing.

3.0 MAIN CONTENT

3.1 Tissue Processing

The aim of tissue processing is to embed the tissue in a solid material firm enough to support the tissue and give it sufficient rigidity to enable thin sections to be cut, and yet soft enough not to damage the knife or tissue.

The five main stages in the preparation of histological slides are:

1. Fixation
2. Dehydration
3. Clearing
4. Sectioning
5. Staining

3.1.1 Fixation

The aim of fixation is to preserve the tissue in a state that most reflect the living cell. Choice of fixative is generally dependent on the tissue of interest as different fixatives better preserve particular tissue elements.
Aim of fixation:

i. To prevent autolysis and bacterial attack.
ii. To fix the tissues so they will not change their volume and shape during processing.
iii. To prepare tissue and leave it in a condition which allow clear staining of sections.
iv. To leave tissue as close as their living state as possible and no small molecules should be lost.

3.1.1.1 Conventional Chemical Fixation

Chemical fixatives are used to preserve tissue from degradation, and to maintain the structure of the cell and of sub-cellular components, such as cell organelles (e.g., nucleus, endoplasmic reticulum, mitochondria). There are many fixatives which have been developed over the years using mixture containing heavy metals or picric acid, but the most

commonly used fixatives for general plant histology are buffered aldehyde and formalin/acid/alcohol mixtures (FAA).
Aldehyde fixatives usually contain a combination of paraformaldehyde and glutaraldehyde: paraformaldehyde rapidly penetrates the tissue while glutaraldehyde gives superior cross linking. Higher concentrations of glutaraldehyde improve morphological preservation but excessive cross linking destroys delicate antigenic sites on proteins. FAA fixes nucleic acids very well but gives poorer morphological preservation and makes the tissue hard and, therefore, more difficult to section.

3.1.1.2 Frozen Section Fixation

Frozen section is the rapid way to fix and mount histological sections. Fresh or fixed tissues are snap-frozen in liquid nitrogen, or with a high-pressure jet of CO_2, and sectioned using a refrigeration device called a cryostat. The frozen tissue is sliced and the frozen slices are mounted on a glass slide and stained. The tissues are infiltrated in 40% sucrose prior to freezing and sectioning to help protect the tissue during freezing.

Advantages of fixation by frozen sections:

- Give better preservation of antigenicity
- Minimal exposure to fixative
- Not exposed to the organic solvents
- Disadvantages of fixation by frozen sections
- Lack morphological detail
- Possibility of biohazard

3.1.2 Dehydration

Dehydration removes fixative and water from the tissue and replaces them with dehydrating fluid. There are a variety of compounds used, many of which are alcohols. To minimise tissue distortion from diffusion currents, delicate specimens are dehydrated in a graded ethanol series from water through 10%-20%-50%-95%-100% ethanol. In the paraffin wax method, following any necessary post fixation treatment, dehydration from aqueous fixatives is usually initiated in 60% -70% ethanol, progressing through 90%-95% ethanol, and then two or three changes of absolute ethanol before proceeding to the clearing stage. Examples of dehydrating agents used are ethanol, methanol, and acetone. The duration of dehydration should be kept to the minimum consistent with the tissues being processed. Tissue blocks 1 mm thick should receive up to 30 minutes in each alcohol, blocks 5 mm thick require up to 90 minutes or longer in each change. Tissues may be held and stored indefinitely in 70% ethanol without harm.

3.1.3 Clearing

Clearing is the process of replacing the dehydrating fluid with another fluid that is totally miscible with both the dehydrating fluid and the embedding medium. The choice of a clearing agent depends upon the following:

i. The type of tissues to be processed and the type of processing to be undertaken
ii. Intended processing conditions, such as temperature, vacuum and pressure
iii. Safety factors
iv. Cost and convenience
v. Speedy removal of dehydrating agent
vi. Ease of removal by molten paraffin wax
vii. Minimal tissue damage.

Commonly used clearing agents include toluene, xylene, chloroform, benzene, etc.

3.1.4. Embedding and Sectioning

After tissues have been dehydrated and before they can be "sectioned" i.e. sliced very thinly, they must be secured in a very hard solid block in such a way that the hardened material secure all parts of the biological tissues and is transparent to the optical method used for viewing the finished samples. Generally, wax, polyethylene glycol (PEG), or resins (e.g. LR white) are used as embedding material for histology. During this process the tissue samples are placed into moulds along with liquid embedding material which is then hardened. Paraffin wax is probably the most commonly used embedding material, being easy to section. It is a polycrystalline mixture containing solid hydrocarbons produced during the refining of coal and mineral oils. It is about two thirds of the density and slightly more elastic than dried protein. Wax and polyethylene glycol (PEG) are removable matrix; PEG is water soluble and requires lower infiltrating temperature than wax. Resins are not easily removable, however, they produce a much harder block and, therefore, thinner sections can be obtained.

Sectioning an embedded tissue sample is the next step necessary to produce sufficiently thin slices of sample that the detail of the microstructure of the cells/tissue can be clearly observed using microscopy techniques (either light microscopy or electron microscopy). Sectioning of embedded tissues depends on the type of microscopy that will be used to observe it and, hence, the thickness of sample required. In the case of samples to be studied using light microscopy, a steel knife

mounted in a microtome may be used to cut 10μm thin sections, which are then mounted on a glass microscope slide. In case of samples to be studied using transmission electron microscopy, a diamond knife mounted in an ultramicrotome may be used to cut 50 nm thin sections, which are then mounted on a 3-millimeter-diameter copper grid. A microtome is a mechanical device utilised to slice biological specimens into very thin segments for microscopic examination. Most microtomes use a steel blade and are used to prepare sections of plant tissues for histology.

3.1.5 Staining

Most biological tissues have very little contrast, and cellular details are hard to discern with the ordinary light microscope. Staining is employed to give both contrasts to the tissue as well as highlighting particular features of interest. Plant histologists have been staining tissues with natural dyes but today most dyes are synthetic. Dyes are extensively used in histology as they can enhance and improve the visibility of the specimen and often have an affinity for a specific tissue element, allowing quick and easy identification of specific cell types and cellular components. Although some dyes can be used on their own to stain tissues, specific dyes are often used with a counter stain to contrast various components within the tissues. Prior to staining, it is necessary to de-wax sections; resin sections can be stained directly.

Tolonium chloride (also known as toluidine blue) is a cationic dye that binds to negatively charged groups. An aqueous solution of this dye is blue, but different colors are generated when the dye binds with different anionic groups in the cell. For example, a pinkish purple color will appear when the dye reacts with carboxylated polysaccharides, such as pectic acid; green, greenish blue or bright blue with polyphenolic substances, such as lignin and tannins; and purplish or greenish blue with nucleic acids. Acridine orange is a nucleic acid selective fluorescent cationic dye that interacts with DNA and RNA by intercalation and electrostatic attractions, respectively. It is useful as a non-specific stain for backlighting conventionally stained cells. However, extreme care should be taken when using Acridine orange as it is carcinogenic. Haematoxylin is extracted from the heartwood of the logwood tree. It is one of the most commonly used stains in histology. It is generally utilized with a second counter stain, where haematoxylin as the primary stain for nuclei and contrasting or counterstaining with Orange G, safranin, or Fast Green.

3.1.5.I Cell Wall Stains

Although cell walls can be visualised using general tissue stains such as Toluidine blue, there are a number of quick and simple methods which can be used to specifically stain individual cell wall components, such as pectin using Ruthenium red, cellulose using Calcofluor, lignin using Phloroglucinol or safranin and callose using Aniline blue, Resorcinol blue or Astra-blue.

3.1.5. ii Carbohydrates and Starch Stains

Total carbohydrates can be stained in tissue sections using the periodic acid Schiff's or PAS technique. The classic stain for starch uses iodine in potassium iodide.

3.1.5.iii Stains for Cell Viability

There are a number of fluorescent stains for assessing cell viability, however, the two fluorochromes, fluorescein diacetate (FDA) and propidium iodide are commonly used to detect living and dead cell, respectively, in tissues or cell suspensions. FDA is taken up by live cells and de-esterified to fluorescein, which fluoresces green with blue excitation. Conversely, propidium iodide is taken up by damaged or dead cells which fluoresces red with green excitation.

3.1.5.iv Stains for Microorganisms in Plant Tissues

Bacteria are particularly difficult to detect with the light microscope as high magnification and high resolution optics are required. Fungi are generally detected in plant tissues using the dyes methyl blue or thionin. Bacteria can be detected using Gram staining technique. The Gram stain is widely performed on dried, heat fixed smears and gives blue stain with Gram positive microorganisms and red stain with Gram negative microorganisms.

3.1.5.v Nucleic Acid Stains

The earliest method to stain nucleic acid (both DNA and RNA) in section is by using acridine orange, but this requires a fluorescence microscope. For bright field optics, methyl green-pyronin method is used. However, background pyronin staining is often a problem. DNA in nuclei, mitochondria and chloroplasts can be stained using DAPI (4',6-diamidino-2-phenylindole), Hoechst 33258, Hoechst 33342 or propidium iodide.

3.1.5.vi Lipid Stains

Dyes like Sudan IV or Sudan black are generally used for staining of lipids. Nile blue is used preferentially to stain acid lipids, like phospholipids and works best on fresh tissues.

4.0 CONCLUSION

To study tissues, the specimen is generally sliced into thin sections then contrast within tissues is induced using dyes, heavy metals, or fluorochromes. Specific staining is obtained by using a dye which has an affinity for a particular cell type or tissue element, or by the use of specific probes, such as labelled antibodies or labelled RNA or DNA probes.

5.0 SUMMARY

Plant histology is the branch of biology concerned with the composition and structure of plant tissues in relation to their specialised functions. Its aim is to determine how tissues are organised at all structural levels, from cells and intercellular substances to organs. It provides a realistic interpretation of morphology, physiology, and phylogeny of the structure of cells and tissues. A variety of techniques are used for histological studies by using various fixatives, stains, the use of microtome for preparing thin sections, light microscopy, electron microscopy, and X-ray diffraction.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is plant histology?
2. What do you mean by tissue processing?
3. Why fixation required for histology?
4. What are the advantages and disadvantages of fixation by frozen sections?
5. Give the name of stains used for staining of following:
   A. Cell wall
   B. Carbohydrate and starch
   C. Cell viability test
   D. Microorganism in plant tissues
   E. Nucleic acid
   F. Lipid.

Write short notes on:

A. Fixation
B. Dehydration
C. Clearing

D. Sectioning
E. Staining.

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MODULE 4 INTRODUCTION TO PLANT GENETIC ENGINEERING

UNIT 1 GENETIC MATERIAL OF PLANT CELLS

CONTENTS

1.0 INTRODUCTION

What is genetic material?

The research at the end of the 19th century had verified Mendelian inheritance and it was also believed that the genetic material is in the chromosome. However, scientists still didn't know the true features of the genetic material. In the early twentieth century, biologists believed that proteins carried genetic information. But the Griffith experiment with *Streptococcus pneumonia* (1928), Avery, MacLeod and McCarty experiment (1944) on transforming principle and Hershey-Chase experiment (1952) on bacteriophage T2, confirms that DNA is genetic material. Genetic material is the material that determines the inherited characteristics of a functional organism. It has the following properties:

- It must be stable
- It must be capable of being expressed when needed
- It must be capable of accurate replication
- It must be transmitted from parent to progeny without change

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain what genetic material is.
- explain the transforming principle
- discuss DNA as the transforming principle
- know meaning of Hershey and chase experiment
- define DNA
- understand chloroplast genome.

3.0 MAIN CONTENT

3.1 The Transforming Principle

In the year 1928, Griffith used two types of strains of pneumonia-causing bacterium, *Streptococcus pneumoniae*, using mice for his experiment. One was S-type (smooth) strain having a polysaccharide coat and produces smooth, shiny colonies on a lab plate. The polysaccharide coat of S-type makes it resistant to the immune system of mice. The other strain, R-type (rough) strain, lacks the coat and produces colonies that look rough and irregular. The R-type lacks the polysaccharide coat and thus it will be destroyed by the immune system of the host. Griffith discovered that there was 'something' that causes the convertion of the R-strain to virulent S- strain.

In the first stage of the Griffith's experiment, he showed that when mouse was injected with S-type strain, mouse died of pneumonia but when injected with R-type strain, mouse lived. The next stage showed that if heat-killed S-type strain was injected to mouse, all mice lived, and this result suggested that the bacteria had been rendered ineffective. The attractive results came with the third part of the experiment, where mice were injected with a mixture of heat-killed S-type strain and non-virulent R-type strains; interestingly all mice developed pneumonia and died. (Figure 1.1). In their blood, Griffith found live bacteria of the deadly S- type. The S strain extract somehow had "transformed" the R-strain bacteria to S- form.

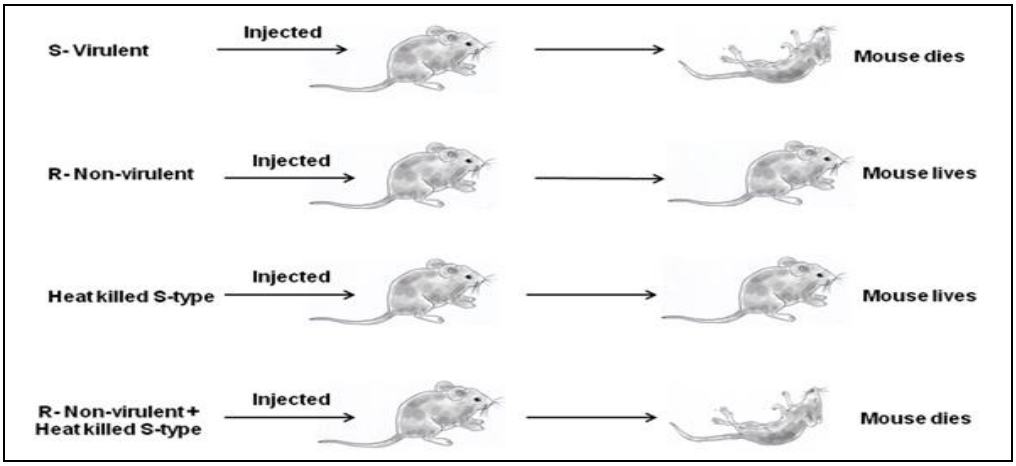

Figure 1.1: Griffith's experiment discovering the "transforming principle" in *Pneumococcus* bacteria

3.2 DNA as the Transforming Principle

Bacteriologists were interested in the difference between the two strains of Streptococci that Frederick Griffith had identified in 1928. The bacteriologists suspected the transforming factor was some kind of protein. The transforming principle could be involved with alcohol, which showed that it was not a carbohydrate, like the polysaccharide coat itself. But Avery and MacCarty observed that proteases, enzymes that degrade proteins, did not destroy the transforming principle. Neither did lipases, enzymes that digest lipids. They found that the transforming substance was rich in nucleic acids, but ribonuclease, which digests RNA, did not inactivate the substance. Avery and McCarty also found that the transforming principle had a high molecular weight. In 1944, Oswald Avery, Colin MacLeod and MaclynMacCarty showed in their experiments (Figure 1.2) that DNA (not proteins) can transform the properties of cells. They had isolated DNA which was the agent to produce an enduring, heritable change in an organism. Thus, clarifying the chemical nature of the genes and proving that DNA as the "transforming principle" while studying *Streptococcus pneumonia*, bacteria that can cause pneumonia. Until then, biochemists had assumed that deoxyribonucleic acid was a relatively unimportant, structural chemical in chromosomes and that protein, with their greater chemical complexity, transmitted genetic traits.

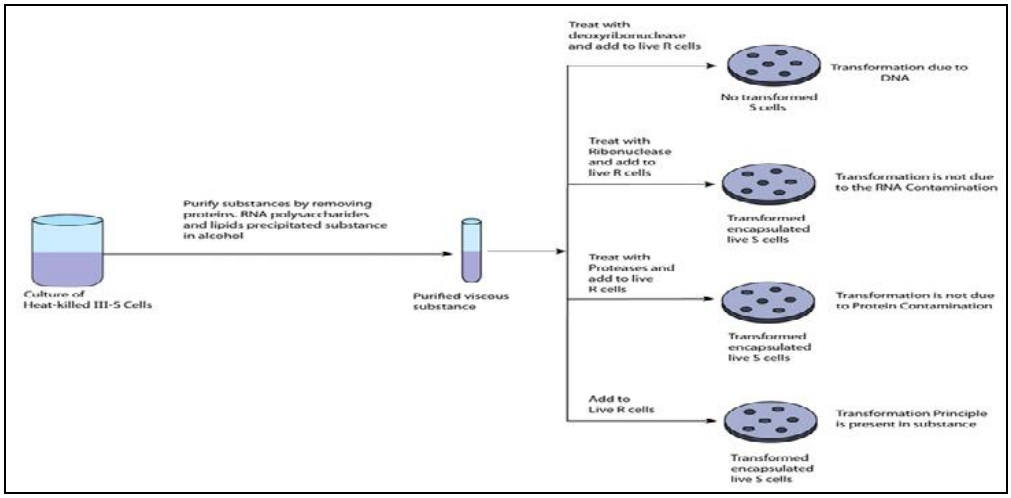

Figure 1.2: Avery, Macleod and McCarty's experiment demonstrating the Griffith's transformation

3.3 Hershey and Chase Experiment

Hershey and Chase in their experiments used the T2 bacteriophage as the vehicle for delivering genetic material. Like all bacterial viruses, T2 is comprised of only a protein-based outer wall and a DNA core, its simple structure making it the perfect research candidate. The virus protein contains sulfur but no phosphorus and the virus DNA contain phosphorus but no sulfur. They tagged the T2 phage DNA with radioactive phosphorous (P^{32}) and proteins with radioactive sulfur (S^{35}). The researchers could track the location of DNA and protein according to the radiation concentrations. Then they allowed the tagged phages for infection to *E.coli*. After introducing phage culture to the bacterial sample, it was agitated in blender to brutally disturb the infected bacteria, causing the protein shells to detach from their hosts. Then, for separation of bacterium from the phages and proteins, they used a centrifuge. Once the separation was complete, they measured the radiation concentrations in the *E.coli* cells and the protein shells. The most of the P^{32} label appeared in large quantities within the bacterial sample, demonstrating that DNA was transferred from the bacteriophage to the host organism whereas most of the S^{35} label had remained outside of the cells. Further, despite the protein shells were detached, reproduction of the phage was taking place and the virus was still copied in each of the host cells. This suggested that the proteins shell itself was not necessary to the replication process following the initial insertion of genetic material Figure 1.3.

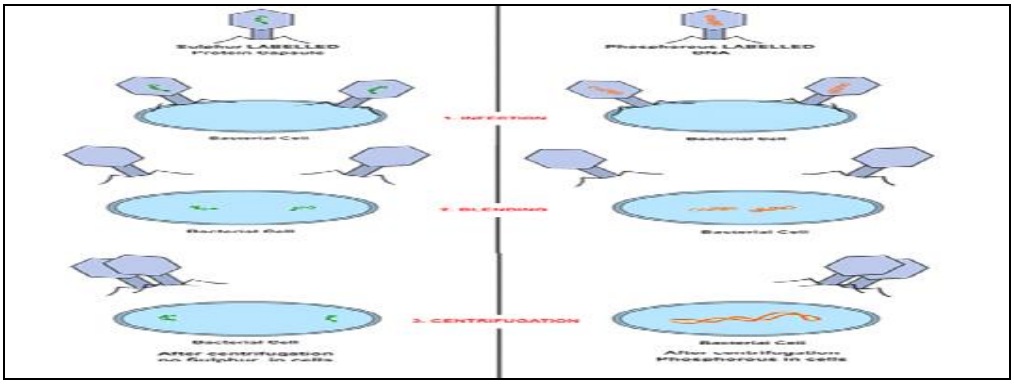

Figure 1.3: Hersey and Chase's experiment with T2 Bacteriophase in *E. coili*

So from previous explanation we can define the genetic material "*The genetic material of a cell or a plant refers to those materials found in the nucleus, mitochondria and chloroplast, which play a fundamental role in determining the structure and nature of cell substances, and capable of self-propagating and variation. It can be a gene, a part of a gene, a group of genes, a DNA molecule, a fragment of DNA, a group of DNA molecules, or the entire genome of an organism*."

3.4 DNA

Deoxyribonucleic Acid, known as DNA, is the genetic material found in the cells of nearly all living organisms. DNA is the fundamental building blocks of life. Nearly every cell (with a nucleus) in a person's body has the same DNA. Most DNA is located in the cell nucleus (nuclear DNA), but DNA can also be found in the mitochondria (mitochondrial DNA or mt-DNA) and in chloroplast (chloroplast DNA or ctDNA). In 1929 Phoebus Levene at the Rockefeller Institute identified the components that make up a DNA Molecule. The information in DNA is made up of four bases which combine to form chains. These bases include two purines (Adenine and Guanine) and two pyrimidines (Cytosine and Thymine). These are commonly referred to as A, G, C and T, respectively. Each base is attached to a Sugar (S) molecule and a Phosphate (P) molecule (Figure 1.4.A-C). Sugar and phosphate are back bone of nucleotides (Figure 1.5 A). Together; a base and a sugar are called a nucleoside (Figure 1.5B)**.** Together, a base, sugar, and phosphate are called a nucleotide (Figure 1.5C). Nucleotides are arranged in two long strands that form a spiral called a double helix (Figure 1.6 A). The structure of the double helix is somewhat like a ladder, with the base pairs (Figure 1.6B) forming the ladder's rungs and the sugar and phosphate molecules forming the vertical side pieces of the ladder. He showed that the components of DNA were linked in the order phosphate-sugar-base. He called each of these units a nucleotide and suggested the DNA molecule consisted of a string of nucleotide

units linked together through the phosphate groups, which are the 'backbone' of the molecule.

However,Levene thought the chain was short and that the bases repeated in the same fixed order. TorbjornCaspersson and Einar Hammersten showed that DNA was a polymer. This was only accepted after the structure of DNA was elucidated by James D. Watson and Francis Crick in their 1953 Nature publication. Watson and Crick proposed the central dogma of molecular biology in 1957, describing the process whereby proteins are produced from nucleic DNA. In 1962 Watson, Crick, and Maurice Wilkins jointly received the Nobel Prize for their determination of the structure of DNA. The number of purine bases in DNA is equal to the number of pyrimidines. This is due to the law of complimentary base pairing; where Thymine (T) can only pair with Adenine (A), and Guanine (G) can only pair with Cytosine (C). Knowing this rule, we could predict the base sequence of one DNA strand if we knew the sequence of bases in the complimentary strand.

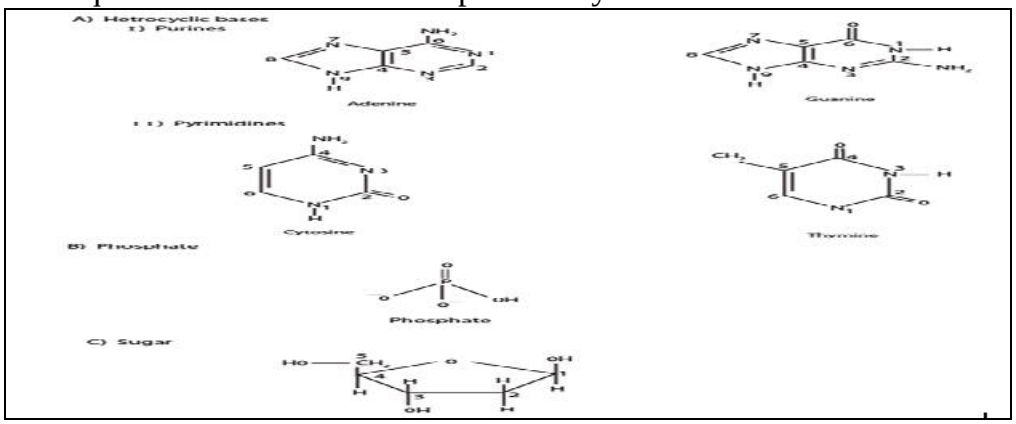

Figure 1.4: Components of nucleotides and nucleic acids

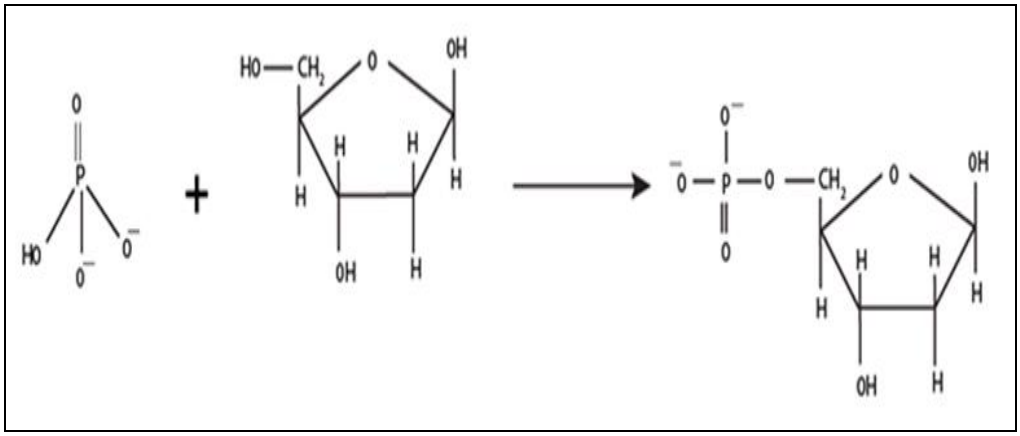

Figure 1.5A: Sugar phosphate backbone of common nucleotides

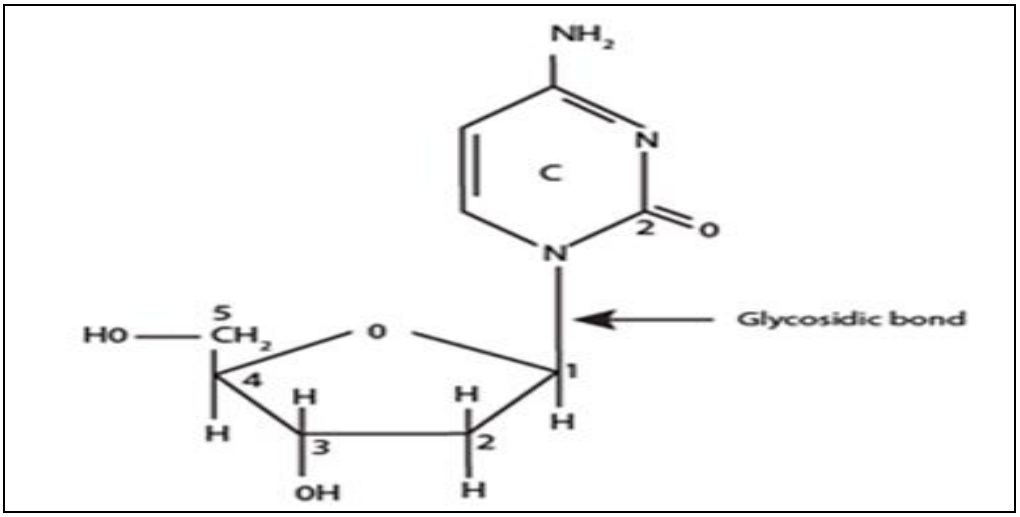

Figure 1.5B: Nucleosides (C=Cytosine)

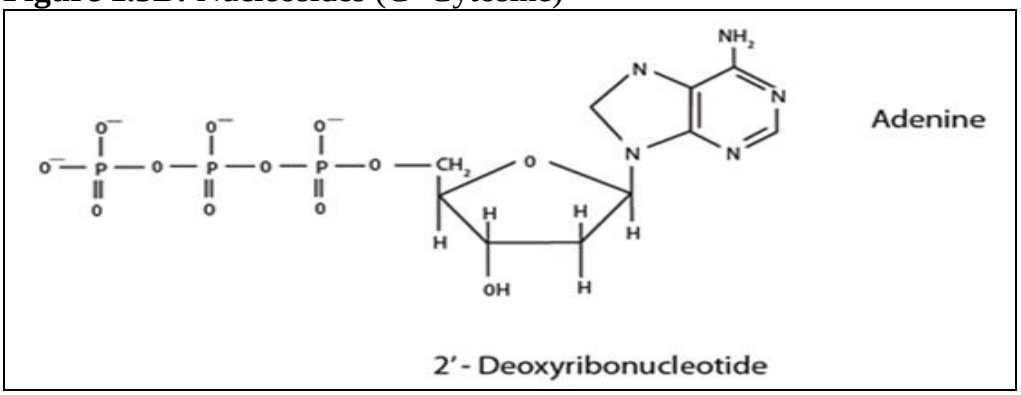

Figure 1.5C: Nucleotides

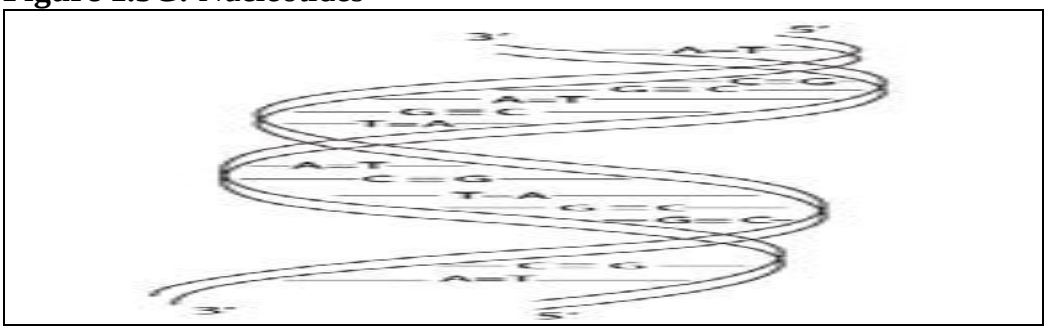

Figure 1.6A: DNA double helix structure

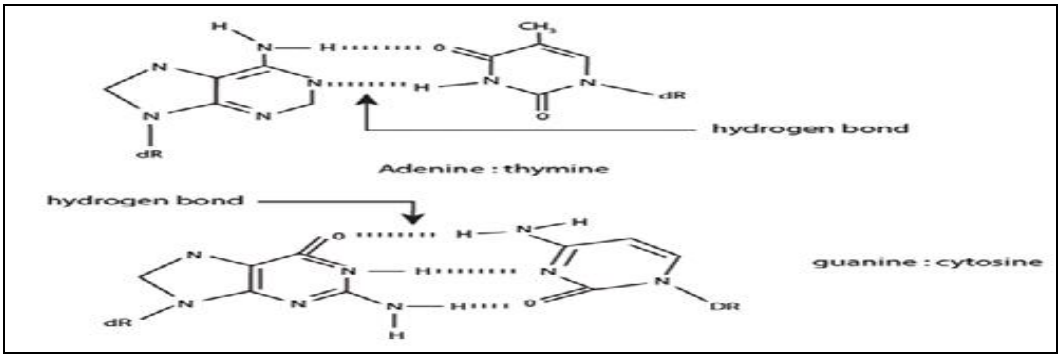

Figure 1.6B: DNA Base pairing by hydrogen bond

The endosymbiotic theory concerns the origins of mitochondria and plastids (e.g. chloroplasts), which are organelles of eukaryotic cells. According to this theory, these organelles originated as separate prokaryotic organisms that were taken inside the cell as endosymbionts. Mitochondria developed from proteobacteria (in particular, Rickettsiales or close relatives) and chloroplasts from cyanobacteria. Mitochondrial and chloroplast genomes do not contain a full set of housekeeping genes, and lack many that other descendants of their speculative ancestors share, there must have been a loss of genes. However, some of these genes likely migrated to the nucleus, where analogues of these genes are now found.

3.5 Chloroplast Genome

The chloroplast is the green plastid in land plants, algae and some protists. As the site in the cell where photosynthesis takes place, chloroplasts are responsible for much of the world's primary productivity, making chloroplasts essential to the lives of plants and animals alike. Agriculture, animal farming, and fossil fuels such as coal and oil are all "products" of photosynthesis that took place in chloroplasts. Other important activities that occur in chloroplasts (and several non-photosynthetic plastid types) include the production of starch, certain amino acids and lipids, some of the colorful pigments in flowers, and some key aspects of sulfur and nitrogen metabolism. The interactions between plastid and nuclear encoded transcription and translation process is elaborated in Figure 21.7.
All plastids considered to date contain their own DNA, which is actually a reduced "genome" derived from a cyanobacterial ancestor that was captured early in the evolution of the eukaryotic cell. The chloroplast genome encodes for all the rRNA & tRNA species required for protein synthesis. The ribosomes contain two small rRNAs in addition to the major species. The chloroplast genome codes for ~50 proteins, including RNA polymerase & some ribosomal proteins. Again the rule is that organelle genes are transcribed & translated the apparatus of the organelle. The chloroplast genome of the higher plants varies in length, but displays a characteristic landmark. It has a lengthening sequence, 10-24kb depending on the plants, that is present in two identical copies as an inverted repeat (Gene that are coded within the inverted repeats are present in two copies per genome & include the rRNA genes).

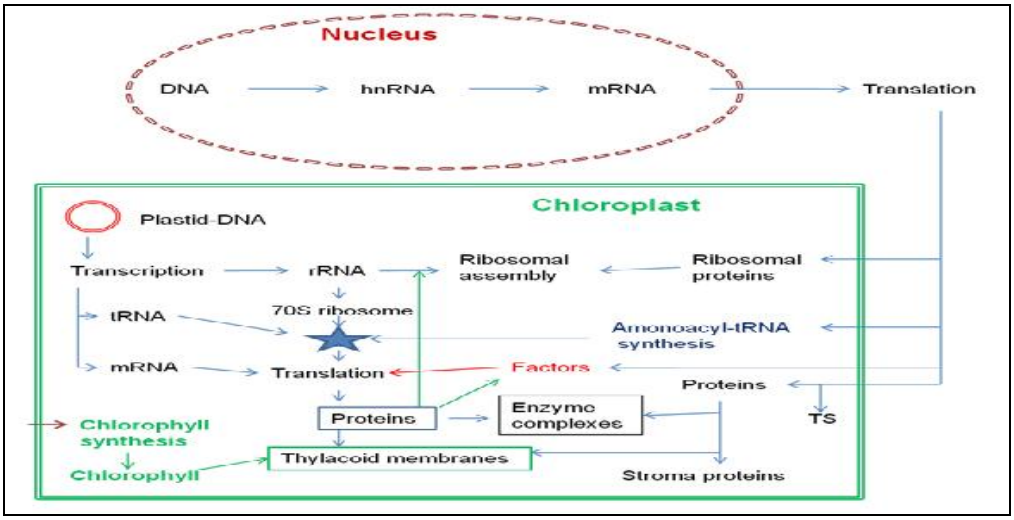

Figure 1.7: Model of the interactions between plastid and nuclear encoded transcription and translation products. TS: transit sequence: a N-terminal section of the polypeptide chain, essential for the penetration of the polypeptide across the membrane, subsequently being cleaved off proteolytically.

3.6 Chloroplast Genome

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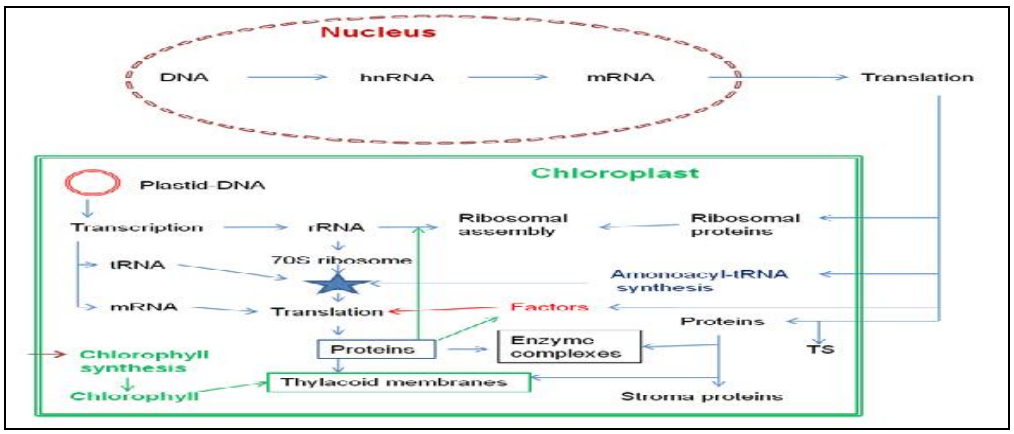

Figure 1.7: Model of the interactions between plastid and nuclear encoded transcription and translation products. TS: transit sequence: a N-terminal section of the polypeptide chain, essential for the penetration of the polypeptide across the membrane, subsequently being cleaved off proteolytically.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) is DNA that is present in Mitochondria. Mitochondrion is the part of organic cells that produce most of the cellular energy by converting organic materials mode into Adenosine Tri-phosphate (ATP) mode via the process of oxidative phosphorylation. The details of mitochondrial functions are elaborated in Figure 1.8. Typically, nuclear DNA determines the function of a cell; however, mitochondria have their own DNA and are assumed to have evolved separately (Endosymbiotic theory). Mitochondria have their own genome, usually multiple copies in one mitochondrion, in circular form, located in several nucleoid regions, with no histone association (naked). Mitochondrial genome size varies with organism to organism, plants have mitochondrial average 150-200 kb, but human mitochondria genome is only 16 kb. Mitochondrial DNA encodes enzymes required for oxidative phosphorylation and mitochondrial electron transfer. A cell can have different types of mitochondria (heteroplasmy) or same type of mitochondria (homoplasmy). Mitochondrial DNA analysis is helpful in forensic cases in which nuclear DNA is insufficient for short tandem repeat (STR) typing. Shed body, head, and pubic hairs with no cellular material (hair follicle) attached to the root bulb and aged skeletal remains are the samples most commonly analyzed for mtDNA because nuclear DNA is not recoverable from these tissues. Usually a cell has hundreds or thousands of mitochondria which can occupy up to 25% of the cell's cytoplasm, and each mitochondrion contains 1-10 mtDNA molecules. The high copy number of mtDNA molecules found in each cell is one reason why mtDNA is recoverable from hairs and old skeletal remains.

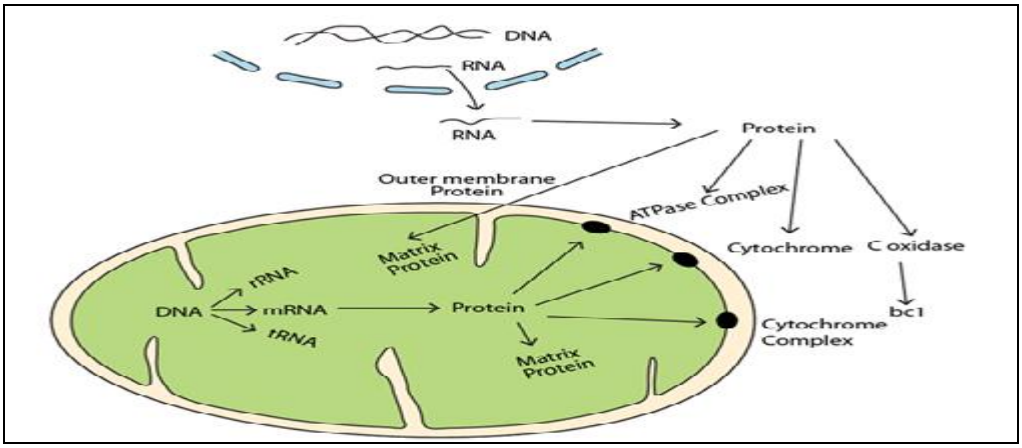

Figure 1.8: Mitochondrial genome functions

4.0 CONCLUSION

The research at the end of the 19th century had verified Mendelian inheritance and it was also believed that the genetic material is in the chromosome. However, scientists still didn't know the true features of the genetic material. In the early twentieth century, biologists believed that proteins carried genetic information. But the Griffith experiment with *Streptococcus pneumonia* (1928), Avery, MacLeod and McCarty experiment (1944) on transforming principle and Hershey-Chase experiment (1952) on bacteriophage T2, confirms that DNA is genetic material.

5.0 SUMMARY

Genetic material is the material that determines the inherited characteristics of a functional organism. It has the following properties:

- It must be stable
- It must be capable of being expressed when needed
- It must be capable of accurate replication
- It must be transmitted from parent to progeny without change.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is a genetic material?
2. What is transformation?
3. Why DNA is considered as genetic materials?
4. What are the components of DNA?
5. Write short notes on:
   A. Griffith's experiment
   B. Oswald Avery, Colin MacLeod and Maclyn McCarty experiment
   C. Hershey and Chase experiment

D. Nucleosides
E. DNA double helix

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UNIT 2 MODE OF GENE DELIVERY IN PLANT

CONTENTS

1.0 INTRODUCTION

Different systems are now available for gene transfer and successive regeneration of transgenic plants and the most common being *Agrobacterium* -mediated transformation. However, the preferred host of *Agrobacterium* is the dicot plants and its efficiency to transfer genes in monocots is still unsatisfactory. The alternative to this, is the introduction of DNA into plants cells without the involvement of a biological agent like, *Agrobacterium* , and leading to stable transformation is known as direct gene transfer. The most often applied direct methods are microprojectile bombardment or protoplast transformation.

The direct DNA transfer methods have been subdivided into three categories:

1. Physical gene transfer method
2. Chemical gene transfer method
3. DNA imbibitions by cell, tissue and organ.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain physical gene transfer method
- explain chemical gene transfer method
- discuss direct gene transformation through imbibition.

3.0 MAIN CONTENT

3.1 Physical Gene Transfer Method

3.1.1 Particle Bombardment

The Particle bombardment device, well known as the gene gun, was developed to enable penetration of the cell wall so that genetic material containing a gene of interest can be transferred into the cell. This physical direct gene transfer method, gene gun (Figure 2.1) is used for genetic transformation of several organisms to introduce a diverse range of desirable traits. Plant transformation using particle bombardment follows the same steps as in *Agrobacterium* mediated transformation method:

i. Isolation of desired genes from the source organism
ii. To develop a functional transgenic construct including the selected gene of interest; promoters to drive expression; modification of codon, if needed, to increase successful protein production; and marker genes to facilitate tracking of the introduced genes in the host plant
iii. Insertion of transgenic construct into a useful plasmid
iv. Introduce the transgenes into plant cells
v. Regenerate the plants cells, and
vi. Test the performance of traits or gene expression under *in vitro*, greenhouse and field conditions.

Figure 2.1: A gene gun apparatus

In particle bombardment method, 1-2 µm tungsten or gold particles (called micro-projectiles) coated with genetically engineered DNA are accelerated with air pressure at high velocities and shot into plant tissues on a Petri-plate, as shown in Figure 2.2. This is the second most widely used method, after *Agrobacterium* mediated transformation, for plant

genetic transformation. The device accelerates particles in one of the two ways: (1) by means of pressurized helium gas or (2) by the electrostatic energy released by a droplet of water exposed to high voltage. The earlier devices used blank cartridges in a modified firing mechanism to provide the energy for particle acceleration, and thus, the name particle gun. It is also called Biolistics, Ballistics or Bioblaster).
The microcarriers (or microprojectiles), the tungsten or gold particles coated with DNA, are carried by macrocarriers (macro projectiles) which are then inserted into the apparatus and pushed downward at high velocities. The Macro-projectile is stopped by a perforated plate, while allowing the microprojectiles to propelled at a high speed into the plant cells on the other side. As the micro-projectiles enter the plant cells, the transgenes are free from the particle surface and may inserted into the chromosomal DNA of the plant cells. Selectable markers help in identifying those cells that take up the transgene or are transformed. The transformed plant cells are then regenerated and developed into whole plants by using tissue culture technique.

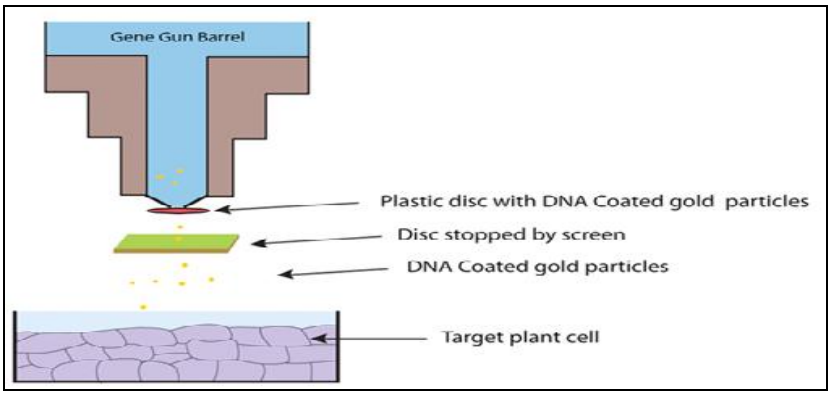

Figure 2.2: Diagrammatic illustration of gene transfer using Gene Gun method

The technique has many advantages and can be used to deliver DNA into virtually all the tissues, like immature and mature embryos, shoot-apical meristem, leaves, roots etc. Particle bombardment methods are also useful in the transformation of organelles, such as chloroplasts, which enables engineering of organelle-encoded herbicide or pesticide resistance in crop plants and to study photosynthetic processes.
Limitations to the particle bombardment method, compared to *Agrobacterium*-mediated transformation, include frequent incorporation of multiple copies of the transgene at a single insertion site, rearrangement of the inserted genes, and insertion of the transgene at multiple insertion sites. These multiple copies can be associated with silencing of the transgene in subsequent progeny. The target tissue may often get damaged due to lack of control of bombardment velocity.

3.1.2 Electroporation

Electroporation is another popular physical method for introducing new genes directly into the protoplasts. In this method, electric field is playing important role. Due to the electric field protoplast get temporarily permeable to DNA. In electroporation, plant cell protoplasts are kept in an ionic solution containing the vector DNA in a small chamber that has electrodes at opposite ends. A pulse of high voltage is applied to the electrode which makes the transient pores (ca. 30 nm) in the plasma membrane, allowing the DNA to diffuse into the cell (Figure 2.3). Immediately, the membrane reseals. If appropriately treated, the cells can regenerate cell wall, divide to form callus and, finally, regenerate complete plants in suitable medium. The critical part of the procedure is to determine conditions which produce pores that are sufficiently large and remain open long enough to allow for DNA diffusion. At the same time, the conditions should make pores that are temporary. With a 1 cm gap between the electrodes and protoplasts of 40-44µm diameter, 1-1.5 kVcm $^{-2}$ of field strength for 10µs is required for efficient introduction of DNA. It was seen that presence of 13% PEG (added after DNA) during electroporation significantly raised the transformation frequency. The other factors which may improve the transformation frequency by electroporation are linearizing of plasmid, use of carrier DNA, and heat shock (45 ~ for 5 min) prior to addition of vector, and placing on ice after pulsing. Under optimal conditions transformation frequencies of up to 2% have been reported. Stably transformed cell lines and full plants of a number of cereals have been produced through electroporation.

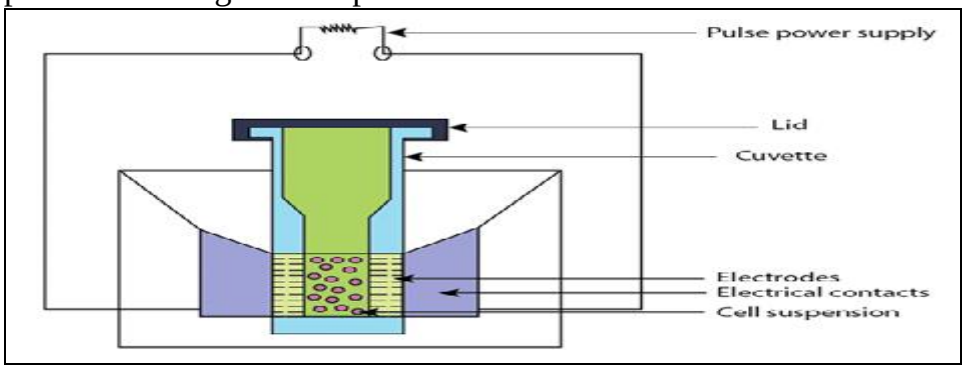

Figure 2.3: Electroporation

There are some parameters that can be considered when performing *in vitro* electroporation:

1. Cell size

Cell size is inversely correlated to the size of the external field needed to generate permeabilisation. Consequently, optimisation for each cell type is essential. Likewise, cell orientation matters for cells that are not spherical.

2. Temperature
It has been observed that plant membrane resealing is effectively temperature dependent and shows slow closure at low temperatures. For DNA transfer, it has been found that cooling at the time of permeabilisation and subsequent heating in incubator increases transfer efficacy and cell viability.

3. Post-pulse manipulation
Cells are susceptible when in the permeabilised state, and it has been shown that waiting for 15min after electroporation in order to allow resealing before pipetting cells, increases cell viability.

4. Composition of electrodes and pulsing medium
For short pulses is needed for release of metal from the standard aluminium electrodes used in standard disposable cuvettes. Some authors advocate the use of low conductivity or more resistance media for DNA transfer in order to increase viability and increase transfection efficacy.

3.1. 3 Microinjection

The microinjection technique is a direct physical approach to inject DNA directly into the plant protoplasts or cells (specifically into the nucleus or cytoplasm) using fine tipped (0.5-1.0 µm diameter) capillary glass needle or micropipettes. Through microinjection technique, the desired gene introduce into large cells, such as oocytes, eggs, and the cells of early embryo (Figure 2.4).

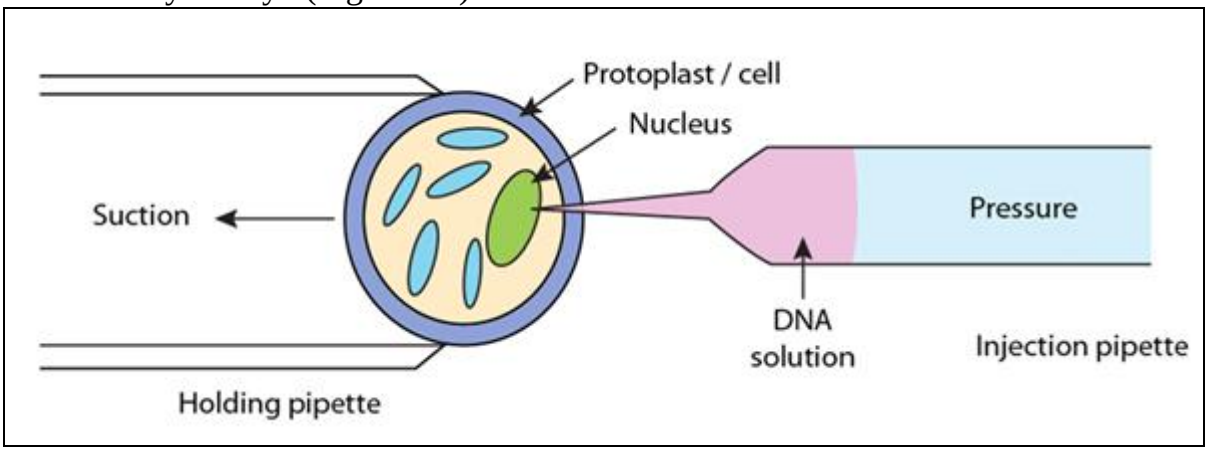

Figure 2.4: Microinjection

3.1.4 Liposome-Mediated Transformation

The idea of a method of direct plant transformation elaborated in the middle eighties was to introduce DNA into the cell by means of liposomes. Liposomes are microscopic spherical vesicles that form

when phospholipids are hydrated. Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids. Liposomes encapsulate the DNA fragments and then adhere to the cell membranes and fuse with them to transfer DNA fragments. Thus, the DNA enters the cell and then to the nucleus. Lipofection is a very efficient technique used to transfer genes in bacterial, animal and plant cells. They can be loaded with a great variety of molecules, including DNA. In the case of protoplasts, the transfection (lipofection) occurs through the membrane fusion and endocytosis. When pollen grains are transformed, liposomes are delivered inside through pores. The efficiency of bioactive-beads-mediated plant transformation was improved using DNA-lipofection complex as the entrapped genetic material instead of naked DNA used in the conventional method. Liposome-mediated transformation is far from routine, in spite of the low expense and equipment requirement. A probable reason is its laboriousness and low efficiency. Only few reports on the integration of genes introduced by means of liposomes followed by transgenic plant regeneration for tobacco and wheat have been published thus far.

3.1.5 Silicon Carbide Fiber Mediated Transformation (SCMT)

SCMT is one of the least complicated methods of plant transformation. Silicon carbide fibers are simply added to a suspension containing plant tissue (cell clusters, immature embryos, callus) and plasmid DNA, and then mixed in a vortex, or in other laboratory apparatus such as, commercial shakers, blenders etc. DNA-coated fibers penetrate the cell wall in the presence of small holes created in collisions between the plant cells and fibers. The most often used fibers in this procedure are single crystals of silica organic minerals like, siliconcarbide, which have an elongated shape, a length of 10–80 mm, and a diameter of 0.6 mm, and which show a high resistance to expandability. Fiber size, the parameters of vortexing, the shape of the vessels used, the plant material and the characteristics of the plant cells, especially the thickness of the cell wall are the factors depending on the efficiency of SCMT. There are several known examples of deriving transgenic forms, cell colonies or plants in maize, rice, Wheat, bacco, *Loliummultiflorum , Loliumperenne, Festuca arundinacea , and Agrostis stolonifera* by SCMT.
SCMT is an easy, fast and inexpensive procedure. Therefore, it could be an attractive alternative method of plant transformation in particular situations, e.g. when a gene gun is not available and *Agrobacterium* - mediated transformation is difficult or not possible (as in the case of numerous monocots). The other advantages of the SCF-mediated method over other procedures include the ability to transform walled cells, thus, avoiding protoplast isolation.

The main disadvantages of this method are low transformation efficiency, damage to cells, thus, negatively influencing their further regeneration capability. Another disadvantage is that silicon fibers have similar properties to asbestos fibers and care must be taken when working with them as breathing the fibers can lead to serious sicknesses. Silicon carbide has some carcinogenic properties as well.

3.1.6 The Pollen-Tube Pathway Method

The transformation method via pollen-tube pathway has great function in agriculture molecular breeding. Foreign DNA can be applied to cut styles shortly after pollination. The DNA reaches the ovule by flowing down the pollen-tube. This procedure, the so-called pollen-tube pathway (PTP), was applied first time for the transformation of rice. The authors obtained transgenic plants at remarkably high frequency. Afterward PTP was used for other species e.g. wheat, soybean, *Petunia hybrida* and watermelon. A bacterial inoculum or plasmid DNA can also be injected into inflorescence with pollen mother cells in the pre- meiotic stage without removing the stigma. In that case, it is expected that foreign DNA will be integrated with the gamete genome. Such an approach has been employed for rye. Pollen collected from inflorescences injected with a suspension of genetically engineered *A. tumefaciens* strain was predestined for the pollination of the emasculated spikes of the maternal plant. But the transformation efficiency was about 10-fold lower than that approximately reached for this species via microprojectile bombardment. Shou et al. (2002) also reported they were unable to reproduce the pollen-tube pathway transformation for delivering plasmid DNA into soybean. They concluded that the pollen-tube pathway transformation in cotton and soybean was not reproducible. This might have been because of the manipulation of transformation, the growth stage of plants, the effects of environment and weather

3.2. Chemical Gene Transfer Method

This involves plasma membrane destabilizing and/or precipitating agents. Protoplasts are mainly used which are incubated with DNA in buffers containing PEG, poly L-ornithine, polyvinyl alcohol or divalent ions. The chemical transformation techniques work for a broadspectrum of plants.

3.2.1 Polybrene–Spermidine Treatment

The combination polybrene–spermidine treatment greatly enhanced the uptake and expression of DNA and, hence, the recovery of nonchimeric germline transgenic cotton plants. The major advantages of using the

polybrene–spermidine treatment for plant genetic transformation are that polybrene is less toxic than the other polycations; spermidine protects DNA from shearing because of its condensation effect; and because no carrier DNA is used, and the integration of plasmid DNA into the host genome should enable direct analysis of the sequences surrounding the site of integration. To deliver plasmid DNA into cotton suspension culture obtained from cotyledon-induced callus, polybrene and/or spermidine treatments were used. The transforming plasmid (pBI221.23) contained the selectable hpt gene for hygromycin resistance and the screenablegus gene. Primary transformant cotton plants were regenerated and analysed by DNA hybridisation and b-glucuronidase assay.

3.2.2 PEG Mediated Gene Transfer

In this method protoplasts are isolated and a particular concentration of protoplast suspension is taken in a tube followed by addition of plasmid DNA (donor or carrier). To this 40% PEG4000(w/v) dissolved in mannitol and calcium nitrate solution is slowly added because of high viscosity, and this mixture is incubated for few minutes (ca 5 min.). As per the requirements of the experiments, transient or stable transformation studies are conducted. Among the most important parameters that affect the efficiency of PEG-mediated gene transfer are the concentration of calcium and magnesium ions in the incubation mixture, and the presence of carrier DNA. The linearised dsDNA are more efficiently expressed and integrated in the genome than the supercoiled forms. The advantage of the method is that the form of DNA applied to the protoplast is controlled entirely by the experimenter and not by intermediate biological vector. Main disadvantage is that the system requires a protoplast.

3.2.3 Calcium-Phosphate Co-Precipitation

DNA when mixed with calcium chloride solution isotonic phosphate buffer DNA-CaPO 4 precipitate. The precipitate is allowed to react with actively dividing cells for several hours, washed and then incubated in the fresh medium. Giving them a physiological shock with DMSO can increase the efficiency of transformation to a certain extent. Relative success depends on high DNA concentration and its apparent protection in the precipitate.

3.2.4 DEAE Dextran Procedure

Transformation of cells with DNA complexed to the high molecular weight diethyl amino ethyl (DEAE) dextran is used to obtain efficient

transient expression. The efficiency increasewhen 80% DMSO shock is given. But this technique does not produce stable transformants.

3.2.5 The Polycation DMSO Technique

It involves use of a polycation, polybrene, to increase the absorption of DNA to the surface followed by a brief treatment by 25-30% DMSO to increase the membrane permeability and enhance the uptake. The major advantage of polybrene is that it is less toxic than other polycations and a high transformation efficiency requires very small quantities of plasmid DNA to be used.

3.3 Direct Gene Transformation through Imbibition

During imbibition the uptake of exogenous DNA of dehydrated plant tissues is a direct gene transfer method which has been studied since the 260s and for which the literature contains a number of both claims and refutations. The physical and biochemical changes which are already known occur in plant tissues during dehydration (e.g. a large water potential between the dry tissue and external solution, rapid cell expansion, cell wall rupture, cell membrane structural changes and leakiness; suggest that under these conditions DNA uptake might be possible. DNA uptake and expression was observed under simple dehydration conditions, but was stimulated by the presence of 20% DMSO, suggesting that membrane permeabity was an important factor in the process. A number of lines of evidence supported the conclusion that reporter gene expression was the result DNA uptake into cells and plants were recovered from treated embryos, but no evidence of stable transformation was presented. Subsequent research on the imbibition transformation has extended its application to dessicated somatic embryos of alfalfa, which showed transient GUS expression at frequencies upto 70%. The stable transformation of rice by embryo imbibition was also reported.

The frequency of transient expression of gusA and hpt genes using the CaMV35S promoter was about 30 to 50%. The main sites of gusA gene expression were meristems of roots and vascular bundles of leaves. Also, DNA uptake, integration and expression of the hpt gene in selected rice were investigated by various PCR methods and Southern blot analysis of genomic DNA. It was shown that the hygromycin phosphotransferase (HPT) DNA was present in the rice genome in an integrated form and not as a plasmid form. These methods are technically the simplest of DGT methods, as they require no specialist equipment and the preparation of target plant tissues are generally simple. This simplicity constitutes the advantage of these techniques, while their limitations are i) they can be applied only to very specific

organs or tissues (i.e. newly pollinated flowers or hydrating embryos) and ii) it is still not clear that they lead to stable, and heritable transformation. While they add support to the observation that many different plant cells may be amenable to DNA uptake and expression, at present these techniques are subjects to further analysis and development rather than usable gene transfer methods.

4.0 CONCLUSION

Different systems are now available for gene transfer and successive regeneration of transgenic plants and the most common being *Agrobacterium* -mediated transformation. However, the preferred host of *Agrobacterium* is the dicot plants and its efficiency to transfer genes in monocots is still unsatisfactory. The alternative to this, is the introduction of DNA into plants cells without the involvement of a biological agent like, *Agrobacterium* , and leading to stable transformation is known as direct gene transfer. The most often applied direct methods are microprojectile bombardment or protoplast transformation.

5.0 SUMMARY

The direct DNA transfer methods have been subdivided into three categories:

1. Physical gene transfer method
2. Chemical gene transfer method
3. DNA imbibitions by cell, tissue and organ.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is the basic principle of particle bombardment?
2. Describes the parameters considered in *in vitro* electroporation techniques.
3. What is the role of electric field in electroporation techniques?
4. What are the materials used in microinjection?
5. Write short notes on:
   a. Gene gun
   b. Electroporation
   c. Microinjection.

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UNIT 3 SELECTION AND SCREENING OF TRANSFORMATIONS

CONTENTS

1.0 INTRODUCTION

Genetic selection of transformed cells is a significant step of any plant transformation. Screening of transformed cells or plants for gene integration and expression in transformed cells or plants is a process that involves several techniques, including DNA and RNA blot hybridization analysis, PCR, ELISA analysis. In the absence of a correct selection system one would face with the option of screening every shoot that regenerates in a transformation experiment. In cases where transformation frequency is high this may be possible but for plant species that transform with low frequencies this would be a laborious, if not impossible, task. Therefore, a selectable marker gene (Table 3.1) is incorporated into the plant transformation vectors and an appropriate selecting agent is added to the culture medium which favors the growth of only transformed cells. The genes used as selectable markers are dominant and typically of bacterial origin. For successful selection, the target plant cells must be susceptible to moderately low concentrations of the selecting agent in a non-leaky way. The compound that inhibits the growth but does not kill the wild type cells is preferred as a selecting agent in plant transformation. The concentration of the selecting agent used varies widely depending on the sensitivity of the plant species and/or explant source.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- know the history and evolution
- explain the initiation and establishment of cell suspension cultures

- distinguish between Plant cell cultures vs. microbial cultures.

3.0 MAIN CONTENT

3.1 Selectable Marker Genes Used in Plant Transformation

Table 3.1: Selectable marker genes used in plant transformation

Gene	Enzyme encoded	Selective agent(s)
Antibiotics		
ble	Enzymic activity not known	Bleomycin
dhfr	Dihydrofolate reductase	Methotrexate Trimethoprim
hpt	Hygromycin phosphotransferase	Hygromycin B
npt II	Neomycin phosphotransferase	G418 Kanamycin
Herbicides		
als	Mutant forms of acetolactate synthase	Chlorsulfuron Imidazolinones
Aro A	5-Enolpyruvylshikimate 3-phosphate synthase	Glyphosate (Roundup)
bar	Phosphinothricin acetyltransferase	Phosphinothricin (Bialaphos)

A screening can also be possible by screening or scorable or reporter gene, incorporated into the transformation vectors, which allows for the detection of transformed cells, tissues or plants (Table3.2). The essential features of an ideal reporter gene are:

i. An efficient and easy detection with high sensitivity
ii. Lack of endogenous activity in plant cells
iii. A relatively rapid degradation of the enzyme

The screening markers presently used are mostly derived from bacterial genes coding for an enzyme that is readily detected by the use of chromogenic, fluorigenic, photon emitting or radioactive substrates. A screening marker gene is functional only if an enzyme with comparable activity is not present in non-transformed cells. The utility of any particular gene construct as a transformation marker varies depending on the plant species and the tissue involved. The kanamycin resistance gene is probably the most extensively used selectable marker phenotype and *Uid* A gene (also referred to as *gus*), which encodes β-glucuronidase, is the most versatile reporter gene. The screened cells and the plants regenerated from transformation are further subjected to biochemical analyses, such as Southern hybridization, PCR and Northern hybridization. The former determines the presence and the number of copies of the introduced gene while the latter demonstrates the presence of transcripts of the transgene.

3.2 Screenable Marker Genes Used in Plant Transformation

Table 3.2:Screenable marker genes used in plant transformation

Gene	Enzyme encoded	Substrate(s) and assays
CAT	Chloramphenicol acetyl transferase	[^{14}C]chloramphenicol and acetyl CoA; TLC separation of acetylated [^{14}C]chloramphenicol - detection by autoradiography
lac Z	β-galactosidase	As β-glucuronidase; problems with background activity in some species
GUS	β-glucuronidase	Range of substrates depending on assay; colourimetric, fluorometric, and histochemical techniques available
lux	Luciferase: bacterial insect	Decanal and $FMNH_2$ ATP and O_2 and luciferin Bioluminescent assays: quantitative tests on extracts or in situ tissue assays with activity detected by exposure of X-ray film
npt-II	Neomycin phosphoryltransferase	Kanamycin and [^{32}P]ATP In situ assay on enzyme fractionated by non-denaturing PAGE; enzyme detected by autoradiography Quantitative dot-binding assay on reaction products

4.0 CONCLUSION

Genetic selection of transformed cells is a significant step of any plant transformation. Screening of transformed cells or plants for gene integration and expression in transformed cells or plants is a process that involves several techniques, including DNA and RNA blot hybridization analysis, PCR, ELISA analysis. In the absence of a correct selection system one would face with the option of screening every shoot that regenerates in a transformation experiment.

5.0 SUMMARY

For successful selection, the target plant cells must be susceptible to moderately low concentrations of the selecting agent in a non-leaky way. The compound that inhibits the growth but does not kill the wild type cells is preferred as a selecting agent in plant transformation. The concentration of the selecting agent used varies widely depending on the sensitivity of the plant species and/or explant source.

6.0 TUTOR-MARKED ASSIGNMENT

1. How will you confirm that a particular gene transformed in plant cell?
2. What is selectable marker, describe with example?
3. Give some example of herbicides uses as selectable marker.
4. Give some example of antibiotics which are used as selectable marker in plant transformation.
5. What is screenable marker?
6. Give some example of reporter gene?

UNIT 4 GENE SILENCING

CONTENTS

1.0 INTRODUCTION

The term gene silencing is commonly used to describe the "switching off" of a gene by a mechanism without genetic modification. The term gene silencing refers to an epigenetic phenomenon, the heritable inactivation of gene expression that does not involve any changes to the deoxyribonucleic acid (DNA) sequence. While this phenomenon has initially been studied in transgenic plants, its relevance in the regulation of endogenous plant genes has become increasingly apparent. Below some cellular components are mentioned where gene silencing occurred:

- Chromatin and heterochromatin
- Dicer
- dsRNA
- Histones
- MicroRNA
- siRNA
- Transposons

Gene silencing has following two major subdivisions by which genes are regulated:

1. Transcriptional Gene Silencing (TGS) and
2. Posttranscriptional Gene Silencing (PTGS)

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- Explain Transcriptional Gene Silencing (TGS)
- State Post-Transcriptional Gene Silencing (PTGS).

3.0 MAIN CONTENT

3.1 Transcriptional Gene Silencing (TGS)

Transcriptional gene silencing is the product of chromosomal histone modifications, creating an environment of heterochromatin, which is surrounded to a gene that makes it inaccessible to transcriptional machinery (RNA polymerase, transcription factors, etc.). TGS blocks primary transcription from nuclear DNA and is in most cases associated with DNA methylation and chromatin condensation in nearly all organisms that possess a DNA methylation system (Figure 23.1).

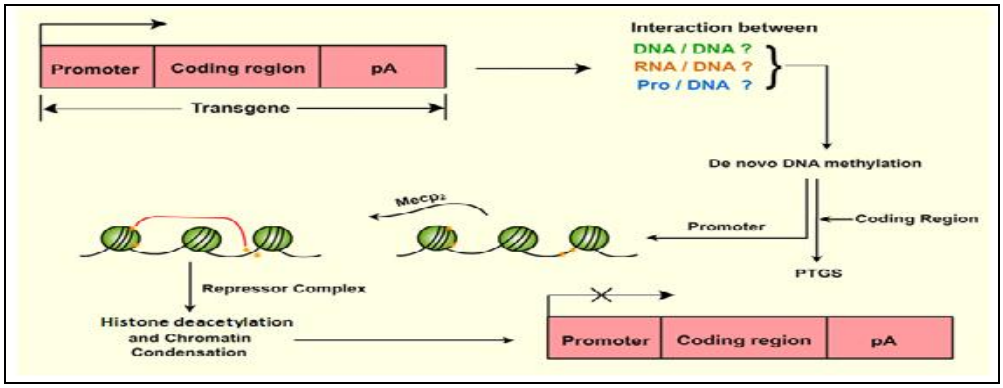

Figure 4.1: Schematic representation of DNA methylation – mediated transcriptional gene silencing (TGS)

3.2 Post-Transcriptional Gene Silencing (PTGS)

Post-transcriptional gene silencing is the product of transcribed mRNA of a specific gene being silenced. When mRNA was destructed, then translation to form an active gene product (in most cases, a protein) will be prevented. A general process of post-transcriptional gene silencing is by RNAi. PTGS involves a cytoplasmic, target sequence-specific RNA degradation process that is possibly activated by double-stranded RNA (dsRNA). This dsRNA is independent of ongoing translation. TGS can be transmitted generation to generation by meiosis whereas PTGS is usually lost during meiosis. In PTGS, double stranded RNA is interred into a cell and gets chopped up by the enzyme known as dicer to form siRNA. siRNA then binds to the RNA-induced silencing complex (RISC) and is unwound. The anitsense RNA complexed with RISC protein and binds to its corresponding mRNA, which is then cleaved by the enzyme slicer rendering it inactive (Figure 23.2).

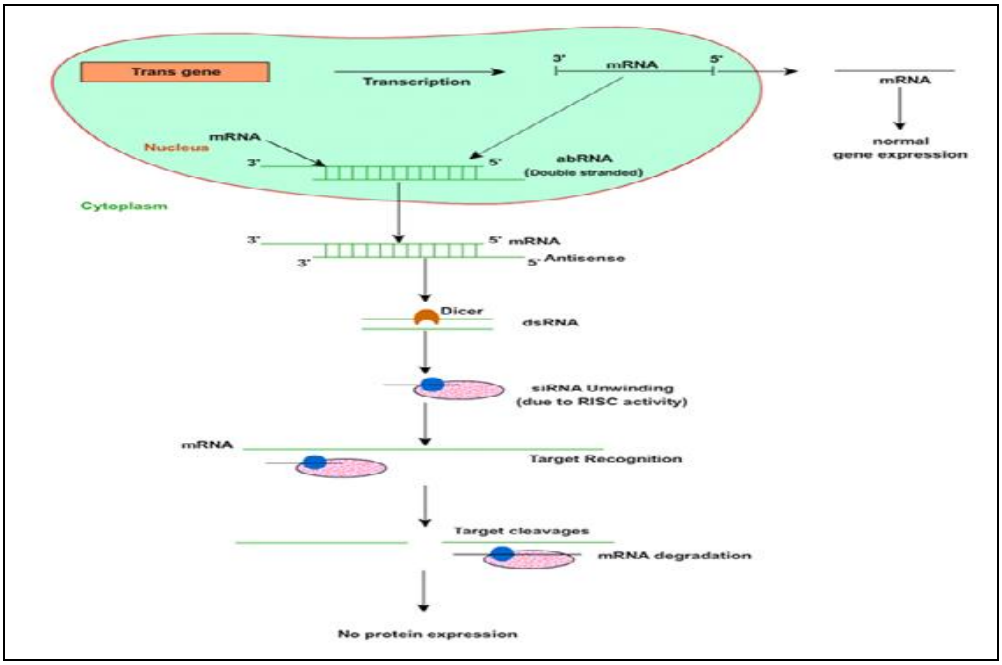

Figure 4.2: Schematic representation of post translational gene silencing (PTGS)

4.0 CONCLUSION

The term gene silencing is commonly used to describe the "switching off" of a gene by a mechanism without genetic modification. The term gene silencing refers to an epigenetic phenomenon, the heritable inactivation of gene expression that does not involve any changes to the deoxyribonucleic acid (DNA) sequence.

5.0 SUMMARY

While this phenomenon has initially been studied in transgenic plants, its relevance in the regulation of endogenous plant genes has become increasingly apparent. Below some cellular components are mentioned where gene silencing occurred:

- Chromatin and heterochromatin
- Dicer
- dsRNA
- Histones
- MicroRNA
- siRNA
- Transposons

6.0 TUTOR-MARKED ASSIGNMENT

1. What is gene silencing?
2. What are the cellular components where gene silencing occurred?
3. How many types of gene silencing is found?
4. Describe the mechanism of gene silencing?
5. Write short notes on:
   A. DNA methylation
   B. Post translational gene silencing
   C. Transcriptional gene silencing.

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UNIT 5 APPLICATIONS OF GENETIC ENGINEERING

CONTENTS

1.0 INTRODUCTION

The knowledge of the molecular basis of diseases caused by various pathogens has allowed testing different strategies to produce disease resistant transgenic plants. Genetic engineering has also been successful in producing herbicide resistance plants. Some other applications are to develop high degree of tolerance or resistance to pests (insects, nematodes, etc.) and diseases. Below are some examples of genetic engineering applications in agriculture:

- Virus resistance
- Insect resistance
- Golden rice
- Long lasting tomatoes

2.0 OBJECTIVE

By the end of this unit, you will be able to:

- know of some genetic engineering applications in agriculture.

3.0 MAIN CONTENT

3.1 Virus Resistance

Plant viruses can cause severe damage to crops by substantially reducing vigor, yield, and product quality. Viruses cause more than 1400 plant diseases and thus, decreasing the agricultural productivity. Unfortunately, there is no viracidal compound to control these diseases. Some diseases, such as rice tungro disease, are caused by two or more

distinct viruses and attempts to incorporate genes for resistance against them have not met with success. Virus resistance is achieved usually through the antiviral pathways of RNA silencing, a natural defense mechanism of plants against viruses. The experimental approach consists of isolating a segment of the viral genome itself and transferring it into the genome of a susceptible plant. Integrating a viral gene fragment into a host genome does not cause disease (the entire viral genome is needed to cause disease). Instead, the plant's natural antiviral mechanism that acts against a virus by degrading its genetic material in a nucleotide sequence specific manner via a cascade of events involving numerous proteins, including ribonucleases (enzymes that cleave RNA), is activated. This targeted degradation of the genome of an invader virus protects plants from virus infection.

Three hypotheses have been investigated to engineer development of virus resistance plants:

- Expression of the virus Coat Protein (CP) gene
- Expression of satellite RNAs and
- Use of antisense viral RNA

3.1.1 Expression of the Virus Coat Protein (CP) Gene

This technique is most common. In this CP-mediated resistance (CP-MR) is developed, based on the well-known process of cross protection. It is protected against super infection by a severe strain of related virus. This method has been commonly used in agriculture to confer protection against severe virus infection. However, the technique has following disadvantages:

- Due to synergistic interaction, infection of cross protected plants with a second unrelated virus may cause a severe disease,
- The suspicious virus strain might mutate to a more severe form, leading to extensive crop losses,
- Protecting virus strain may cause a small but significant decrease in yields, and
- In cross protection, the protecting virus must be applied each growing season.

Most of these problems can be offset by genetic engineering of CP-MR in plants. CP-MR produced a c-DNA encoding the capsid protein (CP) sequences of TMV, ligated it to a strong transcriptional promoter (CaMV 35S promoter) and transport sequences to provide constitutive expression of the gene throughout the transgenic plant, and flanked on the 3' end by poly A signal from the nopaline synthase gene. This

chimeric gene was introduced into a disarmed plasmid of *A. tumefaciens* and the modified bacterium was used.

3.1.2 Expression of Satellite RNAs

Some viruses have specific feature to contain, in addition to their genomic RNA, a small RNA molecule known as satellite RNA (S-RNA). The S-RNAs require the company of a specific 'helper' virus (closely related virus) for their replication. S-RNA does not have sequence to encode CP. They are encapsulated in the coat protein of their helper virus or satellite vi-ruses which encode their own coat protein. Due to ability to modify disease symptoms S-RNAs now have point of attention in genetic engineering. Most of the S-RNAs decrease the severity of viral infection, presumably through interference with viral replication. By this method, tomato, a number of pepper varieties, cucumber, eggplant, cabbage and tobacco plants against CMV have been protected. The first time S-RNA induced attenuation of viral symptoms involved the introduction of cDNA copies of CMV S-RNA into the genome of tobacco plants.

3.1.3 Use of Antisense Viral RNA

Here using the antisense RNA, which is a single stranded RNA molecule complementary to the mRNA (sense RNA), transcribed by a given gene, is another approach suggested for introducing viral resistance in plants. The sense RNA carries codons to translate to a specified sequence of amino acids. The antisense RNA, on the other hand, does not contain the functional protein sequences. When both sense and antisense RNA are present together in cytoplasm, they anneal to form a duplex RNA molecule which cannot be translated. Using this methodology, transgenic plants expressing 3' region of antisense RNA, including CP gene of TMV or CMV. RNAs were produced which have property to protect against infection with respective viruses or viral RNA.

3.2 Insect Resistance

Insects cause serious losses in agricultural products in the field at the time of cultivation and during storage. Insects belonging to the orders, Coleoptera, Lepidoptera and Diptera, are the most serious plant pests which cause agricultural damages. Use of insecticides, bio-pesticides has several harmful side effects. *Bacillus thuringiensis* (Bt), a free-living, Gram-positive soil bacterium, has been employed as insecticide specificity towards lepidopteran pests. It is environmentally safe and thus, is high in demand. On the other hand, the major problems in using Bt sprays for controlling the insect attack on plants

- The high cost of production of Bt insecticide and
- The instability of the protoxin crystal proteins under field conditions, necessitating multiple applications.

To avoid these problems transgenic plants expressing Bt toxin genes have been engineered. Insect resistant transgenic plants have also been created by introducing trypsin inhibitor gene.

3.2.1 Bt Cotton

Two Bt proteins have been recognized as being of particular use for the control of the major pests of cotton and the genes encoding for these proteins have been incorporated into cotton plants by Monsanto. In the 280's a lot work was undertaken by Monsanto to identify and extract the Bt genes and during this decade the gene encoding for the Bt protein Cry1Ac was successfully inserted into a cotton plant. Nowadays, several plant genes are transformed and used as insect resistant plants.

3.3 Golden Rice

Golden rice is genetically modified rice which contains a large number of A-vitamins. Or more correctly, the rice contains the constituent beta-carotene which is converted in the body into Vitamin-A. So, when you eat golden rice, so can get more amount of vitamin of A. Beta-carotene is orange colour so genetically modified rice is golden color. For the making of golden for synthesis of beta-carotene three new genes are implanted: two from daffodils and the third from a bacterium.

Advantages:

- The rice can be considered for poor people in underdeveloped countries. They eat only an extremely limited diet lacking in the essential bodily vitamins.

Disadvantage:

- Critics terror that poor people in underdeveloped countries are becoming too dependent on the rich western world. Generally, genetically modified plants are developed by the large private companies in the West.
- The customers who buy patented transgenic seeds from the company may need to sign a contract not to save or sell the seeds from their harvest, which raises concerns that this technology might lead to dependence for small farmers.

3.4 Long-Lasting Tomatoes

Long-lasting, genetically modified tomatoes now came in to the market. This is the first genetically modified food available to consumers. The genetically modified tomato produces less of the substance that causes tomatoes to rot, so remains firm and fresh for a long time.

4.0 CONCLUSION

The knowledge of the molecular basis of diseases caused by various pathogens has allowed testing different strategies to produce disease resistant transgenic plants. Genetic engineering has also been successful in producing herbicide resistance plants. Some other applications are to develop high degree of tolerance or resistance to pests (insects, nematodes, etc.) and diseases.

5.0 SUMMARY

Some examples of genetic engineering applications in agriculture are:

- Virus resistance
- Insect resistance
- Golden rice
- Long lasting tomatoes

6.0 TUTOR-MARKED ASSIGNMENT

1. What are the strategies for producing virus resistance plant?
2. Which gene responsible for insect resistance?
3. Which vitamin contains enhanced in golden rice?
4. Write short notes on
   A. Virus resistance
   B. Insect resistance
   C. Bt cotton
   D. Golden rice

7.0 REFERENCES/FURTHER READING

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MODULE 5 APPLICATION OF CELL CULTURE SYSTEMS IN METABOLIC ENGINEERING

UNIT 1 HAIRY ROOT CULTURES

CONTENTS

1.0 INTRODUCTION

Plant remains major source of pharmaceuticals and fine chemicals and cell cultures have been viewed as promising alternatives to whole plant extraction for obtaining valuable chemicals. The major constraint with the cell culture is that they are genetically unstable and tend to produce low yield of secondary metabolites. A new method for enhancing secondary metabolite production is by transformation of cells or tissues using the natural vector system. *Agrobacterium rhizogenes*, the

causative agent of hairy root disease, is a soil dwelling gram negative bacterium capable of entering a plant through a wound and causing a proliferation of secondary roots. The mechanism of transformation is elaborated in Figure 1.1. The biosynthetic capacity of the hairy root cultures is equivalent or sometimes more to the corresponding plant roots. Therefore, hairy root cultures have been developed as an alternate source for the production of root biomass and to obtain root derived compounds.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain establishment of hairy root cultures
- state the genetics of transformation
- state the genes responsible for hairy root formation
- state the factors influencing the transformation
- discuss confirmation of transformation
- explain screening of transformation
- explain and state properties of hairy roots
- state the application of hairy root cultures.

3.0 MAIN CONTENT

3.1 Establishment of Hairy Root Cultures

For the production of hairy root cultures, the explant material is inoculated with a suspension of *A. rhizogenes.* The bacterial suspension is generated by growing bacteria in Yeast Mannitol Broth (YMB) medium for two days at 25°C under shaking conditions. Thereafter, pelleting by centrifugation (5 x 10 rpm; 20 min) and resuspending the bacteria in YMB medium to form a thick suspension (approx. 10^{10} viable bacteria/ml). Transformation may be inducesd in aseptic seedlings or surface sterilised detached leaves, leaf-discs, petioles, stem segments, from greenhouse grown plants by scratching the leaf midrib or the stem of a plantlet with the needle of a hypodermic syringe containing a small (about 5-10 ul) droplet of thick bacterial suspension of *A. rhizogenes.*

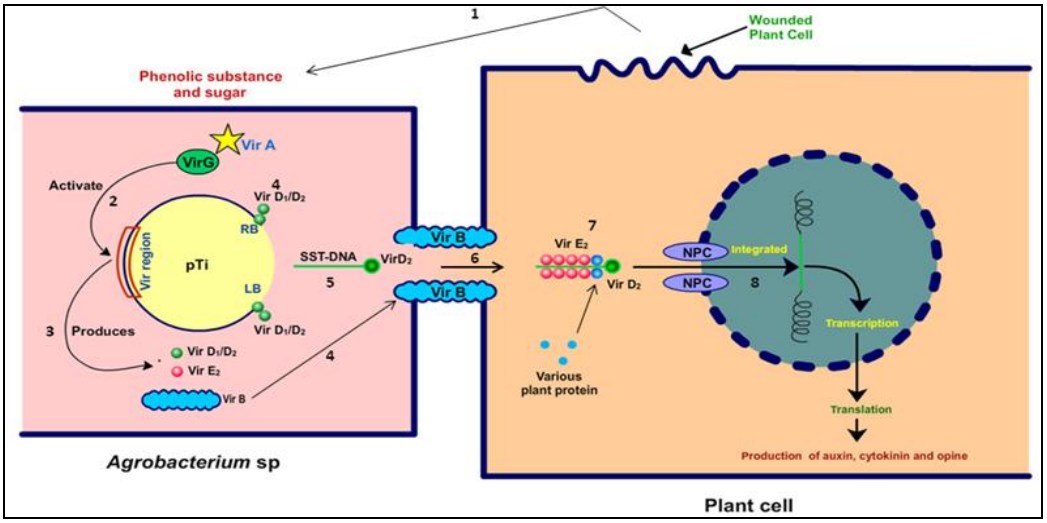

Figure 1.1: The *Agrobacterium* injects a plasmid (naked circular DNA) into the host cells

Wounded plant cell releases phenolic substances and sugar (1); which are sensed by *Vir* A, *Vir* A activates *Vir* G, *Vir* G induced for expression of *Vir* gene of Ri-plasmid (2); *Vir* gene produces all the *Vir* -protein (3); *Vir* D_1 and *Vir* D_2 are involved in ssT-DNA production from Ri-plasmid and its export (4) and (5); the ssT-DNA (associated with *Vir* D_1 and *Vir* D_2) with *Vir* E_2 are exported through transfer apparatus *Vir* B (6); in plant cell, T-DNA coated with *Vir* E_2 (7); various plant proteins influence the transfer of T-DNA + *Vir* D_1 + *Vir* D_2 + *Vir* E_2 complex and integration of T-DNA to plant nuclear DNA(8). (LB= left border; RB= Right border; pRi = Ri plasmid, NPC = nuclear pore complex).

3.2 Genetics of Transformation

Ri plasmids contain one or two regions of T-DNA and a *Vir* (Virulence) region, all of which are necessary for tumorigenesis (Figure 1.2). The Ri plasmid is very similar to Ti plasmid except that their T-DNAs have homology only for auxin and opine synthesis sequences. The T-DNA of Ri plasmid lacks genes for cytokinin synthesis. The T-regions of Ti and Ri plasmids contain oncogenes that are expressed in the plants. Another type, present in Ri plasmids only, appears to impose a high hormone sensitivity on the infected tissue. The T-DNA of Ri plasmids codes for at least three genes that each can induce root formation, and that together cause hairy root formation from plant tissue. Current results indicate that the products of these genes induce a potential for increased auxin sensitivity that is expressed when the transformed cells are subjected to a certain level of auxin. After this stage the transformed roots can be grown in culture without exogenous supply of hormones.

The Ri-plasmids are classified into two main classes according to the opines formed in transformed roots. First, agropine-type strains induce roots to synthesiseagropine, mannopine and the related acids. Second, mannopine-type strains which induce roots to produce mannopine and the related acids. The agropine-type Ri-plasmids are very similar as a group and a quite distinct group from the mannopine-type plasmids. Perhaps the most studied Ri-plasmids are agropine-type strains, which are considered to be the most virulent and, therefore, more often used in the establishment of hairy root cultures.

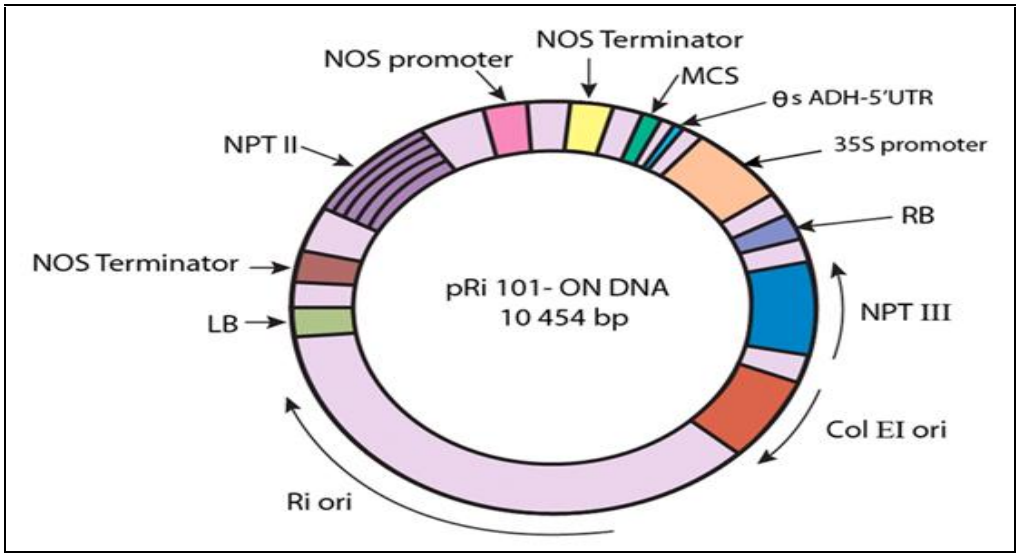

Figure 1.2: Structure of Ri-plasmid

3.3 The Genes Responsible for Hairy Root Formation

The agropine-type Ri-plasmid consists of two separate T-DNA regions known as the TL-DNA and TR-DNA. Each of the T-DNA fragments is separated from each other by at least 15 kb of non-integrated plasmid DNA. These two fragments can be transferred separately during the infection procedure. The TR-DNA of the agropine type Ri-plasmid carries genes encoding auxin synthesis (*tms* 1 and *tms* 2) and agropine synthesis (*ags*). The mannopine type Ri-plasmids contain only one T-DNA. TL-DNA region consists of four root locus (*rol*) genetic loci, *rol* A, *rol* B, *rol* C, and *rol* D, which affect hairy root induction. In particular, *rol* B seems to be the most important in the differentiation process of transformed cells and also function as induction of hairy roots by hydrolyzing bound auxins leading to an increase in the intracellular levels of indole-3-acetic acid. Gene *rol* A involved in development of hairy root morphology, *rol* B is responsible for protruding stigmas and reduced length of stamens; *rol* C causes internode shortening and reduced apical dominance.

3.4 Factors Influencing the Transformation

Following factors influence the transformation process:

- Virulence of *A. rhizogenes* strains
- Medium
- Age of the explant
- Nature of the explant

3.5 Confirmation of Transformation

Confirmation of transformation can be performed on the basis of following markers:

- Biochemical markers
- Opines
- Mannopines
- Genetic markers
- Southern hybridisation
- Polymerase chain reaction.

3.6 Screening of Transformation

Screening of transformation can be performed by GUS assay, leaf callus assay, rooting and bleaching assays.

3.7 Properties of Hairy Roots

3.7.1 Hairy Roots

Hairy roots have following properties

- high degree of lateral branching
- profusion of root hairs
- absence of geotropism
- they have high growth rates in culture, due to their extensive branching, resulting in the presence of many meristems
- they do not require conditioning of the medium .

3.7.2 Hairy Roots are Genetically Stable

Hairy roots are genetically stable consequently they exhibit biochemical stability that leads to stable and high-level production of secondary metabolites. Hairy root cultures apparently retain diploidy in all species so far studied. The stable production of hairy root cultures is dependent on the maintenance of organised states. The factors which promote

disorganisation and callus formation depress secondary metabolite production. The productivity of hairy root cultures is stable over many generations in contrast to disorganised cell cultures. This stability is reflected in both the growth rate and the level pattern of secondary metabolite production.

3.8 Application of Hairy Root Cultures

3.8.1 Production of Secondary Metabolites

The hairy root system is stable and highly productive under hormone-free culture conditions. The fast growth, low doubling time, easy maintenance, and ability to synthesise a range of chemical compounds of hairy root cultures gives additional advantages as continuous sources for the production of plant secondary metabolites. Usually root cultures require an exogenous phytohormone supply and grow very gradually, resulting in the poor or insignificant synthesis of secondary metabolites. Hairy roots are also a valuable source of photochemical that is useful as pharmaceuticals, cosmetics, and food additives. These roots synthesize more than a single metabolite; prove economical for commercial production purposes. Many medicinal plants have been transformed successfully by *A. rhizogenes* and the hairy roots induced show a relatively high productivity of secondary metabolites, which are important pharmaceutical products. Sevon has summarised the most important alkaloids produced by hairy roots, including *Atropa belladonna* L., *Catharanthustrichophyllus* L., and *Datura candida* L.
Metabolic engineering offers new perspectives for improving the production of secondary metabolites by the over expression of single genes. This approach may lead to an increase of some enzymes involved in metabolism and, consequently, results in the accumulation of the target products. This method utilises the foreign genes that encode enzyme activities not normally present in a plant. This may cause the modification of plant metabolic pathways. Two direct repeats of a bacterial lysine decarboxylase gene, expressed in the hairy roots of *Nicotiana tabacum,* have markedly increased the production of cadaverine and anabasine (Feckeret al . 293). The production of anthraquinone and alizarin in hairy roots of *Rubia peregrina* L. was enhanced by the introduction of isochorismate synthase. *Catharanthus roseus* hairy roots harboring hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) cDNA without the membrane-binding domain were found to produce more ajmalicine and catharanthine or serpentine and campesterol than the control.

3.8.2 Production of Compounds Not Found in Untransformed Roots

Transformation may affect the metabolic pathway and produce new compounds that cannot be produced normally in untransformed roots. For example, the transformed hairy roots of *Scutellariabaicalensis* Georgi accumulated glucoside conjugates of flavonoids instead of the glucose conjugates accumulated in untransformed roots.

3.8.3. Changing Composition of Metabolites

Bavage et al. (297) reported the expression of an *Antirrhinum* dihydroflavonol reductase gene which resulted in changes in condensed tannin structure and its accumulation in root cultures of *L. corniculatus*. The analysis of selected root culture lines indicated the alteration of monomer levels during growth and development without changes in composition.

Table 1.1: Pharmaceutical products produced using hairy root cultures

Plant species	Product
Bidens spp.	Polyacetylenes
Cinchona ledgeriana	Quinoline alkaloids
Datura spp.	Tropane
Cassia spp.	Anthraquinones
Echinacea purpurea	Alkaloids

4.0 CONCLUSION

Plant remains major source of pharmaceuticals and fine chemicals and cell cultures have been viewed as promising alternatives to whole plant extraction for obtaining valuable chemicals. The major constraint with the cell culture is that they are genetically unstable and tend to produce low yield of secondary metabolites.

5.0 SUMMARY

A new method for enhancing secondary metabolite production is by transformation of cells or tissues using the natural vector system. *Agrobacterium rhizogenes*, the causative agent of hairy root disease, is a soil dwelling gram negative bacterium capable of entering a plant through a wound and causing a proliferation of secondary roots.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is hairy root culture?
2. Describe the establishment of hairy root culture.
3. What are the genes responsible for hairy root formation?
4. What are the factors which influence the transformation of hairy root culture?
5. What are the properties of hairy roots?
6. Give some example of pharmaceutical products from hairy root culture?

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UNIT 2 SCREENING OF HIGH YIELDING CELL LINES AND EXTRACTION OF HIGH VALUE INDUSTRIAL PRODUCTS

CONTENTS

1.0 INTRODUCTION

Plant-derived substances are of great interest due to their versatile applications. These plants derived substance are richest bio-resource of drugs of traditional systems of medicine, modern medicines, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Extraction is the separation of medicinally active compounds from plant tissues using selective solvents through standard protocol. The products so obtained from plants are complex mixtures of metabolites, in liquid or semisolid state or in dry powder form, and are intended for oral or external use. These include decoctions, infusions, fluid extracts, tinctures, pilular extracts or powdered extracts. Plant cell culture is a genetically heterogeneous system. In addition, epigenetic changes cause genetic instability leading to product accumulation only in some population of cells. The overall production of secondary metabolites in a cell culture depends on the rate of accumulation within the productive cells. By cell culture system only low yields of desired secondary metabolites are obtained.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain screening and selection of highly productive cell lines
- state procedure for extraction of high value industrial products

3.0 MAIN CONTENT

3.1 Screening and Selection of Highly Productive Cell Lines

3.1.1 Screening and Selection

Callus culture is an easiest system for screening and selection program. Callus which shows the desired coloration is picked up and subcultured until pure cell line is established. The heterogeneity in the biochemical activity of cells has been exploited to obtain highly productive cell lines. The selection with cell suspension cultures can also be performed with a fine, rapidly growing suspension of cells consisting of small aggregates of up to 50 cells. Selection procedure can be easily achieved if the product of interest is a pigment. For example, in cultures of *Lithospermumerythrorhizon*, screening of a number of clones resulted in 13–20-fold increase in shikonin production. Increased production of anthocyanin by clonal selection and visual screening has been reported in *Euphorbia milii* and *Daucus carota*. High performance liquid chromatography (HPLC), radioimmuno assays (RIA) and mutation strategies have also been employed in order to obtain overproducing cell lines. The use of selective agents can be employed as an alternative approach to select high yielding cell lines. In this method, a large population of cells is exposed to a toxic (or cytotoxic) inhibitor or environmental stress and only cells that are able to resist the selection procedures will grow. P-Fluorophenylalanine, an analogue of phenylalanine, was extensively used to select high-yielding cell lines with respect to phenolics. Other selective agents consist of 5-methyltryptophan; glyphosate and biotin have also been used to select high-yielding cell lines.

3.2 Procedure for Extraction of High Value Industrial Products

3.2.1 Plant Material

Plants are the source of effective phytomedicines since times immemorial. Man is able to obtain from them a wondrous variety of industrial chemicals. Plant based natural constituents can be derived from the plant part, like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components. Fresh or dried plant materials can be used as a source for the extraction of secondary plant components. Many authors had reported about plant extract preparation from the fresh plant tissues. The logic behind this came from the ethno medicinal uses of fresh plant materials among the traditional and tribal people. But as many plants are used in the dry form by conventional healers and due to differences in water content within

different plant tissues, plants are generally air dried to a constant weight before extraction. Other researchers dry the plants in the oven at about 40°C for 72 h. In most of the reported works, underground parts (roots, tuber, rhizome, bulb etc.) of a plant were used extensively compared with other above ground parts in search for bioactive compounds possessing antimicrobial properties.

3.2.2 Choice of Solvents

Properties of a good solvent in plant extractions include, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability of the extract to complex or dissociate. The factors affecting the choice of solvent are, quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractant.

The various solvents that are used in the extraction procedures are:

3.2.2.1 Water

Antimicrobial activity. Though traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extract. Reports indicates that water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics are important as only antioxidant compounds.

3.2.2.2 Acetone

Acetone dissolves many hydrophilic and lipophilic components from the plants and is miscible with water. It is volatile and has a low toxicity to the bioassay used. It is a very useful extractant, especially for antimicrobial studies where more phenolic compounds are required to be extracted. A study reported that extraction of tannins and other phenolics was better in aqueous acetone than in aqueous methanol.

3.2.2.3 Alcohol

The higher activity of the ethanolic extracts compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols. More useful explanation for the decrease in the activity of aqueous extract can be ascribed to the enzyme polyphenol oxidase, which degrade polyphenols in water extracts, whereas in methanol and ethanol they are inactive.

3.2.2.4 Chloroform

Terpene lactones have been obtained by following extractions of dried barks with hexane, chloroform and methanol with activity concentrating in chloroform fraction. Rarely tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents.

3.2.2.5 Ether

Ether is commonly used for the selective extraction of coumarins and fatty acids.

3.2.2.6 Dichloromethane

It is another solvent used for carrying out the extraction procedures. It is specially used for the selective extraction of only terpenoids.

3.2.3 Extraction Procedures

3.2.3.1 Plant Tissue Homogenisation

Plant tissue homogenisation in solvent has been generally used by researchers. Dried or wet, plant parts are grinded in a blender, with certain quantity of solvent, to fine particles, and shaken vigorously for 5-10 min or left overnight before filtering the extract. The filtrate then may be dried under reduced pressure and redissolved in small amount of solvent during quantification by HPLC.

3.2.3.2 Soxhlet Extraction

Soxhlet extraction is only necessary where the preferred compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the preferred compound has a high solubility in a solvent then a simple filtration procedure can be used to separate the compound from the insoluble substance. The benefit of this system (Figure 2.1) is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds.

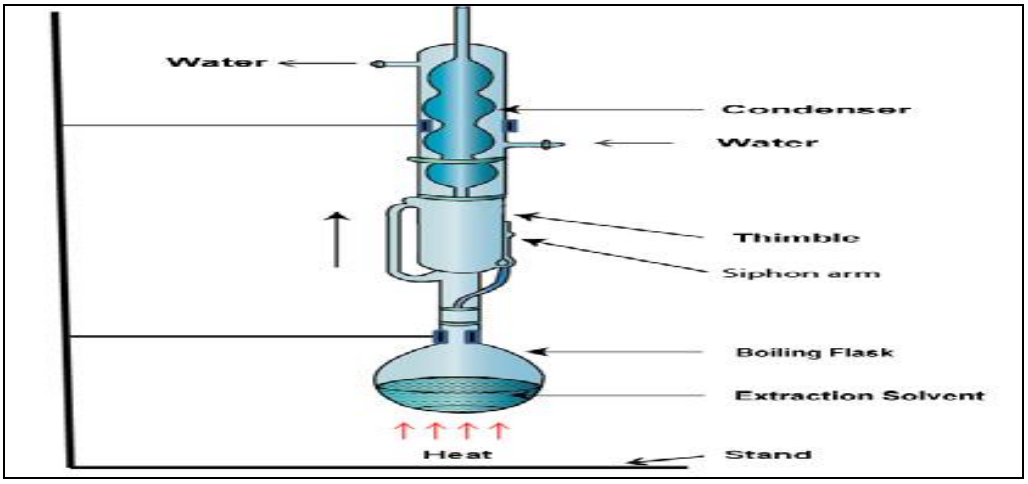

Figure 2.1: A Soxhlet apparatus

3.2.3.3 Maceration

In maceration (for fluid extract), whole or coarsely powdered plant-drug is kept in contact with the solvent in a container with stopper for a defined period with frequent agitation until soluble matter is dissolved. This method is best suitable for use in case of the thermolabile compounds.

3.2.3.4 Decoction

This method is used for the extraction of the water soluble and heat stable constituents from crude drug by boiling it in water for 15 minutes, cooling, straining and passing sufficient cold water through the drug to produce the required volume.

3.2.3.5 Percolation

This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstruum and allowed to stand for approximately 4 h in a closed container, after which the mass is packed and the top of the percolator is closed. Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained, therein, is allowed to drip slowly. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

3.2.3.6 Sonication

The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz. This increases the permeability of cell walls and produces cavitations. Although the process is useful in some cases, like extraction of Rauwolfi root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.

3.2.4 Steps Involved in the Extraction

For extraction from plant explants, most important steps are size reduction, extraction, filtration, concentration and drying.

3.2.4.1 Size Reduction

The objective for powdering the plant material is to rupture its organ, tissue and cell structures so that its medicinal ingredients are exposed to the extraction solvent. Furthermore, size reduction maximizes the surface area, which in turn enhances the mass transfer of active principle from plant material to the solvent.

3.2.4.2 Extraction

Extraction of the plant material is carried out in three ways:

3.2.4.3 Cold Aqueous Percolation

The powdered material is macerated with water and then poured into a tall column. Cold water is added until the powdered material is completely immersed. It is allowed to stand for 24 h so that water-soluble ingredients attain equilibrium in the water. The enriched aqueous extract then can be concentrated in multiple-effect evaporators to a particular concentration.

3.2.4.4 Hot Aqueous Extraction

This is done in an open-type extractor. The extractor is a cylindrical vessel made from type 316 stainless steel and has a diameter (D) greater than the height (H), i.e. the H/D ratio is approximately 0.5. The bottom of the vessel is welded to the dished end and is provided with an inside false bottom with a filter cloth. The outside vessel has a steam jacket and a discharge valve at the bottom. One part powdered plant material and sixteen parts demineralized water is fed into the extractor. Heating

is done by injecting steam into the jacket. The material is allowed to boil until the volume of water is reduced to one-fourth its original volume.

3.2.4.5 Solvent Extraction

The principle of solid-liquid extraction is that when a solid material comes in contact with a solvent, the soluble components in the solid material move to the solvent. Thus, solvent extraction of plant material results in the mass transfer of soluble active principle (medicinal ingredient) to the solvent, and this takes place in a concentration gradient. The rate of mass transfer decreases as the concentration of active principle in the solvent increases, until equilibrium is reached, i.e. the concentrations of active principle in the solid material and the solvent are the same. Thereafter, there will no longer be a mass transfer of the active principle from plant material to the solvent. Since mass transfer of the active principle also depends on its solubility in the solvent, heating the solvent can enhances the mass transfer. Furthermore, if the solvent in equilibrium with the plant material is replaced with fresh solvent, the concentration gradient is altered. This gives rise to different types of extractions: cold percolation, hot percolation and concentration.

3.2.4.6 Filtration

The extract so obtained is separated out from the marc (exhausted plant material) by allowing it to trickle into a holding tank through the built-in false bottom of the extractor, which is covered with a filter cloth. The marc is retained at the false bottom, and the extract is received in the holding tank. From the holding tank, the extract is pumped into a sparkler filter to remove fine or colloidal particles from the extract. The filtered extract is subjected to spray drying with a high pressure pump at a controlled feed rate and temperature, to get dry powder. The desired particle size of the product is obtained by controlling the inside temperature of the chamber and by varying the pressure of the pump.

3.2.4.7 Concentration

The enriched extract from percolators or extractors, known as miscella, is fed into a wiped film evaporator where it is concentrated under vacuum to produce a thick concentrated extract. The concentrated extract is further fed into a vacuum chamber dryer to produce a solid mass free from solvent. The solvent recovered from the wiped film evaporator and vacuum chamber dryer is recycled back to the percolator or extractor for the next batch of plant material. The solid mass, thus, obtained is pulverised and used directly for the desired pharmaceutical formulations or further processed for isolation of its phytoconstituents.

3.2.4.8 Drying

Drying process is final extraction step. Drying is a mass transfer process consisting of removal of water or another solvent by evaporation from a solid, semi-solid or liquid. This process is frequently used as a final production step before packaging products. Freeze drying is a drying method where the solvent is frozen prior to drying and is then sublimed, below the melting point of the solvent.

4.0 CONCLUSION

Plant-derived substances are of great interest due to their versatile applications. These plants derived substance are richest bio-resource of drugs of traditional systems of medicine, modern medicines, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Extraction is the separation of medicinally active compounds from plant tissues using selective solvents through standard protocol. The products so obtained from plants are complex mixtures of metabolites, in liquid or semisolid state or in dry powder form, and are intended for oral or external use. These include decoctions, infusions, fluid extracts, tinctures, pilular extracts or powdered extracts.

5.0 SUMMARY

Plant cell culture is a genetically heterogeneous system. In addition, epigenetic changes cause genetic instability leading to product accumulation only in some population of cells. The overall production of secondary metabolites in a cell culture depends on the rate of accumulation within the productive cells. By cell culture system only low yields of desired secondary metabolites are obtained. The reason for low production in culture may be due to:

- Competition between primary and secondary pathways for common intermediates.

- Low levels of expression of key enzymes at rate limiting steps in a pathway.

Screening and selection are often used as exchangeable terms. Screening is a passive technique by which a great number of cells alone analyzed for a certain trait and those showing the desired features are cultivated and screened. Selection process is an active process, which deliberately favor only the survival of the desired variants while wild type cells are killed.

6.0 TUTOR-MARKED ASSIGNMENT

1. How do you screen and select cell line for high production of cell secondary metabolites?
2. What is the region of low production of secondary metabolites?
3. Describe the extraction process.
4. What are the factors that affect chemical extraction?
5. Describe the solvents required for extraction.
6. Draw diagram of Soxhlet apparatus.
7. Write short notes on:
   A. Tissue homogenisation
   B. Maceration
   C. Percolation
   D. Sonication
   E. Extraction steps.

7.0 REFERENCES/FURTHER READING

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UNIT 3 FRACTIONATION AND BIOASSAYS OF PLANT EXTRACT

CONTENTS

1.0 INTRODUCTION

Fractionation is a separation method in which a certain quantity of a mixture (solid, liquid, solute, suspension or isotope) is divided into number of smaller quantities (fractions) in which the composition changes according to a gradient. Fractions are collected based on differences in a specific property of the individual components. Bioassays are generally conducted to measure the potency of a substance by its effect on living cells and are useful in the development of new drugs. Biological assays must be carried out in order to identify plant extracts, to guide the separation and isolation, and to evaluate lead compounds.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain the concept of fractionation
- know the impotance of bioassays.

3.0 MAIN CONTENT

3.1 Fractionation

Fractionation is a separation method in which a certain quantity of a mixture (solid, liquid, solute, suspension or isotope) is divided into number of smaller quantities (fractions) in which the composition changes according to a gradient. Fractions are collected based on differences in a specific property of the individual components. Fractionation process makes it possible to separate more than two

components in a mixture in a single run. It is generally carried out by suspending each extract in water separately and partitioning with different organic solvents, such as hexane, chloroform, ethyl acetate, and methanol in order of increasing polarity by using separating funnel. A simple fractionation unit is shown in Figure 3.1. All the fractions of plant extract can be dried by evaporating respective solvent using rotary evaporator and can be stored at 4°C till further analysis.

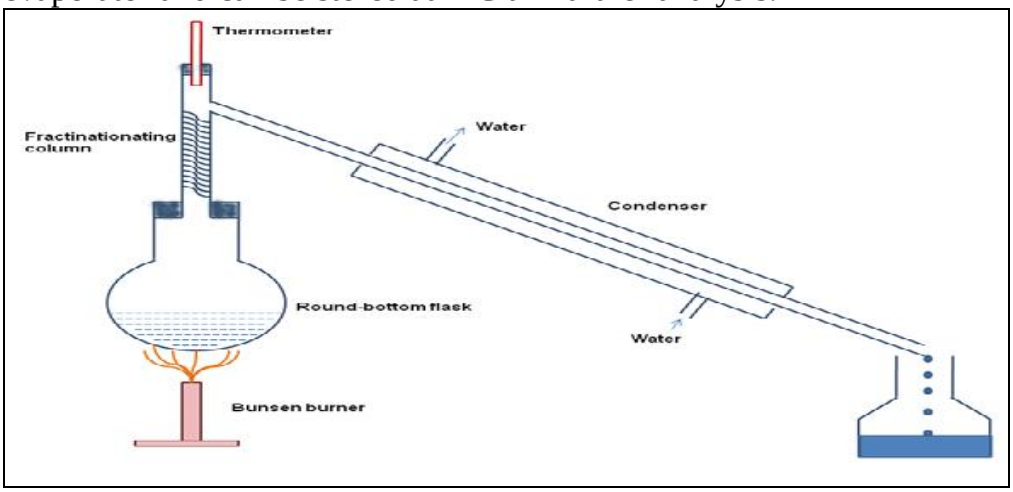

Figure 3.1: A simple fraction distillation apparatus

Qualitative phytochemical analysis can be done for various phytoconstituents, like alkaloids, tannins, glycosides, steroids and saponins, by thin layer chromatography (TLC) of obtained fractions in different solvent system. High performance liquid chromatography (HPLC) is an important device for quantitative analysis of phytochemicals, their monitoring and quality assurance. The method enables complex mixtures to be separated into individual compounds, which can be identified and quantified by suitable detectors and data handling systems. Separation and detection occur at ambient temperature or slightly above. Therefore, the method is preferably suited for compounds of limited thermal stability.

3.2 Bioassays

Bioassays are generally conducted to measure the potency of a substance by its effect on living cells and are useful in the development of new drugs. Biological assays must be carried out in order to identify plant extracts, to guide the separation and isolation, and to evaluate lead compounds. These assays may be conducted both *in vitro* or *in vivo.*

3.2.1 Antiviral Assays

A number of compounds extracted from different species of higher plants have shown antiviral activity. Examples included tannins, flavones, alkaloids that displayed *in vitro* activity against numerous

viruses. Antiviral assays are basically an extension of cytotoxicity assays. To perform this, the cultures of mammalian cells are infected with virus, test compounds are added, and the fate of the cells is assessed. It was reported that the antiviral activity detected was due to phytoalexins produced by the plant as a protection against plant viruses. These assays simultaneously allow an estimation of cytotoxicity (loss of the cell monolayer in which the plaques are normally formed). Active extracts or compounds then become candidates for testing against tumor cell lines, for example, P388 or L1210.

The methanol extracts of the aerial parts of *Hypericum mysorense* and *Hypericum hookerianum,* exhibited detectable antiviral effect towards HSV-1 with an inhibitory concentration at IC_{50} value of 50µg/ml. The acetone extract of *Usneacomplanata* also showed antiviral activity at an IC_{50} value of 100µg/ml. Other examples of plant extracts exhibiting anti-HSV activity are *Acnistusarborescens, Cupania glabra, Dichapetalum axillar, Drypeteslasiogyna, Mallotusmollissimus.*
The pentacyclic triterpenoids, betulinic acid, oleanolic acid and ursolic acid, are widespread plant metabolites. All the three triterpenes inhibit HIV-1 protease activity *in vitro*. Betulinic acid was found to be active in vivo as well using athymic mice carrying human melanomas. Further biological studies suggest that betulinic acid works by induction of apoptosis.

3.2.2 Cell Cytotoxicity Assays

Cytotoxicity assays are used by the pharmaceutical industry to screen for cytotoxic compounds. Cell membrane integrity is one of the most common methods to measure cell viability and cytotoxic effects. Compounds having cytotoxic effects often have compromised cell membrane integrity. Cytotoxicity can be monitored using the MTT or MTS assay. The aqueous extract isolated from *in vitro* derived cell cultures, raised from leaf-discs of *Lantana camara,* exhibited promising anti-proliferative activity on HeLa cells (Figure 3.2, 3.3). The minimal activity of the extract on normal BHK-21 cells verifies it's potential as a feasible anti-cancer agent.

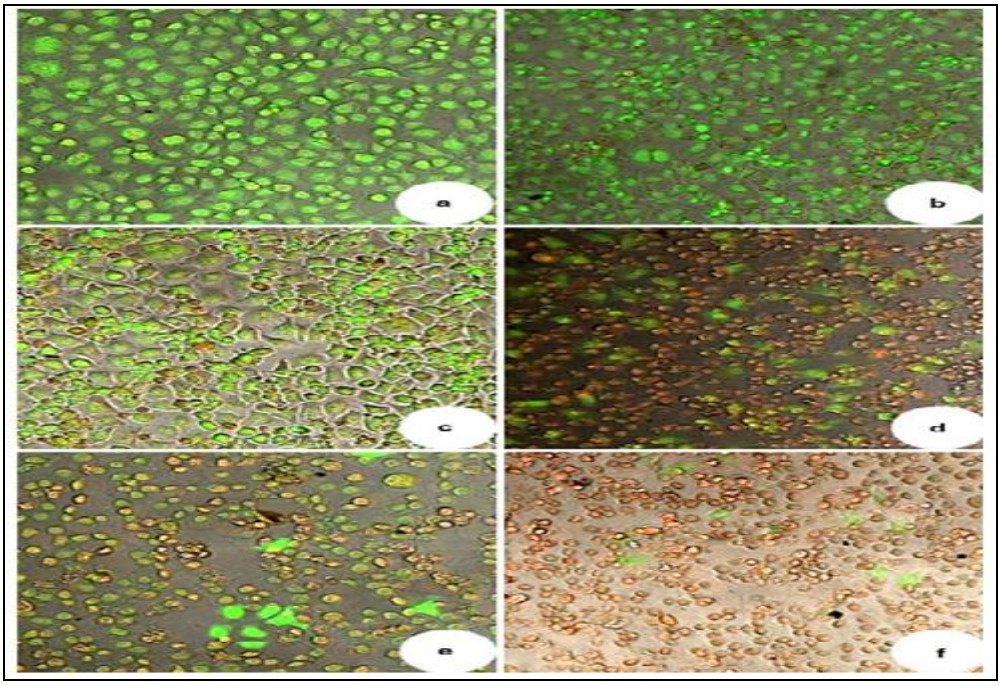

Figure 3.2: HeLa cells are stained with Acridine Orange/Ethidium Bromide and viewed under confocal laser scanning microscope. **a.** Control healthy cells, **b.** HeLa cells treated with aqueous extract of *L. camara* for 24 h showing bright green nuclei indicating initiation of chromatin condensation, **c.** same as **b** , treated for 36 h, showing zones of cleared monolayer, **d, e and f.** same as **b,** treated for 48, 60 and 72 h, respectively, showing gradual increase in frequency of dead cells taken the orange color.

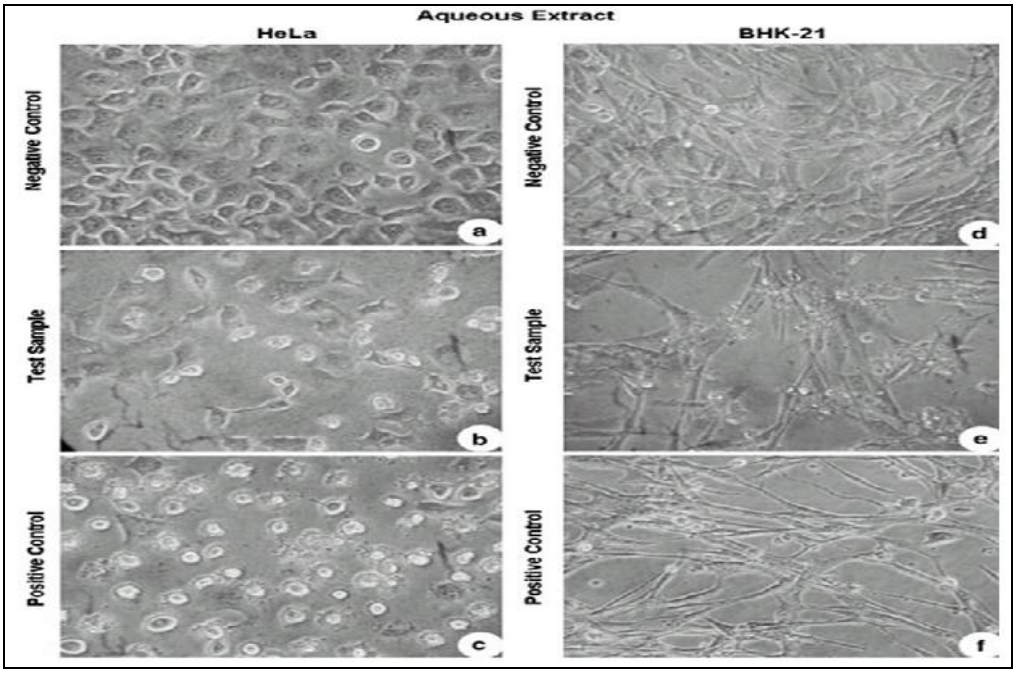

Figure 3.3: Morphological observations of HeLa and BHK-21 cells under light microscopy.

a. untreated HeLa cells, **b.** HeLa cells treated with aqueous extract of *L. camara* , showing rounded apoptotic bodies, **c.** HeLa cells treated with curcumin, showing similar cell death patterns as in **b, d.**untreated BHK-21 cells, **e.** BHK-21 cells treated with aqueous extract of *L. camara*, showing low frequency of apoptotic bodies when compared to HeLa cells, **f.** BHK-21 cells treated with curcumin, showing similar cell death patterns as in **e.**

3.2.3 Antimicrobial Assays

An anti-microbial substance either kills or inhibits the growth of microorganisms, such as fungi, bacteria. Plants have been investigated scientifically for antimicrobial activity, and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms. Antimicrobial activity of the crude extracts can be determined by these two methods:

A. Agar well method
B. Disc diffusion assay method

Nutrient agar can be prepared for bacteria according to the manufacturers' instructions. Immediately after autoclaving, allow the media to cool at 45°C to 50°C in water bath. Pour the freshly prepared cooled media (approximately 4 mm depth) into flat-bottomed Petri dishes (90mm in diameter). Spread about 0.2ml of the test inoculum of bacteria uniformly on the surface of the solidified agar media using a sterile inoculation L-shaped glass rod. Make four equidistant wells of 5mm diameter and 4mm in depth on the agar using a sterile corn borer. Make two more wells for positive and negative controls at the middle of the agar. Fill about 3 µl of the plant extracts (concentrations ranged from 3–200mg/ml) and controls into the wells. The positive controls will be antibiotic (e.g. Ampicillin). The negative controls will be DMSO for organic solvent extracts and distilled water for aqueous extracts. Label the wells to correspond with the code numbers of the test crude extracts and controls. Store the treated plates in a refrigerator at 4°C for at least six hours to allow diffusion of the extracts into the agar while arresting the growth of the test microbes. The plates were then incubated for 24 hours at favorable condition for a particular organism. The test was carried out in triplicates. Antimicrobial activity was determined by measuring the diameters of zones of inhibition in mm.

A. Agar well method
B. Disk diffusion assay

The preparation of media and inoculation of the test microbes are same as described in the agar well method. However, instead of punching out wells on the agar, sterile 5 mm Whatman No. 1 filter paper discs will be

used in the disc diffusion method. Soak the discs into the dissolved crude extracts for minimum of two hours. Blank discs impregnated generally with Ampicillin (10µg/disc) for gram positive bacteria, Gentamicin (15µg/disc) for gram negative bacteria and Fluconazole (0.4mg/disc) for Candida are used as positive controls. For negative control, discs will be soaked in DMSO and distilled water, for organic solvent and water extracts, respectively. By use of sterile forceps, place four seeded discs of the plant extracts equidistantly onto each of the inoculated plates. Place two extra discs for positive and negative controls at the middle of plate. Store the treated plates in a refrigerator at 4°C for minimum of six hours and then transfer to incubator for 24 hours at favorable conditions for a particular organism (37°C for bacteria and for 48 hours at 30°C for Candida). Perform the test in triplicates. Antimicrobial activities were determined by measuring the diameters of zones of inhibition in mm.

3.2.4 Anthelmintic Assay

Plant-derived compounds have played significant role in the field of anthelmintic drugs, such as Santonin, the main active substance isolated from wormwood (*Artemisia maritima* L). The protocol for anthelmintic screening using *Caenorhabditis elegans* (Maupas) developed by Simpkin and Coles (281). The test is carried out in a 24-well tissue culture plate with a well volume of 2.5 ml. Each well is filled with 2 ml of M_9 liquid medium. M_9 solution consists of 6g Na_2HPO_4, 3 g KH_2PO_4, 5g NaCl, and 0.3g $MgSO_4$. $7H_2O$ dissolved in 1000 ml water and autoclaved at 120°C for 20 min. Extract solutions, dissolved in DMSO, are added to make 500 ppm solutions, and then 10 µl *C. elegans* suspensions containing 30 to 40 larvae are subsequently introduced into each cell. The plates are incubated at 20°C for five day, and the number of dead nematodes is recorded using a phase contrast microscope, and anthelmintic activity is graded as shown below:

- Nematode counts and the motility of the nematodes correspond to the control
+ 0 to 20% fewer nematodes than the control; nematodes move slowly
++ Slightly higher nematode counts than the initial counts; nematode counts 20% less than the
control; nematodes move very slowly
+++ Same nematode counts as the initial counts, all dead

Active fractions/compounds are repeated with lower concentrations and compared to known anthelmintics, such as santonin.

4.0 CONCLUSION

Fractionation is a separation method in which a certain quantity of a mixture (solid, liquid, solute, suspension or isotope) is divided into number of smaller quantities (fractions) in which the composition changes according to a gradient. Fractions are collected based on differences in a specific property of the individual components. Bioassays are generally conducted to measure the potency of a substance by its effect on living cells and are useful in the development of new drugs. Biological assays must be carried out in order to identify plant extracts, to guide the separation and isolation, and to evaluate lead compounds.

5.0 SUMMARY

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6.0 TUTOR-MARKED ASSIGNMENT

1. What do you mean by fractionation?
2. What do you mean about bioassay and give the name of some bioassay techniques?
3. Describe anti-viral assay.
4. What is cell cytotoxicity and how it is done?
5. What is antimicrobial assay?
6. Write short note on:

A. Agar well method
B. Disc diffusion assay method
C. Anthelmintic assay.

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UNIT 4 GROWTH AND PRODUCTION KINETICS OF CELL CULTURES IN SHAKE FLASKS

CONTENTS

1.0 INTRODUCTION

For the development of plant cell culture technology, accurate and rapid measurement of cell growth and assessment of growth-related bioprocess kinetics are essential to the rational development of plant cell bioprocess engineering. Although the plant cell culture system appears to be similar to a microbial cell culture system, there are important differences between the two. The major differences include cell size, aggregation of plant cells, change in plant cell physiology for its primary and secondary metabolisms, rheological properties of the medium, and requirement of plant cells for complex nutrients. Therefore, the best method of assessing the plant cell growth kinetics should be carefully examined and evaluated. The cell suspension culture is much more amenable for biochemical studies and process development than callus cultures. The success in the establishment of a cell suspension culture depends, to a great extent, on the availability of “friable” callus tissue (i.e., a tissue that, when stirred in liquid medium, rapidly disaggregate into single cells and small clusters). The cell suspension culture generally grows at a faster rate and allows cells to be in direct contact with the medium nutrients. Suspension culture could be run as batch culture or continuous culture.

In batch culture, the culture environment continuously changes and growth, product formation, substrate utilisation, all terminate after a certain time interval.

But in continuous culture, fresh nutrient medium is continually supplied to a well-mixed culture, and products and cells are simultaneously withdrawn. Growth and product formation can be maintained for prolonged periods of time in continuous culture.

The reasons proposed for predominant use of batch culture is:

- Many secondary products are not growth associated
- Genetic instability of cultured cells
- Operability and reliability
- Economic considerations

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- state procedure of growth measurement of plant cell suspension cultures
- explain parameters of growth efficiency
- know determination of the concentrations of nutrients or metabolites
- explain the conductivity method
- explain and state cell viability assay.

3.0 MAIN CONTENT

3.1 Procedure of Growth Measurement of Plant Cell Suspension Cultures

Growth of suspension cultures is generally assessed as the settled cell volume, the packed cell volume, fresh cell weight, dry cell weight. Medium residual conductivity and pH measurements are other indirect evaluation methods. Finally, parameters describing growth efficiency, such as specific growth rate (μ), doubling time (t_d), and growth index, is determined.

3.1.1 Growth Curve

It is commonly accepted that growth of a cell suspension culture with respect to time is best described by the sigmoid curve theory. At the beginning, the cell population grows relatively very slow (lag phase). As the population size of plant cell approaches one half of the carrying capacity (defined by the nutrient status of the culture medium), the growth of culture per time unit increases. The growth rate is measured by the steepness of the curve, and it is the steepest when the population

density reaches one-half of the carrying capacity (in the middle of the sigmoid). After that the steepness of the curve decreases until it reaches the carrying capacity (stationary phase). At this time the growth rate slowly decreases due to limitation of nutrients as described in Figure 4.1.

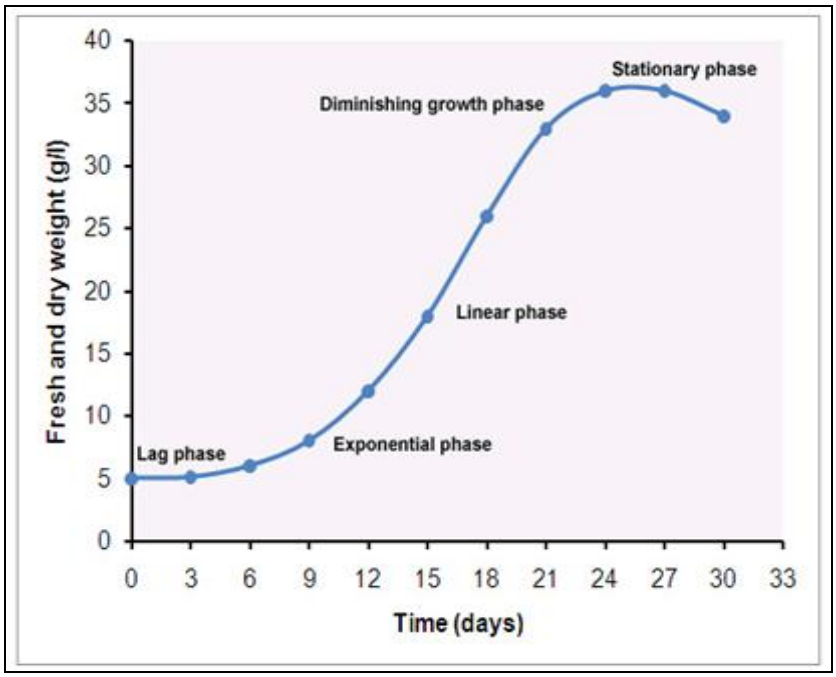

Figure 4.1: Graph showing different growth phases of a typical plant cell suspension culture.

The method needs harvesting cells at frequent intervals to determine the fresh weight and dry weight of cells per volume of cell suspension culture, thus, this is an invasive method. The method is also used to understand the nutrient uptake by the cells in suspension culture. For example, in cell suspension cultures of *Lantana camara*, it was observed that the cultures remained in the lag phase till the second day. Biomass increased till the 12^{th} day following which the stationary phase started (Figure 4.2). There are several methods of evaluating growth kinetics of plant cells. Selected examples include, fresh cell weight, settled volume, packed cell volume, cell optical density, cell size, nitrogen content, protein content, nucleic acid content, mitotic index, electrical conductivity, respiration, and pH measurement. In addition, concentrations of substrate and extracellular product have also been used for such purpose of selecting the best method of studying growth kinetics, especially from the bioprocess engineering point of view.

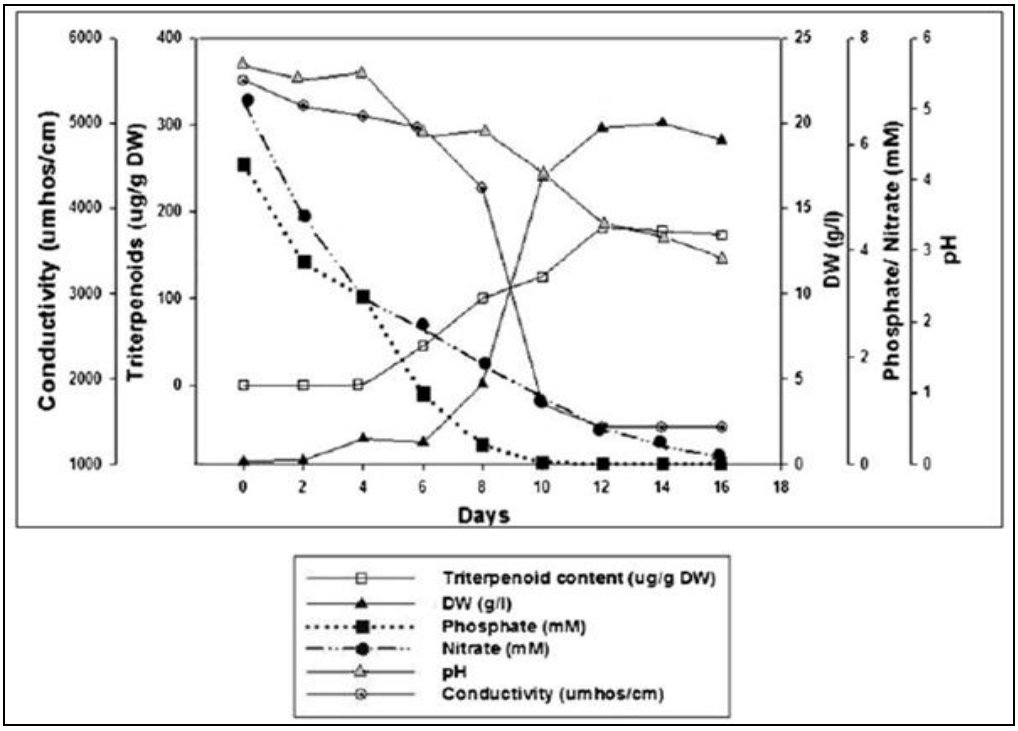

Figure 4.2: Cell growth and nutrient uptake in *L. camara* cells

3.2 Parameters of Growth Efficiency

3.2.1 Growth Index

As described by Loyola-Vargas and V á zquez-Flota (2006) that at a given sampling time, both fresh and dry weights are measurements of complete biomasses of the tissues. No reference to the actual growth capacity is taken in consideration. Growth index (GI) is a relative estimation of such capacity as it correlates the biomass data at the sampling time to that of the initial condition. It is calculated as the ratio of the accumulated and the initial biomass. The accumulated biomass corresponds to the difference between the final and the initial masses.

$$GI = \frac{W_f - W_i}{W_i}$$

Where in this equation, GI is growth index while W_f is final cell mass and W_i is the initial cell mass. Both W_f and W_i are taken either as fresh or dry weight.

3.2.2 Specific Growth Rate

As explained by Loyola-Vargas and V á zquez-Flota (2006), the specific growth rate (μ) refers to the steepness of a curve, and it is defined as the rate of increase of biomass of a cell population per unit of biomass concentration. It can be calculated in batch cultures, since during a defined period of time, the rate of increase in biomass per unit of

biomass concentration is constant and measurable. This period of time occurs between the lag phase and stationary phases. During this period, the increase in the cell population fits a straight-line equation between ln x and t.

$$\ln x = \mu t + \ln x_0$$

$$\mu = \frac{\ln x - \ln x_0}{t}$$

Where, x_o is the initial biomass (or cell density), x is the biomass (or cell density) at time t, and μ is the specific growth rate. Mu can be calculated from the above relationship, which is the slope of the line between ln x and t.

3.2.3 Doubling Time

Doubling time (t_d) is the time required for the concentration of biomass of a population of suspension cells to double. One of the greatest contrasts between the growths of cultured plant cells refers to their respective growth rates. The doubling time (dt) can be calculated according to the following equation (Loyola-Vargas and V á zquez-Flota, 2006)

$$td = \frac{\ln 2}{\mu}$$

n this equation, μ is the specific growth rate. By using the above equation, the specific growth rate of the suspended cells of *L. camara* was found to be 0.1072/day as shown in Figure 4.2.

3.3 Determination of the concentrations of nutrients or metabolites

Some nutrients in the cell suspension culture medium shows correlation with growth in a single culture flask. For example, total nitrate and phosphate levels in the medium can be used to understand the cell growth. Uptake of NH_4^+ ions may result in decrease in pH due to liberation of H^+ ions. Its uptake may be at a slower or faster rate in comparison to phosphate. Complete utilisation of phosphate from culture medium results in the onset of stationary phase sometimes for example, in *L. camara*, it was a major limiting nutrient for growth.

3.4 The Conductivity Method

The conductivity method of measuring growth kinetics of plant cell lines was used especially with the purpose of bioprocess engineering

applications of plant cell cultures. The major advantages of using conductometry as the biosensing technique for measurement of plant cell growth kinetics are:

i. The method is very economical and efficient.
ii. It gives accurate, reliable and reproducible measurements, while amenable to continuous on-line monitoring and process control.
iii. It is a noninvasive method which does not adversely affect the plant cells or the bioreactor operation.
iv. The kind of plant cell lines or their morphology does not affect the method itself.

3.5 Cell Viability Assay

At different parameters, cell viability in suspension cultures can be checked by using 1% fluorescein diacetate (FDA) solution. For an example, cell suspension of *L. camara* cultures were maintained at different agitation speed of 60-150 rpm, but 120 rpm only favored the fine suspension of live and healthy viable cells with small cell aggregates as is observed in fluorescein staining (Figure 4.3).

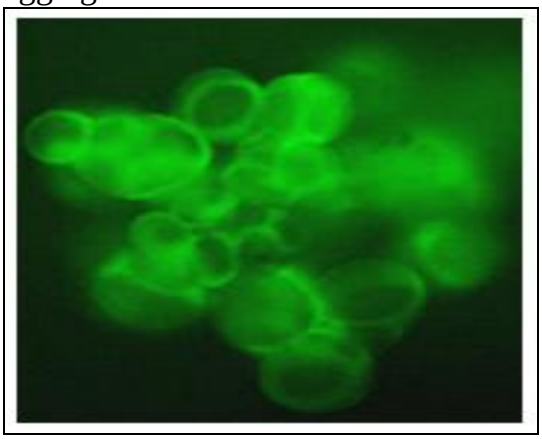

Figure 4.3: 3-week-old cells of *L. camara* stained with 1% fluorescein diacetate solution showing live dividing cells with intact cell wall.

4.0 CONCLUSION

For the development of plant cell culture technology, accurate and rapid measurement of cell growth and assessment of growth-related bioprocess kinetics are essential to the rational development of plant cell bioprocess engineering. Although the plant cell culture system appears to be similar to a microbial cell culture system, there are important differences between the two.

5.0 SUMMARY

The major differences include cell size, aggregation of plant cells, change in plant cell physiology for its primary and secondary metabolisms, rheological properties of the medium, and requirement of plant cells for complex nutrients. Therefore, the best method of assessing the plant cell growth kinetics should be carefully examined and evaluated. The cell suspension culture is much more amenable for biochemical studies and process development than callus cultures. The success in the establishment of a cell suspension culture depends, to a great extent, on the availability of "friable" callus tissue (i.e., a tissue that, when stirred in liquid medium, rapidly disaggregate into single cells and small clusters)

6.0 TUTOR-MARKED ASSIGNMENT

1. For establishment of cell suspesnsion cultures which type of callus tissue is generally considered?
2. Why suspension culture is generally considered for biochemical study and process development?
3. What are the differences between batch culture and continuous culture?
4. Why batch cultures are predominantly used?
5. How growth curve is prepared for suspension culture?
6. Describe the parameters of growth efficiency.
7. Write short notes on:
   A. Growth index
   B. Specific growth rate
   C. Doubling time
   D. Cell viability assay
   E. Advantages of using conductometry as the biosensing technique.

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UNIT 5 BIOREACTORS FOR PLANT ENGINEERING

CONTENTS

1.0 INTRODUCTION

Plant tissue culture is the key method of propagation for a large number of commercially important plants including important vegetatively propagated crops. Over 600 million plants can be produced in a year by tissue culture methods. Agar culture is the main culture technique generally used for commercial tissue culture propagation. It requires a large number of small culture vessels and labor, and results in the requirement of many laminar-air-flow clean benches, large autoclaves, and large culture spaces equipped with illuminated shelves, electric energy, etc. Thus, it is the cause for both limited propagation efficiency and high production costs. In order to overcome these problems, many attempts for establishing large-scale production of propagules with simple production facilities and techniques have been made including robotics, photoautotrophic cultures, bioreactor techniques, etc. Bioreactor technique seems to be the most promising technique among them in reducing the labour, and providing low production cost, which will be sufficient for establishing a practical system for *in vitro* mass propagation and commercialisation of plants. A bioreactor may be referred to as any manufactured or engineered device that supports a biologically active environment. Bioreactors are widely used for industrial production of microbial, animal and plant metabolites as by allowing large-scale cultivation of cells. A simple diagram of a bioreactor is shown in Figure 5.1.

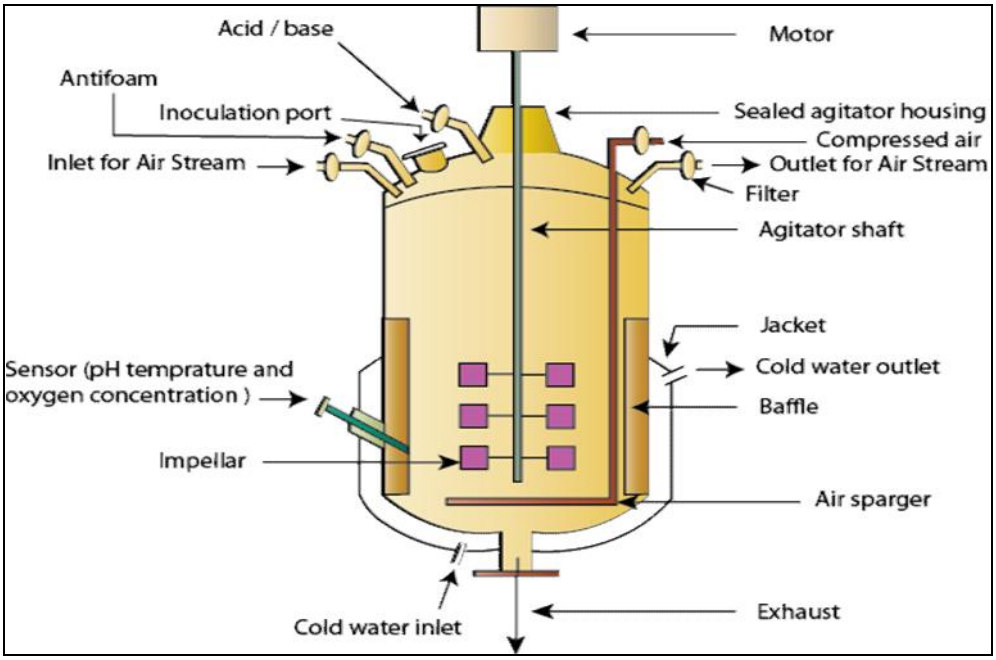

Figure 5.1: Diagram of a typical bioreactor.

2.0 OBJECTIVE

By the end of this unit, you will be able to:

- explain process format
- state applications of bioreactors in plant propagation
- idenfitify scale-up process
- state process design considerations
- outline types of bioreactors
- explain bioreactors for hairy roots.

3.0 MAIN CONTENT

3.1 Process Format

The choice of culture system influences the productivity. There are three main methods for the mass culture of plant cells, **i.** batch culture system, **ii.** continuous culture system and **iii.** semi-continuous (draw-fill). Out of these, the most studies on plant cells have been based on batch cultures system grown in fermentors (or bioreactors) (Fig.5.1). In batch cultivation, an inoculum of known cell density is "seeded" into a specified volume of preconditioned medium in the bioreactor and allowed to grow for a definite period under controlled conditions. The cells containing the desired intracellular metabolite are harvested from the fermentor at the end of the culture period. Typically, nothing is added or removed from the bioreactor during the course of cultivation, except addition of acid/base to control the pH and air to supply oxygen

to respiring plant cells. To start a new process, the bioreactor is cleaned, sterilised and filled with sterile medium and inoculated with the desired plant cells. Various environmental parameters such as pH, temperature and dissolved oxygen can be controlled with the use of pH, temperature and dissolved oxygen probes and the corresponding control systems. In batch culture, the time taken for cleaning etc can be considerable but can be eliminated by the use of continuous culture. In a continuous system, the nutrients consumed by the tissues are continuously replenished by an inflow of fresh medium to the bioreactor. A constant inflow of fresh medium is maintained by a constant efflux of equivalent volume of spent medium plus cells. However, continuous culture of plant cells is difficult because of the presence of aggregates which give a non-homogeneous culture and make sampling difficult. The adhesion of the cells to the walls of the bioreactor also gives problems. The slow growth of the culture means that the supply of fresh medium has to be at a slow rate, which in practice, is difficult. One method of avoiding continuous culture is ‘ draw-fill’ or ‘semi-continuous ' culture where, at the end of the culture period, 90% of the culture is removed and the remaining 10% topped up with fresh, sterile medium. This avoids the cleaning and sterilizing the vessels between the runs.

The continuous or draw-fill cultures are suitable for the production of secondary products that are ‘growth related’. However, secondary products are generally non-growth related and accumulate only after growth has ceased. To improve or stimulate secondary product yield, the medium or culture conditions are often changed; thus, continuous culture or draw-fill are not suitable. The non-growth-related accumulation requires a two-stage process, which can be organised by using a batch culture. In such a system, in the first stage growth of plant cells is optimised and the cells are transferred to a second stage. The second stage contains the nutrients in which product formation takes place in the cells. The culture conditions in the second stage are normally different from those in the first stage.

3.2 Applications of Bioreactors in Plant Propagation

i. Large number of plantlets which are free from physiological disorder can easily be produced in one batch in the bioreactor.
ii. Handling of cultures, such as inoculation and harvest, is easy. It also reduces the number of culture vessels and the area of culture space, which further reduces the overall cost of the production.
iii. Nutrient uptake and growth rate is increased because the surface of the cultures is always in contact with medium.
iv. Forced aeration is performed which improves the growth rate and final biomass.

Many plant species have been cultured in the bioreactor and the responses of cultures in bioreactors may vary from species to species. Following are the list of plant species where bioreactors are used for large scale propagation:

i. Shoots: *Atropa belladonna, Chrysanthemum morifolium, Dianthus caryophyllus, Fragaria ananassa, Nicotiana tabacum, Petunia hybrida, Primula obconica, Zoysia japonica, Scopolia japonica, Spathiphyllum, Stevia rebaudiana*, etc.
ii. Bulbs: *Fritillaria thunbergii, Hippeastrumhybridum, Hyacinthus orientalis, Lilium,* etc.
iii. Corms: *Caladium sp., Colocasia esculenta, Pinelliaternata,* etc.
iv. Tubers: *Solanum tuberosum*
v. Embryos or adventitious buds: *Atropa belladonna*

3.3 Scale-Up Process

Scale-up generally involves taking a lab-scale bioprocess and replicating it as closely as possible to produce larger amounts of product. A typical scale-up sequence in plant cell and tissue culture studies starts with jars, moves to 1 litre shake flasks, after that to 1-10 litre glass bioreactors, then scale-up through to stainless steel vessels of varying sizes from 30-150 litre to 1000 litre. The large-scale cultivation of plant cell and tissue culture is an alternative to the traditional methods of plantation. As compared to microbial cultures, plant cell suspensions, shoot and root cultures pose many different problems in bioreactors during scale-up. Plant cells grow slowly, the cells are large and form clumps, which make them more sensitive to shear associated with agitation and exhibit long processing times. Organ cultures are far more sensitive to shear. These characteristics lead to the necessity to design alternative bioreactor configurations, particularly those that reduce shear within the large-scale bioreactor. Various culture conditions must be monitored to control plant morphogenesis and biomass growth in bioreactors, such as the morphology, oxygen supply and CO_2 exchange, mixing, pH and temperature.

3.4 Process Design Considerations

3.4.1 Aggregation

Due to large size (length up to 200 μm) and slow growing nature, compare to the microbial cells, plant cells are although capable of withstanding tensile strain but are sensitive to shear stress. They have a very rigid cell wall and a culture will contain a wide range of cell shapes and sizes. Unlike many microorganisms, plant cells in suspension culture occur as groups or aggregates. Whether these aggregates arise

due to failure of the cells to separate after division or by cell aggregation is unknown but they are loose structure whose average size and size distribution vary with culture conditions. Further, the secretion of extracellular polysaccharides, particularly in the later stages of growth, may contribute to increased adhesion. A consequence of these characteristics results in sedimentation, insufficient mixing and diffusion-limited biochemical reaction. On the other hand, the aggregate structure has also been implicated in secondary product accumulation, as it provide cell-cell contact and so form micro-environment within the aggregate, which stimulate secondary product synthesis. Hence controlled aggregation of plant cells is of interest from process engineering point of view.

3.4.2 Mixing

Mixing favors cell growth by promoting nutrient transfer from liquid and gaseous phases to cells. It also helps dispersion of air bubbles for effective oxygenation. Although plant cells have higher tensile strength, compare to microbial cells, their shear sensitivity towards hydrodynamic stresses restricts the use of high agitation for efficient mixing. Mixing decrease the mean aggregate size but have an unfavorable effect on cell viability. Plant cells are often grown in stirred tank bioreactors at very low agitation speeds. Sufficient mixing can be achieved by proper design of the impeller; helical-ribbon impeller has been reported to enhance mixing at the high density of plant cell suspension cultures.

3.4.3 Oxygen and Aeration Effects

Plant cells require comparatively lower oxygen than that of microbial cells due to their low growth rates. High oxygen concentration has proved toxic to the cells, metabolic activities, etc. and may strip nutrients, such as carbon dioxide from the culture broth. Carbon dioxide is often considered as an essential nutrient in the culture of plant cells and has a positive effect on cell growth. Hence, the factors that influence efficient oxygen transfer in plant cell cultures must be carefully analysed when a bioreactor system is being selected. The intensity of culture broth mixing, the extent of air bubble dispersion, and the hydrodynamic pressure inside the culture vessel influence suitable aeration of the culture.

High aeration may result into severe foaming, which has significant influence on the cell growth and secondary metabolite production. Foaming of plant cell suspensions is associated with aeration rates and extracellular polysaccharides, fatty acids and high sugar concentrations in the plant cell culture medium. This can result in the wall growth phenomenon and clogging of air exhaust filter and lead to high rate of

contamination. A number of antifoams such as, polypropylene glycol 1025 and 2025, Pluronic PE 6100, and Antifoam-C have often been employed to control foaming; however, in some cases this resulted in reduction in cell growth and product formation.

3.4.4 Shear Sensitivity

Sensitivity of plant cells to hydrodynamic stress related with aeration and agitation can be ascribed to the physical characteristics of the suspended cells, their size, the presence of thick cellulose based cell wall, and presence of large vacuoles. Aeration and mixing system, aeration rate and impeller tip speed generally decide the shear-related effects on plant cells. Mechanically agitated vessels may over-aerate plant cultures, in addition to damaging and breaking the cells through the hydrodynamic stress generated by aeration, agitation, shaking, pumping, and other operations. Low agitation and high aeration provide oxygen in a reasonable mixing range.

3.4.5 Optimisation of Process Parameter

Nutrients directly influence the yield and productivity of metabolites in plant cell suspension cultures. Therefore, it is important to study and quantify the effect of selected key medium components on growth as well as product accumulation and strike a balance between the two to enhance the yield and productivity. This is essential for secondary metabolites production as conditions suitable for growth may adversely affect the product formation and vice versa. The first step in bioprocess media optimization is the identification of relatively significant media components, such as sugars, nitrogen compounds, minerals and growth factors as well as culture conditions and then to determine their optimum levels. The growth of cells in the bioreactor is controlled by using concentration of the growth-limiting nutrient. At steady-state the cell density and substrate concentration are constant. At steady state, $\mu = D$, where $D = F / V$ (F = medium flow rate, V = culture volume).

3.5 Types of Bioreactors

In vitro plant cell culture is currently carried in a diverse range of bioreactor designs, ranging from batch, airlift, and stirred tank to perfusion and continuous flow systems. For a small-scale operation, both the conventional and novel bioreactor designs are relatively easy to operate. For a larger scale of operation, problems of maintaining bioreactor sterility and providing adequate oxygen supply to the cells have yet to be resolved. The bioreactors used for plant cell cultures are classified as under:

- Mechanically agitated bioreactors: stirred tank reactor equipped with various propellers (spin, helix, bladed, paddle), rotary drum tank reactor, etc.
- Air driven bioreactors: bubble column, concentric tube airlift reactor, external loop airlift reactor, propeller loop reactor, jet loop reactor, etc.
- Non-agitated bioreactors: (a) packed bed, (b) fluidized bed, (c) membrane reactor.

Three important scientific and practical issues are involved in bioreactor design and operation for plant cells:

- Cell growth and product formation assessment
- Modeling of the culture dynamics, including the integration of biosynthesis and product separation
- Studies involving the flow, mixing and mass transfer between the phases, in order to define criteria for bioreactor design and scale up.

3.5.1 Mechanically Agitated Bioreactors

The various plant bioreactors designs are proposed by various authors depending upon the plant species used (*see* Bisaria et at, 2002). The most common and popular bioreactor is the stirred tank bioreactor and sufficient knowledge exists about its design and applications. Although it has gained much popularity, stirred tank bioreactors have numerous limitations, such as high-power consumption, high shear, and problems with sealing and stability of shafts in tall bioreactors. In order to diminish the shear forces, numerous modifications have been developed by employing a variety of impeller designs and seals (Figure 5.2. Aa).

Horizontal vessels or rotary drum reactors (Figure 5.2. Ab) have significantly higher surface area to volume ratio than other reactor types. Therefore, mass transfer is achieved with comparably less power consumption. Horizontal vessels used for the cultivation of high-density plant suspensions have shown advantages in terms of suspension homogeneity, low shear environment and reduced wall growth, over either airlift or stirred tank reactors. However, the drawback is their comparatively high energy consumption in large scale operations.

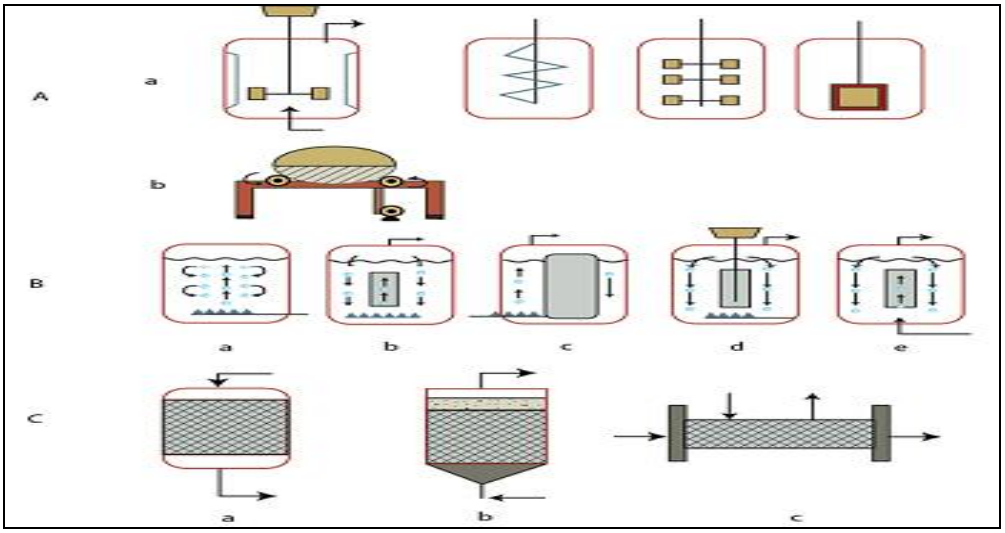

Figure 5.2: Bioreactor types for plant cell, tissue and organ cultures: (A) Mechanically agitated bioreactors: (a) stirred tank reactor equipped with various propellers (spin, helix, bladed, paddle), (b) rotary drum tank reactor; (B) Air driven bioreactors: (a) bubble column, (b) concentric tube airlift reactor (IL ALR), (c) external loop airlift reactor (EL ALR), (d) propeller loop reactor, (e) jet loop reactor; (C) Non-agitated bioreactors: (a) packed bed, (b) fluidised bed, (c) membrane reactor.

3.5.2 Air Driven Bioreactors

A bubble column bioreactor (Figure 5.2. Ba) is a reactor, in the shape of a column, in which the reaction medium is kept mixed and aerated by the introduction of air at the bottom (IUPAC, 297). The major advantages of bubble column bioreactors are the low capital costs, uncomplicated mechanical configurations and less operational costs due to low energy requirements. Alternatively, they are less suitable for the processes where highly viscous liquids exist. In an airlift bioreactor (Figure 5.2. Bb), the reaction medium is agitated and aerated by the introduction of air or another gas mixture and the circulation is improved by internal draught tubes or external loops. Thus, the reactor volume is separated into gassed and degassed regions generating a vertically circulating flow (IUPAC, 297). Airlift bioreactors fulfill the low O_2 demands of plant cell cultures with low shear effects. Airlift bioreactors have a number of advantages, such as combining high loading of solid particles, providing good mass transfer, relatively low shear rate, low energy requirements, and simple design. The main disadvantage is their unsuitability for high density plant cultures. Hence stirred tank bioreactors are preferred for culturing plant cell suspensions at high densities. A further problem in air lift bioreactors is extensive foaming which can clog the air exhaust filters and increase the risk of contamination. To overcome these problems, sparger rings for plant

cells at high aeration rates, bubble free aeration, antifoam agents, etc. can be applied.

3.5.3 Non-Agitated Bioreactors

For the immobilisation of a large number of cells per unit volume packed bed (Figure 5.2. Ca) and membrane reactors (Figure 5.2.Cc) are advantageous. However, diffusional limitations of mass transfer and difficulties in handling gaseous components can limit the use of both configurations (Sajc et al., 2000). Fluidised bed reactor (Figure 5.2. Cb) is based on the utilisation of the energy of the flowing fluid to suspend the particles. This type of reactors provides the major benefit of mass transfer of the small particles.

3.6 Bioreactors for Hairy Roots

While designing a suitable bioreactor for hairy root cultures the physiology and morphology of the hairy roots should be taken into consideration. The major problem in bioreactor cultivation of hairy roots is their tendency to form clumps resulting from the bridging of primary and secondary roots. This results in densely packed root beds and reduces mass transfer (both oxygen and nutrients). Root thickness, root length, the number of root hairs and root branching frequency are some of the factors which should be taken into consideration for hairy root cultures in bioreactors. Immobilisation of hairy roots by horizontal or vertical meshes as well as by cages or polyurethane foam promotes their growth in submerged stirred bioreactors, bubble columns, air lift reactors and drum reactors where the roots are immersed in the culture medium. Isolation of the roots from the impeller also rules out the possibility of root damage even at low tip speeds in stirred bioreactors. Also the oxygen transfer limitation in hairy root cultures in bioreactors can be reduced by growing them in gas phase bioreactors, spray or droplet reactors and mist reactors. Here the roots are exposed to humidified air or a gas mixture and nutrients are delivered as droplets by spray nozzles. Spray and mist reactors also provide the added advantage of low hydrodynamic stress.

4.0 CONCLUSION

High productivity, high product yield and high product concentration are the major objectives of plant tissue process development. A variety of bioreactor types providing growth and expression of bioactive substances are available today for plant cell and tissue cultures. Low biomass and product level can be achieved in any type of bioreactors. However, an improved understanding of the manifold interactions between cultivated cells, product formation and the specific designs for

different bioreactor types will enhance and sustain high productivity and also reduce the process costs.

5.0 SUMMARY

A variety of bioreactor types providing growth and expression of bioactive substances are available today for plant cell and tissue cultures. Low biomass and product level can be achieved in any type of bioreactors. However, an improved understanding of the manifold interactions between cultivated cells, product formation and the specific designs for different bioreactor types will enhance and sustain high productivity and also reduce the process costs.

6.0 TUTOR-MARKED ASSIGNMENT

1. What are the advantages of plant bioreactor?
2. How is batch culture different from continuous or semi-continuous culture of plant cell suspension?
3. What are important parameters need to be considered while designing the suitable plant bioreactor process?
4. Select the two main problems caused by high biomass concentrations in plant cell culture:
   a) rapid settling of the culture
   b) difficult to control temperature
   c) oxygen supply
   d) harvesting the cells
   e) mixing
5. Write short notes on
   a) Mechanically agitated bioreactors
   b) Air driven bioreactors
   c) Non-agitated bioreactors
   d) Bioreactors for hairy roots.

UNIT 6 MANIPULATION IN PRODUCTION PROFILE BY ABIOTIC AND BIOTIC ELICITATION

CONTENTS

1. INTRODUCTION

Treatment with biotic and abiotic compounds helps in the stimulation of particular facets of plant metabolism and enhances the yield of desired secondary metabolites. “Phytoalexins” are the secondary compounds accumulated in response to microbial attack. Accumulation of phytoalexins, which results in chemical resistance, is an important factor in plant defense and has been demonstrated for wide variety of species. Many higher plants are major sources of natural products which are used in pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides. Plant tissue culture is a potential supplement to traditional agriculture in the industrial production of bioactive plant secondary metabolites, which is an alternative to production of desirable medicinal compounds from plants.

Tissue culture is an *in vitro* propagation technique of a wide range of excised plant parts, through which a mass of cells (callus) is produced from an explant tissue. The callus produced, can be utilised to regenerate plantlets or to extract or manipulate primary and secondary metabolites. The signals triggering the formation of phytoalexins are called elicitors. Elicitors have also been shown to induce a range of other plant secondary metabolites. The production of these compounds is a dynamic defense response exhibited by plant cells when challenged by an elicitor. The most commonly used biotic elicitors include fungal homogenates of the genus, like *Phytophthora*, *Aspergillus*, and *Alternaria*, and abiotic elicitors, e.g., inorganic salts of cadmium, copper, and vanadyl, jasmonates (plant hormones that have a dual effect on plant growth and development). By cell culture system only low yields of desired secondary metabolites can be obtained, and efforts are

required to improve the productivity of plant cell cultures by means of elicitation.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain elicitor- induced effects in plant cells
- state the mechanism of elicitation
- outline the special features of elicitors
- explain and state classification of elicitors.

3.0 MAIN CONTENT

3.1 Elicitor- Induced Effects in Plant Cells

Different types of effects are induced in plant cells by the use of elicitors, which is reflected by the influenced cell metabolism.

- Ca^{2+} metabolism
- Massive variation in membrane integrities, respiration, protein and phosphate metabolism, ethylene production and peroxidase activity.
- Differential gene expression, consequently forming enzymes concerned in the synthesis of polysaccharides as callose, hydroxyproline rich glycoproteins (HRGP) in cell walls via induction of proline hydroxylase, lignin and polyphenolics (deposited in cell walls) chitinases and protein inhibitors, specific proteins against pathogenic infections, phytoalexins.

3.2 Mechanism of Elicitation

Elicitors bind to a specific receptor probably located in the plasma membrane. The elicitor-receptor interactions generate signals that then activate nuclear genes involved in plant defense reactions, such as the biosynthesis of phytoalexins. The pathway is initiated by the action of local and systemic signal molecules and putative plasma membrane receptors. Wound signal molecules include polygalacturonic acid, chitosan, physical signals, abscisic acid and systemin. Plasma membrane receptors include a β-glucan-elicitor-binding protein (GEBP), a systemin binding protein of 160 kDa and an unidentified receptor for oligosaccharide elicitors. A lipase translates the wound signal and releases linolenic acid from membrane phospholipid, a process stimulated by ABA, volicitin and β- glucosidase from the oral secretion of insects is converted to jasmonic acid through the octadecanoid pathway.

3.3 Special Features of Elicitors

1. The products which accumulate in plant cell cultures due to elicitation may be antimicrobial in nature, but they should not be grouped in phytoalexins unless there is sufficient proof that the source of plant respond to pathogens is with the rapid accumulation of the same product. Therefore a new term that has been coined for those compounds, which in cell cultures are inducible by way of elicitation, is "Elicitation Product" or "Elicitation Metabolite".
2. Elicitors can be regarded as substitute of production media (optimum cultural conditions).
3. Optimum employment of elicitors depends upon:
   a) Elicitor specificity
   b) Elicitor concentration
   c) Duration of elicitor contact
   d) Elicitor of cell line (clones)
   e) Time course of elicitation
   f) Growth stage of culture
   g) Growth regulation
   h) Nutrient composition
4. Response of the cells to elicitation in suspension cultures may be in the following ways:
   - In a given cell line, different products may show highest level of accumulation, at different times and stages of growth.
   - Product accumulation may be observed in cell lines where area and pathway of synthesis is not known.
   - Elicitation may not cause an additive effect when applied to cells in production media but may shorten the culture period required for maximum product accumulation.
5. Product accumulation due to elicitation has also been observed in growth media. Such occurrence may be due to excretion or leakage caused by cell breakdown

3.4 Classification of Elicitors

Elicitors produced within plant cells are termed as endogenous elicitors, while those produced by microorganisms are called exogenous elicitors. Depending upon their nature, they are classified as biotic and abiotic elicitors.

3.4.1 Biotic Elicitors

Biotic elicitors are either pathogen or host origin that can stimulate defense responses (such as phytoalexin accumulation) in plant tissues. From earlier studies carbohydrates have been known for the overproduction of secondary metabolites in plant cell cultures. Albersheim et al. (277) first isolated oligosaccharides that activate a variety of plant defense genes. The signal transfer triggered by carbohydrate elicitors has been studied with regard to calcium influx, pH shifts and production of H_2O_2 in tobacco cell cultures.

Examples of Elicitors

- The combination of oligosaccharides and methyl jasmonate has been employed to induce phytoalexins in rice systems (Nojiri *et al.* 296).
- Production of paclitaxel in *Taxus canadensis* cell suspension cultures was enhanced when the cultures were treated with a combination of N-acetyl ketohexose and methyl jasmonate (Linden *et al.* 2000).
- Methyl jasmonate, a lipid-derived elicitor, was also applied as an elicitor in combination with chitopentaose to *J. chinensis* cell suspension cultures for the enhancement of podophyllotoxin production. (Premjet *et al*, 2002).
- Fungal elicitor is normally one which is derived from a fungus (*Phytophthora, Botrytis* , *Verticilium, Alternaria, Fusarium* , etc.) pathogenic to the plant species. Although preparations derived from non-pathogenic (*Aspergillus, Micromucor, Rhodotorula*, etc) fungus have also been successfully employed for the elicitation purpose.

The latter type of elicitors is often released by mechanical wounding or enzymatic hydrolysis of polymeric compounds in plants which are polysaccharides of plant cell walls. These are of two types: β-linked glucans and chitosan. Another group of elicitors are enzymes with polygalactomarase activity, which releases pectic fragments from plant cell walls. It has been observed that very small amount of such water-soluble oligomers lead to rapid induction of phytoalexins in cell cultures. Some more examples of biotic elicitor are listed in table 37.1.

Table 6.1: Examples of biotic elicitors

Biotic elicitors	Plant species	product
Chitosan	*Lupinus albus*	Isoflavonoids
N-Acetylchitohexaose	*Taxus canadensis*	Taxol
Mannan	*Hypericum perforatum*	Hypericins
Oligogalacturonic acid	*Panax ginseng*	Saponin
Yeast elicitor, Methyl jasmonate	*Rauvolfia canescens*	Raucaffricine
Methyl jasmonate	*Glycyrrhiza glabra*	Soyasaponin 5-deoxyflavonoid
Yeast elicitor	*Coleus blumei*	Rosmarinic acid
Polysaccharide	*Allium cepa*	Tcibulin1, Tcibulin2
Polysaccharides, fungal elicitor, Methyl jasmonate	*Lithospermum erythrorhizon*	Shikonin, Rosmarinic acid

3.4.2 Abiotic Elicitors

All the factors which cannot be regarded as natural components of the environment of a plant cell are considered as abiotic elicitors. Abiotic elicitors are of non-biological origin mainly the metal ions. Also the abiotic elicitors are of physical or chemical nature working via endogenously formed biotic elicitors. Some examples of abiotic elicitor are listed in table 6.2. Salicylic acid, methyl jasmonate, calcium chloride, silver nitrate, copper sulphate, cinnamic acids, etc. Acids can be employed for eliciting the plant cell cultures.

The use of metal ions as elicitors offers many advantages over their biotic counterparts, these include:

- their ready availability
- relatively low cost
- ease of use
- they are chemically defined

Table 6.2: Examples of abiotic elicitors

Abiotic elicitors	Plant species	Product
Arachidonic acid	*Capsicum annuum*	Capsidol, Rishitin
Copper chloride	*Matricaria chamomilla*	Hemiarin, Umbelliferone
Copper sulphate	*Hyoscyamus albus*	Phytoalexin
Cd^{2+}, Cu^{2+}	*Atropa belladonna*	Casaicin
Curdlan, Xanthan	*Capsicum frutescence*	Indole alkaloids
Salicylic acid	*Daucus carota*	Chitinase
Vanadium sulphate	*Catharanthus roseus*	Catharanthine

4.0 CONCLUSION

Treatment with biotic and abiotic compounds helps in the stimulation of particular facets of plant metabolism and enhances the yield of desired secondary metabolites.

5.0 SUMMARY

“Phytoalexins” are the secondary compounds accumulated in response to microbial attack. Accumulation of phytoalexins, which results in chemical resistance, is an important factor in plant defense and has been demonstrated for wide variety of species. Many higher plants are major sources of natural products which are used in pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides. Plant tissue culture is a potential supplement to traditional agriculture in the industrial production of bioactive plant secondary metabolites, which is an alternative to production of desirable medicinal compounds from plants.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is elicitor?
2. What are the effects of elicitor in plant cell?
3. Describe mechanism of elicitation.
4. Write down the features of elicitors?
5. What are the factors affecting elicitation?
6. What are the responses of the cells to elicitation in suspension cultures?
7. How many type of elicitor are found?
8. Write short notes on:
   A. Elicitor
   B. Biotic elicitor
   C. Abiotic elicitor.

UNIT 7 BIOTRANSFORMATION

CONTENTS

1.0 INTRODUCTION

Biotransformation is chemical reactions catalyzed by cells, organs or enzymes. It is defined as a process through which the functional groups of organic compounds are modified by living cells to a chemically different product. Biotransformation explores the unique properties of biocatalysts, namely their stereo-and region-specificity and their ability to carry out reactions at no extreme pH values and temperatures. Biotransformation may be used to carry out specific conversions of complex substrates using plant, animal or microbial cells or purified enzymes as catalyst. Biotransformation is different from biosynthesis where complex products are assembled from simple substrates by whole cells, organs or organisms. They are also different from biodegradations in which complex substances are broken down to simple ones. Biotransformation has great potential to generate novel products or to produce known products more efficiently.

The production of food metabolites, fine chemicals and pharmaceuticals can be achieved by biotransformation using biological catalysts. Cell suspension cultures, immobilised cells, hairy root cultures can be useful for the production of food additives and pharmaceuticals by biotransformation process. Plant cells for biotransformation purposes are selected because of two main reasons. Plant cells are usually able to catalyse the reactions stereospecifically, resulting in chirally pure products. They can carry out regiospecific modifications that are not easily carried out by chemical synthesis or by microorganisms. These reactions include reduction, oxidation, hydroxylation, acetylation,

esterification, glucosylation, isomerisation, methylation, demethylation, epoxidation, etc.
However, for a successful and viable process, the following prerequisites must be met:

- The culture must have the essential enzymes.
- The substrate or precursor must not be toxic to the cell culture.
- The substrate must reach the appropriate cellular compartment of the cell.
- The rate of product formation must be faster than its further metabolism.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain biotransformation using plant cells and organ cultures
- explain biotransformation using immobilized cell culture
- identify genetic engineering approaches towards biotransformation
- list advantages of biotransformation
- state factors influencing biotransformation.

3.0 MAIN CONTENT

3.1 Biotransformation Using Plant Cells and Organ Cultures

The biotransformation rates by plant cells and organs are depend on a variety of factors including the solubility of precursors, the amount of enzyme activity present, localisation of enzymes, presence of side reactions producing undesired byproduct and presence of enzymes degrading the desired product. Elicitation, permeabilisation, pH variation and osmotic effects can also influence biotransformation capacity of cells. Some examples of biotransformation reactions performed by *in vitro* plant cell and organ cultures are given below:

- *Peganum harmala* cell culture converted geranyl acetate to geraniol and linalyl acetate to linalool and α-terpineol.
- The alkaloid nitrosamine, which contains seven stereogenic centers, is present in *Nitrariaschoberi*as a racemate. Isolation of a chiral metabolite might be due to spontaneous nonenzymatic reactions starting from an achiral precursor followed by enzyme-catalysed metabolism of one of the enantiomers.

- *Catharanthus roseus* suspension cell cultures can oxidise the phenylsulphonyl group from completely synthetic molecules to phenylsulfonyl derivatives.

Biotransformation of cinobufagin by *C. roseus* cell suspension cultures is shown in **Figure 7.1**.

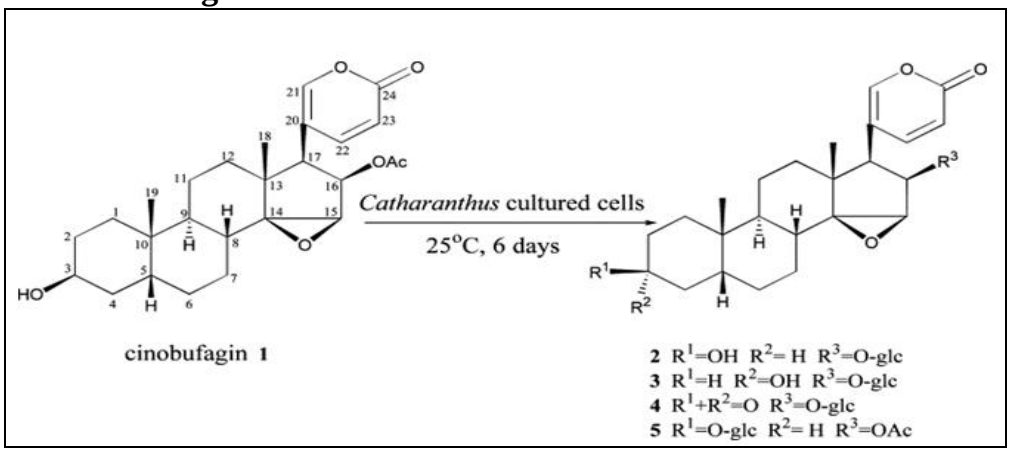

Figure 7.1: Biotransformation of cinobufagin by *C. roseus* cell suspension cultures

Table 7.1: Examples of biotransformation

Substrate	Main product	Types of reaction	Plant species
Nerol	Geranial, neral	Oxidation of OH	*Rosa centifolia*
Neryl acetate	Geranial, neral	Oxidation of OH	*Rosa centifolia*
Valencene	Nootkatone	Hydroxylation, Dehydrogenation	*Citrus paradisi*
Steviol	Rubusoside	Glucosylation	*Coffea arabica*

3.2 Biotransformation Using Immobilised Cell Culture

Entire cells offer the chance to implement multistep biotransformation and to utilise and recycle essential cofactors and co-enzymes. Isolated enzymes may be sensitive to denaturing conditions, including pH, heat and specific organic solvents. In order to be useful in biotransformation reactions, biocatalysts need to be stable and reusable. Use of whole cell immobilised system may help overcome some stability problems. Immobilised plant cells have some additional advantages over freely suspended cells. They are more resistant to shear damage and can be used repeatedly over a prolonged period. Complete cell immobilisation may also create adverse conditions under which secondary metabolite production may be improved. A very common method for immobilisation of plant cells are gel entrapment by ion exchange, precipitation, polymerisation and in preformed structures. For

adsorption of plant cells, solid surfaces can be used. Enzymes may be adsorbed to insoluble supports by hydrogen bonding, dipole–dipole interactions and hydrophobic interactions. Commonly used supports are polypropylene and diatomaceous celite.

3.3 Genetic Engineering Approaches Towards Biotransformation

Bioconversion/biotransformation capacity of cell cultures can be further improved by changing the following parameters:

- Cell selection
- Elicitation
- Permeabilisation
- Radiation
- pH of medium
- Osmotic shock

A more basic approach is the transfer of genes that code for the key enzymes catalysing the desired biosynthetic reactions into a fungal or bacterial cell because of their ability to produce high amounts of enzymes (Pras et al., 295). Hashimoto et al. (293) explain the expression of hyoscyamine 6-b-hydroxylase in *Escherichia coli* . This recombinant bacterium was capable to convert hyoscyamine to scopolamine. Subsequently, this cloned gene has been transferred to *Atropa belladonna* and expressed constitutively. Additionally, he also found that transformed hairy roots with increased efficiency of conversion of hyoscyamine to scopolamine. Cloning and expression of bacterial lysine decarboxylase under the control of a 35S promoter fused to the coding sequences of the small subunit of rubisco transit peptide in tobacco root cultures was found to affect two secondary metabolic pathways (Berlin et al., 298).

3.4 Advantages of Biotransformation

The advantages consist of the production of novel compounds, improvement in the productivity of desired compound and overcoming the problems related with chemical synthesis. Biotransformation studies lead to basic information to elucidate the biosynthetic pathway, and catalysis can be carried out under mild conditions, thus reducing undesired by-products, energy, safety and costs.

3.5 Factors Influencing Biotransformation

3.5.1 Improvement of Cell Viability

Many substances are harmful to cultured cells. So it is necessary to decrease the toxicity in order to increase the yield of the product. Sugar can increase cell viability during glycosylation of phenolic compounds. Antioxidants can improve cell viability and increase product formation in the biotransformation of phenolics. Yokoyama (291) reported that antioxidant, such as gallic acid, ascorbic acid; cystein and tannins could increase the production of arbutin when hydroquinone was added to the cell culture.

3.5.2 Selection of Plant Species

The capacity for biotransformation is diverse among plant species. Tabata et.al (288) reported that among seven species of plant cell cultures, only *Datura* had capacity to biotransform coumarins, flavonoids, phenolic acids and anthraquinones.

3.5.3 Immobilised Plant Cells

It has distinct advantages e.g. reuse of the expensive biocatalyst, continuous process, and process control is simplified.

3.5.4 Root Culture

Cell suspension culture has excellent biotransformation capacity for glucosylation. Furuya et al. (289) have found that the root culture showed higher glycosylation activity than cell culture.

4.0 CONCLUSION

Biotransformation is chemical reactions catalysed by cells, organs or enzymes. It is defined as a process through which the functional groups of organic compounds are modified by living cells to a chemically different product. Biotransformation explores the unique properties of biocatalysts, namely their stereo-and region-specificity and their ability to carry out reactions at no extreme pH values and temperatures. Biotransformation may be used to carry out specific conversions of complex substrates using plant, animal or microbial cells or purified enzymes as catalyst.

5.0 SUMMARY

Biotransformation is different from biosynthesis where complex products are assembled from simple substrates by whole cells, organs or organisms. They are also different from biodegradations in which complex substances are broken down to simple ones. Biotransformation has great potential to generate novel products or to produce known products more efficiently. The production of food metabolites, fine chemicals and pharmaceuticals can be achieved by biotransformation using biological catalysts. Cell suspension cultures, immobilised cells, hairy root cultures can be useful for the production of food additives and pharmaceuticals by biotransformation process. Plant cells for biotransformation purposes are selected because of two main reasons. Plant cells are usually able to catalyse the reactions stereospecifically, resulting in chirally pure products. They can carry out regiospecific modifications that are not easily carried out by chemical synthesis or by microorganisms. These reactions include reduction, oxidation, hydroxylation, acetylation, esterification, glucosylation, isomerisation, methylation, demethylation, epoxidation, etc.

However, for a successful and viable process, the following prerequisites must be met:

- The culture must have the essential enzymes.
- The substrate or precursor must not be toxic to the cell culture.
- The substrate must reach the appropriate cellular compartment of the cell.
- The rate of product formation must be faster than its further metabolism.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is biotransformation?
2. What are the factors involves in plant biotransformation?
3. Give some examples of plant biotransformation using plant cell culture.
4. Describe about biotransformation using immobilised cell culture.
5. Describe genetic engineering approaches towards biotransformation.
6. What are the advantages of biotransformation?

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UNIT 8 ADVANTAGES OF PLANT CELL, TISSUE AND ORGAN CULTURE AS SOURCE OF SECONDARY METABOLITES

CONTENTS

1.0 INTRODUCTION

Plant tissue culture can be defined as the *in vitro* manipulation of plant cells and tissues and is a keystone in the foundation of plant biotechnology. It is useful for plant propagation and in the study of plant growth regulators. It is generally required to manipulate and regenerate transgenic plants. Whole plants can be regenerated under *in vitro* conditions using plant organs, tissues or single cells, by inoculating them in an appropriate nutrient medium under sterile environment. Plant tissue culture relies on the fact that many plant cells have the capacity to regenerate into a whole plant–a phenomena known as tot potency. Plant cells, cells without cell walls (protoplasts), leaves, or roots can be used to generate a new plant on culture media containing the necessary nutrients and plant growth regulators. Plant tissue culture was first attempted by Haberlandt (202). He grew palisade cells from leaves of various plants but they did not divide. In 234, White generated continuously growing cultures of meristematic cells of tomato on medium containing salts, yeast extract and sucrose and vitamin B (pyridoxine, thiamine and nicotinic acid) and established the importance of additives. In 253, Miller and Skoog, University of Wisconsin – Madison discovered Kinetin, a cytokine that plays an active role in organogenesis. Plant cell cultures are an attractive alternative source to whole plants for the production of high-value secondary metabolites.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- state advantages of plant tissue culture over conventional agricultural production
- explain plant secondary metabolites
- outline strategies for enhanced production of secondary metabolites in plant cell cultures
- state advantages of cell, tissue and organ cultures as sources of secondary metabolites.

3.0 MAIN CONTENT

3.1 Advantages of Plant Tissue Culture over Conventional Agricultural Production

The most important advantage of *in vitro* grown plants is that it is independent of geographical variations, seasonal variations and also environmental factors. It offers a defined production system, continuous supply of products with uniform quality and yield. Novel compounds which are not generally found in the parent plants can be produced in the in vitro grown plants through plant tissue culture. In addition, stereo- and region- specific biotransformation of the plant cells can be performed for the production of bioactive compounds from economical precursors. It is also independent of any political interference. Efficient downstream recovery of products and rapidity of production are its added advantages (Figure 8.1).

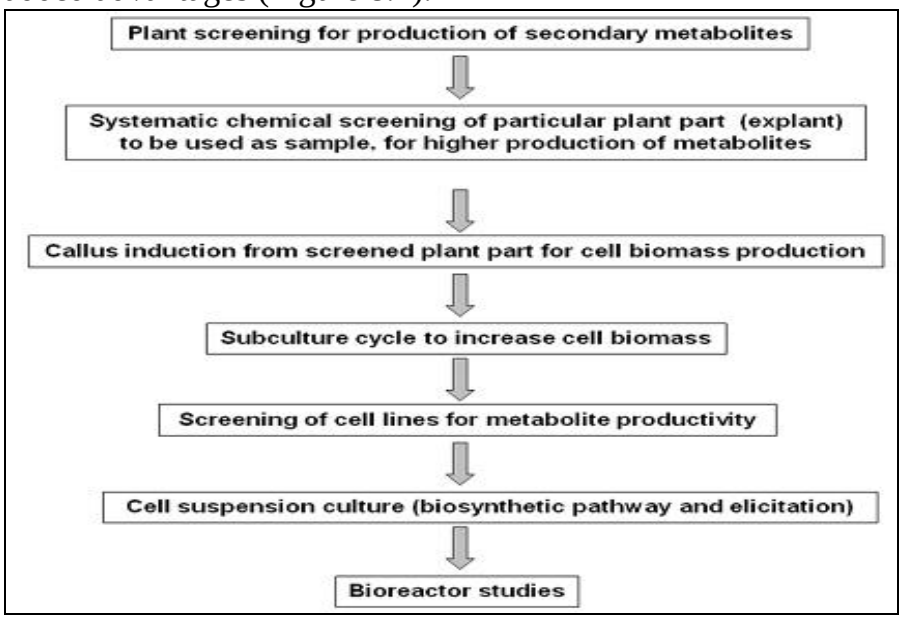

Figure 8.1: *Steps involved in the production of secondary metabolites from plant cell*

3.2 Plant Secondary Metabolites

Plant products can be classified into primary plant metabolites and secondary metabolites. Primary plant metabolites are essential for the survival of the plant. It consists of sugars, amino acids and nucleotides synthesized by plants and are used to produce essential polymers. Typically, primary metabolites are found in all species within broad phylogenetic groupings, and are produced using the same metabolic pathway. Secondary metabolites are the chemicals, which are not directly involved in the normal growth and development, or reproduction of an organism. Secondary metabolites are not indispensable for the plants but play a significant role in plant defense mechanisms. Primary metabolites essentially provide the basis for normal growth and reproduction, while secondary metabolites for adaptation and interaction with the environment. The economic importance of secondary metabolites lies in the fact that they can be used as sources of industrially important natural products like colours, insecticides, antimicrobials, fragrances and therapeutics. Therefore, plant tissue culture is being potentially used as an alternative for plant secondary metabolite production. Majority of the plant secondary metabolites of interest to humankind fit into categories which categorise secondary metabolites based on their biosynthetic origin. Secondary metabolism in plants is activated only in particular stages of growth and development or during periods of stress, limitation of nutrients or attack by micro-organisms.

Plants produce several bioactive compounds that are of importance in the healthcare, food, flavor and cosmetics industries. Many pharmaceuticals are produced from the plant secondary metabolites. Currently, many natural products are produced solely from massive quantities of whole plant parts. The source plants are cultured in tropical, subtropical, geographically remote areas, which are subject to drought, disease and changing land use patterns and other environmental factors. Secondary metabolites can be derived from primary metabolites through modifications, like methylation, hydroxylation and glycosylation. Secondary metabolites are naturally more complex than primary metabolites and are classified on the basis of chemical structure (e.g., aromatic rings, sugar), composition (containing nitrogen or not), their solubility in various solvents or the pathway by which they are synthesized (Table 8.1). They have been classified into terpenes (composed entirely of carbon and hydrogen), phenolics (composed of simple sugars, benzene rings, hydrogen and oxygen) and nitrogen and/or sulphur containing compounds (Figure 8.2). It has been observed that each plant family, genus and species produces a characteristic mix of these bioactive compounds.

All plants produce secondary metabolites, which are specific to an individual species, genus and are produced during specific environmental conditions which makes their extraction and purification difficult. As a result, commercially available secondary metabolites, for example, pharmaceuticals, flavours, fragrances and pesticides etc. are generally considered high value products as compared to primary metabolites and they are considered to be fine chemicals.

Table 8.1: Classification of secondary metabolites

Terpenes		Phenols		Nitrogen and /or sulphur containing compounds	
Type	Example	Type	Example	Type	Example
Monoterpenes	Farnessol	Lignan	lignan	Alkaloids	Nicotine
Sespuiterpenes	Limonene	Tannins	gallotannin	Atropine	
Diterpenes	Taxol	Flavonoids	anthocyanin	Glucosinolates	Sinigrin
Triterpenes	Digitogenin	Coumarins	Umbelliferone		
Tetraterpenoids	Carotene				
Sterols	Spinasterol				

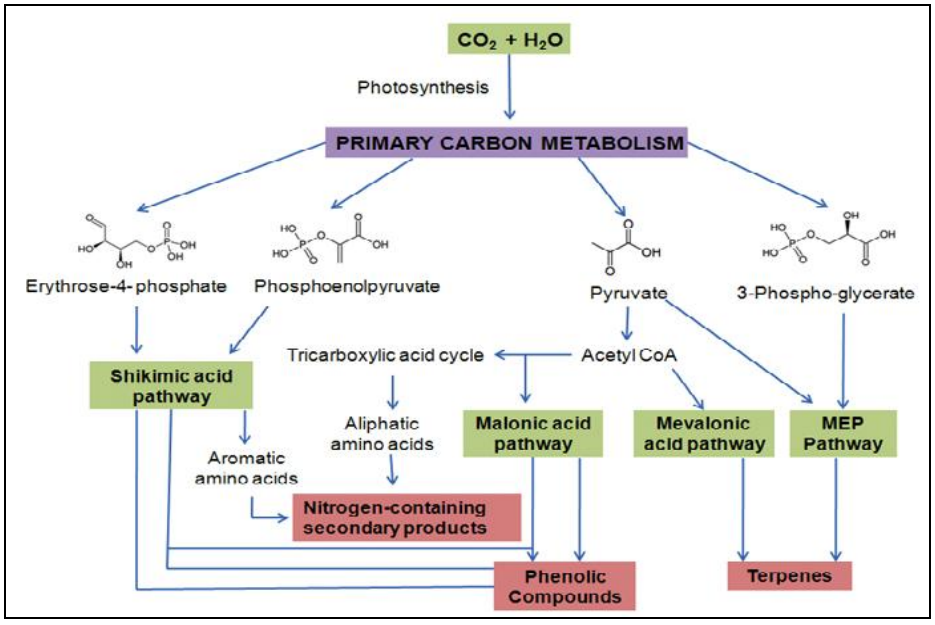

Figure 8.2: *The production of secondary metabolites is tightly associated with the pathways of primary/central metabolism, such as glycolysis, shikimate and production of aliphatic amino acids.*

3.3 Strategies for Enhanced Production of Secondary Metabolites in Plant Cell Cultures

3.3.1 Proper Selection of Cell Lines

The heterogeneity within the cell population can be screened by selecting cell lines capable of accumulating higher level of metabolites.

3.3.2 Manipulation of Medium

The constituents of culture medium, like nutrients, phytohormones and also the culture conditions, like temperature, light etc. influence the production of secondary metabolites. For e.g., if sucrose concentration is increased from 3% to 5%, production of rosamarinic acid is increased by five times. In case of shikonin production, IAA enhances the yield whereas 2,4-D and NAA are inhibitory.

3.3.3 Addition of Elicitors

Elicitors are the compounds which induce the production and accumulation of secondary metabolites in plant cells. Elicitors produced within the plant cells include cell wall derived polysaccharides, like pectin, pectic acid, cellulose etc. Product accumulation also occurs under stress conditions caused by physical or chemical agents like UV, low or high temperature, antibiotics, salts of heavy metals, high salt concentrations which are grouped under abiotic elicitors. Addition of these elicitors to the medium in low concentration enhances the production of secondary metabolites.

3.3.4 Addition of Precursors

Precursors are the compounds, whether exogenous or endogenous, that can be converted by living system into useful compounds or secondary metabolites. It has been possible to enhance the biosynthesis of specific secondary metabolites by feeding precursors to cell cultures. For example, amino acids have been added to suspension culture media for production of tropane alkaloids, indole alkaloids. The amount of precursors is usually lower in callus and cell cultures than in differentiated tissues. Phenylalanine acts as a precursor of rosmarinic acid; addition of phenylalanine to *Salvia officinalis* suspension cultures stimulated the production of rosmarinic acid and decreased the production time as well. Phenylalanine also acts as precursor of the N-benzoylphenylisoserine side chain of taxol; supplementation of *Taxus cuspidata* cultures with phenylalanine resulted in increased yields of taxol. The timing of precursor addition is critical for an optimum effect.

The effects of feedback inhibition must surely be considered when adding products of a metabolic pathway to cultured cells.

3.3.5 Permeabilisation

Secondary metabolites produced in cells are often blocked in the vacuole. By manipulating the permeability of cell membrane, they can be secreted out to the media. Permeabilisation can be achieved by electric pulse, UV, pressure, sonication, heat, etc. Even charcoal can be added to medium to absorb secondary metabolites.

3.3.6 Immobilisation

Cell cultures encapsulated in agarose and calcium alginate gels or entrapped in membranes are called immobilised plant cell cultures. Immobilisation of plant cells allows better cell to cell contact and the cells are also protected from high shear stresses. These immobilized systems can effectively increase the productivity of secondary metabolites in a number of species. Elicitors can also be added to these systems to stimulate secondary metabolism.

3.3.7 Limitations

- Production cost is often very high.
- Lack of information of the biosynthetic pathways of many compounds is a major drawback in the improvement of their production.
- Trained technical manpower is required to operate bioreactors.

3.4 Advantages of Cell, Tissue and Organ Cultures as Sources of Secondary Metabolites

3.4.1 Plant Cell Cultures

Once interesting bioactive compounds have been were identified from plant extracts, the first part of the work consisted in collecting the largest genetic pool of plant individuals that produce the corresponding bioactive substances. However, a major characteristic of secondary compounds is that their synthesis is highly inducible; therefore, it is not certain, if a given extract is a good indicator of the plant potential for producing the compounds. The ability of plant cell cultures to produce secondary metabolites came quite late in the history of *in vitro* techniques. For a long time, it was believed that undifferentiated cells, such as callus or cell suspension cultures were not able to produce secondary compounds, unlike differentiated cells or specialized organs.

3.4.2 Callus Culture

Callus is a mass of undifferentiated cells derived from plant tissues for use in biological research and biotechnology. In plant biology, callus cells are those cells that cover a plant wound. To induce callus development, plant tissues are surface sterilized and then plated onto *in vitro* tissue culture medium. Different plant growth regulators, such as auxins, cytokinins, and gibberellins, are supplemented into the medium to initiate callus formation. It is well known that callus can undergo somaclonal variations, usually during several subculture cycles. This is a critical period where, due to *in vitro* variations, production of secondary metabolite often varies from one subculture cycle to another. When genetic stability is reached, it is necessary to screen the different cell (callus) lines according to their aptitudes to provide an efficient secondary metabolite production. Hence, each callus must be assessed separately for its growth rate as well as intracellular and extracellular metabolite concentrations. This allows an evaluation of the productivity of each cell line so that only the best ones will be taken for further studies, for example, for production of the desired compound in suspensions cultures.

3.4.3 Cell suspension cultures

Cell suspension cultures represent a good biological material for studying biosynthetic pathways. They allow the recovery of a large amount of cells from which enzymes can be easily separated. Compared to cell growth kinetics, which is usually an exponential curve, most secondary metabolites are often produced during the stationary phase. This lack of production of compounds during the early stages can be explained by carbon allocation mainly distributed for primary metabolism when growth is very active. On the other hand, when growth stops, carbon is no longer required in large quantities for primary metabolism and secondary compounds are more actively synthesized. However, some of the secondary plant products are known to be growth-associated with undifferentiated cells, such as betalains and carotenoids.

3.4.4 Organ cultures

Plant organs are alternative to cell cultures for the production of plant secondary metabolites. Two types of organs are generally considered for this objective: hairy roots and shoot cultures. A schematic representation of various organised cultures, induced under *in vitro* conditions, is given in Figure 8.3.

3.4.4.1 Shoot Cultures

Shoots exhibit some comparable properties to hairy roots, genetic stability and good capacities for secondary metabolite production. They also provide the possibility of gaining a link between growth and the production of secondary compounds.

3.4.4.2 Hairy Root Cultures

Hairy roots are obtained after the successful transformation of a plant with *Agrobacterium rhizogenes*. They have received considerable attention of plant biotechnologists, for the production of secondary compounds. They can be subcultured and indefinitely propagated on a synthetic medium without phytohormones and usually display interesting growth capacities owing to the profusion of lateral roots. This growth can be assimilated to an exponential model, when the number of generations of lateral roots becomes large.

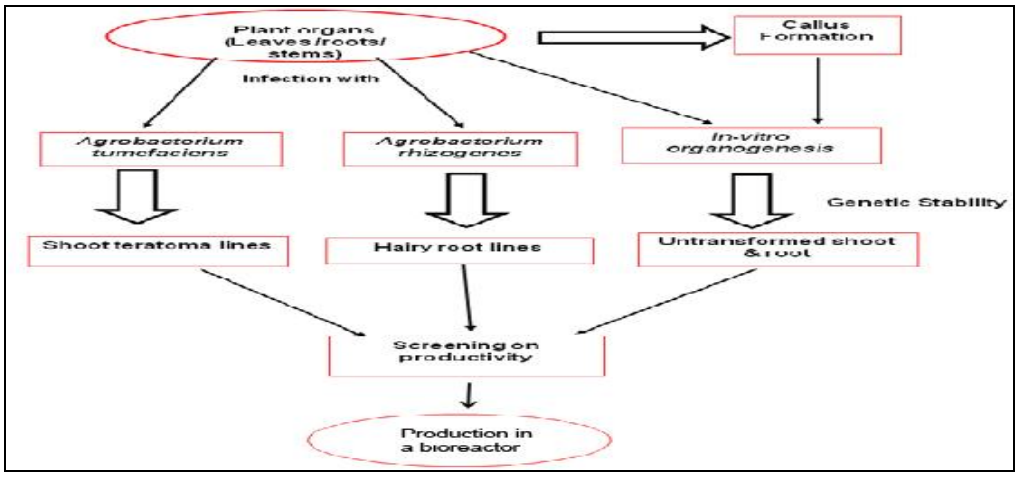

Figure 8.3: Guidelines for the production of secondary metabolites from plant organ cultures.

4.0 CONCLUSION

Plant tissue culture can be defined as the *in vitro* manipulation of plant cells and tissues and is a keystone in the foundation of plant biotechnology. It is useful for plant propagation and in the study of plant growth regulators. It is generally required to manipulate and regenerate transgenic plants. Whole plants can be regenerated under *in vitro* conditions using plant organs, tissues or single cells, by inoculating them in an appropriate nutrient medium under sterile environment. Plant tissue culture relies on the fact that many plant cells have the capacity to regenerate into a whole plant–a phenomena known as tot potency.

5.0 SUMMARY

Plant cells, cells without cell walls (protoplasts), leaves, or roots can be used to generate a new plant on culture media containing the necessary nutrients and plant growth regulators. Plant tissue culture was first attempted by Haberlandt (202). He grew palisade cells from leaves of various plants but they did not divide. In 234, White generated continuously growing cultures of meristematic cells of tomato on medium containing salts, yeast extract and sucrose and vitamin B (pyridoxine, thiamine and nicotinic acid) and established the importance of additives. In 253, Miller and Skoog, University of Wisconsin – Madison discovered Kinetin, a cytokine that plays an active role in organogenesis. Plant cell cultures are an attractive alternative source to whole plants for the production of high-value secondary metabolites.

6.0 TUTOR-MARKED ASSIGNMENT

1. How secondary metabolites are produced?
2. What is the importance of plant cell culture in production of secondary metabolites?
3. What are the steps involved in the production of secondary metabolites from plant cell?
4. How production of secondary metabolites is associated with primary metabolites?
5. What are the strategies for the enhancement of production of secondary metabolites?
6. Give examples of following:
   A. Alkaloid B. Coumarin C. Flavonoid D. Sterol E. Triterpens
7. Following compounds are which class of terpenes?
   A. Taxol B. Digitogenin C. Farnesol D. Spinasterol.

7.0 REFERENCES/FURTHER READING

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MODULE 5 APPLICATION OF CELL CULTURE SYSTEMS IN METABOLIC ENGINEERING

UNIT 1 HAIRY ROOT CULTURES

CONTENTS

1.0 INTRODUCTION

Plant remains major source of pharmaceuticals and fine chemicals and cell cultures have been viewed as promising alternatives to whole plant extraction for obtaining valuable chemicals. The major constraint with the cell culture is that they are genetically unstable and tend to produce low yield of secondary metabolites. A new method for enhancing secondary metabolite production is by transformation of cells or tissues using the natural vector system. *Agrobacterium rhizogenes*, the

causative agent of hairy root disease, is a soil dwelling gram negative bacterium capable of entering a plant through a wound and causing a proliferation of secondary roots. The mechanism of transformation is elaborated in Figure 1.1. The biosynthetic capacity of the hairy root cultures is equivalent or sometimes more to the corresponding plant roots. Therefore, hairy root cultures have been developed as an alternate source for the production of root biomass and to obtain root derived compounds.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain establishment of hairy root cultures
- state the genetics of transformation
- state the genes responsible for hairy root formation
- state the factors influencing the transformation
- discuss confirmation of transformation
- explain screening of transformation
- explain and state properties of hairy roots
- state the application of hairy root cultures.

3.0 MAIN CONTENT

3.1 Establishment of Hairy Root Cultures

For the production of hairy root cultures, the explant material is inoculated with a suspension of *A. rhizogenes.* The bacterial suspension is generated by growing bacteria in Yeast Mannitol Broth (YMB) medium for two days at 25°C under shaking conditions. Thereafter, pelleting by centrifugation (5 x 10 rpm; 20 min) and resuspending the bacteria in YMB medium to form a thick suspension (approx. 10^{10} viable bacteria/ml). Transformation may be induced in aseptic seedlings or surface sterilised detached leaves, leaf-discs, petioles, stem segments, from greenhouse grown plants by scratching the leaf midrib or the stem of a plantlet with the needle of a hypodermic syringe containing a small (about 5-10 ul) droplet of thick bacterial suspension of *A. rhizogenes.*

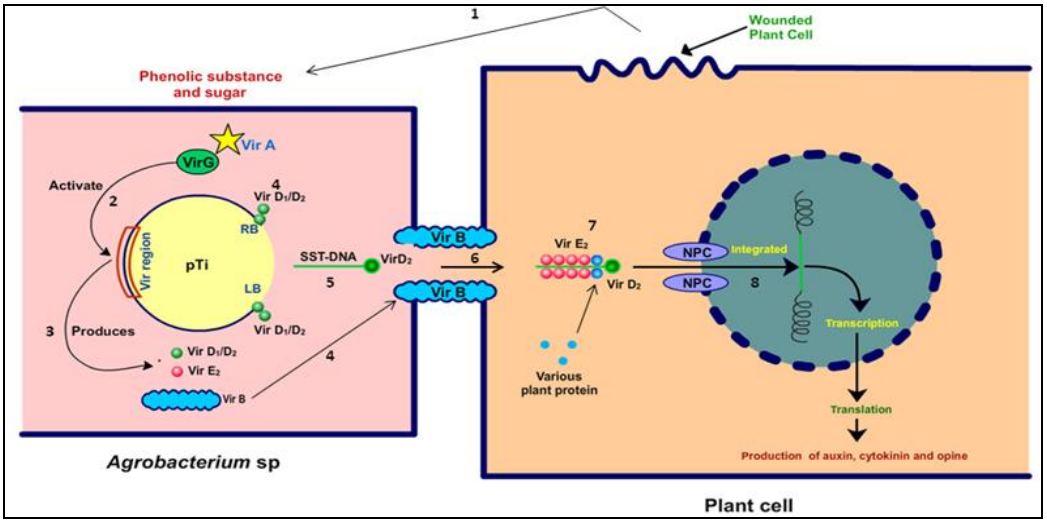

Figure 1.1: The *Agrobacterium* injects a plasmid (naked circular DNA) into the host cells

Wounded plant cell releases phenolic substances and sugar (1); which are sensed by *Vir* A, *Vir* A activates *Vir* G, *Vir* G induced for expression of *Vir* gene of Ri-plasmid (2); *Vir* gene produces all the *Vir* -protein (3); *Vir* D_1 and *Vir* D_2 are involved in ssT-DNA production from Ri-plasmid and its export (4) and (5); the ssT-DNA (associated with *Vir* D_1 and *Vir* D_2) with *Vir* E_2 are exported through transfer apparatus *Vir* B (6); in plant cell, T-DNA coated with *Vir* E_2 (7); various plant proteins influence the transfer of T-DNA + *Vir* D_1 + *Vir* D_2 + *Vir* E_2 complex and integration of T-DNA to plant nuclear DNA(8). (LB= left border; RB= Right border; pRi = Ri plasmid, NPC = nuclear pore complex).

3.2 Genetics of Transformation

Ri plasmids contain one or two regions of T-DNA and a *Vir* (Virulence) region, all of which are necessary for tumorigenesis (Figure 1.2). The Ri plasmid is very similar to Ti plasmid except that their T-DNAs have homology only for auxin and opine synthesis sequences. The T-DNA of Ri plasmid lacks genes for cytokinin synthesis. The T-regions of Ti and Ri plasmids contain oncogenes that are expressed in the plants. Another type, present in Ri plasmids only, appears to impose a high hormone sensitivity on the infected tissue. The T-DNA of Ri plasmids codes for at least three genes that each can induce root formation, and that together cause hairy root formation from plant tissue. Current results indicate that the products of these genes induce a potential for increased auxin sensitivity that is expressed when the transformed cells are subjected to a certain level of auxin. After this stage the transformed roots can be grown in culture without exogenous supply of hormones.

The Ri-plasmids are classified into two main classes according to the opines formed in transformed roots. First, agropine-type strains induce roots to synthesiseagropine, mannopine and the related acids. Second, mannopine-type strains which induce roots to produce mannopine and the related acids. The agropine-type Ri-plasmids are very similar as a group and a quite distinct group from the mannopine-type plasmids. Perhaps the most studied Ri-plasmids are agropine-type strains, which are considered to be the most virulent and, therefore, more often used in the establishment of hairy root cultures.

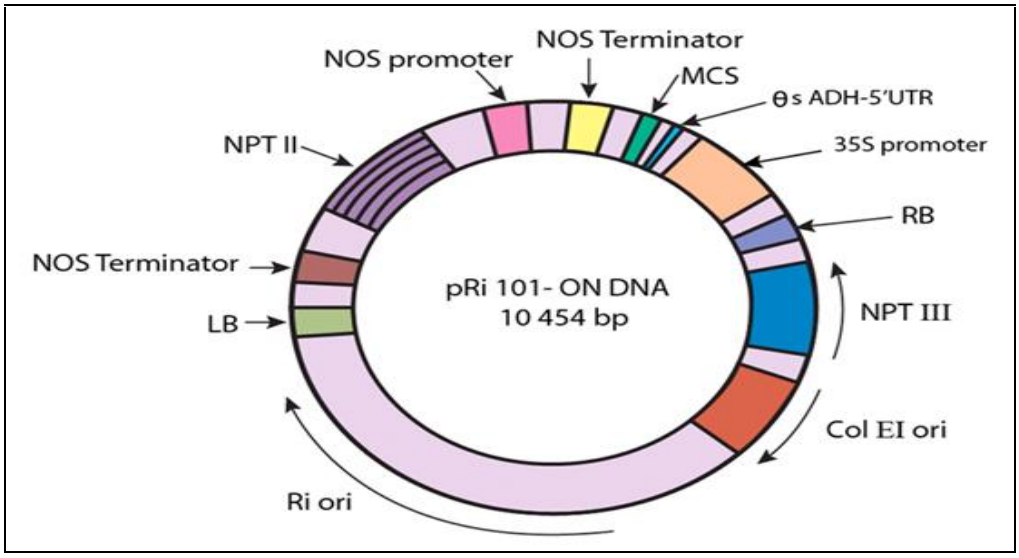

Figure 1.2: Structure of Ri-plasmid

3.3 The Genes Responsible for Hairy Root Formation

The agropine-type Ri-plasmid consists of two separate T-DNA regions known as the TL-DNA and TR-DNA. Each of the T-DNA fragments is separated from each other by at least 15 kb of non-integrated plasmid DNA. These two fragments can be transferred separately during the infection procedure. The TR-DNA of the agropine type Ri-plasmid carries genes encoding auxin synthesis (*tms* 1 and *tms* 2) and agropine synthesis (*ags*). The mannopine type Ri-plasmids contain only one T-DNA. TL-DNA region consists of four root locus (*rol*) genetic loci, *rol* A, *rol* B, *rol* C, and *rol* D, which affect hairy root induction. In particular, *rol* B seems to be the most important in the differentiation process of transformed cells and also function as induction of hairy roots by hydrolyzing bound auxins leading to an increase in the intracellular levels of indole-3-acetic acid. Gene *rol* A involved in development of hairy root morphology, *rol* B is responsible for protruding stigmas and reduced length of stamens; *rol* C causes internode shortening and reduced apical dominance.

3.4 Factors Influencing the Transformation

Following factors influence the transformation process:

- Virulence of *A. rhizogenes* strains
- Medium
- Age of the explant
- Nature of the explant

3.5 Confirmation of Transformation

Confirmation of transformation can be performed on the basis of following markers:

- Biochemical markers
- Opines
- Mannopines
- Genetic markers
- Southern hybridisation
- Polymerase chain reaction.

3.6 Screening of Transformation

Screening of transformation can be performed by GUS assay, leaf callus assay, rooting and bleaching assays.

3.7 Properties of Hairy Roots

3.7.1 Hairy Roots

Hairy roots have following properties

- high degree of lateral branching
- profusion of root hairs
- absence of geotropism
- they have high growth rates in culture, due to their extensive branching, resulting in the presence of many meristems
- they do not require conditioning of the medium .

3.7.2 Hairy Roots are Genetically Stable

Hairy roots are genetically stable consequently they exhibit biochemical stability that leads to stable and high-level production of secondary metabolites. Hairy root cultures apparently retain diploidy in all species so far studied. The stable production of hairy root cultures is dependent on the maintenance of organised states. The factors which promote

disorganisation and callus formation depress secondary metabolite production. The productivity of hairy root cultures is stable over many generations in contrast to disorganised cell cultures. This stability is reflected in both the growth rate and the level pattern of secondary metabolite production.

3.8 Application of Hairy Root Cultures

3.8.1 Production of Secondary Metabolites

The hairy root system is stable and highly productive under hormone-free culture conditions. The fast growth, low doubling time, easy maintenance, and ability to synthesise a range of chemical compounds of hairy root cultures gives additional advantages as continuous sources for the production of plant secondary metabolites. Usually root cultures require an exogenous phytohormone supply and grow very gradually, resulting in the poor or insignificant synthesis of secondary metabolites. Hairy roots are also a valuable source of photochemical that is useful as pharmaceuticals, cosmetics, and food additives. These roots synthesize more than a single metabolite; prove economical for commercial production purposes. Many medicinal plants have been transformed successfully by *A. rhizogenes* and the hairy roots induced show a relatively high productivity of secondary metabolites, which are important pharmaceutical products. Sevon has summarised the most important alkaloids produced by hairy roots, including *Atropa belladonna* L., *Catharanthustrichophyllus* L., and *Datura candida* L.
Metabolic engineering offers new perspectives for improving the production of secondary metabolites by the over expression of single genes. This approach may lead to an increase of some enzymes involved in metabolism and, consequently, results in the accumulation of the target products. This method utilises the foreign genes that encode enzyme activities not normally present in a plant. This may cause the modification of plant metabolic pathways. Two direct repeats of a bacterial lysine decarboxylase gene, expressed in the hairy roots of *Nicotiana tabacum,* have markedly increased the production of cadaverine and anabasine (Feckeret al . 293). The production of anthraquinone and alizarin in hairy roots of *Rubia peregrina* L. was enhanced by the introduction of isochorismate synthase. *Catharanthus roseus* hairy roots harboring hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) cDNA without the membrane-binding domain were found to produce more ajmalicine and catharanthine or serpentine and campesterol than the control.

3.8.2 Production of Compounds Not Found in Untransformed Roots

Transformation may affect the metabolic pathway and produce new compounds that cannot be produced normally in untransformed roots. For example, the transformed hairy roots of *Scutellariabaicalensis* Georgi accumulated glucoside conjugates of flavonoids instead of the glucose conjugates accumulated in untransformed roots.

3.8.3. Changing Composition of Metabolites

Bavage et al. (297) reported the expression of an *Antirrhinum* dihydroflavonol reductase gene which resulted in changes in condensed tannin structure and its accumulation in root cultures of *L. corniculatus*. The analysis of selected root culture lines indicated the alteration of monomer levels during growth and development without changes in composition.

Table 1.1: Pharmaceutical products produced using hairy root cultures

Plant species	Product
Bidens spp.	Polyacetylenes
Cinchona ledgeriana	Quinoline alkaloids
Datura spp.	Tropane
Cassia spp.	Anthraquinones
Echinacea purpurea	Alkaloids

4.0 CONCLUSION

Plant remains major source of pharmaceuticals and fine chemicals and cell cultures have been viewed as promising alternatives to whole plant extraction for obtaining valuable chemicals. The major constraint with the cell culture is that they are genetically unstable and tend to produce low yield of secondary metabolites.

5.0 SUMMARY

A new method for enhancing secondary metabolite production is by transformation of cells or tissues using the natural vector system. *Agrobacterium rhizogenes*, the causative agent of hairy root disease, is a soil dwelling gram negative bacterium capable of entering a plant through a wound and causing a proliferation of secondary roots.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is hairy root culture?
2. Describe the establishment of hairy root culture.
3. What are the genes responsible for hairy root formation?
4. What are the factors which influence the transformation of hairy root culture?
5. What are the properties of hairy roots?
6. Give some example of pharmaceutical products from hairy root culture?

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UNIT 2 SCREENING OF HIGH YIELDING CELL LINES AND EXTRACTION OF HIGH VALUE INDUSTRIAL PRODUCTS

CONTENTS

1.0 INTRODUCTION

Plant-derived substances are of great interest due to their versatile applications. These plants derived substance are richest bio-resource of drugs of traditional systems of medicine, modern medicines, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Extraction is the separation of medicinally active compounds from plant tissues using selective solvents through standard protocol. The products so obtained from plants are complex mixtures of metabolites, in liquid or semisolid state or in dry powder form, and are intended for oral or external use. These include decoctions, infusions, fluid extracts, tinctures, pilular extracts or powdered extracts. Plant cell culture is a genetically heterogeneous system. In addition, epigenetic changes cause genetic instability leading to product accumulation only in some population of cells. The overall production of secondary metabolites in a cell culture depends on the rate of accumulation within the productive cells. By cell culture system only low yields of desired secondary metabolites are obtained.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain screening and selection of highly productive cell lines
- state procedure for extraction of high value industrial products

3.0 MAIN CONTENT

3.1 Screening and Selection of Highly Productive Cell Lines

3.1.1 Screening and Selection

Callus culture is an easiest system for screening and selection program. Callus which shows the desired coloration is picked up and subcultured until pure cell line is established. The heterogeneity in the biochemical activity of cells has been exploited to obtain highly productive cell lines. The selection with cell suspension cultures can also be performed with a fine, rapidly growing suspension of cells consisting of small aggregates of up to 50 cells. Selection procedure can be easily achieved if the product of interest is a pigment. For example, in cultures of *Lithospermumerythrorhizon*, screening of a number of clones resulted in 13–20-fold increase in shikonin production. Increased production of anthocyanin by clonal selection and visual screening has been reported in *Euphorbia milii* and *Daucus carota*. High performance liquid chromatography (HPLC), radioimmuno assays (RIA) and mutation strategies have also been employed in order to obtain overproducing cell lines. The use of selective agents can be employed as an alternative approach to select high yielding cell lines. In this method, a large population of cells is exposed to a toxic (or cytotoxic) inhibitor or environmental stress and only cells that are able to resist the selection procedures will grow. P-Fluorophenylalanine, an analogue of phenylalanine, was extensively used to select high-yielding cell lines with respect to phenolics. Other selective agents consist of 5-methyltryptophan; glyphosate and biotin have also been used to select high-yielding cell lines.

3.2 Procedure for Extraction of High Value Industrial Products

3.2.1 Plant Material

Plants are the source of effective phytomedicines since times immemorial. Man is able to obtain from them a wondrous variety of industrial chemicals. Plant based natural constituents can be derived from the plant part, like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components. Fresh or dried plant materials can be used as a source for the extraction of secondary plant components. Many authors had reported about plant extract preparation from the fresh plant tissues. The logic behind this came from the ethno medicinal uses of fresh plant materials among the traditional and tribal people. But as many plants are used in the dry form by conventional healers and due to differences in water content within

different plant tissues, plants are generally air dried to a constant weight before extraction. Other researchers dry the plants in the oven at about 40°C for 72 h. In most of the reported works, underground parts (roots, tuber, rhizome, bulb etc.) of a plant were used extensively compared with other above ground parts in search for bioactive compounds possessing antimicrobial properties.

3.2.2 Choice of Solvents

Properties of a good solvent in plant extractions include, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability of the extract to complex or dissociate. The factors affecting the choice of solvent are, quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractant.

The various solvents that are used in the extraction procedures are:

3.2.2.1 Water

Antimicrobial activity. Though traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extract. Reports indicates that water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics are important as only antioxidant compounds.

3.2.2.2 Acetone

Acetone dissolves many hydrophilic and lipophilic components from the plants and is miscible with water. It is volatile and has a low toxicity to the bioassay used. It is a very useful extractant, especially for antimicrobial studies where more phenolic compounds are required to be extracted. A study reported that extraction of tannins and other phenolics was better in aqueous acetone than in aqueous methanol.

3.2.2.3 Alcohol

The higher activity of the ethanolic extracts compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols. More useful explanation for the decrease in the activity of aqueous extract can be ascribed to the enzyme polyphenol oxidase, which degrade polyphenols in water extracts, whereas in methanol and ethanol they are inactive.

3.2.2.4 Chloroform

Terpene lactones have been obtained by following extractions of dried barks with hexane, chloroform and methanol with activity concentrating in chloroform fraction. Rarely tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents.

3.2.2.5 Ether

Ether is commonly used for the selective extraction of coumarins and fatty acids.

3.2.2.6 Dichloromethane

It is another solvent used for carrying out the extraction procedures. It is specially used for the selective extraction of only terpenoids.

3.2.3 Extraction Procedures

3.2.3.1 Plant Tissue Homogenisation

Plant tissue homogenisation in solvent has been generally used by researchers. Dried or wet, plant parts are grinded in a blender, with certain quantity of solvent, to fine particles, and shaken vigorously for 5-10 min or left overnight before filtering the extract. The filtrate then may be dried under reduced pressure and redissolved in small amount of solvent during quantification by HPLC.

3.2.3.2 Soxhlet Extraction

Soxhlet extraction is only necessary where the preferred compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the preferred compound has a high solubility in a solvent then a simple filtration procedure can be used to separate the compound from the insoluble substance. The benefit of this system (Figure 2.1) is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds.

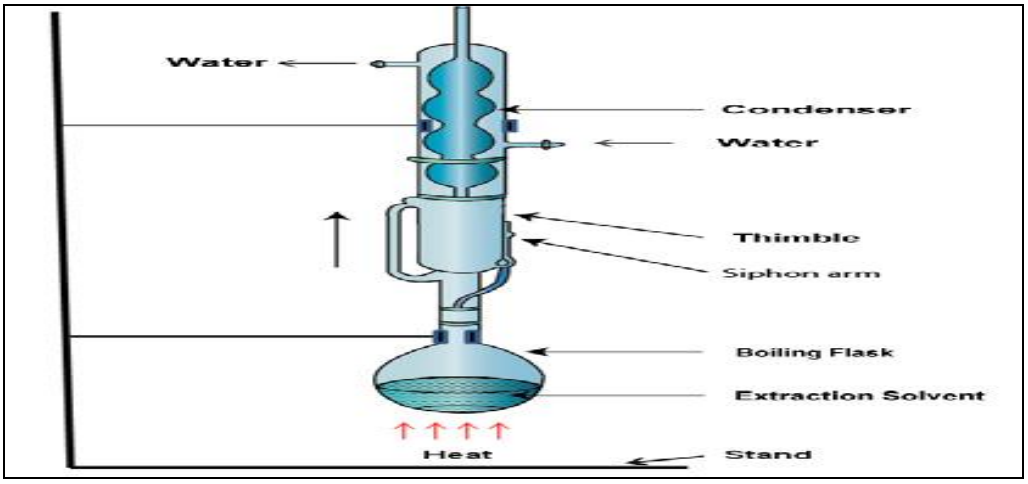

Figure 2.1: A Soxhlet apparatus

3.2.3.3 Maceration

In maceration (for fluid extract), whole or coarsely powdered plant-drug is kept in contact with the solvent in a container with stopper for a defined period with frequent agitation until soluble matter is dissolved. This method is best suitable for use in case of the thermolabile compounds.

3.2.3.4 Decoction

This method is used for the extraction of the water soluble and heat stable constituents from crude drug by boiling it in water for 15 minutes, cooling, straining and passing sufficient cold water through the drug to produce the required volume.

3.2.3.5 Percolation

This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstruum and allowed to stand for approximately 4 h in a closed container, after which the mass is packed and the top of the percolator is closed. Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained, therein, is allowed to drip slowly. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

3.2.3.6 Sonication

The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz. This increases the permeability of cell walls and produces cavitations. Although the process is useful in some cases, like extraction of Rauwolfi root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.

3.2.4 Steps Involved in the Extraction

For extraction from plant explants, most important steps are size reduction, extraction, filtration, concentration and drying.

3.2.4.1 Size Reduction

The objective for powdering the plant material is to rupture its organ, tissue and cell structures so that its medicinal ingredients are exposed to the extraction solvent. Furthermore, size reduction maximizes the surface area, which in turn enhances the mass transfer of active principle from plant material to the solvent.

3.2.4.2 Extraction

Extraction of the plant material is carried out in three ways:

3.2.4.3 Cold Aqueous Percolation

The powdered material is macerated with water and then poured into a tall column. Cold water is added until the powdered material is completely immersed. It is allowed to stand for 24 h so that water-soluble ingredients attain equilibrium in the water. The enriched aqueous extract then can be concentrated in multiple-effect evaporators to a particular concentration.

3.2.4.4 Hot Aqueous Extraction

This is done in an open-type extractor. The extractor is a cylindrical vessel made from type 316 stainless steel and has a diameter (D) greater than the height (H), i.e. the H/D ratio is approximately 0.5. The bottom of the vessel is welded to the dished end and is provided with an inside false bottom with a filter cloth. The outside vessel has a steam jacket and a discharge valve at the bottom. One part powdered plant material and sixteen parts demineralized water is fed into the extractor. Heating

is done by injecting steam into the jacket. The material is allowed to boil until the volume of water is reduced to one-fourth its original volume.

3.2.4.5 Solvent Extraction

The principle of solid-liquid extraction is that when a solid material comes in contact with a solvent, the soluble components in the solid material move to the solvent. Thus, solvent extraction of plant material results in the mass transfer of soluble active principle (medicinal ingredient) to the solvent, and this takes place in a concentration gradient. The rate of mass transfer decreases as the concentration of active principle in the solvent increases, until equilibrium is reached, i.e. the concentrations of active principle in the solid material and the solvent are the same. Thereafter, there will no longer be a mass transfer of the active principle from plant material to the solvent. Since mass transfer of the active principle also depends on its solubility in the solvent, heating the solvent can enhances the mass transfer. Furthermore, if the solvent in equilibrium with the plant material is replaced with fresh solvent, the concentration gradient is altered. This gives rise to different types of extractions: cold percolation, hot percolation and concentration.

3.2.4.6 Filtration

The extract so obtained is separated out from the marc (exhausted plant material) by allowing it to trickle into a holding tank through the built-in false bottom of the extractor, which is covered with a filter cloth. The marc is retained at the false bottom, and the extract is received in the holding tank. From the holding tank, the extract is pumped into a sparkler filter to remove fine or colloidal particles from the extract. The filtered extract is subjected to spray drying with a high pressure pump at a controlled feed rate and temperature, to get dry powder. The desired particle size of the product is obtained by controlling the inside temperature of the chamber and by varying the pressure of the pump.

3.2.4.7 Concentration

The enriched extract from percolators or extractors, known as miscella, is fed into a wiped film evaporator where it is concentrated under vacuum to produce a thick concentrated extract. The concentrated extract is further fed into a vacuum chamber dryer to produce a solid mass free from solvent. The solvent recovered from the wiped film evaporator and vacuum chamber dryer is recycled back to the percolator or extractor for the next batch of plant material. The solid mass, thus, obtained is pulverised and used directly for the desired pharmaceutical formulations or further processed for isolation of its phytoconstituents.

3.2.4.8 Drying

Drying process is final extraction step. Drying is a mass transfer process consisting of removal of water or another solvent by evaporation from a solid, semi-solid or liquid. This process is frequently used as a final production step before packaging products. Freeze drying is a drying method where the solvent is frozen prior to drying and is then sublimed, below the melting point of the solvent.

4.0 CONCLUSION

Plant-derived substances are of great interest due to their versatile applications. These plants derived substance are richest bio-resource of drugs of traditional systems of medicine, modern medicines, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Extraction is the separation of medicinally active compounds from plant tissues using selective solvents through standard protocol. The products so obtained from plants are complex mixtures of metabolites, in liquid or semisolid state or in dry powder form, and are intended for oral or external use. These include decoctions, infusions, fluid extracts, tinctures, pilular extracts or powdered extracts.

5.0 SUMMARY

Plant cell culture is a genetically heterogeneous system. In addition, epigenetic changes cause genetic instability leading to product accumulation only in some population of cells. The overall production of secondary metabolites in a cell culture depends on the rate of accumulation within the productive cells. By cell culture system only low yields of desired secondary metabolites are obtained. The reason for low production in culture may be due to:

- Competition between primary and secondary pathways for common intermediates.

- Low levels of expression of key enzymes at rate limiting steps in a pathway.

Screening and selection are often used as exchangeable terms. Screening is a passive technique by which a great number of cells alone analyzed for a certain trait and those showing the desired features are cultivated and screened. Selection process is an active process, which deliberately favor only the survival of the desired variants while wild type cells are killed.

6.0 TUTOR-MARKED ASSIGNMENT

1. How do you screen and select cell line for high production of cell secondary metabolites?
2. What is the region of low production of secondary metabolites?
3. Describe the extraction process.
4. What are the factors that affect chemical extraction?
5. Describe the solvents required for extraction.
6. Draw diagram of Soxhlet apparatus.
7. Write short notes on:
   A. Tissue homogenisation
   B. Maceration
   C. Percolation
   D. Sonication
   E. Extraction steps.

7.0 REFERENCES/FURTHER READING

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UNIT 3 FRACTIONATION AND BIOASSAYS OF PLANT EXTRACT

CONTENTS

1.0 INTRODUCTION

Fractionation is a separation method in which a certain quantity of a mixture (solid, liquid, solute, suspension or isotope) is divided into number of smaller quantities (fractions) in which the composition changes according to a gradient. Fractions are collected based on differences in a specific property of the individual components. Bioassays are generally conducted to measure the potency of a substance by its effect on living cells and are useful in the development of new drugs. Biological assays must be carried out in order to identify plant extracts, to guide the separation and isolation, and to evaluate lead compounds.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain the concept of fractionation
- know the impotance of bioassays.

3.0 MAIN CONTENT

3.1 Fractionation

Fractionation is a separation method in which a certain quantity of a mixture (solid, liquid, solute, suspension or isotope) is divided into number of smaller quantities (fractions) in which the composition changes according to a gradient. Fractions are collected based on differences in a specific property of the individual components. Fractionation process makes it possible to separate more than two

components in a mixture in a single run. It is generally carried out by suspending each extract in water separately and partitioning with different organic solvents, such as hexane, chloroform, ethyl acetate, and methanol in order of increasing polarity by using separating funnel. A simple fractionation unit is shown in Figure 3.1. All the fractions of plant extract can be dried by evaporating respective solvent using rotary evaporator and can be stored at 4°C till further analysis.

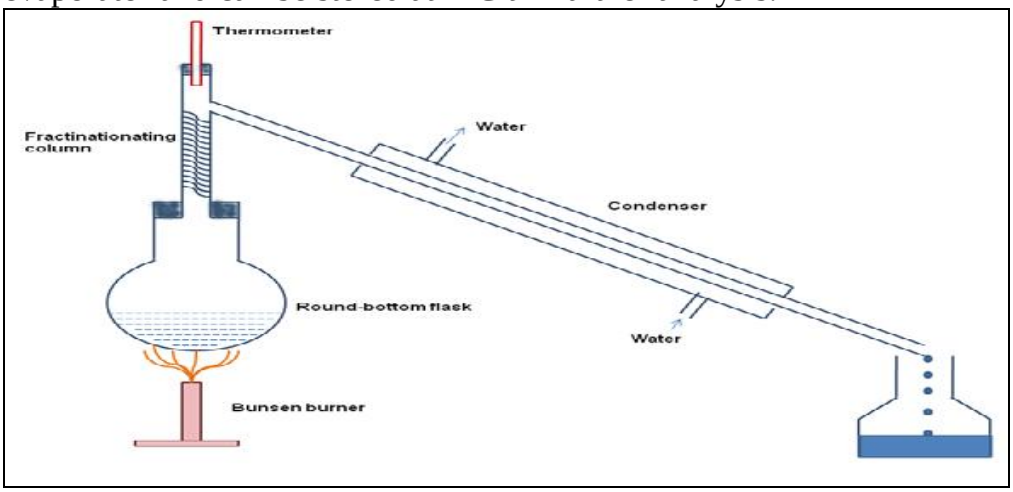

Figure 3.1: A simple fraction distillation apparatus

Qualitative phytochemical analysis can be done for various phytoconstituents, like alkaloids, tannins, glycosides, steroids and saponins, by thin layer chromatography (TLC) of obtained fractions in different solvent system. High performance liquid chromatography (HPLC) is an important device for quantitative analysis of phytochemicals, their monitoring and quality assurance. The method enables complex mixtures to be separated into individual compounds, which can be identified and quantified by suitable detectors and data handling systems. Separation and detection occur at ambient temperature or slightly above. Therefore, the method is preferably suited for compounds of limited thermal stability.

3.2 Bioassays

Bioassays are generally conducted to measure the potency of a substance by its effect on living cells and are useful in the development of new drugs. Biological assays must be carried out in order to identify plant extracts, to guide the separation and isolation, and to evaluate lead compounds. These assays may be conducted both *in vitro* or *in vivo.*

3.2.1 Antiviral Assays

A number of compounds extracted from different species of higher plants have shown antiviral activity. Examples included tannins, flavones, alkaloids that displayed *in vitro* activity against numerous

viruses. Antiviral assays are basically an extension of cytotoxicity assays. To perform this, the cultures of mammalian cells are infected with virus, test compounds are added, and the fate of the cells is assessed. It was reported that the antiviral activity detected was due to phytoalexins produced by the plant as a protection against plant viruses. These assays simultaneously allow an estimation of cytotoxicity (loss of the cell monolayer in which the plaques are normally formed). Active extracts or compounds then become candidates for testing against tumor cell lines, for example, P388 or L1210.

The methanol extracts of the aerial parts of *Hypericum mysorense* and *Hypericum hookerianum,* exhibited detectable antiviral effect towards HSV-1 with an inhibitory concentration at IC_{50} value of 50µg/ml. The acetone extract of *Usneacomplanata* also showed antiviral activity at an IC_{50} value of 100µg/ml. Other examples of plant extracts exhibiting anti-HSV activity are *Acnistusarborescens, Cupania glabra, Dichapetalum axillar, Drypeteslasiogyna, Mallotusmollissimus.*
The pentacyclic triterpenoids, betulinic acid, oleanolic acid and ursolic acid, are widespread plant metabolites. All the three triterpenes inhibit HIV-1 protease activity *in vitro*. Betulinic acid was found to be active in vivo as well using athymic mice carrying human melanomas. Further biological studies suggest that betulinic acid works by induction of apoptosis.

3.2.2 Cell Cytotoxicity Assays

Cytotoxicity assays are used by the pharmaceutical industry to screen for cytotoxic compounds. Cell membrane integrity is one of the most common methods to measure cell viability and cytotoxic effects. Compounds having cytotoxic effects often have compromised cell membrane integrity. Cytotoxicity can be monitored using the MTT or MTS assay. The aqueous extract isolated from *in vitro* derived cell cultures, raised from leaf-discs of *Lantana camara,* exhibited promising anti-proliferative activity on HeLa cells (Figure 3.2, 3.3). The minimal activity of the extract on normal BHK-21 cells verifies it's potential as a feasible anti-cancer agent.

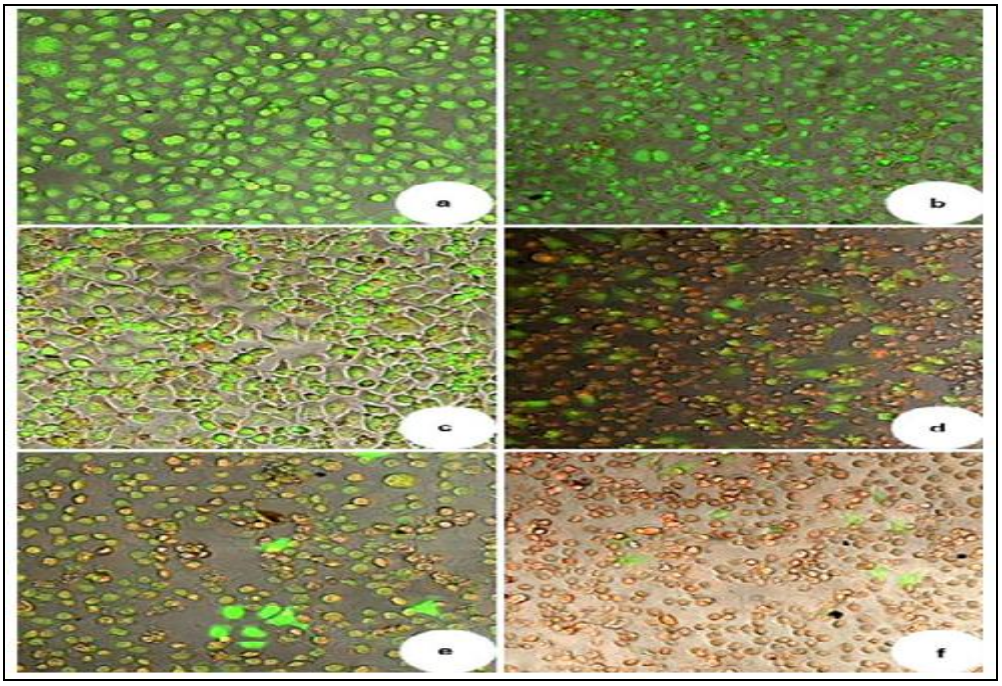

Figure 3.2: HeLa cells are stained with Acridine Orange/Ethidium Bromide and viewed under confocal laser scanning microscope. **a.** Control healthy cells, **b.** HeLa cells treated with aqueous extract of *L. camara* for 24 h showing bright green nuclei indicating initiation of chromatin condensation, **c.** same as **b** , treated for 36 h, showing zones of cleared monolayer, **d, e and f.** same as **b,** treated for 48, 60 and 72 h, respectively, showing gradual increase in frequency of dead cells taken the orange color.

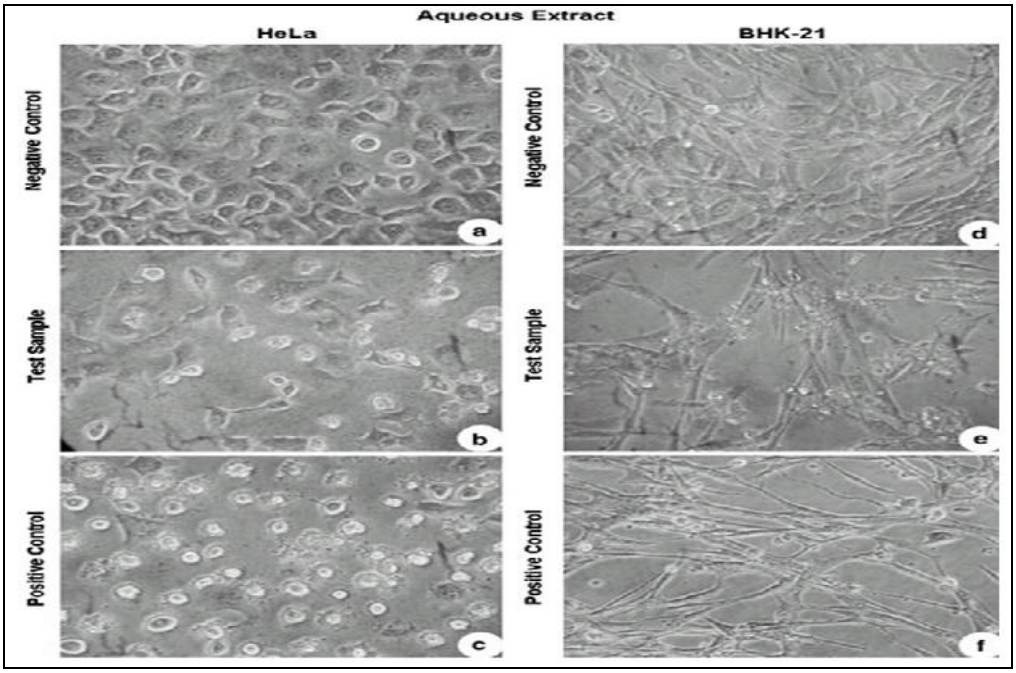

Figure 3.3: Morphological observations of HeLa and BHK-21 cells under light microscopy.

a. untreated HeLa cells, **b.** HeLa cells treated with aqueous extract of *L. camara* , showing rounded apoptotic bodies, **c.** HeLa cells treated with curcumin, showing similar cell death patterns as in **b, d.**untreated BHK-21 cells, **e.** BHK-21 cells treated with aqueous extract of *L. camara*, showing low frequency of apoptotic bodies when compared to HeLa cells, **f.** BHK-21 cells treated with curcumin, showing similar cell death patterns as in **e.**

3.2.3 Antimicrobial Assays

An anti-microbial substance either kills or inhibits the growth of microorganisms, such as fungi, bacteria. Plants have been investigated scientifically for antimicrobial activity, and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms. Antimicrobial activity of the crude extracts can be determined by these two methods:

A. Agar well method
B. Disc diffusion assay method

Nutrient agar can be prepared for bacteria according to the manufacturers' instructions. Immediately after autoclaving, allow the media to cool at 45°C to 50°C in water bath. Pour the freshly prepared cooled media (approximately 4 mm depth) into flat-bottomed Petri dishes (90mm in diameter). Spread about 0.2ml of the test inoculum of bacteria uniformly on the surface of the solidified agar media using a sterile inoculation L-shaped glass rod. Make four equidistant wells of 5mm diameter and 4mm in depth on the agar using a sterile corn borer. Make two more wells for positive and negative controls at the middle of the agar. Fill about 3 µl of the plant extracts (concentrations ranged from 3–200mg/ml) and controls into the wells. The positive controls will be antibiotic (e.g. Ampicillin). The negative controls will be DMSO for organic solvent extracts and distilled water for aqueous extracts. Label the wells to correspond with the code numbers of the test crude extracts and controls. Store the treated plates in a refrigerator at 4°C for at least six hours to allow diffusion of the extracts into the agar while arresting the growth of the test microbes. The plates were then incubated for 24 hours at favorable condition for a particular organism. The test was carried out in triplicates. Antimicrobial activity was determined by measuring the diameters of zones of inhibition in mm.

A. Agar well method
B. Disk diffusion assay

The preparation of media and inoculation of the test microbes are same as described in the agar well method. However, instead of punching out wells on the agar, sterile 5 mm Whatman No. 1 filter paper discs will be

used in the disc diffusion method. Soak the discs into the dissolved crude extracts for minimum of two hours. Blank discs impregnated generally with Ampicillin (10µg/disc) for gram positive bacteria, Gentamicin (15µg/disc) for gram negative bacteria and Fluconazole (0.4mg/disc) for Candida are used as positive controls. For negative control, discs will be soaked in DMSO and distilled water, for organic solvent and water extracts, respectively. By use of sterile forceps, place four seeded discs of the plant extracts equidistantly onto each of the inoculated plates. Place two extra discs for positive and negative controls at the middle of plate. Store the treated plates in a refrigerator at 4°C for minimum of six hours and then transfer to incubator for 24 hours at favorable conditions for a particular organism (37°C for bacteria and for 48 hours at 30°C for Candida). Perform the test in triplicates. Antimicrobial activities were determined by measuring the diameters of zones of inhibition in mm.

3.2.4 Anthelmintic Assay

Plant-derived compounds have played significant role in the field of anthelmintic drugs, such as Santonin, the main active substance isolated from wormwood (*Artemisia maritima* L). The protocol for anthelmintic screening using *Caenorhabditis elegans* (Maupas) developed by Simpkin and Coles (281). The test is carried out in a 24-well tissue culture plate with a well volume of 2.5 ml. Each well is filled with 2 ml of M_9 liquid medium. M_9 solution consists of 6g Na_2HPO_4, 3 g KH_2PO_4, 5g NaCl, and 0.3g $MgSO_4$. $7H_2O$ dissolved in 1000 ml water and autoclaved at 120°C for 20 min. Extract solutions, dissolved in DMSO, are added to make 500 ppm solutions, and then 10 µl *C. elegans* suspensions containing 30 to 40 larvae are subsequently introduced into each cell. The plates are incubated at 20°C for five day, and the number of dead nematodes is recorded using a phase contrast microscope, and anthelmintic activity is graded as shown below:

- Nematode counts and the motility of the nematodes correspond to the control
+ 0 to 20% fewer nematodes than the control; nematodes move slowly
++ Slightly higher nematode counts than the initial counts; nematode counts 20% less than the
control; nematodes move very slowly
+++ Same nematode counts as the initial counts, all dead

Active fractions/compounds are repeated with lower concentrations and compared to known anthelmintics, such as santonin.

4.0 CONCLUSION

Fractionation is a separation method in which a certain quantity of a mixture (solid, liquid, solute, suspension or isotope) is divided into number of smaller quantities (fractions) in which the composition changes according to a gradient. Fractions are collected based on differences in a specific property of the individual components. Bioassays are generally conducted to measure the potency of a substance by its effect on living cells and are useful in the development of new drugs. Biological assays must be carried out in order to identify plant extracts, to guide the separation and isolation, and to evaluate lead compounds.

5.0 SUMMARY

Fractionation is a separation method in which a certain quantity of a mixture (solid, liquid, solute, suspension or isotope) is divided into number of smaller quantities (fractions) in which the composition changes according to a gradient. Fractions are collected based on differences in a specific property of the individual components. Bioassays are generally conducted to measure the potency of a substance by its effect on living cells and are useful in the development of new drugs. Biological assays must be carried out in order to identify plant extracts, to guide the separation and isolation, and to evaluate lead compounds.

6.0 TUTOR-MARKED ASSIGNMENT

1. What do you mean by fractionation?
2. What do you mean about bioassay and give the name of some bioassay techniques?
3. Describe anti-viral assay.
4. What is cell cytotoxicity and how it is done?
5. What is antimicrobial assay?
6. Write short note on:

A. Agar well method
B. Disc diffusion assay method
C. Anthelmintic assay.

7.0 REFERENCES/FURTHER READING

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UNIT 4 GROWTH AND PRODUCTION KINETICS OF CELL CULTURES IN SHAKE FLASKS

CONTENTS

1.0 INTRODUCTION

For the development of plant cell culture technology, accurate and rapid measurement of cell growth and assessment of growth-related bioprocess kinetics are essential to the rational development of plant cell bioprocess engineering. Although the plant cell culture system appears to be similar to a microbial cell culture system, there are important differences between the two. The major differences include cell size, aggregation of plant cells, change in plant cell physiology for its primary and secondary metabolisms, rheological properties of the medium, and requirement of plant cells for complex nutrients. Therefore, the best method of assessing the plant cell growth kinetics should be carefully examined and evaluated. The cell suspension culture is much more amenable for biochemical studies and process development than callus cultures. The success in the establishment of a cell suspension culture depends, to a great extent, on the availability of “friable” callus tissue (i.e., a tissue that, when stirred in liquid medium, rapidly disaggregate into single cells and small clusters). The cell suspension culture generally grows at a faster rate and allows cells to be in direct contact with the medium nutrients. Suspension culture could be run as batch culture or continuous culture.

In batch culture, the culture environment continuously changes and growth, product formation, substrate utilisation, all terminate after a certain time interval.

But in continuous culture, fresh nutrient medium is continually supplied to a well-mixed culture, and products and cells are simultaneously withdrawn. Growth and product formation can be maintained for prolonged periods of time in continuous culture.

The reasons proposed for predominant use of batch culture is:

- Many secondary products are not growth associated
- Genetic instability of cultured cells
- Operability and reliability
- Economic considerations

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- state procedure of growth measurement of plant cell suspension cultures
- explain parameters of growth efficiency
- know determination of the concentrations of nutrients or metabolites
- explain the conductivity method
- explain and state cell viability assay.

3.0 MAIN CONTENT

3.1 Procedure of Growth Measurement of Plant Cell Suspension Cultures

Growth of suspension cultures is generally assessed as the settled cell volume, the packed cell volume, fresh cell weight, dry cell weight. Medium residual conductivity and pH measurements are other indirect evaluation methods. Finally, parameters describing growth efficiency, such as specific growth rate (μ), doubling time (t_d), and growth index, is determined.

3.1.1 Growth Curve

It is commonly accepted that growth of a cell suspension culture with respect to time is best described by the sigmoid curve theory. At the beginning, the cell population grows relatively very slow (lag phase). As the population size of plant cell approaches one half of the carrying capacity (defined by the nutrient status of the culture medium), the growth of culture per time unit increases. The growth rate is measured by the steepness of the curve, and it is the steepest when the population

density reaches one-half of the carrying capacity (in the middle of the sigmoid). After that the steepness of the curve decreases until it reaches the carrying capacity (stationary phase). At this time the growth rate slowly decreases due to limitation of nutrients as described in Figure 4.1.

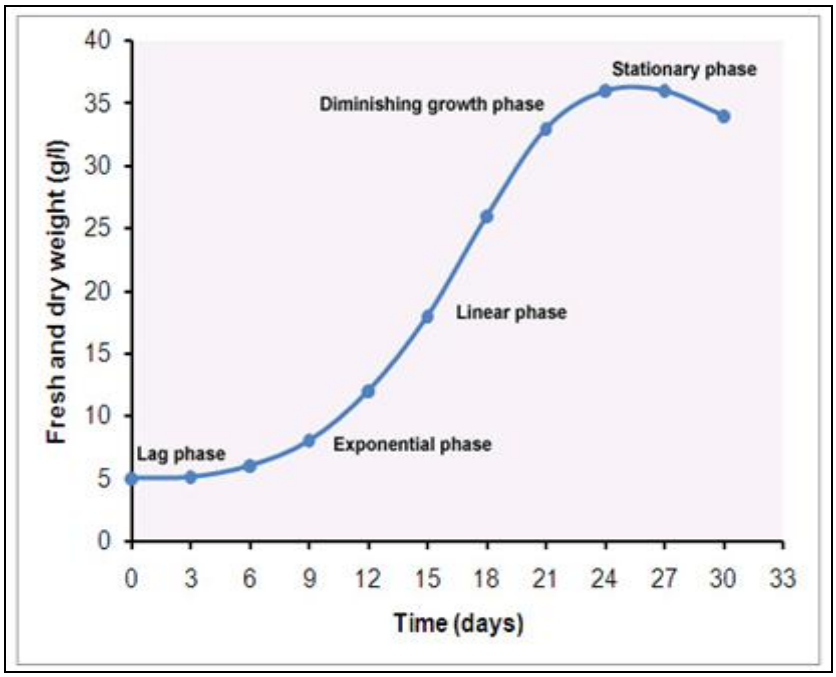

Figure 4.1: Graph showing different growth phases of a typical plant cell suspension culture.

The method needs harvesting cells at frequent intervals to determine the fresh weight and dry weight of cells per volume of cell suspension culture, thus, this is an invasive method. The method is also used to understand the nutrient uptake by the cells in suspension culture. For example, in cell suspension cultures of *Lantana camara*, it was observed that the cultures remained in the lag phase till the second day. Biomass increased till the 12^{th} day following which the stationary phase started (Figure 4.2). There are several methods of evaluating growth kinetics of plant cells. Selected examples include, fresh cell weight, settled volume, packed cell volume, cell optical density, cell size, nitrogen content, protein content, nucleic acid content, mitotic index, electrical conductivity, respiration, and pH measurement. In addition, concentrations of substrate and extracellular product have also been used for such purpose of selecting the best method of studying growth kinetics, especially from the bioprocess engineering point of view.

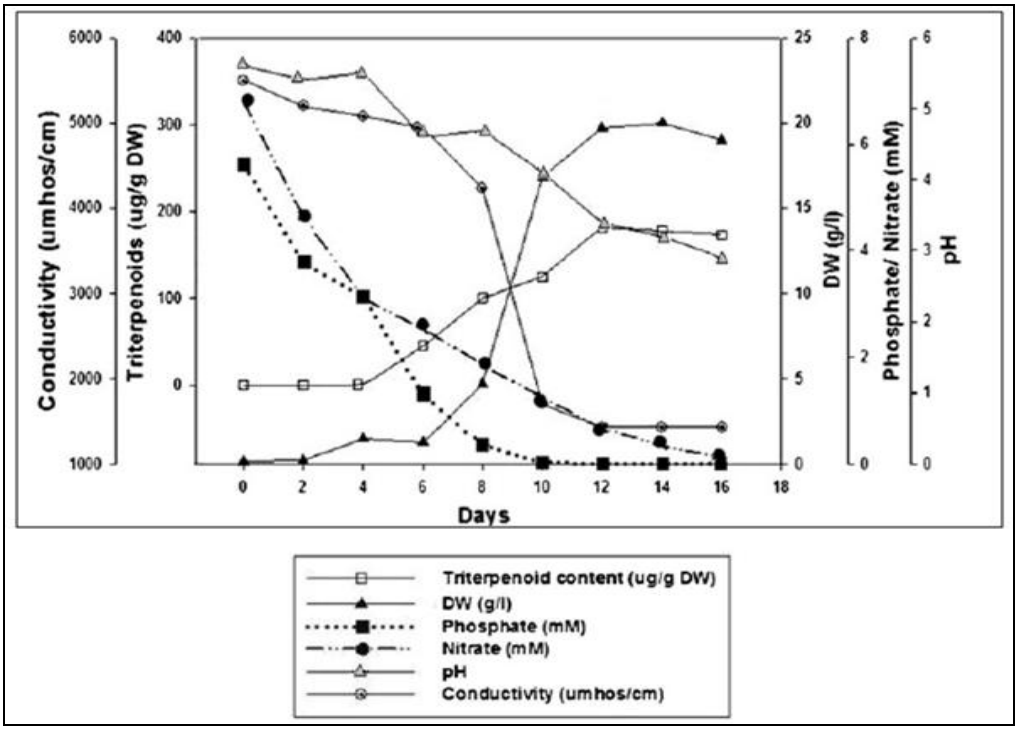

Figure 4.2: Cell growth and nutrient uptake in *L. camara* cells

3.2 Parameters of Growth Efficiency

3.2.1 Growth Index

As described by Loyola-Vargas and V á zquez-Flota (2006) that at a given sampling time, both fresh and dry weights are measurements of complete biomasses of the tissues. No reference to the actual growth capacity is taken in consideration. Growth index (GI) is a relative estimation of such capacity as it correlates the biomass data at the sampling time to that of the initial condition. It is calculated as the ratio of the accumulated and the initial biomass. The accumulated biomass corresponds to the difference between the final and the initial masses.

$$GI = \frac{W_f - W_i}{W_i}$$

Where in this equation, GI is growth index while W_f is final cell mass and W_i is the initial cell mass. Both W_f and W_i are taken either as fresh or dry weight.

3.2.2 Specific Growth Rate

As explained by Loyola-Vargas and V á zquez-Flota (2006), the specific growth rate (μ) refers to the steepness of a curve, and it is defined as the rate of increase of biomass of a cell population per unit of biomass concentration. It can be calculated in batch cultures, since during a defined period of time, the rate of increase in biomass per unit of

biomass concentration is constant and measurable. This period of time occurs between the lag phase and stationary phases. During this period, the increase in the cell population fits a straight-line equation between ln x and t.

$$\ln x = \mu t + \ln x_0$$

$$\mu = \frac{\ln x - \ln x_0}{t}$$

Where, x_o is the initial biomass (or cell density), x is the biomass (or cell density) at time t, and μ is the specific growth rate. Mu can be calculated from the above relationship, which is the slope of the line between ln x and t.

3.2.3 Doubling Time

Doubling time (t_d) is the time required for the concentration of biomass of a population of suspension cells to double. One of the greatest contrasts between the growths of cultured plant cells refers to their respective growth rates. The doubling time (dt) can be calculated according to the following equation (Loyola-Vargas and V á zquez-Flota, 2006)

$$td = \frac{\ln 2}{\mu}$$

n this equation, μ is the specific growth rate. By using the above equation, the specific growth rate of the suspended cells of *L. camara* was found to be 0.1072/day as shown in Figure 4.2.

3.3 Determination of the concentrations of nutrients or metabolites

Some nutrients in the cell suspension culture medium shows correlation with growth in a single culture flask. For example, total nitrate and phosphate levels in the medium can be used to understand the cell growth. Uptake of NH_4^+ ions may result in decrease in pH due to liberation of H^+ ions. Its uptake may be at a slower or faster rate in comparison to phosphate. Complete utilisation of phosphate from culture medium results in the onset of stationary phase sometimes for example, in *L. camara*, it was a major limiting nutrient for growth.

3.4 The Conductivity Method

The conductivity method of measuring growth kinetics of plant cell lines was used especially with the purpose of bioprocess engineering

applications of plant cell cultures. The major advantages of using conductometry as the biosensing technique for measurement of plant cell growth kinetics are:

i. The method is very economical and efficient.
ii. It gives accurate, reliable and reproducible measurements, while amenable to continuous on-line monitoring and process control.
iii. It is a noninvasive method which does not adversely affect the plant cells or the bioreactor operation.
iv. The kind of plant cell lines or their morphology does not affect the method itself.

3.5 Cell Viability Assay

At different parameters, cell viability in suspension cultures can be checked by using 1% fluorescein diacetate (FDA) solution. For an example, cell suspension of *L. camara* cultures were maintained at different agitation speed of 60-150 rpm, but 120 rpm only favored the fine suspension of live and healthy viable cells with small cell aggregates as is observed in fluorescein staining (Figure 4.3).

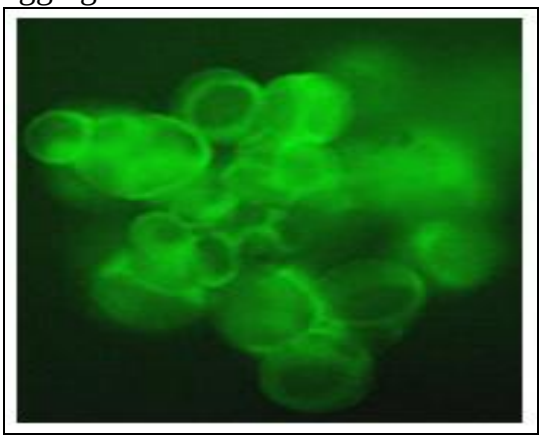

Figure 4.3: 3-week-old cells of *L. camara* stained with 1% fluorescein diacetate solution showing live dividing cells with intact cell wall.

4.0 CONCLUSION

For the development of plant cell culture technology, accurate and rapid measurement of cell growth and assessment of growth-related bioprocess kinetics are essential to the rational development of plant cell bioprocess engineering. Although the plant cell culture system appears to be similar to a microbial cell culture system, there are important differences between the two.

5.0 SUMMARY

The major differences include cell size, aggregation of plant cells, change in plant cell physiology for its primary and secondary metabolisms, rheological properties of the medium, and requirement of plant cells for complex nutrients. Therefore, the best method of assessing the plant cell growth kinetics should be carefully examined and evaluated. The cell suspension culture is much more amenable for biochemical studies and process development than callus cultures. The success in the establishment of a cell suspension culture depends, to a great extent, on the availability of "friable" callus tissue (i.e., a tissue that, when stirred in liquid medium, rapidly disaggregate into single cells and small clusters)

6.0 TUTOR-MARKED ASSIGNMENT

1. For establishment of cell suspesnsion cultures which type of callus tissue is generally considered?
2. Why suspension culture is generally considered for biochemical study and process development?
3. What are the differences between batch culture and continuous culture?
4. Why batch cultures are predominantly used?
5. How growth curve is prepared for suspension culture?
6. Describe the parameters of growth efficiency.
7. Write short notes on:
   A. Growth index
   B. Specific growth rate
   C. Doubling time
   D. Cell viability assay
   E. Advantages of using conductometry as the biosensing technique.

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UNIT 5 BIOREACTORS FOR PLANT ENGINEERING

CONTENTS

1.0 INTRODUCTION

Plant tissue culture is the key method of propagation for a large number of commercially important plants including important vegetatively propagated crops. Over 600 million plants can be produced in a year by tissue culture methods. Agar culture is the main culture technique generally used for commercial tissue culture propagation. It requires a large number of small culture vessels and labor, and results in the requirement of many laminar-air-flow clean benches, large autoclaves, and large culture spaces equipped with illuminated shelves, electric energy, etc. Thus, it is the cause for both limited propagation efficiency and high production costs. In order to overcome these problems, many attempts for establishing large-scale production of propagules with simple production facilities and techniques have been made including robotics, photoautotrophic cultures, bioreactor techniques, etc. Bioreactor technique seems to be the most promising technique among them in reducing the labour, and providing low production cost, which will be sufficient for establishing a practical system for *in vitro* mass propagation and commercialisation of plants. A bioreactor may be referred to as any manufactured or engineered device that supports a biologically active environment. Bioreactors are widely used for industrial production of microbial, animal and plant metabolites as by allowing large-scale cultivation of cells. A simple diagram of a bioreactor is shown in Figure 5.1.

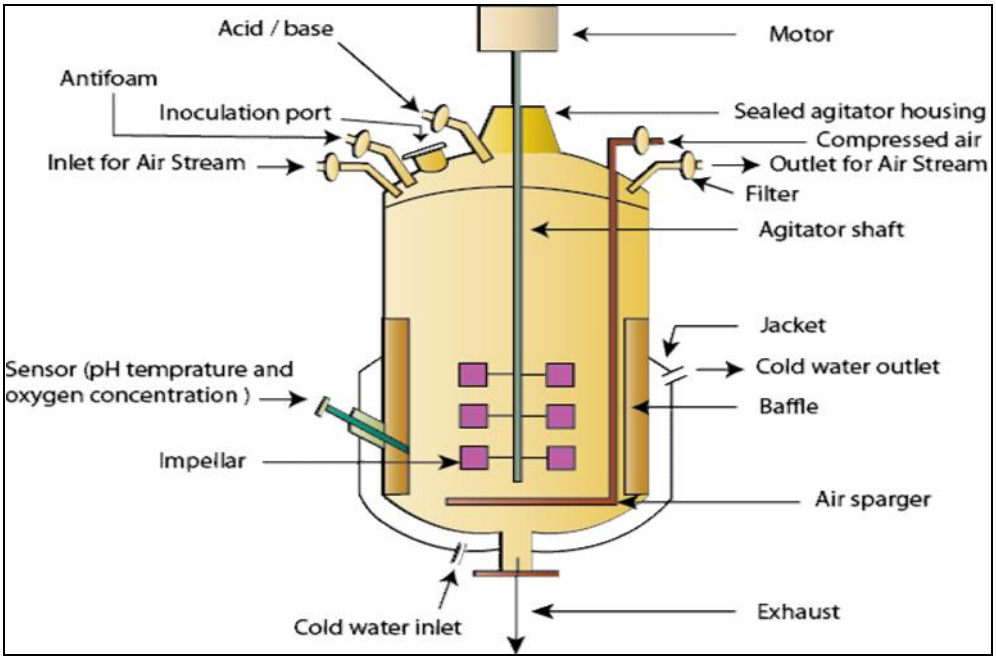

Figure 5.1: Diagram of a typical bioreactor.

2.0 OBJECTIVE

By the end of this unit, you will be able to:

- explain process format
- state applications of bioreactors in plant propagation
- idenfitify scale-up process
- state process design considerations
- outline types of bioreactors
- explain bioreactors for hairy roots.

3.0 MAIN CONTENT

3.1 Process Format

The choice of culture system influences the productivity. There are three main methods for the mass culture of plant cells, **i.** batch culture system, **ii.** continuous culture system and **iii.** semi-continuous (draw-fill). Out of these, the most studies on plant cells have been based on batch cultures system grown in fermentors (or bioreactors) (Fig.5.1). In batch cultivation, an inoculum of known cell density is "seeded" into a specified volume of preconditioned medium in the bioreactor and allowed to grow for a definite period under controlled conditions. The cells containing the desired intracellular metabolite are harvested from the fermentor at the end of the culture period. Typically, nothing is added or removed from the bioreactor during the course of cultivation, except addition of acid/base to control the pH and air to supply oxygen

to respiring plant cells. To start a new process, the bioreactor is cleaned, sterilised and filled with sterile medium and inoculated with the desired plant cells. Various environmental parameters such as pH, temperature and dissolved oxygen can be controlled with the use of pH, temperature and dissolved oxygen probes and the corresponding control systems. In batch culture, the time taken for cleaning etc can be considerable but can be eliminated by the use of continuous culture. In a continuous system, the nutrients consumed by the tissues are continuously replenished by an inflow of fresh medium to the bioreactor. A constant inflow of fresh medium is maintained by a constant efflux of equivalent volume of spent medium plus cells. However, continuous culture of plant cells is difficult because of the presence of aggregates which give a non-homogeneous culture and make sampling difficult. The adhesion of the cells to the walls of the bioreactor also gives problems. The slow growth of the culture means that the supply of fresh medium has to be at a slow rate, which in practice, is difficult. One method of avoiding continuous culture is ‘ draw-fill’ or ‘semi-continuous ' culture where, at the end of the culture period, 90% of the culture is removed and the remaining 10% topped up with fresh, sterile medium. This avoids the cleaning and sterilizing the vessels between the runs.

The continuous or draw-fill cultures are suitable for the production of secondary products that are ‘growth related’. However, secondary products are generally non-growth related and accumulate only after growth has ceased. To improve or stimulate secondary product yield, the medium or culture conditions are often changed; thus, continuous culture or draw-fill are not suitable. The non-growth-related accumulation requires a two-stage process, which can be organised by using a batch culture. In such a system, in the first stage growth of plant cells is optimised and the cells are transferred to a second stage. The second stage contains the nutrients in which product formation takes place in the cells. The culture conditions in the second stage are normally different from those in the first stage.

3.2 Applications of Bioreactors in Plant Propagation

i. Large number of plantlets which are free from physiological disorder can easily be produced in one batch in the bioreactor.
ii. Handling of cultures, such as inoculation and harvest, is easy. It also reduces the number of culture vessels and the area of culture space, which further reduces the overall cost of the production.
iii. Nutrient uptake and growth rate is increased because the surface of the cultures is always in contact with medium.
iv. Forced aeration is performed which improves the growth rate and final biomass.

Many plant species have been cultured in the bioreactor and the responses of cultures in bioreactors may vary from species to species. Following are the list of plant species where bioreactors are used for large scale propagation:

i. Shoots: *Atropa belladonna, Chrysanthemum morifolium, Dianthus caryophyllus, Fragaria ananassa, Nicotiana tabacum, Petunia hybrida, Primula obconica, Zoysia japonica, Scopolia japonica, Spathiphyllum, Stevia rebaudiana*, etc.
ii. Bulbs: *Fritillaria thunbergii, Hippeastrumhybridum, Hyacinthus orientalis, Lilium,* etc.
iii. Corms: *Caladium sp., Colocasia esculenta, Pinelliaternata,* etc.
iv. Tubers: *Solanum tuberosum*
v. Embryos or adventitious buds: *Atropa belladonna*

3.3 Scale-Up Process

Scale-up generally involves taking a lab-scale bioprocess and replicating it as closely as possible to produce larger amounts of product. A typical scale-up sequence in plant cell and tissue culture studies starts with jars, moves to 1 litre shake flasks, after that to 1-10 litre glass bioreactors, then scale-up through to stainless steel vessels of varying sizes from 30-150 litre to 1000 litre. The large-scale cultivation of plant cell and tissue culture is an alternative to the traditional methods of plantation. As compared to microbial cultures, plant cell suspensions, shoot and root cultures pose many different problems in bioreactors during scale-up. Plant cells grow slowly, the cells are large and form clumps, which make them more sensitive to shear associated with agitation and exhibit long processing times. Organ cultures are far more sensitive to shear. These characteristics lead to the necessity to design alternative bioreactor configurations, particularly those that reduce shear within the large-scale bioreactor. Various culture conditions must be monitored to control plant morphogenesis and biomass growth in bioreactors, such as the morphology, oxygen supply and CO_2 exchange, mixing, pH and temperature.

3.4 Process Design Considerations

3.4.1 Aggregation

Due to large size (length up to 200 μm) and slow growing nature, compare to the microbial cells, plant cells are although capable of withstanding tensile strain but are sensitive to shear stress. They have a very rigid cell wall and a culture will contain a wide range of cell shapes and sizes. Unlike many microorganisms, plant cells in suspension culture occur as groups or aggregates. Whether these aggregates arise

due to failure of the cells to separate after division or by cell aggregation is unknown but they are loose structure whose average size and size distribution vary with culture conditions. Further, the secretion of extracellular polysaccharides, particularly in the later stages of growth, may contribute to increased adhesion. A consequence of these characteristics results in sedimentation, insufficient mixing and diffusion-limited biochemical reaction. On the other hand, the aggregate structure has also been implicated in secondary product accumulation, as it provide cell-cell contact and so form micro-environment within the aggregate, which stimulate secondary product synthesis. Hence controlled aggregation of plant cells is of interest from process engineering point of view.

3.4.2 Mixing

Mixing favors cell growth by promoting nutrient transfer from liquid and gaseous phases to cells. It also helps dispersion of air bubbles for effective oxygenation. Although plant cells have higher tensile strength, compare to microbial cells, their shear sensitivity towards hydrodynamic stresses restricts the use of high agitation for efficient mixing. Mixing decrease the mean aggregate size but have an unfavorable effect on cell viability. Plant cells are often grown in stirred tank bioreactors at very low agitation speeds. Sufficient mixing can be achieved by proper design of the impeller; helical-ribbon impeller has been reported to enhance mixing at the high density of plant cell suspension cultures.

3.4.3 Oxygen and Aeration Effects

Plant cells require comparatively lower oxygen than that of microbial cells due to their low growth rates. High oxygen concentration has proved toxic to the cells, metabolic activities, etc. and may strip nutrients, such as carbon dioxide from the culture broth. Carbon dioxide is often considered as an essential nutrient in the culture of plant cells and has a positive effect on cell growth. Hence, the factors that influence efficient oxygen transfer in plant cell cultures must be carefully analysed when a bioreactor system is being selected. The intensity of culture broth mixing, the extent of air bubble dispersion, and the hydrodynamic pressure inside the culture vessel influence suitable aeration of the culture.

High aeration may result into severe foaming, which has significant influence on the cell growth and secondary metabolite production. Foaming of plant cell suspensions is associated with aeration rates and extracellular polysaccharides, fatty acids and high sugar concentrations in the plant cell culture medium. This can result in the wall growth phenomenon and clogging of air exhaust filter and lead to high rate of

contamination. A number of antifoams such as, polypropylene glycol 1025 and 2025, Pluronic PE 6100, and Antifoam-C have often been employed to control foaming; however, in some cases this resulted in reduction in cell growth and product formation.

3.4.4 Shear Sensitivity

Sensitivity of plant cells to hydrodynamic stress related with aeration and agitation can be ascribed to the physical characteristics of the suspended cells, their size, the presence of thick cellulose based cell wall, and presence of large vacuoles. Aeration and mixing system, aeration rate and impeller tip speed generally decide the shear-related effects on plant cells. Mechanically agitated vessels may over-aerate plant cultures, in addition to damaging and breaking the cells through the hydrodynamic stress generated by aeration, agitation, shaking, pumping, and other operations. Low agitation and high aeration provide oxygen in a reasonable mixing range.

3.4.5 Optimisation of Process Parameter

Nutrients directly influence the yield and productivity of metabolites in plant cell suspension cultures. Therefore, it is important to study and quantify the effect of selected key medium components on growth as well as product accumulation and strike a balance between the two to enhance the yield and productivity. This is essential for secondary metabolites production as conditions suitable for growth may adversely affect the product formation and vice versa. The first step in bioprocess media optimization is the identification of relatively significant media components, such as sugars, nitrogen compounds, minerals and growth factors as well as culture conditions and then to determine their optimum levels. The growth of cells in the bioreactor is controlled by using concentration of the growth-limiting nutrient. At steady-state the cell density and substrate concentration are constant. At steady state, $\mu = D$, where $D = F / V$ (F = medium flow rate, V = culture volume).

3.5 Types of Bioreactors

In vitro plant cell culture is currently carried in a diverse range of bioreactor designs, ranging from batch, airlift, and stirred tank to perfusion and continuous flow systems. For a small-scale operation, both the conventional and novel bioreactor designs are relatively easy to operate. For a larger scale of operation, problems of maintaining bioreactor sterility and providing adequate oxygen supply to the cells have yet to be resolved. The bioreactors used for plant cell cultures are classified as under:

- Mechanically agitated bioreactors: stirred tank reactor equipped with various propellers (spin, helix, bladed, paddle), rotary drum tank reactor, etc.
- Air driven bioreactors: bubble column, concentric tube airlift reactor, external loop airlift reactor, propeller loop reactor, jet loop reactor, etc.
- Non-agitated bioreactors: (a) packed bed, (b) fluidized bed, (c) membrane reactor.

Three important scientific and practical issues are involved in bioreactor design and operation for plant cells:

- Cell growth and product formation assessment
- Modeling of the culture dynamics, including the integration of biosynthesis and product separation
- Studies involving the flow, mixing and mass transfer between the phases, in order to define criteria for bioreactor design and scale up.

3.5.1 Mechanically Agitated Bioreactors

The various plant bioreactors designs are proposed by various authors depending upon the plant species used (*see* Bisaria et at, 2002). The most common and popular bioreactor is the stirred tank bioreactor and sufficient knowledge exists about its design and applications. Although it has gained much popularity, stirred tank bioreactors have numerous limitations, such as high-power consumption, high shear, and problems with sealing and stability of shafts in tall bioreactors. In order to diminish the shear forces, numerous modifications have been developed by employing a variety of impeller designs and seals (Figure 5.2. Aa).

Horizontal vessels or rotary drum reactors (Figure 5.2. Ab) have significantly higher surface area to volume ratio than other reactor types. Therefore, mass transfer is achieved with comparably less power consumption. Horizontal vessels used for the cultivation of high-density plant suspensions have shown advantages in terms of suspension homogeneity, low shear environment and reduced wall growth, over either airlift or stirred tank reactors. However, the drawback is their comparatively high energy consumption in large scale operations.

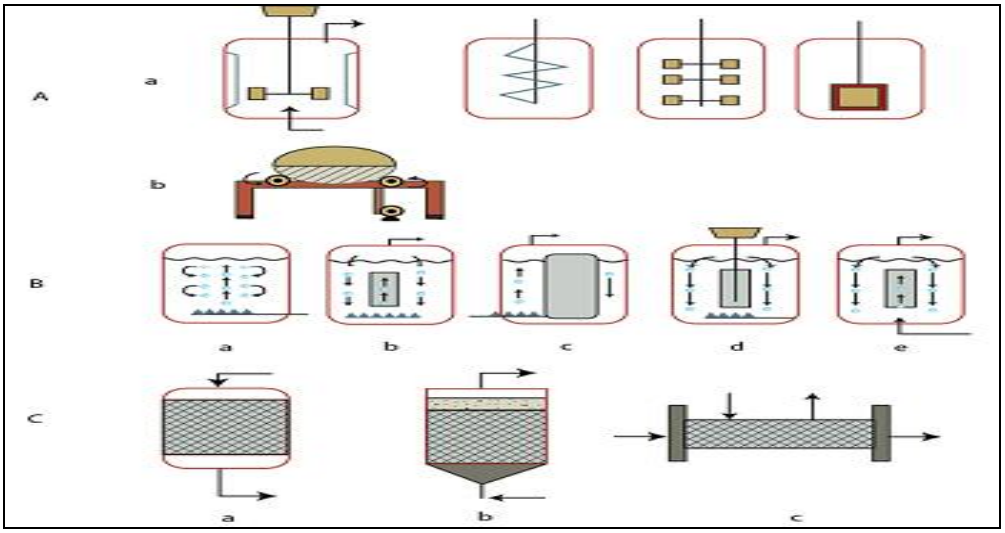

Figure 5.2: Bioreactor types for plant cell, tissue and organ cultures: (A) Mechanically agitated bioreactors: (a) stirred tank reactor equipped with various propellers (spin, helix, bladed, paddle), (b) rotary drum tank reactor; (B) Air driven bioreactors: (a) bubble column, (b) concentric tube airlift reactor (IL ALR), (c) external loop airlift reactor (EL ALR), (d) propeller loop reactor, (e) jet loop reactor; (C) Non-agitated bioreactors: (a) packed bed, (b) fluidised bed, (c) membrane reactor.

3.5.2 Air Driven Bioreactors

A bubble column bioreactor (Figure 5.2. Ba) is a reactor, in the shape of a column, in which the reaction medium is kept mixed and aerated by the introduction of air at the bottom (IUPAC, 297). The major advantages of bubble column bioreactors are the low capital costs, uncomplicated mechanical configurations and less operational costs due to low energy requirements. Alternatively, they are less suitable for the processes where highly viscous liquids exist. In an airlift bioreactor (Figure 5.2. Bb), the reaction medium is agitated and aerated by the introduction of air or another gas mixture and the circulation is improved by internal draught tubes or external loops. Thus, the reactor volume is separated into gassed and degassed regions generating a vertically circulating flow (IUPAC, 297). Airlift bioreactors fulfill the low O_2 demands of plant cell cultures with low shear effects. Airlift bioreactors have a number of advantages, such as combining high loading of solid particles, providing good mass transfer, relatively low shear rate, low energy requirements, and simple design. The main disadvantage is their unsuitability for high density plant cultures. Hence stirred tank bioreactors are preferred for culturing plant cell suspensions at high densities. A further problem in air lift bioreactors is extensive foaming which can clog the air exhaust filters and increase the risk of contamination. To overcome these problems, sparger rings for plant

cells at high aeration rates, bubble free aeration, antifoam agents, etc. can be applied.

3.5.3 Non-Agitated Bioreactors

For the immobilisation of a large number of cells per unit volume packed bed (Figure 5.2. Ca) and membrane reactors (Figure 5.2.Cc) are advantageous. However, diffusional limitations of mass transfer and difficulties in handling gaseous components can limit the use of both configurations (Sajc et al., 2000). Fluidised bed reactor (Figure 5.2. Cb) is based on the utilisation of the energy of the flowing fluid to suspend the particles. This type of reactors provides the major benefit of mass transfer of the small particles.

3.6 Bioreactors for Hairy Roots

While designing a suitable bioreactor for hairy root cultures the physiology and morphology of the hairy roots should be taken into consideration. The major problem in bioreactor cultivation of hairy roots is their tendency to form clumps resulting from the bridging of primary and secondary roots. This results in densely packed root beds and reduces mass transfer (both oxygen and nutrients). Root thickness, root length, the number of root hairs and root branching frequency are some of the factors which should be taken into consideration for hairy root cultures in bioreactors. Immobilisation of hairy roots by horizontal or vertical meshes as well as by cages or polyurethane foam promotes their growth in submerged stirred bioreactors, bubble columns, air lift reactors and drum reactors where the roots are immersed in the culture medium. Isolation of the roots from the impeller also rules out the possibility of root damage even at low tip speeds in stirred bioreactors. Also the oxygen transfer limitation in hairy root cultures in bioreactors can be reduced by growing them in gas phase bioreactors, spray or droplet reactors and mist reactors. Here the roots are exposed to humidified air or a gas mixture and nutrients are delivered as droplets by spray nozzles. Spray and mist reactors also provide the added advantage of low hydrodynamic stress.

4.0 CONCLUSION

High productivity, high product yield and high product concentration are the major objectives of plant tissue process development. A variety of bioreactor types providing growth and expression of bioactive substances are available today for plant cell and tissue cultures. Low biomass and product level can be achieved in any type of bioreactors. However, an improved understanding of the manifold interactions between cultivated cells, product formation and the specific designs for

different bioreactor types will enhance and sustain high productivity and also reduce the process costs.

5.0 SUMMARY

A variety of bioreactor types providing growth and expression of bioactive substances are available today for plant cell and tissue cultures. Low biomass and product level can be achieved in any type of bioreactors. However, an improved understanding of the manifold interactions between cultivated cells, product formation and the specific designs for different bioreactor types will enhance and sustain high productivity and also reduce the process costs.

6.0 TUTOR-MARKED ASSIGNMENT

1. What are the advantages of plant bioreactor?
2. How is batch culture different from continuous or semi-continuous culture of plant cell suspension?
3. What are important parameters need to be considered while designing the suitable plant bioreactor process?
4. Select the two main problems caused by high biomass concentrations in plant cell culture:
   a) rapid settling of the culture
   b) difficult to control temperature
   c) oxygen supply
   d) harvesting the cells
   e) mixing
5. Write short notes on
   a) Mechanically agitated bioreactors
   b) Air driven bioreactors
   c) Non-agitated bioreactors
   d) Bioreactors for hairy roots.

UNIT 6 MANIPULATION IN PRODUCTION PROFILE BY ABIOTIC AND BIOTIC ELICITATION

CONTENTS

1. INTRODUCTION

Treatment with biotic and abiotic compounds helps in the stimulation of particular facets of plant metabolism and enhances the yield of desired secondary metabolites. “Phytoalexins” are the secondary compounds accumulated in response to microbial attack. Accumulation of phytoalexins, which results in chemical resistance, is an important factor in plant defense and has been demonstrated for wide variety of species. Many higher plants are major sources of natural products which are used in pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides. Plant tissue culture is a potential supplement to traditional agriculture in the industrial production of bioactive plant secondary metabolites, which is an alternative to production of desirable medicinal compounds from plants.

Tissue culture is an *in vitro* propagation technique of a wide range of excised plant parts, through which a mass of cells (callus) is produced from an explant tissue. The callus produced, can be utilised to regenerate plantlets or to extract or manipulate primary and secondary metabolites. The signals triggering the formation of phytoalexins are called elicitors. Elicitors have also been shown to induce a range of other plant secondary metabolites. The production of these compounds is a dynamic defense response exhibited by plant cells when challenged by an elicitor. The most commonly used biotic elicitors include fungal homogenates of the genus, like *Phytophthora*, *Aspergillus*, and *Alternaria*, and abiotic elicitors, e.g., inorganic salts of cadmium, copper, and vanadyl, jasmonates (plant hormones that have a dual effect on plant growth and development). By cell culture system only low yields of desired secondary metabolites can be obtained, and efforts are

required to improve the productivity of plant cell cultures by means of elicitation.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain elicitor- induced effects in plant cells
- state the mechanism of elicitation
- outline the special features of elicitors
- explain and state classification of elicitors.

3.0 MAIN CONTENT

3.1 Elicitor- Induced Effects in Plant Cells

Different types of effects are induced in plant cells by the use of elicitors, which is reflected by the influenced cell metabolism.

- Ca^{2+} metabolism
- Massive variation in membrane integrities, respiration, protein and phosphate metabolism, ethylene production and peroxidase activity.
- Differential gene expression, consequently forming enzymes concerned in the synthesis of polysaccharides as callose, hydroxyproline rich glycoproteins (HRGP) in cell walls via induction of proline hydroxylase, lignin and polyphenolics (deposited in cell walls) chitinases and protein inhibitors, specific proteins against pathogenic infections, phytoalexins.

3.2 Mechanism of Elicitation

Elicitors bind to a specific receptor probably located in the plasma membrane. The elicitor-receptor interactions generate signals that then activate nuclear genes involved in plant defense reactions, such as the biosynthesis of phytoalexins. The pathway is initiated by the action of local and systemic signal molecules and putative plasma membrane receptors. Wound signal molecules include polygalacturonic acid, chitosan, physical signals, abscisic acid and systemin. Plasma membrane receptors include a β-glucan-elicitor-binding protein (GEBP), a systemin binding protein of 160 kDa and an unidentified receptor for oligosaccharide elicitors. A lipase translates the wound signal and releases linolenic acid from membrane phospholipid, a process stimulated by ABA, volicitin and β- glucosidase from the oral secretion of insects is converted to jasmonic acid through the octadecanoid pathway.

3.3 Special Features of Elicitors

1. The products which accumulate in plant cell cultures due to elicitation may be antimicrobial in nature, but they should not be grouped in phytoalexins unless there is sufficient proof that the source of plant respond to pathogens is with the rapid accumulation of the same product. Therefore a new term that has been coined for those compounds, which in cell cultures are inducible by way of elicitation, is "Elicitation Product" or "Elicitation Metabolite".
2. Elicitors can be regarded as substitute of production media (optimum cultural conditions).
3. Optimum employment of elicitors depends upon:
   a) Elicitor specificity
   b) Elicitor concentration
   c) Duration of elicitor contact
   d) Elicitor of cell line (clones)
   e) Time course of elicitation
   f) Growth stage of culture
   g) Growth regulation
   h) Nutrient composition
4. Response of the cells to elicitation in suspension cultures may be in the following ways:
   - In a given cell line, different products may show highest level of accumulation, at different times and stages of growth.
   - Product accumulation may be observed in cell lines where area and pathway of synthesis is not known.
   - Elicitation may not cause an additive effect when applied to cells in production media but may shorten the culture period required for maximum product accumulation.
5. Product accumulation due to elicitation has also been observed in growth media. Such occurrence may be due to excretion or leakage caused by cell breakdown

3.4 Classification of Elicitors

Elicitors produced within plant cells are termed as endogenous elicitors, while those produced by microorganisms are called exogenous elicitors. Depending upon their nature, they are classified as biotic and abiotic elicitors.

3.4.1 Biotic Elicitors

Biotic elicitors are either pathogen or host origin that can stimulate defense responses (such as phytoalexin accumulation) in plant tissues. From earlier studies carbohydrates have been known for the overproduction of secondary metabolites in plant cell cultures. Albersheim et al. (277) first isolated oligosaccharides that activate a variety of plant defense genes. The signal transfer triggered by carbohydrate elicitors has been studied with regard to calcium influx, pH shifts and production of H_2O_2 in tobacco cell cultures.

Examples of Elicitors

- The combination of oligosaccharides and methyl jasmonate has been employed to induce phytoalexins in rice systems (Nojiri *et al.* 296).
- Production of paclitaxel in *Taxus canadensis* cell suspension cultures was enhanced when the cultures were treated with a combination of N-acetyl ketohexose and methyl jasmonate (Linden *et al.* 2000).
- Methyl jasmonate, a lipid-derived elicitor, was also applied as an elicitor in combination with chitopentaose to *J. chinensis* cell suspension cultures for the enhancement of podophyllotoxin production. (Premjet *et al*, 2002).
- Fungal elicitor is normally one which is derived from a fungus (*Phytophthora, Botrytis* , *Verticilium, Alternaria, Fusarium* , etc.) pathogenic to the plant species. Although preparations derived from non-pathogenic (*Aspergillus, Micromucor, Rhodotorula*, etc) fungus have also been successfully employed for the elicitation purpose.

The latter type of elicitors is often released by mechanical wounding or enzymatic hydrolysis of polymeric compounds in plants which are polysaccharides of plant cell walls. These are of two types: β-linked glucans and chitosan. Another group of elicitors are enzymes with polygalactomarase activity, which releases pectic fragments from plant cell walls. It has been observed that very small amount of such water-soluble oligomers lead to rapid induction of phytoalexins in cell cultures. Some more examples of biotic elicitor are listed in table 37.1.

Table 6.1: Examples of biotic elicitors

Biotic elicitors	Plant species	product
Chitosan	*Lupinus albus*	Isoflavonoids
N-Acetylchitohexaose	*Taxus canadensis*	Taxol
Mannan	*Hypericum perforatum*	Hypericins
Oligogalacturonic acid	*Panax ginseng*	Saponin
Yeast elicitor, Methyl jasmonate	*Rauvolfia canescens*	Raucaffricine
Methyl jasmonate	*Glycyrrhiza glabra*	Soyasaponin 5-deoxyflavonoid
Yeast elicitor	*Coleus blumei*	Rosmarinic acid
Polysaccharide	*Allium cepa*	Tcibulin1, Tcibulin2
Polysaccharides, fungal elicitor, Methyl jasmonate	*Lithospermum erythrorhizon*	Shikonin, Rosmarinic acid

3.4.2 Abiotic Elicitors

All the factors which cannot be regarded as natural components of the environment of a plant cell are considered as abiotic elicitors. Abiotic elicitors are of non-biological origin mainly the metal ions. Also the abiotic elicitors are of physical or chemical nature working via endogenously formed biotic elicitors. Some examples of abiotic elicitor are listed in table 6.2. Salicylic acid, methyl jasmonate, calcium chloride, silver nitrate, copper sulphate, cinnamic acids, etc. Acids can be employed for eliciting the plant cell cultures.

The use of metal ions as elicitors offers many advantages over their biotic counterparts, these include:

- their ready availability
- relatively low cost
- ease of use
- they are chemically defined

Table 6.2: Examples of abiotic elicitors

Abiotic elicitors	Plant species	Product
Arachidonic acid	*Capsicum annuum*	Capsidol, Rishitin
Copper chloride	*Matricaria chamomilla*	Hemiarin, Umbelliferone
Copper sulphate	*Hyoscyamus albus*	Phytoalexin
Cd^{2+}, Cu^{2+}	*Atropa belladonna*	Casaicin
Curdlan, Xanthan	*Capsicum frutescence*	Indole alkaloids
Salicylic acid	*Daucus carota*	Chitinase
Vanadium sulphate	*Catharanthus roseus*	Catharanthine

4.0 CONCLUSION

Treatment with biotic and abiotic compounds helps in the stimulation of particular facets of plant metabolism and enhances the yield of desired secondary metabolites.

5.0 SUMMARY

“Phytoalexins” are the secondary compounds accumulated in response to microbial attack. Accumulation of phytoalexins, which results in chemical resistance, is an important factor in plant defense and has been demonstrated for wide variety of species. Many higher plants are major sources of natural products which are used in pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides. Plant tissue culture is a potential supplement to traditional agriculture in the industrial production of bioactive plant secondary metabolites, which is an alternative to production of desirable medicinal compounds from plants.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is elicitor?
2. What are the effects of elicitor in plant cell?
3. Describe mechanism of elicitation.
4. Write down the features of elicitors?
5. What are the factors affecting elicitation?
6. What are the responses of the cells to elicitation in suspension cultures?
7. How many type of elicitor are found?
8. Write short notes on:
   A. Elicitor
   B. Biotic elicitor
   C. Abiotic elicitor.

UNIT 7 BIOTRANSFORMATION

CONTENTS

1.0 INTRODUCTION

Biotransformation is chemical reactions catalyzed by cells, organs or enzymes. It is defined as a process through which the functional groups of organic compounds are modified by living cells to a chemically different product. Biotransformation explores the unique properties of biocatalysts, namely their stereo-and region-specificity and their ability to carry out reactions at no extreme pH values and temperatures. Biotransformation may be used to carry out specific conversions of complex substrates using plant, animal or microbial cells or purified enzymes as catalyst. Biotransformation is different from biosynthesis where complex products are assembled from simple substrates by whole cells, organs or organisms. They are also different from biodegradations in which complex substances are broken down to simple ones. Biotransformation has great potential to generate novel products or to produce known products more efficiently.

The production of food metabolites, fine chemicals and pharmaceuticals can be achieved by biotransformation using biological catalysts. Cell suspension cultures, immobilised cells, hairy root cultures can be useful for the production of food additives and pharmaceuticals by biotransformation process. Plant cells for biotransformation purposes are selected because of two main reasons. Plant cells are usually able to catalyse the reactions stereospecifically, resulting in chirally pure products. They can carry out regiospecific modifications that are not easily carried out by chemical synthesis or by microorganisms. These reactions include reduction, oxidation, hydroxylation, acetylation,

esterification, glucosylation, isomerisation, methylation, demethylation, epoxidation, etc.
However, for a successful and viable process, the following prerequisites must be met:

- The culture must have the essential enzymes.
- The substrate or precursor must not be toxic to the cell culture.
- The substrate must reach the appropriate cellular compartment of the cell.
- The rate of product formation must be faster than its further metabolism.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain biotransformation using plant cells and organ cultures
- explain biotransformation using immobilized cell culture
- identify genetic engineering approaches towards biotransformation
- list advantages of biotransformation
- state factors influencing biotransformation.

3.0 MAIN CONTENT

3.1 Biotransformation Using Plant Cells and Organ Cultures

The biotransformation rates by plant cells and organs are depend on a variety of factors including the solubility of precursors, the amount of enzyme activity present, localisation of enzymes, presence of side reactions producing undesired byproduct and presence of enzymes degrading the desired product. Elicitation, permeabilisation, pH variation and osmotic effects can also influence biotransformation capacity of cells. Some examples of biotransformation reactions performed by *in vitro* plant cell and organ cultures are given below:

- *Peganum harmala* cell culture converted geranyl acetate to geraniol and linalyl acetate to linalool and α-terpineol.
- The alkaloid nitrosamine, which contains seven stereogenic centers, is present in *Nitrariaschoberi*as a racemate. Isolation of a chiral metabolite might be due to spontaneous nonenzymatic reactions starting from an achiral precursor followed by enzyme-catalysed metabolism of one of the enantiomers.

- *Catharanthus roseus* suspension cell cultures can oxidise the phenylsulphonyl group from completely synthetic molecules to phenylsulfonyl derivatives.

Biotransformation of cinobufagin by *C. roseus* cell suspension cultures is shown in **Figure 7.1**.

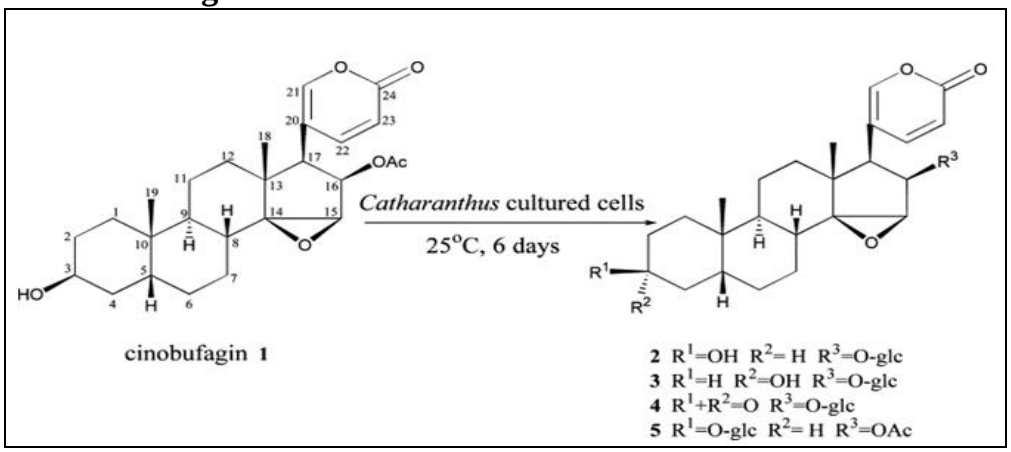

Figure 7.1: Biotransformation of cinobufagin by *C. roseus* cell suspension cultures

Table 7.1: Examples of biotransformation

Substrate	Main product	Types of reaction	Plant species
Nerol	Geranial, neral	Oxidation of OH	*Rosa centifolia*
Neryl acetate	Geranial, neral	Oxidation of OH	*Rosa centifolia*
Valencene	Nootkatone	Hydroxylation, Dehydrogenation	*Citrus paradisi*
Steviol	Rubusoside	Glucosylation	*Coffea arabica*

3.2 Biotransformation Using Immobilised Cell Culture

Entire cells offer the chance to implement multistep biotransformation and to utilise and recycle essential cofactors and co-enzymes. Isolated enzymes may be sensitive to denaturing conditions, including pH, heat and specific organic solvents. In order to be useful in biotransformation reactions, biocatalysts need to be stable and reusable. Use of whole cell immobilised system may help overcome some stability problems. Immobilised plant cells have some additional advantages over freely suspended cells. They are more resistant to shear damage and can be used repeatedly over a prolonged period. Complete cell immobilisation may also create adverse conditions under which secondary metabolite production may be improved. A very common method for immobilisation of plant cells are gel entrapment by ion exchange, precipitation, polymerisation and in preformed structures. For

adsorption of plant cells, solid surfaces can be used. Enzymes may be adsorbed to insoluble supports by hydrogen bonding, dipole–dipole interactions and hydrophobic interactions. Commonly used supports are polypropylene and diatomaceous celite.

3.3 Genetic Engineering Approaches Towards Biotransformation

Bioconversion/biotransformation capacity of cell cultures can be further improved by changing the following parameters:

- Cell selection
- Elicitation
- Permeabilisation
- Radiation
- pH of medium
- Osmotic shock

A more basic approach is the transfer of genes that code for the key enzymes catalysing the desired biosynthetic reactions into a fungal or bacterial cell because of their ability to produce high amounts of enzymes (Pras et al., 295). Hashimoto et al. (293) explain the expression of hyoscyamine 6-b-hydroxylase in *Escherichia coli* . This recombinant bacterium was capable to convert hyoscyamine to scopolamine. Subsequently, this cloned gene has been transferred to *Atropa belladonna* and expressed constitutively. Additionally, he also found that transformed hairy roots with increased efficiency of conversion of hyoscyamine to scopolamine. Cloning and expression of bacterial lysine decarboxylase under the control of a 35S promoter fused to the coding sequences of the small subunit of rubisco transit peptide in tobacco root cultures was found to affect two secondary metabolic pathways (Berlin et al., 298).

3.4 Advantages of Biotransformation

The advantages consist of the production of novel compounds, improvement in the productivity of desired compound and overcoming the problems related with chemical synthesis. Biotransformation studies lead to basic information to elucidate the biosynthetic pathway, and catalysis can be carried out under mild conditions, thus reducing undesired by-products, energy, safety and costs.

3.5 Factors Influencing Biotransformation

3.5.1 Improvement of Cell Viability

Many substances are harmful to cultured cells. So it is necessary to decrease the toxicity in order to increase the yield of the product. Sugar can increase cell viability during glycosylation of phenolic compounds. Antioxidants can improve cell viability and increase product formation in the biotransformation of phenolics. Yokoyama (291) reported that antioxidant, such as gallic acid, ascorbic acid; cystein and tannins could increase the production of arbutin when hydroquinone was added to the cell culture.

3.5.2 Selection of Plant Species

The capacity for biotransformation is diverse among plant species. Tabata et.al (288) reported that among seven species of plant cell cultures, only *Datura* had capacity to biotransform coumarins, flavonoids, phenolic acids and anthraquinones.

3.5.3 Immobilised Plant Cells

It has distinct advantages e.g. reuse of the expensive biocatalyst, continuous process, and process control is simplified.

3.5.4 Root Culture

Cell suspension culture has excellent biotransformation capacity for glucosylation. Furuya et al. (289) have found that the root culture showed higher glycosylation activity than cell culture.

4.0 CONCLUSION

Biotransformation is chemical reactions catalysed by cells, organs or enzymes. It is defined as a process through which the functional groups of organic compounds are modified by living cells to a chemically different product. Biotransformation explores the unique properties of biocatalysts, namely their stereo-and region-specificity and their ability to carry out reactions at no extreme pH values and temperatures. Biotransformation may be used to carry out specific conversions of complex substrates using plant, animal or microbial cells or purified enzymes as catalyst.

5.0 SUMMARY

Biotransformation is different from biosynthesis where complex products are assembled from simple substrates by whole cells, organs or organisms. They are also different from biodegradations in which complex substances are broken down to simple ones. Biotransformation has great potential to generate novel products or to produce known products more efficiently. The production of food metabolites, fine chemicals and pharmaceuticals can be achieved by biotransformation using biological catalysts. Cell suspension cultures, immobilised cells, hairy root cultures can be useful for the production of food additives and pharmaceuticals by biotransformation process. Plant cells for biotransformation purposes are selected because of two main reasons. Plant cells are usually able to catalyse the reactions stereospecifically, resulting in chirally pure products. They can carry out regiospecific modifications that are not easily carried out by chemical synthesis or by microorganisms. These reactions include reduction, oxidation, hydroxylation, acetylation, esterification, glucosylation, isomerisation, methylation, demethylation, epoxidation, etc.

However, for a successful and viable process, the following prerequisites must be met:

- The culture must have the essential enzymes.
- The substrate or precursor must not be toxic to the cell culture.
- The substrate must reach the appropriate cellular compartment of the cell.
- The rate of product formation must be faster than its further metabolism.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is biotransformation?
2. What are the factors involves in plant biotransformation?
3. Give some examples of plant biotransformation using plant cell culture.
4. Describe about biotransformation using immobilised cell culture.
5. Describe genetic engineering approaches towards biotransformation.
6. What are the advantages of biotransformation?

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UNIT 8 ADVANTAGES OF PLANT CELL, TISSUE AND ORGAN CULTURE AS SOURCE OF SECONDARY METABOLITES

CONTENTS

1.0 INTRODUCTION

Plant tissue culture can be defined as the *in vitro* manipulation of plant cells and tissues and is a keystone in the foundation of plant biotechnology. It is useful for plant propagation and in the study of plant growth regulators. It is generally required to manipulate and regenerate transgenic plants. Whole plants can be regenerated under *in vitro* conditions using plant organs, tissues or single cells, by inoculating them in an appropriate nutrient medium under sterile environment. Plant tissue culture relies on the fact that many plant cells have the capacity to regenerate into a whole plant–a phenomena known as tot potency. Plant cells, cells without cell walls (protoplasts), leaves, or roots can be used to generate a new plant on culture media containing the necessary nutrients and plant growth regulators. Plant tissue culture was first attempted by Haberlandt (202). He grew palisade cells from leaves of various plants but they did not divide. In 234, White generated continuously growing cultures of meristematic cells of tomato on medium containing salts, yeast extract and sucrose and vitamin B (pyridoxine, thiamine and nicotinic acid) and established the importance of additives. In 253, Miller and Skoog, University of Wisconsin – Madison discovered Kinetin, a cytokine that plays an active role in organogenesis. Plant cell cultures are an attractive alternative source to whole plants for the production of high-value secondary metabolites.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- state advantages of plant tissue culture over conventional agricultural production
- explain plant secondary metabolites
- outline strategies for enhanced production of secondary metabolites in plant cell cultures
- state advantages of cell, tissue and organ cultures as sources of secondary metabolites.

3.0 MAIN CONTENT

3.1 Advantages of Plant Tissue Culture over Conventional Agricultural Production

The most important advantage of *in vitro* grown plants is that it is independent of geographical variations, seasonal variations and also environmental factors. It offers a defined production system, continuous supply of products with uniform quality and yield. Novel compounds which are not generally found in the parent plants can be produced in the in vitro grown plants through plant tissue culture. In addition, stereo- and region- specific biotransformation of the plant cells can be performed for the production of bioactive compounds from economical precursors. It is also independent of any political interference. Efficient downstream recovery of products and rapidity of production are its added advantages (Figure 8.1).

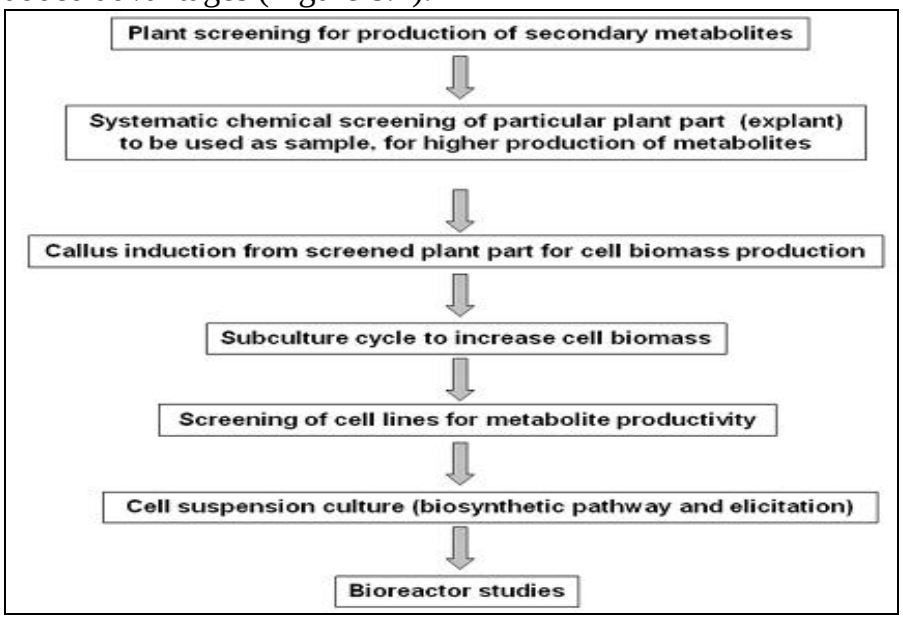

Figure 8.1: *Steps involved in the production of secondary metabolites from plant cell*

3.2 Plant Secondary Metabolites

Plant products can be classified into primary plant metabolites and secondary metabolites. Primary plant metabolites are essential for the survival of the plant. It consists of sugars, amino acids and nucleotides synthesized by plants and are used to produce essential polymers. Typically, primary metabolites are found in all species within broad phylogenetic groupings, and are produced using the same metabolic pathway. Secondary metabolites are the chemicals, which are not directly involved in the normal growth and development, or reproduction of an organism. Secondary metabolites are not indispensable for the plants but play a significant role in plant defense mechanisms. Primary metabolites essentially provide the basis for normal growth and reproduction, while secondary metabolites for adaptation and interaction with the environment. The economic importance of secondary metabolites lies in the fact that they can be used as sources of industrially important natural products like colours, insecticides, antimicrobials, fragrances and therapeutics. Therefore, plant tissue culture is being potentially used as an alternative for plant secondary metabolite production. Majority of the plant secondary metabolites of interest to humankind fit into categories which categorise secondary metabolites based on their biosynthetic origin. Secondary metabolism in plants is activated only in particular stages of growth and development or during periods of stress, limitation of nutrients or attack by micro-organisms.

Plants produce several bioactive compounds that are of importance in the healthcare, food, flavor and cosmetics industries. Many pharmaceuticals are produced from the plant secondary metabolites. Currently, many natural products are produced solely from massive quantities of whole plant parts. The source plants are cultured in tropical, subtropical, geographically remote areas, which are subject to drought, disease and changing land use patterns and other environmental factors. Secondary metabolites can be derived from primary metabolites through modifications, like methylation, hydroxylation and glycosylation. Secondary metabolites are naturally more complex than primary metabolites and are classified on the basis of chemical structure (e.g., aromatic rings, sugar), composition (containing nitrogen or not), their solubility in various solvents or the pathway by which they are synthesized (Table 8.1). They have been classified into terpenes (composed entirely of carbon and hydrogen), phenolics (composed of simple sugars, benzene rings, hydrogen and oxygen) and nitrogen and/or sulphur containing compounds (Figure 8.2). It has been observed that each plant family, genus and species produces a characteristic mix of these bioactive compounds.

All plants produce secondary metabolites, which are specific to an individual species, genus and are produced during specific environmental conditions which makes their extraction and purification difficult. As a result, commercially available secondary metabolites, for example, pharmaceuticals, flavours, fragrances and pesticides etc. are generally considered high value products as compared to primary metabolites and they are considered to be fine chemicals.

Table 8.1: Classification of secondary metabolites

Terpenes		Phenols		Nitrogen and /or sulphur containing compounds	
Type	Example	Type	Example	Type	Example
Monoterpenes	Farnessol	Lignan	lignan	Alkaloids	Nicotine
Sespuiterpenes	Limonene	Tannins	gallotannin	Atropine	
Diterpenes	Taxol	Flavonoids	anthocyanin	Glucosinolates	Sinigrin
Triterpenes	Digitogenin	Coumarins	Umbelliferone		
Tetraterpenoids	Carotene				
Sterols	Spinasterol				

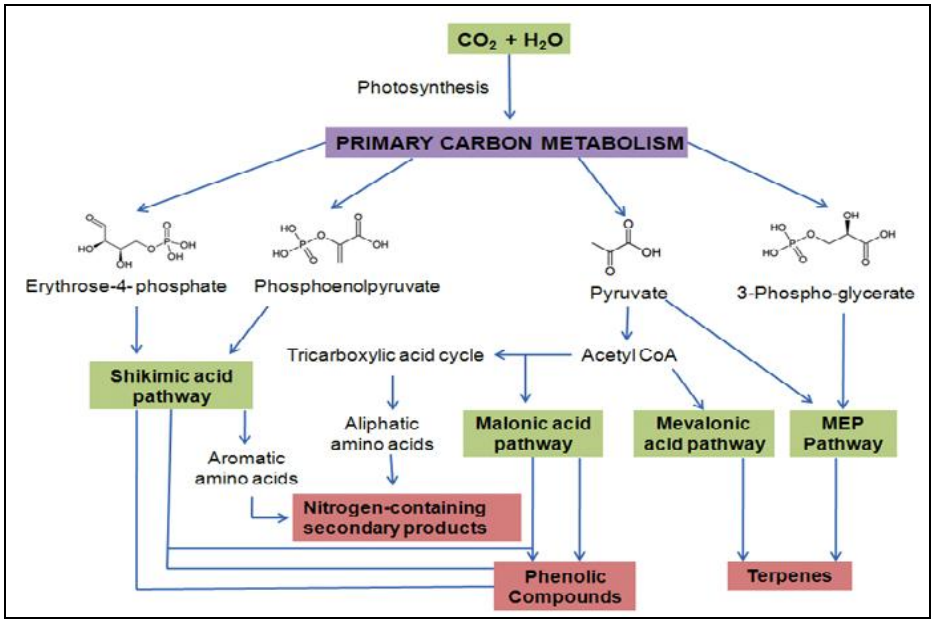

Figure 8.2: *The production of secondary metabolites is tightly associated with the pathways of primary/central metabolism, such as glycolysis, shikimate and production of aliphatic amino acids.*

3.3 Strategies for Enhanced Production of Secondary Metabolites in Plant Cell Cultures

3.3.1 Proper Selection of Cell Lines

The heterogeneity within the cell population can be screened by selecting cell lines capable of accumulating higher level of metabolites.

3.3.2 Manipulation of Medium

The constituents of culture medium, like nutrients, phytohormones and also the culture conditions, like temperature, light etc. influence the production of secondary metabolites. For e.g., if sucrose concentration is increased from 3% to 5%, production of rosamarinic acid is increased by five times. In case of shikonin production, IAA enhances the yield whereas 2,4-D and NAA are inhibitory.

3.3.3 Addition of Elicitors

Elicitors are the compounds which induce the production and accumulation of secondary metabolites in plant cells. Elicitors produced within the plant cells include cell wall derived polysaccharides, like pectin, pectic acid, cellulose etc. Product accumulation also occurs under stress conditions caused by physical or chemical agents like UV, low or high temperature, antibiotics, salts of heavy metals, high salt concentrations which are grouped under abiotic elicitors. Addition of these elicitors to the medium in low concentration enhances the production of secondary metabolites.

3.3.4 Addition of Precursors

Precursors are the compounds, whether exogenous or endogenous, that can be converted by living system into useful compounds or secondary metabolites. It has been possible to enhance the biosynthesis of specific secondary metabolites by feeding precursors to cell cultures. For example, amino acids have been added to suspension culture media for production of tropane alkaloids, indole alkaloids. The amount of precursors is usually lower in callus and cell cultures than in differentiated tissues. Phenylalanine acts as a precursor of rosmarinic acid; addition of phenylalanine to *Salvia officinalis* suspension cultures stimulated the production of rosmarinic acid and decreased the production time as well. Phenylalanine also acts as precursor of the N-benzoylphenylisoserine side chain of taxol; supplementation of *Taxus cuspidata* cultures with phenylalanine resulted in increased yields of taxol. The timing of precursor addition is critical for an optimum effect.

The effects of feedback inhibition must surely be considered when adding products of a metabolic pathway to cultured cells.

3.3.5 Permeabilisation

Secondary metabolites produced in cells are often blocked in the vacuole. By manipulating the permeability of cell membrane, they can be secreted out to the media. Permeabilisation can be achieved by electric pulse, UV, pressure, sonication, heat, etc. Even charcoal can be added to medium to absorb secondary metabolites.

3.3.6 Immobilisation

Cell cultures encapsulated in agarose and calcium alginate gels or entrapped in membranes are called immobilised plant cell cultures. Immobilisation of plant cells allows better cell to cell contact and the cells are also protected from high shear stresses. These immobilized systems can effectively increase the productivity of secondary metabolites in a number of species. Elicitors can also be added to these systems to stimulate secondary metabolism.

3.3.7 Limitations

- Production cost is often very high.
- Lack of information of the biosynthetic pathways of many compounds is a major drawback in the improvement of their production.
- Trained technical manpower is required to operate bioreactors.

3.4 Advantages of Cell, Tissue and Organ Cultures as Sources of Secondary Metabolites

3.4.1 Plant Cell Cultures

Once interesting bioactive compounds have been were identified from plant extracts, the first part of the work consisted in collecting the largest genetic pool of plant individuals that produce the corresponding bioactive substances. However, a major characteristic of secondary compounds is that their synthesis is highly inducible; therefore, it is not certain, if a given extract is a good indicator of the plant potential for producing the compounds. The ability of plant cell cultures to produce secondary metabolites came quite late in the history of *in vitro* techniques. For a long time, it was believed that undifferentiated cells, such as callus or cell suspension cultures were not able to produce secondary compounds, unlike differentiated cells or specialized organs.

3.4.2 Callus Culture

Callus is a mass of undifferentiated cells derived from plant tissues for use in biological research and biotechnology. In plant biology, callus cells are those cells that cover a plant wound. To induce callus development, plant tissues are surface sterilized and then plated onto *in vitro* tissue culture medium. Different plant growth regulators, such as auxins, cytokinins, and gibberellins, are supplemented into the medium to initiate callus formation. It is well known that callus can undergo somaclonal variations, usually during several subculture cycles. This is a critical period where, due to *in vitro* variations, production of secondary metabolite often varies from one subculture cycle to another. When genetic stability is reached, it is necessary to screen the different cell (callus) lines according to their aptitudes to provide an efficient secondary metabolite production. Hence, each callus must be assessed separately for its growth rate as well as intracellular and extracellular metabolite concentrations. This allows an evaluation of the productivity of each cell line so that only the best ones will be taken for further studies, for example, for production of the desired compound in suspensions cultures.

3.4.3 Cell suspension cultures

Cell suspension cultures represent a good biological material for studying biosynthetic pathways. They allow the recovery of a large amount of cells from which enzymes can be easily separated. Compared to cell growth kinetics, which is usually an exponential curve, most secondary metabolites are often produced during the stationary phase. This lack of production of compounds during the early stages can be explained by carbon allocation mainly distributed for primary metabolism when growth is very active. On the other hand, when growth stops, carbon is no longer required in large quantities for primary metabolism and secondary compounds are more actively synthesized. However, some of the secondary plant products are known to be growth-associated with undifferentiated cells, such as betalains and carotenoids.

3.4.4 Organ cultures

Plant organs are alternative to cell cultures for the production of plant secondary metabolites. Two types of organs are generally considered for this objective: hairy roots and shoot cultures. A schematic representation of various organised cultures, induced under *in vitro* conditions, is given in Figure 8.3.

3.4.4.1 Shoot Cultures

Shoots exhibit some comparable properties to hairy roots, genetic stability and good capacities for secondary metabolite production. They also provide the possibility of gaining a link between growth and the production of secondary compounds.

3.4.4.2 Hairy Root Cultures

Hairy roots are obtained after the successful transformation of a plant with *Agrobacterium rhizogenes*. They have received considerable attention of plant biotechnologists, for the production of secondary compounds. They can be subcultured and indefinitely propagated on a synthetic medium without phytohormones and usually display interesting growth capacities owing to the profusion of lateral roots. This growth can be assimilated to an exponential model, when the number of generations of lateral roots becomes large.

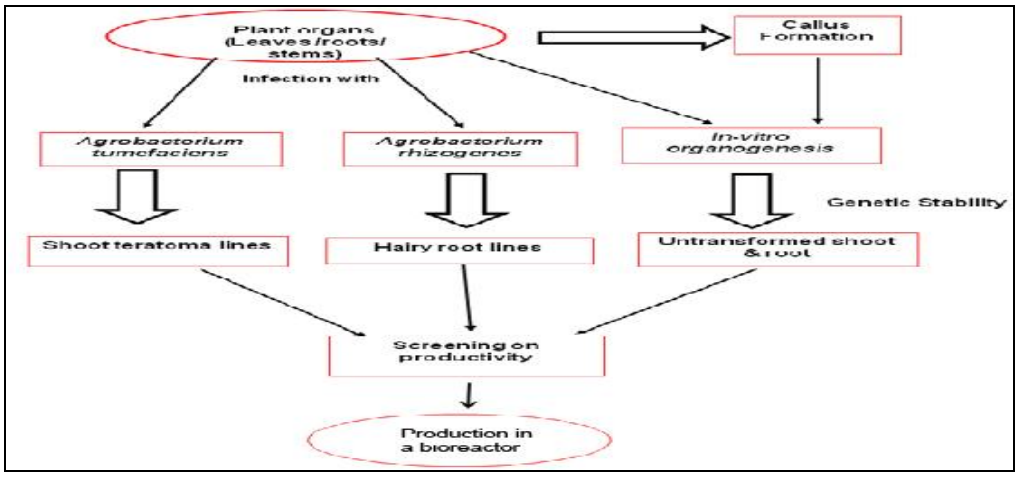

Figure 8.3: Guidelines for the production of secondary metabolites from plant organ cultures.

4.0 CONCLUSION

Plant tissue culture can be defined as the *in vitro* manipulation of plant cells and tissues and is a keystone in the foundation of plant biotechnology. It is useful for plant propagation and in the study of plant growth regulators. It is generally required to manipulate and regenerate transgenic plants. Whole plants can be regenerated under *in vitro* conditions using plant organs, tissues or single cells, by inoculating them in an appropriate nutrient medium under sterile environment. Plant tissue culture relies on the fact that many plant cells have the capacity to regenerate into a whole plant–a phenomena known as tot potency.

5.0 SUMMARY

Plant cells, cells without cell walls (protoplasts), leaves, or roots can be used to generate a new plant on culture media containing the necessary nutrients and plant growth regulators. Plant tissue culture was first attempted by Haberlandt (202). He grew palisade cells from leaves of various plants but they did not divide. In 234, White generated continuously growing cultures of meristematic cells of tomato on medium containing salts, yeast extract and sucrose and vitamin B (pyridoxine, thiamine and nicotinic acid) and established the importance of additives. In 253, Miller and Skoog, University of Wisconsin – Madison discovered Kinetin, a cytokine that plays an active role in organogenesis. Plant cell cultures are an attractive alternative source to whole plants for the production of high-value secondary metabolites.

6.0 TUTOR-MARKED ASSIGNMENT

1. How secondary metabolites are produced?
2. What is the importance of plant cell culture in production of secondary metabolites?
3. What are the steps involved in the production of secondary metabolites from plant cell?
4. How production of secondary metabolites is associated with primary metabolites?
5. What are the strategies for the enhancement of production of secondary metabolites?
6. Give examples of following:
   A. Alkaloid B. Coumarin C. Flavonoid D. Sterol E. Triterpens
7. Following compounds are which class of terpenes?
   A. Taxol B. Digitogenin C. Farnesol D. Spinasterol.

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MODULE 6 MOLECULAR FARMING AND APPLICATIONS

UNIT 1 AIMS SCOPE AND BOTTLENECKS OF MOLECULAR FARMING

CONTENTS

1.0 INTRODUCTION

The large-scale production of recombinant proteins in plants is known as molecular farming. Its objective is to harness the power of agriculture to cultivate and harvest plants for the production of recombinant therapeutics, diagnostics, industrial enzymes and green chemicals. Molecular farming has the potential to provide unlimited quantities of recombinant proteins and, thus, represents a powerful alternative to conventional protein production systems. Plants have several advantages over traditional expression systems, such as bacteria, yeast, mammalian cell lines and transgenic animals. Transgenic plants can be maintained, harvested and processed using normal agricultural practices. There is a structural and functional similarity between plant-derived recombinant protein and native protein. Plants have been used to produce a wide range of products, including therapeutic human proteins, recombinant antibodies, subunit vaccines, nutraceuticals, biopolymers and industrial enzymes.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- state and explain aims and scope of molecular farming
- identify bottlenecks.

3.0 MAIN CONTENT

3.1 Aims and Scope of Molecular Farming

The large-scale production of recombinant proteins in plants is known as molecular farming. Its objective is to harness the power of agriculture to cultivate and harvest plants for the production of recombinant therapeutics, diagnostics, industrial enzymes and green chemicals. Molecular farming has the potential to provide unlimited quantities of recombinant proteins and, thus, represents a powerful alternative to conventional protein production systems. Plants have several advantages over traditional expression systems, such as bacteria, yeast, mammalian cell lines and transgenic animals. Transgenic plants can be maintained, harvested and processed using normal agricultural practices. There is a structural and functional similarity between plant-derived recombinant protein and native protein. Plants have been used to produce a wide range of products, including therapeutic human proteins, recombinant antibodies, subunit vaccines, nutraceuticals, biopolymers and industrial enzymes.

3.1.1 High-Value Pharmaceutical Proteins

Most of the recombinant proteins produced from plants are high-value pharmaceuticals for human use. This can be further classified into 3 categories:

1. Human or animal proteins used for replacement therapies
   These include hormones, growth factors and enzymes, which are mainly expressed in tobacco. Rice has been used for the production of human interferon and antitrypsin.
2. Recombinant antibodies
   Many different antibodies have been expressed in a variety of plant-based expression systems. Recombinant antibodies are used to prevent, diagnose and treat disease. These molecules range in complexity from simple polypeptides to secretory immunoglobulins in which 10 polypeptide chains are covalently assembled through disulphide bridges.
3. Recombinant vaccines

A large number of vaccines are under development, many expressed in tobacco or potato. Potato has the advantage that it can be used for the production of edible vaccines. Vaccines against hepatitis B virus and rabies virus are produced in transgenic lettuce and tomato, respectively.

3.1.2 Plant-Derived Feed Additives and Processing Enzymes

Plants have been used to produce feed additives and catabolic enzymes. One application is the use of feed plants to produce vaccines against animal diseases. Pigs fed on transgenic corn expressing gastroenteritis vaccine are protected from the disease. Plants engineered to express catabolic enzymes can be used to increase the nutritional value of feed. Several plant species have been engineered to express amylases and cellulases to help break down starch and cellulose which increases their utilisable carbohydrate content.

3.1.3 Technical Proteins from Plants

A variety of specialized proteins such as molecular biology reagents are synthesized by transgenic plants. Technical proteins that have been produced in plants include enzymes, avidin, bovine elastin, human collagen and spider silk. Molecular farming can be economical even when the natural source of a protein is abundant (egg whites for avidin).

3.2 Bottlenecks

There are several challenges that need to be tackled in order to improve and establish molecular farming as an alternative production technology. These include technical, economic, safety and regulatory challenges.

3.2.1. Techno-Economic Challenges

1. Expression of the protein

The technology of molecular farming has been tremendously improved over years. The transient production in tobacco leaves have been achieved of five grams of the target protein per kilogram of plant biomass. Still there is a further demand to improve yield to compete with improved mammalian cell lines and microbial fermentations.

2. Downstream processing

The most important thing which has to be taken into consideration is to improve the downstream processing and purification which constitutes 80% of total production costs. Some substances like lignin, fibers, waxes, phenolic compounds, pigments and endogenous proteases c reat e s problems in downstream processing. The large amount of biomass is, thereby, causing technical and economic problems. For example, fiber

rich tissue can clog up chromatography columns and pigment can make it difficult to clean them. Other challenges include the scale up of filtration steps, proteolytic degradation of the target protein during downstream processing and the designing of suitable virus clearance technique.

3. Glycosylation

More than half of human proteins and biopharmaceuticals are glycosylated. Glycosylation could affect their function including half life, tissue targeting and biological activity. Proper glycosylation is therefore an important requirement in terms of safety and efficacy. Biopharmaceuticals are subjected to *in vitro* glycoengineering including the knock-out elimination of unwanted sugars and knock-in glycosylation of proteins. Glycoengineering is particularly difficult in tobacco because tobacco-derived glycan structures are highly heterogeneous, which results in a mixture of different glycol-variants.

3.2.2 Safety and Regulatory Challenges

- Higher concentration of pharmacologically active proteins in plant tissue could be a source of health and environmental risks:
- Risk for human health
- Unintended exposure to material from farm plants might occur via pollen, debris from leaves, stem and flowers and ground water. It is considered to be problematic in case of pharmaceutical substances which might exert toxic, allergic or hormonal effects.
- Environmental risks
- Open field production in plant molecular farming is of concern because of potential effects on non-target organisms and environment including soil flora and fauna. Even in case of strictly confined fields, pollinators and herbivorous insects will frequent visit these fields. With genes expressing pharmacologically active substances introgression into wild types via pollen is considered a particular problem.
- The main regulatory challenges are

1. The establishment of an appropriate quality assurance system for upstream production to establish a consistent production.
2. The choice of proper cultivation, harvest, storage and primary processing procedures including control measures.
3. The establishment of an appropriate banking system (cell bank, seed bank, virus bank).

4.0 CONCLUSION

The large-scale production of recombinant proteins in plants is known as molecular farming. Its objective is to harness the power of agriculture to cultivate and harvest plants for the production of recombinant therapeutics, diagnostics, industrial enzymes and green chemicals. Molecular farming has the potential to provide unlimited quantities of recombinant proteins and, thus, represents a powerful alternative to conventional protein production systems.

5.0 SUMMARY

Plants have several advantages over traditional expression systems, such as bacteria, yeast, mammalian cell lines and transgenic animals. Transgenic plants can be maintained, harvested and processed using normal agricultural practices. There is a structural and functional similarity between plant-derived recombinant protein and native protein. Plants have been used to produce a wide range of products, including therapeutic human proteins, recombinant antibodies, subunit vaccines, nutraceuticals, biopolymers and industrial enzymes.

6.0 TUTOR-MARKED ASSIGNMENT

1. What is the main aim of molecular farming?
2. Describe the bottleneck of molecular farming.
3. Write short notes on:
   i. Glycosylation
   ii. Downstream processing.

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UNIT 2 PRODUCTION OF INDUSTRIAL ENZYMES AND BIODEGRADABLE PLASTICS

CONTENTS

1.0 INTRODUCTION

The first commercialised 'industrial proteins' produced from transgenic plants were avidin and β- glucuronidase, both of which were produced in maize. After this ProdiGene Inc. company went for the large-scale production of trypsin, which is difficult to produce in conventional recombinant systems.

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- analyses production of industrial enzymes
- explain production of biodegradable plastics
- identify production of bioplastics i n cotton fibers
- state production of PHA copolymers
- state applications of PHAs.

3.0 MAIN CONTENT

3.1 Production of Industrial Enzymes

The first commercialised 'industrial proteins' produced from transgenic plants were avidin and β- glucuronidase, both of which were produced in maize. After this ProdiGene Inc. company went for the large-scale production of trypsin, which is difficult to produce in conventional

recombinant systems. A selected list of industrial enzymes produced in transgenic plants and their important application is given in Table 31.1.

Table 31.1: Some example of industrial enzymes produced in transgenic plants and their important applications

Enzyme	Applications
α-Amylase	Food processing
Avidin	In diagnostic kits
Cellulase	Production of alcohol from cellulose
β-glucanase	In brewing industry
β-glucuronidase	In diagnostic kits
Lignin peroxidase	In paper manufacture
Phytase	Improved phosphate utilization
Trypsin	Pharmaceutical
Xylanase	Biomass processing, paper and textile industries

3.1.1 Trypsin

Trypsin is an important proteolytic enzyme and its production by conventional recombinant approaches is rather difficult. This protein is currently harvested from bovine and protein pancreases. It has wide range of applications including the production of pharmaceuticals, such as insulin, vaccine production and wound care. Transgenic maize plants were used for the expression of bovine pancreatic trypsin. Expression levels were much higher for trypsinogen, the inactive precursor for trypsin, than those for active trypsin. Seed preferred expression of the zymogen form yields the highest expression level of a protease in transgenic plants.

3.1.2 Avidin

Avidin was the first commercial transgenic protein produced. Until plant derived avidin entered the market, the source for commercial production of avidin was chicken egg white. Avidin is a medium-sized, glycosylated protein. Recombinant avidin was produced in transgenic maize. Glycosylation of maize-derived avidin is similar to that seen in native avidin although some glycosyl residues are added to recombinant avidin. Avidin system provides a good example of the economic promise of transgenic plants for large scale production of heterologous proteins.

3.1.3 Cellulase and Xylanase

Cellulases and xylanases, which are normally produced by gut microorganisms in ruminants, have been produced in number of different plants. These enzymes are used in the bioethanol, textile, pulp and paper industries and for the production of animal feed. In all these processes, they are basically involved in the degradation of plant material (cellulose). To avoid the risk of autodigestion of plant cells (by these enzymes), engineered, thermostable, forms of the enzymes with high temperature optima were used. Thus, cellulase and xylanase, produced by transgenic plants, are inactive at the temperatures at which plants normally grow. The activity of these enzymes is restored on heating the plant extracts. An interesting point to note is that these enzymes would require only the minimum of purification.

3.1.4 Phytase

Phytase is a hydrolytic enzyme that catalyses the hydrolysis of phytate (inositol hexaphosphate) to inositol and inorganic phosphate (Figure 31.1). Phytate is present in high quantities in many plant seeds used as a feed to pigs and poultry. These animals do not possess the enzyme phytase; hence they cannot derive the nutrient phosphate from phytate. The undigested phytate gets excreted and accumulates in the soil and water, leading to eutrophication. Transgenic plants capable of synthesizing phytase in their seeds have been developed. These seeds are used in the feed of animals. The phytase enzyme has successfully solved nutritional (phosphate) and environmental (eutrophication) problems.

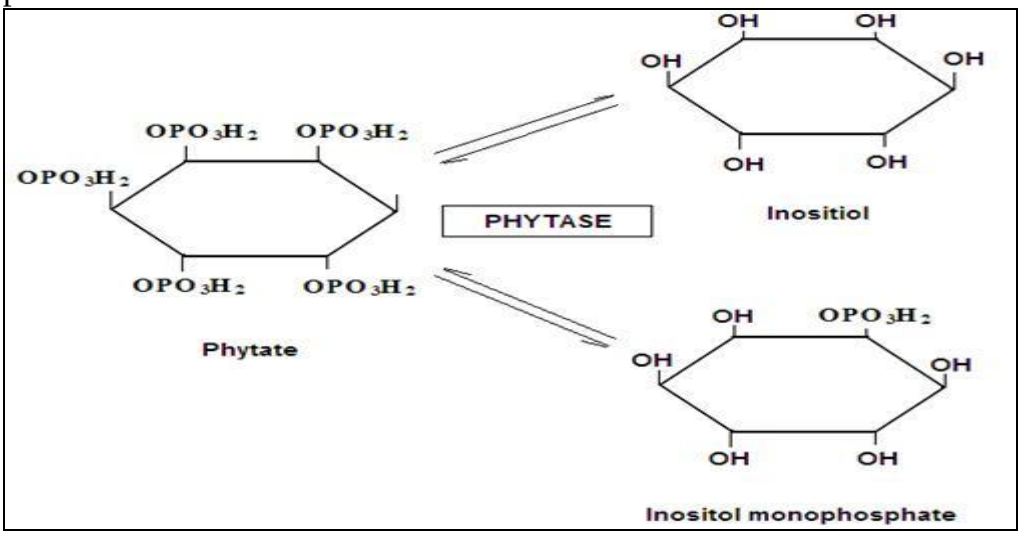

Figure 31.1: The action of phytase

3.2 Production of Biodegradable Plastics

Biodegradable plastics (Bioplastics) are chemically polyhydroxyalkanoates (PHAs). They are intracellular carbon and energy storage compounds, produced by many microorganisms. They are biodegradable polymers, and are elastic in nature. These compounds are currently produced by microbial fermentation. Several experimental studies are in progress to produce bulk quantities of bioplastics in plants. Among the PHAs polyhydroxy butyrate (PHB) is the most important one.

3.2.1 PHA-Chemistry and Properties

PHA serves as lipid reserve material in bacteria. The granules of PHA, stored within the cells are clearly visible under electron microscope. PHAs are linear polyester polymers composed of hydroxyacid monomers. Structures of PHAs are given in Figure 31.2. The most commonly found monomers are 3 hydroxy acids with a carbon length ranging from C_3 to C_{14}.

3.2.1.1 Homopolymer of PHA – Polyhydroxybutyrate

The most common PHA is polyhydroxybutyrate. It is a polyester with 3-hydroxybutyrate as the repeating unit. PHB is a homopolymer PHA. It is hard and inflexible. Being a high molecular weight compound, the accumulation of PHB in huge quantities does not affect the osmotic pressure within the cell. The reserve carbon compound PHB can be oxidized to carbon dioxide and water, releasing large amount of energy. Bacteria need energy to maintain pH gradient and concentration gradient of several compounds. This energy called maintenance energy essential for the survival of cells, is met by the reserve material PHB.

3.2.1.2 Heteropolymers of PHA

Majority of polyhydroxyalkanoates except PHB contains 2 or more different monomers called as heteropolymers. These heteropolymers are usually composed of a random sequence of monomers in different chains. Beside 3 hydroxy acids several other hydroxyl acids are found in the structures of PHA (eg. 4-hydroxybutyrate). It depends on the organism and the nature of carbon source supplied during accumulation of the polymer. The properties of PHA mostly depend on the nature of the monomers it contains. In general, PHA with longer side chains and hetero polymeric PHA are more flexible and softer.

3.2.1.3 3-Hydroxybutyrate-co-3-Hydroxyvalerate (PHB/V)

By changing the medium composition and selecting a specific organism, the chemical structure of PHA can be altered. In the presence of glucose and propionic acid, the organism *Ralstoniaeutropha* produces a copolymer of 3-hydroxy-butyrate and 3-hydroxyvalerate. The presence of 3-hydroxyvalerate monomers makes PHB/V flexible and stronger. The properties of PHB/V are similar to those of polypropylene, and therefore it is commercially more useful.

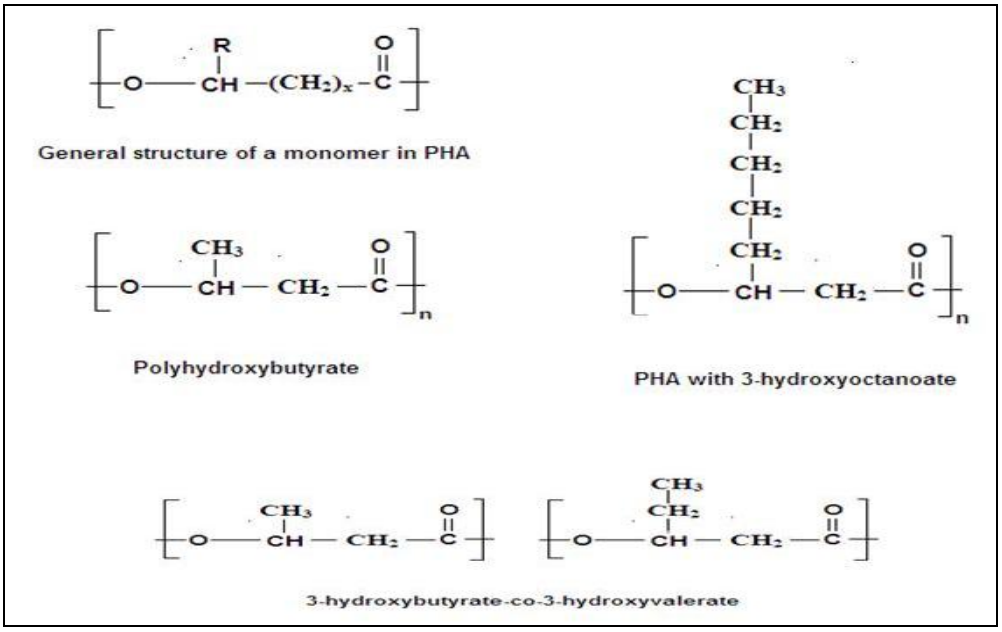

Figure 31.2: Structures of polyhydroxyalkanoates

3.2.2 Biosynthesis of PHB/V

Some strains of *Ralstoniaeutropha* are capable of synthesizing polyhydroxybutyrate-co-hydroxy-valerate (PHB/V). For the formation of PHB/V, glucose and propionic acid are required as substrates. Propionyl CoA is responsible for the synthesis of 3-hydroxyvalerate. The three enzymes involved in the synthesis of 3-hydroxy butyrate also participate in the formation of 3-hydroxyvalerate.The polymer PHB/V contains 3-HB and 3-HV monomers in a random sequence. The relative concentrations of glucose and propionic acid in the culture medium determine the chemical composition of PHB/V. The biosynthetic pathways are described in Figure 31.3.

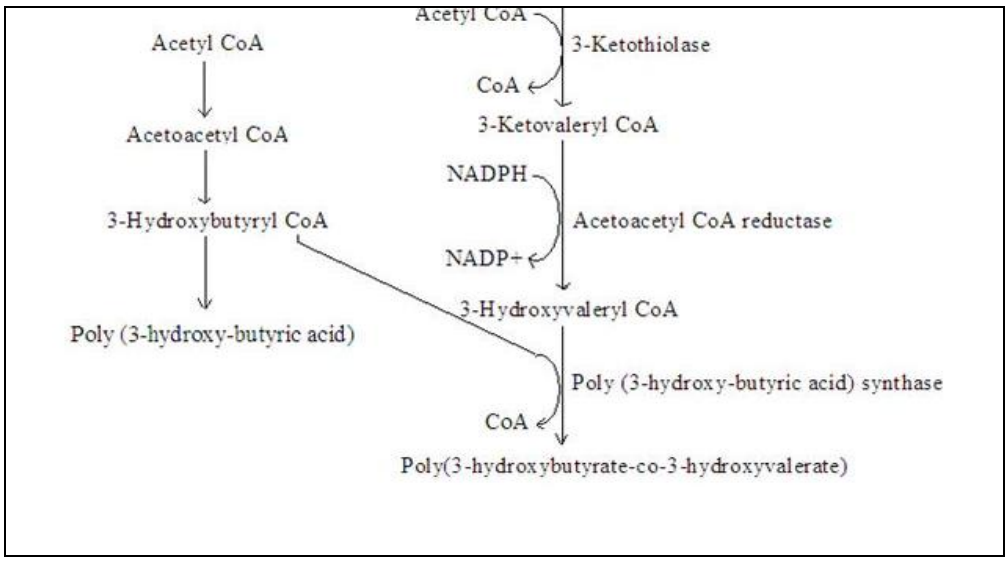

Figure 31.3: The biosynthetic pathways

3.2.3 Polyhydroxy Butyrate (PHB)

3.2.3.1 Biosynthesis of PHB

PHB is synthesized in 3 reaction steps starting with acetyl CoA. Acetyl CoA is converted to acetoacetyl CoA by the enzyme 3–ketothiolase which is then reduced to 3- hydroxybutyryl CoA by acetoacetyl CoA reductase (Figure 40.3). The reducing equivalents are supplied by NADPH. The enzyme PHA synthase is responsible for the addition of 3-hydroxy butyrate residues to the growing PHB chain. Majorly, PHP synthesize in cytoplasm and plastid of cell.

1. PHB production in cytoplasm

Starting from acetyl CoA, polyhydroxy butyrate production is a 3 stage pathway (Figure 40. 3) involving the following enzymes (with corresponding genes).

- 3-Ketothiolase (phaA)
- Acetoacetyl-CoA reductase (phaB)
- PHB synthase (phaC)

The three genes coding the respective enzymes have been isolated from *Alcaligenes eutrophus* and cloned. The cytoplasm of plant cell contains 3-Ketothiolase. Therefore, only two genes (phaB and phaC) coding acetoacetyl CoA reductase and PHB synthase were transferred to develop *Arabidopsis.* By this approach the quantity of PHB produced was very low.

2. PHB production in plastid

In this case all three genes (phaA, PhaB, PhaC) of PHB synthesis was separately fused with a coding sequence of transit peptide bound to N-terminal fragment of Rubisco (ribulose 1,5- bisphosphate carboxylase oxygenase) subunit protein (Figure 31.4). These genes were then directed to chloroplast. The genes expression was carried out by CaMV 35S promoter. Transgenic *Arabidopsis* plants with each gene construct were first developed. Then a series of sexual crossings were carried out between the individual transformants. The transgenic plants developed by this approach yielded good quantity of bioplastics and there was no adverse effect on the growth and fertility of these plants.

3.3 Production of Bioplastics in Cotton Fibers

Cotton fibres contain the enzyme β-ketothiolase. Therefore, the genes for the other two enzymes of PHB pathway (phaB and phaC) from *Alcaligenes eutrophus* were transferred into meristems of cotton plant by particle bombardment. In this case large quantities of PHB were produced in the fibres of transgenic cotton plants.

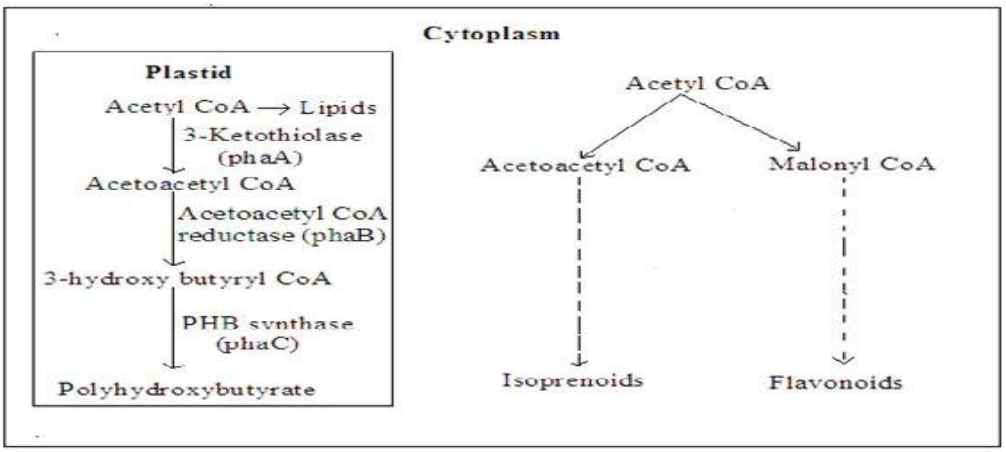

Figure 31.4: Chloroplast synthesis of PHB

3.4 Production of PHA Copolymers

The other bioplastic, composed of polyhydroxyalkanoate copolymer is a polymer made up of longer monomers. It is less crystalline and more flexible compared to PHB. The PHAs are produced from the intermediates of β oxidation of fatty acids like 3-hydroxy acyl-CoA. PHAs have also been produced through genetic manipulations of peroxisomes and glyoxisomes.

3.5 Applications of PHAs

There are several applications for PHA produced by micro-organisms within the medical and pharmaceutical industries, chiefly due to their biodegradable properties.

- In Fixation and orthopaedic applications, including sutures, suture fasteners, meniscus repair devices, rivets , tacks, staples, screws (including interference screws).
- It also used in making of bone plates and bone plating systems, surgical mesh, repair patches, slings, cardiovascular patches, orthopedic pins (including bone.lling augmentation material), adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices.
- PHAs can also be useful in making of skin substitutes, dural substitutes, bone graft substitutes, bone dowels, wound dressings, and hemostats .
- PHB can be implanted in the human body without rejection. This is because PHB does not produce any immune response and, thus, it is biocompatible. PHB has several medical applications like, durable bone implants and wound dressing.

4.0 CONCLUSION

The first commercialized 'industrial proteins' produced from transgenic plants were avidin and β- glucuronidase, both of which were produced in maize. After this ProdiGene Inc. company went for the large-scale production of trypsin, which is difficult to produce in conventional recombinant systems.

5.0 SUMMARY

Biodegradable plastics (Bioplastics) are chemically polyhydroxyalkanoates (PHAs). They are intracellular carbon and energy storage compounds, produced by many microorganisms. They are biodegradable polymers, and are elastic in nature. These compounds are currently produced by microbial fermentation.

6.0 TUTOR-MARKED ASSIGNMENT

1. Give the name of industrial proteins which are produced from transgenic plant and are commercialised first.
2. Give some examples of industrial enzymes which are produced from transgenic plant. Write down their applications.
3. Describe the production of biodegradable plastics.

4. Describe the chemistry and biosynthesis of polyhydroxybutyrate.
5. Describe the biosynthesis of polyhydroxy butyrate.
6. Describes the steps of cytoplasmic synthesis of PHB.
7. Write short notes on:
   A. Trypsin
   B. Avidin
   C. Cellulase and xylanase
   D. Phytase
   E. Polyhydroxy butyrate
   F. Chloroplast synthesis of PHB.

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UNIT 3 PRODUCTION OF ANTIBODIES

CONTENTS

1.0 INTRODUCTION

Antibodies or immunoglobulins are the defense proteins produced in mammals. Immunoglobulins have been divided into five major classes on the basis of their physical, chemical and immunological properties. The five main classes of immunoglobulins are IgA, IgD, IgG, IgE and IgM. Immunoglobulins are composed of two heavy chains and two light chains (Figure 32.1). Each class of Ig has its own class of H (heavy) chain, termed α, δ, ε, γ, μ respectively. Each Ig also has two light chains which are either κ or λ. The main antibody found in secretions, IgA has a more complicated structure. In secretions, IgA exists as a dimer of two IgA molecules joined by a J chain and a secretory component. The two antigen binding sites in the antibody molecule are formed from the variable regions of the light and heavy chains. Since these variable regions are responsible for the antigen binding, simpler, smaller molecules that still bind antigens can be produced. Single chain variable fragment (ScFv) antibodies are produced from synthetic genes made by fusing the sequence for light and heavy chain variable regions. ScFv antibodies are the commonly used most successful antibodies.

The use of plants for commercial production of antibodies, referred to as plantibodies, is a new approach in biotechnology. The first successful functional antibody produced in plant was mouse immunoglobulin IgG1. For these two transgenic tobacco plants, one synthesizing heavy γ chain and the other light κ chain, are crossed to generate progeny that can produce an assembled functional antibody. Some examples of antibodies produced in transgenic plants were given in Table 32.1.

Table 32.1: Antibodies produced in transgenic plants

Antibody	Targeted against	Transgenic plant	Application
sIgA(hybrid)	*Streptococcus mutans*	Tobacco	Dental caries
IgG(guy's 13)	*Streptococcus mutans*	Tobacco	Dental caries
IgG (Co 17-1A)	Surface antigen	Tobacco	Colon cancer
IgG (anti HSV-2)	Viral antigen	Soybean	Herpes simplex virus

2.0 OBJECTIVE

By the end of this unit, you will be able to:

- explain about the different types of antibodies and how they are produced.

3.0 MAIN CONTENT

3.1 Production of Secretory IgA (sIgA)

Secretory IgA is an immunoglobulin that protects against dental caries produced by *Streptococcus mutans*. For the production of sIgA various antibody subunits are produced in different plant lines that are subsequently crossed to produce a functional antibody. The antibody does this by recognizing the native streptococcus antigen cell-surface adhesion molecule, which prevent colonisation. Four separate transgenic plants synthesizing four distinct pieces of antibody (H, L, J chains and secretory component) were developed. In cross 1 the plants expressing H and L chains were crossed to give plants producing IgA. In the second cross these plants were crossed with plants expressing J chain to give the progeny producing dimeric IgA. The dimeric IgA producing plants were then crossed (cross3) with the plants expressing secretory component, the functional sIgA could be produced.

Secretory antibodies have many advantages. Their yield in plants is substantially higher since they are resistant to proteolytic degradation. sIgA are the major antibodies that protect against mucosal infections of microorganisms. They bind to antigen with more avidity giving good protection. These secretory antibodies have now been tested on humans. They have been topically applied to teeth and found to be effective in preventing colonisation by *Streptococcus mutans* upto 4 months. This is comparable to the protection offered by immunoglobulins produced through hybridoma technology. This was possible despite some structural differences between plantibodies and monoclonal antibodies.

It has been found that there is no difference in the binding properties between the two types of antibodies. Plants have been used to produce a variety of antibodies, including whole antibodies, antigen binding fragments and single chain variable fragment antibodies. Despite the difference in the glycosylation pattern seen between antibodies produced in plants and mammalian expression systems, antibodies produced in plants generally seem to exhibit similar properties to antibodies produced in other mammalian systems. Production of secondary IgA molecule is described in Figure 41.2.

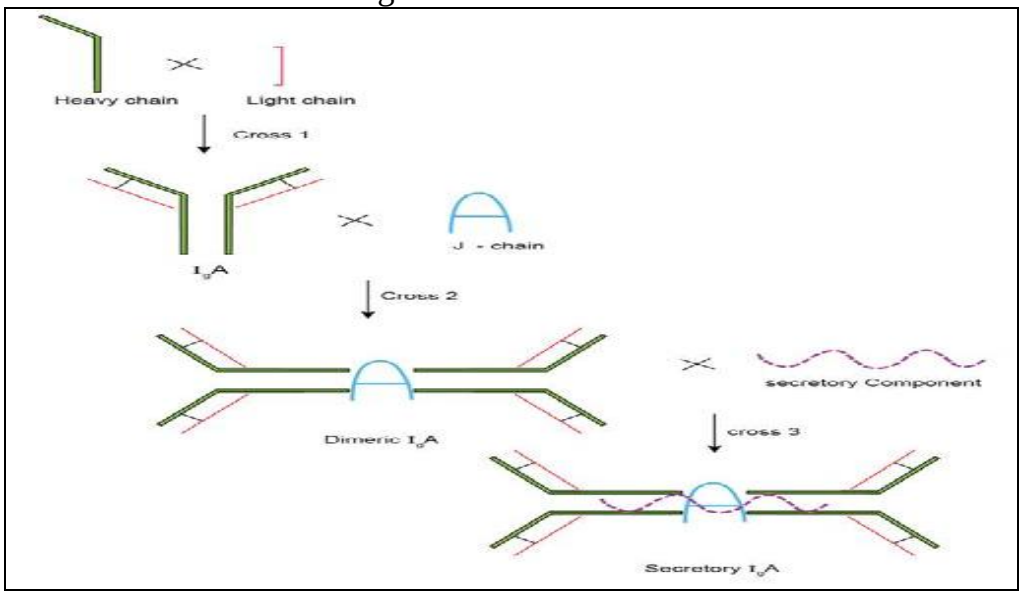

Figure 32.2: Production of secondary IgA molecule

3.2 Custom-Made Antibodies

Most of the antibodies are produced by generating stably transformed plants that express the protein. For the development of an idiotype vaccine another approach has been used. A tobacco mosaic *tobamovirus* (TMV) based vector has been developed for the production of a secreted ScFv protein during virus infection of non-transgenic tobacco plants. In this vector a virus promoter regulates the expression of the ScFv gene and the antibody coding sequence is inserted downstream from a rice α-amylase leader sequence, which target the protein to the extracellular compartment of the plant (Figur2e 32.3).

This antibody produced can be used for the treatment of B-cell lymphoma. In this disease, these cells produce a particular antibody that is unique to each patient. In this idiotype vaccine system the patient is treated with a copy of the lymphoma antibody to generate immunity against the original antibody producing lymphoma cells. Each individual antibody has a variable region that has a unique antigenic constitution, the idiotype. Thus, the idiotype vaccine will only raise a specific response against the lymphoma cells and not against other cells. Using a

TMV vector system, it is now possible to generate the idiotype region of a tumor specific immunoglobulin as a ScFv. The system was first developed using mice as model, but it has now entered clinical trials. It is interesting that each of the antibodies in the clinical trials is unique, and can be considered as a single drug.

The advantage of this transient expression system is that it is very rapid and can be used to produce small batches of customised antibody. Another advantage is that it is possible to harvest enough material for clinical use from green house grown plants, thus ensuring high levels of containment.

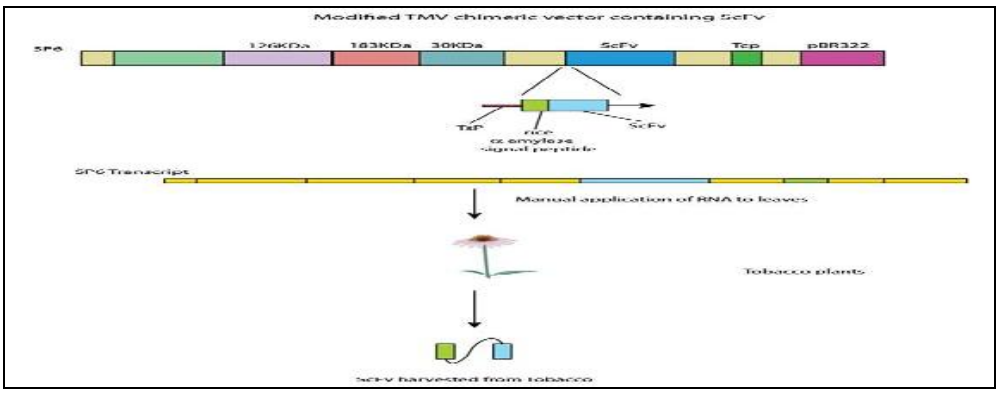

Figure 32.3: The engineered TMV vector for the expression of pharmaceutical proteins

TsP-translation start position; TcP-tomato coat proteins; pBR322-bacteria replication sequence

3.3 Production of Edible Vaccines

Transgenic plants provide an alternative system for the production of recombinant vaccines. The major advantage of vaccine production in plants is the direct use of edible plants tissue for oral administration. By the use of edible vaccines the problems associated with the purification of vaccines can be avoided. The stable or transient expression system can be used to produce vaccines in plants. Transgenic plants have been developed for expressing antigens derived from animal viruses. The need for use of edible vaccines comes from the fact that larger numbers of people are the victims of enteric diseases. Edible vaccine provides mucosal immunity against infectious agents. Some of the edible vaccines are mentioned in Table 32.2.

Table 32.2: Examples of plant edible subunit vaccines

Recombinant protein (vaccine)	Transgenic plant	Protection against
Rabies glycoprotein	Tomato	Rabies virus
Foot and mouth virus (VPI)	Arabidopsis	Foot and mouth virus
Herpes virus B surface antigen	Tobacco	Herpes simplex virus
Cholera toxin B subunit	Potato	*Vibrio cholerae*
Human cytomegalovirus glycoprotein B	Tobacco	Human cytomegalovirus

3.3.1 Choice of Plants for Edible Vaccines

Most of the vaccines production was carried out in tobacco plant that is not edible. These vaccines are now being produced in edible plants such as banana, tomato and potato. For use in animals the common fodder crops are used. Banana is an ideal system for the production of edible vaccine since it is grown in most part of the world and eaten raw.

3.3.2 Edible Vaccine Production and Use

The bacterium, *Agrobacterium tumefaciens* is commonly used to deliver the DNA for bacterial or viral antigens. A plasmid carrying the antigen gene and an antibiotic resistance gene are incorporated into the bacterial cells. The cut pieces of potato leaves are exposed to an antibiotic to kill the cells that lack the new genes. The surviving cells (gene altered ones) can multiply and form a callus. This callus will sprout and form shoots and roots, which are grown in soil to form plants. After 3 weeks the plant produces potatoes containing antigen vaccines (Figure 41.4). The first clinical trials in humans involved the ingestion of transgenic potatoes with a toxin of *E.coli* causing diarrhoea.

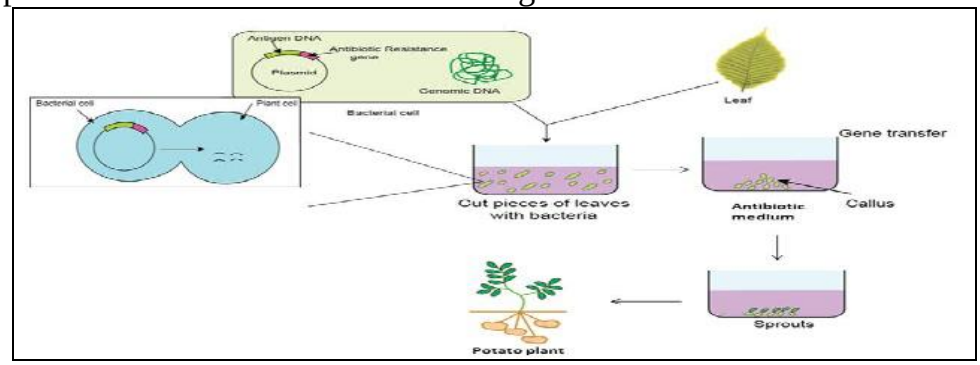

Figure 32.4: Schematic representation of production of edible vaccine

3.3.3 Delivery of Vaccine to the Gut

Vaccines, being protein are likely to be degraded in the stomach. But in the case of edible vaccine it has been found that orally administered plant material can induce immune response. There is a difficulty of dose adjustment when edible vaccines are consumed as a part of food stuff. Instead of the direct use of plant material, a food-based tablet containing a known dose of vaccine has been produced. This approach is being applied to vaccines produced in tomatoes.

3.3.4 Limitations of Edible Vaccines

Direct consumption of transgenic fruit or vegetable or food-based tablets have some problems.

- The risk of loss of vaccines by the action of enzymes in stomach and intestine.
- The possibility of allergic reactions as they enter circulation.

4.0 CONCLUSION

Antibodies or immunoglobulins are the defense proteins produced in mammals. Immunoglobulins have been divided into five major classes on the basis of their physical, chemical and immunological properties. The five main classes of immunoglobulins are IgA, IgD, IgG, IgE and IgM. Immunoglobulins are composed of two heavy chains and two light chains.

5.0 SUMMARY

In this unit we have learnt:

1. The use of plants for commercial production of antibodies, referred to as plantibodies, is a new approach in biotechnology.
2. The first successful functional antibody produced in plant was mouse immunoglobulin IgG1.
3. For these two transgenic tobacco plants, one synthesising heavy γ chain and the other light κ chain, are crossed to generate progeny that can produce an assembled functional antibody.

6.0 TUTOR-MARKED ASSIGNMENT

1. Give some example of antibodies produced in transgenic plants.
2. Draw the structure of IgG antibody.
3. Describe the production of secretory antibody (IgA).

4. Describe any vector used for the expression of pharmaceutical proteins.
5. Give some example of plant edible vaccines and its transgenic plant.
6. How edible vaccine is produced?
7. Write short note on:
   A. Immunoglobulins
   B. Secretory IgA
   C. Edible vaccines
   D. Custom-made antibodies.

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UNIT 4 METABOLIC ENGINEERING FOR PRODUCTION OF FATTY ACIDS, INDUSTRIAL OILS, TERPENOIDS AND FLAVONOIDS

CONTENTS

1.0 INTRODUCTION

Plant oils are used in foods and for industrial purposes. The industrial applications of plant oils include their use in the manufacture of soaps, detergents, lubricants and biofuels. Plastid, cytoplasm and endoplasmic reticulum are involved in the production of oils and these oils are stored lipid bodies after synthesis. Acetate from the cytoplasm is taken up by the plastids and converted to acetyl-CoA and malonyl CoA

2.0 OBJECTIVES

By the end of this unit, you will be able to:

- explain the meaning of metabolic engineering for production of fatty acids
- analyses metabolic engineering of terpenoids
- state metabolic engineering of flavonoids.

3.0 MAIN CONTENT

3.1 Metabolic Engineering for Production of Fatty Acids

Plant oils are used in foods and for industrial purposes. The industrial applications of plant oils include their use in the manufacture of soaps, detergents, lubricants and biofuels. Plastid, cytoplasm and endoplasmic reticulum are involved in the production of oils and these oils are stored lipid bodies after synthesis. Acetate from the cytoplasm is taken up by

the plastids and converted to acetyl-CoA and malonyl CoA (Figure 33.1). These two molecules undergo a series of reactions, catalysed by the enzyme fatty acid synthase, to produce fatty acyl carrier proteins with different carbon atoms.

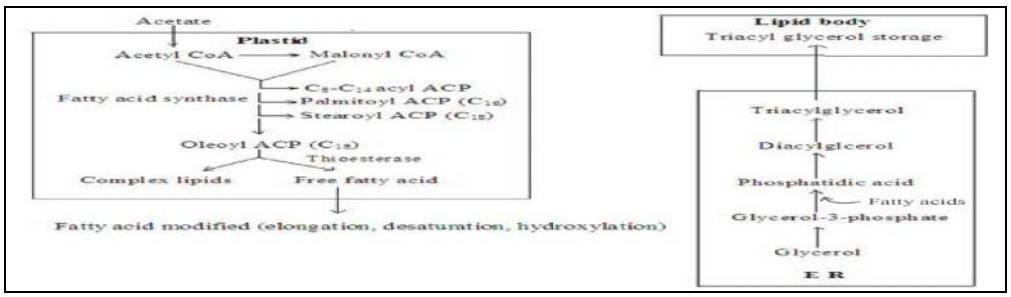

Figure 4.1: Biosynthesis of triacylglycerol in plants

The fatty acid thus produced are released and exported to the cytoplasm by the action of acyl-ACP thioesterases. Here they undergo certain modifications like elongation, desaturation, hydroxylation etc. These modified fatty acids react with glycerol 3-phosphate to finally form triacylglycerols. Triacylglycerols are transported to lipid oil bodies and stored. The oil body of seeds also contains proteins called oleosin, in the lipid monolayers. In Fig 4.1, please show that glycerol-3-phosphate reacts with fatty acids to form phosphatidic acids, which are converted to various triacylglycerols. (Please check this). Triacylglycerol is one word, not two (triacyl glycerol is wrong).

3.1.1 Production of Shorter Chain Fatty Acids

Majority of the plant oils contain more than 16 carbons (eg. palmitic, stearic and oleic acids). Shorter chain fatty acids (C_8-C_{14}) are more useful in industries for the production of soaps, detergents, cosmetics etc. By terminating the hydrolysis of acyl ACP by specific thioesterases higher amount of selected fatty acids can be produced. Acyl-ACP thioesterase that specifically hydrolyses lauroyl-ACP has been isolated from California bay tree. The gene encoding this enzyme has been cloned and transferred to oil seed rape. The transgenic plants were found to produce oils with high proportion lauric acid.

3.1.2 Production Of Longer Chain Fatty Acids

Triacylglycerols with longer chain fatty acids are preferred for use as industrial oils. In oil seed rape there is two step elongation pathway from oleoyl-CoA to erucoyl-CoA such that euric acid is one of the constituents of rape seed oils. Euric acid is valuable as an industrial oleochemical, but unsuitable for human consumption. Conventional breeding has led to the development of two distinct oilseed rape crops. High euric acid rape seed is used for industrial purposes and low euric acid with virtually no euric acid for food products. However the highest

euric acid content of high euric acid rape seed is about 50% of the total fatty acids which makes the process of separating out and disposing of the other fatty acids difficult. Some attempts are made to over express genes encoding the enzymes elongases and transfer of genes to produce enzyme that can preferentially incorporate euric acid into triacylglycerols.

3.1.3 Production of Unsaturated Fatty Acids

Oleic acid (C_{18}) rich oils are useful as food, feed and in other industries. Some attempts are made to transfer antisense gene encoding the enzyme desaturase in oilseed rape and soybean plants. The antisense gene was under the control of the napin (a seed specific protein of *Brassica*) gene promoter. There was a marked decrease in the amount of the desaturase enzyme, resulting in a decreased formation of oleic acid and a rise in the production of stearic acid. This high stearic acid has a potential as a cocoa butter substitute.

3.1.4 Production of Saturated Fatty Acids

Oils with high contents of saturated fatty acids have been produced by using anti sense RNA approach. The fatty acid stearate (C_{18}) was reduced to unsaturated fatty acid such as oleic acid, linoleic acid and linolenic acid. The enzyme stearoyl-ACP desaturase catalyses the conversion of stearoyl- ACP to oleoyl-ACP. The gene encoding the enzyme stearoyl-ACP desaturase has been isolated from *Brassica rapa* and its complementary sequence cloned. The transgenic plants developed by this approach were found to contain high contents of saturated fatty acid namely stearic acid and low concentration of oleic acid.

3.1.5 Production of Rare Fatty Acids

There are some rare fatty acids, which are industrially important, but normally synthesised in the plants. Some plants producing the rare fatty acids have been identified and the genes transferred for their overproduction. One such fatty acid is petroselenic acid, which is found in coriander and has been used as a raw material for industry. Oxidation of petroselenic acid by ozone produces lauric acid and adipic acid, which can be used for nylon production. Transformation of tobacco with a coriander acyl-ACP desaturase cDNA led to the production of petroselenic acid in calli to a level of 5% of total fatty acids.

Production of several unsaturated fatty acids can be increased through genetic manipulation by incorporating genes coding special enzymes called front-end desaturase. These enzymes insert additional double

bonds between existing bonds and the carboxyl end of the fatty acid. Those poly unsaturated fatty acids produced by using this approach have pharmaceutical and nutraceutical value eg. γ-linolenic acid, arachidonic acid.

Ricolenic acid is produced in castor beans to a level of 90 % of the total fatty acids. However, the castor oil crop has a number of problems, including the presence of toxic compounds, such as ricin in the residual meal. The synthesis of ricolenic acid involves the direct hydroxylation of oleic acid bound to phosphatidylcholine on the ER membrane. The cDNA for the 12-hydroxylase has been cloned from castor bean and could be used to produce castor oil in major oil crops. Another fatty acid modifying enzyme has been cloned from *Crepisacetylenics.* This enzyme catalyses triple bond and epoxy group formation in fatty acids, and would be a valuable way of inserting chemically reactive sites into oils.

3.2 Metabolic Engineering of Terpenoids

Terpenoids are organic compounds possessing branched chains constructed from the isoprene unit, 2-methyl butadiene, which is composed of five carbon atoms. There are more than 25000 terpenoids. Depending on the number of isoprene units they are classified as hemiterpenes, monoterpenes, sesquiterpenes, diterpenes and triterpenes. Steroids are also biosynthetically classified as terpenoids because their basic structure originates from the common precursor of triterpenes and squalene. Terpenoids are distributed in microorganisms, plants and animals. On the basis of their chemical structure, terpenoids may possibly be the most diverse group of plant secondary metabolites.
Monoterpenoids are known as fragrant components of the essential oils of flowers, herbs and spices, and they are extracted for use as flavours and perfumes. Sesqui and diterpenoids function as phytoalexins to protect plants from herbivores or microbial pathogens. Some plant hormones like abscisic acid and gibberellins also belong to this class. Triterpenes can be divided into phytosterols and saponins.

3.2.1 Biosynthesis of Isoprene Unit

All terpenoids are biosynthesised from dimethylallyl diphosphate and its isomer isopentenyl diphosphate. Higher plants possess two distinct biosynthetic pathways for isopentenyl diphosphate biosynthesis, the classic acetate/mevalonate pathway and the non-mevalonate pathway. Mevalonate pathway is responsible for the biosynthesis of sterols in cytosol, whereas the plastidial isoprenoids, such as carotenoids, plastoquinone and phytol are biosynthesised by the mevalonate independent pathway. Many industrially important terpenoids and other

natural compounds having isoprenoid molecules, like monoterpenoids and taxol are synthesised by this mevalonate independent pathway.

3.2.2 Mevalonate –Independent Pathway

In plants, the mevalonate independent pathway is localized in the plastids (Figure 33.2). The first enzymatic condensation reaction in this pathway is catalysed by 1-deoxy-D-xylulose 5- phosphate synthase in the presence of the glycolysis products thiamin pyrophosphate D-glyceraldehyde 3-phosphate and pyruvate, to yield 1-deoxy-D-xylulose 5-phosphate (DXP), which is why this biosynthetic route is often described as the 'DXP pathway'. The next step is the intramolecular rearrangement and reduction of DXP to give 2-C-methyl-erythritol 4-phosphate, which is catalysed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase. Because the reaction converting DXP to MEP by DXR is specific to this pathway it is also sometimes called 'the MEP pathway'. The third reaction takes place in the presence of CTP, when the 4-phosphate residue of MEP is cytidylated by 4-diphosphocytidil-2C-methyl-D-erythritol synthase to give 4-diphosphocytidil-2C-methyl-D-erythritol.Subsequently the 2- hydroxyl group of CDP-ME is phosphorylated by CDP-ME kinase with ATP to give 4-diphosphocytidil-2C-methyl-D-erythritol phosphate. Further, CDP-ME2P is converted to 2C-methyl-D-erythritol 2,4-cyclodiphosphate. The last step in this reaction is the conversion of this substance to IPP and DMAPP by the enzymatic reaction catalysed by lytB in *E.coli* .

Most of the plant-derived genes involved in the DXP/MEP pathway have been isolated. Unlike the microbial enzymes, the plant derived enzymes involved in the DXP/MEP pathway encode polypeptides bearing a N-terminal plastidial transit peptide that directs the enzymes to the plastids, which is where the mevalonate independent pathway operates in higher plants. The enzyme DXS control a regulatory step for plastidialisoprenoid biosynthesis in tomato fruit. The next step controlled by the enzyme DXR, seems likely to be non-limiting.

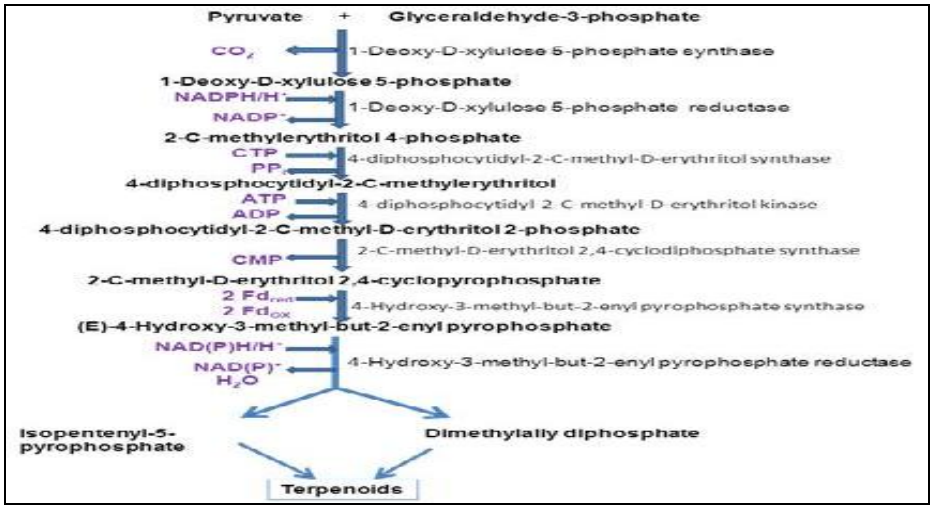

Figure 4.2: DXP/MEP pathway in bacteria and plants

The DXP pathway mentioned above is restricted to plastid-bearing eukaryotes. All of the enzymes involved in this isoprenoid pathway are potential targets for new classes of herbicides. Since this pathway occurs in several bacteria and the malaria parasite, as well as in plants, they also display potential as drugs against these pathogens. An important physiological role of DXP/MEP pathway in plants has been demonstrated in tomato. The inhibition of DXR activity with fosmidomycin, a specific inhibitor of this enzyme, caused the reduction of carotenoids that seems to affect abscisic acid biosynthesis, adding to the arrest of seedling development.

3.2.3 Mevalonate Pathway

A common biosynthetic precursor of sesquiterpenoids and triterpenoids is produced by the mevalonate pathway (Figure 4.3), which is localized in the plant cytosol of plants. The biosynthetic route of the mevalonate pathway has been established in bacteria and mammals. The cytosolic IPP pathway involves the condensation of three molecules of acetyl CoA in two steps, which are catalysed by thiolase and hydroxymethylglutaryl CoA synthae. HMG CoA is subsequently reduced by HMG-CoA reductase in the presence of NADPH to form mevalonic acid. After two sequential phosphorylation of MVA, with ATP at fifth position, the resulting MVA 5-diphosphate (DPMVA) is converted in to IPP by DPMVA decarboxylase in the presence of ATP.
In this pathway the first enzyme that is responsible for IPP supply is HMGR. The cDNA cloning of HMGR from *Arabidopsis* showed that there are two HMGR genes in *Arabidopsis*, HMGR1 and HMGR2. Both of these have two highly conserved hydrophobic domains in the N termini, by which HMGR is inserted into the microsomal membrane, whereas the C-terminal domains are assumed to face the cytosolic space and function as the catalytic domain.

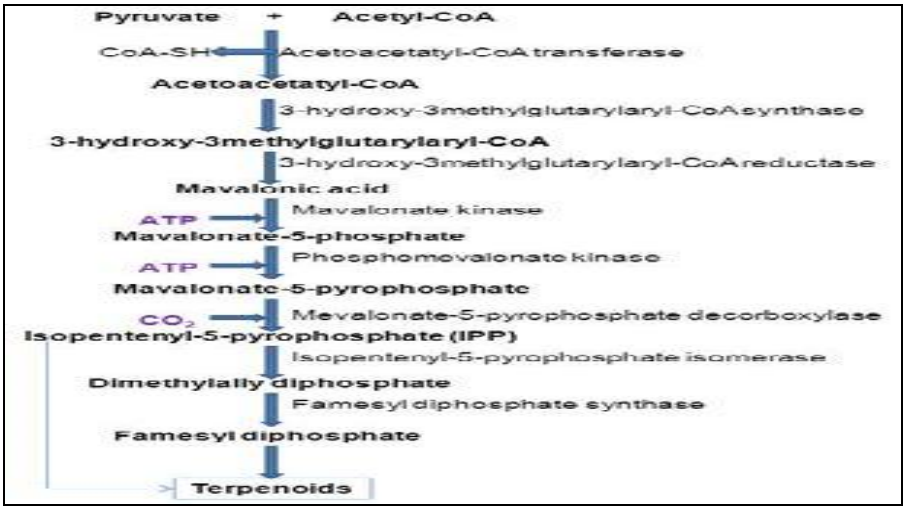

Figure 4.3: Mevalonate pathway in plants and mammals

In this pathway the first enzyme that is responsible for IPP supply is HMGR. The cDNA cloning of HMGR from *Arabidopsis* showed that there are two HMGR genes in *Arabidopsis*, HMGR1 and HMGR2. Both of these have two highly conserved hydrophobic domains in the N termini, by which HMGR is inserted into the microsomal membrane, whereas the C-terminal domains are assumed to face the cytosolic space and function as the catalytic domain. Both polypeptide show similar enzymatic function, although their expression within tissues is different for the two molecular species ie.HMGR1 is detectable in all tissues where as HMGR2 is restricted to meristematic and floral tissues. Unlike the chemical suppression of the mevalonate pathway in plants with a specific inhibitor of HMGR, mevinolin, there have been few attempts to increase the expression of the homologous HMGR by genetic engineering in plants. One example was the over-expression of the homologous HMGR cDNA, driven by the CaMV 35S promoter, reported in *Arabidopsis*. However only a modest increase in enzyme activity was found, although a striking elevation of mRNA was observed in some transgenic plants.

3.3 Metabolic Engineering of Flavanoids

Flavanoids are some of the most extensively studied secondary metabolites. Flavanoids are biosynthesized via the general phenylpropanoid pathway (Figure 42.4). 4-Coumaroyl–CoA is converted to chalcone by chalcone synthase (CHS), the first enzyme in biosynthesis of flavanoid. This enzyme catalyses the step wise condensation of three acetate units, malonyl-CoA, to 4-coumaroyl –CoA to yield tetrahydroxychalcone. In addition to its functional importance, this enzyme plays a crucial role in the induction of flavonoid production by light irradiation.

Tetrahydroxychalcone is cyclised by chalcone isomerase (CHI), and which is further converted to dihydrokaempferol, a typical flavanonol,

by flavanonone 3-hydroxylase. If one hydroxyl group is introduced to the B-ring of dihydrokaempferol, by Flavanoid 3 '-hydroxylase, dihydroquercetin is formed, where as if two hydroxyl groups are introduced by Flavanoid3',5'-hydroxylase dihydromyricetin is formed. Dihydroflavanol 4- reductase (DFR) catalyses the reduction of these three dihydroflavonols to the corresponding leucoanthocyanidins, which are then converted to anthocyanidins by anthocyanidin synthase (ANS), another 2 – oxoglutarate-dependent dioxygenase. Anthocyanidins are usually glucosylated at the third position of the flavonoid structure by UDP-glucose: flavonoid 3-glucosyltrasferase. The fifth position is often glucosylated. This is catalysed by another enzyme, 5-0-glucosyltransferase.

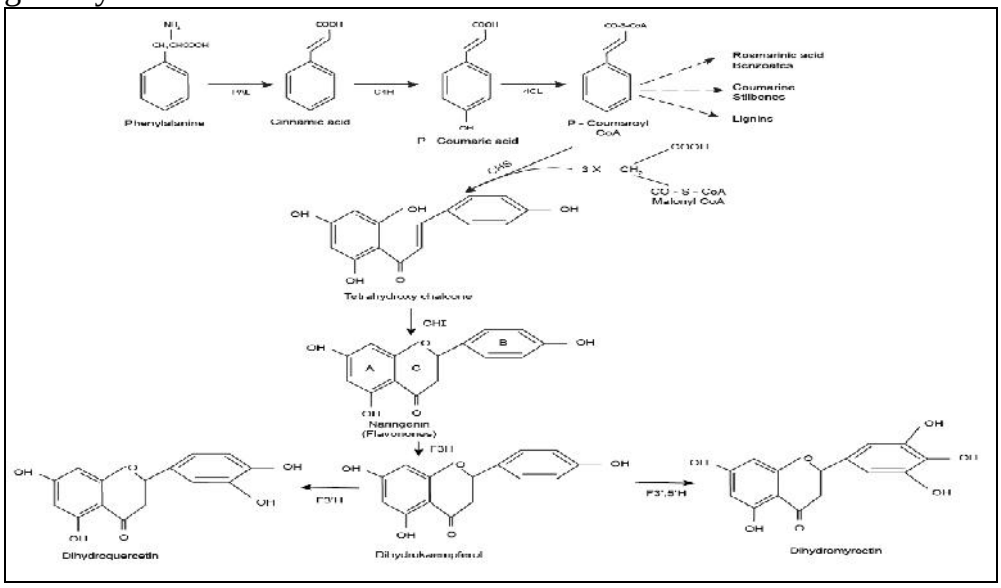

Figure 4.4: Biosynthetic pathway of flavonoids (PAL- phenyl alanine ammoia-lyase; C4H- cinnamate 4-hydroxylase; 4CL- 4-coumarate CoA ligase; CHS- chalcone synthase; CHI- Chalcone isomerase; F3H- flavonone-3-hydroxylase; F3'H- flavonoids-3'-hydroxylase; F-3',5'-hydroxylase)

Along with carotenoids, flavonoids are largely responsible for the colour of flower. Flavanoid show a particularly wide range of colour tone, i.e, yellow, red, purple and blue. From the view point of molecular breeding, alteration of flower colour by the manipulation of the anthocyanin biosynthetic pathway has long been a goal of many researchers working on horticultural plants. Most of the gene involved in anthocyanin biosynthesis has been cloned for use as molecular tool in metabolic engineering of the flower colour of various plant species. In early studies, biosynthetic route was blocked by the introduction of antisense of such genes in petunia, eg.CHS and Flavanol synthase. In this work reduced anthocyanin content of the flower petals was achieved.

The creation of blue flowers in species in which that colour does not occur has been studied. The formation of blue colour is usually controlled by complicated mechanisms, eg. Higher vacuolar pH and co-pigmentation, these are some important factors involved in achieving blue colours in flower petals. Most blue colours contain aromatic acylated delphinidin derivatives, ie, three vicinal hydroxyl groups in the B ring and p-coumaroylation with a sugar residue are necessary factors in the production of blue colour. Therefore, two hydroxylase F3'H and F3',5'H, which belong to the same cytochrome P-450 family, are key enzymes in the alteration of flower colour. Florigen and Suntory succesfully developed transgenic violet carnations by introducing petunia F3', 5'H and DFR genes in to DFR-deficient white carnations. The blue colour was conferred to the petals of the transgenic carnations by delphinidin, which is not produced by wild dianthus species. Violet carnations, named Moondust are the first example of a transgenic floricultural crop to be sold. A darker version, named moon shadow has also been produced and marketed. Another important gene involved in changing flower colour to blue is aromatic acyltransferase (AAT).

Flavanoids also play an important role as phytoalexins. The biosynthetic route of isoflavanoids is the same as that of anthocyanin up to flavonones, such as naringenin or linquiritigenin, which are converted to isoflavonoids via the specific biosynthetic reaction of 2-hydroxyisoflavanone synthase, a cytochrome (P450) enzyme. Genes of HIS have been cloned from liquorice, soyabean and chickpea. This enzyme catalyses a unique 1, 2 aryl migration of flavanones to yield the isoflavone skeletons ,eg.Daizein and genistein, which are often methylated by isoflavanone, 0-methyltransferase (IOMT). Isflavanone 2'-hydroxylase (I2'H) plays an important role in isoflavone phytoalexin production. For instance, the hydroxylation of the 2' position of the beta ring of the isoflavanoidcatalysed by another (P450) enzyme, isoflavonone 2'-hydroxylase, is essential for the formation of the five membered ring structure of medicarpin. Because 2HIS and IOMT are involved in phytoalexin production, the expression of these genes is reported to be strongly induced by treatment with an elicitor, both in whole plants and cell cultures.

Genetic manipulation of isoflavanoids has been done by generating transgenic alfalfa ectopically expressing IOMT, which showed 7-0-methyltransferase activity *in vitro* as well as *in vivo* in uninfected leaves. A key reaction for the phytoalexin formation was 4'-0-methylation of isoflavanoids. The transgenic plants exhibited increased induction of the phenylpropanoid/isoflavanoid pathway gene transcripts after infection, although the basal expression of these genes was less affected. Plants over-expressing IOMT display resistance to the pathogen, *Phomamedicaginis* .

4.0 CONCLUSION

Plant oils are used in foods and for industrial purposes. The industrial applications of plant oils include their use in the manufacture of soaps, detergents, lubricants and biofuels. Plastid, cytoplasm and endoplasmic reticulum are involved in the production of oils and these oils are stored lipid bodies after synthesis. Acetate from the cytoplasm is taken up by the plastids and converted to acetyl-CoA and malonyl CoA.

5.0 SUMMARY

In this unit we have learnt:

1. Metabolic engineering for production of fatty acids.
2. Metabolic engineering of terpenoids.
3. Metabolic engineering of flavonoids.

6.0 TUTOR-MARKED ASSIGNMENT

1. Describe the steps involves in biosynthesis of triacylglycerol in plants.
2. Describe the production of following:
   A. Shorter chain fatty acids
   B. Longer chain fatty acids
   C. Unsaturated fatty acids
   D. Saturated fatty acids
   E. Rare fatty acids.

7.0 REFERENCES/FURTHER READING

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