BOT(N)-121 & BOT(N)-121L

B.Sc. II Semester

BIOFERTILIZERS

DEPARTMENT OF BOTANY

SCHOOL OF SCIENCES

UTTARAKHAND OPEN UNIVERSITY, HALDWANI

BIOFERTILIZERS

DEPARTMENT OF BOTANY
SCHOOL OF SCIENCES
UTTARAKHAND OPEN UNIVERSITY

Phone No. 05946-261122, 261123
Toll free No. 18001804025
Fax No. 05946-264232, E. mail info@uou.ac.in
htpp://uou.ac.in

Expert Committee

Board of Studies

Programme Coordinator

Unit Writers	Unit No.

BIOFERTILIZERS: BOT(N)-121

1- Dr. Pooja Juyal
Department of Botany
Uttarakhand Open University, Haldwani

1 & 2

2. Dr. Prabha Bisht Dhondiyal
Assistant Professor (AC)
Department of Botany
Uttarakhand Open University, Haldwani

3 & 7

4- Dr. S.N.Ojha
Assistant Professor
Department of Botany, School of Sciences
Uttarakhand Open University, Haldwani

4 & 5

3-Dr. Kirtika Padalia
Assistant Professor (AC)
Department of Botany
Uttarakhand Open University, Haldwani

6 & 8

LABORATORY COURSE: BOT(N)-121L

4- Dr. S.N.Ojha
Assistant Professor
Department of Botany, School of Sciences
Uttarakhand Open University, Haldwani

1 & 2

1. Dr. Prabha Bisht Dhondiyal
Assistant Professor (AC)
Department of Botany
Uttarakhand Open University, Haldwani

3 & 6

2-Dr. Kirtika Padalia
Assistant Professor (AC)
Department of Botany
Uttarakhand Open University, Haldwani

4

3- Dr. Pooja Juyal
Department of Botany
Uttarakhand Open University, Haldwani

5

Chief Course Editor

Dr. S.N. Ojha
Assistant Professor,
Department of Botany, School of Sciences
Uttarakhand Open University, Haldwani

Dr. Pooja Juyal
Assistant Professor (AC)
Department of Botany, School of Sciences
Uttarakhand Open University, Haldwani

Co-Editor

Dr. Prabha Dhondiyal
Assistant Professor (AC)
Department of Botany, School of Sciences
Uttarakhand Open University, Haldwani

Dr. Kirtika Padalia
Assistant Professor (AC)
Department of Botany, School of Sciences,
Uttarakhand Open University, Haldwani

Title	**:**	**Biofertilizers**
ISBN No.	**:**	
Copyright	**:**	**Uttarakhand Open University**
Edition	**:**	**2024**

Published By: Uttarakhand Open University, Haldwani, Nainital-263139

CONTENTS

BIOFERTILIZERS: BOT(N)- 121

BLOCK-1 INTRODUCTION TO BIOFERTILIZERS

UNIT-1 BIOFERTILIZERS: DEFINITION, DIFFERENT SOURCES AND IMPORTANCE

Contents

1.1-OBJECTIVES

After reading this unit students will be able to:

- define biofertilizers and their characteristics
- What are the sources of biofertilizers
- discuss about the importance of biofertilizers
- understand about the Comparison with conventional fertilizers

1.2-INTRODUCTION

The agricultural practices have undergone significant change over the years. Due to the constant rise in population every year, the food system faces significant challenges to supplying enough food and agricultural products, especially in developing countries. The way that agriculture is practiced around the world has evolved over time. The green revolution is credited with many significant breakthroughs, but it has also resulted in an overuse of synthetic fertilisers, which are currently a major source of concern because they not only pollute the environment and soil but also threaten human and animal health. The green revolution failed to consider sustainability despite remarkable growth in the food supply. Over time, people have come to understand that biofertilizers are effective alternatives to the hazardous synthetic fertilizers and made the decision to adopt sustainable agricultural practices. While conducted major research they found one of the effective way to reduce usage of synthetic fertilizers, this happened with the discovery of Biofertilizers by humans.

Generally, Long-term farming usually results in agricultural land becoming impoverished if inputs are not properly supplemented. Under conventional farming systems, we need to use significant quantities of agrochemicals to increase the soil nutrient content, which pollutes the ecosystem. Therefore, it is essential to use a balanced and responsible usage of organic agriculture in order to make agriculture sustainable. All of these factors have led to a shift in favour of using biofertilizers, which protect plants from various plant diseases and stressful environments while providing nutrition through natural processes like nitrogen fixation, hormone production, siderophore, various hydrolytic enzymes and the solubilization of zinc, potassium, and phosphorus.

Biofertilizers are a necessity for Indian farmers because Indian economy is based on agriculture. Utilising a high-quality biofertilizer can improve soil fertility and yield better crops for farmers, even if the majority of agricultural activities depend on the monsoon season. A large population of a specific or a group of beneficial microorganisms helps improve the soil productivity either by solubilising earth phosphorus or promoting plant growth through the synthesis of growth improvement substances. A biologically active substance known as biofertilizers helps in improving the fertility of the soil.

1.3-DEFINITION OF BIOFERTILIZER

The term biofertilizer or which can be more appropriately called ‘microbial inoculants’ can be generally defined preparations that contain living or dormant cells of effective strains of microorganisms that help in crop plants' uptake of nutrients through interactions in the rhizosphere when applied through seed or soil. They accelerate some microbial activities in the soil that increase the amount of nutrients available in a form that is simple for plants to absorb.
If we split the term "biofertilizers" into its component, "bio" and "fertilisers," bio stands for "living life," while fertiliser refers to improving the quality of nutrients. Biofertilizer describes biological products that contain living microorganisms and that, when applied to soil, plant surfaces, or seed, promote growth in a number of ways, such as by improving plant nutrient uptake, increasing root biomass or area, and increasing nutrient availability. Microbes or microbial products are used as biofertilizers. Biofertilizers contributes to the protection of the ecosystem by reducing the usage of chemical fertilisers.

Simply said, biofertilizers are combination of particular strains of microorganisms. Biofertilizers are substances that are enriched with bacteria that help in the growth of trees and plants by providing them with more essential nutrients. It is made up of organisms such as bacteria, blue-green algae, and mycorrhizal fungus. Mycorrhizal fungi are responsible for the uptake of minerals by plants from organic matter, while cyanobacteria are characterised by their ability to fix nitrogen. Nitrogen fixation is the process of converting atmospheric nitrogen into nitrogenous compounds in soil that are suitable for plant absorption through a series of reactions. For example, some bacteria convert nitrogen to ammonia. As a result, nitrogen becomes available for plants. Furthermore, the bacteria may be phosphate solubilizers or nitrogen fixers. They change soil phosphorus from its insoluble to soluble forms. As a result, phosphorus will be available for plants.

Biofertilizers are economical, effective, and renewable sources of plant nutrients. It has a specifically important role in agricultural production. When these prepared biofertilizers are mixed with seeds, setts, seedlings or soil, they improve crop productivity and soil health, by the biological nitrogen fixation process, solubilisation and uptake of other nutrients and synthesis of growth-promoting substances such as vitamins and plant growth hormones. In order to prevent soil erosion, they also produce capsular polysaccharides. Using biofertilizers not only encourages the growth of healthy crops but also prevents the spread of pathogenic microorganisms. Biofertilizers is an essential component of integrated nutrient management since they provide an affordable and sustainable source of plant nutrients that can be added to chemical fertilisers for sustainable farming. Various microorganisms and their relationship with crop plants are being used in the production of biofertilizers.

1.4-SOURCES OF BIOFERTILIZERS

Biofertilizers add nutrients to the soil through the natural processes of fixing atmospheric nitrogen, solubilizing phosphorus, and enhancing plant growth through the synthesis of growth-promoting substances. Depending on their nature and function, they may be classified into different categories.

1- Nitrogen-fixing biofertilizers

2- Phosphorus Solubilising Microorganisms (PSM)

3- Phosphate mobilizing microbes: Mycorrhizae

4- Mineral-Solubilizing Biofertilizers

5- Plant Growth Promoting Rhizobacteria (PGPR)

1-Nitrogen-fixing biofertilizers: Although nitrogen is most common and abundant in the air, its difficulty in being fixed and absorbed by plants makes it a limiting nutrient. But some microbes can fix a significant amount of nitrogen, and some of them may also develop different kinds of relationships with plants. All the nitrogen-fixing organisms are prokaryotes. The nitrogen-fixing microorganisms known as diazotrophs are found in nature in various groups.

These are broadly divided into three categories:

(i) Free living (Asymbiotic or Non-symbiotic)

(ii) Associative Symbiosis

(iii) Symbiotic microorganism

(i) Free-Living Nitrogen-Fixing Bacteria (Asymbiotic or Non-symbiotic): They live freely in the soil and fix nitrogen fixation. Some of them are saprotrophic, living on organic remains, for example- *Azotobacter, Bacillus polymyxa, Clostridium, Beijerinckia*. They are further distinguished into aerobic and anaerobic forms.

They live freely in the soil and perform nitrogen fixation. Some of them are saprotrophic, living on organic remains, for example- *Azotobacter, Bacillus polymyxa, Clostridium, Beijerinckia*. They can be further divided into anaerobic and aerobic forms.

(ii) Loose Association of Nitrogen-Fixing Bacteria: This type of bacterial group lives both the inside and outside of the root. Both the bacterium and the host show a significant amount of symbiosis. Hence, they are known as **Associative symbiotic bacteria.** Azospirillum is an important bacterium in this group, suggests for millets, grass, wheat, maize, sorghum, rice etc.

(iii) Symbiotic Nitrogen-Fixing Bacteria: They develop a mutually beneficial relationship with the plants. Plants provide the bacteria with shelter and food. They supply some part of their

fixed nitrogen to the plants in exchange. Rhizobia (e.g., *Rhizobium*) are the most important group of symbiotic nitrogen-fixing bacteria. On the roots of legume plants, they develop nodules. Legume plants use their roots to fix and utilise this nitrogen. The host plants give bacteria a place to live and the energy to fix atmospheric N2, which is in return plant, receives fixed N2 (as protein). About a dozen species of *Rhizobium*, including R. leguminosarum, R. lupini, R. trifolii, R. meliloti, and R. phaseoli, develop relationships with the roots of various legumes. Rhizobia nodulates non-legumes such as *Trema* or *Parasponi*a sp., etc. Depending on the crop, nodulated legumes contribute significantly to the amount of N2 fixed in the biosphere. In 1888, Beijerinck first isolated and cultivated a microbe from the roots of legumes, which he named this as Bacillus radicola and later modified as Rhizobium.

Table-1 Biofertilizers and the crops on which it is used

Biofertilizer	**Crops Commonly Used On**
Rhizobium	Legumes (e.g., soybean, chickpea, lentils, groundnut)
Azotobacter	Cereals (e.g., wheat, rice, maize), vegetables, cotton, spices, tobacco, ornamental flowers
Azospirillum	Cereals, millets, oilseeds, vegetables
Phosphobacteria	Cereals, oilseeds, pulses, vegetables, sugarcane, fruits and flowers
Blue Green Algae (Cyanobacteria)	Paddy, banana
VAM (Mycorrhizae)	Various crops for enhanced nutrient uptake
Phosphate solubilizing bacteria and fungi	All crops
Azolla	Paddy

Here we will discuss some of the important types of biofertilizers.

(a) Cynobacteria: Blue-green algae are also known as Cynobacteria, Cyano means blue that means it is blue bacteria. The first description of BGA's agronomic potential in rice was presented by P.K. De (1939), who attributing the natural fertility of tropical rice fields to these N2-fixing organisms. N2-fixing potential of BGA can be estimated by evaluating its biomass, N-content and N2-fixing activity. As with Azolla or any other organic source, the nitrogen fixed by BGA becomes available to the rice crop after their decomposition. BGA can contribute about 20-30 kg N/ha. One of the characteristics of many free-living blue-green algae, or cyanobacteria, is their ability to fix nitrogen. In addition, BGA can enrich the soil with vitamins, minerals, 172 proteins, and natural growth hormones. For example, *Anabaena, Nostoc, Aulosira, Cylindrospermum, Totypothrix,* and *Stigonema.* Cyanobacteria are photosynthetic microorganisms. So, they enrich the soil with more nitrogen and organic matter. These prokaryotic organisms containing chlorophyll fix nitrogen from the atmosphere.

The photosynthetic Cyanobacteria usually coexist with lichens as symbionts in various pioneer habitats or as free-living organisms in pioneer ecosystems like desert soils. They also establish symbiotic relationships with other organisms, like cycads and the water fern *Azolla*. The relatioship with *Azolla*, where cyanobacteria are having in the leaves, has sometimes been shown to be important for nitrogen inputs in rice paddies, especially if the fern is allowed to grow and then ploughed into the soil to release nitrogen before the rice crop is sown.

(b) *Azotobacter*: Azotobacter is a genus of heterotrophic free-living nitrogen-fixing bacteria found in alkaline and neutral soils. It is recommended for non-leguminous crops such as paddy, millets, cotton, tomato, cabbage, and other monocotyledonous crops because of its aerobic nature. Azotobacter also produces growth-promoting compounds viz., auxins, and gibberellins and also to lesser extent the vitamins. A high level of organic matter in the soil is beneficial to Azotobacter performance. Many Azotobactor strains also exhibit fungicidal properties against specific species of fungus. The ability of the soil to fix nitrogen and multiply is enhanced by the presence of organic materials. The most common species found in India's arable soils is *Azotobactor chrococcum.*

(c) *Acetobactor*: *Acetobactor diazotrophicus* is a newly discovered nitrogen fixing bacteria related with sugarcane crop. The alpha group of proteobacteria includes this particular bacterium. It was isolated from samples of sugarcane leaves, roots, buds, and stems. Acetobactor is found in apoplastic fluid of sugarcane stem and, to a lesser extent, in xylem vessels. It is an acid and high salt tolerant and sucrose loving bacteria and capable of fixing up to 200 kg nitrogen per hectare. After being inoculated, sugarcane yielded more under field conditions. After its application, auxin and antibiotic type substance production have also been observed.

(d) *Azospirillum*: It is a nitrogen-fixing bacteria that live around the roots of higher plants but do not develop an close relationship with plants. It is commonly referred to as a rhizosphere association as this bacteria collects plant fluid and uses it as food. This process is termed as associative mutualism. Azospirillum is frequently found in the roots of grasses and cereals. Azospirillum is tolerant of high temperatures (30–40^{0}C), has a low energy need, and is widely established in cereal roots. Azospirillum are mesophilic and reported in crops grown in acidic to alkaline range. Inoculums of Azospirillum can survive in saline, acidic conditions. Thus, it works well in tropical environments. It can be shown that total nutrient assimilation (NPK) in plants inoculated with Azospirillum is seen to be higher than in plants that are uninoculated. Bacterization resulted to higher yields, which could be attributed to compounds which promote growth of plants and increased nitrogen uptake by plants.

(e) *Rhizobium*: The name *Rhizobium* was established by Frank in 1889. Rhizobium is an important nitrogen-fixing bacteria. Rhizobium coexists symbiotically with leguminous plants, especially in the nodules found in their roots. However they enter into symbiotic relationship only with leguminous plants, by infecting their roots and developing nodules on them. Only

when they are present inside the root nodules they develop the ability to fix nitrogen. In the nodule cells, bacteria (bacteroids) lie in groups surrounded by the membrane of the host cells, which is lined by a pink-red pigment called leghemoglobin. The term "effective nodules" refers to nodules that are filled with pink sap (leghaemoglobin pigment). This pigment keeps the rhythm of oxygen supply to the bacteria and helps the activity of nitrogenase enzyme. It traps the atmospheric nitrogen and transforms it into usable forms that increase the plants growth. In terms of the quantity of fixed nitrogen, they are the most effective biofertilizer. There are seven genera that are highly specific in forming nodules in legumes, referred to as a cross-inoculation group. Different species of rhizobium are classified into two groups (i) slow growing rhizobium (under the genus Bradyrhizobiun) and (ii) the fast growing groups (under the genus Rhizobium).

(f) *Frankia*: Frankia is a genus of Actinomycetes that has the ability to fix nitrogen gas in association with several tree species. The genera Alnus in the family Betulaceae and Casuarina and Allocasuarina in the family Casuarinaceae are examples of such trees. The relationship formed by species of Frankia are generally host-plant specific, but the same bacteria can nodulate closely related plant species. Frankia associations with plants range from those that are highly effective to those that are relatively ineffective at fixing nitrogen. Nitrogen deficiency in Casuarina can be resolve by inoculation with strains of Frankia that are effective at fixing nitrogen. Leaves of some plants (e.g., *Ardisia*) develop special internal cavities for providing space to symbiotic nitrogen-fixing bacteria, *Xanthomonas* and *Mycobacterium*. Such leaves are a constant source of nitrogen fertilizer to the soil. These leaves are a constant source of nitrogen fertilizer to the soil.

(g) *Azolla*: Azolla is known as free floating water fern (or pteridophyte), which contains as endosymbiont the nitrogen fixing cyanobacterium *Anabaena azollae*. Azolla is grown as a dual crop with rice or mixed into the soil before rice transplanting. In symbiotic relationships with blue green algae (*Anabaena azollae*), it fixes atmospheric nitrogen in rice fields. This association is a live, floating N, factory using energy from photosynthesis to fix atmospheric N, amounting to 40-60 kg N/ha/rice crop. *Azolla pinnata* is the most widely distributed species in India among the seven species of Azolla. It can be grown both as a green manure before transplanting and as a dual crop after transplanting of rice.

2- Phosphorus Solubilising Microorganisms (PSM)

Another most important primary nutrient for plants, after nitrogen, is phosphorus. Phosphate solubilizing microorganisms (PSMs) are group of beneficial microorganisms capable of hydrolyzing organic and inorganic phosphorus compounds from insoluble compounds. Among these PSMs, strains from bacterial genera (*Bacillus*, *Pseudomonas*, and *Rhizobium*), fungal genera (*Penicillium* and *Aspergillus*), actinomycetes, and arbuscular mycorrhizal (AM) are notable. Crops only recover 15 to 20 percent of the applied phosphorus; the remaining has been

fixed in the soil. The fixed form does not contribute to the available phosphorous content in the soil. A group of heterotrophic microorganisms solubilize this fixed phosphorous by producing organic acids and enzymes and make them available to the crops. These organisms solubilize the fixed soil phosphorus and release the citrate and water soluble phosphorus when applied at rock phosphate. Microorganisms present in organic wastes also help in the mineralization of organic phosphate compounds. They can be utilised to speed up the composting process once the thermophilic phase is complete. Their use in biofertilizers aims to increase the yields of legume, cereals, vegetables and fruit crops. The phosphorus-solubilizing bacteria (PSB) by releasing organic acids with a lower pH in their surroundings dissolve bound phosphates in soil and increase the amount of phosphorus available to plants. For example Bacillus spp., Paenibacillus spp., Pseudomonas spp. etc. The use of fungi in acidic soils and bacteria in neutral to alkaline soils increases the efficacy of applied soil phosphorus.

3- Phosphate mobilizing microbes: Mycorrhizae

The term mycorrhizae (Gr. myces = fungus, rhizo = roots) was coined for symbiotic associations formed by fungi with roots. The term mycorrhiza was taken from Greek language meaning 'fungus root'. This term was coined by Frank in 1885. This type of biofertilizers contains mycorrhizal fungi also known as phosphate absorbers. The mycorrhizal fungi form obligate or facultative functional mutualistic symbioses with more than 80% of all terrestrial plants, in which the fungus is dependent on host for photosynthesis and energy and in return provides a plethora of benefits to its host. The mycelium of the fungus extends from host plant root surfaces into soil, thereby increasing the surface area for more efficient nutrient access and acquisition for the plant, especially from insoluble phosphorus sources and others like calcium, copper, zinc, etc, e.g. ectomycorrhiza (*Laccaria* spp., *Pisolithus* spp., *Boletus* spp., *Amanita* spp.), endomycorrhiza (e.g. arbuscular mycorrhiza- *Glomus* sp., *Gigaspora* sp., *Acaulospora* sp., *Scutellospora* sp., and *Sclerocystis* sp.). The most common fungal partners of mycorrhiza are *Glomus* species. Mycorrhizal roots show a sparse or dense woolly growth of fungal hyphae on their surface. Root cap and root hairs are absent. Mycorrhiza is a potential biofertilizer which mobilizes P, Fe, Zn, B and other trace elements. It supplies moisture from far-off inches and is useful for long duration crops. It is resistant to dry powder and can be stored for up to two years. Mycorrhiza is classified into two main groups: (i) Ecto-mycorrhiza (ii) Endo-mycorrhiza. In the ecto-mycorrhiza, the hyphae develop a layer both outside and within the root in the intercellular spaces of epidermis and cortex, whereas endomycorrhiza is divided into three sub groups. Among these VAM are most common. They develop an internal network of hyphae between cortical cells which reaches to the soil and absorb nutrients and water. Several crop plants, whether dicot, monocot, annual, or perennial, associate with VAM.

Ectomycorrhiza (Ectotrophic Mycorrhiza): On the surface of the root, the fungus grows a mantle. Internally, it occurs in the intercellular spaces of the cortex. The fungal hyphae are nourished by the sugars and other food elements secreted by the root cells into the intercellular

gaps. The exposed fungal hyphae increase the surface of the root to multiple times. They perform several functions for the plant such as- Water absorption Solubilisation of organic matter of the soil humus, release inorganic nutrients, absorb nutrients and transport them to the roots, direct minerals absorption from the soil over a large area and handing over the same to the root. It is known that plants with ectomycorrhiza may absorb nitrogen, phosphate, potassium, and calcium two-three times more. The fungus secretes antimicrobial compounds which prevent young roots from pathogens. Ectomycorrhiza occurs in trees such as Peach, Pine, Eucalyptus, Oak (*Quercus*), etc. In most cases, the fungal partner is particular. It belongs to Basidiomycetes.

Endomycorrhiza (Endotrophic Mycorrhiza): There are fewer fungal hyphae lie on the surface. The others, which include grasses, crop plants, orchids, and certain woody plants, live in the cortex of the root, mostly in the intercellular spaces with some hyphal tips passing within the cortical cells. At the orchid's seedling stage, the fungal hyphae also provide nourishment by developing nutrient-rich cells called pelotons. Intracellular growth occurs in order to get nourishment because, unlike ectomycorrhiza, the cortical cells do not secrete sugars in the intercellular spaces.

Vesicular Arbuscular Mycorrhizal (VAM): Fungi have special structures called vesicles and arbusculars. VAM fungi are intercellular, obligate endosymbionts and, on establishment on the root system, they function as an extended root system. Along with harvesting moisture from deeper and faraway niches in the soil, they also harvest various micronutrients and provide them to the host plants. VAM is an endotrophic (live inside) mycorrhiza formed by aseptated phycomycetous fungi. VAM promote the phosphorus nutrition by not only increasing its availability, but also increasing its mobility. VAM help in the transport of nutrient mainly of phosphorus, zinc and sulfur. In addition, they transfer many nutrients from the soil to the roots of plants, including Cu (copper), K (potassium), Al (aluminium), Mn (manganese), Fe (iron), and Mg (magnesium). They have sac-like structures called vesicles for storing nutrients, and they also have another structure called an arbuscule which helps in transporting distant nutrients to the vesicules and roots. The VAM forms an association with plant roots. It enters the root cortex and spreads throughout the plant's roots. A single fungus may form a mycorrhizal association with several plants, for e.g., *Glomus.*

4- Mineral-Solubilizing Biofertilizers: Specific rhizobacteria can solubilize insoluble potassium forms, which is another important nutrient necessary for plant growth. For example- *Bacillus edaphicus, B. mucilaginosus,* and *Paenibacillus glucanolyticus*. Another important mineral is zinc, which is present at a less amount in the Earth's crust, due to which it is externally applied as the costlier soluble zinc sulphate to overcome its deficiencies in plant. Certain microorganisms such as *B. subtilis, T. thioxidans,* and *Saccharomyces* spp., can solubilize cheaper, insoluble zinc compounds such as zinc oxide, zinc carbonate, and zinc sulphide in soil. These microbes prove to be very useful in helping the plant in absorbing zinc as very less percentage (1-4%) of zinc which is manually applied to the plant is absorbed. Microbes

can also degrade silicates and aluminum silicates. Bacteria produce various organic acids that play a dual role in silicate weathering. They promote hydrolysis by adding H+ ions to the medium. In addition, the hydroxy carbolic acid, oxalic acid, keto acid, and citric acid from complexes with cations encourage the removal and retention of these cations in a dissolved state in the medium. Many Bacillus species that are present in various types of soils are widely used to dissolve silicate.

5- Plant Growth Promoting Rhizobacteria (PGPR): Plant growth promoting rhizobacteria (PGPR) are bacteria that colonize roots or rhizosphere soil (the soil zone surrounding the plant roots) and are beneficial to plants. They are also known as microbial pesticides e.g. Bacillus spp. and Pseudomonas fluorescence. Plant growth is improved by Serratia spp. and Ochrobactrum spp. Other than nitrogen-fixing, phosphorus and minerals solubilizing microbes, there are microbes that are appropriate to be utilized as biofertilizers as these promote plant growth by synthesizing growth-promoting compounds like growth hormones- auxins, gibberellin etc. These bacteria shows different mechanisms that promote plant growth, including nitrogen fixation, phosphorus solubilization, production of antibiotics, cytokinins, chitinase, and other hydrolytic enzymes, as well as the improvement of soil porosity. For example, *Alcaligenes, Achromobacter, Arthrobacter, Azotobacter, Actinoplanes, Bacillus, Pseudomonas fluorescens, Rhizobium, Bradyrhizobium* etc.

Table-2: Specification and categorisation of fertilizers based on their nature and function

S.No.	Groups	Example
N2 fixing biofertilizers		
1.	Free-living	*Azotobacter, Clostridium, Anabaena, Nostoc,*
2.	Symbiotic	*Rhizobium, Frankia, Anabaena azollae*
3.	Associative symbiotic	*Azospirillum*
P Solubilizing biofertilizers		
1.	Bacteria	*Bacillus megateriumvar.* phosphaticum *Bacillus circulans, Pseudomonas striata*
2.	Fungi	*Penicillium* sp., *Aspergillus awamori*
P Mobilizing biofertilizers		
1.	Arbuscular mycorrhiza	*Glomus* sp., *Gigaspora* sp., *Acaulospora* sp., *Scutellospora* sp. and *Sclerocystis* sp.
2.	Ectomycorrhiza	*Laccaria* sp.,*Pisolithus* sp., *Boletus* sp., *Amanita* sp.
3.	Orchid	Mycorrhiza *Rhizoctonia solani*
Biofertilizers for micro nutrients		
1.	Silicate and zinc solubilizers	*Bacillus* sp.
Plant growth promoting Rhizobacteria		
1.	Pseudomonas	*Pseudomonas fluorescens*

Nowadays, commercially available PGPR inoculants seem to enhance growth by preventing the spread of plant disease (known as Bio-protectants), or enhance nutrient uptake (known as Bio-fertilizers), or stimulation of phytohormones (known as Bio-stimulants). Pseudomonas and Bacillus produce phytohormones and growth regulators that increase the root surface area (more fine roots) of plants, for water and nutrients absorption. They are known as bio-stimulants, and the phytohormones they release include gibberellins, cytokinins, indole-acetic acid and inhibitors of ethylene production. It was found that a combination of the arbuscular mycorrhizal fungi *Glomus aggregatum*, the PGPR *Bacillus polymyxa* and *Azospirillum brasilense* maximized biomass and P content of the aromatic grass palmarosa (*Cymbopogon martinii*) when cultivated with an insoluble inorganic phosphate.

1.4.1 Applications of Biofertilizer to Crops

The biofertilizers can be inoculated on seeds as well as in the roots of various crop plants under appropriate conditions. They can also be applied directly to the soil. There are specific methods for applying biofertilizers, which are explained below:

1-Seed treatment: The process involves mixing of phosphorus and nitrogen fertilizers in the water. After that, seeds are dipped in this mixture. The seeds are dried after being applied with this mixture. They must be sowed as soon as possible once they dry out to prevent harmful microorganisms from damaging them.

2-Seedling root dip: This process is applied to the rice crop. The area where the crop has to grow is covered with a water bed. After being planted, the rice seedlings remain in water for eight to ten hours. The application of dipping seedling roots in a water suspension of biofertilizer nitrogen-fixing *Azotobacter* or *Azospirillum* and phosphorus-solubilizing microbial biofertilizer for a sufficient period of time is common for plantation crops like cereals, vegetables, fruits, trees, sugarcane, cotton, grapes, bananas, and tobacco.

3-Soil treatment: Bio fertilizers can be used to treat soil since they restore the original fertility of the soil. All of the compost fertilizers and biofertilizers are mixed. They are kept for one night. The mixture is then applied to the soil where seeds are to be sowed the next day. A few examples of soil-applied biofertilizers are *Rhizobium* (for leguminous plants or trees) and *Azotobacter* (for tea, coffee, rubber, coconuts, and all fruit/agroforestry plants); BGA and azolla in rice fields; and mycorrhiza in nursery beds.

4-Self Inoculation or Tubez Inoculation: This method is mainly suitable for use with *Azotobactor*. In this process, 50 litres of water are stored in a drum, to which 4-5 kg of *Azotobacter* biofertilizer is added and thoroughly mixed. This mixture is used to the planting materials required for an acre of land. In a similar manner, if we are treating potatoes, then dip the tubers in the mixture and then plant them after keeping the materials dry in the shade.

1.5- IMPORTANCE OF BIOFERTILIZER

Biofertilizers are products made of beneficial free-living bacteria from the soil. They strengthen different parts of plants, such as the roots, stem, and foliage, and help to increase overall productivity by reducing the quantity of synthetic fertiliser used in agricultural farms.

1.5.1 Advantages of Biofertilizers: Bio-fertilizers are becoming more and more widely used, because chemical fertilisers cause irreversible damage to the soil. Following are the benefits of using bio-fertilizers are:

1-Biofertilizers improve the growth of crops and improve the yield of plants by 20–30%.

2- Even in semi-arid conditions, these work well. These biofertilizers function well even in extremely dry environments with low rainfall.

3-Biofertilizers contribute to the better structure, increased water-holding capacity, and increased nutrient availability for plants in soils. Additionally, they increase the soil's capacity to hold carbon and reduce pests and plant diseases.

4-Bio-fertilizers function by releasing specific chemicals that inhibit harmful microorganisms, preventing them from surviving in the soil.

5- Along with producing vital nutrients like nitrogen, potassium, and phosphorus, they also release several minor micronutrients that improve soil fertility. In addition, they produce vitamins, minerals, and many compounds that promote growth in the soil, including as gibberllic acid and cytokinins.

6- An additional benefit of biofertilizers is that farmers can produce biofertilizers on their farms by inoculating bacterial cultures, provided they have sufficient knowledge and expertise with the procedure.

7-They do not pollute the environment. In comparison to synthetic fertilisers, which permanently degrade soil condition, these fertilisers do not leave behind any residues or polluting compounds when they are applied to soil. As a result, biofertilizers are becoming more important in sustainable agricultural practices.

8- Since biofertilizers reduce phosphate by 25% and are therefore more environmentally friendly than chemical fertilisers, they protect the environment from pollution.

9- Biofertilizers are an essential component of sustainable agriculture and have a significant effect on crop productivity and soil health. They are used to improve the biological, chemical, and physical characteristics of soils and are produced from natural resources such plant leftovers, animal manures, compost, and sludge.

1.5.2 Disadvantage of Biofertilizers: A few drawbacks of biofertilizers are listed below:

1-Biofertilizers only yield a 20–30% increase in crop yield. In comparison to chemical fertilisers, they do not considerably raise productivity.

2- Specific crops require a special type of fertiliser. This is more applicable to symbiotic microbes. It is not possible to enhance crop production or root nodulation by using non-specific Rhizobium as a fertiliser.

3- Production of microbial fertiliser needs to happen in an extremely aseptic environment. A common problem in microbial mass production is contamination.

4- Microbes are light-sensitive, therefore when they come into contact with sunlight, they are ultimately destroyed.

5- After being kept at room temperature for six months, and after being kept at a cooling temperature for two years, microbial fertilisers must be used.

6-The quantity of nutrients in compost products varies greatly. Moreover, the costs of implementation are greater than those of some chemical fertilisers.

7- Long-term and extensive application may lead to the deposition of nutrients, salts, and heavy metals, which could have a negative impact on plant growth, soil organism development, water quality, and human health.

8-Due to the lower nutrient contents compared to chemical fertilisers, large volumes must be applied to the soil.

9- Plant growth and development may be limited by insufficient availability of essential macronutrients.

10- There may be nutritional deficiencies as a result of low macro- and micronutrient transfer.

11-Biofertilizers have not been widely accepted, primarily because to their slow and unimpressive responses.

12- Demand the production and application expertise; additionally, these are difficult to store.

1.6- COMPARISON WITH CONVENTIONAL FERTILIZERS

Depending on the types and contents of materials added to promote plant growth, fertilisers can be divided into two groups: chemical fertilisers and bio-fertilizers.

Chemical Fertilizer: Chemical fertilisers, commonly referred to as synthetic fertilisers, are made of chemicals and are produced in factories by using natural elements applying them to chemical changes. These are manufactured in large quantities and include one or more essential nutrients that enhance plant development and increase yields in agriculture. This leads to a faster, healthier, and larger crop production. Chemical-based fertilisers, in contrast to biofertlizers, are

extracted and highly processed. To put it simply, the purpose of adding them to the soil is to support plant growth by means of inorganic chemicals.

Biofertilizer: Biofertilizers, sometimes referred to as microbial fertilisers or microbial inoculants, are substances that contain that contain living or latent microorganisms. These compounds are given to soil, plant surfaces, or seeds to increase soil fertility and improve plant growth. Biofertilizers basically increase the nutrients of host plants by increasing the content of the soil. They are generally preferred over chemical fertilisers because to their friendliness to the environment, less cause pollution, and most importantly, their ability to prevent pathogens from growing. Biofertilizers may contain nitrogen fixing microbes or phosphate soluble microbes or VAM fungal spores. These are spread into the soil through seed treatment or field-wide application during cultivation. The word "biofertilizer" comes from the fact that biological wastes are utilised to increase the fertility of the land

1.6.1Difference between Biofertilizer and Chemical Fertilizer:

1- Based on Type: Fertilisers can be categorised as chemical or bio-fertilizers based on the types of materials that are added to the soil to provide one or more nutrients that are necessary for plant growth. Bio-fertilizers are substances containing inactive or living cells of microorganisms that, when mixed with soil, plant surfaces, or seeds, improve soil fertility and increase the growth of plants. On the other hand, chemical fertilisers are made from chemical bases and are produced in factories by applying natural elements by making them to undergone chemical processes.

2- Composition: The nitrogen-fixing microorganisms, potassium solubilizers, phosphate solubilizers, or phosphorous mobilizers present in bio-fertilizers are only used in combination with the microbes or spores of VAM fungi. The three essential nutrients that make up modern chemical fertilisers are potassium, phosphorus, and nitrogen. These are mainly industrially synthesised inorganic chemicals and are extracted and highly purified, compared to their organic substitutes.

3- Nutrient Distribution: Chemical fertilisers include one or more of the three consistently distributed essential nutrients needed for plant growth. According to studies, the best crop production may result from replacing an equal amount of chemical fertiliser. They are therefore more costly. On the other hand, bio-fertilizers maintain an uneven distribution of essential nutrients, which keeps the soil rich in various kinds of micro- and macronutrients. As a result, these are less costly than their inorganic equivalents.

4- Effectiveness: Biofertilizers are applied to the soil either by seed treatment or field-wide application during cultivation. Biofertilizers take time than their chemical substitutes to provide the necessary nutrients to the plants, but they are not toxic because they contain organic materials. Compared to chemical fertilisers, which have a longer shelf life and a higher nutrient density, biofertilizers have a lower nutrient density and a significantly shorter shelf life.

Therefore, using chemical fertilisers would require less amounts to achieve the same goals as using biofertilizers.

It is true that the application of biofertilizers greatly enhances crop availability and nutrient supply in upland crop production. Because biofertilizers are more environmentally friendly, sustainable, and biodegradable than chemical fertilisers, they lower production costs and the amount of fertiliser used in agriculture. However, even with all of these benefits, a lot of farmers continue to use chemical fertilisers since they produce better and faster crops and function more quickly than their organic counterparts because they dissolve almost instantly in water. In addition to their drawbacks, chemical fertilisers are the main source of food production worldwide and are a preferred choice of farming in many nations.

1.7- SUMMARY

There are numerous microorganisms that can be used to enhance agricultural production. These are known as biofertilizers. Biofertilizer consists of living microorganisms such as mycorrhizal fungus, blue-green algae, and bacteria that are incorporated into fertilisers to enhance plant growth in a sustainable manner. Biofertilizers perform an important role in agriculture. It is a safer choice for humans to solve the problem of feeding a growing global population at a time when agriculture faces a number of environmental challenges. The rhizosphere of crop soil is affected by biofertilizers and helps in boosting the growth of the crop.

Biofertilizers perform on the principle of natural relationship between flora and fauna. Humans use and improve naturally occurring processes such as nitrogen fixation by microbes, nutrition mobilisation and solubilisation by microorganisms, micronutrient and macronutrient absorption, and stimulation of plant natural growth hormones or phytohormones. The role of Biofertilizers is to fix nitrogen, boost plant growth and yield, and mineralize the soil. Blue-green algae, *Azotobacter*, and *Rhizobium* are a few common microbe examples. Fertilisers can be applied using a variety of methods, including seedling root dipping, soil, seed treatment, and self inoculation. Biofertilizers are extremely beneficial than chemical fertilisers and do not pollute the environment. Frequent use of chemical fertilisers and toxic pesticides has increased the release of hazardous substances that are damaging to human health and upset the ecological balance. Biofertilizers differ from usual fertilisers in that they do not include harmful chemicals that could be dangerous to crops and humans.

1.8- GLOSSARY

Biofertilisers: Biofertilizers are microorganisms (such as bacteria, algae, and fungi) or biologically active compounds used to improve soil fertility, either alone or in combination.

Chemical fertilizer: A chemical fertilizer is defined as any inorganic material of completely or partially synthetic origin that is applied to the soil to sustain plant growth.

Diazotrophs: are bacteria that convert atmospheric nitrogen gas into a more usable form such as ammonia.

Rhizobia: Rhizobia are special bacteria that can live in the soil or in nodules developed on the roots of legumes.

***Rhizobium*:** *Rhizobium* is a genus of Gram-negative soil bacteria that helps in nitrogen fixation in leguminous plants.

Vesicular Arbuscular Mycorrhiza (VAM): VAM fungi are mycorrhizal species of fungus that live in the roots of various higher-order plants.

Plant Growth Promoting Rhizobacteria (PGPR): The term "PGPR" refers to a group of rhizosphere bacteria that promote plant growth.

1.9- SELF ASSESSMENT QUESTION

1.9.1 Multiple Choice Questions:

1-Which of the following fern is a biofertilizer?
(a) *Salvinia* (b) *Pteridium*
(c) *Azolla* (d) *Marsilea*

2- Which of the following is an endomycorrhiza?
(a) *Glomus* (b) *Agaricus*
(c) *Rhizobium* (d) *Nostoc*

3-Which of the following is a nitrogen fixer in the root nodules of *Alnus*?
(a) *Bradyrhizobium* (b) *Clostridium*
(c) *Azorhizobium* (d) *Frankia*

4. Which of the following is incorrectly matched?
(a) Alfalfa – *Rhizobium*
(b) Alnus – *Frankia*
(c) Nitrogen fixer – *Anabaena*
(d) Mycorrhiza – *Rhodospirrilum*

5-What are biofertilizers?
(a) Pesticides (b) Natural fertilizers
(c) Synthetic chemicals (d) Weed killers

6-Biofertilizers are beneficial for:
(a) Killing beneficial microorganisms (b) Increasing soil erosion
(c) Depleting soil nutrients (d) Enhancing soil fertility

7-Azotobacter is a type of biofertilizer that provide to the enrichment of soil with which nutrient?
(a) Calcium (b) Nitrogen
(c) Potassium (d) Phosphorus

8-Biofertilizer which helps in fixing atmospheric nitrogen in leguminous plants is-
(a) Rhizobium (b) Cyanobacteria
(c) Azospirillum (d) Phosphobacteria

9- Process of converting atmospheric nitrogen into a usable form by biofertilizers is called:
(a) Denitrification (b) Ammonification
(c) Nitrogen fixation (d) Nitrification

10-How many molecules of ATP are required to fix one molecule of nitrogen?
(a) 12 (b) 20
(c) 6 (d) 16

11- Which of the following is a non-symbiotic biofertiliser?
(a) VAM (b) Azotobacter
(c) Anabaena (d) Rhizobium

12-Biofertilisers are the living organisms which
(a) Bring about soil nutrient enrichment (b) maximise the ecological benefits
(c) minimise the environmental hazards (d) all of these

Answer Keys: 1.9.1: 1-(c), 2-(a), 3(d), 4-(d), 5-(b), 6-(d), 7-(b), 8-(a), 9-(c), 10-(d), 11-(b), 12-(d)

1.10- REFERENCES

- A handbook of organic farming by Arun.K.Sharma published by Agrobios (India), Jodhpur.
- A text book of Soil Science by R.K. Mehra published by Indian Council of Agricultural Research, Pusa, New Delhi.
- Handbook of Composite Organic Farming by Himadri Panda and Dharamvir Hota, published by Gene-Tech Books, New Delhi

- https://www.onlinebiologynotes.com/biofertilizer-advantages-types-methods-of-application-and-disadvantages/
- http://eagri.org/eagri50/SSAC222/lec17.pdf
- https://unacademy.com/content/neet-ug/study-material/biology/biofertilizers-components/
- https://www.embibe.com/exams/microbes-as-biofertilizers/
- https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9445558/
- https://www.bio-fit.eu/q8/lo1-why-biofertilizers?start=4
- https://www.bio-fit.eu/q1/lo3-common-used-bio-fertilizers?start=1
- https://gyansanchay.csjmu.ac.in/wp-content/uploads/2022/08/Biofertilizers.pdf
- https://tractorgyan.com/tractor-industry-news-blogs/1202/biofertilizers-types-importance-and-benefits
- https://infinitylearn.com/surge/biology/biofertilizers/
- http://www.differencebetween.net/technology/difference-between-biofertilizer-and-chemical-fertilizer/
- http://www.differencebetween.net/technology/difference-between-biofertilizer-and-chemical-fertilizer/
- https://www.hindawi.com/journals/ija/2019/4917256/
- https://www.slideshare.net/AbdullahSaleem24/biofertillizersgroups-of-biofertillizersscope-and-future-perspective-of-biofertillizers.
- https://agrotexglobal.com/what-is-bio-fertilizer-its-types-and-uses/
- http://eagri.org/eagri50/AMBE101/lec25.html
- https://geolifeshop.com/blogs/news/importance-of-biofertilizer
- https://scienceagribio.com/biofertilizers-definition-types-uses-importance/
- https://jaihokisan.in/important-biofertilizers-and-their-usage-in-different-crops.html
- https://www.scholarsresearchlibrary.com/articles/importance-of-biofertilizers-in-agriculture-biotechnology-15125.html

1.11-SUGGESTED READINGS

- A handbook of organic farming by Arun.K.Sharma published by Agrobios (India), Jodhpur.
- A text book of Soil Science by R.K. Mehra published by Indian Council of Agricultural Research, Pusa, New Delhi.
- Handbook of Composite Organic Farming by Himadri Panda and Dharamvir Hota, published by Gene-Tech Books, New Delhi.

1.12-TERMINAL QUESTIONS

1.12.1 Short answer type Questions

1-How do biofertilizers promote plant growth?

2-What are the main sources of biofertilizers?

3- Write short notes on:

(a) VAM

(b) Phosphate mobilizing microbes

(c) Acetobactor

1.12.2 Long answer type Questions

1- Explain in detail Comparison of Chemical Fertilizers with Biofertilizers.

2- What is biofertilizers? Discuss in detail classification of biofertilizers.

3- Give a detail account on Nitrogen-fixing biofertilizers.

UNIT-2 BIOLOGICAL NITROGEN FIXATION

Contents

2.1-OBJECTIVES

After reading this unit, learner will be able to:

- Know about for nitrogen fixation.
- Discuss the types of nitrogen fixation
- Describe about the biological nitrogen fixation
- Explain the nitrogen cycle

2.2-INTRODUCTION

The chemical element Nitrogen has the atomic number seven and the symbol N. Nitrogen is a colourless, odourless element that is categorised as a non-metal and usually found in gas form. All living systems have nitrogen as a component of their biological compounds. It is vital for all living things because it makes up a large portion of amino acids, which are the building blocks of proteins, and nucleic acids like DNA, which carry genetic information to next generations of organisms, Although gaseous nitrogen makes up around 78% of the atmosphere, it cannot be bioavailable for plants or animals.

Plants can only use reduced forms of nitrogen, even though it is one of the most readily available elements. Nitrogen cannot be taken directly from the atmosphere. Thus, all life forms require nitrogen fixation, whether it comes from natural or artificial sources. The nitrogen gas in the atmosphere or air is converted by living organisms, mainly bacteria, into nitrogen compounds that plants may use. In other words, nitrogen fixation is the process of converting atmospheric nitrogen into related nitrogenous compounds in soil, such as ammonia, which is what plants need.

2.3-NITROGEN FIXATION

Nitrogen is the most common vital macroelement in living things, after carbon, hydrogen, and oxygen. In order for plants to manufacture amino acids, proteins, nucleic acids, cytochromes, chlorophylls, alkaloids, phytohormones, and several types of vitamins, they need nitrogen. Plants get most of their nitrogen from the environment. In the atmosphere, it occurs as free diatomic (N_2) molecules. This is an extremely inert gas. Since the higher plants cannot directly use it, it needs to be reformed. Plants compete with microbes for limited amount of nitrogen present in the soil. Nitrogen is mostly taken up by plants as ammonium ions (NH_4+) or nitrate ($NO_3–$) from the soil.

The nitrate is more common in well oxygenated, non-acidic soils, whereas ammonium is more common in acidic or water logged soils. The other potential sources of available soil nitrogen include synthetic fertilisers that plants can directly absorb, animal excrement (urea), and amino acids from decomposing organic materials.

Nitrogen fixation refers to the process of converting free nitrogen—both elemental and molecular—into nitrogenous compounds so that plants can absorb it. Nitrogen fixation is vital to life, because fixed inorganic nitrogen molecules are necessary for the biosynthesis of all nitrogen-containing organic compounds, including proteins, nucleoside triphosphates, amino acids, and nucleic acids.

Nitrogen fixation is of two types:

1-Abiological nitrogen fixation

2- Biological nitrogen fixation

1-Abiological nitrogen fixation

This is also known as physical nitrogen fixation. Abiological nitrogen fixation is the process of converting free nitrogen into nitrogenous compounds without the support of any biological organism. There are two types of it:

(i) Natural

(ii) Industrial/Artificial

(i) Natural Nitrogen Fixation: A natural phenomenon during which lightning energy converts nitrogen into nitrogen oxides, which are then utilised by plants. In other words, nitric oxide (NO) is formed when the N2 and O2 of the air interact with lightning (i.e. an electric discharge in the clouds) and thunder. Nitrogen peroxide (NO_2) is formed when the nitric oxides undergo further oxidation with oxygen.

The reactions are as follows:

$$N_2 + O_2 \text{ Lightning} \rightarrow \text{Thunder } 2NO \text{ (Nitric Oxide)}$$

$$2NO + O_2 \rightarrow 2NO_2 \text{ Oxidation (Nitrogen peroxide)}$$

During the rain, Nitric acid (HNO_3) and nitrous acid (HNO_2) form when NO_2 reacts with rain water. Rainwater and acids reach on the soil, where they combine with alkaline radicals to produce nitrates (NO_3^-) and nitrites ($N0_2^-$), which are soluble in water.

$$2NO_2 + H_2O \rightarrow HNO_2 + HNO_3$$

$$HNO_3 + \text{Ca or K salts} \rightarrow \text{Ca or K nitrates}$$

The nitrates are soluble in water and are absorbed up by the plants through their roots.

(ii) Industrial nitrogen fixation: It is a synthetic alternative that uses ammonia to help in nitrogen fixation. Nitrogen and hydrogen react directly to form ammonia. It is later transformed into several fertilisers, including urea.

The Haber-Bosch process: Ammonia is produced directly by this technique from nitrogen and hydrogen. The German chemist Fritz Haber discovered in 1909 that atmospheric nitrogen could react with hydrogen under extremely high pressure and temperature conditions. This reaction is catalysed by an iron catalyst and results in an extremely high proportion of ammonia, which is the starting point to the synthesis of a wide variety of nitrogen compounds. Carl Bosch made this process commercially viable, and it is now referred to as the synthetic ammonia process or the Haber-Bosch method. Nowadays, the Haber-Bosch process is among the biggest and most fundamental in the global chemical industry.

$$N_2 + 3H_2 \rightleftharpoons 2NH_3 \text{ (only 20\% conversion)}$$

The NH3 that is formed thus can be utilised as fertiliser directly, but the majority of it is converted into urea and ammonium nitrate (NH_4NO_3).

Besides this, combustion of fossil fuels (natural gas, coal, crude oil) and products produced from crude oil (petrol, diesel, gasoline) contributes to nitrogen fixation.

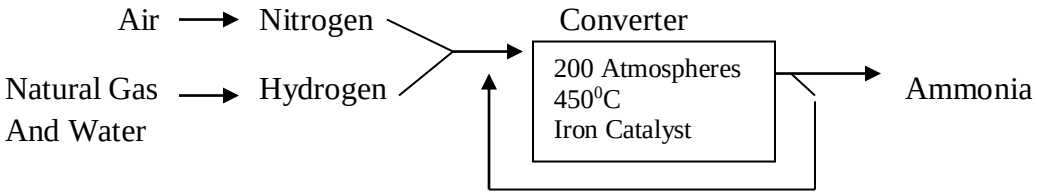


Fig.2.1: Haber-Bosch process

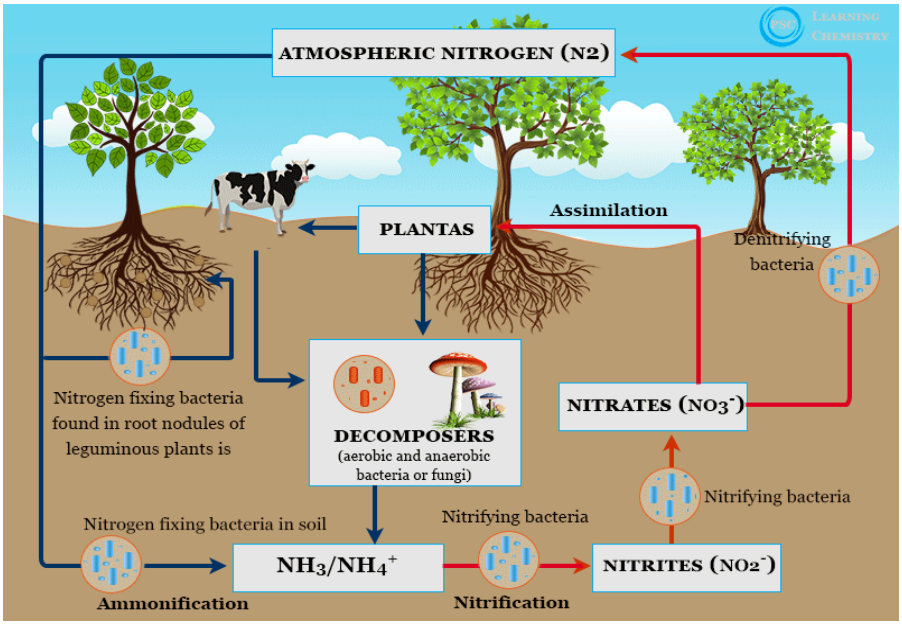


Fig.2.2: Nitrogen fixation

(Source: https://www.priyamstudycentre.com/2022/03/nitrogen-fixation.html)

Challenges related to Industrial Nitrogen Fixation

The following are the drawbacks of using industrial nitrogen fixation

1- The cost of producing NH_3 industrially is high.

2-Fertiliser distribution and transportation require a lot of labour.

3-Nitrogen application to the soil usually contaminates nearby water supplies.

4- When fossil fuels are used to provide the energy needed to make this fertiliser, carbon dioxide emissions occur, which contributes to global warming.

5-The nitrogen cycle is disturbed by the overuse of these artificial fertilisers, which pollutes groundwater and surface water and harm ecosystem.

6-Increased amounts of nitrogen fertiliser to fresh water, as well as marine ecosystems have led to eutrophication, which is the process by which these systems promote the growth of microorganisms, especially algae.

2- Biological Nitrogen Fixation

It is already known that plants and animals do not absorb nitrogen directly from the atmosphere. Blue-green algae and bacteria such as Rhizobium convert nitrogen from its unusable form into beneficial forms. These microorganisms fix these nitrogenous compounds in the soil.

Following are the essential requirement for N_2-fixation

i) Nitrogenase is the name of the enzyme that catalyses a conversion from molecular N_2 to NH_3. It is generated by a set of genes known as the nif genes. Thus, one of the essential needs is the expression of the nif genes, which results in the formation of functional nitrogenase.

ii) Nitrogenase's activity is highly sensitive to oxygen. Therefore, the aerobic N_2-fixing organisms need a cellular defence mechanism to protect nitrorgenase activity from oxygen inhibition or the formation of an anaerobic environment.

iii) The process of reducing N2 to NH3 requires a lot of energy; each N2 reduced requires 16 to 24 ATP molecules. Therefore, the organism needs to be able to provide a large supply of ATP.

iv) The presence of Mo and Fe is essential for the development of the nitrogenase enzyme complex in plants, as they are essential components of the enzyme.

v) The production of the nitrogenase enzyme occurs in bacteria rather than in cells with access to fixed nitrogen sources like ammonia or nitrate. Therefore, in circumstances when there are no such fixed nitrogen sources, the N2-fixation process takes place.

2.4 BIOLOGICAL NITROGEN FIXATION

Biological nitrogen fixation was discovered by German agronomist Hermann Hellriegel and Dutch microbiologist Martinus Beigerinck. The process by which different types of microbes change atmospheric nitrogen into nitrogen that can be used, such as ammonia, is known as "Biological Nitrogen Fixation." Animals and plants use ammonia to produce organic substances like proteins and nucleic acids. Biological nitrogen fixation is a beneficial alternative to nitrogen fertiliser. Biological nitrogen fixation is occurring naturally in soil by microorganism termed Diazotrophs that comprise of bacteria such as Azotobacter and Archaea. All biological nitrogen fixation is affected by enzymes called nitrogenises. Iron is present in these enzymes together a second metal, generally molybdenum but occasionally vanadium.

In simpler terms, the process by which atmospheric nitrogen is converted to ammonia in the presence of nitrogenizes is known as biological nitrogen fixation, or BNF. Nitrogenize is a naturally occurring biological catalyst that is only present in particular microbes, such as the free-living Azospirillum, Azotobacter, and BGA, or the symbiotic Rhizobium and Frankia. The process is performed by Two main types of microorganisms—those that coexist symbiotically with other plants and those that are "free living" or non-symbiotic.

The ability of the root nodule associations to fix atmospheric N_2 was first identified. The first group of organisms known to have the ability to fix nitrogen are rhizobia. A unique class of prokaryotes is responsible for biological nitrogen fixation (BNF), which was identified by Beijerinck in 1901. These organisms catalyse the transformation of atmospheric nitrogen (N_2) into ammonia (NH_3) by using the enzyme nitrogenase. Only certain prokaryotes, such as Rhizobium, Azotobacter, and Cyanobacteria, are capable of fixing atmospheric nitrogen among all the organisms on Earth. They are referred to as diazotrophs or nitrogen fixers. They fix over 95 percent of the total world nitrogen yearly (-200 million matric tones) by natural processes. Diazotrophs coexist in symbiotic partnerships with plants, living as nodules on the roots of legume plant including clover, peas, and beans. Nitrogen from the atmosphere is taken up by the bacteria and transformed into ammonia, which the plant uses for growth and development. In non-symbiotic relationships, Diazotrophs that are free-living fix nitrogen in soil or water, providing it to other species within the ecosystem.

The nitrogen found in approximately 70 percent of the earth's atmosphere is very inert di-nitrogen (n = n), which is not used by most plants. Two nitrogen atoms joined by a triple covalent bond formed atmospheric di-nitrogen (N_2). This triple bond is hard to break; it takes about 225 kcal of energy to do it. The process by which inert gaseous di-nitrogen (N_2) is reduced into ammonia (NH_3) by means of certain microbes so that plants can use is known as Biological nitrogen fixation, or diazotrophy.

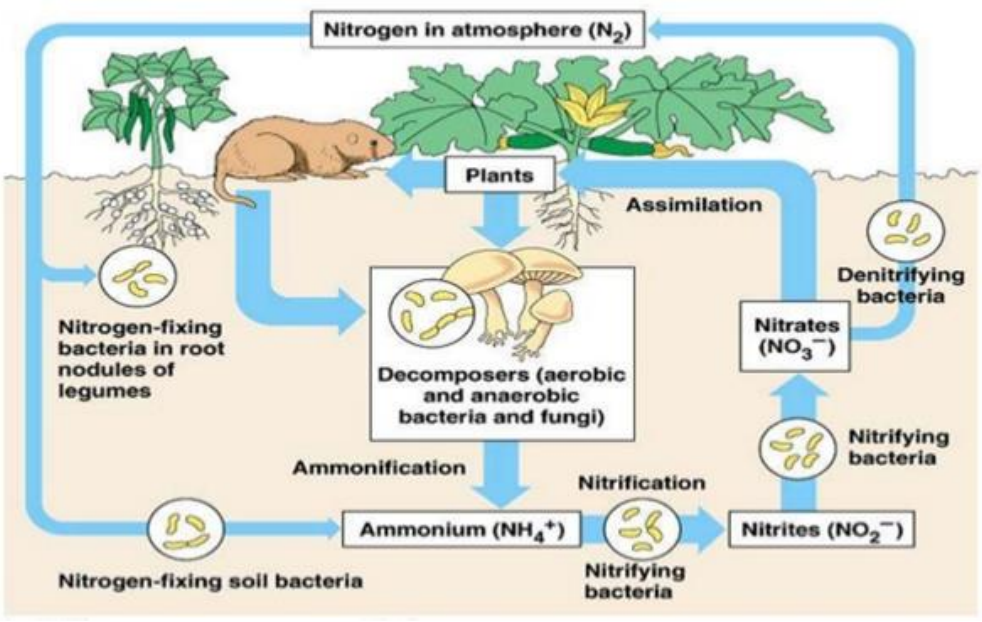


Fig.2.3 Biological nitrogen fixation
(Source: https://onlinesciencenotes.com/wp-content/uploads/2023/04/Biologican-nitrogen-fixation.jpg)

Process of Biological Nitrogen Fixation

In this process, nitrogen from the atmosphere is converted by microbes to ammonia, which is attackable by nearby plants. These microorganisms oxidise organic molecules to produce the 16 moles of Adenosine Triphosphate (ATP) required for the reduction of one mole of nitrogen. Microorganisms that do not undergo photosynthesis and are free-living obtain these molecules from other organisms, whereas microorganisms that do photosynthesis, such Cyanobacteria, utilise sugars that are produced during photosynthesis.

$$N_2 + 8H+ + 8e- + 16\ Mg\text{-}ATP\ 2NH_3 + H_2 + 16\ Mg\text{-}ADP + 16\ Pi$$

Biological nitrogen fixation is a process that takes place in 2 stages those are:

Stage 1: *Rhizobium* contacts a susceptible root hair

The roots of legume plants release compounds, such as flavonoids, organic acids, and amino acids that attract *Rhizobium*. This type of attraction is known as chemotactic attraction.

Stage 2: Bacteria invade the root hair

Hairs of root curled because to unique complex polysaccharides present on the rhizobium surface detected by lectins. The bacteria stuck, entered the root cells, and multiplied. PGRs are necessary for cortical and pericycle cells to dedifferentiate. The root provides auxin, while bacteria produce cytokinin.

Biological Nitrogen fixation may be categorized into following types:

A. Non- Symbiotic/ Asymbiotic Biological Nitrogen Fixation.

B. Associative Biological Nitrogen Fixation.

C. Symbiotic Biological Nitrogen Fixation.

A-Non- Symbiotic/Asymbiotic Biological Nitrogen Fixation

The biological nitrogen fixation process carried out by microorganisms living outside of plant cells is known as non-symbiotic nitrogen fixation. Numerous free-living, nitrogen-fixing microbes are found in soil. Several anaerobic and aerobic microorganisms as well as blue-green algae are among them. The non-symbiotic (asymbiotic) nitrogen fixers can be classified as follows:

1. **Free-living aerobic Nitrogen-fixing bacteria:**
    - Photosynthetic: Chlorobium, Chromatium.
    - Non-Photosynthetic: Azotobacter, Azomonas, Dexia, Beijerinckia.
2. **Free-living anaerobic Nitrogen-fixing bacteria:**
    - Photosynthetic: Rhodospirillum.
    - Non-Photosynthetic: Clostridium.
3. **Free-living chemosynthetic bacteria:**
    - Heterotrophic: Desulfovibrio.
4. **Cyanobacteria or Blue-green algae:**
    - Heterocyst bearing: Nostoc, Anabaena, Calothrix, Rivularia.
    - Non-Heterocyst bearing: Oscillatoria, Gloeocapsa, Plectonema, Lyngbya.
5. **Free-living Fungi:** Yeasts and Pullularia. The free-living, symbiotic nitrogen fixers are primitive. Fixation is a reduction process that does not require respiration. Under low aeration, these organisms fix nitrogen more actively, as long as no hydrogen gas is produced.

B-Associative Symbiotic Nitrogen Fixation

Bacteria that are close to grain and grass roots fix nitrogen. This adaptable mutualism is called associative symbiosis. The rhizosphere, or the area between soil and roots, is home to the bacteria, which occasionally enter the roots. The roots absorb part of the fixed nitrogen, while the bacteria get their nourishment from the roots' production of carbohydrates.

Some examples are:

1- Azospirillum brasilense in association with cereal roots.
2- Beijerinckia in association with the roots of Sugarcane.
3- *Azotobacter paspali* in association with roots of tropical grass- *Paspalum notatum.*

C-Symbiotic Biological Nitrogen Fixation

The exchange in which one plant provides a niche and the other produces fixed nitrogen is known as symbiotic nitrogen fixation. The process involves symbiotic bacteria found in root nodules fixing atmospheric nitrogen. Plants that bear pods, such peas, beans, alfalfa, and clovers, and legumes have mutually beneficial associations with these bacteria. In the same soil, the following generation of legumes can utilise the fixed nitrogen.

Symbiotic biological nitrogen fixation can be categorised into the following groups:

1. Nitrogen Fixation in leguminous plants: A number of legume plants have symbiotic nitrogen fixers, primarily belonging to the genus Rhizobium. Within specialised structures on the roots known as root nodules, they established their home. The bacteria fix nitrogen only when they are within the nodules. The relationship is considered to be symbiotic since the host plant provides the nodule bacteria with organic carbon (carbohydrates). The host plant is then given fixed nitrogen by the microbes. A symbiont of soybeans, Bradyrhizobium japonicum grows slowly. The symbiont Azorhizobium caulinodans forms stem nodules in Sesbania species.

(a) Chemical interaction and entry: The bacteria first grow in the soil close to the roots of larger plants, where they are unable to fix nitrogen. These bacteria enter the roots of leguminous plants by root hairs and interact chemically with the roots when they come into contact with them. Multiple chemical signals sent by the root hair are required for bacterial entry into the root hair.

(b) Formation of infection thread: The root hair cell wall is broken down by the enzymes that produced by bacteria resulting in the formation of a thread like structure called infection thread. These bacteria multiply and enter the infection thread, and ultimately move up to the inner cortex, where they penetrate tetraploid cells and promote them to divide.

(c) Formation of root nodule and bacteroids: A knob-like outgrowth known as a root nodule is formed by the multiplying cells. When the available cytosol is filled, these bacteria multiply and colonise inside the tetraploid cells, at whichpoint they go dormant. The term "bacteroid" refers to any larger, dormant, non-motile bacterium. Several thousand of bacteroids are present in a typical root nodule cell.

(d) Peribacteroid membrane and peribacteroid space: Within the cytoplasm, the bacteroids generally exist in groups. A membrane known as the peribacteroid membrane surrounds each group. Peribacteroid space is the area inside the cell that is surrounded by the peribacteroid membrane. Within the cytosol of nodule cells, a red pigment known as Leghaemoglobin is filled in the area outside the peribacteroid space. Leghemoglobin functions as a highly efficient O2 scavenger because it combines with oxygen quickly, just like haemoglobin does in our red blood cells.

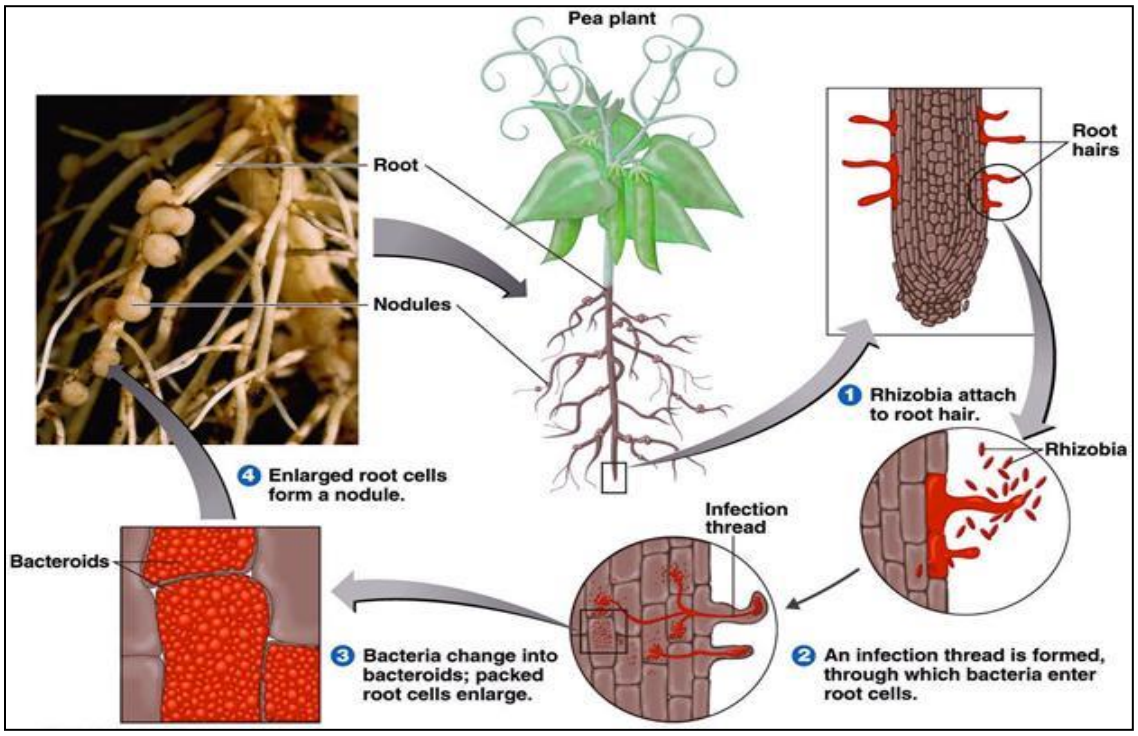


Fig.2.4: Nitrogen Fixation in leguminous plants
(Source: http://eagri.org/eagri50/AMBE101/lec18.html)

2. Nitrogen Fixation via nodule formation in the non-leguminous plants: Root nodules are known to be produced by a wide variety of plants in families other than Leguminosae. Trees and shrubs are the most significant among them. The following are some example of non-leguminous plants that fix nitrogen and develop root nodules:

1-Genus Frankia produces root nodules in association with *Alnus, Myrica gale, Casuarina equisetifolia*, etc.

2-Rhizobium also has root nodules in the genus Parasponia.

3- Leaf nodules are formed by bacteria Klebsiella in the genus Psychotria and by bacteria Burkholderia in genus *Pavetta zimermanniana.*

3- Nitrogen Fixation through Non-nodulation: Symbiotic nitrogen fixation takes place in certain plants, however nodules do not develop. These relationships are a type of pseudosymbiosis. Following are some examples-

1. Lichens, a relationship of fungi and algae (cyanobacteria or green algae).
2. Anthoceros is a bryophyte that is associated with Nostoc.
3. Azolla, a fern, is associated with Anabaena.
4. Cycas, a Gymnosperm, in association with Anabaena or Nostoc, blue green algae in its coralloid roots.

Importance of Biological Nitrogen fixation

All plants, especially forage crops, need a lot of nitrogen (N) for proper growth and development. Biological nitrogen fixation (BNF) is the process by which certain plants absorb atmospheric nitrogen gas (N2) into their tissues. This process is limited to a small number of plants. Among forage plants, legumes (plants in the Fabaceae family) are well known for their ability to absorb nitrogen from the atmosphere.

This process is crucial for the production of forage because it suggests that three sources could supply the essential nitrogen: the soil, fertilisers, and the atmosphere through BNF. Fertiliser costs can be reduced while retaining soil fertility, high levels of protein in forage, and high yields for forage growers that figure out how to get as much nitrogen from the atmosphere as possible with BNF. Some forage crops cannot absorb N_2 from the air into their tissues without a host plant, which is referred to as a microsymbiont. A symbiotic relationship is one in which two creatures learn to collaborate for their mutual benefit. A naturally occurring soil bacterium is the second organism found in the majority of forage crops. The most widely acknowledged bacterium linked to fodder crops is called rhizobia because it belongs to the bacterial genus *Rhizobium*.

The plant's roots become infected by these soil bacteria, resulting in the growth of nodules. The BNF process, which is a chemical reaction, takes place in the nodules. The process can be explained very simply using the following equation, even though it involves many complicated biological processes:

N2 + 8H2+ 16ATP ——> 2 NH3 + 2H2+ 16ADP + 16 Pi

As shown in the above equation, one molecule of nitrogen gas (N_2) combine with eight hydrogen ions (also referred as protons) to produce two molecules of ammonia ($2NH_3$) and two molecules of hydrogen gas (8H+) ($2H_2$).

This process is carried out by an enzyme known as nitrogenase. The 16 ATP molecules (ATP stands for adenosine triphosphate, a chemical that stores energy) show the energy required to complete the BNF process. 16 ATP is a significant quantity of plant energy in biochemical terms. Thus, the BNF process is "expensive" for the facility in terms of energy usage. Nitrogen is one of the amino acids that a plant can use to produce the proteins it needs for growth and development.

Benefits of Biological Nitrogen Fixation

Biological nitrogen fixation is important for Retaining soil fertility and minimising the use of synthetic fertilisers. Moreover, it is essential to the global nitrogen cycle because it affects the quantity of nitrogen available for plant development and, in addition, the productivity of aquatic and terrestrial ecosystems. A lot of interest has been focused on improving BNF during the

production of various legumes and forages in recent decades. In comparison to conventional N fertilisers, BNF provide a type of nitrogen (N) that may be more affordable and sustainable. The following points provide an outline and discussion of some of the major benefits:

1-Efficiency: The distribution and production of legume inoculants do not require a lot of energy. Application on the seed is easier as compared to adding fertilizers on the field. Legume tree crops may sustain itself over time with BNF.

2-Economics: Increased use of BNF can help producers generate greater profits by reducing production costs. Crop rotations utilising nitrogen-fixing crops may greatly reduce the amount of N fertiliser required for the next crops in the cycle. For example, corn can be planted in a rotation with alfalfa to produce beneficial yields while requiring less funds to buy and apply fertiliser.

It can be costly to use synthetic fertilisers, especially for small-scale farmers. Farmers can save money by using biological nitrogen fixation as a means of reducing their dependency on expensive fertilisers.

3-Environment: Water resource contamination from excess fertiliser leaching and runoff is prevented by using inoculants as an alternative to N fertiliser. A responsible approach to conserving natural resources includes the use of BNF. Heavily uses synthetic fertilization for N fixation, dangerous for the environment. On the other hand, Biological nitrogen fixation proves beneficial to both farmers and the environment. It is efficiently performed through N-fixing crop species and a specific microbial balance, which contributes to organic farming.

4-Sustainability: Applying BNF frequently can improve soil fertility and tilth, which in turn can improve sustainable food production. A more sustainable fertiliser substitute for synthetic fertilisers that are purchased is the usage of so-called green manure crops, according to certain producers. Green manure crops are crops grown mainly to be incorporated into the soil rather than for harvest. N levels and organic matter content in soil may rise over time if green manure crops are used to restore atmospheric N_2.

5-Enhanced Soil fertility: With the natural increase in nitrogen content, biological nitrogen fixation enhances soil fertility. As for non-legumes, nitrogen fixing bacteria inoculants are an efficient solution. They allow farmers to get the job done without artificial means. N-fixing legumes can help non-legume crops by improving soil fertility, permeability, and organic matter through practices like crop rotations, green manuring, and alley cropping. Legumes naturally form symbiotic interactions with bacteria that fix nitrogen. Examples of legumes include peas, lentils, and soybeans. Legumes can help in restoring the soil's nitrogen content when added to crop plants.

6-Increased soil health and biodiversity: The health of the soil is improved by nitrogen-fixing organisms. When they convert atmospheric nitrogen, organic matter is released, which enriches

the soil with compounds that are high in nutrients. This promotes the development of beneficial microbes and increases soil biodiversity overall, forming a durable and sustainable ecosystem.

7-Enhanced Nutrient cycling: Nitrogen fixation restores the available nitrogen in the soil, which promotes nutrient cycle. Nitrogen-fixing plants absorb atmospheric nitrogen in self-sufficient systems such as cover crops and intercropping. This nitrogen is then released during the plants' decomposition, providing the soil with essential nutrients for the next crop.

2.5 NITROGEN CYCLE

Biological nitrogen fixation plays an essential part in providing nitrogen for other forms of life on earth, since it contributes about 60% of the total N_2 fixed in the biogeochemical nitrogen cycle. Biologically available nitrogen is restored and reused during the metabolic processes of different species in the process called nitrogen cycle (Fig-2.5).

The nitrogen cycle is a biogeochemical process in which nitrogen is transferred from the atmosphere to living organisms and then back to the atmosphere in various forms. The nitrogen cycle is a cyclical process in which nitrogen moves from an inorganic form in the atmosphere to an organic form in living organisms. The atmosphere is the biggest reservoir of nitrogen, predominantly as nitrogen gas (N2). All living things require nitrogen since it is essential to plant growth. If there is insufficient nitrogen, plants will not thrive and provide low crop yields. If there is an excess of nitrogen, plants may become toxic. Nitrogen is an essential component of the nucleic acids DNA and RNA, which are the most significant biological molecules and are essential to all living beings. Plants cannot generate amino acids (substances that contain nitrogen and hydrogen that are found in many living cells, muscles, and tissue) if they do not receive adequate nitrogen. Plants cannot produce the specific proteins required by plant cells if amino acids are not present. Plant growth suffers when there is insufficient nitrogen. Nitrogen is the most crucial ingredient for plant growth that plants acquire from the soil. Green plants get nitrogen from soil solution in the form of ammonium, nitrate, and nitrite ions, but atmospheric nitrogen is the primary source of all these nitrogen molecules.

Nitrogen undergoes several changes during the nitrogen cycle. The nitrogen cycle consists of the following steps:

1- Nitrogen fixation
2-Ammonification
3-Nitrification
4-Nitrogen assimilation
5-Denitrification
6-Sedimentation
The details are as follows:

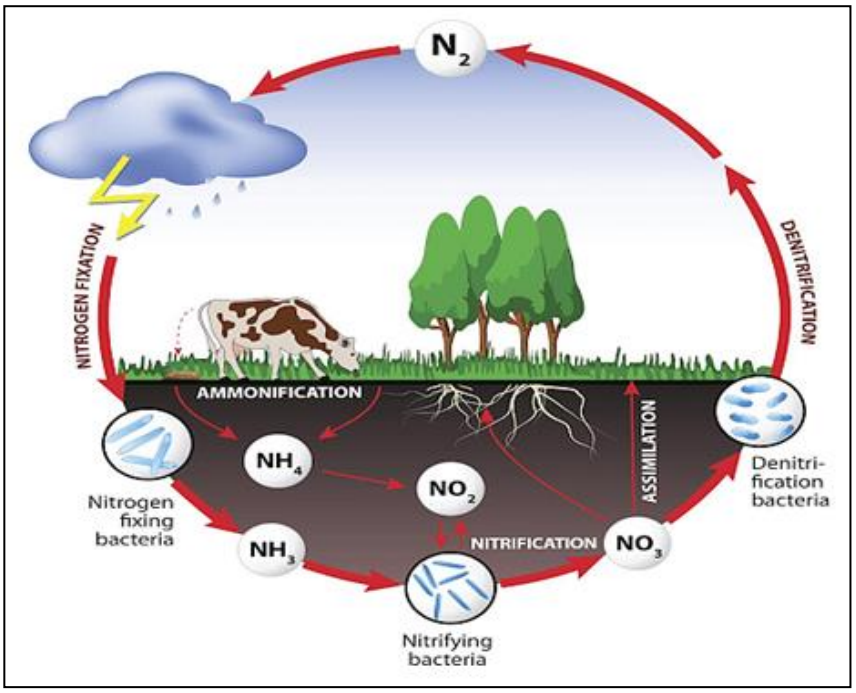


Fig.2.5: Nitrogen Cycle (Source: https://prepp.in/news/e-492-nitrogen-cycle-environment-notes)

(i) Nitrogen fixation: The majority of nitrogen enters ecosystems via certain bacteria in soil and plant roots that convert nitrogen gas into ammonia (NH_3). Nitrogen fixation is the conversion of free nitrogen from the environment into biologically suitable nitrogenous molecules. This is the first stage of the nitrogen cycle. The nitrogen cycle starts with this phase. The transformation of atmospheric N_2 into ammonia (NH3) defines this stage. In this process, bacteria like Azotobacter and Rhizobium play a significant role. They live in the roots of leguminous plants and contribute to the conversion of inert nitrogen to ammonia. This process is of two types:

(a) Physicochemical or non-biological fixation
(b) Biological nitrogen fixation

(a) Physicochemical or non-biological fixation: During the physicochemical process of nitrogen fixation, atmospheric nitrogen mixes with oxygen during lightning or electrical discharges in the clouds, forming nitrogen oxide, NO, and nitrogen dioxide, NO_2. These nitrogen forms are then washed into soils by rain or snow.

$$N_2 + 2(O) \xrightarrow[\text{Discharge}]{\text{Electric}} 2NO$$

$$2NO + 2(O) \longrightarrow 2NO_2$$

$$2NO_2 + (O) \longrightarrow N_2O_5$$

Nitrogen oxides dissolve in rainwater and react with mineral components to generate nitrates and other nitrogenous compounds when they reach the earth's surface:

$$N_2O_5 + H_2O \longrightarrow 2HNO_3$$

$$2HNO_3 + CaCO_2 \longrightarrow Ca(NO_3)_2 + CO_2 + H_2O$$

Nitrogenous chemicals are produced through various types of combustion and are rinsed down with rain water. Nitrogen and hydrogen combine at high pressure and temperature to form ammonia (industrial nitrogen fixation).

(b) Biological nitrogen fixation: Biological nitrogen fixation is the process by which nitrogen is fixed in plants with the help of anaerobic bacteria. Certain prokaryotes are responsible for it. Cyanobacteria (blue-green algae) fix huge amounts of nitrogen in the oceans, lakes, and soils. Symbiotic bacteria (Rhizobium) found in the root nodules of legumes and symbiotic cyanobacteria (Nostoc, Anabaena, etc.) found in the free state or in the thalli of Anthoceros (bryophytes), Azolla (water fern), coralloid roots of Cycas (gymnosperm) fix atmospheric nitrogen. Natural processes alone fix 95% of the total world nitrogen. They use nitrogenase, an enzyme, to catalyse the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). Plants may easily absorb NH3 and use it to make the aforementioned nitrogenous biomolecules.

Certain free living nitrogen fixing bacteria, such as Azotobacter, Clostridium, Beijerinckia, and others, fix atmospheric free nitrogen in the soil. Frankia, a nitrogen-fixing actinomycetous fungus found in the roots of higher plants such as Alnus and Casuarina, is another nitrogen-fixing actinomycetous fungus. Nitrogen-fixing organisms mix atmospheric nitrogen with hydrogen from the respiratory system to generate ammonia, which in turn interacts with organic acids to form amino acids.

(ii) Ammonification: This is another method of producing ammonia. Dead Organic remains of plants and animals are broken down in the soil by bacteria, releasing ammonia into the soil. These bacteria, particularly actinomycetes and bacilli (Bacillus ramosus, B. vulgaris, and B. mesenterilus), feed on dead and waste material and release ammonia into the soil. This ammonia production process is known as ammonification. Ammonia is already present in the soil due to nitrogen-fixing bacteria. Ammonification raises the concentration of ammonia in the ground. Bacteria (Pseudomonas, Bacillus, Clostridium, Serratia), fungus (Alternaria, Aspergillus, Mucor, Penicillium), and actinomycetes (Streptomyces) can convert organic nitrogen compounds to ammonia. Ammonification is essential because it provides nitrogen to the soil in a form that plants can use and distribute through the food chain. Ammonification is the most effective method of getting nitrogen for many types of plants that grow in acidic soils.

(iii) Nitrification: Nitrification is the process by which ammonia is transformed into nitrites and then into nitrates. Nitrification is a natural process in which ammonia (NH4+) is transformed to nitrites (NO2-) and then to nitrates (NO3-) by a group of specialised bacteria. Nitrosomonas are a type of specialised bacteria that convert ammonia to nitrates. This takes place in two steps:

(a) Conversion of Ammonia into Nitrites: The first phase involves converting NH3 (ammonia) to NO3- (nitrates). Nitrosomonas bacteria cause this. They oxidise soil ammonia and convert it to nitrites. The following chemical equation is used to represent the reaction:

$$2NH_4^+ + 2O_2 \longrightarrow NO_2^- + 2H_2O + \text{energy}$$

(b) **Conversion of Nitrites to Nitrates:** Several microorganisms, including Penicillium species, Nitrobacter, Nitrocystis, and others, convert nitrites to nitrates. Nitrocystis oceanus is a common marine autotroph that uses nitrification to generate energy. The chemical equation of the reaction is given below.

$$2NO^-_2 + O_2 \longrightarrow 2NO_3^- + \text{energy}$$

Some nitrates are also become available through weathering of nitrate-containing rocks.

(iv) Nitrogen assimilation: Green plants absorb inorganic nitrogen in the form of nitrates, nitrites, and ammonia, which is then transformed into nitrogenous organic molecules. Nitrates first convert to ammonia, which then reacts with organic acids to generate amino acids. Amino acids are necessary for the formation of proteins, enzymes, chlorophylls, nucleic acids, and other compounds. Plant proteins provide the nitrogen required by animals. Animals do not directly consume plant proteins. During digestion, they are broken down into amino acids, which are then absorbed and processed into animal proteins, nucleic acids, and so on. Plants can take nitrogen through their roots once it has been fixed in the soil. Assimilation is the term used to describe the process of absorption.
In the process of nitrogen assimilation, plants utilise the nitrates produced by soil bacteria to produce nucleotides, amino acids, and other essential molecules for life. Nitrates are absorbed by plants through their roots, where they are used for producing nucleic acids and amino acids. These amino acids and nucleic acids are then utilised by the animals that consume the plants in their own cells.

Plants obtain nitrogen molecules from the soil via their roots, which are available in the form of ammonia, nitrite ions, nitrate ions, or ammonium ions and are utilised to produce plant and animal proteins. Plants enter the food web in this way when they are consumed by primary consumers. It first becomes the nitrite ion, then the ammonium ion, which adds amino acids, nucleic acids, chlorophyll, and other compounds. Plants and rhizobia have a symbiotic relationship in which the plant provides nutrients to the bacteria and the bacteria provide nitrogen to the plant.

(v) Denitrification: Some nitrates are not absorbed by the plants. They are transformed into atmospheric nitrogen by pseudomonas and clostridium. It is a biological reduction process in which nitrogen molecules are released back into the environment in their gaseous state - nitrogen gas (N) - by the conversion of nitrate (NO3-). This is the final phase in which the nitrogen compounds in the soil return to the atmosphere. Denitrifying bacteria include Pseudomonas denitrificans, Bacillus licheniformis, Thiobacillus denitrificans, Hypomicrobium, and Chromobacterium etc.

$2NO^-_3 \longrightarrow 2NO^-_2 \longrightarrow 2NO \longrightarrow N_2O \longrightarrow N_2$

(vi) Sedimentation: Soil nitrates are washed down to the sea or leached deep into the earth with percolating water. Nitrates lost from the soil surface are thus trapped up in the rocks. This is called nitrogen sedimentation. Rock nitrogen is only released when it is exposed and weathered. As a result, a significant amount of nitrogen gets fixed and stored in plants, animals, and microorganisms. Nitrogen leaves the biological system in the same proportion that it enters from the atmosphere, and nitrogen input and output are balanced throughout the ecosystem.

2.6 SUMMARY

Nitrogen is an essential nutrient for the growth and development of all living things. Nitrogen cannot be taken directly from the atmosphere. Thus, all life forms require nitrogen fixation. Nitrogen fixation is a biological process of converting free nitrogen-both elemental and molecular into nitrogenous compounds so that plants can absorb it. Nitrogen fixation is of two types- Abiological nitrogen fixation and natural nitrogen fixation. Biological nitrogen fixation is the conversion of atmospheric nitrogen into ammonia and organic derivative by natural means especially such conversion, by microorganisms in the soil, into a form that can be assimilated by plants, by an enzyme called nitrogenise. Abiological fixation of nitrogen differs from biological fixation where nitrogen is reduced to ammonia without the involvement of any bio-catalyst or bacteria's. Biological nitrogen fixation may be categorised into non-symbiotic biological nitrogen fixation, associative biological nitrogen fixation and symbiotic biological nitrogen fixation. BNF provides a natural means of supplying nitrogen for plants. BNF is important for retaining soil fertility and minimising the use of synthetic fertilizers. Nitrogen cycle involves ammonification, nitrification, nitrogen fixation, nitrogen assimilation, denitrification and sedimentation.

2.7 GLOSSARY

Diazotrophs: Diazotrophs are organisms, such as bacteria and archaea that fix atmospheric nitrogen gas into a more usable form such as ammonia. These microbes are able to grow without external sources of fixed nitrogen.

Biological nitrogen fixation: The process by which certain anaerobic bacteria help plants in fixing nitrogen is known as "biological nitrogen fixation."

Nif genes: Genes responsible for N fixation are called Nif genes.

Prokaryotes: A prokaryotes is a single-cell organism whose cell lacks a nucleus and other membrane-bound organelles.

Catalyze: To speed up a process, particularly a biochemical or chemical reaction.

Nitrogenize: Nitrogenases are enzymes used by some organisms to fix atmospheric nitrogen gas (N_2).

Rhizobium: Rhizobium is a bacterium present in soil which helps in fixing nitrogen in leguminous plants.

Legume: Any plant belonging to the Fabaceae family, including its leaves, stems, and pods, is referred to as a legume.

Cytosol: It is the fluid in which the cell's organelles occur.

Tetraploid: Any organism that contains four copies of its chromosomes (symbolized as 4n) examples include- cotton, wheat and Brussel sprouts)

Forages: The edible parts of plants other than separated grain that used as fodder for grazing animals or can be harvested for feeding.

Clostridium: is a genus of anaerobic, Gram-positive bacteria.

Pseudomonas: is a gram-negative, rod-shaped bacterium with polar flagella.

Tilth: It refers to favourable physical properties of soil including proper drainage, water holding capacity, aeration and structure.

2.8 SELF ASSESSMENT QUESTION

2.8.1 Multiple Choice Questions:

1-Ammonification is the formation of-
(a) Ammonia from nitrates by decomposers
(b) Ammonia from nitrogen
(c) Ammonia from amino acids
(d) Ammonia from nitrates by nitrogen fixers

2-Process of conversion of soil nitrates (NO_3) to nitrogen (N_2) is called -
(a) Ammonification (b) Nitrification
(c) Nitrogenfixation (d) Denitrification

3- Conversion of nitrites to nitrates is called -
(a) Nitrosococcus (b) Clostridium
(c) Nitrobacter (d) Nitrosomonas

4- Conversion of ammonia to nitrite and then to nitrates is called
(a) Ammonification (b) Denitrification
(c) Assimilation (d) Nitrification

5-Plants cannot absorb molecular N2 in the atmosphere because
(a) N2 has double bonds making it highly stable
(b) Abundance in the atmosphere inhibits absorption
(c) N2 has triple bonds making it highly stable
(d) None of these

6-Industrial fixation is accomplished by
(a) Helmonts process (b) Haber-Bosch process
(c) Friedel-Crafts reaction (d) ReimerTiemann Reaction

7-Enzyme nitrogenise is
(a) Mo-Fe protein (b) Mo-Mn protein
(c) Mn-Fe protein (d) Cu-Fe protein

8-The root nodules of legumes contain a pink pigment which has high affinity for oxygen is-
(a) Nod haemoglobin (b) haemoglobin
(c) Bacterial haemoglobin (d) leghaemoglobin

9- Which from the following organism is capable of carrying out denitrification?
(a) Nitrosomonas (b) Pseudomonas
(c) Beijernickia (d) Nitrobacter

10- Which one of the following are symbiotic nitrogen-fixing bacteria?
(a) *Rhizobium trifolii* (b) *Escherichia coli*
(c) *Clostridium pasteurianum* (d) *Azotobacter* sp.

2.8.2 True and false

1-Enzymes for nitrogen fixation are found exclusively in eukaryotes.
2- Nitrogen-fixing process is done by bacteria in the presence of oxygen.
3- The bacteria responsible for biological nitrogen fixation are called **diazotrophs.**

4- Nitrosomonas are a type of specialised bacteria that convert ammonia to nitrates.
5- Biological nitrogen fixation is the process of converting free nitrogen into nitrogenous compounds without the support of any biological organism.

Answer Keys:

2.8.1: 1-(c), 2-(d), 3-(c), 4-(d), 5-(c), 6-(b), 7-(a), 8-(d), 9-(b), 10-(a)

2.8.2: 1-False, 2-False, 3-True, 4-True, 5-False

2.9 REFERENCES

- A handbook of organic farming by Arun.K.Sharma published by Agrobios (India), Jodhpur.
- A text book of Soil Science by R.K. Mehra published by Indian Council of Agricultural Research, Pusa, New Delhi.
- Handbook of Composite Organic Farming by Himadri Panda and Dharamvir Hota, published by Gene-Tech Books, New Delhi
- https://unacademy.com/content/cbse-class-11/study-material/biology/biological-nitrogen-fixation/
- https://www.geeksforgeeks.org/what-is-biological-nitrogen-fixation/
- https://testbook.com/ias-preparation/nitrogen-fixing-bacteria
- https://forages.oregonstate.edu>b..

2.10 SUGGESTED READINGS

- A handbook of organic farming by Arun.K.Sharma published by Agrobios (India), Jodhpur.
- A text book of Soil Science by R.K. Mehra published by Indian Council of Agricultural Research, Pusa, New Delhi.
- Handbook of Composite Organic Farming by Himadri Panda and Dharamvir Hota, published by Gene-Tech Books, New Delhi.

2.11 TERMINAL QUESTIONS

2.11.1 Short answer type Questions

1- Discuss in brief about Nitrogen fixation.
2- Differentiate between Non- Symbiotic and Symbiotic Biological Nitrogen Fixation.
3- Differentiate between Natural and Industrial Nitrogen fixation.
4- Write short notes on the following:
   (a) Associative Symbiotic Nitrogen Fixation
   (b) Ammonification
   (c) Nitrification

(d) Nitrogen assimilation
(e) Denitrification
(f) Sedimentation

2.11.2 Long answer type Questions

1- Explain in detail about Biological nitrogen fixation.
2- Define Nitrogen fixation. Describe in detail about the types of Nitrogen fixation.
3- Give a detail note about the importance of Biological nitrogen fixation.
4- Define Nitrogen cycle. Discuss the steps involved in nitrogen cycle.

UNIT-3 GENERAL ACCOUNT OF MICROBES COMMONLY USED AS BIOFERTILIZER

Contents

3.1 OBJECTIVES

After reading this unit the learners are able to

- Know about various microbes used as biofertilizer
- Know about algae used as biofertilizer
- Know about mychorrhiza used as biofertilizer

3.2 INTRODUCTION

Long-term farming generally results in agricultural land becoming deprived of nutrients if inputs are not properly supplemented. Under conventional farming systems, we need to use significant quantities of agrochemicals to augment the soil nutrient content, which in turn pollutes the ecosystem. Therefore, implementing a responsible and balanced use of organic agriculture is essential to making agriculture sustainable. Similar ideas, such as enhancing soil health and biodiversity to support plant growth throughout time, are outlined in the principles of organic farming.

Enhancing the nutrient supply and their availability in crop cultivation is a major function of biofertilizer. *Rhizobium* is the most thoroughly studied biofertilizer currently available. In comparison to external application, the nutrients fixed by the soil bacteria are more efficient. Considering that the atmosphere contains 78% nitrogen, it has been predicted that roughly 80,000 tonnes of nitrogen are available over a one hectare piece of land. A portion of this nitrogen is captured by the biofertilizers and fixed in the soil, which is beneficial for the plant. The use of biofertilizer improves soil nutrient availability in multiple ways since it contains a variety of beneficial bacteria.In addition to other organic manure applications, the use of biofertilizers during crop production contributes to the development of the natural qualities of the soil under organic farming. Certain microorganisms, such as fungi, algae and bacteria are used as biofertilizers. These have the ability to fix nitrogen from the atmosphere, dissolve additional and natural nutrients (such as phosphorus) in the soil and transform them into forms that plants can use. They are a cheap, sustainable, and environmentally friendly source of plant nutrients. They may be extremely important for preserving the long-term fertility and sustainability of the soil. In order to offer future generations a healthy future, biofertilizers are essential.

Biofertilizer is a substance that includes living microorganisms that, when added to soil, plant surfaces or seeds encourages development by making more primary nutrients available to the hos plant. Through the natural processes of nitrogen fixation, phosphorus solubilization and growth-promoting substance synthesis, it adds nutrients to the soil and promotes plant development.

As we know "Biofertilizer" is made up of microorganisms that aid in the growth of trees and plants by providing them with more vital nutrients. It is made up of living things, such as bacteria, mycorrhizal fungus and blue-green algae. While cyanobacteria are distinguished by

their ability to fix nitrogen, mycorrhizal fungi extract minerals from organic matter in a manner that is preferred by the plant.

3.3 MICROBES AS BIOFERTILIZERS

The live microorganisms found inside plant sections or the root zone are known as biofertilizers. These microorganisms are also known as plant growth-promoting rhizobacteria because they either directly or indirectly enhance the physiological characteristics, growth and production of plants. By increasing the availability of mineral nutrients, biologically fixing nitrogen, solubilizing phosphorus and producing growth hormones, biofertilizers help plants grow and develop more. Furthermore, these microorganisms and their byproducts are seen to be the greatest substitute for synthetic fertilisers because they are organic, environmentally friendly agro-inputs that improve soil health and sustainability. They break down more quickly, are effective in very small amounts, and are less likely to cause infections and other pests to develop resistance. They can be categorised in different ways based on their nature and function.

Biofertilizers that contain nitrogen aid in adjusting the soil's nitrogen content. Because plants require a specific amount of nitrogen in the soil to grow properly, nitrogen is a factor that limits the growth of plants. The best nitrogen biofertilizer to employ will depend on the crop being grown, as different biofertilizers work best in different types of soil. For legume crops, *Rhizobium* is utilised; for non-legume crops, *Azotobacter* or *Azospirillum*; for sugarcane blue-green algae; and *Azolla* for low land rice paddies.

3.3.1 *Rhizobium*

Legumes (Fabaceae) grow better when exposed to a specific kind of soil bacteria called *Rhizobia*. Without the ability to fix nitrogen on their own, *Rhizobia* are motile, gram-negative, non-sporulating rods that express genes involved in nitrogen fixation. The ability of R*hizobia* to form nodules and reside in the soil or within the root hairs of legumes makes them amazing bacteria. *Rhizobia* and legumes come into contact during the germination of legume seeds in the soil. If the two are compatible, a complicated procedure begins that permits the *Rhizobia* to penetrate the plant's root hairs. The plant produces a root nodule close to the entrance. The nodules filled with pink sap (leghaemoglobin pigment) are called the effective nodules. This pigment maintains the rhythm of oxygen supply to the bacteria and helps the activity of nitrogenase enzyme. The nitrogenase is responsible for reduction of nitrogen to ammonia in the process of nitrogen fixation.

This bacterium was first identified in 1888 from legume nodules by the Dutchman Beijirinck. Subsequently, the bacterium was identified as *Rhizobium* in Bergey's Manual of Determinative Bacteriology. Frank came up with the name *Rhizobium* in 1889. Seven unique species make up this genus according to the "Cross Inoculation Group Concept". Thus far, over twenty cross-inoculation groups have been formed. Some of them are given in Table 3.2.

Table 3.2: *Rhizobium* Cross Inoculation Groups

Rhizobium Spp.	**Cross Inoculation Grouping**	**Legume types**
R. leguminosarum	Pea group	*Pisum, Visia, Lens*
R. phaseoli	Bean group	*Phaseolus*
R. trifolii	Clover group	*Trifolium*
R. meliloti	Alfalfa group	*Melilotus, Medicago, Trigonella*
R. lupine	Lupine group	*Lupinus, Orinthopus*
R. japonicum	Soybean group	Glycine
Rhizobium spp.	Cowpea group	*Vigna, Arachis*

There is a new *Rhizobium* classification now in place. This group of *Rhizobia* is referred to as "slow growing" *Bradyrhizobium*, while *Rhizobium* is the name of the "fast growing" *Rhizobia*. Nevertheless, because germs from one group can spread to another, this classification is still not completely distinct. The concept known as "the principle of cross inoculation" is predicated on the idea that another type of bacterium that forms nodules may nodulate legumes within a certain infection group.

Rhizobia are soil bacteria, as you may know. They are able to fix nitrogen in the atmosphere. They form symbiotic relationships with legumes, including some non legumes such as *Parasponia*. Root hairs allow *Rhizobium* bacteria to access the roots. They develop nodules and emit specific stimulatory root exudates (as seen in the figure 3.1). *Rhizobia* enter the enlarged cortical cells inside the root and undergo a differentiation into "bacteroids" that fix nitrogen. When the bacteria and plant are living apart, neither can fix nitrogen. The term "effective nodules" refers to nodules that contain pink sap, or the pigment leghaemoglobin. This pigment supports the action of the nitrogenase enzyme and keeps the oxygen supply to the bacteria in sync. In the nitrogen fixation process, nitrogen is reduced to ammonia by the enzyme nitrogenase. The details of their species have given in the (Table 3.3).

Table 3.3: Classification of *Rhizobium* Biofertilizers (Source: Jordon, 1984)

***Rhizobium* Species**	**Principal Plant Inoculated**
Rhizobium leguminosarum	
Biovar phaseoli	*Phaseolus* (Bean)
Biovar viceae	*Vicea* (Vetch)
Biovar trifolii	*Trifolium* (Berseem)
Rhizobium meliloti	*Melilotus* (Senji)
	Trigonella (Fenugreek)
	Medicago (Lucerne)
Rhizobium loti	Lotus(Trefoils)
Bradyrhizobium japonicum	*Glycine* (Soybean)
Bradyrhizobium species	*Lupinus* (Lupin), *Vigna* (Cowpea), *Cicer* (Gram)

The family Rhizobiaceae now includes two more genera. The plants that are nodulating the soybean and daincha (*Sesbania*) are called *Sinorhizobium* and *Azorhizobium*, respectively. It was possible to extract *Azorhizobium caulinodans* from *Sesbania rostrata* stem nodules. Moreover, this can colonise rice roots and leave behind nodules. It has also been discovered that *A. caulinodans* responds to maize. In its free-living stage, it can also fix nitrogen at a high rate.

Fig. 3.1: *Root nodulation in Soybean*

3.3.2 BLUE GREEN ALGAE

Blue green algae (BGA) also known as cyanobacteria form non-fibrous slimy growth on the ground, and on the surface of soil and water. Under a microscope, blue-green algae can be observed as single cells, huge numbers of cells called colonies, or strings of cells called trichomes. Certain accumulations might be big enough to be visible to the unaided eye. Other names for blue green algae include cyanophytes, cyanobacteria and most recently, cyanoprokaryotes. Their exterior appearance is similar to that of algae, and they have comparable needs in terms of light, nutrients, and carbon dioxide. Tiny gas vesicles in the cells of some blue-green algae species control whether the algae float to the water's surface or sink to the bottom in response to changes in light and nutrition availability.

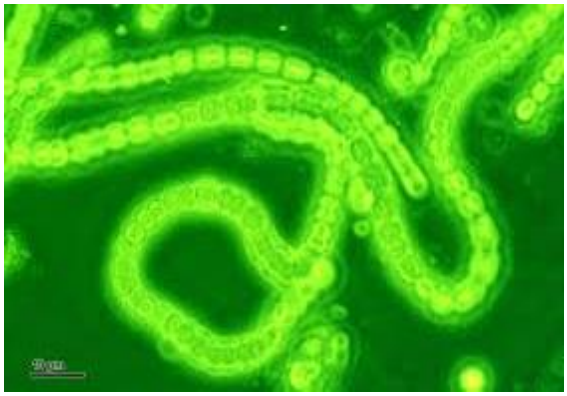


Fig 3.2: Cyanobacteria

They have striking similarities with bacteria except they have oxygen evolving photosynthesis. They are cosmopolitan in distribution and have ability to survive in extreme climatic conditions. They predominantly multiply vegetatively through fragmentation. A sexual reproduction is brought about by formation of exospores, endospores and akinetes. None of the BGA has sexual reproduction and form motile stages. Unlike the green algae, their food reserve is glycogen, which does not react with iodine to form violet colour. They belong to 8 different families, phototrophic in nature and produce auxin, indole-acetic acid and gibberellic acid, fix 20-30 kg N/ha in wet land rice fields. Application of BGA increases paddy yields by 15-20%. The species of blue-green algae which are known to fix atmospheric nitrogen are classified into 3 groups

(i) Heterocystous- aerobic forms

(ii) Aerobic unicellular forms and
(iii) Non- heterocystous filamentous, microaerophilic forms.

The composite inoculation of blue green algae may consist of the cultures viz. *Nostoc*, *Anabaena*, *Calothrix*, *Tolypotrix*, *Plectonema*, *Gloeocapasa*, *Oscillatoria*, *Clindrospermum*, *Aulosira* and *Scytonema*. The term algal fertilizer was coined in early 60s to embody such blue green algae which have the capacity to metabolize molecular nitrogen and bring about an addition to the nitrogen level of soil.

In a symbiotic partnership with the aquatic fern *Azolla*, the blue-green algae *Anabaena azollae* fixes atmospheric nitrogen. The *Azolla* that lives in a ventral pore in each vegetative leaf's dorsal lobe is linked to BGA. The endophyte inhabits the water fern's tissue and fixes atmospheric nitrogen. We can also use *Azolla*, BGA on paddy fields. In a flooded field of rice, BGA have the ability to both fix atmospheric nitrogen and carry out photosynthetic activities. *Azolla* is a water fern that grows quickly in just one week, it can quadruple in weight. *Azolla* also contains rich organic manure. It quickly turns soil nitrogen into a mineral that the crop can use very quickly. Apart from fixing atmospheric nitrogen, BGA also synthesises and releases certain molecules that promote growth, such as amino compounds and auxin, which in turn encourage the growth of rice plants. After a week of transplanting, the inoculant can be dispersed at a pace of around 10 kg algal cultures in order to multiply the algae in the paddy field.

3.3.3 *Azospirillum*

The alphaproteobacterium *Azospirillum* is an associative symbiotic bacteria that forms associations by residing in the rhizosphere rather than forming nodules. *Azospirillum* species form associations with a wide range of plants, especially C_4 plants like vegetables, rice, cereals, millets and forage grasses. A nitrogen-fixing bacteria was first described by Beijerinck in 1925 under the name *Spirillum lipoferum*. Tarrand et al. (1978) subsequently renamed this organism as *Azospirillum* (Nitrogen fixing *Spirillum*). It is well known that *Azospirillum* is a dominant soil bacterium. Additionally, *Azospirillum* and higher plants have a close associative relationship. Although the bacteria do not form any outgrowths or apparent nodules on the root tissue, they reside on the surface of the roots and occasionally even enter the root tissues.

The nitrogen they fix is between 10 and 40 kg/ha. By reducing the need for nitrogenous fertilizers by 25–30%, the *Azospirillum* inoculation aids in the improved vegetative growth of plants. There are now just four recognised species of *Azospirillum*. The species in question are *A. lipoferum, A. brasilense, A. amazonense,* and *A. iraquense*. Both *A. lipoferum* and *A. brasilense* are relatively widespread in Indian soils. More yields of several vegetable crops have been obtained with *Azospirillum* inoculation. (A.K. Sharma 2001); Subbiah (1994) observed that applying *Azospirillum* at a rate of 2 kg/ha increases the yield and nutrient content of bellary onions and chillies.

From various plant roots and soils, nearly 19 species were identified and separated; of these, *Azospirillum lipoferum* and *A. brasilense* were thoroughly investigated for use in agricultural crops. In addition to fixing nitrogen, a number of strains are also capable of producing plant growth hormone, solubilizing phosphorus, mitigating drought through the action of ACC deaminase, sequestering iron through siderophores, and induced systemic resistance in order to guard against diseases.

3.3.4 *Acetobactor*

Recently, the nitrogen-fixing bacterium *Acetobactor diazotrophicus* was discovered and connected to the sugarcane crop. The proteobacteria alpha group includes this bacterium. Sugarcane leaf, root, bud, and stem samples were used to extract it. *Acetobactor* is present in xylem vessels to a lesser degree and in the apoplastic fluid of sugarcane stems. This particular kind of bacterium is resistant to acid, high salt, and sugar and has the ability to fix up to 200 kg of nitrogen per hectare. Under field conditions, sugarcane yielded more after being inoculated.

3.3.5 *Frankia*

The genus *Frankia* contains nitrogen-fixing mycelial bacteria (actinomycetes) that coexist symbiotically with non-leguminous plants. It infects and nodulate almost eight families of mostly woody plants which are known as actinorhizal plants (Benson and Silvester, 1993). It creates root nodules in *Alnus* and *Casuarina*. Growing *Casuarina* will increase the fertility of the soil in desolate areas. When soils lack nitrogen, *Frankia* makes *Casuarina* trees appropriate for agro-forestry systems. *Frankia* first appears as a tiny lateral swelling on roots during nodulation, and as it develops, it forms new lobes at the apices of the roots, which eventually form a cluster coralloid structure. The inoculation of *Casuarina* and *Alnus* plants results in an increase in growth, nodulation, nodule dry weight and nitrogenase activity.

There is non-leguminous symbiosis present. Nitrogen-fixing bacteria that are not *Rhizobia* are known to be present in the root nodules of about 120 species, most of which are trees and shrubs. The causative organism is called *Frankia* and it is known that an actinomycete forms associations with non-leguminous plants such as *Alnus, Casuarina, Ceanothus, Eleagnus, Myrica, Hippophae, Purshia* etc. Due to a lack of knowledge about their potential, non-leguminous nitrogen fixing systems have a limited agricultural significance. Depending on the type of soil, the environment, and the age of the plants, these Angiosperms can acquire quite different amounts of nitrogen.

3.3.6 *Azotobactor*

Alkaline and neutral soils contain heterotrophic free-living nitrogen-fixing bacteria called *Azotobactor*. *Azotobactor chrococcum* is the species that grows most frequently in India's fertile soils. In addition to fixing atmospheric nitrogen in soils, it can also synthesise compounds that

promote growth, such as auxins and gibberellins, and to a lesser extent, vitamins. Numerous *Azotobactor* strains have the ability to kill specific fungal species. Rice, maize, cotton, sugarcane, pearl millet, vegetables, and certain plantation crops have all shown signs of *Azotobactor* response. In uncultivated areas, its population is quite low. The ability of the soil to fix nitrogen and multiply is enhanced by the presence of organic materials.

Field studies on *Azotobacter* have shown that this is appropriate for inoculation under various ago-climatic circumstances with crop plants such as onion, brinjal, tomato and cabbage seeds or seedlings. Under typical field circumstances, *Azotobacter* inoculation reduces the need for nitrogenous fertilizers by 10 to 20%. Part of the mild advantages is attributed to *Azotobacter*. The heaviest breathing bacteria, *Azotobacter*, needs a lot of organic carbon to develop. It is a weak competitor for soil nutrients. It can help crops by fixing nitrogen and releasing chemicals that promote growth and inhibit fungus.

In soils with low levels of organic matter, *Azotobacter* is less effective. Even in alkaline soils, it grows well. Plant growth and seed germination are both enhanced by it. The majority of crops are grown with the use of *Azotobactor*, which are free-living, chemo heterotrophic bacteria that fix nitrogen. *Azotobacter* use contributes to 10–20 kg N/ha of nitrogen savings. It generates compounds that encourage growth, which enhances the germination of seeds and the development of longer root systems. It creates polysaccharides that enhance the aggregation of soil. Near the roots of agricultural plants, *Azotobacter* inhibits the growth of pathogenic and saprophytic microorganisms. In western countries where farmers heavily apply chemical fertilisers, *Azotobacter* technology has not been well embraced.

On the other hand, the advantages of using *Azotobacter* have been documented in a nation like India, where the use of artificial fertilizers in rainfed agricultural areas is minimal and in irrigated areas is far less than in wealthy nations. It will be of enormous benefit to small and marginal farmers to save 10–20 kg of nitrogen per hectare as chemical fertiliser. *Azotobacter* will be of great relief and aid in the maintenance of healthier plant populations and growth. *Azotobacter* contributes to the higher growth, population, and productivity of crops such as cotton, sorghum, mustard, and maize, as well as vegetable crops (tomato, potato, etc.). Often, yield improvements in cash crops range from 10 to 20%, whereas in cereals they are often between 15 and 30%.

3.3.7 Mycorrhiza

A fungus and the root of a higher plant form a mutually beneficial or symbiotic relationship known as a mycorrhiza. *Glomus* species are the most prevalent fungus partners of mycorrhiza. The surface of mycorrhizal roots exhibits a hairy growth of fungal hyphae that can be sparse or dense. There is no root hair or root cap. Potential biofertilizer mycorrhiza can mobilise trace elements including P, Fe, Zn, and B. For crops that require an extended growing season, it provides moisture from far sources. There are two types of mycorrhizae: ectomycorrhiza and endomycorrhiza, depending on where the fungus is located.

(a) Ectotrophic Mycorrhiza, (Ectomycorrhiza)

Over the surface of the root, the fungus grows a mantle. It is located inside the cortex's intercellular gaps. The fungal hyphae are fed by the sugars and other food elements secreted by the root cells into the intercellular gaps. The root's surface is increased by several times due to the exposed fungal hyphae. They serve several purposes for the plants such as (i) absorption of water, (ii) The soil humus containing organic matter is dissolved, inorganic nutrients are released, absorbed, and transferred to the roots; (iii) Minerals are directly absorbed from the soil across a broad surface area and given to the roots. Plants that have ectomycorrhiza are known to absorb nitrogen, phosphate, potassium, and calcium two to three times more. (iv)The fungus secretes antimicrobial compounds that shield the immature roots from disease attack. Trees including eucalyptus, peach, pine, oak (*Quercus*) and others have ectomycorrhiza. In most cases, the fungal companion is specific. It belongs to Basidiomycetes.

(b) *Endomycorrhiza (Endotrophic Mycorrhiza)*

On the root surface, there are less fungal hyphae. The rest of the fungi reside in the cortex of the root, primarily in the intercellular gaps with some hyphal ends running into the cortical cells in grasses, crop plants, orchids and certain woody plants. Fungal hyphae also nourish orchid seedlings during this period by forming nutrient-rich cells known as pelotons. Since cortical cells do not secrete sugars into the intercellular spaces like ectomycorrhiza do, intracellular growth is necessary to acquire food.

The fungus known as Vesicular Arbuscular Mycorrhizal (VAM) have unique structures called vesicles and arbusculars. When they establish themselves on the root system, VAM fungi function as an expanded root system. They are intercellular, obligatory endosymbionts. In addition to gathering moisture from remote and deeper soil pockets, they also gather and supply the host plants with a variety of micronutrients. By making phosphorus more mobile and more readily available, VAM helps with phosphorus nutrition. Obligate symbionts, VAM enhance H_2O, Zn, Co, and P uptake. Only transplanted crops and perennial crops can use it on a wide basis. Many plants, can create a mycorrhizal relationship with a single fungus e.g., Glomus. The various forms of biofertilizers are products of naturally occurring helpful bacteria. All plants, animals, and people can safely use them. They help save chemical inputs and are good for crops and natural nutrient cycles. They are also environmentally friendly.

3.4 SUMMARY

Any substance that contains one or more of the essential elements—nitrogen, phosphorus, potassium, sulphur, calcium, magnesium, iron, manganese, molybdenum, copper, boron, zinc, chlorine, sodium, cobalt, vanadium, and silicon is generally referred to as a "fertiliser." In order to increase the land's fertility, fertilisers are applied. Over the past 20 years, the word

"biofertilizer" has been defined in a variety of ways due to advances in our understanding of the interactions that take place between rhizosphere bacteria and plants. When applied to soils, seeds, or plant surfaces, biofertilizers are compounds that include living microorganisms that colonise the rhizosphere or the interior of the plants and encourage development by boosting the supply or availability of primary nutrients to the target crops.

As per Vessey's definition, biofertilizer refers to a material that possesses living microorganisms that when applied on soil, plant surfaces, or seeds, invade the plant's interior or rhizosphere and stimulate growth by augmenting the host plant's availability or supply of primary nutrients. "A product that contains living microorganisms, which exert direct or indirect beneficial effects on plant growth and crop yield through different mechanisms" was the definition of biofertilizer in 2005. Since the bacteria were employed to manage plant infections, the term was expanded. However, microorganisms that control hazardous organisms to encourage plant growth such as biofungicides, bionematocides, bioinsecticides, or any other items with similar activities that support plant health are more commonly classified as biopesticides than as biofertilizers.

Additionally, biofertilizers are living, biologically active products or microbial inoculants of bacteria, algae, and fungi that can be added singly or in combination to the soil to enrich it with nutrients like organic matter, nitrogen, and phosphorus. Biofertilizers work as a substance that improves the soil's nutrient quality by utilising microorganisms that coexist freely with plants.

3.5 GLOSSARY

Azolla: A group of aquatic ferns capable of fixing high level of nitrogen from atmosphere and are widely grown as a fertilizer crop in low land rice cultivaiion system.

Azospirillum: Nitrogen fixing root and soil inhabiting bacterium in tropics.

Azotobactor: An aerobic, non symbiotic nitrogen fixing bacteria.

Biofertilizer: Biofertilizers are substances that are enriched with bacteria that aid in the growth of trees and plants by providing them with more essential nutrients.

Blue Green Algae (BGA): A heterogeneous group of prokaryotic photosynthetic nitrogen fixing organisms which contain chlorophyll 'a'. They are obligate phototrophs and store cyanophycean starch.

Gram negative: Gram-negative bacteria are bacteria that unlike gram-positive bacteria do not retain the crystal violet stain used in the Gram staining method of bacterial differentiation.

Inoculation: the action of immunizing someone against a disease by introducing infective material, microorganisms, or vaccine into the body.

Legumes: Legumes are plants in the family Fabaceae (or Leguminosae), or the fruit or seeds of such plants.

Mychorrizae: mycorrhizae are fungi that have a symbiotic relationship with the roots of many plants. The fungi which commonly form mycorrhizal relationships with plants are ubiquitous in the soil.

Nitrogen fixation: Conversion of dinitrogen gas (N,) in to a combined form (e.g. NH,, NH,).

Nitrogenase: Enzyme concerned with conversion of molecular nitrogen in to ammonia.

Nodules: Root nodules are the knob-like structures formed especially on and from roots of leguminous plants, as a result of symbiotic infection by nitrogen-fixing bacteria such as Rhizobium.

Nutrient: Substance taken by a cell from its environment and used in catabolic or anabolic reactions.

Obligate: Essential or necessary

Obligate symbionts: These are mutualists that tend to have a nutritional function and typically occur in insects that feed on imbalanced diets such as plant saps or cellulose

Phototrophic: (of an organism) obtaining energy from sunlight to synthesize organic compounds for nutrition.

3.6 SELF ASSESSMENT QUESTION

3.6.1Multiple Choice Questions

1. Which of the following cyanobacteria can fix atmospheric nitrogen?
   a) Nostoc b) Anabaena
   c) Oscillatoria d) All of the above

2. Which of the following fern is a biofertilizer?
   a) *Salvinia* b) *Pteridium*
   c) *Azolla* d) *Marsilea*

3. Which of the following is not used as a biofertiliser?
   a) Bacteria b) Algae
   c) Cyanobacteria d) Fungi

4. What is the full form of VAM?
   a) Vesicular-arbuscular mycorrhizae b) Venom Azolla mycorrhiza
   c) Venom-arbuscular mycorrhizae d) Vesicular-azollae mycorrhizae

5. The symbiotic association between fungi and roots of higher plants is called
   a) Lichen b) Mychorrhiza
   c) Biofertilizeer d) BOD

6. Which of the following serve as biofertilizer in paddy feilds?
   a) Bacteria b) Yeast
   c) Cyanobacteria d) Fungi

7. Which of the following cannot be a biofertilizer
   a) Cyanobacteria b) Fungi
   c) Virus d) bacteria

8. In mychorrhiza association the fungal symbiont help in
   a) Phosphorus nutrition b) Resistance to root borne pathogen
   c) Tolerance to salinity and drought d) All of the above

9. Which of the following is not a biofertilizer?
   a) Rhizobium b) Nostoc
   c) Mycorrhiza d) Agrobacterium

10. An organism used as a biofertilizer for raising soybean crop is
   a) *Azospirillum* b) *Rhizobium*
   c) *Nostoc* d) *Azotobactor*

Answer Key:

3.6.1: 1-(d), 2-(c), 3(d), 4-(a), 5-(b), 6-(c), 7-(c), 8-(d), 9-(d), 10-(b).

3.7 REFERENCES

- file:///C:/Users/User/Downloads/Azospirillum%20Liquid%20Biofertilizer%20for%20Major%20crops%20of%20Tamil%20Nadu.pdf
- https://www.ctahr.hawaii.edu/bnf/Downloads/Training/BNF%20technology/Rhizobia.PDF
- https://www.sanfoundry.com/biology-questions-answers-microbes-biofertilizers/
- https://www.bio-fit.eu/upload/Bio-Fit-Book/EN/Bio-FIT_Book_EN.pdf
- https://epgp.inflibnet.ac.in/epgpdata/uploads/epgp_content/S000014ER/P000284/M025288/ET/1512044777Paper15_Module27__etext.pdf
- https://en.wikipedia.org/wiki/Biofertilizer
- https://egyankosh.ac.in/bitstream/123456789/8896/1/Unit-3.pdf

- Vessey, J. Kevin (2003). "Plant growth promoting rhizobacteria as biofertilizers". Plant and Soil. 255 (2): 571–586. doi:10.1023/A:1026037216893. S2CID 37031212
- Jordon, D.C.11984) in Bergey 's Manual of ~ ~ s t e m a i i cBacteriology, Vol. 1 (eds)J.G. Holt and N.R. Krieg): 234, Williams and Wilkins

3.8 SUGGESTED READINGS

- https://www.bio-fit.eu/upload/Bio-Fit-Book/EN/Bio-FIT_Book_EN.pdf
- https://epgp.inflibnet.ac.in/epgpdata/uploads/epgp_content/S000014ER/P000284/M025288/ET/1512044777Paper15_Module27__etext.pdf

3.9 TERMINAL QUESTIONS

3.9.1 Short answer type Questions

1. Write a note in nitrogen fixing biofertilzer.
2. Write briefly on mycorrhizae.
3. Write a short note on *Rhizobium* as biofertilizer.
4. Write briefly about BGA (*cyanobacteria*) as biofertilizer.

3.9.2 Long answer type Questions

1. Write an essay on microorganisms that are used as biofertilizers.
2. Write a detailed note on mycorrhizae as bioferlilizer.

UNIT-04 *RHIZOBIUM*: ISOLATION, IDENTIFICATION AND MASS MULTIPLICATION

Contents

4.1 OBJECTIVES

After reading this unit you would be able to:

- To know about the *Rhizobium*.
- Define the isolation, identification, mass multiplication of *Rhizobium*
- Discuss about the carrier based inoculants and actinorrhizal symbiosis.

4.2 INTRODUCTION

Rhizobium plays a significant role in agricultural ecosystem services by forming symbiotic relationships with various kinds of leguminous plants, which facilitates nitrogen fixation through biological means. Around 80% of the biologically fixed nitrogen in agriculture comes from symbiotic relationships between ruminant plants and bacteria from the Rhizobiaceae family. There are currently six genera in the family Rhizobiaceae like *Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium,* and *Bradyrhizobium*, which are generally referred to as Rhizobia (Vance, 1998).

Rhizobia are gram negative, motile rod shaped bacteria that live in symbiotic partnerships with legumes, forming nodules on their roots or stems and fixing atmospheric nitrogen (Quatrini et al. 2002). This mutually beneficial relationship reduces the requirement for nitrogenous fertilizers when leguminous crops are growing and enriches the soil with nitrogen. Its function in fixing nitrogen was initially identified in 1888 in the legume root nodule (Hirsch et al., 2001). They are motile nature and they can travel through the water films around the soil particles in moist soil (Hamdi, 1971).

Legumes' symbiotic nitrogen fixation process is typically the main way to add nitrogen to soil for fertility goals. Some rhizobial inoculants have been applied to enhance mineral uptake, boost phytohormone production, and prevent the negative effects of metals. These activities enhance the sustainability and soil quality while also gradually promoting plant growth and development. Therefore, the study of *Rhizobium* and its relationship with leguminous plants is a topic of major interest to agricultural scientists and researchers. Improved agricultural techniques, such as inoculating crops with particular strains of *Rhizobium* to increase nitrogen fixation and crop output, can be developed by understanding the mechanisms of symbiosis and nitrogen fixation. Thus, in this unit we will highlight and cover the isolation, identification, mass multiplication of *Rhizobium*. We will also give emphasis to carrier based inoculants and actinorrhizal symbiosis.

4.3 RHIZOBIUM

Rhizobium is a symbiotic diazotrophs. It is rod shaped non-spore forming, a Gram (-) soil bacteria known as is essential to the fixation of nitrogen in the roots of leguminous plants. Endo-symbiotic association with legumes, invade legume root through root hairs and to form efficient

red colored LHb (Leghemoglobin) in the root nodules and fix atmospheric nitrogen. Leguminous plants like peas, beans, cowpeas, and soybeans have a symbiotic connection with *Rhizobium* bacteria that is vital to both the nitrogen cycle and sustainable agriculture (Fig. 4.1).

This bacterium was first identified in 1888 from legume nodules by the 'Dutchman Beijirinck'. Subsequently, the bacteria were identified as *Rhizobium* in Bergey's Manual of Determinative Bacteriology. Frank came up with the name *Rhizobium* in 1889.

Fig. 4.1: Root nodules in leguminous plant

Rhizobium is a Gram-negative soil-dwelling bacteria that may colonies the roots of legume plants and symbiotically fix atmospheric nitrogen.

The bacteria of the genus *Rhizobium* belongs to family Rhizobiaceae are a genetically diverse and physiologically heterogeneous group of microorganisms that despite this belong to the same class since they can nodulate groups of Leguminosae plants. This classification system is commonly known as "cross-inoculation" grouping. A cross-inoculation group is a group of legumes in which one species of *Rhizobium* nodulates all the legumes within that group. According to the former system, *Rhizobium* species are classified into two groups according to the traits of their growth.

Group I: The *Rhizobium* spp of Group I are the fast-growing acid producers which develop considerable turbidity in liquid media within 2-3 days and have a mean doubling time of 2-4 hours. The cells are rod-shaped to pleomorphic, 0.5 to 0.9 microns in diameter and 1.2 to 3.0 microns long, and are motile by 2-6 peritrichous flagella. A variety of carbohydrates can support their growth, while glucose, mannitol, and sucrose are usually the best alternatives. Members of this group normally infect temperate legumes with their Rhizobia.

Group II: The rhizobia that grow slowly and produce alkali are falls under group II. They take three to five days to create moderate turbidity in liquid media, and their mean doubling time is six to seven hours. The majority of these strains grow most effectively when fed pentoses as a carbon source. The cells are primarily rod-shaped and have a single polar or subpolar flagellum that makes them motile. Trophic legume species are nodulated by this group.

Seven unique species make up this genus according to the "Cross Inoculation Group Concept". Thus far, over twenty cross-inoculation groups have been formed. Out of this, only seven are well-known as listed in Table 4.1.

Table 4.1: *Rhizobium* with their Cross Inoculation Groups and legume types

S.N.	Name of *Rhizobium* Spp	Cross Inoculation Grouping	Legume Types
A	**Group-I**		
1	*R. leguminosarum*	Pea group	*Vicia* spp, *Pisum* spp, Visia, *Lens culinaris*
2	*R. phaseoli*	Bean group	*Phaseolus vulgaris, P. coccineus*
3	*R. trifolii*	Clover group	*Trifolium subterraneum, T. Semipilosum, T. repens,* and Other *Trifolium* spp
4	*R. meliloti*	Alfalfa group	*Melilotus* spp, *Medicago* spp, *Trigonella* spp
B	**Group-II**		
5	*R. lupine*	Lupine group	*Lupinus* spp, *Orinthopus* spp
6	*R. japonicum*	Soybean group	*Glycine* spp
7	*Rhizobium* spp	Cowpea group	*Vigna* spp, *Arachis* spp, *Desmodium* spp., *Macroptilium* spp., *Lablab* sp., etc

4.3.1 *Rhizobium* - Legume Symbiosis

You are aware that, rhizobia are soil bacteria. They have an ability to fix nitrogen from the atmosphere. They form a symbiotic association with legumes and some non-legumes like *Parasponia, Casuarina* etc. This is symbiotic association between plant and bacteria, initiated when bacteria in the soil attach to root hairs. This highly attachment process is mediated by plant proteins, the lectins that bind the bacteria to the surface of the root hairs and then penetrated by the microbes. Inside the root, *rhizobia* invade expanded cells of cortex, and then differentiate into nitrogen-fixing "bacteroids". The infected root cells divide and form a nitrogen fixing nodule which provides the anaerobic environment necessary for nitrogen fixation. Neither the plant nor *the* bacteria can fix nitrogen when live separately. The nodules filled with pink sap (leghaemoglobin pigment) are called the effective nodules (Fig. 4.2). This pigment maintains the rhythm of oxygen supply to the bacteria and helps the activity of nitrogenase enzyme. The nitrogenase is responsible for reduction of nitrogen to ammonia in the process of nitrogen fixation. This bacterium is classified into two genera, *Rhizobium* and *Bradyrhizobium.* The details of their species have given in the (Table 4.2).

Fig 4.2: Pink sap (leghaemoglobin pigment) effective nodules (Source: Syed Ismail et al., 2021)

Table 4.2: Classification of *Rhizobium* Biofertilizers (Source: Jordon (1984)

S.N.	*Rhizobium* Species	Strain	Principal Plant Inoculated
1	*Rhizobium leguminosarum*	*Biovar*	*Phaseolus* (Bean)
		Biovar viceae	*Vicea* (Vetch)
		Biovar trifolii	*Trifolium* (Berseem)
2	*Rhizobium meliloti*		*Melilotus* (Senji); *Trigonella* (Fenugreek); *Medicago* (Lucerne)
3	*Rhizobium loti*		*Lotus* (Trefolis)
4	*Bradyrhizobium*		*Glycine* (Soybean)
5	*Bradyrhizobium species*		*Cicer* (Gram); *Lupinus* (Lupin); *Vigna*

The amount of atmospheric nitrogen fixed varies with the strains of *Rhizobium*, plant species and environmental factors. *Rhizobium* host interaction and annual nitrogen fixation by different group of host plant are given below in Table 4.3 and 4.4.

Table 4.3: Nitrogen fixation (Kg/Year) by *Rhizobium* host interactions (Source: Rai et al., 2019)

Group of host plant	Name of the host plant	Name of *Rhizobium* species	Nitrogen fixation kg/year
Pea Group	Pea (*Pisum sativum*), Lentil (*Lens culinaris*)	*R. leguminosorium*	62-132
Soybean Group	Soybean (*Glycine max)*	*R. Japonica*	57-105
Lupine Group	*Lupinus arcticus*	*R. lupini*	70-90
Alfalfa group	*Melilotus indicus*	*R. meliloti*	100-150
Beans Group	*Phaseolus munga*	*R. phaseoli*	80-110
Clover group	*Trifolium repens*	*R. trifolii*	130
Cowpea Group	*Cicer arietinum, Phaseolus munga*	*R. species*	57-105

Table 4.4: Symbiotic Nitrogen Fixation by legumes and non-legumes in different regions

S.N.	Legumes	Kg/ha
I	**Tropical and sub-tropical region**	
	Cowpea	73-354
	Pigeon pea	168-280
	Green gram	61-342
	Soybean	1-168
	Groundnut	77-124
	Clover	100-150
	Stylosanthes	34-320
	Lentil	88-114
	Cluster bean	34-320
II	**Temperate region**	
	Broad bean	
	Garden pea	52-77
	Lupin	145-208
	Lucerne	184-463
	Chickpea	85-100
	Fenugreek	44
III	**Non-legumes**	
	Casuarina	52
	Alnus	139

(Source: https://egyankosh.ac.in)

The Rhizobiaceae family now includes two more genera recently. These are the *Sinorhizobium* and *Azorhizobium* fungi, which are nodding Soybeans and Dhaincha (Sesbania), respectively. *Azorhizobium caulinodans* were isolated from the stem nodules of *Sesbania rostrata*. Additionally, rice roots may become colonised by this and develop nodules. It has also been found that *A. caulinodans* responds to maize. It is also capable of high nitrogen fixation in the free living state.

4.3.2 Root Nodule

Leguminous plants frequently have nodules in their roots. Their formation is due to its association with *Rhizobium*, a bacterium that fixes nitrogen. Rhizobia are the general term used for different genera of nitrogen-fixing bacteria, e.g. *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, etc.

4.3.2.1 Types of Root Nodule

There are mainly two types of root nodules: Indeterminate root nodules and determinate type (Fig. 4.3). The type of root nodule that will be formed depends on the host plant and not on the rhizobial strain (Hirsch, 1992). Differences between the two nodule types are the site of first internal cell divisions, nodule growth, and the form of the mature nodule (Hirsch, 1992).

Determinate nodules are formed on tropical and subtropical legumes such as *L. japonicus*, soybean and bean and indeterminate nodules are formed on temperate legumes such as pea, alfalfa and vetch. A comparison between the two nodule types is given in Table 4.5.

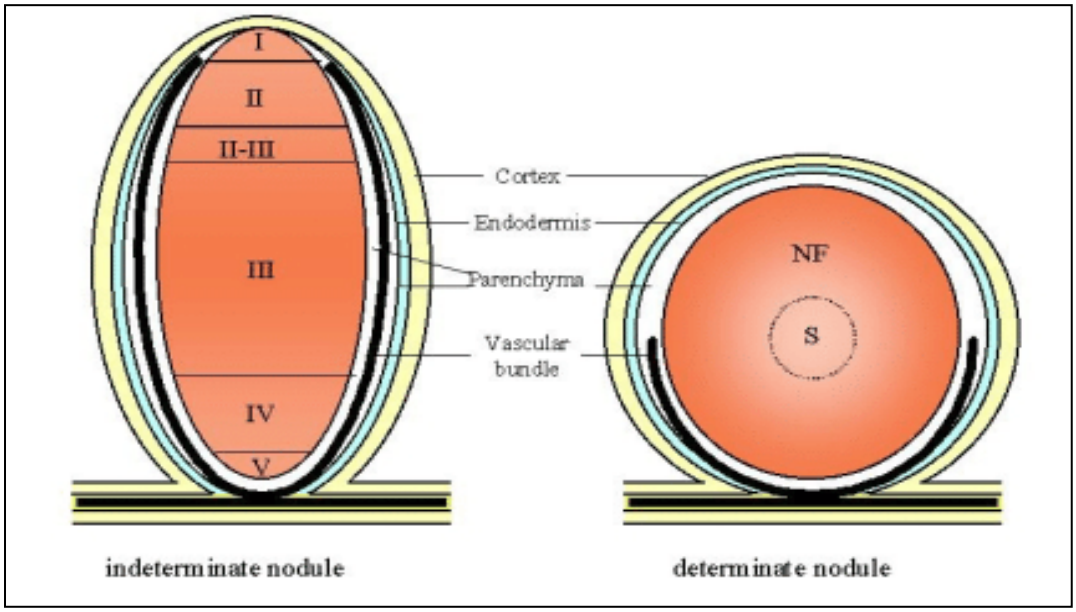


Fig: 4.3: Structure of indeterminate and determinate types of root nodules

I: Meristem zone, II: Prefixation zone, II-III: Interzone, III: Nitrogen fixation zone, IV: Senescent zone, V: Saprophytic zone, S: Senescent zone, NF: Nitrogen fixation zone (Source: Pawslowski and Bisseling, 1996).

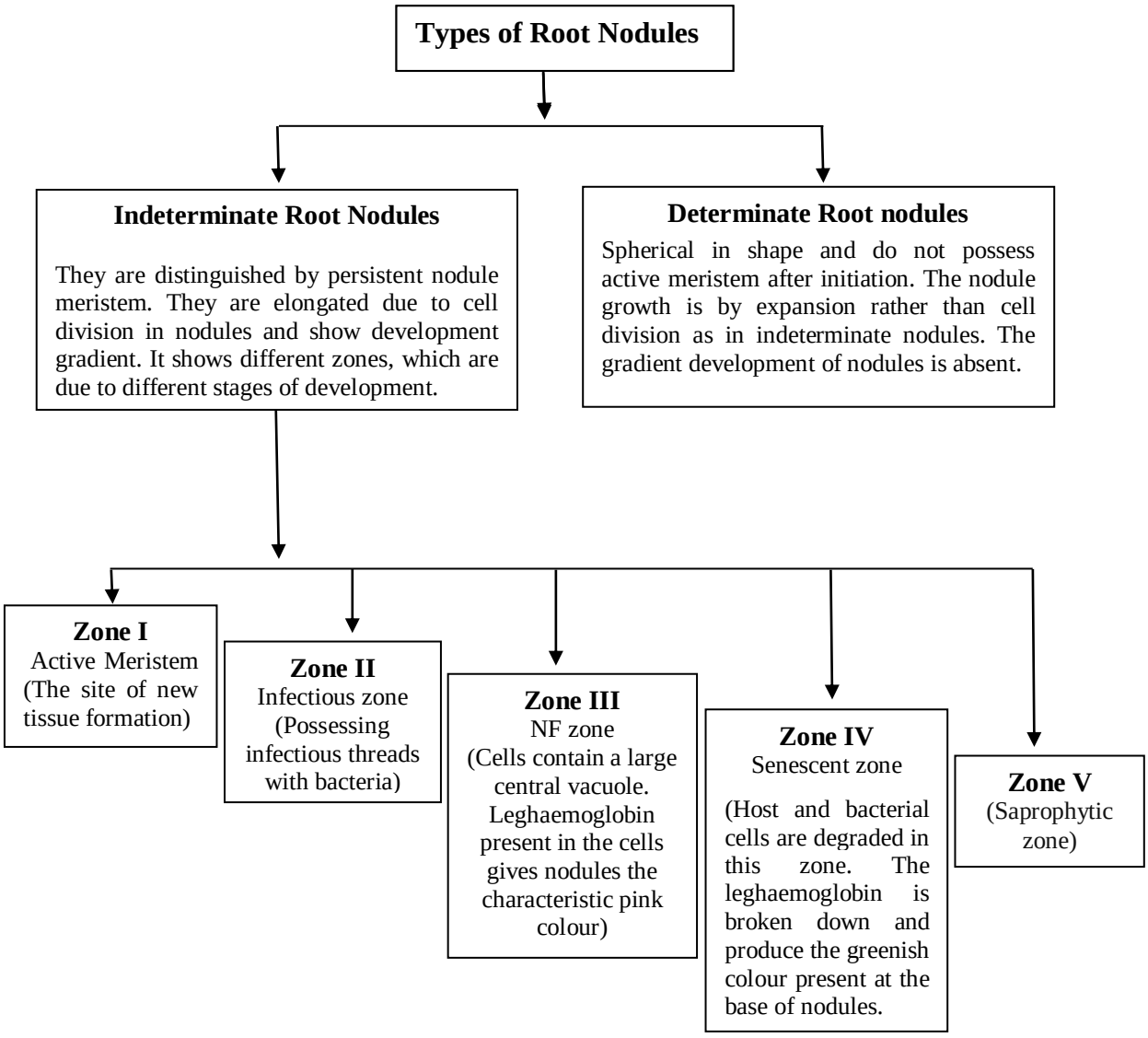
Table 4.5: Comparison of several characteristics of indeterminate and determinate nodule types

S.N.	Parameter	Indeterminate nodule	Determinate nodule
1	Site of initial cell divisions	Inner cortex	Outer cortex
2	Meristem	Persistent meristem	No persistent meristem
3	Nodule form	Elongated	Round shaped
4	Geographic region of plant origin	Temperate regions	Subtropical and tropical
5	Example of symbiotic association	*Medicago-Sinorhizobium*	*Glycine-Bradyrhizobium*

(adapted from Hirsch, 1992)

4.3.2.2 Root Nodule Formation Steps

Root nodule formation is initiated, when the soil contains a low level of nitrogen. The two symbiotic partners use cell signalling for the association and developing nodules. Steps of nodulation are:



4.3.2.3 Steps in the Formation of Root Nodules

Low levels of nitrogen in the soil induce the formation of root nodules. Cell signalling is used by the two symbiotic partners to form the relationship and grow nodules. The nodulation steps are as follows (Fig 4.4):

- Legumes' roots secrete flavonoids, which attracts Rhizobia towards the roots and Rhizobia accumulate around root hairs.
- Root hairs around Rhizobia curl as a result of the nodulation or nod factors secreted by them.
- Many developmental processes, including membrane depolarization, root hair curling, root cortex cell division, and intracellular calcium transport, are stimulated by nod factors.

- The infection thread is formed when the nod factor binds to receptors on the root hairs' plasma membrane. Additionally, Rhizobia can also penetrate through cracks in the root cells.
- Infection threads provide the channel or way to bacteria to enter epidermal cells.
- After, Rhizobia enter cortex cells; each bacterium becomes wrapped in a membrane called a symbiosome that is produced from plants.
- Rhizobia produce chemicals that start nodule formation. It is caused by a signal transduction pathway that is dependent on calcium, which sets off metabolic reactions that lead to in cell proliferation and the formation of nodules.
- Another crucial factor in the development of nodules is Cytokinin.
- Bacteria within nodules differentiate into bacteroids, which fix nitrogen. Nodules generate vascular tissues to facilitate nutrition exchange.

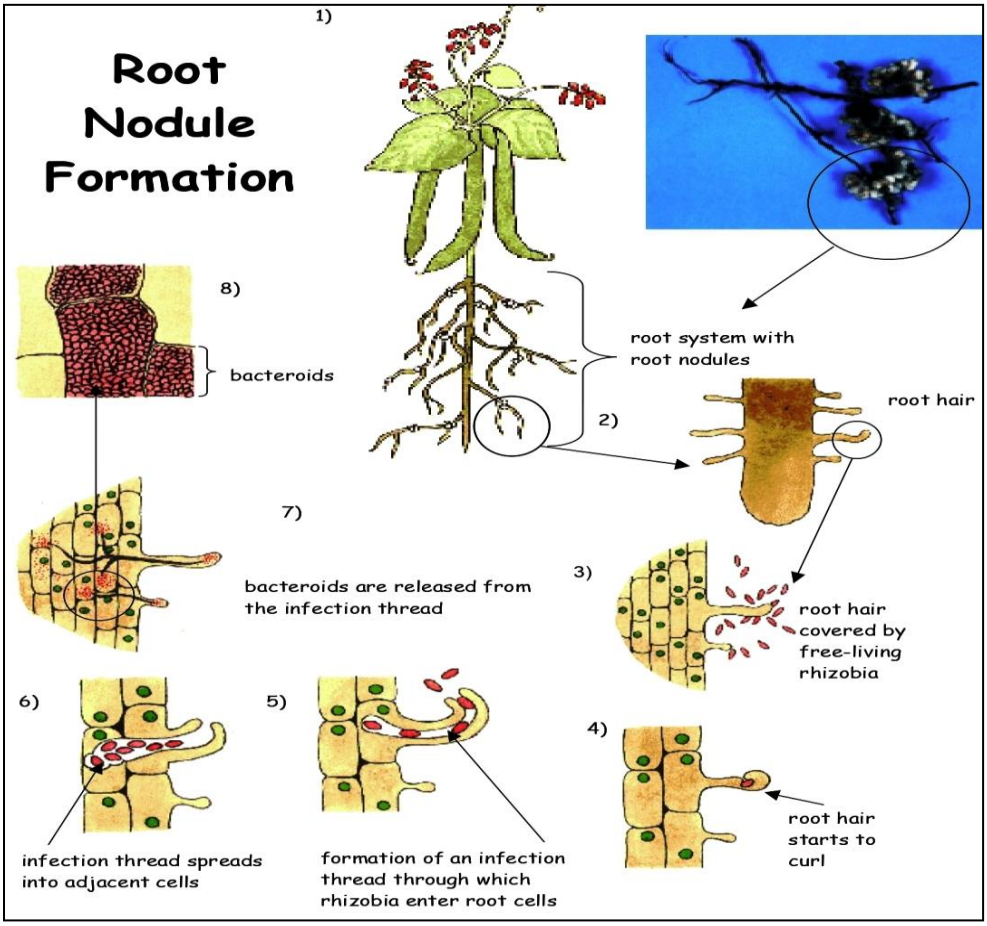


Fig 4.4: Steps of root nodule formation by Rhizobia (Source: Yadav *et al.*, 2021)

4.3.2.4 Factors Affecting Nodule Formation

There are many internal and external factors, which affect the nodule formation such as:

- Some external factors include heat, acidity, nitrate content of the soil, etc. If plants already have enough nitrogen and do not require more, high nitrogen soil prevents nodule formation and symbiotic relationships.
- The process of nitrogen fixation is oxygen-sensitive. The heme pigment leghaemoglobin, which is present in root nodules, promotes oxygen transfer.
- Leaf tissues autonomously control nodule formation.

Internal nodule formation is also regulated by ethylene. It has been discovered that the external application of ethylene inhibits the formation of nodules.

4.4 ISOLATION AND IDENTIFICATION OF RHIZOBIUM

The isolation of *Rhizobium* from root nodules of leguminous plants, involves a series of steps to obtain pure cultures of these nitrogen-fixing bacteria. Here we will study about different steps of the isolation of *Rhizobium* from the fresh nodules of Legumes (Fig 4.5).

4.4.1 Isolation from fresh nodule

- Fresh roots of legume crops collected from field are washed with tap water to remove soil and organic particles.
- The roots with the associated nodules are cut 2-3 mm on either side of the nodules, which are kept in place using forceps.
- Whole and, undamaged nodules are immersed for 10 second in ethanol (95%) or isopropanol (to release the tissue's surface tension and remove air bubbles); transferred to a 2.5-3.0% (v/v) solution of sodium hypochlorite or chlorox (commercial bleach) 1:1 (v/v) and soaked for 4-5 min.
- The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse the sterilizing chemicals (Lupwayi and Haque, 1994). Forceps may be sterilized quickly by dipping in alcohol and flaming. Sterile glass or plastic Petri dishes may be used as containers for the alcohol, sodium hypochlorite, and water. Otherwise, nodules may be placed into a 125 ml conical flask.
- Mercuric chloride solution (0.1% weight/volume) or solution of hydrogen peroxide (3% W/V) can be used for the sterilization of nodule.
- Followed by nodules were transferred into sterile Petri-dishes and crushed with alcohol flamed sterile glass rod in a drop of normal saline solution (0.85% NaCl) inside a laminar air flow hood (Somasegaran and Hoben, 1994). Then after 0.1ml (loopful) of the suspensions

were streaked on plate containing Yeast Extract Mannitol Agar (YEMA) and incubated at 28 ± 2^0C from 3-5 days.

- Yeast Extract Mannitol Agar (YEMA) (Vincent, 1970) composition contains:-

Mannitol	:	10 g/l
K2HP04	:	0.5 g/l
MgS04 .7H20	:	0.2 g/l
NaCl	:	0.1 g/l
Yeast Extract	:	0.5 g/l
Agar	:	15 g/l
Distilled Water	:	1000 ml
PH		7±0.1

- They were autoclaved at 121^{0}C for 15 minutes.

4.4.1.1 Purification and preservation of isolates

After 3-5 days of growth, single dome-shaped colonies were chosen with sterile inoculating loop and streaked on sterile YEMA plates and incubated at 28 ± 20C. After a thorough examination of the purity and homogeneity of the colony types by repeated re-streaking, one well-isolated colony was selected, moved on a YEMA slant containing 0.3% (W/V) CaCO3, and incubated at 28±2^{0}C. When sufficient growth was observed, the culture was transferred to be preserved at 4^{0}C for future use (Vincent, 1970). Then, in accordance with Jordan (1984), the isolated native strains were categorised based on morphological, biochemical and physiological traits.

4.4.1.2 Preliminary examination of pure cultures:

According to Somasegaren and Hoben, (1994), each isolate should examine for presumptive purity using Peptone-Glucose Test (PGT), gram staining and growth response to YEMA-CR medium (Congo red test).

- **Congo red absorption test**

Congo red stock solution was prepared by dissolving 0.25g of Congo red in 100ml of sterile distilled water. From stock solution, 10ml was added to a liter of YEMA and autoclaved. To determine Congo red absorption by the colonies, a loop full of test isolates was streaked over the medium, covered with aluminium foil to create a dark condition, and incubated at 28±20C for three to seven days (Vicent, 1970).

- **Peptone-glucose test (PGT)**

According to Lupwayi and Haque (1994), the Peptone Glucose Test (PGT) was prepared by dissolving 5g of glucose, 10g of peptone, 15g of agar and 10ml of bromocresol purple (BCP) in a liter of distilled water and the pH was adjusted to 6.8 with 1N NaoH and HCl. For preparation of BCP stock solution, dissolving 1g of BCP in 100ml of ethanol. Three days old yeast extract

manitol broth culture containing approximate number of cells (104 cells ml-1) was streaked on to the PGT to observe the growth after having incubated at 28±2^{0}C for 3 to 7 days.

- **Gram reaction test**

According to Lupwayi and Haque (1994), all isolates were examined using the Gram reaction to determine if they were gramme negative or not in order to quickly identify contaminants.

- **Designation of the isolates**

  According to Solomon and Fassil (2014), all the isolates require designation of their identification with abbreviation and/or numbers at this stage.

4.4.1.3 Cultural characteristics

- **Colony morphology**

Lupwayi and Haque's (1994) morphological criteria (size, shape, color, and texture) were used to determine the isolates. Morphologically *Rhizobium* can be identified on YEMA as a White, glistening, and mucoid colonies. Each isolate was given a loopful of a 48-hour-old broth culture, which was then inoculated onto YEMA and cultured for 3–7 days at 28±2^{0}C. According to the information in Martinez-Romero et al. (1991), colony diameter, morphology, and texture were measured after 7 days.

- **Acid-Base production Test**

To determine the ability of the *Rhizobial* isolates to produce acid or alkaline in the medium, YEMA containing bromothymol blue (BTB) (0.025 w/v) was used. In order to record the colour changes in the media, a loopfull of isolates from a culture broth that had been cultured for 48 hours was streaked onto the YEMA-BTB medium and incubated for 3–7 days (Jordan, 1984).

- **Growth and Eco-physiological tests**

A loop full of a 48-hour-old broth culture was inoculated onto the YEMA medium for each biochemical and physiological test. The inoculated YEMA plates were incubated at 28 ± 2^{0}C for 3-5 days (Somasegaren and Hoben, 1994). Ultimately, the growth of each rhizobial isolate was determined as (+) for positive growth and (-) for no growth (Solomon and Fassil, 2014).

- **Temperature tolerance**

Each isolate was inoculated on YEMA plates to assess its growth at various incubation temperatures. According to Lupwayi and Haque (1994), the inoculation plates were incubated at 4^{0}C, 10^{0}C, 15^{0}C, 35^{0}C, 40^{0}C and above or below 4^{0}C.

- **Salt tolerance**

The ability of the isolates to grow at different level of salt concentrations was determined by inoculating each isolate on the YEMA media containing 0.1%, 0.3%, 0.5%, 0.8%, 1%, 2%, 3%, 4%, 5% and above or below 0.1% of NaCl as indicated in Lupwayi and Haque (1994).

pH tolerance

The capacity of each rhizobial isolate to grow on acidic and alkaline media was determined by inoculating each isolate on YEMA adjusted at a pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and above or below 4.0, using NaOH and HCl adjustments as described by Bernal and Graham (2001).

Phosphorous solubilization

All isolates were tested for their ability to solubilize tri-calcium phosphate according to Lupway and Haque (1994).

Amino acid utilization

Different types of amino acids including methionine, L-glutamate, L-tryptophane, L-tyrosine, lysine, aspargine, alanine, L-phenylalanine, L-leucine, Proline, Urea, Argenine, L-arginine and other types of aminoacides were used in this experiment in order to determine the ability of the isolates to utilize the amino acids as a nitrogen source.

Carbohydrate utilization

Isolates were checked for their ability to utilize different carbohydrate sources at this stage. For this matter, different sources of carbohydrate can be checked up. This may include monosaccharides (D-glucose, D-fructose, D- galactose, D-arabinose, D-mannose and xylose), disaccharides (maltose, lactose and sucrose) and sugar alcohols (sorbitol, inositol and glycerol). The tests were carried out according to Somasegaran and Hoben (1994).

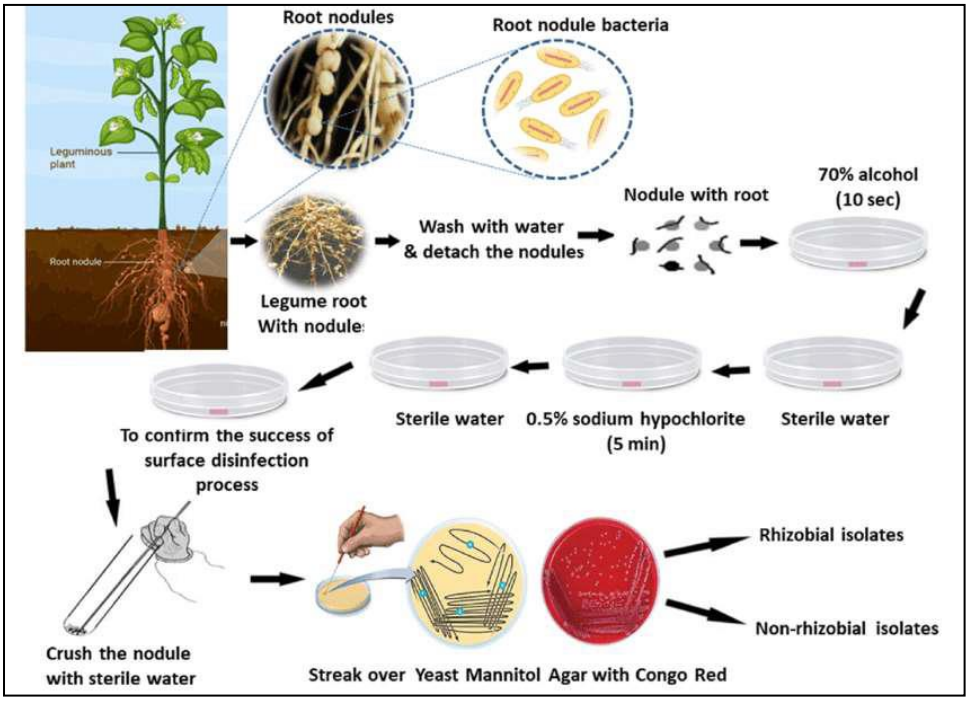


Fig 4.5: Different steps of the isolation of *Rhizobium* from the fresh nodules of Legume (Source: Etesami, 2022)

4.5 MASS MULTIPLIATION OF RHIZOBIUM

The multiplication of the *Rhizobium* is essential for the large scale inoculation of the microbes in the host tissue to get better the plant health by making the possibilities available to the standing crops. Only the inoculums of this specific species are not sufficient, additionally processing and packing are also required for the transfer of the material from the lab to the fields. The following procedures are involved in the large-scale, successful multiplication of *Rhizobium* bacteria in mass production:

- Inoculum preparation
- Processing of carrier material,
- Selection of ideal carrier material,
- Preparation of carrier material,
- Mixing the carrier & broth culture and packing,
- Preparation of the inoculants packing

The inoculums preparation requires careful preparation and in-depth understanding of microbial pure culture procedures, along with the application of the following tools:

- **Autoclave for sterilization**: Used for making the media & glass goods along with desired materials contamination free,
- **Hot air oven:** For growth of the microbes at desired temperature
- **Laminar Air Flow chamber**: For transfer of Inoculums in a sterile environment
- **Incubator**: Incubation of the microbes
- **Rotary and Shake:** Aeration of the growth of the microbes
- **pH meter:** To maintain the desired pH of the microbes
- **Refrigerator**: To preserve the inoculants at low temperature
- **Fermentor:** A large vessel for mass production of microbes in controlled condition.

Moreover to these, the aseptic atmosphere must be maintained for the entire microbiological process to be carried out successfully. This requires the preparation of the media, the appropriate growth components, and a constant electric supply.

4.5.1 Inoculums Preparation:

The following phases of the *Rhizobium* isolation from the root nodules must be understood in order to prepare the inoculums.

- Required Materials: Legume plants root, sterile distilled water, pipettes, test tubes, YEMA plates, 95% ethanol, 0.1% Mercuric chloride solution, aseptic conditions by employing sterilising agents, disinfectants, and the basic parts of microbiological parameters.

- The root nodules are collected, washed with the running tap water and treated with 0.1% mercuric chloride or 3-5% Hydrogen peroxide,
- After repeated running with the tap water, the nodules are passed through 70% ethyl alcohol,
- YEMA plates are prepared with the sterilized environment,
- 01gm of nodular extract is prepared with 10 ml of distilled water and mixed properly,
- Serial dilution is made upto$10^{-}8$. Suspension of 0.1 ml from 10^{-8} is taken and poured over the plates prepared previously and made it, spreads throughout the plate,
- After that, in the suitable environmental conditions at 32℃ is incubated for further analysis.

4.5.2 Preparation of Stock Culture

The *Rhizobium* colony will form on the plate four to five days after incubation, and it can be detected using Congo red dye. The majority of the *Rhizobium* colony took on the colour of white after being stained with Congo red. The pH of the stock culture to be kept at 6.8. For large scale production requires- production of starter culture; the starter culture is prepared in suitable broth. In the starter culture, the conical flasks are kept at the suitable temperature for the same in the large scale production.

4.5.3 Production of carrier based inoculums

After the production of semi-liquid inoculums from the fermentor, the carrier materials are taken. The carrier materials may be pit, lignite, charcoal of the farmyard manure and vermiculite. The carrier materials must be cheap, easily available, low toxic contents along with high organic contents to address the economic feasibility. Water holding capacity of the carrier materials must be more than 50%. The carrier materials must be transformed into dust and the pH must be maintained with calcium carbonate powder. All materials must be adequately autoclaved in order to remove contamination from the substances. For the carrier materials to adhere to the root surface where the inoculation is to be administered, they must possess a kind of adhesive quality.

4.5.4 Packing

The bacterial culture must be packaged in a metallic tray with a suitable carrier and with the help of mixture or using hand gloves, the mixture must be mixed properly before storage. The polythene bags should be low density grade with a thickness of 50-75micron. The appropriate steps that should be taken to ensure that the process is properly sterilised in order to get the maximum benefits from the expected results.

The following information ought to be included in the package such as the name of the manufacturer, name of the strain, the crop that is advised in addition to the inoculation procedure, date of manufacturer, date, batch number, price etc.; full address of the manufacturer along with storage; instructions to the farmers for its uses and application.

4.5.6 Storgae of *Rhizobium* Bio-fertilizer in Packet

The packets ought to be kept out of direct sunlight and heat in a cool location. The packets should be kept in batches in plastic crates or gunny bags, either at room temperature or under cold storage conditions. For best use, the inoculant population has to be checked at 15-day intervals.

4.6 CARRIER-BASED INOCULANTS

Carrier-based inoculants are mixtures that contain beneficial microorganisms, like bacteria or fungi, which are applied to plants, seeds, or soil to support plant growth and health. These inoculants use carriers to transport and maintain the viability of the microorganisms until they are applied to the plants. The carriers provide a medium that promotes the survival, propagation, and effective delivery of these beneficial microbes. Here we will an in-depth review of the numerous aspects of carrier-based inoculants:

- **Microorganisms:** These are one of the major beneficial part of the carrier-based inoculants which includes nitrogen-fixing bacteria, like *Rhizobium, Azospirillum*, and *Azotobacter*, which convert atmospheric nitrogen into a form that plants can absorb and use; **Phosphate-solubilizing bacteria such as** *Bacillus* and *Pseudomonas* (these bacteria convert convert insoluble phosphorus in the soil into a soluble form); **Mycorrhizal fungi (**enhancing nutrient and water uptake**) and Biocontrol agents** like *Trichoderma* species that help control plant diseases and pests.
- **Carrier material:** Various types of material are used as carrier for seed, soil and foliar inoculation such as organic materials (Peat, compost, charcoal, lignite, and humus, which provide a nutrient-rich environment for the microorganisms); **Inorganic materials (**Vermiculite, perlite, bentonite, and clay, which offer stability and support for microbial cells**) and Synthetic materials** (Polymers and other synthetic substances that can enhance the shelf life and stability of the inoculants).

For preparation of seed inoculant, the carrier material is milled to fine powder with particle size of 10 -40 μm. According to "Handbook for Rhizobia", a good carrier material for seed inoculation should have the following qualities, (Somasegaran and Hoben, Springer, 1994). These are non-toxic to the bacterial strain used as the inoculant; good moisture absorption capacity; easy to process and free of lump-forming materials; easy to sterilise by autoclaving or gamma-irradiation; available in sufficient amounts; inexpensive; good adhesion to seeds; good pH buffering capacity and non-toxicity to plants. Peat is the commonly used carrier material for seed inoculation. For soil inoculation, carrier material with granular form (0.5–1.5 mm) is generally used. Granular forms of peat, perlite, charcoal or soil aggregates are suitable for soil inoculation.

- **Types of carrier-based inoculants:** There are three types of carrier based inoculants such as seed, soil and foliar inoculants. **Seed inoculants** applied directly to seeds before planting e.g. *Rhizobium* inoculants for leguminous plants. **Soil inoculants mixed** into the soil, typically around the root zone (mycorrhizal fungi inoculants for enhancing nutrient uptake.). **Foliar inoculants** sprayed onto the leaves of plants e.g. biocontrol agents like *Bacillus thuringiensis* for pest management.
- **Benefits carrier-based inoculants:** The use of carrier-based inoculants in agriculture has several benefits, which are frequently utilised to enhance plant growth and health. Some important benefits of carrier-based inoculants are given below:
  - **i. Enhanced Nutrient Availability**: Improves nitrogen fixation, phosphate solubilization, and nutrient uptake. Due to which dependence on chemical fertilizers is reduced.
  - **ii. Improved Soil Health**: Enhances microbial diversity and activity. Improve soil structure and fertility.
  - **iii. Disease Suppression**: Inhibits pathogens through competitive exclusion and antagonism. Reduce dependence on chemical pesticides.
  - **iv. Environmental Sustainability**: Promotes sustainable agricultural practices. Minimize environmental pollution and degradation.

As a result, carrier-based inoculants are essential to sustainable agriculture because they increase the availability of nutrients, encourage plant development, improve soil health, and provide inexpensive and ecologically friendly agricultural methods.

4.7 ACTINORRHIZAL SYMBIOSIS

Actinorrhizal symbiosis is a mutualistic relationship between certain non-leguminous plants and nitrogen-fixing bacteria belonging to the genus *Frankia*. This symbiosis allows the plants to thrive in nitrogen-poor soils by enabling them to access atmospheric nitrogen. Here are the key components and features of actinorrhizal symbiosis:

1. **Host Plants**: The plants involved in this symbiosis are known as actinorrhizal plants. These include members of several plant families, such as Betulaceae (e.g., alders), Casuarinaceae (e.g., she-oaks), and Myricaceae (e.g., wax myrtles).
2. **Symbiotic Bacteria**: The bacteria involved are actinomycetes, specifically from the genus *Frankia*. These soil-dwelling bacteria can form nodules on the roots of the host plants.
3. **Nodule Formation**: When *Frankia* bacteria come into contact with the roots of a compatible host plant, they infect the roots and induce the formation of specialized root nodules. These nodules house the bacteria and provide an anaerobic environment necessary for nitrogen fixation.

4. **Nitrogen Fixation**: Inside the nodules, *Frankia* bacteria convert atmospheric nitrogen (N2) into ammonia (NH3), a form of nitrogen that plants can use for growth. This process is catalyzed by the enzyme nitrogenase, which is produced by the bacteria.
5. **Mutual Benefits**: The plant provides carbohydrates and a protective niche to the bacteria, while the bacteria supply the plant with bioavailable nitrogen. This exchange allows actinorrhizal plants to grow in environments with low soil nitrogen availability, enhancing their survival and competitive advantage.
6. **Ecological Significance**: Actinorrhizal plants often play crucial roles in their ecosystems. They can improve soil fertility by increasing nitrogen content, facilitate the establishment of other plant species, and are important in succession processes in disturbed areas.

The term "*Actinorhiza*" describes the root nodules that grow on the roots of some non-leguminous plants as a result of a symbiotic relationship with bacteria of the genus *Frankia* that fix nitrogen. The word itself derived from two Greek words: "actino," which means "ray" or "radiate," and "rhiza," which means "root," which reflecting to the nodules' frequently radiating or lobed form. These nodules are specialized structures where the nitrogen fixation process takes place, enabling the host plant to absorb nitrogen from the atmosphere and transform it into a form that can be used by the plant for growth.

Actinorhizal plants are a diverse group of non-leguminous plants that form symbiotic relationships with nitrogen-fixing bacteria and belong to eight different families and about 25 genera, including **Betulaceae** (e.g., Alders such as *Alnus* spp.); **Casuarinaceae** (e.g., She-oaks such as *Casuarina* spp.); **Myricaceae** (e.g., Wax myrtles such as *Myrica* spp.); **Elaeagnaceae** (e.g., Sea buckthorn such as *Hippophae* spp.); **Rosaceae** (e.g., Dryas such as *Dryas* spp.); **Rhamnaceae** (e.g., Ceanothus such as *Ceanothus* spp.). These plants are typically found in nitrogen-deficient environments, including disturbed sites, volcanic soils, and riverbanks.

4.7.1 Ecological and Environmental Significance of Actinorrhizal plants

Actinorrhizal plants are crucial to sustaining biodiversity, stabilising soils, improving soil fertility through nitrogen fixation, supporting in ecosystem restoration, and assisting in the sequestration of carbon and the mitigation of climate change. Because of their vast ecological and environmental value, they are important resources for conservation and sustainable land management.

4.8 SUMMARY

Rhizobium bacteria play a vital role in sustainable agriculture by forming symbiotic relationships with leguminous plants and facilitating nitrogen fixation. Around 80% of the biologically fixed nitrogen in agriculture comes from symbiotic relationships between ruminant plants and bacteria from the Rhizobiaceae family. There are currently six genera in the family Rhizobiaceae like

Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium, and *Bradyrhizobium*, which are generally referred to as Rhizobia (Vance, 1998). This bacterium was first identified in 1888 from legume nodules by the 'Dutchman Beijirinck'. Subsequently, the bacteria were identified as *Rhizobium* in Bergey's Manual of Determinative Bacteriology. Frank came up with the name *Rhizobium* in 1889.

Legumes' symbiotic nitrogen fixation process is typically the main way to add nitrogen to soil for fertility goals. Some rhizobial inoculants have been applied to enhance mineral uptake, boost phytohormone production, and prevent the negative effects of metals. These activities enhance the sustainability and soil quality while also gradually promoting plant growth and development. Through their interactions with plants, *Rhizobium* bacteria contribute to soil fertility, nutrient cycling, and agricultural productivity, making them indispensable allies in the quest for sustainable food production.

The isolation of *Rhizobium* from root nodules of leguminous plants, involves a series of steps to obtain pure cultures of these nitrogen-fixing bacteria. The different steps of the isolation of *Rhizobium* from the fresh nodules of legumes are purification and preservation of isolates; preliminary examination of pure cultures, cultural characteristics etc.

The multiplication of the *Rhizobium* is essential for the large scale inoculation of the microbes in the host tissue to get better the plant health by making the possibilities available to the standing crops. Only the inoculums of this specific species are not sufficient, additionally processing and packing are also required for the transfer of the material from the lab to the fields. The following procedures are involved in the large-scale, successful multiplication of *Rhizobium* bacteria in mass production:

- Inoculum preparation
- Processing of carrier material,
- Selection of ideal carrier material,
- Preparation of carrier material,
- Mixing the carrier & broth culture and packing,
- Preparation of the inoculants packing

Carrier-based inoculants are mixtures that contain beneficial microorganisms, like bacteria or fungi, which are applied to plants, seeds, or soil to support plant growth and health. These inoculants use carriers to transport and maintain the viability of the microorganisms until they are applied to the plants. The carriers provide a medium that promotes the survival, propagation, and effective delivery of these beneficial microbes. Therefore, carrier-based inoculants are essential to sustainable agriculture because they increase the availability of nutrients, encourage plant development, improve soil health, and provide inexpensive and ecologically friendly agricultural methods.

Actinorrhizal symbiosis is a mutualistic relationship between certain non-leguminous plants and nitrogen-fixing bacteria belonging to the genus *Frankia*. This symbiosis allows the plants to thrive in nitrogen-poor soils by enabling them to access atmospheric nitrogen.

4.9 SELF ASSESSMENT QUESTIONS

4.9.1 Multiple Choice Questions

1. Which of the following media is commonly used for the isolation of *Rhizobium*?
a) MacConkey Agar b) Nutrient Agar
c) Potato Dextrose Agar d) Yeast Extract Mannitol Agar (YEMA)

2. Which technique is commonly used for the initial isolation of *Rhizobium* from root nodules?
a) Serial dilution b) Pour plate technique
c) Spread plate technique d) Streak plate technique

3. What is the primary source for isolating *Rhizobium*?
a) Plant leaves b) Animal liver
c) Root nodules of legumes d) Soil samples

4. Which of the following is an essential component in the medium for mass multiplication of *Rhizobium*?
a) Antibiotics b) Heavy metals
c) High salt concentration d) Carbohydrates (e.g., mannitol)

5. In the context of *Rhizobium* inoculant production, what does the term 'carrier' refer to?
a) The bacteria themselves
b) The substrate used to culture the bacteria
c) The laboratory equipment used
d) The inert material used to deliver the bacteria to the field

6. Which sterilization method is commonly used for the carriers in *Rhizobium* inoculant production?
a) Autoclaving b) UV irradiation
c) Filtration d) Microwave heating

7. What is the primary benefit of using *Rhizobium* inoculants in agriculture?
a) Increased resistance to pests b) Enhanced nitrogen fixation
c) Improved photosynthesis d) Faster seed germination

8. *Rhizobium* inoculants are primarily used with which type of crops?
a) Cereal crops b) Leguminous crops
c) Root crops d) Fruit crops

9. Which of the following is NOT a method of applying *Rhizobium* inoculants to seeds?
a) Seed coating b) Soil drenching
c) Seed pelleting d) Foliar spraying

10. What is the ideal pH range for the growth of *Rhizobium* in culture media?
a) 4.0 - 5.0 b) 5.5 - 7.0
c) 7.5 - 9.0 d) 9.5 - 11.0

11. Which piece of equipment is essential for maintaining a sterile environment during the isolation of *Rhizobium*?
a) Autoclave b) Centrifuge
c) Incubator d) Laminar flow

4.9.2 Fill in the Blanks

1) *Rhizobium* is a Gram-negative soil-dwelling ……..

2) The bacteria of the genus *Rhizobium* belongs to family……..

3) This bacterium was first identified in…… from legume nodules by the 'Dutchman Beijirinck'

4) The *Rhizobium* spp of Group I are the fast-growing…….

5) The ……..that grow slowly and produce alkali are falls under group II.

6) There are mainly …… types of root nodules.

7) …….nodules are formed on tropical and subtropical legumes.

8) Actinorrhizal symbiosis is a……..between certain non-leguminous plants and nitrogen-fixing bacteria

9) Carrier-based inoculants are mixtures that contain beneficial…….

10) In actinorrhizal symbiosis nitrogen-fixing bacteria belongs to the genus…….

Answer key- 4.9.1: 1-(d); 2-(d); 3-(c); 4-(d); 5-(d); 6-(a); 7-(b); 8-(b); 9-(d); 10-(b);11-(d)

Answer key- 4.9.2: 1) Bacteria; 2) Rhizobiaceae; 3) 1988; 4) acid producers; 5) rhizobia; 6) two; 7) Determinate; 8) mutualistic relationship; 9) microorganisms; 10) *Frankia*.

4.10 REFERENCES

- Bernal, G. and Graham, P.H. (2001). Diversity in the rhizobia associated with Phaseolus vulgaris L. in Ecuadore, and comparisons with Mexican bean rhizobia. Can. J. Microbiol. 47: 526-534.
- Chenn, P., Micro-organisms in Agriculture. Biological Sciences Review, May 1999, Vol. 11, pp2-4.
- Etesami, H. (2022). Root nodules of legumes: A suitable ecological niche for isolating nonrhizobial bacteria with biotechnological potential in agriculture. Current Research in Biotechnology. 4: 78-86 (doi.org/10.1016/j.crbiot.2022.01.003)**.**
- Hamdi, Y. A. (1971). Soil-water tension and the movement of rhizobia. Soil Biol. Biochem. 3, 121–126. 10.1016/0038-0717(71)90004-6.
- Hirsch, A.M., Lum, M.R., Downie, J.A. (2001). What makes the rhizobia legume symbiosis so special? Plant Physiol. 127, 1484–1492.
- https://egyankosh.ac.in/bitstream/123456789/8896/1/Unit-3.pdf.
- https://www.legumehub.eu/is_article/biological-nitrogen-fixation-in-legumes/
- Indge, B., The Nitrogen Cycle. Biological Sciences Review, Nov. 2000, Vol. 13, pp25-27.
- Jordan, D. (1984). Rhizobaceae. In: Bergey's Manual of Systematic Bacteriology, pp. 234-256 (Hendricks, D.P., Sneath, H.A. and Halt, J.H., eds). Orient Longman, New York.
- Legesse, S. (2016). Isolation, Identification and Authentication of Root Nodule Bacteria (Rhizobia) in Promoting Sustainable Agricultural Productivity: A Review. Developing Country Studies (E-journal). 6(1): 87-91
- Lupwayi, N. and Haque, I. (1994). Legume-Rhizobium Technology Manual: Environmental sciences division, international livestock center for Africa, Addis Ababa, Ethiopia. pp.1-93.
- Madigan, M.T., Martinko, J.M. & Parker, J., Brock Biology of Micro-organisms, 9th ed., 2000, Prentice-Hall, pp 709-717.
- Martinez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P. and Pardo, M. A. (1991). Rhizobium tropici, a novel species nodulating Phaseolus vulgaris L beans and Leucaena sp trees. Int. J. Syst. Bacteriol. 41:417-426.
- Moran, R., The Little Nitrogen Factories. Biological Sciences Review, Nov.1997 Vol. 10, pp2-6.
- Quatrini, P., Scaglion, G., Cardinale, M., Caradonna, F. and Puglia, A.M. (2002). *Bradyrhizobium* sp. nodulatig the mediterranean shrub, Spanis broom (*Spartium junceum* L.). J .Appl.Microbiol.92:13-21.
- Rai, P.K., Rai, G.K., Tandon, V., Sharma, S., Viaks, V. (2019). Application of Bio-Fertilizer for Sustainable Agriculture. In: Gupta, V. and Razdan, V.K. (edt.). Recent Advances in

Production of Bio-Fertilizers and Bio-Pesticides, ICAR Short Course. November, 13th - 22th 2019. SKUAST- Jammu, Jammu-Kashmir, India.

- Solomon Legesse and Fassil Assefa (2014). Symbiotic and phenotypic characteristics of rhizobia nodulating faba bean (vicia faba) from tahtay koraro, northwestern zone of Tigray Regional State, Ethiopia. IJTEEE: 2(11), 15-23.
- Somasegaran, P. and Hoben, H. J. (1985). Hand Book for Rhizobia – Methods in Legume Rhizobium Technology. Springer-Verlag, Heidelberg, Germany.
- Somasegaran, P. and Hoben, H.J. (1994). Handbook for Rhizobia. Springer-Verlag, p.380.
- Syed Ismail, Dhamak, A. L., Mohanty, S. R. 2021. Technical bulletin - *Rhizobium* biofertilizer technology for legumes of Maharashtra. AINP SBB technical bulletin VNMKV, Parbhani, India.
- Vance, C.P. (1998). Legume Symbiotic nitrogen fixation: Agronomical aspects. Kluver Academic Publishers, Dorderecht, 509- 530.
- Vincent, J.M. (1970). A Manual for the Practical Study of Root Nodule Bacteria. Blackwell, Oxford and Edinburgh, pp.164.
- Yadav, G.K., Sawale, D.D., Jagdhani, A.D., Yadav, K., Gurjar, L.S. and Chopra, M.L. (2021). Biofertilizer as an important component of soil fertility management. In: Kumar, N. (edt.), Current Research in Soil Science. Akinik Publication, New Delhi, pp 109-118.

4.11 SUGGESTED READINGS

- Chenn, P., Micro-organisms in Agriculture. Biological Sciences Review, May 1999, Vol. 11, pp2-4.
- Dubey, R. C. and Maheshwari, D. K. (2012). A textbook of Microbiology. Publisher: S Chand & Company P Ltd, New Delhi-55 (India).
- https://egyankosh.ac.in/bitstream/123456789/8896/1/Unit-3.pdf.
- Indge, B., The Nitrogen Cycle. Biological Sciences Review, Nov. 2000, Vol. 13, pp25-27.
- Vashishta B.R. and Sinha A.K. (2010). Botany for Degree Students: Fungi. S Chand Publication, New Delhi.

4.12 TERMINAL QUESTIONS

4.12.1 Short Answer Type Questions

1. Explain in brief about Carrier-based inoculants.
2. Write a short note on types of root nodules.
3. Describe in brief about Actinorrhizal symbiosis.
4. Write a short note on ecological and environmental significance of actinorrhizal plants

4.12.2 Long Answer Type Questions

1. What are the key steps involved in the isolation of *Rhizobium* from legume root nodules?
2. Give a detail account on mass multiplication of *Rhizobium*.
3. What is root nodule? Describe its types and steps in the formation of root nodules.
4. Give a detailed account on isolation, identification, mass multiplication of *Rhizobium*

BLOCK-2 COMMON BIO-FERTILIZERS AND FIXERS

UNIT-05- *AZOSPIRILLUM*: ISOLATION, IMPORTANT CHARACTERISTICS AND MASS MULTIPLICATION

Contents:

5.1 OBJECTIVES

- After reading this unit you would be able to:
  - Define a free-living nitrogen-fixing bacteria *Azospirillum* and its characteristics,
  - Explain the isolation, identification and mass multiplication of *Azospirillum*,
  - Describe the Carrier-based inoculants of *Azospirillum* and its associative effect of different microorganisms.

5.2 INTRODUCTION

Azospirillum is one of the most commonly known and commercially used plant growth-promoting rhizobacteria (PGPR) in crop production system. It is found in association with many plants worldwide in microaerophilic (low oxygen condition) and free-living habitats. *Azospirillum* is known for their plant growth promoting (PGP) activities like nitrogen fixation; production of the phytohormones such as indole 3-acetic acid (IAA), abscisic acid (ABA), gibberellic acid, cytokinin, zeatin (type of cytokinin) and ethylene; plant growth regulatory substances such as polyamines; osmotic stress response in plants and siderophore production. In addition to their diverse potential to stimulate plant growth, these bacteria show more competitive characteristics and are capable of adapting to most rhizospheric environments. Due to their microaerophilic nature, these bacteria function most effectively in soils with medium to heavy textures.

5.3 TAXONOMY AND CHARACTERISTICS OF AZOSPIRILLUM

Azospirillum, a free-living nitrogen-fixing bacteria closely associated with cereals and grasses. It is a Gram-negative motile bacteria belonging to the family Rhodospirillaceae and associated with roots of monocots, including important cereals such as wheat, rice and corn.

5.3.1Taxonomy:

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Alphaproteobacteria
Order: Rhodospirillales
Family: Rhodospirillaceae
Genus: *Azospirillum*

5.3.2 Characteristics

Azospirillum species are rod to spirillum shaped, Gram-negative bacteria and freely lives in soil forming nonspecific symbiotic associations with various plants in particular, corn. They are motile, typically possessing peritrichous or polar flagella for movement. They are facultative

anaerobes, means they can grow in both the presence and absence of oxygen. It can fix atmospheric nitrogen, converting it into ammonia, which plants can then use as a nitrogen source. These bacteria thrive in the rhizosphere of various plants, particularly cereals and grasses. They can colonize the root surface as well as the interior of roots (endophytes). This genus includes the following species: *A. lipoferum, A. halopraeferans, A. nitrocaptans, A. amazonense, A. brasilense, and A. seropedica*. In 1922, Beijerinck first identified and named *Azospirillum lipoferum* as *Spirillum lipoferum*.

Azospirillum characteristically develops white, dense, and undulating pellicles on a semi-solid malate containing enrichment medium. The pellicle is formed 2 mm below the surface of the medium indicating the microaerophilic nature of the bacterium. The gram-negative bacterium *Azospirillum* exhibits spirillar motility, polymorphism, and poly-β-hydroxy butyrate (PHB) granules.

It fixes atmospheric nitrogen (dinitrogen) in microaerophilic surroundings (low oxygen conditions) but possesses ability to grow profusely in ammonium- rich environment without fixing nitrogen. The bacterium also produces growth substances such as indole acetic acid (IAA), kinetins, and gibberellins.

5.3.3 Mechanisms of Plant Growth Promotion

Azospirillum promotes plant growth in a number of ways, many of which enhance the nutrient uptake, root growth, and stress tolerance of the host plants. The following are the main ways that *Azospirillum* promotes plant growth:

1. Biological Nitrogen Fixation (BNF): *Azospirillum* fixes atmospheric nitrogen (N_2) into ammonia (NH_3) with the help of enzyme nitrogenase. This fixed nitrogen is then available for plant uptake, providing a vital nutrient that is often limiting in soils. As a result, increases nitrogen availability in the soil, reducing the need for chemical nitrogen fertilizers and enhanced plant growth, particularly in nitrogen-poor soils.

2. Phytohormone Production: *Azospirillum* synthesizes auxine-IAA (Indole-3-Acetic Acid) that promotes root elongation and branching; Gibberellins (promote stem elongation, seed germination), and cytokinins (influence cell division and shoot formation). These phytohormones stimulate root development, increasing root surface area for nutrient and water absorption and Enhanced overall plant growth and development.

3. Enhanced Nutrient Uptake: *Azospirillum* solubilizes insoluble phosphate compounds in the soil, making phosphorus more available to plants and it also increases the production of siderophores. (ron-chelating compounds solubilize iron, facilitating its uptake by plants).

4. Improvement of Root Architecture: *Azospirillum* alters the architecture of roots by generating phytohormones such IAA, which promotes the growth of adventitious and lateral roots as well as longer roots. A larger root system facilitates better uptake of nutrients and water, which improves plant development and stress tolerance.

5. Stress Tolerance: By increasing root water intake and producing stress-related hormones and osmolytes, *Azospirillum* helps plants adapt to drought more effectively. In saline environments, the bacteria help the plant regulate its osmotic pressure and ionic balance, increases the resilience and yield of plants in harsh environmental circumstances, like excessive salt and drought.

6. Production of Antimicrobial Compounds: It is produces various antimicrobial compounds that inhibit the growth of plant pathogens. So it reduces the incidence of soil-borne diseases, promoting healthier plant growth.

7. Biofilm Formation: *Azospirillum* forms biofilms on root surfaces, which provide a protective environment and facilitate close interactions with plant roots and enhances the colonization efficiency and stability of *Azospirillum* in the rhizosphere, leading to more consistent plant growth promotion.

Therefore, *Azospirillum* promotes plant growth through a combination of nitrogen fixation, phytohormone production, enhanced nutrient uptake, improved root architecture, and increased stress tolerance. These mechanisms work synergistically to improve plant health, increase crop yields, and contribute to sustainable agricultural practices. The versatility and efficiency of *Azospirillum* make it an essential component of biofertilizers and a valuable tool for enhancing agricultural productivity.

5.3.4 Applications of *Azospirillum* in Agriculture

As you know, *Azospirillum* is a genus of bacteria known for its plant growth-promoting properties, particularly in agriculture. These bacteria are known to associate with the roots of various plants, especially cereals and grasses, where they provide multiple benefits that enhance plant growth and yield. Here are the key applications of *Azospirillum* in agriculture:

1. **As a Biofertilizers:** *Azospirillum* is widely used in biofertilizer formulations to improve soil fertility and promote plant growth. These biofertilizers reduce the need for chemical fertilizers, contributing to sustainable agriculture.
2. **Nitrogen Fixation:** It can fix atmospheric nitrogen into a form that plants can use. This process is particularly beneficial for crops grown in nitrogen-deficient soils. The bacteria convert atmospheric nitrogen (N_2) into ammonia (NH_3), which plants can then uptake and use for their growth.
3. **Production of Phytohormones:** Phytohormones such gibberellins, cytokinins, and indole-3-acetic acid (IAA) are produced by *Azospirillum*. These hormones improve a plant's overall development by promoting root growth.
4. **Improvement of root Architecture:** The bacteria influence root morphology by increasing root length and the number of root hairs. This results in a larger root surface area, allowing plants to access water and nutrients more efficiently.

5. **Enhanced Nutrient Uptake:** It can improve the absorption of many micronutrients, potassium, and phosphorus. This is a result of both the better root architecture and the production of substances that solubilize soil nutrients and increase plant availability.
6. **Biocontrol of Pathogens:** Due to antimicrobial production capacity of some strains of *Azospirillum* they can inhibit the growth of plant pathogens. This biocontrol property helps in reducing the incidence of diseases, thereby supporting healthier plant growth.
7. **Stress Tolerance:** The bacteria enhance the plant's stress resistance mechanisms, such as osmotic adjustment and the scavenging of reactive oxygen species. It can help plants tolerate abiotic stresses such as drought, salinity, and heavy metal toxicity.
8. **Yield Improvement:** *Azospirillum* increases agricultural yields through enhancing root formation, nitrogen uptake, and general plant health. When inoculated with *Azospirillum*, studies have demonstrated considerable yield benefits in a variety of crops, including wheat, maize, rice, and millet.
9. **Seed Inoculants:** Seeds can be coated with *Azospirillum* inoculants before planting. This practice ensures early root colonization, leading to better seedling establishment and growth.
10. **Soil Amendments:** It can be introduced into the soil through various organic amendments such as compost and manure. This promotes a healthy soil microbiome and enhances soil fertility.
11. **Plant Growth-Promoting Rhizobacteria (PGPR) Consortiums:** It is often used in combination with other PGPRs to create synergistic effects that enhance nutrient availability, improve plant health, and increase yields.

Thus, using *Azospirillum* in agriculture is a viable way to increase crop output in a sustainable way. Farmer dependence on chemical inputs can be decreased while increasing soil fertility, plant development, and yields are increased by utilising the natural capabilities of these helpful bacteria.

Azospirillum, a nitrogen-fixing biofertilizer is called a biofertilizer. *Azospirillum lipofereum* is an extremely helpful bacterium for roots and soil. It is a nitrogen-fixing associative symbiotic bacterium. It is present in the soil around and on the surface of plant roots.

Azospirillum bacterium fixes the atmospheric nitrogen and makes it available to plants in non-symbiotic manner that can replace 50-90% of the nitrogen fertilizer required by plants. It is applicable for all crops including field crops, potting soil, vegetable and flower gardens, orchards and turf grass.

5.4 ISOLATION OF AZOSPIRILLUM

Isolation of *Azospirillum* from soil or plant roots in in-vitro condition involves a series of controlled laboratory procedures to ensure the accurate identification and cultivation of this beneficial bacterium. Here, you will be study about step-by-step protocol of isolation of *Azospirillum* from soil or plant roots.

5.4.1 Required Materials:

- Root or Soil samples
- Sterile distilled water; Sterile glassware (Beakers, Test tubes, Petri dishes, pipettes and spreaders); Homogenizer or mortar and pestle; Incubator; Microscope; Autoclave, Gram staining kit
- **Nutrient media:** Nitrogen-Free Malate (NFM) agar or Dobereiner's medium.
- **Chemicals:** Biochemical test reagents**;** Ethanol (70%)**;** Sodium hypochlorite (1%)
- **Composition of Nitrogen-Free Malate (NFM) Agar:** Nitrogen-Free Malate (NFM) agar is a selective medium used for the isolation and cultivation of nitrogen-fixing bacteria *Azospirillum*. NFM medium normally contains the necessary nutrients for bacterial, excluding nitrogen sources to select for organisms capable of nitrogen fixation. Here's the detailed composition for preparing NFM agar:
- **Ingredients:** Malic Acid: 2 g/L (Provides a carbon source for bacterial metabolism); Sodium Chloride (NaCl): 1 g/L (Maintains osmotic balance); Magnesium Sulfate ($MgSO4·7H2O$): 0.2 g/L (Supplies magnesium ions, essential for various enzymatic processes); Calcium Chloride ($CaCl2·2H2O$): 0.02 g/L (Provides calcium ions necessary for cell wall stability and function); Potassium Phosphate (K_2HPO_4): 0.5 g/L (Acts as a buffer to maintain pH and provides phosphate ions); Ferrous Sulfate ($FeSO4·7H2O$): 0.01 g/L (Provides iron, a crucial component of nitrogenase enzyme involved in nitrogen fixation) Agar: 15 g/L (for solidification); Distilled Water: Adjust to 1 liter (Solvent for all the components).

Note:

- The pH of the medium should be adjusted to around 6.8-7.0 before autoclaving, as malate and other components can slightly alter the pH.
- Ensure that all equipment and media are sterile to avoid contamination (autoclaving at 121°C for 15-20 minutes). Use aseptic techniques throughout the process.
- Allow the medium to cool to about 50-55°C. Pour the molten medium into sterile Petri dishes and allow it to solidify at room temperature.

5.4.2 Procedure for Isolation of *Azospirillum*:

1. **Sample Collection:**

- Collect samples of plant roots or soil from the rhizosphere. Root samples should be collected carefully to include soil adhering to the roots.
- Use sterile containers to transfer samples to the lab.

2. **Sample Preparation:**

- Weigh 1 gram of soil and suspend it in 10 mL of sterile distilled water in a test tube. Vortex or shake vigorously to ensure thorough mixing.
- Gently wash the roots in sterile distilled water to remove loosely adhering soil. Cut root segments into 1-2 cm pieces. Surface sterilizes root segments by immersing in 70% ethanol for 1 minute, followed by 1% sodium hypochlorite for 2-3 minutes.
- Rinse thoroughly with sterile distilled water to remove any sterilizing agent residues.
- Homogenize the root part in sterile distilled water using a mortar and pestle or homogenizer.

3. Enrichment Culture:

- **Media Preparation:** Prepare Nitrogen-Free Malate (NFM) agar plates, which is selective for nitrogen-fixing bacteria. Autoclave the media and pour into sterile Petri dishes. Allow to solidify.
- **Inoculation:** Pipette 0.1 mL of the soil suspension or root homogenate onto the surface of NFM agar plates. Spread the inoculum evenly using a sterile spreader.

4. Incubation:

- Incubate the inoculated plates at 30°C for 3-5 days. Azospirillum colonies typically appear as white, translucent, and mucoid.

5. **Isolation and Purification:**

- Select distinct colonies and streak onto fresh NFM agar plates to obtain pure cultures.
- Incubate the streaked plates at 30°C for another 3-5 days to confirm the purity of the cultures.

6. **Identification:**

i) **Microscopic Examination:** Perform Gram staining on isolated colonies. *Azospirillum* species are Gram-negative and will appear as pink to red rod-shaped cells under the microscope.

ii) **Biochemical Tests:** Perform further biochemical testing to support the results, such as:

- **Motility Test:** *Azospirillum* is typically motile with a characteristic spiral shape in liquid culture.
- **Catalase Test:** *Azospirillum* is catalase-positive.
- **Indole Test:** It may produce indole.
- **Nitrate Reduction Test:** Tests for the reduction of nitrate to nitrite.
- Additional tests can include carbohydrate utilization and oxidative/fermentative metabolism.

Azospirillum can be successfully isolated and identified in vitro by following these detailed steps, which allowing for further study and potential application in agricultural practices.

5.5 MASS MULTIPLICATION OF AZOSPIRILLUM

Mass multiplication of *Azospirillum* involves several important steps to produce a high volume of viable bacteria which is suitable for agricultural application as a biofertilizer. Here you will study in detailed points of mass multiplication of *Azospirillum*.

i) **Isolation and maintenance of pure culture:** Isolate Azospirillum from soil samples using selective media like nitrogen-free malate (NFM) agar. For short-term storage and frequent sub-culturing, keep pure cultures on NFM agar slants at 4°C.

ii) **Preparation of seed inoculum:** Transfer a loopful of the pure culture into a 250 ml Erlenmeyer flask containing 100 ml of NFM broth. Incubate the flask at 28-30°C on a rotary shaker at 120-150 rpm for 48-72 hours. Check the optical density (OD) at 600 nm to ensure the bacterial growth has reached an appropriate level (usually OD600 of 0.8-1.0).

iii) **Preparation of Fermentation Medium:** Prepare a fermentation medium optimized for *Azospirillum* growth. A common composition for that will be- Sucrose: 20 g/L, K2HPO4: 0.5 g/L, MgSO4•7H2O: 0.2 g/L, NaCl: 0.1 g/L, CaCO3: 5 g/L). After that sterilize the medium by autoclaving at 121°C for 15-20 minutes.

iv) **Inoculation and Fermentation:** Transfer the seed inoculum (5-10% v/v) into a large fermenter containing the sterilized fermentation medium. Maintain the fermenter conditions at 28-30°C with continuous aeration and agitation. Monitor the pH, dissolved oxygen, and other parameters to ensure optimal growth conditions. Allow the fermentation to proceed for 48-72 hours or until the bacterial population reaches 10^8-10^9 CFU/ml.

v) **Harvesting:** After reaching the desired cell density, harvest the bacterial cells by centrifugation at 6000 rpm for 15 minutes or by filtration. Re-suspend the bacterial pellet in a suitable carrier likes sterilized peat, lignite, or talc powder.

vi) **Formulation and Packaging:** Mix the bacterial suspension with the carrier to achieve a final moisture content of about 30-40%. Pack the formulated product into polyethylene bags or bottles. Make sure proper labeling with details for instance strain, cell count, production date, and expiration date.

vii) **Quality Control:** Perform quality control tests to ensure the viability and purity of the *Azospirillum* formulation. Tests may include cell count, contamination check, pH, and moisture content.

viii) **Storage and Distribution:** Store the final product in a cool, dry place away from direct sunlight. Distribute with guidelines on application rates and methods.

Note: Ensure all equipment and media are properly sterilized to prevent contamination. Regularly monitor the fermentation parameters and make adjustments as necessary. Choose an appropriate carrier that supports the survival and activity of *Azospirillum.* Train personnel in aseptic techniques and proper handling of microbial cultures.

Azospirillum is an effective biofertilizer. Therefore, you can increase soil fertility and crop productivity in agriculture by mass multiplying of *Azospirillum* by following these procedures.

5.6 Carrier-Based Inoculants of Azospirillum

Carrier-based inoculants are an effective and useful way to deliver the helpful nitrogen-fixing bacterium *Azospirillum* to crops. These inoculants play a crucial role in sustainable agriculture by enhancing soil fertility and promoting plant growth. The use of carrier materials helps to stabilize the bacterial cells, making them easier to handle, store, and apply.

5.6.1 Benefits of Carrier-Based Inoculants

Some of the benefits of using carrier-based inoculants of *Azospirillum* in agriculture will be discussed here, which are as follows:

- **Enhanced Viability and Longevity:** The carrier material provides a protective environment that enhances the survival and longevity of *Azospirillum* cells during storage and transportation.
- **Simpleness of Use**: Farmers can employ carrier-based formulations in a variety of agricultural contexts since they are simple to work with and apply.
- **Cost-Effectiveness**: Using locally available and inexpensive carrier materials can reduce the total cost of the inoculant production process. Common carrier materials like peat, lignite, talc, and charcoal are inexpensive and readily available, making the production of inoculants cost-effective.
- **Reduced Chemical Fertilizer Usage:** By promoting natural nitrogen fixation, *Azospirillum* inoculants reduce the need for chemical nitrogen fertilizers, lowering input costs for farmers
- **Extended Shelf Life**: Properly formulated carrier-based inoculants can have a shelf life of 6-12 months, making them more practical for commercial distribution.
- **Environmental Benefits**: Using *Azospirillum* inoculants promotes sustainable agricultural practices by reducing dependence on chemical fertilizers and minimizing

their environmental impact. It also contributes to improved soil structure and fertility over time, fostering a healthy soil microbiome.

5.6.2 Common Carrier Materials:

Some of the common carrier materials like peat, lignite. talc, charcoal, vermiculite etc., which are useful carrier to deliver the helpful nitrogen-fixing bacterium *Azospirillum* to crops. **Sterilized peat** is widely used due to its high organic matter content and excellent moisture-holding capacity; **Lignite** supports microbial growth and is also an effective carrier. **Talc** is an inert mineral; talc provides a good surface area for bacterial adhesion. Sterilized **charcoal** is porous and has good moisture retention properties, making it suitable for bacterial inoculants. **Vermiculite** is an expanded mineral that provides good aeration and moisture retention, supporting bacterial survival.

5.6.3 Preparation Process of Carrier-Based Inoculants of *Azospirillum*

A number of important steps must be followed in order to ensure the survival and effectiveness of the bacterial cells used to prepare *Azospirillum* carrier-based inoculants. Here you will be study about detailed step by step procedure:

1. Selection and Preparation of Carrier Material

- **Criteria for Carrier Material**: The selected carrier material should be non-toxic and non-pathogenic, high water-holding capacity, good aeration properties, easily sterilizable, readily available and cost-effective.
- **Common Carrier Materials**: Common Carrier Materials: Some of the common carrier materials which have some specific potency such as peat (high organic matter content, good moisture retention); lignite (to supports microbial growth); Talc (inert mineral, good surface area); Charcoal (porous, good moisture retention); Vermiculite (provides good aeration and moisture retention) to used as a carrier material of *Azospirillum*
- **Preparation of Carrier**: Sterilize the carrier material to eliminate any unwanted microorganisms. Peat can be autoclaved at 121°C for 15-20 minutes or gamma-irradiated. Talc and lignite are typically autoclaved. Adjust the moisture content to about 30-40%.

2. Cultivation of *Azospirillum*

- **Culture Medium**: Use nitrogen-free malate (NFM) broth. Inoculate a loopful of *Azospirillum* from a pure culture into a 250 ml Erlenmeyer flask containing 100 ml of NFM broth. Incubate at 28-30°C on a rotary shaker at 120-150 rpm for 48-72 hours. Monitor bacterial growth by measuring the optical density (OD) at 600 nm, aiming for an OD600 of 0.8-1.0.

3. Harvesting and Resuspension: After reaching the desired cell density, harvest the bacterial cells by centrifugation at 6000 rpm for 15 minutes or by filtration. Resuspend the bacterial pellet in a small volume of sterile saline or another suitable suspension medium to achieve a high concentration of *Azospirillum* cells.

4. Mixing Bacteria with Carrier: Mix the bacterial suspension thoroughly with the sterilized carrier material. Usually, 1 part bacterial suspension is mixed with 10 parts carrier by weight, but this can vary depending on the carrier and desired final cell concentration.

5. Curing: In order to provide the mixture to cure for 24-48 hours to enable the bacteria to attach to the carrier particles and stabilize. Maintain in a controlled environment (e.g., 25-30°C) to support bacterial survival.

6. Packaging: Pack the treated inoculant into polyethylene bags, bottles, or other suitable containers and ensure proper labeling with details such as strain, cell count, production date, and expiration date.

7. Quality Control

- **Viability Testing**: Check for viable cell count to ensure the inoculant contains the desired number of *Azospirillum* cells (typically 10^8-10^9 CFU/g of carrier).
- **Contamination Check**: Ensure the absence of contaminating microorganisms.
- **Moisture Content**: Verify that the moisture content is within the desired range (30-40%).

8. Storage and Distribution: The inoculant is stored in a cool, dry place away from direct sunlight. If inoculants are prepared and stored properly, their shelf life might range from six to twelve months. After that, distribute to farmers with guidelines on application rates and methods.

Therefore, carrier-based inoculants of *Azospirillum* are a key component in modern sustainable agriculture. They enhance plant growth, improve soil health, and contribute to increased crop yields. By using effective carrier materials and following proper preparation protocols, high-quality *Azospirillum* inoculants can be produced and utilized to benefit agricultural practices globally.

5.7 ASSOCIATIVE EFFECT OF DIFFERENT MICROORGANISMS

Azospirillum is a well-known genus of nitrogen-fixing bacteria that form beneficial associations with a wide range of plants, especially cereals and grasses. The interaction of *Azospirillum* with other microorganisms in the rhizosphere can have significant effects on soil health, plant growth, and microbial community dynamics. Here in this section you will be study the relationship of *Azospirillum* with different microorganisms.

1. **Association with Rhizobium**: When *Azospirillum* is associated with *Rhizobium*, several synergistic effects can enhance plant growth and nitrogen fixation:

- **Nitrogen Fixation Synergy:** *Azospirillum* and *Rhizobium* can simultaneously inoculate leguminous plants, and improves nitrogen fixation and plant growth. In the rhizosphere, *Azospirillum* fixes nitrogen independently, but *Rhizobium* usually forms root nodules and fixes nitrogen symbiotically.
- This association provides a more and regular supply of nitrogen to the plant and improves growth and yield.
- **Phytohormone Production:** Indole-3-acetic acid (IAA), a phytohormone, is produced by both bacteria, which promotes root development and increase nutrient uptake. Plant health and productivity are improved by this synergistic action.
- **Complementary Nutrient Uptake:** *Rhizobium* primarily focuses on nitrogen fixation, while *Azospirillum*'s ability to solubilize other nutrients can provide a more balanced nutrient supply to the plant.

2. Association with Mycorrhizal Fungi: When *Azospirillum* associates with mycorrhizal fungi, several plant-microbe interactions can benefit plant growth and health like:

- **Enhanced Root Growth and Development:** The phytohormones produced by *Azospirillum* encourage root growth, providing a more widespread root system for mycorrhizal colonization.
- **Improved Nutrient Uptake**: *Azospirillum* fixes atmospheric nitrogen, resulting in a more balanced nutrient supply, whereas mycorrhizal fungi improve the absorption of nutrients like phosphorus.
- **Increased Plant Biomass and Yield**: The combined effects of better nutrient uptake and enhanced root development lead to increased plant growth and higher crop yields.
- **Stress Resilience**: The combination of *Azospirillum* and mycorrhizal fungi can enhance plant resistance to abiotic stresses such as drought and salinity. Mycorrhizal fungi improve water absorption, while *Azospirillum* increases nitrogen availability and produces stress-mitigating phytohormones.
- **Soil Health and Fertility**: The presence of both microorganisms improves soil structure, microbial diversity, and overall soil health.

3. Interaction with Phosphate-Solubilizing Bacteria (PSB): *Azospirillum* and phosphate-Solubilizing bacteria (PSB) are two types of beneficial soil microorganisms that play vital roles in promoting plant growth and soil fertility. Their interaction is particularly fascinating because it can lead to synergistic effects that improve plant nutrient uptake and overall health. An overview of their interactions and benefits is as follows:

- **Improved Nutrient Cycling:** Phosphate-solubilizing bacteria (PSB) convert insoluble phosphorus in the soil into forms that plants can absorb. *Azospirillum* can enhance this process by producing organic acids that help solubilize phosphorus.
- The dual inoculation can improve nitrogen and phosphorus availability, which will promote vigorous growth of plants and increased yields.
- **Microbial Synergy:** The presence of both *Azospirillum* and PSB can makes a more diverse and stable microbial community in the rhizosphere, enhancing overall soil health and fertility.

4. Association with Plant Growth-Promoting Rhizobacteria (PGPR): These interactions are complex and multifaceted, involving various mechanisms that benefit plants directly and indirectly. Some important beneficial associations are given as:

- Multiple Growth-Promoting Activities: PGPR, such as Bacillus and Pseudomonas species, can promote plant growth through various mechanisms, including nitrogen fixation, phosphate solubilization, production of phytohormones, and suppression of plant pathogens.
- Co-inoculation with *Azospirillum* can amplify these benefits, leading to improved nutrient uptake, enhanced root growth, and increased plant resistance to diseases.
- Disease Suppression: The combined action of *Azospirillum* and other PGPR can help suppress soil-borne pathogens through the production of antimicrobial compounds and competition for resources, thereby reducing the incidence of plant diseases.

5. Interaction with Decomposer Microorganisms: The interaction between *Azospirillum* and decomposer microorganisms represents a key component of soil health and plant productivity. By working together, these microorganisms enhance nutrient cycling, improve soil structure, and promote plant growth, leading to more sustainable and productive agricultural system. This synergy not only benefits plant health but also contributes to long-term soil fertility and ecosystem stability. Here's an in-depth look at how *Azospirillum* interacts with decomposer microorganisms:

- **Organic Matter Decomposition:** The decomposer microorganisms, which include bacteria and fungi, are essential to the cycling of nutrients because they break down organic materials. *Azospirillum* can enhance this process by increasing the availability of nitrogen, which is essential for the microbial breakdown of organic material.
- This interaction can lead to improved soil structure and fertility, promoting healthier plant growth.

- **Enhanced Soil Health:** The presence of decomposer microorganisms along with *Azospirillum* can contribute to a more balanced and productive soil ecosystem, fostering sustainable agricultural practices.

Azospirillum's associations with various rhizosphere bacteria have a significant impact on sustainable agriculture. Plant growth, soil fertility, and stress resistance can all be considerably increased by co-inoculating with *Rhizobium*, mycorrhizal fungi, phosphate-solubilizing bacteria, rhizobacteria that promote plant development, and decomposer microorganisms. In order to maximise agricultural output and environmental sustainability, it is crucial to address soil and plant health holistically and to take advantage of the synergistic effects of various microbial populations.

5.8 SUMMARY

Azospirillum is one of the most commonly known and commercially used plant growth-promoting rhizobacteria (PGPR) in crop production system. It is found in association with many plants worldwide in microaerophilic (low oxygen condition) and free-living habitats. It is a Gram-negative motile bacteria belonging to the family Rhodospirillaceae and associated with roots of monocots, including important cereals such as wheat, and rice and corn. *Azospirillum* species are rod to spirillum shaped, Gram-negative bacteria and freely lives in soil forming nonspecific symbiotic associations with various plants in particular, corn. They are motile, typically possessing peritrichous or polar flagella for movement. It can fix atmospheric nitrogen, converting it into ammonia, which plants can then use as a nitrogen source. These bacteria thrive in the rhizosphere of various plants, particularly cereals and grasses. They can colonize the root surface as well as the interior of roots (endophytes).

This genus includes the following species: *A. lipoferum, A. halopraeferans, A. nitrocaptans, A. amazonense, A. brasilense, and A. seropedica*. *Azospirillum* promotes plant growth through a combination of nitrogen fixation, phytohormone production (auxine-IAA, **g**ibberellins**,** cytokinins), enhanced nutrient uptake, improved root architecture, and increased stress tolerance. These mechanisms work synergistically to improve plant health, increase crop yields, and contribute to sustainable agricultural practices.

Azospirillum, a nitrogen-fixing biofertilizer is called a biofertilizer. An essential ingredient for all plants is nitrogen. *Azospirillum lipofereum* is an extremely helpful bacterium for roots and soil. It is a nitrogen-fixing associative symbiotic bacterium. It is present in the soil around and on the surface of plant roots.

Isolation and mass multiplication of *Azospirillum* from soil or plant roots in *in-vitro* condition involves a series of controlled laboratory procedures to ensure the accurate identification and cultivation of this beneficial bacterium. Carrier-based inoculants of *Azospirillum* are a key component in modern sustainable agriculture. They enhance plant growth, improve soil health,

and contribute to increased crop yields. By using effective carrier materials and following proper preparation protocols, high-quality *Azospirillum* inoculants can be produced and utilized to benefit agricultural practices globally.

Azospirillum's associations with various rhizosphere bacteria have a significant impact on sustainable agriculture. Plant growth, soil fertility, and stress resistance can all be considerably increased by co-inoculating with *Rhizobium*, mycorrhizal fungi, phosphate-solubilizing bacteria, rhizobacteria that promote plant development, and decomposer microorganisms.

5.9 SELF ASSESSMENT QUESTIONS

5.9.1 Multiple Choice Questions

1. Which of the following is the primary function of *Azospirillum* in agriculture?

    a) Nitrogen fixation
    b) Phosphate solubilization
    c) Production of antibiotics
    d) Decomposition of organic matter

2. *Azospirillum* is mainly associated with the rhizosphere of which type of plants?

    a) Aquatic plants
    b) Cereal crops
    c) Fruit crops
    d) Leguminous crops

3. Which of the following media is commonly used for the isolation of *Azospirillum*?

    a) MacConkey Agar
    b) Potato Dextrose Agar (PDA)
    c) Sabouraud Dextrose Agar
    d) Nitrogen-Free Malate (NFM) medium

4. What is the optimal temperature range for the growth of *Azospirillum*?

    a) 10-15°C
    b) 20-30°C
    c) 35-40°C
    d) 45-50°C

5. Which of the following carrier materials is NOT commonly used for *Azospirillum* inoculants?

    a. Charcoal
    b. Gelatin

c. Peat
d. Talc

6. What is the typical shelf life of properly stored *Azospirillum* inoculants?

a) 1-2 months
b) 3-4 months
c) 6-12 months
d) 18-24 months

7. Which plant hormone is primarily produced by *Azospirillum* to promote plant growth?

a) Auxins
b) Gibberellins
c) Cytokinins
d) Ethylene

8. Which method is commonly used to ensure the sterility of carrier materials before inoculation with *Azospirillum*?

a) Autoclaving
b) Boiling
c) Dry heat sterilization
d) Sun drying

9. Which of the following is a benefit of using *Azospirillum* inoculants in agriculture?

a) Increased pest resistance
b) Enhanced nitrogen availability
c) Reduced soil erosion
d) Improved water retention in soil

10. During the mass multiplication of Azospirillum, what is the purpose of using a rotary shaker?

a) To maintain temperature
b) To sterilize the medium
c) To mix the carrier material
d) To provide aeration and agitation

5.9.2 Fill in the Blanks

1) *Azospirillum* is known for their ……. activities.

2) *Azospirillum*, a …….. bacteria closely associated with cereals and grasses.

3) It is a Gram-negative motile bacteria belonging to the family ……

4) In the rhizosphere, *Azospirillum* fixes nitrogen ……

5) Carrier-based inoculants of *Azospirillum* are a key component in ……...

Answer key

5.9.1: *1-(a); 2-(b); 3-(d); 4-(b); 5-(b); 6-(c); 7-(a); 8-(a); 9-(b); 10-(d)*

5.9.2: 1) plant growth promoting (PGP); 2) free-living nitrogen-fixing; 3) Rhodospirillaceae; 4) independently; 5) modern sustainable agriculture

5.10 REFERENCES

- Baldani, J.I., Reis, V.M., Videira, S.S., Boddey, L.H., & Baldani, V.L.D. (2014). The art of isolating nitrogen-fixing bacteria from grasses: a practical approach. In Handbook for *Azospirillum* (pp. 1-22). Springer, Cham.
- Bashan, Y., Holguin, G., & de-Bashan, L.E. (2004). *Azospirillum*-plant relationships: Physiological, molecular, agricultural, and environmental advances (1997-2003). *Canadian Journal of Microbiology*, 50(8), 521-577.
- Bhattacharyya, P.N., & Jha, D K. (2012). Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. *World Journal of Microbiology and Biotechnology*, 28, 1327-1350.
- Fages, J. (1994). *Azospirillum* inoculants and field experiments. In Y. Okon (Ed.), *Azospirillum*/Plant Associations (pp. 87-110). CRC Press.
- Okon, Y., & Labandera-Gonzalez, C.A. (1994). Agronomic applications of *Azospirillum*: An evaluation of 20 years worldwide field inoculation. *Soil Biology and Biochemistry*, 26(12), 1591-1601.
- Sharma, A. K., & Sharma, R. (2012). Biofertilizers for sustainable agriculture: A review. *Agricultural Reviews*, 33(2), 113-128.
- Verma, J.P., Yadav, J., Tiwari, K.N., & Lavakush. (2010). Impact of plant growth promoting rhizobacteria on crop production. *International Journal of Agricultural Research*, 5(11), 954-983.
- Vessey, J.K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil*, 255(2), 571-586.

5.11 SUGGESTED READINGS

- Kannaiyan, S. (2002). *Biofertilizers Technology*. Scientific Publishers.
- Kochhar, S.L., Economic Botany: A comprehensive study, Cambridge University Press, 2016 680 pages.
- Mishra, A. K., & Prasad, K. (2006). Isolation and characterization of Azospirillum from maize rhizosphere. *Journal of the Indian Society of Soil Science*, 54(4), 568-574.

- Rai, R., Singh, N.P., & Sachan, R.C. (2001). Mass multiplication and shelf life of Azospirillum biofertilizer. *Indian Journal of Agricultural Sciences*, 71(10), 645-647.
- Subba Rao, N.S. (1993). *Biofertilizers in Agriculture and Forestry*. Oxford & IBH Publishing Co. Pvt. Ltd.

5.12 TERMINAL QUESTIONS

5.12.1 Short Answer Type Questions

5. Explain in brief about applications of *Azospirillum* in Agriculture.
6. Which selective media is commonly used for isolating *Azospirillum*?
7. Describe in brief about mass multiplication of *Azospirillum*?
8. How does *Azospirillum* contribute to plant growth and soil fertility?
9. How does *Azospirillum* improve soil fertility?

5.12.2 Long Answer Type Questions

5. What are the methods for isolating *Azospirillum* species from soil samples?
6. What is *Azospirillum*? Describe in detail its characteristics and Mechanisms of Plant Growth Promotion.
7. Give a detailed account on associative effect of *Azospirillum* with different microorganisms.
8. Describe in detail about carrier-based inoculants of *Azospirillum.*

UNIT-06 *AZOTOBACTER*: CLASSIFICATION AND IMPORTANT CHARACTERISTICS

Contents

6.1 OBJECTIVES

After reading this unit learners will be able:

- To know the classification and important characteristics of *Azotobacter*
- To know the crop response to *Azotobacter* inoculums
- To know the maintenance and mass multiplication of *Azotobacter*

6.2 INTRODUCTION

Azotobacter is a genus of free-living or motile diazotrophic bacteria that are oval or spherical in shape, form thick-walled cysts, and create huge amounts of capsular slime. They are aerobic, free-living soil microorganisms that play an important role in nitrogen fixation by binding atmospheric nitrogen that plants cannot access and releasing it in the form of ammonium ions into the soil. *Azotobacter* is found largely in alkaline soils and aquatic habitats, as well as on some plants (Gandora et al. 1998; Tejera et al. 2005; Kumar et al. 2007). The usage of *Azotobacter* biofertilizer is well-known throughout the world. In addition to being great biofertilizers, *Azotobacter* is employed to make food additives and biopolymers (Nongthombam et al. 2021).

The first representative of the genus *Azotobacter chroococcum* was discovered and described in 1901 by Dutch microbiologist and botanist Martinus Beijerinck (Beijerinck 1901). The genus *Azotobacter* has 7 different known species which includes *A. croococcum, A. beijerinckii, A. salinestris, A. paspali, A. armeniacus, A. nigricans* and *A. vinelandii* **(**Garrity et al. 2015; Alhia and Hassan 2010). These bacteria use atmospheric nitrogen gas to synthesise cell proteins (Bishop et al. 1986). This cell protein will be mineralized once the *Azotobacter* cell dies, contributing nitrogen to crop plants (Tchan et al. 1983). *Azotobacter* spp. have been reported to be sensitive to temperatures above 35^{o}C, acidic pH, and high salt levels (Baral and Adhikari 2013). This bacterium improves crop growth and yield by activating rhizospheric microorganisms (Parmar and Dufresne 2011), biosynthesizing active substances and generating phytopathogenic inhibitors (Franche et al. 2009).

6.3 CLASSIFICATION

According to Becking (1974), the eighth edition of Bergey's handbook defines the family *Azotobacter*aceae as aerobic, heterotrophic, gram-negative bacteria that may fix nitrogen nonsymbiotically under normal atmospheric partial pressure of oxygen. Thompson and Skerman (1979) deduced from the numerical taxonomy that "the Gram negative bacterial genera that are non-spore former, free nitrogen fixer and not found similarity with genera of other families" make up the Azotobacteraceae family. By 1901, Beijerinck Martinus had identified and

characterised the most notable separate genus, *A. chroococcum*. He identified and named *A. chroococcum* as the leading aerobic nitrogen fixer that is free-living (Beijerinck, 1901). Following Lipman's 1903 description of *A. vinelandii*, the organism was renamed *A. beijerinckii* (Lipman, 1903) in Beijerinck's honour. The Russian scientist Nikolai Krasilnikov discovered *A. nigricans* in 1949 (Krassilnikov, 1949). *A. nigricans subsp. achromogenes* and *A. nigricans subsp. nigricans* are the two subspecies that Thompson and Skerman (1981) separated it. *A. armeniacus* was proposed by Thompson and Skerman in 1981. Page and Shivprasad (1991) reported on the air-tolerant and microaerophilic form of *A. salinestris* that was dependent on sodium ions. The taxonomic classification of *Azotobacter* is:

Kingdom Bacteria

Phylum Proteobacteria

Class Gammaproteobacteria

Order Pseudomonadales

Family *Azotobacter*aceae

Genus *Azotobacter*

Azotobacter is a type of nonendospore-forming, Gram-negative bacteria. The genera of *Azomonas, Beijerinckia, Derxia*, and *Azotobacter* are primarily responsible for some of the species that produce the inactive structures known as cysts. The *Azotobacter* exhibits both motile and nonmotile characteristics and it is catalase and oxidase positive nature.

In 1901, Beijerinck, who, studying the Chemolithotrophy, was attracted by nitrogen fixer *Azotobacter* and extensively studied the melanin producing coccus, *A. chroococcum* and the extensive capsule producing nitrogen fixer *A. agilis*, which was found in Holland soil. The Beijerinck isolated the *A. chroococcum*, the first species of *Azotobacter* from the Holland soil (Beijerinck, 1901). Thereafter in the succeeding period, numerous other types of *Azotobacter* collection have been isolated from rhizosphere and soil which were categorized under the family Azotobacteraceae. Lipman (1904) reported *A. vinelandii*, *A. beijerinckii*, respectively, while Krassilnikov (1949) and Dobereiner (1966), isolated and characterized unique *Azotobacter* having specific association with wild grassroots *Paspalum notatum*, i.e., *A. paspali*. Thompson and Skerman in 1981, reported *A. armeniacus* and in 1991 *A. salinestris* by William Page and Shivprasad (1991).

Based on a number of characteristics, the *Azotobacter*aceae family was divided into two main genera: *Azotobacter* and Azomonas. Six main species of the genus *Azotobacter* are distinguished by their ability to fix nitrogen in the atmosphere. *Azotobacter* is an aerobic bacteria that can grow in low oxygen environments or in media deficient in nitrogen. The genus has six primary species

have been recognised and thoroughly investigated. These are: *A. chroococcum*, *A. vinelandii*, *A. beijerinckii*, *A. nigricans*, *A. armeniacus* and *A. paspali*.

In addition to the species already described, Page and Shivprasad (1991) discovered a nitrogen fixer that forms cysts from the saline soil surface of Alberta, Canada. The organism was identified as *A. salinetris* after exhibiting particular additional characteristics, such as the presence of Na+/succinic acid efflux. *A. chroococcum* is the most often found species in soil. Based on the production of microcysts, the genus *Azotobacter* distinguished itself from Azomonas. Whereas Azomonas never produced the microcyst, which is a dormant spore-like structure while *Azotobacter* does. In addition, *Azotobacter* consistently exhibits a higher GC content—63–67.5%—than Azomonas, which had a GC concentration of 52–59 mol%.

The typical genus was allocated to the family Azotobacteraceae (Pribram, 1933), but after the 16S rRNA sequencing study, they were shifted to the family Pseudomonadaceae. The phylogenetic study in 2004 discovered that *A. vinelandii* fits the equal clade as *Pseudomonas aeruginosa* (Rediers et al., 2004) bring about with the concept that the genera *Azotobacter*, *Pseudomonas* and *Azomonas* are correlated and might be alternative (Young and Park, 2007). The taxonomic dispute led to the use of the immunological attractions that exist between the various Azotobacteraceae family species through the Immunoelectrophoresis technology. Compared to the strains of *A. paspali* and *A. vinelandii*, the *A. chroococcum* was immunologically heterogeneous (Tchan et al., 1983).

6.4 IMPORTANT CHARACTERISTICS

Azotobacter has distinct physiological and morphological properties (Table 6.1) that distinguish it from other Gram-negative and nitrogen fixers. *Azotobacter* is Gram-negative, blunt to oval short rods with 1.5-2 mm or more in diameter, having soil as common habitat, besides that aquatic plant rhizospheric and phylospheric were also the identified habitat. They are generally aerobic and capable to fix the atmospheric nitrogen in presence of suitable carbon sources.

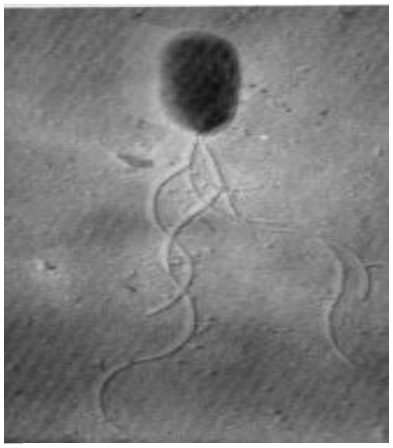


Fig.6.1: Electron micrograph of Azatobacter chroococcum cell (Bisset and Hale 1953)

***Habitat*:** They can be found in rocky areas, hot steppes, dry deserts, foothill peaks, valleys, and dry sands. They are also find in the soil of many different topographical regions since they can withstand cold and thrive in aquatic environments, such as some species have been reported in the Arctic and Antarctic (Garg et al., 2001). *Azotobacter* species exist in a variety of soil habitats from slightly acidic to alkaline soil while some species such as *A. paspali* is associated with plant roots. However, *Azotobacter* species demand high minerals such as

phosphates, their populations are often abundant in fertile soil. According to Ramaswamy et al. (1977), black soil had a higher concentration of *Azotobacter* than red soil, and as depth increased, the number of *Azotobacter* was found to decrease. The *Azotobacter* population in soil is mostly affected by other soil microbiota.

***Colonies*:** *Azotobacter* species are chemoheterotrophic, nitrogen fixers, and motile, except for *A. beijerinckii* and *A. nigricans*, which have peritrichous and polar flagella. They often form diffusible and nondiffusible large colonies on a nitrogen-free substrate containing sugar or alcohol as carbon sources. The colonies are generally smooth, opaque, and somewhat convex glistening, though the nature of the colony varies depending on the medium and type of carbon sources used (Thompson and Skerman, 1979); for example, larger, more transparent and viscous colonies appeared on media containing sucrose and raffinose than nitrogen-free media with glucose.

Table 6.1: Primary morphological characters on Burks medium

***Azotobacter* species/ Gram nature**	**Cell shape**	**Cell size (L×W) mm**	**Pigment production**	**Motility**
A. vinelandii/ (gram −ve)	Round-ended rods	3.0-4.5×1.5-2.4	Yellow-green, fluorescent, water-soluble pigment	+ ve
A. beijerinckii/ (gram −ve)	Rods or ellipsoidal	3.2-5.3×1.7-2.7	Yellowish or cinnamon	−ve
A. chroococcum/ (gram −ve)	Rod-, oval-void, or coccus	3.0-7.0× 1.5-2.3	Brown or blackish-brown	+ve
A. paspali/ (gram −ve)	Long filaments	7-12×1.3-1.7	Yellow-green, fluorescent or red-violet, water-soluble pigment	+ve
A. armeniacus/ (gram −ve)	Bluntly rounded rods	5.0-5.7 × 1.7-2.0	Diffusible brown-black or red-violet	+ve
A. nigricans/ (gram −ve)	Bluntly rounded rods	4.1-4.9 × 1.5-2.7	Yellow non diffusible pigment	−ve
A. salinetris/ (gram −ve)	Rods	2-4×4.5-5.0	Black brown	+ve

(Source: Nongthombam et al. 2021)

***Pigments*:** The species is further distinguished by the development of grey-brown and black nondiffusible pigments. The pigment generation is also discovered to be media component dependent; for example, *A. chroococcum* produce a nondiffusible brown-gray pigment. Pigment synthesis has also been found to be reliant on media components. For example, *A. chroococcum* produces a non-diffusible brown-grey pigment on Stainers media (Pribram, 1933) fortified with 0.2% gluconate and a black diffusible pigment on benzoate-enriched medium. However, *A. nigricans* and *A. armeniacus* don't produce brown diffusible pigment when exposed to benzoate, but *A. vinelandii* develops brown-black pigment.

Nutrition: *Azotobacter* is a chemoheterotroph which utilises sugars like glucose, fructose, ethanol, acetate, carbinol fumarate, pyruvate, and other organic acids as a carbon source. It is capable of using other nitrogen compounds but has poor or no ability to consume nitrate. *Azotobacter* does not require organic growth factors, instead, it requires minerals such as vanadium and molybdenum, which are key components of the nitrogen fixation mechanism.

Growth: The optimum temperature of growth for most *Azotobacter* members is 28-37^{o}C, but another cardinal temperature varies depending on the species. For example, some species require a minimum temperature of 14^{o}C for growth, while *A. beijerinckii* and *A. nigricans* require a minimum temperature of 9^{o}C and *A. armeniacus* requires a minimum growth temperature of 28^{o}C. Although the optimal temperature for most *Azotobacter* is 32^{o}C, *A. paspali* and *A. vinelandii* prefer 37^{o}C. The optimal temperature has also been observed to vary by strain; for example, certain strains of *A. chroococcum* have an optimum temperature of 37°C. Temperature tolerance was also shown to vary depending on the strain isolated from the subtropical and temperature regions. It was discovered that all *Azotobacter* survive at 50^{o}C for 10 minutes, however no species can survive for 10 minutes at 60^{o}C treatment or incubation. Similarly, *A. nigricans* and *A. armeniacus* cannot develop at 37 degrees Celsius. *Azotobacter* growth has been recorded at pH values ranging from acidic to alkaline, 4.8-8.5.

Azotobacter thrives best in a nitrogen-free medium with an adequate carbon supply. Although the organism is catalase positive and aerobic in nature, a low oxygen concentration condition is required for better nitrogen fixation because nitrogen fixation is classified as a reductive process involving the major oxygen labile enzymes, which are inactivated in the presence of oxygen.

Reproduction**:** Like all other bacteria, *Azotobacter* reproduces via simple division (fission) and respires aerobically to produce energy. After the unfavourable conditions are once again met, the cysts germinate and become vegetative cells, which subsequently multiply by straightforward cell division. During the time that the cysts are forming, they are unable to reproduce.

6.5 CROP RESPONSE TO AZOTOBACTER INOCULUM

In 1902, two researchers Gerlach and Voel first described the use of *Azotobacter* as a biofertilizer to provide nitrogen to soil that has biologically fixed as one of this microbial activities (Tien et al. 1979). As a potential substitute for inorganic nitrogen sources (such as urea), *Azotobacter* can be a valuable addition to chemical fertilisers since it supplies nitrogen in the form of ammonia, nitrate and amino acids without posing a risk of overdosing. The aforementioned bacterium is involved in a variety of processes that promote plant growth, including the fixing of atmospheric N_2 and the production of PGRs, including auxins, cytokinins, gibberellins, amino acids and vitamins, as well as phosphate solubilization (Barea et al. 1974; Apte and Shende 1981; Damir et al. 2011; Chahal and Chahal 1988; Mishra et al. 2013). A report shows that this bacterium possesses a high range of N_2 fixation in the amount of 2-15mg N fixed

per gram of glucose consumed and also has a high acetylene reduction assay (Apte and Shende1981). *Azotobacter chroococcum* helps in reducing the infection of nematodes by 48% which is followed by *Azospirillum* (4%) and *Pseudomonas* (11%) (Mishra et al. 2013). *Azotobacter* stimulates rhizospheric microorganisms, produces phyopathogenic inhibitors, and produces physiologically active chemicals, all of which have positive effects on crop growth and yield (Table 6.2) (Lenart 2012). By accelerating up the mineralization of organic wastes in the soil and preventing the absorption of heavy metals, *Azotobacter* makes certain nutrients such as carbon, nitrogen, phosphorus, and sulphur, available to the soil (Levai et al. 2008).

Table 6.2: Azotobacter effects on crop yield

SN	**Crop**	**Yield increased over yield obtained from chemical fertilizers (%)**
1	Rice	5
2	Sorghum	15-20
3	Wheat	8-10
4	Maize	15-20
5	Potato	13
6	Tomato	2-24
7	Carrot	16
8	Cauliflower	40
9	Cotton	7.27
10	Sugarcane	9-24

(Source: Bhattacherjee and Dey 2014)

6.5.1 Effects of Azotobacter on growth and yield of crops

The deposition of dry matter is increasing in plants that have been treated with *Azotobacter*. The production of a chemical like a plant growth regulator and fixed nitrogen triggers the development of leaves, roots, branching, blooming and fruiting. Moreover, it makes plants more resilient to dry conditions and water shortages (Zena and Peru 1986). The rate at which a plant's leaf area grows impacts its photosynthetic capability, which improves produce assimilation and increases yield. For example in a research, potato yields have risen by 33.3% and 38.3% when *Azotobacter* sp is used (Zena and Peru 1986). In a research conducted by Triplett (1996) concluded that a maize plant that doesn't need nitrogen fertiliser for maximum growth and output is most likely to be accomplished through the establishment of the diazotrophic endophytic association in maize. After *Azotobacter* inoculation, yield increases vary from 2 to 45% in vegetables, 9 to 24% in sugarcane, and 0 to 31% in maize, sorghum, mustard, etc. (Pandey and Kumar 1989a, b). In contrast to uninoculated treatments, the other study found that seed inoculation with *Azotobacter* increased grain and stover yield (Laxminarayan 2001). According to Singh and Dutta (2006), inoculation with *Azotobacter* resulted in a considerable increase in seed production (7.86q ha^{-1}) in mustard and rapeseed.

The growth of rice plants was affected by the dual inoculation of *A. chroococcum* and *P. indica*, which resulted in beneficial responses on shoot length, root length, fresh shoot and root weight, dry shoot and root weight, and panicle number (Kamil et al. 2008). Although a significant body of experimental research has been conducted on the stimulation of plant development in general by *Azotobacter*, the precise mechanism by which *Azotobacter* promotes plant growth remains unclear. Three possible mechanisms have been proposed to explain the action: (i) N_2 fixation; delivering combined nitrogen to the plant; (ii). the production of phytohormone – like substances that change the plant growth and (iii). morphology and bacterial nitrate reduction, thereby increasing nitrogen accumulation in inoculated plants.

6.5.2 Nitrogen fixation

Biological nitrogen fixation plays an important role in maintaining soil fertility (Vance and Graham 1995). *Azotobacter* is used for studying nitrogen fixation and inoculation of plants due to its rapid growth and high level of nitrogen fixation. They are extremely tolerant to oxygen while fixing nitrogen and this is due to respiration protection of nitrogenase (Hakeem et al. 2016). They have respiratory protection, uptake of hydrogenases and switch on-off mechanisms for protection of nitrogenase enzyme from oxygen (Chhonkar et al. 2009). *Azotobacter chroococcum* is revealed to have uptake hydrogenase which metabolises hydrogen (H_2) evolved during nitrogen fixation (Partridge et al. 1980). Nitrogen can be transformed by *Azotobacter* into ammonia, which plants can then absorb easily. It has been established by Iswaran and Sen (1960) that optimal calcium nutrition levels are necessary for growth of *Azotobacter* and their nitrogen fixing capacity. However, it was discovered that as the N level rose, the bacteria effectiveness decreased (Soleimanzadeh 2013). As non-symbiotic heterotrophic bacteria, *Azotobacter* spp. may fix up to 20 kg of nitrogen per hectare annually (Kizilkaya 2009). This means that they can partially replace mineral nitrogen fertilisers in crop production (Hajnal et al. 2004). The role of *Azotobacter* in nitrogen fixation involved in nitrogen cycle in the biosphere is well known.

6.5.3 Growth promoting and other substances produced by Azotobacter

In addition to their capacity to fix nitrogen, *Azotobacter* species are known for their production of various growth hormones (IAA and other auxins, including gibberllins and cytokinins), vitamins, antibacterial and antifungal compounds, and siderophores (Barea and Brown 1974; Pandey and Kumar 1989b). These compounds can either directly or indirectly affect microbial activity and plant growth. Natural compounds called growth substances or plant hormones are produced by both microbes and plants. In plants and microbes, they can stimulate or inhibit specific physiological and metabolic processes. The growth of the closely related higher plants is influenced by these hormones that come from the rhizosphere or root surface. Auxins, cytokinins and GA-like compounds are produced by *Azotobacter*, and these growth materials are the main factor regulating the accelerated growth of plants (Azcorn and Barea 1975). In order to combat

numerous plant diseases, *Azotobacter* species are capable of producing antifungal substances (Jen-Hshuan 2006).

It has been observed that a variety of *Azotobacter* strains produces pigments that are essential to microbial metabolism. For instance, *A. chroococcum* produces the water-soluble, dark-brown pigment melanin. It is believed that this mechanism, which takes place at high metabolic levels during nitrogen fixation, shields the nitrogenase system from oxidation (Shivprasad and Page 1989).

Azotobacter increases soil microbial activity by acting as an indicator. According to reports on *Azotobacter*, dehydrogenase activity—which indicates the degree of oxidation reduction, or the degree of metabolic activity of microorganisms—is a more accurate measure of the overall microbiological activity of soil (Jafari et al. 2012). Mutant *A. vinelandi* is more suitable for the biosynthesis of alginate in view of its latent utilization as a food stabilizer has better qualitative properties (Chen et al. 1985). Various strains of *Azotobacter* have the ability to produce amino acids when cultivated in culture media supplemented with various sources of carbon and nitrogen (Lopez et al. 2005). Substances like amino acid produced by these rhizobacteria are involved in many processes that explain plant-grown promotion. In contrast to non-inoculated control plants, *Azotobacter*-inoculated plants had increased levels of chlorophyll, nitrogen, phosphorus, potassium, and protein content (Naseri et al. 2013).

6.5.4 Azotobacter in seed inoculation and nutrient uptake

When *Azotobacter* is inoculated into seeds, it aids in the absorption of certain micronutrients such as zinc and iron as well as macronutrients like phosphorus and nitrogen (Sahoo et al. 2014). Additionally, these bacterial strains are employed to enhance the nutritional value of maize, wheat and rice. Due to the assistance of *Azotobacter* in providing nitrogen to standing crops, crop yields are significantly boosted (Basel and Hassan 2010). In a greenhouse experiment, it was discovered that the protein and carbohydrate content of two types of maize (Inra 260 and Inra 210) with *Azotobacter* inoculated seeds increased (Yasari and Patwardhan 2007). The crop's biomass increases when manure and *Azotobacter* are applied together (Bhattacherjee and Dey 2014).

6.6 MASS MULTIPLICATION AND MAINTENANCE OF AZOTOBACTER

Azotobacter is mass-multiplicated by an artificial inoculation procedure. The mass manufacturing of *Azotobacter* involves the following procedures.

1. **Inoculants:** There are two methods to obtain the appropriate bacterial strains: (A) directly purchase the desired strains from the identified authentic sources; (B) Isolation of bacteria from soil:

A. Purchase of desired strains direct from the identified authentic sources: The bacterial strain which have the highest market demand is identified first. Purchase of the intended strains from the approved sources, such as regional MOA biofertilizer labs, IARI, Agricultural Universities, and select ICAR institutions. International suppliers including NifTAL, IRRI, and others are also available. These sources uphold pure cultures. Using standard procedures, they must be further subcultured and kept exclusively for mass manufacturing.

B. Isolation of bacteria: It is comparatively long method to get the bacterial strains. In this method following steps are required:

i. *Collection of the bacteria*: The soil samples should be collected near the rhizosphere region of the plants. Under aseptic conditions, the rhizospheric soil samples should collect carefully and put in to the labeled plastic bags.

ii. *Incubation:* The air dried soils should prepared to isolate the bacteria by spread plate technique in Burk's culture media which is a most suitable medium for growth and cultivation of nitrogen fixing bacteria as well as diazotrophs such as *Azotobacter* species (Samal et al. 2020). Isolation of the bacteria from the rhizosphere soil by making a series of dilution of soil from 10-1 to 10-7 on *Azotobacter* media and incubation for 48 h at 30ºC. Bacterial culture should repeat for three to four times to obtaining the purity of the cultured of bacteria (Whitman 2010).

iii. *Identification and characterization*: Further consideration is given to the biochemical and enzymatic activities of the isolated bacterial strains from the rhizospheric soil samples. Using the cell Gram stain and the colony test (which measures the form, colour, margin, texture and nature of the colony) isolates should be described. Several biochemical examines including nitrate reductase, citrate utilisation, methyl red, VP, catalase (cover-slip method), oxidation test etc are used to identify the biochemical features of the strain.

2. Growth: *Azotobacter* is often cultured on a nitrogen-free solid medium. The mother culture of *Azotobacter* is frequently (6 months) transferred to a new solid medium to revive the growth. To ensure that the culture is kept in good condition, this process is performed on a regular basis.

3. Production: In the large scale production following procedure is required:

i. **Mother culture**: A pure growth of any organism on a small scale is called as a mother culture. Mother culture is always prepared in 500 or 1000 ml capacity conical flask and then this mother culture is used for further production. This is accomplished by taking 1L conical flasks, filling them with 500 ml of nitrogen-free medium of broth, plugging them with non-absorbent cotton, then sterilising

them in an auto slave for 15–20 minutes at 75 lbs of pressure. Flasks are then inoculated with mother culture with the help of inoculating needle aseptically.

The flasks are placed in a shaker and stirred for duration of 72 to 90 hours in order to get the maximum growth of bacteria in the broth. Bacteria are multiplied by binary method i.e. cell division. After about 90 days, the number of bacterial cell is estimated to become 100 crores per milliliters. The entire bacterial development in this broth indicates the presence of mother culture, which needs to be handled carefully because the preparation of mother culture affects the biofertilizer's quality and purity.

ii. **Production on a large scale:** *Azotobacter* is multiplied on a large scale by two ways viz. Fermenter and Shaker. Any microorganism can be multiplied most automatically and precisely in a fermenter. The medium is taken in a fermenter and subsequently sterilised in this technique. 1% mother culture is then introduced when the medium's pH has been adjusted. Concentrated broth is prepared by adjusting the temperature and oxygen supply to cause the *Azotobacter* to grow to its maximum potential. Once the carrier has been sterilised, the concentrated culture broth is combined with it to create biofertilizers. A suitable fermenter is chosen in accordance with supply and demand.

In the 2nd method i.e., shake method, a suitable medium is prepared transferred to conical flask of suitable capacity. These flasks are then sterilized in an autoclave at 15 lbs pressure for 15 minutes. Each flask is inoculated with 10 ml mother culture and they are transferred to shaker for multiplication where they are kept for 72-90 hours. This broth is mixed with a suitable carrier previously sterilized. Thus biofertilizer is prepared, filled in plastic bags and stored in cool place.

iii. **Selection of carrier**: A carrier is nothing but a substance which has high organic matter, higher water holding capacity and supports the growth of organism. In order to transport the biofertilizer and becomes easy to use the suitable carrier is selected. Generally Lignite cool, compost and peat soil are suitable carriers for *Azotobacter*. Out of these carriers lignite is most suitable for this organism, since it is cheaper, keeps organism living for longer period and does not lower the quality of bio-fertilizers.

4. **Mixing and packing:** There are 2-3 alternatives depending upon the sophistication and automation of the unit.
    i. **Under non sterile system:** The broth is harvested from the fermenter into sterilized carrier - the mixing is done manually under aseptic condition and packed in polythene bags of desired quantity.
    ii. **In a slightly upgraded method: T**he broth and sterilized carrier are mixed mechanically in a blender and the material is packed using semiautomatic packing

and sealing machine. In a slightly modified method some units are packing by delivering desired quantities of carrier and broth simultaneously from separate pipe conveyance system into the polythene bags.

iii. **Under a completely sterile system:** The carrier is taken in autoclavable polypropylene bags and pre sealed - into which the broth from fermenter is directly injected with the help of dispenser. The injection hole is immediately sealed. The packets are kept in incubation room for about a week before transferring to store room.

Sterile system of packing using auto syringe and dispenser is recommended to be the best method.

Maintenance of *Azotobacter* cultures

- For routine maintenance *Azotobacter* should be subcultured at monthly or bimonthly with sucrose as carbon source.
- The culture can also be preserved by use of heavy mineral oil (paraffin). Usually *Azotobacter* survives for many months. Routine sub-culturing at 6-monthly interval is adequate.
- The cultures are grown on slants in tubes. Sterile mineral oil is added to these tubes after growth has occurred to completely cover the slope. These can be maintained on the lower most rack of refrigerator.
- Mineral oil is sterilized in an oven for 3 days for 90 min each day at 160^{o}C.
- *Azotobacter* can also be maintained as glycerol based cultures in a deep freezer.
- Broth cultures are prepared. Glycerol solution (60%) is sterilized by autoclaving and is added to the broth culture to get a final glycerol concentration of 15% v/v.

As per Bureau of Indian Standards (BIS) specifications, certain tests are required to be conducted, like no. of cells, colony character, reaction etc. Cell number at the time of manufacture should not be less than 10^7 per gram of carrier material for *Azotobacter*. Similarly, the number of cell count and permissible contamination at expiry dates are also specified. As certification arrangements are not in place at present, legislation for quality monitoring and accredited labs for testing may be needed in future to ensure proper quality and to promote this product.

As per Indian Standards Institution (ISI) (ISI) standards, one gram of biofertilizer immediately after it is prepared should have one crore cells of bacteria and 15 days before expiry date one gram of biofertilizer should have 10 lakh bacteria. If biofertilizer is stored at 15-20^{o}C then it will remain effective for 6 months. However, at 0-4^{o}C (cold storage) the bacteria will remain active for 2 years. The storage periods are decided after testing the biofertilizer for that particular storage conditions, such temperature and humidity (Table 6.3).

Table 6.3: Indian standard specification for Azotobacter

SN	Parameters	*Azotobacter* inoculation IS- 9138-2002
1.	Base	Carrier based
2.	Viable cells	10^7 cells/g of carrier within 15 days manufacture
3.	Cell no at the time of expiry	10^6 cells/g within 15 days before expire date
4.	Shelf life or expire period	6 month from the date of manufacture
5.	Permission contamination	No contamination at 10^5 dilution
6.	pH	6.0-7.5
7.	Moisture	35-40%
8.	Strain	e.g., *A. chrococcum*
9.	Carrier	Should pass through 100 micron IS sieve
10.	Efficiency test	Minimum amount of N-fixation not less than 10 mg/g of source utilization

6.7 SUMMARY

The present unit discuss about classification and important characteristics of *Azotobacter,* their response in crop yield and maintenance and mass multiplication of *Azotobacter* as biofertilizer. *Azotobacter* is a genus of free-living or motile diazotrophic bacteria that are oval or spherical in shape, form thick-walled cysts and create huge amounts of capsular slime. The bacterium belongs to family Azotobacteraceae and is aerobic, heterotrophic, gram-negative bacteria that play an important role in nitrogen fixation. The usage of *Azotobacter* as biofertilizer is well-known throughout the world. The genus *Azotobacter* has 7 different known species. This bacterium improves crop growth and yield by activating rhizospheric microorganisms, biosynthesizing active substances and generating phytopathogenic inhibitors. *Azotobacter* has distinct physiological and morphological properties that distinguish it from other Gram-negative and nitrogen fixers. They can be found in rocky areas, hot steppes, dry deserts, foothill peaks, valleys, and dry sands. Like all other bacteria, *Azotobacter* reproduces via fission.

The bacterium is involved in a variety of processes that promote plant growth, including the fixing of atmospheric N_2 and the production of PGRs, including auxins, cytokinins, gibberellins, amino acids, and vitamins, as well as phosphate solubilisation. *Azotobacter* is mass-multiplicated by an artificial inoculation procedure. The mass manufacturing of *Azotobacter* involves the following steps: inoculants, Growth, Production, Mixing and packing. As per Bureau of Indian Standards (BIS) and Indian Standards Institution (ISI) specifications, certain tests are required to

be conducted, like no. of cells, colony character, reaction etc. Cell number at the time of manufacture should not be less than 10^7 per gram of carrier material for *Azotobacter*. Similarly, the number of cell count and permissible contamination at expiry dates are also specified. As certification arrangements are not in place at present, legislation for quality monitoring and accredited labs for testing may be needed in future to ensure proper quality and to promote this product. If biofertilizer is stored at 15-20^{o}C then it will remain effective for 6 months. However, at 0-4^{o}C (cold storage) the bacteria will remain active for 2 years. The storage periods are decided after testing the biofertilizer for that particular storage conditions, such temperature and humidity.

6.8 GLOSSARY

- **Aerobic organisms:** Organisms that can utilize oxygen as the final electron acceptor during metabolism.
- **Ammonia fertilizer:** A material with a high concentration of nitrogen compounds put on soil to stimulate plant growth.
- **Azotobacter:** free-living or motile diazotrophic bacteria
- **Biofertilizer, biological fertilizer, organic fertilizer:** A biofertilizer is a natural fertilizer that helps to provide all the nutrients required by the plants and to increase the quality of the soil with a natural microorganism environment.
- **Capsule:** A gelatinous structure that surrounds some bacteria.
- **Coccus:** Spherical bacteria.
- **Colony:** A group of bacteria in a culture derived from the multiplication of single cell
- **Crop:** A cultivated plant grown to be harvested either to be used or to be sold
- **Culture medium:** A preparation containing nutrients and growth factors suitable for the cultivation of microorganisms.
- **Culture:** A growth of microorganisms.
- **Habitat:** The place or type of site where species and communities normally live or grow, usually characterized by relatively uniform physical features or by consistent plant forms, e.g. deserts, lakes and forest are all habitats.
- **Nitrification:** The bacterial oxidation of ammonium ($NH4^+$) to nitrate ($NO3^-$).
- **Nitrogen fixation:** The oxidation of nitrogen gas (N_2) to ammonia (NH_3) or nitric oxide (NO).
- **Nutrient cycling:** Transfers and chemical transformations of nutrients in ecosystems, including recycling through decomposition.
- **Nutrition:** The intake of food, and the interplay of biological, social, and economic processes that influence the growth, function and repair of the body.
- **Pest:** Any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products, livestock, food and storage products.

- **Soil fertility:** The ability of a soil to sustain plant growth by providing essential plant nutrients and favorable chemical, physical, and biological characteristics as a habitat for plant growth.
- **Species:** An interbreeding group of organisms that is reproductively isolated from all other organisms, although there are many partial exceptions to this rule in particular taxa.

6.9 SELF ASSESSMENT QUESTIONS

6.9.1 Multiple choice questions

1. Which of the following is not used as a bio-fertiliser?
   a. Bacteria b. Algae
   c. Cynobacteria d. Fungi

2. Which of the following is not a free-living Nitrogen-fixing bacteria?
   a. *Azotobacter* b. *Clostridium*
   c. *Klebsiella* d. *Xanthomonas*

3. Which of the following is an aerobic nitrogen-fixing bacterium?
   a. *Azotobacter* b. *Clostridium*
   c. *Rhodospirillum* d. *Rhodopseudomonas*

4. Presence of which of the following elements is required for nitrogen fixation?
   a. Phosphorus b. Carbon
   c. Silver d. Oxygen

5. The most quickly available source of nitrogen to plants are
   a. Amide fertilizers b. Ammonia fertilizers
   c. Nitrate fertilizers d. Ammonia nitrate fertilizer

6. Pick the correct statement
   a. Legumes do not fix nitrogen
   b. Legumes fix nitrogen independent of bacteria
   c. Legumes fix nitrogen through bacteria in their roots
   d. Legumes fix nitrogen through bacteria in their leaves

7. Which fertilizers when used in excessive amounts cause soil pollution that spreads to neighboring water bodies and cause water pollution too?
   a. Artificial b. Chemical
   c. Compost d. All of the above

8. Major useful product obtained from microbes
   a. Vitamin
   b. Single cell protein
   c. Antibiotic
   d. All of the above

9. Nitrifying bacteria are _____
   a. Gram negative
   b. Minute
   c. Rod shaped
   d. All of the above

10. Bacteria, as a group, are responsible for _____
   a. Nitrogen oxidation
   b. Sulfur oxidation
   c. Nitrogen fixation
   d. All of the above

6.9.2 True and false

1. *Azotobacter* is a genus of free-living or motile diazotrophic bacteria.
2. *Azotobacter* is an example of inorganic fertilizer.
3. In addition to being great biofertilizers, *Azotobacter* is employed to make food additives and biopolymers.
4. *Azotobacter* belongs to order Pseudomonadales.
5. *Azotobacter* cannot inoculated into seeds due to the hard seed coat.

6.9.3 Fill in the blanks

1. The groups of bacteria which have the ability to fix nitrogen from air to soil are _____ and _____.
2. The first representative of the genus _____ was discovered in 1901.
3. *Azotobacter* belongs to class _____
4. *Azotobacter* is a _____ in nutrition.
5. *Azotobacter* thrives best in a _____ medium with an adequate carbon supply.

Answer keys:

6.9.1: 1.d; 2.d; 3.a; 4.a; 5.c; 6.c; 7.b; 8.d; 9.d; 10.d

6.9.2: 1. True; 2. False; 3. True; 4. True; 5. False

6.9.3: 1. Symbiotic, non-symbiotic; 2. *Azotobacter chroococcum;* 3. Gammaproteobacteria; 4. Chemoheterotroph; 5. nitrogen-free

6.10 REFERENCES

- Apte, R. and Shende, S.T., 1981. Studies on *Azotobacter* chroococcum: I. Morphological, biochemical and physiological characteristics of Azotobacter chroococcum. Zentralblatt für

Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Zweite Naturwissenschaftliche Abteilung: Mikrobiologie der Landwirtschaft, der Technologie und des Umweltschutzes, 136(7), pp.548-554.

- Azcón, R. and Barea, J.M., 1975. Synthesis of auxins, gibberellins and cytokinins by Azotobacter vinelandii and Azotobacter beijerinckii related to effects produced on tomato plants. Plant and Soil, 43, pp.609-619.
- Baral, B.R. and Adhikari, P., 2013. Effect of Azotobacter on growth and yield of maize. SAARC Journal of Agriculture, 11(2), pp.141-147.
- Barea, J.M. and Brown, M.E., 1974. Effects on plant growth produced by Azotobacter paspali related to synthesis of plant growth regulating substances. Journal of Applied Bacteriology, 37(4), pp.583-593.
- Becking, S., 1974. Family Azotobacteraceae. In: Bergey's Manual of Systematic Bacteriology, vol. 1. Williams and Wilkins, London, pp. 219e230, 1984.
- Beijerinck, M.W., 1901. On oligonitrophilous bacteria. Koninklijke Nederlandse Akademie van Wetenschappen Proceedings Series B Physical Sciences, vol. 3, pp. 586-595, 3, pp.586-595.
- Bisset, K.A., Hale, C.M.F. 1953. The Cytology and Life-cycle of *Azotobacter chroococcum*. Journal of General Microbiology, 12;8(3):442-448.
- Bhattacharjee, R. and Dey, U., 2014. Biofertilizer, a way towards organic agriculture: A review. African Journal of Microbiology Research, 8(24), pp.2332-2343.
- Bishop, P.E., Premakumar, R., Dean, D.R., Jacobson, M.R., Chisnell, J.R., Rizzo, T.M. and Kopczynski, J., 1986. Nitrogen fixation by Azotobacter vinelandii strains having deletions in structural genes for nitrogenase. Science, 232(4746), pp.92-94.
- Chahal, P.P.K. and Chahal, V.P.S., (1988). Biological control of root-knot nematode of brinjal (Solanummelongena L.) with Azotobacter chroococcum. In US-Pakistan International Workshop on Plant Nematology, Karachi (Pakistan), 6-8 Apr 1986. NNRC.
- Chen, J.H., 2006. The combined use of chemical and organic fertilizers and/or biofertilizer for crop growth and soil fertility. In International workshop on sustained management of the soil-rhizosphere system for efficient crop production and fertilizer use (Vol. 16, No. 20, pp. 1-11). Land Development Department Bangkok Thailand.
- Chen, W.P., Chen, J.Y., Chang, S.C. and Su, C.L., 1985. Bacterial alginate produced by a mutant of Azotobacter vinelandii. Applied and environmental microbiology, 49(3), pp.543-546.
- Damir, O., Mladen, P., Božidar, S. and Srñan, N., 2011. Cultivation of the bacterium Azotobacter chroococcum for preparation of biofertilizers. African Journal of Biotechnology, 10(16), pp.3104-3111.
- Döberainer, J., 1966. Azotobacter paspali sp. nov., uma bacteria fi xadora de nitrogenio na rhizosfera de Paspalum. Pesquisa Agropecuaria Brasileira 1:357–365

- Franche, C., Lindström, K. and Elmerich, C., 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant and soil, 321(1), pp.35-59.
- Gandotra, V., Gupta, R.D. and Bhardwaj, K.K.R., 1998. Abundance of Azotobacter in great soil groups of North-West Himalayas. Journal of the Indian Society of Soil Science, 46(3), pp.379-383.
- Garg, S.K., Bhatnagar, A., Kalla, A. and Narula, N., 2001. In vitro nitrogen fixation, phosphate solubilization, survival and nutrient release by Azotobacter strains in an aquatic system. Bioresource technology, 80(2), pp.101-109.
- Garrity, G.M., Bell, J.A. and Lilburn, T., 2015. Proteobacteria phyl. nov. Bergey's Manual of Systematics of Archaea and Bacteria, pp.1-1.
- González-López, J., Rodelas, B., Pozo, C., Salmerón-López, V., Martínez-Toledo, M.V. and Salmerón, V., 2005. Liberation of amino acids by heterotrophic nitrogen fixing bacteria. Amino acids, 28, pp.363-367.
- Hajnal, T., Jarak, M. and Milosevic, N., 2004. Bacterization of maize: yield response of maize to inoculation. In Book of abstracts of the 10th international symposium on microbial ecology: isme-10, cancun, mexico (Vol. 207).
- Hakeem, K.R., Akhtar, M.S. and Abdullah, S.N.A. eds., 2016. Plant, soil and microbes. Springer.
- Hassan, MBA., 2010. The Effect of Azotobacter Chrococcum as Nitrogen Biofertilizer on the Growth and Yield of Cucumis Sativus. The Islamic University. http://hdl.handle.net/20.500.12358/21551
- Iswaran, V. and Sen, A., 1960. Inactivation of Azotobacter by heat. Curr Sci 27(9):341–342
- Jafari, T.H., Latkovic, D., Duric, S., Mrkovacki, N. and Najdenovska, O., 2012. Research. J Agric Sci, 44(2).
- Kamil, P., Yami, K.D., Singh, A., 2008. Plant growth promotional effect of Azotobacter chroococcum, Piriformospora indica and vermicompost on rice plant. Nepal Journal of Science and Technology, 9, pp.85-90.
- Kizilkaya, R., 2009. Nitrogen fixation capacity of Azotobacter spp. strains isolated from soils in different ecosystems and relationship between them and the microbiological properties of soils. J. Environ. Biol, 30(1), pp.73-82.
- Krassilnikov NA (1949) Opred Zlita baktzrij i aktinomyc etov. Mo skva
- Kumar, R., Bhatia, R., Kukreja, K., Behl, R.K., Dudeja, S.S. and Narula, N., 2007. Establishment of Azotobacter on plant roots: chemotactic response, development and analysis of root exudates of cotton (Gossypium hirsutum L.) and wheat (Triticum aestivum L.). Journal of basic microbiology, 47(5), pp.436-439.
- Laxminarayana, K., 2001. Effect of Azotobacter and Azospirillum on yield performance of maize in hilly regions of Mizoram. Indian J Hill Farm, 14(2), pp.134-137.

- Lenart, A., 2012. Occurrence, characteristics, and genetic diversity of Azotobacter chroococcum in various soils of Southern Poland. Polish Journal of Environmental Studies, 21(2).
- Lévai, L., Veres, S., Bákonyi, N. and Gajdos, É., 2008. Can Wood Ash and Bio-fertilizer Play a Role in Organic Agriculture?. Agronomski glasnik: Glasilo Hrvatskog agronomskog društva, 70(3), pp.263-272.
- Lipman, J.G., 1903. Report on the New Jersey Agricultural Experiment Station.
- Mishra, D., Rajvir, S., Mishra, U. and Kumar, S.S., 2013. Role of bio-fertilizer in organic agriculture: a review. Research Journal of Recent Sciences ISSN, 2277, p.2502.
- Naseri, R., Moghadam, A., Darabi, F., Hatami, A. and Tahmasebei, G.R., 2013. The effect of deficit irrigation and Azotobacter chroococcum and Azospirillum brasilense on grain yield, yield components of maize (SC 704) as a second cropping in western Iran. Bulletin of Environment, Pharmacology and Life Sciences, 2(10), pp.104-112.
- Nongthombam, J., Kumar, A., Sharma, S. and Ahmed, S., 2021. Azotobacter: A complete review. Bull. Env. Pharmacol. Life Sci, 10, pp.72-79.
- Page, W.J. and Shivprasad, S., 1991. Azotobacter salinestris sp. nov., a sodium-dependent, microaerophilic, and aeroadaptive nitrogen-fixing bacterium. International Journal of Systematic and Evolutionary Microbiology, 41(3), pp.369-376.
- Pandey, A. and Kumar, S., 1989. Potential of Azotobacters and Azospirilla as biofertilizers for upland agriculture: a review. J Sci Ind Res 48:134–144
- Parmar, N. and Dufresne, J., 2011. Beneficial interactions of plant growth promoting rhizosphere microorganisms. Bioaugmentation, biostimulation and biocontrol, pp.27-42.
- Partridge, C.D.P., Walker, C.C., Yates, M.G. and Postgate, J.R., 1980. The relationship between hydrogenase and nitrogenase in Azotobacter chroococcum: effect of nitrogen sources on hydrogenase activity. Microbiology, 119(2), pp.313-319.
- Pribram, E., 1933. Klassification der Schizomyceten. F. Deuticke, Leipzig, pp. 1e143.
- Ramaswamy, P.P., Mathan, K.K., Nair, K.S., 1977. Azotobacter population in red and black soils of Tamil Nadu (India). Mysore J. Agric. Sci. 11, 364e366.
- Rediers, H., Vanderleyden, J., De Mot, R., 2004. Azotobacter vinelandii: a Pseudomonas in disguise? Microbiol.-SGM 150, 1117e1119.
- Sahoo, R.K., Ansari, M.W., Dangar, T.K., Mohanty, S. and Tuteja, N., 2014. Phenotypic and molecular characterisation of efficient nitrogen-fixing Azotobacter strains from rice fields for crop improvement. Protoplasma, 251, pp.511-523.
- Samal, D.P.K., Ray, P., Sukla, L.B. and Shukla, V., 2020. Isolation and screening of Azotobacter spp. for plant growth promoting properties and its survival under different environmental stress conditions. Biointerface Res Appl Chem, 10(2), pp.5188-92.

- Shivprasad, S. and Page, W.J., 1989. Catechol formation and melanization by Na+-dependent Azotobacter chroococcum: a protective mechanism for aeroadaptation? Applied and environmental microbiology, 55(7), pp.1811-1817.
- Singh, M.S. and Dutta, S., 2006. Mustard and rapeseed response to Azotobacter-A review. Agricultural Reviews, 27(3), pp.232-234.
- Soleimanzadeh, H. and Gooshchi, F., 2013. Effects of Azotobacter and nitrogen chemical fertilizer on yield and yield components of wheat (Triticum aestivum L.). World Applied Sciences Journal, 21(8), pp.1176-1180.
- Tchan, Y.T., Wyszomirska-Dreher, Z., New, P.B. and Zhou, J.C., 1983. Taxonomy of the Azotobacteraceae determined by using immunoelectrophoresis. International Journal of Systematic and Evolutionary Microbiology, 33(2), pp.147-156.
- Tejera, N.L.C.M.M.G.J., Lluch, C., Martinez-Toledo, M.V. and Gonzalez-Lopez, J., 2005. Isolation and characterization of Azotobacter and Azospirillum strains from the sugarcane rhizosphere. Plant and soil, 270, pp.223-232.
- Thompson, J.P. and Skerman, V.B.D., 1981. Validation list No6. Int. J. Syst. Bacteriol, 31, pp.215-218.
- Tien, T.M., Gaskins, M.H. and Hubbell, D., 1979. Plant growth substances produced by Azospirillum brasilense and their effect on the growth of pearl millet (Pennisetum americanum L.). Applied and environmental microbiology, 37(5), pp.1016-1024.
- Triplett Jr, G.B., Dabney, S.M. and Siefker, J.H., 1996. Tillage systems for cotton on silty upland soils. Agronomy Journal, 88(4), pp.507-512.
- Vance, C.P. and Graham, P.H., 1995, Nitrogen fixation in agriculture: applications and perspectives. In: Tikhonovich IA, Provorov NA, Romanov VI, Newton WE (eds) Nitrogen fixation: fundamentals and applications, current plant science and biotechnology in agriculture 27: 77–86
- Whitman W.B (2010) Bergey's Manual Of Systematic Bacteriology, in N.R. Krieg, J.T. Staley, D.R. Brown, B.P. Hedlund, B.J. Paster, N.L. Ward, W. Ludwig, W.B. Whitman (Eds.) Springer, USA, p. 976
- Yasari, E. and Patwardhan, A.M., 2007. Effects of (Azotobacter and Azosprillium) inoculants and chemical fertilizers on growth and productivity of canola (Brassica napus L.). Asian Journal of Plant Sciences 6(1): 77-82
- Young, J.M. and Park, D.C., 2007. Probable synonymy of the nitrogen-fixing genus Azotobacter and the genus Pseudomonas. International Journal of Systematic and Evolutionary Microbiology, 57(12), pp.2894-2901.
- Zena, G.G. and Peru, C., 1986. Effect of different rates of Azotobacter and frequency of application of Agrispon on yield and quality in the growing of onion (Allium cepa L.) in Cajamarca. National University of Cajamarca Faculty of Agriculture Sciences and Forestry.

- Zulaika, E., Shovitri, M. and Kuswitasary, N.D., 2014. Numerical Taxonomy for Detecting the Azotobacterial Diversity. In The 8th Korean-Asean Joint Symposium on Biomass Utilization and Renewable Energy.
- https://core.ac.uk/download/pdf/158352931.pdf
- https://en.wikipedia.org/wiki/Azotobacter
- https://indiaagronet.com/indiaagronet/manuers_fertilizers/Azotobacter(B).htm
- https://www.sciencedirect.com/science/article/abs/pii/S0065216419300243#:~:text=Azotobacters%20have%20been%20used%20as,or%20reduce%20their%20deleterious%20effect.
- https://www.vedantu.com/biology/Azotobacter.

6.11 SUGGESTED READINGS

- Nongthombam, J., Kumar, A., Sharma, S. and Ahmed, S., 2021. Azotobacter: A complete review. Bull. Env. Pharmacol. Life Sci, 10, pp.72-79.
- Samal, D.P.K., Ray, P., Sukla, L.B. and Shukla, V., 2020. Isolation and screening of Azotobacter spp. for plant growth promoting properties and its survival under different environmental stress conditions. Biointerface Res Appl Chem, 10(2), pp.5188-92.
- Wani, S.A., Chand, S., Wani, M.A., Ramzan, M., Hakeem, K.R. 2016. Azotobacter chroococcum – A Potential Biofertilizer in Agriculture: An Overview. K.R. Hakeem et al. (eds.), Soil Science: Agricultural and Environmental Prospectives, Springer, Switzerland.DOI 10.1007/978-3-319-34451-5_15.

6.12 TERMINAL QUESTIONS

6.12.1 Short answer type questions

1. What is *Azotobacter?*
2. Briefly describe the classification of *Azotobacter.*
3. Write a short note on the habitat of *Azotobacter.*
4. What do you understand by the artificial inoculation of bacteria?
5. How to maintain the *Azotobacter* culture in *in-situ* condition?

6.12.2 Long answer type questions

1. Discuss in detail the importance of *Azotobacter* as biofertilizer.
2. Describe the classification and important characteristics of *Azotobacter*.
3. Write a detailed note on the crop response to *Azotobacter* inoculums.
4. Discuss the procedure of mass multiplication and maintenance of azotobacter in *in-situ* condition.

UNIT- 7 CYANOBACTERIA (BGA), CELL STRUCTURE, FORMS AND CHARACTERISTICS FEATURES

Contents

7.1 OBJECTIVES

After reading this unit the learners are able to

- Know about Morphological forms and cell structure of cyanobacteria
- Know about characteristics features of Cyanobacteria
- Know about role of blue green algae and Azolla in rice cultivation.

7.2 INTRODUCTION

The Cyanobacteria are real bacteria (singular, bacterium). Autotrophic gram-negative bacteria belonging to the phylum Cyanobacteria, sometimes referred to as blue-green algae, Cyanobacteriota, or Cyanophyta, are found naturally in all kinds of water. Fresh, brackish, and marine waters are all capable of supporting these single-celled creatures. Sunlight is used by these organisms to make food. Blooms of cyanobacteria occur on the surface of water in warm, nutrient-rich environments. cyanobacteria are bigger than bacteria, which have chlorophyll-A. Being prokaryotes, they lack membrane-bound organelles like mitochondria and plastids as well as a real nucleus. They have 70s ribosomes, just like other prokaryotes. While other bacteria are also capable of photosynthesis, only cynaobacteria have the pigment chlorophyll a. This pigment, which is also found in plants and algae, is responsible of the evolution of oxygen during photosynthesis. Bacteriochlorophyll, a distinct type of pigment found in photosynthetic bacteria, prevents oxygen from evolving during bacterial photosynthesis.

The term "cyan" in cyanobacteria refers to the colour, blue. Cyanobacteria possess certain accessory pigments such as phycocyanin and phycoerythrin. The presence of these pigments and chlorophyll a together impart characteristic colour to these organisms. It is for this reason that the cyanobacteria are commonly known as blue green algae. Like true algae they also evolve oxygen during photosynthesis and often occupy habitats where algae occur, in fresh, marine and brackish water bodies and on moist soil surface. However, true algae are eukaryotic and the two are not immediately related. Because cyanobacteria have affinities with other bacteria, it is necessary to quickly look at these creatures in order to fully understand cyanobacteria's place in the world of living things. Thus far, almost 4,000 different types of bacteria have been identified. Approximately 1,700 cyanobacteria species are among them. Of all organisms, bacteria are the most prevalent, despite their small number of species. They are the oldest as well.

It is known that bacteria existed as early as 3.5 billion years ago in the fossil record. In terms of morphology and anatomy, bacteria are the most basic living forms. Yet, they differ greatly in terms of metabolism. Numerous bacteria are recognised based on characteristics in culture rather than their individual morphology. The accepted source for information on bacterial categorization is Bergey's Manual of Determinative Bacteriology. The Bergey's Manual lists 19 major families of bacteria, including gliding bacteria, mycoplasma, actinomycetes, spirochaetes,

and Gram-positive cocci, because there is not enough data to classify all bacteria into a hierarchical system.

One such group includes cyanobacteria. Research on the classification of bacteria is continuously occurring. Molecular biologists have examined the composition of ribosomal RNA (rRNA) and the nucleotide sequence of rRNA in bacteria and other species. The archaebacteria and eubacteria, two important bacterial groupings, vary fundamentally from one another, as demonstrated by the present analysis. The chemical nature of the cell membranes of these two bacterial species as well as the eukaryotes has also been found to be different.

According to American scientist Carl Woese, the distinctions between the eubacteria and archaebacteria are just as fundamental as those between these groupings and eukaryotes. Accordingly, there are thought to be three ancient and primary lineages of life on our planet. The cyanobacteria are members of the true bacterial lineage. Members of the archaebacteria can be found in a wide range of strange habitats, including extremely hot and acidic pools and waters with high salinity levels. Several kilometres below the ocean's surface, in deep sea vents, some members of this group reside.

There is a lot of evolutionary exploration in cyanobacteria. The endosymbiont theory states that some ancestral cyanobacterial cells give rise to the plastids of certain algal taxa. Plastids of Red algae and cells of cyanobacteria are similar in that they both have biliproteins and chlorophyll a. Both chlorophylls A and biliproteins are present in green algae and plants. At least three species are known to carry both chlorophylls, despite the fact that the majority of cyanobacteria only contain chlorophyll a. Within the intestinal walls of sea squirts, *Prochloron didemni* reside as symbionts. It was recently found that *Prochlorothrix hollandica* inhabits the lakes of Holland. *Prochlorococcus* has more recently been found to exist in open waters in a free-floating form.

All of these organisms have biliproteins and chlorophylls a, and the chloroplasts found in green algae and plants are similar to the cells of these organisms. Because of this, some scholars classify the three taxa together into a distinct division or class and refer to them as prochlorophytes. It's possible that an endosymbiont related to an ancestral prochlorophyte gave rise to the chloroplast found in green plants.

7.3 CELL STRUCTURE OF CYANOBACTERIA

The cellular structure of cyanobacteria was first observed by renowned scientists Pankratz and Bowen in 1963. It is a prokaryotic cyanobacterium. Understanding the cellular and specialised structure of cyanobacteria is necessary to understand how they were formed.

Compared to bacteria, cyanobacterial cells are bigger and more complex. Generally, prokaryotic cells have a single envelope organisation with a peptidoglycan wall, naked DNA, and 70S

ribosomes; they also lack membrane-bound organelles such as the sap vacuole, mitochondria, endoplasmic reticulum, Golgi bodies, plastids, and lysosomes.

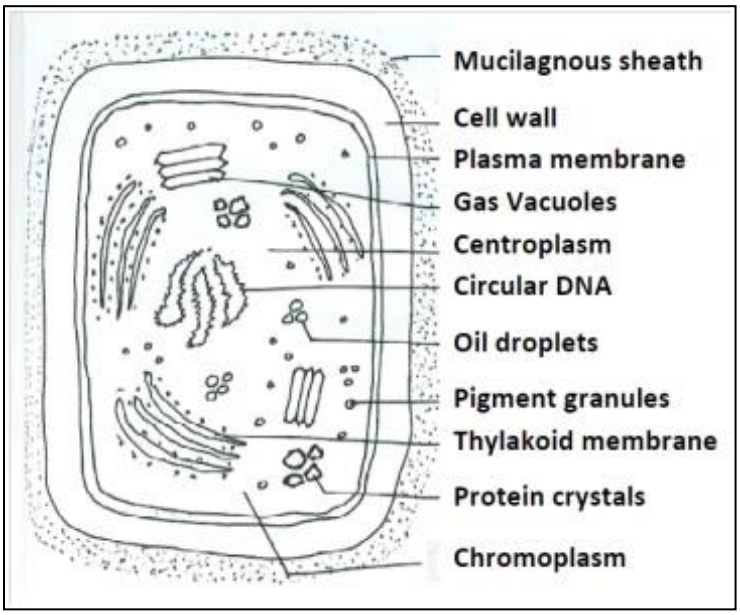


Fig. 7.1: Cell structure of cyanobacteria
*(*Source: https://istudy.pk/algae-cell-structure/*)*

Peptidoglycan is found in the second layer of the four layers that constitute the cell wall. Many photosynthetic thylakoids are found in the outer region of protoplast. We refer to it as chromoplasm. In the cytoplasm, the thylakoids lie freely. They have xanthophylls, carotenes, and chlorophyll an in their membranes. There is no chlorophyll b.

Phycobilisomes are tiny granules that are affixed to the membranes of thylakoids. The latter have pigments called phycobilins, which are auxiliary photosynthetic pigments. There are three different types of phycobilins: phycocyanin (blue), phycoerythrin (red), and allophycocyanin (blue). Differential formation of phycobilins produces specific colouration which is adapted to absorbing maximum amount of solar radiation. Therefore, cyanobacteria are not always blue green. They may appear purplish, violet, brownish, etc.

Instead of typical vacuoles or sap vacuoles, gas vacuoles or pseudo-vacuoles are found. Each gas vacuole consists of a number of submicroscopic units called gas vesicles. Gas vacuoles function as light screen; provide buoyancy regulating mechanism and pneumatic strength.

Centroplasm, the core region of the cytoplasm, has a naked, circular, double-stranded DNA that is usually coiled there. The coiled DNA shares similarities with a chromosome from higher

organisms. Many refer to it as a nucleoid, along with it tiny circular DNA segments can also form, as in bacteria.

They are known as transposons or plasmids. The 70S ribosomes are scattered in nature. A semicircular cluster of coiled membranes frequently affixes the nucleoid to the plasma membrane known as Lamella some. The cells include four different kinds of inclusions. They are polyhedral bodies (ribulose biphosphate carboxylase), β-granules (lipid droplets), α-granules (cyanophycean starch), and volutin granules.

The cells of Cyanobacteria are made up of the plasma membrane, cytoplasm, cell wall, and sheath, a mucilaginous layer similar to that of bacteria.

1. Sheath

Cyanobacteria typically have a hygroscopic, mucilaginous sheath covering them that keeps them moist and shields them from adverse environments (Fig. 7.1). Environmental factors affect the sheath's thickness, consistency and character. The sheath is made of pectic materials and it has an undulating, electron dense fibrillar apearance.

2. Cell Wall

The cyanobacterial cell looks like a multilayered between the plasma membrane and sheath after being examined under an electron microscope. The four layers of the cell wall are referred to as LI, LII, LIII, and LIV. While layers LII and LIV are electron dense, layers LI and LIII are electron transparent.

(i) LI is the innermost layer of cell wall, which is located near the plasma membrane. It is surrounded by LII and has a thickness of 3-10 nm.

(ii) The layer LII is thin and electrons dense layer. Mucopeptides, muramic acid, glucosamine, alanine, glutamic acid and di-amino-pamelic acid are constituents of this layer. The shape and mechanical strength of cell wall are provided by layer LII. This layer ranges in thickness from 10 to 1000 nm.

(iii) LIII is an additional electron-transparent layer with a thickness of nearly 3–10 nm; (iv) LIV, the topmost layer, is a thin layer with a high electron density. It has a wrinkled, flowing or twisted appearance.

Plasmadesmata connect one layer to the next. The cell has several holes that serve as a channel for the secretion of mucilage. Eubacteria and cyanobacteria share a lot of chemical similarities in their cell walls.

The presence of mu-copolymer, a mixture of two sugars (glucosamine) and three amino acids (di-amino-pamelic acid) in the ratio 1:1:1:1:2, is the chemical component of both cyanobacteria and Gram-negative bacteria.

E. coli likewise has a comparable ratio of these components. A variety of cyanobacteria, including *Anacystis nidulans*, *Phormidium uncinatum*, and *Chlorogloea fritschi*, exhibit distinct

ratios of amino acids and carbohydrates. Furthermore, all prokaryotes share the diamino acid. It has been observed that cyanobacteria cells contain lipids and lipopolysaccharides.

3. Plasma Membrane

The plasma membrane known as the plasma lemma, is a bilayer membrane that follows the cell wall. It preserves the physiological integrity of the cell, is 70A thick, and is selectively permeable. A structure known as a lamellosome is created when the plasma membrane locally invades and unites with the photosynthetic lamellae (thylakoids) (Fig.7.1). The other inclusions and the cytoplasm are enclosed by the plasma membrane.

4. Cytoplasm

The cytoplasm is differentiated into two regions i. e., chromoplasm and central colourless region called centroplasm.

(i) Chromoplasm:

The flattened vesicular structures known as photosynthetic lamellae or thylakoids are found in the chromoplasm (Fig.7.1) which may be central, parallel or peripheral in position. Thylakoids are capable of respiration, photophosphorylation, and the Hill reaction in addition to photosynthesis. Inside the lamellae are a number of photosynthetic pigments, including carotenoids, xanthophylls, and chlorophyll a and c. A protein anchors phycobilisomes, or biliproteins, with a diameter of roughly 40 nm on its upper surface.

The three pigments which make phycobilisomes are phycocyanin-C, allophycocyanin, and phycoerythrin-C. The following three pigments use light-harvesting in order: Chlorophylls, allophycocyanin, phycoerythrin, and phytocyanin.

(ii) Centroplasm:

The centroplasm is thought to be a primitive nucleus that lacks a nucleolus and a bilayered nuclear membrane. It is colourless. Centroplasm has a number of stain-tolerant grains. Some scientists believe that the centroplasm is store house for food, while others see it as an emerging nucleus (Fig.7.1).

5. Cytoplasmic Inclusions

There are numerous glycogen granules, oil droplets, and other inclusions scattered throughout the centroplasm and chromoplasm:

(i) Cyanophycin:

(i) Cyanophycin: When cyanobacteria are grown in environments with excess nitrogen, they build up a nitrogenous reserve material known as cyanophycin or cyanophycian granules. Equal amounts of aspartic acid and arginine molecules are used to construct them. They constitute up to 8% of the total dry weight of cells. They can be seen under a light microscope.

(ii) Gas Vacuoles:

Numerous cyanobacteria, such as Gloeotrichia, Anabaena, Microcystis, Oscillatoria, and others, have gas vesicles of viscous pseudo vacuoles of varying diameters.

There are no vacuoles in the cytoplasm. The vesicles are hard, hollow, and elongated cylinders that range in length from 200 to 1000 nm. A 2 nm thick protein border encloses the vesicles. Vacuoles have conical tips (Fig. 7.1). Gases can pass across the protein border freely, but water cannot get through it. They collapse under pressure and lose their refractivity as a result.

Gas vesicles have the responsibility of keeping the cell buoyant so that it can stay at a specific depth of water where it can absorb enough light, oxygen, and nutrients. One important characteristic of free floating cyanobacteria is the process of floating and sinking. They are able to protect themselves from the damaging effects of strong light by using this technique.

(iii) Carboxysomes:

The polyhedral bodies known as carboxysomes are those that contain 1, 5-ribulose biphosphate carboxylase (Rubisco).

(iv) Phosphate Bodies:

They are also known as volutin granules. Minerals including potassium, calcium, and magnesium are found in polyphosphate bodies, which are energy-dense substances that serve as phosphate and energy reserves. Most cyanobacteria include the nitrogen/carbon reserve polymer cyanophycin (CGP).

(v) Phycobilisomes:

In addition to chlorophyll or bacteriochlorophyll pigments, some phototrophic organisms (such as cyanobacteria and red algae) also possess two accessory pigments, such as carotenoids and phycobilins (sometimes called phycobiliproteins). While phycobilins are light-harvesting pigments, carotenoids have a role in photoprotection.

The primary pigments in cyanobacteria that collect light are called phycobilins. Red or blue pigments are called phytobiliproteins. These substances are made up of open-chain tetrapyroles that are biosynthetically produced from closed porphyrin rings. Proteins are connected to the tetrapyroles. Phycobilisomes are large, darkly pigmented, ball-like structures made of aggregated phycobiliproteins which are attached to the outer surface of lamellar membrane.

The lamellar membrane's outer surface is where the phycobilisomes are affixed.

(a) Phycoerythrin, a red pigment that strongly absorbs light at 550 nm; (b) Phycocyanin, a blue pigment that powerfully absorbs light at 620 nm; and (c) Allophycocyanin, a pigment that absorbs light at 650 nm. The arrangement of pigments within phycobilisomes facilitates the

attachment of allophycocyanin to the photosynthetic lamellar membrane. Phycocyanin and phycoerythiin molecules encircle allophycocyanin, respectively.

Shorter (higher energy) wavelengths of light are absorbed by phycocyanin and phycoerythrin, which then transfers energy to allophycocyanin. Chlorophyll is intimately related to allophycocyanin in the reaction centre. Chlorophyll A receives this energy transfer from allophycocyanin. At the lowest light intensity regions, cyanobacterial growth is enabled by the presence of phycobilisomes.

(vi) DNA Matrix:

The centroplasm of cyanobacteria, like that of other prokaryotes, contain DNA fibrils. Like E. *coli*, DNA material lacks nucleoplasm and has a protein that binds to DNA similar to a histone. Although the total number of genomes is unknown, reports of two to three genomes in *Agmenellum* have been reported.

The base composition of DNA differs amongst cyanobacteria, with chroococcales having a base composition of 35–71 moles percent G + C, Oscillatoriales having a base composition of 40–67 moles percent G + C, etc.

The G+C content of pleurocapsales is 39-47 moles and heterocystous forms have 38–47 moles. The molecular weight ranges between 2.2 x 109 to 7.4 x 109 Daltons.

Cyanobacteria have 70S ribosomes, similar to eubacteria. Like bacteria, Cyanobacteria also possess circular plasmid DNA that is covalently closed and non-functional. Since the function of cyanobacterial DNA is not known, these are known as cryptic plasmids.

6. Specialized Structures of Cyanobacteria

Cyanobacteria produce a variety of specialised structures including hormogones, exopores, endospores, Nano cysts, heterocysts, exospores, endospores, akinetes, etc.

(i) Hormogones and Hormocysts:

All filamentous cyanobacteria produce hormogones, which are small segments of trichomes. Hormogones are produced by a variety of processes, including fragmentation and rounding off the end cells (*Nostoc*), delimination of cells into intercalary groups (*Gloeotrichia*) fragmentation of trichomes into pieces (*Oscillatoria*,) and their subsequent degradation (*Oscillatoria, Phormidium*). Hormogones exhibit gliding motion. Every homogone may mature into a new individual.

Similar to hormogones, some other cyanobacteria produce hormocysts or hormospores. Hormocysts can be terminal or intercalary in their production. They have thick mucilaginous sheaths covering their cells and they are heavily granulated. A significant amount of food is deposited in hormocyst cells. Under favourable circumstances, hornocysts grow into new plants.

(ii) Endospores, Exospores and Nanocysts:

Endospores, exospores, and nanocysts are typically produced by non-filamentous cyanobacteria, such as Chamaesiphon, Dermocapsa, and Stichosiphon. Inside the cell, endospores are formed. The cytoplasm of the cell breaks into several pieces during endospore formation, each of which becomes an endospore.

Each endospore germinates into a new plant, such as *Dermocapsa*, after it is liberated. Endospores that are smaller in size but more numerous are referred to as nanospores or nanocysts. Exospores are formed in *Chamaesiphon* by budding exogenously.

(iii) Akinetes:

The members of the families Stigonemataceae, Rivulariaceae, and Nostocaceae grow from vegetative cells into spores like *Gloeotrichia, Nostoc*, and *Rivularia*, or into spherical perennating structures known as akinetes (Fig. 7.2).

Under adverse circumstances, the vegetative cells store a lot of food, grow and have thick walls. They can form in chains or individually. Because akinetes have cyanophycean granules, they have a brown colour. The akinetes develop into vegetative filaments under favourable conditions.

(iv) Heterocysts:

The modified vegetative cells are called heterocysts. The development of heterocysts is dependent on the amount of nitrogen present in the environment. Heterocysts undergo a variety of morphological, physiological, biochemical, and genetic changes during differentiation.

These are pale yellow cells with an extra outer covering that is slightly expanded. They can be formed individually or in chains and they remain in terminal or intercalary positions. These are mostly found in families such as *Oscillatoriaceae, Rivulariaceae, Nostocaceae*, and *Scytonemataceae* (Fig. 7.2).

7.4 MORPHOLOGICAL FORMS OF CYANOBACTERIA

Cyanobacteria are aquatic photosynthetic bacteria that use oxygen to produce food. The morphology of cyanobacteria varies, encompassing unicellular, filamentous, and colonial forms. Filamentous forms, such as akinetes (resting stage cells), hormogonia (reproductive, motile filaments), and heterocysts (for nitrogen fixation), demonstrate functional cell differentiation. Some of the examples are as follows:

Blue-green algae

They belong to several genera, which include *Gloeocapsa, Aulosira, Nostoc, Stigonema, Anabaena, Spirulina, Oscillatoria, Syctonema*, and *Chrococcus.*

(i) Oscillatoria

This blue-green filamentous cyanobacterium is commonly seen in freshwater habitats. As the filaments slide against one another to face the colony towards a light source, they oscillate, giving rise to the name. *Oscillatoria* reproduces by fragmentation which gives rise to separate sections called hormogonia. The hormogonia give rise to new individuals.

(ii) Arthrospira

A genus of filamentous cyanobacteria that floats freely is called Arthrospira. Its multicellular, cylindrical trichomes are arranged in an open left-hand helix. Spirulina, a dietary supplement, is derived from *A. platensis* and *A. maxima*. It is a cyanobacterium with applications in the pharmaceutical and food industries.

(iii) Nostoc

This genus of cyanobacteria is the most widespread and it may be found in a wide range of aquatic and terrestrial habitats. The colonies it forms are made up of filaments of moniliform cells covered in a gelatinous polysaccharide sheath. It may also grow in a symbiotic relationship with plants.

(iv) Anabaena

Anabaena is a genus of filamentous cyanobacteria which have nitrogen fixing capabilities. They live as symbionts with plants.

(v) Rivularia

Around rivers, *Rivularia* grows on damp soils, moist rocks, and submerged stones. It is found in colonies, where the trichomes are organised radially within the colony and partially or completely enclosed in a sheet of gelatin. There is a basal heterocyst in the trichomes. A row of tiny cells makes up the whip- or tail-like, narrow aptic part of each trichome. There are no akinetes in *Rivularia.* The heterocyst and hormogonia help the species reproduce.

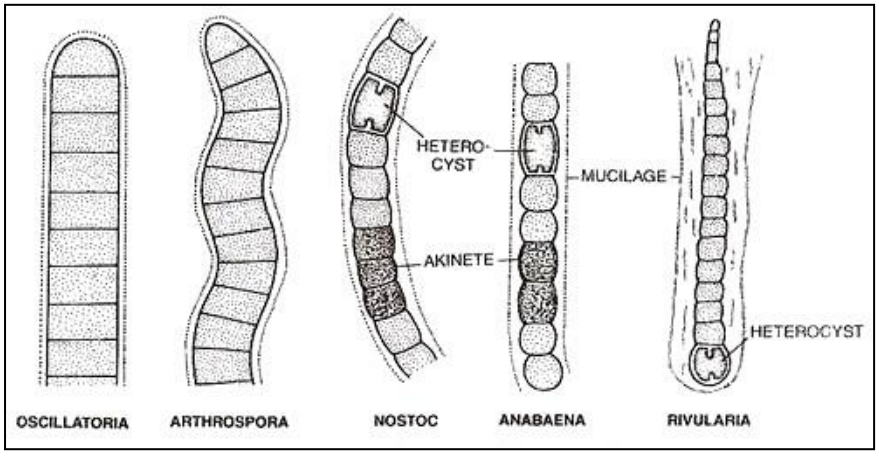


Fig. 7.2: Some common filamentous blue green algae
(Source: https://images.app.goo.gl/T9FGkchMUVQsMdrk9)

7.5 CHARACTERISTICS FEATURES OF CYANOBACTERIA

Photosynthetic prokaryotes are known as cyanobacteria. These organise into single cells or multicellular colonies that produce lengthy filamentous cell chains. These lack pilli and flagella. The following details comprise the characteristics cyanobacteria cell:

1. They are omnipresent and found in most of the habitats.
2. They can be unicellular (*Chroococcus*, *Tetrapedia*, and *Gloeocapsa*), filamentous (*Oscillatoria*), or colonial (*Aphanocapsa*, *Nostoc*, and *Aphanothece*).
3. The pigments in them are Chlorophyll A, P-carotene, Antheraxanthin, Aphanicin, Aphanizophyll, Flavacin, Lutein, Myxoxanthophyll, Oscilloxanthin, Zeaxanthin, Allophycocyanin, Phycocyanin, and Phycoerythrin.
4. The storage products of cyanobacteria are protein and cyanophycean starch.
5. In cyanobacteria, flagella are absent.
6. The first known cyanobacterium to produce chlorophyll f was *Halomicronema hongdechloris.*
7. They can adapt to a variety of environments, including fresh and saltwater, and are single-celled. Hot springs and other harsh situations are also tolerable to them. In addition, cyanobacteria can be found in terrestrial environments such as rocks, deserts, glaciers, and damp soil. They coexist in symbiotic relationships with lichens, plants, and primitive mammals.
8. Nitrogen fixation: The enzyme nitrogenase fixes nitrogen, although it is oxygen-sensitive and requires an almost oxygen-free environment. Cyanobacteria have special defensive mechanisms against oxygen that protect nitrogenase and control the growth of N_2 fixing system.
9. They produce cyanotoxin, which can cause diarrhoea, vomiting, nausea, mouth sores, pneumonia, and stomach pain. Microcystins have the potential to affect the liver, kidneys and reproductive system.

7.6 FACTORS AFFECTING GROWTH OF BLUE GREEN AGLAE

Cyanobacteria form algal blooms in fresh and marine water. The biomass of algae forming algal blooms increases in aquatic environment causing toxicity in water sources. Some of the factors affecting growth of these blue green algae are summarised as following:

I Chemical Factors

1 - Nitrogen:

The Heterocyst-containing blue green algae, in particular, are nitrogen-fixing algae that require less inorganic nitrogen. Only a small amount of nitrogen is required to increase the biomass and protein content of the cells.

2 - Phosphorus:

- In the synthesis of a sequence of phosphorus oxidation reactions (Oxidation Phosphorelation), phosphorus is thought to be a crucial component.
- It is regarded as a crucial component in the synthesis of nucleic acids as well.
- High concentrations of phosphorus are essential for blue-green algae, and this is when cyanobacterial blooms frequently develop. It could be as a result of the abundant uptake of this element by these algae.

3- Micronutrients:

- The microelement needs of blue green algae are similar to those of higher plants,
- Moreover, it is assumed that sodium is necessary for the conversion of nitrogen to ammonium (NH_4), and they require sodium in comparatively high concentrations to maintain osmotic pressure.
- According to reports, cobalt (Co) is also necessary for blue-green algae that coexist with plants or fungi. The role played by this element is still unknown.
- Iron (Fe) and molybdenum (Mo) are required in higher concentrations by blue-green algae that fix atmospheric nitrogen than by those that do not.

4 - pH:

- When light and water are not the limiting factors for algal growth, pH is the most important factor.
- According to some studies Blue-green algae account for 90% of the total algae in alkaline soil (pH 7.5–10), which may help to explain why blue-green algae live in dry soil because it is also an alkaline habitat. The blue green algae do not grow in acidic soil.

II-Physical Factors:

1 - Temperature:

- Temperature variations has an impact on blue-greens' growth, activity, proliferation, and distribution.
- Some varieties of blue-green algae have been discovered in South Antarctica, and many of them are resistant to temperatures well below zero.
- On the other hand, some species have been found to resist temperatures between 40 and 60 degrees Celsius for extended periods of time, and some species have even been known

to survive in drought conditions with temperatures as high as 85°C. The optimal temperature for algal growth is 35°C

2 - Humidity (moisture):

- Droughts or shortages of water have an adverse effect on these organisms' metabolism, or photosynthetic processes.
- As water became more readily available, the number of blue-greens increases.
- Their number also increases in moist soils.

7.7 BIOLOGICAL NITROGEN FIXATION

Microbes called nitrogen-fixing bacteria are able to convert atmospheric nitrogen into fixed nitrogen, which is an inorganic molecule that plants can utilise. These organisms contribute significantly to the nitrogen cycle, accounting for almost 90% of all nitrogen fixation. There are two types of nitrogen-fixing bacteria. The first type of bacteria are the free-living, non symbiotic ones, which include genera like *Azotobacter*, *Beijerinckia*, and *Clostridium* as well as cyanobacteria, such as *Anabaena* and *Nostoc*.

The second type consists of mutualistic (symbiotic) bacteria. Some examples of these are certain species of *Azospirillum*, which are associated to cereal grasses, *Frankia*, which is associated to certain dicotyledonous species (actinorhizal plants), and *Rhizobium*, which is associated to leguminous plants (different pea family members). For details refer to Unit 2 biological nitrogen fixation of this book.

7.8 AZOLLA AND ANABAENA AZOLLAE ASSOCIATION

Water fem *Azolla* is found in temperate and tropical regions in aquatic habitats such as ponds, canals, and paddy fields. *Azolla* is closely related to the Salviniaceae family and is a member of the heterosporous free-floating fem Azollaceae. Of *Azolla*, there are six living species today and 25 known fossil species. Botanists have been interested in this plant because of its rapid growth in nitrogen-deficient environments and symbiotic relationship with a blue-green alga that fixes nitrogen. Recently, the need for less fossil fuel-dependent agricultural technologies has reignited interest in this plant-algae connection. The unique benefits of the *Azolla-Anabaena azollae* symbiotic relations are a result of the morphology and physiology of the association.

The cavity that forms in the proximal region of the dorsal lobes is home to the symbiotic algae. The mouth of the hollow opens up towards the ventral side of dorsal lobes. Gas exchange between the symbiont and the environment most likely occurs at this mouth.

The name *Anabaena azollae* Strasburger was given to the symbiotic alga; however, Fjerdingstad recently suggested that the alga is actually an ecoform of *Anabaena variabilis*. There have been

numerous reports of *Anabaena azollae* being isolated from *azolla*. However, no one has been able to successfully re-inoculate the isolated symbiont into alga-free azolla, therefore it is unknown whether this symbiont is free-living. It has been suggested that *Anabaena azollae* is related to megasporocarps and microsporocarps. Anabaena in immature azolla seedlings may have come from thealgae cells in megasporocarps of the fern.

The cavity that houses the symbiont is formed by the development of epidermal cells during the differentiation of dorsal lobe primordial in *Azolla*. A few of algal cells that were hiding in the shoot apex were taken up by the surrounding epidermal cells and colonized the cavity. The youngest cavity contains hair-like cell. The algal cells in the youngest lobes do not have heterocysts. In the fifteenth leaf, as the lobes are traced back from the shoot apex to the basal parts, the frequency of heterocysts increases. The frequency of heterocysts in a leaf is directly correlated with its ability to fix nitrogen.

Compared to free-living heterocystous blue-green algae, the frequency of heterocysts is higher in symbiotic alga in *azolla*. All cavities include hair-like cells, which are considered to be the location of material exchange between the alga and the host plant.

7.9 ROLE OF BLUE GREEN ALGAE AND AZOLLA IN RICE CULTIVATION

Because *Azolla-Anabena* plants can fix nitrogen from the atmosphere and multiply more quickly than other plants, they are perfect for rice cultivation in tropical climates. It is agronomically remarkable due to the biomass's rather quick breakdown and the nitrogen it quickly becomes available to the standing crop. Because it never causes environmental contamination, *azolla* is environmentally beneficial. Furthermore, it does not compete with rice plants for nutrients or photosynthesis.

These biological systems can supply 1.5–2.0 million tonnes of nitrogen annually to India's crop production, while the same amounts of nitrogen would require at least 3.3–4.4 million tonnes of urea. Azolla is a widely used herb among Chinese and Vietnamese farmers. In actuality, Chinese farmers were the first to mention using azolla as a cheap source of nitrogen, and there are multiple accounts of azolla being used as manure in China and Vietnam from the start of the 17th century. This plant was compared to little factories producing nitrogen fertiliser.

The usage of *Azolla* in rice fields is described in a book authored by Jia Ssu Hsieh in 540 A.D. Singh and associates made the usage of *Azolla* in rice farming mainstream in India. *Azolla* is currently being used as a valuable agricultural input by a large number of progressive farmers and non-governmental organisations, and some of them are actively working to increase its popularity as a biofertilizer, green manure, fodder, and chicken feed. But in order to improve the

system more in line with the needs and demands of the modern world, a few fundamental scientific questions must be resolved.

The following are some advantages of applying *Azolla* on rice fields:

- The application of green manure at a basal rate of 10–12 tonnes per hectare boosts soil nitrogen by 50–60 kg/ha and lowers the 30-35 kg of nitrogenous fertiliser needed for rice crop growth. In low-lying areas, a dense mat prevents weed growth, so *Azolla* inhibits weed growth and supports favourable conditions for rice production. *Azolla* also decreases surface water evaporation, which improves rice water usage efficiency.
- Azolla can be used to reduce ammonia volatilization that occurs with the use of chemical nitrogen fertiliser, control weeds and mosquitoes, and produce hydrogen fuel and biogas.

7.10 SUMMARY

The family of blue green algae, or cyanobacteria, includes approximately 150 genera and 1,500 species in freshwater, marine, and terrestrial environments. The Cyanophyceae, Schizophyceae, and Phycochromophyceae are the synonyms for this class. They are known as cyanobacteria because they are considered as procaryotic bacteria. Larger than bacteria, cyanobacteria have chlorophyll-A. Certain types have unique terminal structures called heterocysts. All cyanobacteria that harbour heterocysts are aerobic photodiazotrophic organisms.

Changes in temperature, salinity, light, and nutrition can all affect blue-green algae. The most independent photosynthetic organisms are cyanobacteria. As a result, they can survive in any kind of environment and on any kind of surface. Their habitats include freshwater, sea, marshes, moist rocks, tree trunks, moist soils, hot springs, frozen rivers and lakes. They also grow well in these diverse environments. The red sea derives its name from *Trichodesmium erythraeum*, a red-colored planktonic cyanobacteria that gives it its colour. This shows that these bacteria are widespread. They are among the first individuals to settle in arid regions. A large number of them are capable of fixing nitrogen.

Probably the most successful class of microorganisms on earth are cyanobacteria. Understanding cyanobacteria is essential since they are ubiquitous in nature and play a major role in ocean primary production. Fixing nitrogen in tropical marine environments is their main function. A unique cyanobacterial flora characterises marine environments, and nature seems to have supplied every possible combination of photosynthetic pigment. This biodiversity is the least understood, but it has the potential to be a significant replenishment resource in the future, especially in the less accessible infralittoral areas.

7.11 GLOSSARY

Azolla: A group of aquatic ferns capable of fixing high level of nitrogen from atmosphere and are widely grown as a fertilizer crop in low land rice cultivaiion system.

Biological nitrogen fixation: The process by which certain anaerobic bacteria help plants in fixing nitrogen is known as biological nitrogen fixation.

Blue Green Algae (BGA): A heterogeneous group of prokaryotic photosynthetic nitrogen fixing organisms which contain chlorophyll 'a'. They are obligate phototrophs and store cyanophycean starch.

Legumes: Legumes are plants in the family Fabaceae (or Leguminosae), or the fruit or seeds of such plants.

Nitrogenase: Enzyme concerned with conversion of molecular nitrogen in to ammonia.

Phototrophic: (of an organism) obtaining energy from sunlight to synthesize organic compounds for nutrition.

Prokaryotes: A prokaryotes is a single-cell organism whose cell lacks a nucleus and other membrane-bound organelles.

Symbiosis: It is any type of a close and long-term biological interaction between two biological organisms of different species.

7.12 SELF ASSESSMENT QUESTION

7.12.1Multiple Choice Questions

1. The Cyanobacteria are also referred to as
   (a) Protists (b) slime moulds
   (c) golden algae (d) blue green algae

2. Oxygenic photosynthesis occurs in
   (a) *Chromatium* (b) *Oscillatoria*
   (c) *Rhodospirillum* (d) *Cholrobium*

3. Which of the following shows the absence of chlorophyll 'b'
   (a) Green algae (b) Red algae
   (c) Blue-green algae (d) Brown algae

4. Which of the following is used as green manure
   (a)Bacteria (b) *Mycoplasma*
   (c) *Nostoc* (d) None of these

5. The matrix around Nostoc colony is
   (a) Gelatinous (b) Hard and corky
   (c) Cartilaginous (d) No matrix at all

6. Which of the following movement may be found in blue-green algae
   (a) Flagellar (b) Ciliary
   (c) Gliding (d) None of the above

7. Which of the following features are found in blue green algae
   (a) abundant secterion of pectin (b) no plastids
   (c) presence of phycocyanin (d) all of the above

8. What is the photosunthetic product in blue green algae
   (a) Starch (b) Glycogen
   (c) Cyanophycean starch (d) None of these

9. Nitrogen fixation takes place in
   (a) Akinetes (b)Vegetative cells
   (c) Heterocysts (d) All of the above

10. Which of the following algae is symbiotic and nitrogen fixing
   (a) *Spirogyra* (b) *Cladophora*
   (c) *Anabaena* (d) *Oedogonium*

7.12.1: Answer Key:

1-(d), 2-(b), 3(c), 4-(c), 5-(a), 6-(c), 7-(d), 8-(c), 9-(c), 10-(c).

7.13 REFERENCES

- Dubey, R. C., Maheshwari D.K. (1999)A textbook of microbiology. S. Chand Publishing Company.
- http://archives.esf.org/fileadmin/Public_documents/Publications/CIANOFIX.pdf
- https://egyankosh.ac.in/bitstream/123456789/16673/1/Unit-2.pdf
- https://egyankosh.ac.in/bitstream/123456789/74482/1/Unit-13.pdf

- https://en.wikipedia.org/wiki/Cyanobacterial_morphology
- https://epgp.inflibnet.ac.in/epgpdata/uploads/epgp_content/biochemistry/11._introductory_selected_aspects_in_microbial,_plant_and_animal_biochemistry/17_nitrogen_fixation_and_cycle/et/4664_et_p04_m17et.pdf
- https://faculty.ksu.edu.sa/sites/default/files/English%205.pdf
- https://www.britannica.com/science/blue-green-algae
- https://www.ncbi.nlm.nih.gov/pmc/articles/PMC542195/pdf/plntphys00163-0003.pdf
- https://www.sciencedirect.com/science/article/abs/pii/B9780323961066000071
- W. Raja et al. / Hacettepe J. Biol. & Chem., 2012, 40 (1), 1–6

7.14 SUGGESTED READINGS

- Dubey, R. C., Maheshwari D.K. (1999)A textbook of microbiology. S. Chand Publishing Company.
- https://egyankosh.ac.in/bitstream/123456789/16673/1/Unit-2.pdf
- https://egyankosh.ac.in/bitstream/123456789/74482/1/Unit-13.pdf

7.15 TERMINAL QUESTIONS

7.15.1 Short answer type Questions

1. Write a short note on morphological forms of Cyanobacteria.
2. Write a short note on characteristic features of Cyanobacteria.
3. Name few factors that influence the growth of Cyanobacteria.
4. Write briefly on *Azolla* and *Anabaena azollae* association.
5. Write a short note on benefits of using azolla in rice fields.

7.15.2 Long answer type Questions

1. Why are cyanobacteria grouped with bacteria rather than with algae?
2. Write a detailed note on cellular structure of cyanobacteria.

UNIT-08 MYCORRHIZAL ASSOCIATION

Contents

8.1 OBJECTIVES

After reading this unit learners will be able:

- To know about general account of mycorrhiza and its types
- To know the taxonomy
- Colonization of VAM – isolation and inoculum production of VAM
- Influence on growth and yield of crop plants

8.2 INTRODUCTION

The mycorrhizal association is a key interest to ecologists and biologists because mycorrhizal fungi influence plant productivity and plant diversity and mycorrhizal fungi connect plants below ground via a hyphal network allowing the movement of resources among coexisting plants. The word mycorrhiza, is derived from a Greek word (i.e., *mýkēs* means "fungus", + *rhiza* means “root”; pl.: mycorrhizae, mycorrhiza or mycorrhizas (Deacon 2019), is a symbiotic association between a fungus and a plant (Kirk et al. 2001). Frank in 1885 was probably the first to recognize the widespread nature of associations between plant roots and mycorrhizal fungi (Frank & Trappe, 2005). The term mycorrhiza refers to the role of the fungus in the plant's rhizosphere and its root system. Certain plants require an association with mycorrhizae, for example, mycorrhizae are necessary for the germination and establishment of *Pinus* seeds.

Plant nutrition, soil biology and soil chemistry are all significantly impacted by mycorrhizae. A fungus called mycorrhiza colonises a plant's root and forms a beneficial (usually) connection with the plant, allowing the fungus to help the plant absorb water and nutrients while the plant gives the fungus fuel, shelter, and nourishment. The fungus provides the plant with mineral nutrients, such as phosphorus, and water, while the plant produces organic molecules through photosynthesis and gives them to the fungus in the form of sugars or lipids. A network of fungus filaments surrounds the developing root or reaches the root cells directly. The hyphae possess a relatively broad surface area, which allows them to collect water and mineral ions from a significantly more amount of soil than the root. The roots receive water and minerals from the fungus and the mycorrhizae receive sugars and N-containing components from the roots. This exchange plays a major role in plant physiology, ecology, evolution, and nutrient cycles.

Not all plants will have mycorrhizal associations. In environments in which water and nutrients are abundant in the soil, plants do not require the assistance of mycorrhizal fungi. The association is normally mutualistic. In particular species, or in particular circumstances, mycorrhizae may have a parasitic association with host plants (Johnson et al. 1997). Mycorrhizas are located in the roots of vascular plants, but mycorrhiza-like associations also occur

in bryophytes (Kottke and Nebel 2005) and there is fossil evidence that early land plants that lacked roots formed arbuscular mycorrhizal associations (Remy et al. 1994).

Mycorrhizal fungi live inside the cortex of plant roots, on the surface of the root, or around the epidermal cells of the root. The hyphae of these fungi also grow out from the roots into the soil where they forage for nutrients that are limiting to plant growth, especially nitrates and phosphates, but organically bound nutrients are also acquired by some mycorrhizal types (e.g. EM and ericoid mycorrhizal fungi) (Read & Perez-Moreno, 2003). These nutrients as well as other benefits are then delivered to their host plants in return for carbohydrates (Smith & Read, 2008). Consequently, the mycorrhizal symbiosis exerts a strong influence on plant growth and fitness.

8.3 TYPES OF MYCORRHIZA, OCCURRENCE AND DISTRIBUTION

The two types of mycorrhizas that are frequently recognised are ectomycorrhizas and endomycorrhizas (Fig 8.1; Table 8.1). The primary difference between these two is: in ectomycorrhiza fungal hyphae do not enter individual cells within the root, whereas, in endomycorrhizal hyphae enter the cell wall and invade the cell membrane, (Harley and Smith 1983; Allen 1991). Ectoendomycorrhiza includes arbutoid mycorrhizas while Endomycorrhiza includes arbuscular, ericoid and orchid mycorrhiza. Monotropoid mycorrhizas form a special category.

1. Ectomycorrhiza

Ectomycorrhiza or EcM is the most advanced type of symbiotic association between fungi and higher plants. EcM usually associates with majority of woody forest tree species (e.g., pine, oak, willow beech, spruce, birch etc) and fungi belonging to the Ascomycota, Basidiomycota and Zygomycota. Ectomycorrhiza are most common in the northern hemisphere, especially in Pinaceae, Betulaceae, Fagaceae and Salicaceae. However, EcM may also occur in some tropical and subtropical forests (Högberg, 1986).

Ectomycorrhizae are able to develop a "wood-wide-web" of submerged fungal mycelium that connects together a cluster of trees. Ectomycorrhizal fungi rely on the host plant for carbon sources because, with few exceptions (ex., *Tricholoma fumosum*), the fungi are unable to utilise cellulose and lignin. In reciprocate, the fungus provides greatly enhanced mineral ion uptake for the plant by the ability to capture nutrients, particularly phosphate and ammonium ions, which the root cannot access. Therefore, they mutually benefited to each other and host plants grow poorly when they lack ectomycorrhizas.

In this relationship the plant root system is completely surrounded by a sheath of fungal tissue which can usually 50-100 µm thick. The hyphae penetrate between the outermost cell layers forming known as **Hartig net**. From this a network of hyphal elements (hyphae, strands and rhizomorphs) extends out to explore the soil domain and interface with the fungal tissue of the root. Some common example of ectomycorrhizal fungi are *Amanita* spp., *Boletus* spp. and *Tricholoma* spp. Ectomycorrhizas can be highly specific, such as many species of *Suillus* and *Leccinum* are symbiotic with only one particular genus of plant, while other fungi, such as the *Amanita*, are generalists that form mycorrhizas with many different plants (den Bakker et al. 2014).

(i) ***Arbutoid mycorrhiza:*** This type of mycorrhiza involves plants of the Ericaceae subfamily Arbutoideae. In this type of association, some hyphae actually penetrate into the root cells, making this type of mycorrhiza an ectendomycorrhiza. In this case, mycorrhizal roots that exhibit characteristics of both ectomycorrhizas and endomycorrhizas. Ectendomycorrhizas are essentially restricted to the plant genera *Pinus* (pine), *Picea* (spruce) and, to a lesser extent. Ectendomycorrhizas have the same characteristics as ectomycorrhizas but show extensive intracellular penetration of the fungal hyphae into living cells of the host root. Arbutoid mycorrhizae are characterized by a hyphal mantel and Hartig net similar to that of ectomycorrhizae, but also an intercellular proliferation of mycelia to form dense hyphal complexes. These hyphal complexes can only be revealed by anatomical investigation. The fungi-forming arbutoid mycorrhizae are mainly basidiomycetes that commonly form ectomycorrhizae in other tree species. These include genera of basidiomycetes such as *Laccaria, Piloderma,* and *Rhizopogon* and ascomycetes such as *Cenococcum*.

2. Endomycorrhiza

Endomycorrhizae are present in more than 80% of plant families, including greenhouse and crop plants such as vegetables, flowers, grasses, and fruit trees. The production of vesicles and arbuscules by the fungus and their penetration of the cortical cells are characteristics of endomycorrhizal relationships. Endomycorrhizas are variable and have been further classified as arbuscular, ericoid, orchid mycorrhizas and monotropoid (Peterson et al 2004).

(i) ***Arbuscular mycorrhiza:*** In an arbuscular mycorrhiza, also known as Vesicular-Arbuscular Mycorrhiza (VAM), the symbiotic fungus reaches the cortical cells of the roots of a vascular plant to produce arbuscules. Only members of the division Glomeromycota of fungi can produce arbuscular mycorrhizas. Arbuscular mycorrhizas, (formerly known as Vesicular Arbuscular Mycorrhizas), have hyphae that penetrate plant cells, producing dichotomously branching invaginations (arbuscules) as a means of nutrient exchange. Often, balloon-like storage structures, termed vesicles, are also produced. In this interaction, fungal hyphae do not in fact penetrate the protoplast (i.e. the interior of the cell), but invaginate the cell membrane, creating a so-called peri-arbuscular membrane. The structure of the arbuscules greatly increases the contact

surface area between the hypha and the host cell cytoplasm to facilitate the transfer of nutrients between them. Arbuscular mycorrhizas are fungi that are obligate biotrophs, meaning that they use the plant host for both growth and reproduction (Lanfranco et al. 2016). Twenty percent of the photosynthetic products made by the plant host are consumed by the fungi, the transfer of carbon from the terrestrial host plant is then exchanged by equal amounts of phosphate from the fungi to the plant host (Kiers et al. 2011). Arbuscular mycorrhizas are found in 85% of all plant families and occur in many crop species (Wang and Qiu 2006)). The hyphae of arbuscular mycorrhizal fungi produce the glycoprotein glomalin, which may be one of the major stores of carbon in the soil.

Table 8.1: Differences between Ectomycorrhiza and Endomycorrhiza

Parameters	Ectomycorrhiza	Endomycorrhiza
Definition	It is a mutualistic association between plants and fungi where the fungal hyphae do not penetrate the plant root cell instead, it extends in between the cells.	It is a mutualistic association between plants and fungi where the fungal hyphae penetrate the host cell wall.
Location	Extracellular	Intracellular
Abundance	It comprises only 3-4% of the mycorrhizal population and is thus scarce compared to the endomycorrhizal population.	It constitutes about 80-90% of the mycorrhizal population and is thus more abundant than ectomycorrhiza.
Development	Mantle and Hartig's net.	Arbuscules and vesicles.
Categories	Arbutoid mycorrhizal associations fall under the category of ectomycorrhiza. Ectomycorhhiza also forms a kind of symbiotic association with the arbuscular mycorrhiza and is termed ectendomycorrhiza.	Endomycorrhiza is of several types, like arbuscular, Ericoid, Orchid and Monotropoid Mycorrhiza.
Associated Fungi	The phylums Ascomycota, Basidiomycota and Zygomycota form ectomycorrhizal interaction with the roots of woody trees.	The phylum Glomeromycota forms an arbuscular mycorrhizal association with vascular plants. However, Basidiomycetes are known to form Orchid Mycorrhiza.

(ii) ***Ericoid mycorrhiza:*** Ericaceae plants and various mycorrhizal fungal lineages come together to produce the mutualistic association known as the ericoid mycorrhiza. Mycorrhizae are formed by most of the genera of plants in the Ericales; these include genera forming important midstory and understory plants such as *Rhododendron* and *Vaccinium*, as well as tree species within the genus *Erica*. The ericoid mycorrhizal root is a delicate structure of only two

cortical layers, an outer hypodermis and an inner endodermis, surrounding a stele consisting of only one or two tracheids, a sieve element and a companion cell. Mycorrhizal colonization of the root is restricted to expanded epidermal cells, in which fungal hyphae penetrate the cell walls and form a dense hyphal profusion, fully filling the colonized cell. Examples of fungi-forming ericoid mycorrhizae have been identified as members of the genera *Hymenoscyphus* and *Oidiodendron*. Soils that support ericaceous vegetation are characteristically nutrient-poor.

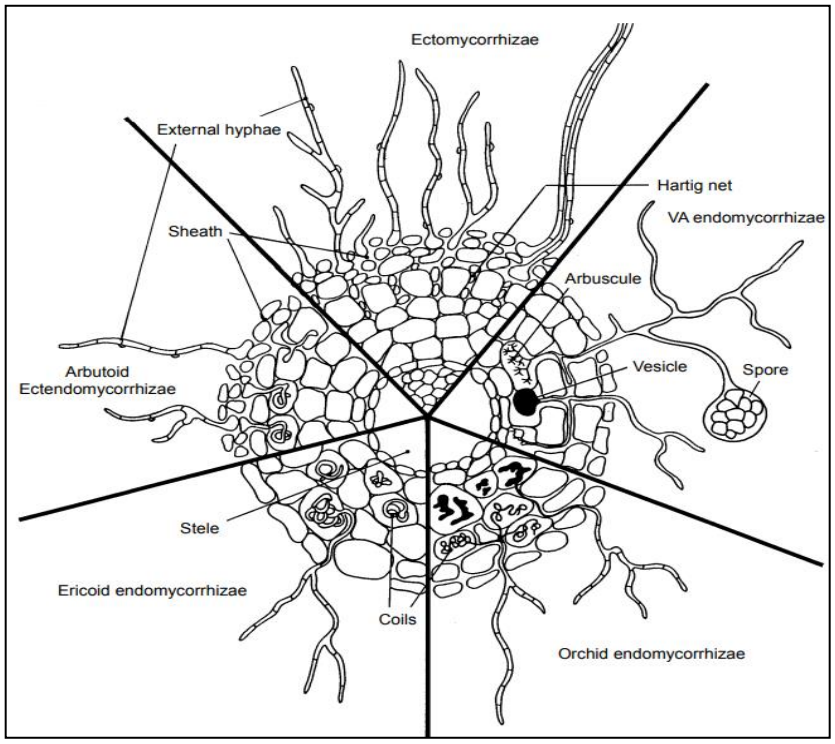


Fig 8.1: The principle structure of the five main types of mycorrhiza (Source: Selosse and Le Tacon,1998)

(iii) ***Orchid Mycorrhiza***: Orchid mycorrhiza is formed between plants of the family Orchidaceae and a variety of fungi. All orchids are myco-heterotrophic at some point in their lifecycle. The colonization by mycorrhizal fungi is critically important during seedling development. Orchid seeds have virtually no energy reserve and seedlings obtain their carbon from the fungal symbiont. Hence, during this stage of the symbiosis, the flow of C is from the fungus to the host which is distinctly different from the other mycorrhiza where C is supplied by the host plant to the fungus. Many adult orchids retain their fungal symbionts, although the benefits to the adult photosynthetic orchid and the fungus remain largely unexplored (Smith and Read, 2008).

(iv) ***Monotropoid mycorrhiza:*** This kind of mycorrhiza can be found in various genera of the Orchidaceae and the Ericaceae subfamily Monotropoideae. These plants get their carbon from the fungal companion and are heterotrophic or mixotrophic. Thus, this type of mycorrhizal relationship is parasitic and non-mutualistic. This type of mycorrhiza occurs in the subfamily Monotropoideae of the Ericaceae, as well as several genera in the Orchidaceae. These plants are heterotrophic or mixotrophic and derive their carbon from the fungus partner. Monotropoid mycorrhiza is that plants forming this type of mycorrhiza are all achlorophyllous, heterotrophic (non-photosynthetic) species; they depend on symbiotic fungal associations that act as linkages to neighboring autotrophic (photosynthetic) trees or shrubs for their carbon acquisition.

8.4 TAXONOMY OF AM FUNGI

AM fungus exhibit unique morphological and physiological characteristics. AM fungus spores are significantly larger than those of other fungi, typically forming in soil and measuring between 50 and 500 μm in diameter. There is no septum present in their hyphae and no stage of sexual development. In the presence of favourable conditions, the spores get germinate and spread their hyphae into the roots of the plants in which they have colonised. AM fungi were long recognised as belonging to the Zygomycota due to physical traits such the absence of a hyphal septum. It has been demonstrated by recent molecular phylogenetic research that Zygomycota is poly-phyletic and that AM fungus need to be distinguished from other Zygomycota. Therefore, the AM fungi have been assigned to a new phylum, Glomeromycota.

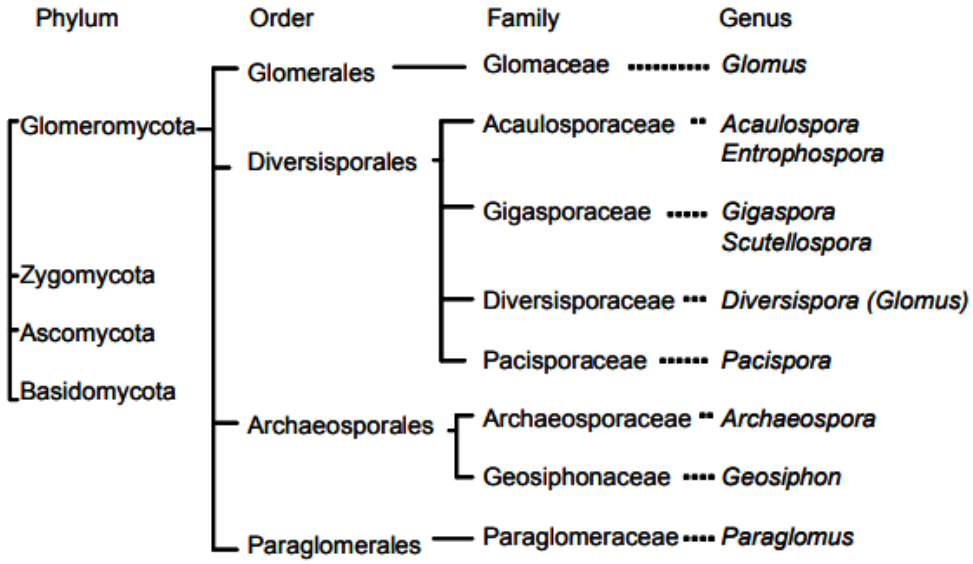


Fig. 8.2: Taxonomy of arbuscular mycorrhizal fungi
(Source: http://www.tu-darmstadt.de/fb/bio/bot/schuessler/amphylo/amphylogeny.htm)

The current classification system is summarized in Figure 8.2. This classification is based primarily on sequence data of rRNA genes. However, some new species have been identified with relatively small numbers of isolates, so further study may lead to revision of the current classification system. Although there is a recent trend to place more emphasis on sequence data of AM fungi for identification, traditional morphological observation is still important and should not be ignored for identification.

8.5 INOCULUM COLONIZATION OF AM: ISOLATION AND INOCULUM PRODUCTION OF AM

Arbuscular Mycorrhiza (AM) formerly known as Vesiclular Arbuscular Mycorrhiza (VAM) is a kind of mycorrhiza that increases the supply of phosphorus to the host plant, which in turn promotes plant development and yield. Compared to non-mycorrhizal plants, mycorrhizal plants have a greater capacity to absorb and accumulate phosphorus from the soil or solution. It has been demonstrated that plants treated with endomycorrhiza exhibit greater resistance to a number diseases.

The Arbuscular Mycorrhizal fungi coexist as symbionts with roots of terrestrial plants. The general consensus today is that they enhance the host plant's growth and phosphorus nutrition, potentially making it more resilient to diseases and drought stress. Consequently, the application of AM fungi to agriculture has been pioneered, and they provide a great deal of potential for sustainable agriculture. In fact, in some countries the AM fungal inocula have been commercialized.

1. Isolation of Arbuscular Mycorrhizal

i. **Isolation of spores from soils and their observation for identification**: The wet sieving method can be used to collect AM fungal spores from soil. Spores have a little lower gravity than soil particles. The spores in the soil can be concentrated by successively pouring the soil suspension and then filtering through a fine mesh screen. The spores in sievings may be identified under a dissecting microscope because they are globular or sub-globular, with a diameter of 50–500 μm. Because the spores of AM fungus have distinct colours and forms, it is easy to distinguish them from one another among the organic detritus gathered on the sieves. Finding the spores concealed by the debris may be challenging in soils like grassland soil that are high in organic detritus. The sucrose density centrifugation technique is frequently employed in such circumstances to isolate spores from organic matter.

ii. **Morphological observation of spores for identification:** Morphology of spores is a basis for identification of AM fungi, because the hyphae and the organs such as arbuscules and vesicles are not specific to species. Spores collected from soil often deteriorate so that they may be used only for tentative identification at genus level. It is necessary to cultivate the target AM fungus in order to conduct extensive observation, and pot culture spores should be utilised. More examination is advised, and at least 30 to 50 spores of the same morphological spore type should be observed.

2. Culturing AM fungi

AM fungi require the symbiotic association with plants for proliferation. Spores extracted from soil can be utilised as the inoculum for the AM fungal strain. Spores in soil, however, are not always effective at colonising plants. As a result, trapping culture is frequently used. Inoculum is either dirt or soil that has been sieved (dirt Trap Culture). Murakoshi et al. (1998) suggest that mycorrhizal plants gathered from the field can be transferred to potting medium as Plant Trap Culture in order to isolate AM fungus colonising roots. (Fig. 8.3).

Potting medium: The most common uses are sterile soil and soil-sand mixtures. Potting medium ingredients ought to be low in readily available phosphate and, ideally, low in organic matter. Certain soil qualities may be necessary for the growth of fungi that have been isolated from particular soil types.

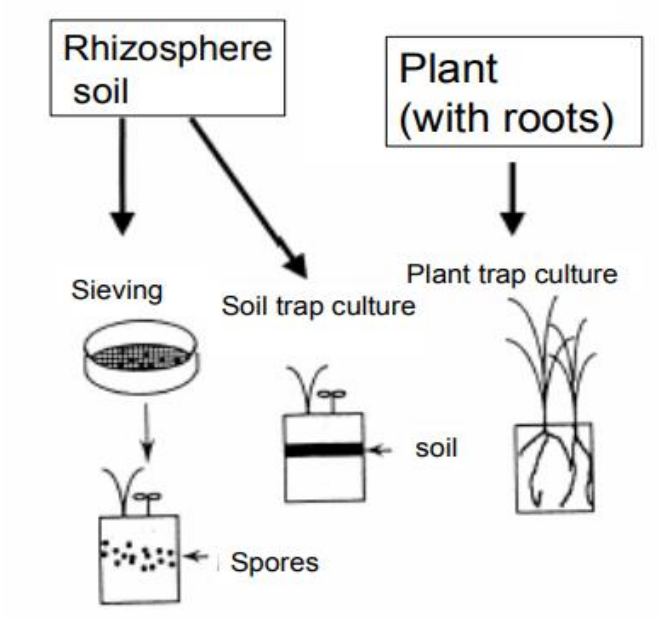


Fig.8.3: Method for trapping AM fungi

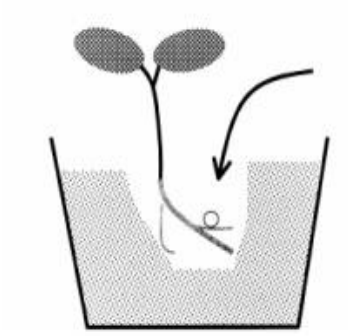


Fig. 8.4: Single spore isolation

Host plant: Numerous mycotrophic plants, such as grass species (*Lolium* spp., *Paspalum notatum*), leguminous species (*Trifolium* spp., *Medicago* spp., *Lotus japonicus*), and other herbaceous species (*Plantago* spp., *Allium* spp.), can be utilised as host species. AM fungi generally do not show host specificity but some species show host preference. Therefore, the plant species from which the target AM fungus is isolated can be used as a host plant.

Growth conditions: Any environment that promotes healthy host plant growth is suitable. It is best to use a growing chamber to prevent contamination. If a greenhouse is used, it needs to be maintained clean. It should be emphasised that even in growth chambers, cross-contamination or dust contamination are unavoidable in open-air environments. In order to avoid cross-contamination with other pot cultures housed in the same chamber, use a plastic bag (Walker & Vestberg 1994).

Single spore isolation: Single spore isolation is required in order to purify an isolated fungus. The spores may have the same morphology, yet they frequently contain contaminants with highly similar morphologies. Such multispore isolates would produce an unanticipated breakout of the contamination through pot culture in succession. Moreover, the culture may consist of groups with different genetic backgrounds even if it only includes one species. Purification by single spore isolation is crucial for these kinds of genetic research and population genetics.

There is no special equipment needed for single spore separation. Two sets of dissecting microscopes are arranged together for easy handling. One spore is taken up and placed under the first microscope, then under the second microscope. The spore is placed on the seedling's fine roots or root tip under the second microscope (Fig. 8.3). In order to ensure the success of culture, meticulous morphological inspection is necessary. You can dry the potting media by not adding any more water to the pot. Spore-containing dry soil can be kept for a year at 4-5°C after the host plant wilts. Reculturing the separate fungus annually is recommended.

3. Inoculant Production

Preparation of trap culture inoculum of arbuscular mycorrhizal fungal spores

It is frequently necessary to catch healthy Arbuscular Mycorrhizal Fungi (AMF) spores in order to establish an inoculum from monospecific cultures. Identification of AMF can also benefit from spore trap cultures. Directly harvested AMF spores from a field plot could seem healthy, but they are not viable. Because of weathering and the inherent, physical, chemical, or biological effects of the soil environment, the spores may appear differently. Trap cultures are important in the following situations:

- In situations where AMF colonisation is high in a plant community's roots but little to no spores are produced, particularly in soil conditions that are both arid and hydrating.

- When there is a lot of microbial activity in the soil, particularly in tropical climates with high relative humidity and moisture content. There may be a lot of organic stuff present at these places. AMF spores may physically change in these conditions, making it hard to identify the species.
- To create monospecific cultures and collect a large number of healthy spores from various species for particular uses.

Procedures

i. Rhizosphere soil is collected, with shoots of trap plant cut at the crown, and roots are finely chopped and mixed with the soils using a sharp chopper.
ii. The chopped roots and soil are mixed 1:1 (v/v) with autoclaved coarse sand in a mechanical mixer, or massaged well in a durable plastic bag.
iii. The soil mix is then transferred to a 15 cm plastic pot.
iv. Plant seeds of suitable trap plant such as tropical signal grass into the pot.
v. The pot cultures are maintained in a greenhouse for at least 3 months, and check sporulation from time to time. By the fourth month AMF sporulation may be at the peak. Sanitary tests may also be carried out to ensure no contamination from parasitic fungi occurs.
vi. Keep fertilizer application to a minimum, to encourage AMF proliferation.
vii. Trap culture pots are later left to dry under shade for up to 2 weeks.
viii. Harvest the spores using the sieving and decanting techniques or the density-gradient centrifugation technique.
ix. The monospecific spores are ready for inoculation onto seedlings of the desired crops

Inoculation of AMF

Two weeks before spore inoculation, the desired seedlings are prepared in suitable containers filled with sandy loam soil.

i. The seedlings are gently uprooted singly on in a small bunch, and have a gentle stream of water sprayed onto the roots so that they stick together.
ii. Spores collected from 3.3.1 are suspended in water and about 200 µl of the spore suspension are pipetted onto the moist roots.
iii. The inoculated seedlings are immediately transplanted into containers of suitable size, containing sterilized soil.
iv. The soil is topped with a sterile growth medium, watered gently under shade, before transferring into the greenhouse.
v. To encourage colonization of AMF onto seedling, fertilizers are not given during the early growth stage of the seedlings.

8.6 INFLUENCES ON GROWTH AND YIELD OF CROP PLANTS

Mycorrhizal fungi allow plants to draw more nutrients and water from the soil. They also increase plant tolerance to different environmental stresses. Moreover, these fungi play a major role in soil aggregation process and stimulate microbial activity. According to the plant species and to the growing practices and conditions, mycorrhizae provide different benefits to the plants and to the environment: Produce more vigorous and healthy plants.

The primary mechanism by which mycorrhizal fungus hyphae are believed to function is by expanding the volume of soil in which nutrients that are available to roots can be absorbed. It is possible for the fungal hyphae to break down and pierce substrates because they produce enzymes such as cellulase, peroxidase, chitinase, and protease. These enzymes are secreted, which facilitates the breakdown of complex organic substrates so that the fungus and/or host plant may absorb and use them as sources of energy and nutrients for growth and reproduction.

The phosphorus and other nutrients that are relatively immobile and present in low concentration in the soil solution are considerably improved by mycorrhizal fungus for plant uptake. The P nutrition of crops can be significantly influenced by arbuscular mycorrhizal fungi, which can increase total uptake and subsequently P use efficiency. Increased crop yield and growth may be associated with it. Crops may occasionally fail to respond to native mycorrhizal fungi's colonisation when arbuscular mycorrhizal fungal colonisation is hindered by cultural manipulation or suppressed because of high soil available P concentrations. In these situations, uptake of P, growth, and yield can all be greatly reduced. The arbuscular mycorrhizal association is primarily responsible for translocating P absorption, but it is also becoming more and more clear that these fungi can play a significant role in the host plant's intake of other nutrients. Although there have been reports of improved uptake of copper (Cu), iron, N, K, Ca, and Mg, zinc nutrition is most frequently said to be impacted by the connection.

Mycorrhizal associations have the potential to enhance water intake and increase resistance to drought conditions. Mycorrhizal plants under stress from drought often absorb more water and nutrients than non-mycorrhizal plants. Increased phosphorus levels generally increase drought resistance. Calculations made from arbuscular mycorrhizal fungi indicate that the amount of water that could travel through the mycelia to the plant is not large enough to influence plant growth or survival.

Research on ectomycorhizae revealed that the fungal strands had the ability to change a plant's water potential. When mycelia threads growing in damp peat were the only source of water, seedlings were kept in a healthy state for at least ten weeks. Arbuscular mycorrhizal fungi, however, are only efficient against mild to moderate drought stress; under more extreme drought circumstances, they lose their effectiveness. The additional benefits of mycorrhizal fungi for crop

production include: Improving flowering and fruiting, increasing plant establishment and survival during seeding or transplanting, increasing yields and crop quality, optimising the use of fertilisers particularly phosphorus, helping to maintain soil quality and nutrient cycling, increase tolerance to soil salinity, reduce disease occurrence etc.

Some examples of crop improvement via ectomycorhizal association are also discussed here. According to Celebi et al. (2010), AMF inoculum is another environmentally friendly agronomic technique for increasing crop output. It is thought to be a viable solution for guaranteeing crop yield and food security in rainfed agriculture. Furthermore, after a triple inoculation of mycorrhizal fungus, the available P status of the soil was shown to have greatly improved so that mycorrhizal associated plant can withstand droughts more easily than those that are not.

According to Yasmeen et al. (2012), applying mix bacterial inoculants and AM fungus together was more effective than using other inoculation treatments. These bioresources are thought to be crucial for efficient bio-inoculants development for productivity. Zaidi and Khan (2005) studied the interactive effect of rhizotrophic microorganisms on growth, yield, and nutrient uptake of wheat (*Triticum aestivum* L.) and found positive effect on plant vigour, nutrient uptake, and yield in wheat plants was recorded in the treatment receiving mixed inoculums of nitrogen-fixing *Azotobacter chroococcum* + phosphate solubilising microorganism (PSM) *Pseudomonas striata* + arbuscular mycorrhizal (AM) fungus.

8.7 SUMMARY

The present unit discuss about the general account of mycorrhiza and its types, taxonomy of arbuscular mycorrhiza (AM), colonization of AM – isolation and inoculum production of VAM and influence on growth and yield of crop plants. The mycorrhiza is a symbiotic association between a fungus and a plant. Frank in 1885 was probably the first to recognize the widespread nature of associations between plant roots and mycorrhizal fungi. Plant nutrition, soil biology and soil chemistry are all significantly impacted by mycorrhizae. In mycorrhiza, the fungus provides the mineral nutrients, such as phosphorus, and water to the plant on the other hand, plant provides shelter and organic molecules in the form of sugars or lipids. The two types of mycorrhizas that are frequently recognised are ectomycorrhizas and endomycorrhizas. The AM fungi were long recognised as belonging to the Zygomycota but with the help of morden techniques now have been assigned to a new phylum, Glomeromycota.

The Arbuscular Mycorrhizal fungi coexist as symbionts with roots of terrestrial plants. The inoculums and commercialized production of mycorrhizal is a systematic and long stepwise process. Consequently, the application of AM fungi to agriculture has been pioneered, and they

provide a great deal of potential for sustainable agriculture. In fact, in some countries the AM fungal inocula have been commercialized.

8.8 GLOSSARY

- **AMF:** This is an abbreviation for "Arbuscular Mycorrhizal Fungi"
- **Arbuscles:** These are intricately branched "treelike" structures within the cortex of a mycorrhizal colonized plant root.
- **Biofertilizer, biological fertilizer, organic fertilizer:** A biofertilizer is a natural fertilizer that helps to provide all the nutrients required by the plants and to increase the quality of the soil with a natural microorganism environment.
- **Hartig net** – a network of inward growing hyphae, that extends into the root, penetrating between the epidermis and cortex.
- **Hyphae:** A hypha (plural hyphae) is a long, branching filamentous structure of a fungus, and also of unrelated in most fungi.
- **Inoculum:** the population of microorganisms or cells that is introduced in the medium or any other suitable medium.
- **Mycorrhiza** (plural – mycorrhizae) – a symbiotic association between a fungus and a plant. The term mycorrhiza refers to the role of the fungus in the plant's root system.
- **Rhizosphere** – a plant's root system, and the. narrow region of soil that is directly influenced by root secretions
- **Spores:** These are the "fruiting bodies" of the fungi and are formed both inside the roots and externally in the soil.
- **Vesicle** – a structure within or outside a cell, consisting of liquid or cytoplasm enclosed by a lipid bilayer

8.9 SELF ASSESSMENT QUESTIONS

8.9.1 Multiple choice questions

1. The term mycorrhiza was coined by
   (a) Frank (b) Robert hook
   (c) Galtin (d) Brown

2. Mycorrhiza is association of
   (a) Fungus with the roots of higher plants (b) Fungus with algae
   (c) Fungus with bacteria (d) None of the above

3. Mycorrhiza is an example of
   (a) Symbiosis (b) Amensalism
   (c) Parasitic (d) Competition

4. Mycorrhiza roots lack
   (a) Root cap (b) Root hair
   (c) Both a and b (d) Carison

5. The major function of ectomycorrhiza
   (a) Absorption of water
   (b) Solubilization of complex organic molecules
   (c) Protection of plants from disease
   (d) All the above

6. Vascular arbuscular mycorrhiza (VAM) is
   (a) ectomycorrhiza (b) endomycorrhiza
   (c) both a and b (d) none of the above

7. One of the following is a bio fertilizer
   (a) CMU (b) DCMU
   (c) VAM (d) Agent Orange

8. In this association, the fungal mycelium forms some special kinds of organs, called vesicles and arbuscules
   (a) VAM (b)ectomycorrhiza
   (c) both a and b (d) none of the above

9. Which type of mycorrhizal association is found in the majority of plants?
   (a) Ectomycorrhiza (b) Endomycorrhiza
   (c) Arbuscular mycorrhiza (d) Orchid mycorrhiza

10. How do mycorrhizal fungi benefit plants in terms of nutrient uptake?
   (a) They produce enzymes to break down organic matter
   (b) They increase the surface area of roots for absorption
   (c) They transfer nutrients from the soil to the plant
   (d) All of the above

8.9.2 True and false

1. In mycorrhizal association, fungi belong mainly belongs to Ascomycetes, Basidiomycetes and zygomycetes.
2. In mycorrhiza, the advantage of plants in association is to only disease protection.
3. Roots associated with algae are known as mycorrhizal roots.
4. Orchid mycorrhiza is formed between plants of the family Orchidaceae and a variety of fungi.
5. *Ericoid* Mycorrhizae are formed by most of the genera of plants in the Ericales.

8.9.3 Fill in the blanks

1- _____ uptake is the main function of mycorrhizal fungi in plant growth?
2- The ectomycorrhiza form an intercellular network in root cortex is known as _____
3- VAM stand for _____
4- Arbuscular Mycorrhiza (AM) formerly known as _____
5- AM fungus have been assigned to a new phylum named _____

Answer keys:

8.9.1: 1.(a); 2.(a); 3. (a); 4.(c); 5.(d); 6.(b); 7.(c); 8.(a); 9.(c); 10.(d)

8.9.2: 1.True; 2. False; 3. False; 4. True; 5. True

8.9.3: 1. Phosphorus; 2. Hartig net; 3. Vesicular Arbuscular Mycorrhiza, 4. Vesiclular Arbuscular Mycorrhiza; 5. Glomeromycota

8.10 REFERENCES

- Allen, M.F., 1991. *The ecology of mycorrhizae*. Cambridge University Press.
- Celebi, S.Z., Demir, S., Celebi, R., Durak, E.D. and Yilmaz, I.H., 2010. The effect of Arbuscular Mycorrhizal Fungi (AMF) applications on the silage maize (Zea mays L.) yield in different irrigation regimes. *European Journal of Soil Biology*, *46*(5), pp.302-305.
- Deacon, J., 2018. The microbial world: Mycorrhizas. bio. ed. ac. uk (archived). *Archived from the original on*, pp.04-27.
- Den Bakker, H.C., Zuccarello, G.C., Kuyper, T.W. and Noordeloos, M.E., 2004. Evolution and host specificity in the ectomycorrhizal genus Leccinum. *New Phytologist*, *163*(1), pp.201-215.
- Frank, B., 2005. On the nutritional dependence of certain trees on root symbiosis with belowground fungi (an English translation of AB Frank's classic paper of 1885). *Mycorrhiza*, *15*(4), pp.267-275.
- Smith, S.E. and Read, D.J., 2010. *Mycorrhizal symbiosis*. Academic press.

- Högberg, P. and Piearce, G.D., 1986. Mycorrhizas in Zambian trees in relation to host taxonomy, vegetation type and successional patterns. *The Journal of Ecology*, pp.775-785.
- Johnson, N.C., Graham, J.H. and Smith, F.A., 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. *The New Phytologist*, *135*(4), pp.575-585.
- Kiers, E.T., Duhamel, M., Beesetty, Y., Mensah, J.A., Franken, O., Verbruggen, E., Fellbaum, C.R., Kowalchuk, G.A., Hart, M.M., Bago, A. and Palmer, T.M., 2011. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *science*, *333*(6044), pp.880-882.
- Kirk, P. M., Cannon, P. F., David, J. C., Stalpers, J. 2001. *Ainsworth and Bisby's Dictionary of the Fungi* (9th ed.). Wallingford, UK: CAB International.
- Kottke, I. and Nebel, M., 2005. The evolution of mycorrhiza-like associations in liverworts: an update. *New Phytologist*, pp.330-334.
- Lanfranco, L., Bonfante, P. and Genre, A., 2016. The mutualistic interaction between plants and arbuscular mycorrhizal fungi. *Microbiology spectrum*, *4*(6), pp.4-6.
- Murakoshi, T., Tojo, M., Walker, C. and Saito, M., 1998. Arbuscular mycorrhizal fungi on adjacent semi-natural grasslands with different vegetation in Japan. *Mycoscience*, *39*(4), pp.455-462.
- Peterson, R.L., Massicotte, H.B. and Melville, L.H., 2004. *Mycorrhizas: anatomy and cell biology*. NRC Research Press.
- Read, D.J. and Perez-Moreno, J., 2003. Mycorrhizas and nutrient cycling in ecosystems–a journey towards relevance?. *New phytologist*, *157*(3), pp.475-492.
- Remy, W., Taylor, T.N., Hass, H. and Kerp, H., 1994. Four hundred-million-year-old vesicular arbuscular mycorrhizae. *Proceedings of the National Academy of Sciences*, *91*(25), pp.11841-11843.
- Selosse, M.A. and Le Tacon, F., 1998. The land flora: a phototroph-fungus partnership?. *Trends in Ecology & Evolution*, *13*(1), pp.15-20.
- Smith, S.E. and Read, D.J., 2008. Mycorrhizal Symbiosis, 3rd editio Edn. Academic Press, London.
- Walker, C. and Vestberg, M., 1994. A simple and inexpensive method for producing and maintaining closed pot cultures of arbuscular mycorrhizal fungi. *Agricultural and Food Science*, *3*(3), pp.233-240.
- Wang, Bin, and YL. Qiu. "Phylogenetic distribution and evolution of mycorrhizas in land plants." *Mycorrhiza* 16 (2006): 299-363.
- Yasmeen, T., Hameed, S., Tariq, M. and Ali, S., 2012. Significance of arbuscular mycorrhizal and bacterial symbionts in a tripartite association with Vigna radiata. *Acta physiologiae plantarum*, *34*, pp.1519-1528.

- Zaidi, A. and Khan, S., 2005. Interactive effect of rhizotrophic microorganisms on growth, yield, and nutrient uptake of wheat. *Journal of plant Nutrition*, *28*(12), pp.2079-2092.
- https://byjus.com/biology/mycorrhizae-an-overview/
- https://en.wikipedia.org/wiki/Mycorrhiza
- https://biologydictionary.net/mycorrhizae/
- https://www.biotechnologynotes.com/biotechnology/vesicular-arbuscular-mycorrhiza-vam-biotechnology/1153
- https://www.davidmoore.org.uk/assets/mostly_mycology/diane_howarth/mycorrhizal%20types.htm#:~:text=Mycorrhizas%20were%20traditionally%20classified%20into,the%20root%2C%20endo%20means%20inside.
- http://www.agriculturejournal.org/volume2number1/influence-of-different-types-mycorrhizal-fungi-on-crop-productivity/
- https://pubmed.ncbi.nlm.nih.gov/35178300/
- https://nph.onlinelibrary.wiley.com/doi/10.1111/nph.13288#:~:text=Four%20major%20mycorrhizal%20types%20have,short%20description%20of%20each%20type).

8.11 SUGGESTED READINGS

- Smith, S.E. and Read, D.J., 2010. *Mycorrhizal symbiosis*. Academic press.
- van Der Heijden, M.G., Martin, F.M., Selosse, M.A. and Sanders, I.R., 2015. Mycorrhizal ecology and evolution: the past, the present, and the future. *New phytologist*, *205*(4), pp.1406-1423.
- Lanfranco, L., Bonfante, P. and Genre, A., 2016. The mutualistic interaction between plants and arbuscular mycorrhizal fungi. *Microbiology spectrum*, *4*(6), pp.4-6.
- Smith, S.E. and Read, D.J., 2008. Mycorrhizal Symbiosis, 3rd editio Edn. Academic Press, London.

8.12 TERMINAL QUESTIONS

8.12.1 Short answer type questions

1- Write a short note on mycorrhiza.
2- Briefly describe the types of mycorrhiza.
3- Write the difference between ectomycorrhiza and endomycorrhiza
4- Describe the taxonomy of Arbuscular mycorrhiza
5- Describe the functions of mycorrhiza in higher plants.

8.12.2 Long answer type questions

1. What is mycorrhiza? Discuss its types, occurrence and distribution in detail.
2. Describe the process of isolation and inoculum of arbuscular mycorrhiza in detail.
3. Discuss the influences on growth and yield of crop plants of mycorrhizal association.

LABORATORY COURSE: BOT(N)-121L

UNIT-01-STUDY THE ROOT SYSTEM OF LEGUMINOUS PLANTS

Contents:

1.1 OBJECTIVES

- After reading this unit you will be able to:
  - Identify some economically important leguminous plants;
  - Explain about the root system of the given legumes;
  - Describe the root nodules of a leguminous plant highlighting its morphological characters.

1.2 INTRODUCTION

Fabaceae, formerly known as Leguminosae, has three subfamilies, many of which are very important to humans as food, fodder, and green manure. One of the main sources of protein is legumes, especially pulses. Legumes use the least amount of nitrogenous fertilisers per unit land area and yield higher protein yields than any other crop. Most leguminous species develop nodules on the surfaces of their roots. This is owing to their capacity to fix atmospheric nitrogen in symbiotic association with the bacterium - *Rhizobium*. This soil bacterium, colonises the roots of legumes and forms nodules from their cortical tissue.

These nodules develop in response to the invasion by specific strains of bacteria. *Rhizobium* colonise **Kidney Bean, Pea, Alfalfa, Clover,** and *Bradyrhizobium* prefers soybean plants. These nodules are functionally active, and are sites of nitrogen fixation. On transverse section of a functional root nodule, the central region invariably has light pink colour due to the presence of a haem protein-leghaemoglobin. The bacteroids are able to fix nitrogen, using energy supplied as carbohydrate produced by the host and delivered via the nodule's phloem link with the root vascular system. The bacteroids in turn supply their host (via the xylem link) with nitrogenous products which are produced in excess of their own needs. Such a relationship of assistance and mutualism is also termed as symbiosis. Both the organisms are benefited from the relationship. It is also a highly specialised one, with the host exhibiting several traits that support and accommodate the bacteria that live in it as a symbiont. Some of these are: nodule structure, diverted vascular strands, leghaemoglobin, and *the Rhizobium*-attracting roots. In this exercise, you will study the root system of some leguminous plants such as Kidney bean and Pea with special emphasis on their structure of the root system, distribution of nodules and symbiotic nitrogen fixation

1.3 STUDY OF THE ROOT SYSTEM OF LEGUMINOUS PLANTS

Leguminous plants, members of the Fabaceae family, are well-known for their ability to improve soil fertility through a symbiotic relationship with nitrogen-fixing bacteria. . In order to understand their significance in sustainable agriculture and the health of ecosystems, it is essential to study their root systems.

1.3.1 Characteristics of Leguminous Root Systems

The root systems of leguminous plants, show several distinct characteristics that contribute to their role in nitrogen fixation, soil enhancement, and overall plant health. The key features of these root systems are following:

1. Taproot System: Leguminous plants typically have a well-developed taproot, which is a primary root that grows directly downward and is larger and thicker than the secondary roots. It is provides strong anchorage for the plant and penetrates deep into the soil, accessing water and nutrients from deeper layers. This deep rooting habit helps legumes survive in dry conditions.

2. Lateral Roots: Lateral roots are secondary roots that emerge from the taproot and spread horizontally. These roots increase the surface area for water and nutrient absorption. They play a crucial role in stabilizing the soil and preventing erosion.

3. Root Nodules: Leguminous plants have specialised structures called root nodules on their roots. These are the homes of rhizobia, which are bacteria that fix nitrogen. The process starts when the legume's root hairs become infected by rhizobia bacteria found in the soil. This leads to the plant and bacteria to exchange a number of metabolic signals that result in the creation of nodules.

Inside the nodules, rhizobia convert atmospheric nitrogen (N_2) into ammonia (NH_3) through the enzyme nitrogenase. This ammonia is then assimilated into amino acids and other nitrogenous compounds essential for plant growth.

4. Root Hairs: Root hairs are tiny hair-like structures that extend from the root epidermis. They significantly increase the root's surface area, enhancing the plant's ability to absorb water and nutrients. Root hairs are also the initial infection sites for rhizobia bacteria during nodule formation.

5. Mycorrhizal Associations: Mycorrhizae are symbiotic associations between plant roots and fungi. In legumes, mycorrhizal fungi enhance nutrient uptake, particularly phosphorus, and improve water absorption. This symbiosis also helps in improving soil structure and fertility.

6. Adventitious Roots: Adventitious roots are roots that grow from non-root tissues, such as stems, leaves, or other parts of the plant. In leguminous plants, adventitious roots are less common than in some other plant families, but they do occur under certain conditions, especially in response to stress or injury. It can help in additional nutrient absorption and stability. *Phaseolus vulgaris* (Common Bean), *Glycine max* (Soybean), *Vicia faba* (Broad Bean), *Arachis hypogaea* (Peanut), *Cajanus cajan* (Pigeon Pea) etc, are examples of leguminous plants that develop adventitious roots.

7. Root Exudates: Root exudates are substances secreted by plant roots into the soil. In legumes, these exudates include organic acids, amino acids, sugars, and other compounds that can alter the

soil environment. Root exudates play a crucial role in attracting rhizobia for nodule formation, deterring pathogens, and facilitating nutrient uptake by changing soil chemical composition

1.3.2 Ecological and Agricultural Significance of Leguminous Roots

The root systems of leguminous plants have profound ecological and agricultural significance due to their unique ability to improve soil fertility, support plant growth, and contribute to ecosystem sustainability. Here are the key aspects of their importance:

- **Soil Fertility**: The nitrogen-fixing ability of leguminous roots enriches soil fertility, reducing the need for synthetic fertilizers and promoting sustainable agriculture.
- **Crop Rotation**: Including legumes in crop rotation systems helps break pest and disease cycles and improves soil health for subsequent crops.
- **Soil Structure**: The extensive root system of legumes improves soil structure, enhancing water infiltration and reducing erosion.
- **Biodiversity**: Leguminous plants contribute to biodiversity by supporting various soil organisms through their root exudates and by providing habitats for beneficial insects and microbes.
- **Reduced Chemical Inputs**: By naturally enriching the soil with nitrogen, leguminous plants reduce the reliance on synthetic fertilizers, promoting more sustainable and eco-friendly farming practices.

1.3.3 Methods of Studying Leguminous Root Systems

Enhancing the agricultural and ecological benefits of leguminous plants needs an understanding of their root systems. These root systems are studied using a variety of methods, from advanced molecular studies to field investigations. Listed here are the main techniques:

1. **Field Observations**: Examining leguminous plants in their natural or agricultural environments provides insights into root architecture, depth, and interactions with soil and other plants.
2. **Laboratory Experiments**: Controlled experiments allow researchers to manipulate environmental factors such as light, water, and nutrients to study their effects on root development and nodulation.
3. **Molecular Techniques**: Techniques like gene expression analysis, RNA sequencing, and genetic modification help in understanding the genetic and biochemical pathways involved in root development and nitrogen fixation.
4. **Imaging Technologies**: Advanced imaging techniques, such as MRI, CT scanning, and fluorescence microscopy, provide detailed visualization of root structures and nodules, aiding in the study of root morphology and function.

1.4 ROOT SYSTEM OF KIDNEY BEAN (*Phaseolus vulgaris* L.)

The kidney bean (*Phaseolus vulgaris* L.) is a type of leguminous plant with a well-developed root system that supports its growth and facilitates its ability to fix atmospheric nitrogen (Fig.1). Here's a detailed look at the root system of the kidney bean, including its structure, functions, and associated symbiotic relationships:

1.4.1 Structure of the Root System

1. **Primary Root (Taproot):** The primary root develops from the radicle of the germinating seed. It grows vertically downward and serves as the main anchor for the plant. The taproot penetrates deep into the soil, providing stability and accessing deeper water and nutrient sources.
2. **Secondary Roots (Lateral Roots):** Secondary or lateral roots emerge from the primary root. These roots spread horizontally, enhancing the surface area for nutrient and water absorption. Lateral roots also contribute to anchoring the plant securely in the soil.
3. **Root Hairs:** Tiny hair-like structures extend from the epidermis of the roots. Root hairs significantly increase the surface area for absorption, facilitating efficient uptake of water and nutrients from the soil.
4. **Root Nodules:** Specialized structures that form on the roots due to the symbiotic relationship with *Rhizobium* bacteria. Nodules house the nitrogen-fixing bacteria, allowing the plant to convert atmospheric nitrogen into a usable form (ammonia).
5. **Morphology of root nodules**: The morphology of root nodules in kidney beans (*Phaseolus vulgaris* L.) involves various aspects including their shape, size, color, and distribution on the root system. These characteristics can vary depending on the specific strain of *Rhizobium* bacteria involved, environmental conditions, and the growth stage of the plant. Here you will be detailed look at these morphological features of the root system of kidney bean.
    - **Shape:** Root nodules are typically spherical to oval in shape. This shape allows for efficient accommodation of the nitrogen-fixing bacteria within the nodule. Occasionally, nodules can have irregular shapes, especially if multiple nodules fuse together or if growth conditions are suboptimal.
    - **Size:** Nodule size can vary widely depending on several factors, including the age of the nodule, the effectiveness of the *Rhizobium* strain, and environmental conditions. Typical sizes range from a few millimeters to over a centimeter in diameter. Newly formed nodules are small, usually less than 1 mm in diameter.
    - As the nodule matures and the bacteria fix more nitrogen, it enlarges, often reaching up to 5-10 mm in diameter.

- **Colour:** Active, healthy nodules have a pink to red interior due to the presence of leghemoglobin, which helps maintain low oxygen levels necessary for nitrogenase activity.
- The pink/red coloration is a key indicator of active nitrogen fixation.
- Inactive or senescent nodules can appear white, green, or brown. White nodules often indicate early stages of formation or lack of effective *Rhizobium* infection.
- Green or brown nodules suggest that the nodule is no longer fixing nitrogen effectively, often due to aging or suboptimal conditions.

Fig.1: Kidney bean (*Phaseolus vulgaris* L.) Plant with nodule in root system

1.4.2 Distribution of Nodules on the Root System

- **Primary and Secondary Roots:** Nodules are typically found on both primary (taproot) and secondary (lateral) roots. They tend to form more abundantly on the secondary roots, which provide a larger surface area for *Rhizobium* infection.
- **Clustered Formation:** Nodules often appear in clusters along the roots rather than being evenly distributed. Clustering can result from localized infection sites where *Rhizobium* bacteria are more concentrated.
- **Proximity to Root Tips:** Nodules usually form near the root tips, where younger, more actively growing root tissues are more susceptible to *Rhizobium* infection. As roots elongate, new nodules can continue to form further along the root.

1.4.3 Symbiotic Nitrogen Fixation

One of the key features of kidney bean roots is their ability to form nodules that fix atmospheric nitrogen. The process of which is given below.

1. **Infection by *Rhizobium*:** *Rhizobium* bacteria in the soil are attracted to the roots by chemical signals (flavonoids). The bacteria attach to root hairs and induce curling of the hairs to form an infection thread.
2. **Nodule Formation:** The infection thread carries *Rhizobium* bacteria into the root cortex, where the bacteria are released into the plant cells. The plant cells proliferate, forming a nodule around the bacteria.
3. **Nitrogen Fixation Process:** Within the nodules, *Rhizobium* bacteria convert atmospheric nitrogen (N_2) into ammonia (NH_3) through the enzyme nitrogenase. The plant uses the ammonia to synthesize amino acids, proteins, and other nitrogen-containing compounds.

1.5 ROOT SYSTEM OF PEA PLANT (Pisum sativum L.)

The root system of the pea plant (*Pisum sativum* L.) is crucial for its overall growth, development, and ability to fix atmospheric nitrogen through symbiosis with *Rhizobium* bacteria. Here's an overview of the pea plant root system (Fig. 2), detailing its structure, functions, and the formation of root nodules:

1.5.1 Structure of the Root System

1. Primary Root (Taproot): The primary tap root system of pea is similar to kidney bean tap root system.

2. Secondary Roots (Lateral Roots): Branch off from the primary root. Spread horizontally and obliquely, increasing the surface area for water and nutrient absorption. Enhance stability and improve nutrient uptake from the soil.

3. Root Hairs: Tiny hair-like extensions from the root epidermis. Significantly increase the surface area for water and nutrient absorption from the soil.

4. Root Nodules: The root nodules of peas (*Pisum sativum* L.) share many similarities with those of other leguminous plants, including kidney beans. However, there are specific characteristics that can vary slightly due to the species and environmental conditions.

5. Morphology of root nodules: Here's a detailed look at the morphology of pea root nodules, focusing on their shape, size, color, and distribution on the root system:

Shape: Pea root nodules are typically spherical to oval in shape. This rounded form allows for optimal space to house the nitrogen-fixing bacteria and provides a stable structure for the

symbiotic relationship. Some nodules may appear lobulated or segmented, particularly as they grow larger or when multiple nodules coalesce.

Size: The size of pea root nodules can vary depending on several factors, including the effectiveness of the *Rhizobium* strain, the age of the nodules, and soil conditions. Generally, nodule sizes range from a few millimeters to about 1-2 centimeters in diameter. Newly formed nodules are small, usually less than 1 mm in diameter. As the nodules mature and increase nitrogen fixation activity, they can grow larger, often reaching up to 5-10 mm or more.

Colour: Active nodules that are fixing nitrogen effectively have a pink to red interior. This coloration is due to leghemoglobin, which helps maintain a low oxygen environment necessary for the nitrogenase enzyme. The pink/red color is a clear indicator of active nitrogen fixation. Inactive or aging nodules can turn white, green, or brown. White nodules might indicate initial stages of formation or ineffective *Rhizobium* infection. Green or brown coloration typically indicates senescence conditions leading to reduced nitrogen fixation.

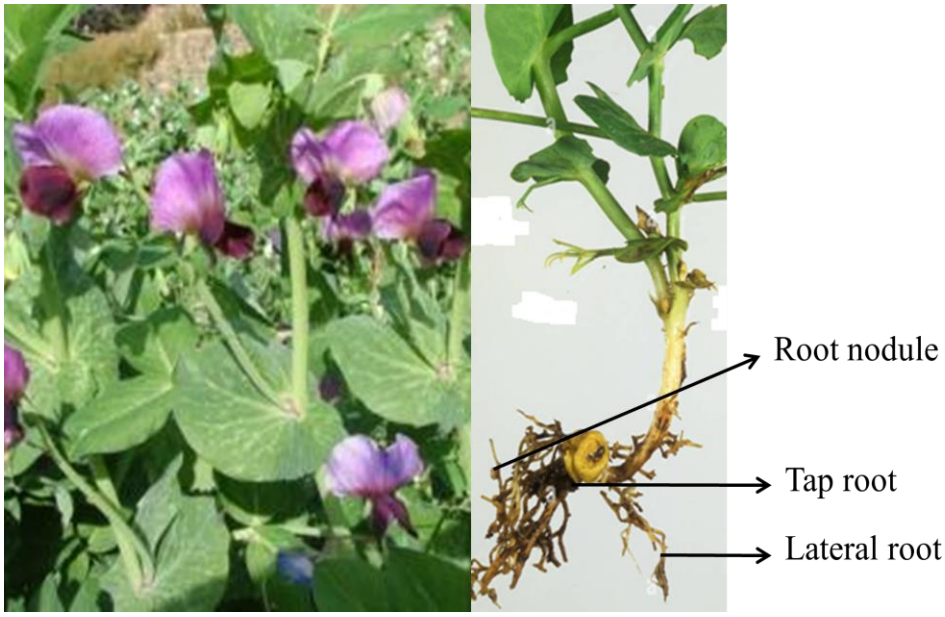


Fig.2: Pea plant (*Pisum sativum* L.) with nodule in root system

1.5.2 Distribution of Nodules on the Root System

- **Primary and Secondary Roots:** Nodules are commonly found on both primary (taproot) and secondary (lateral) roots of pea plants. Secondary roots often bear more nodules due to their extensive surface area and active growth regions.
- **Clustered Formation:** Nodules tend to form in clusters along the roots rather than being evenly distributed. This clustering results from localized sites of *Rhizobium* infection where bacteria are more concentrated.

- **Proximity to Root Tips:** Nodules typically form near root tips, where the root tissue is younger and more susceptible to *Rhizobium* infection. As roots grow and extend, new nodules can continue to form along the root length.

1.5.3 Symbiotic Nitrogen Fixation: Nodules play a crucial role in the pea plant and their ability to fix atmospheric nitrogen, which is essential for its growth and development. Here's a detailed look at the root nodules of peas:

- **Chemical Signaling:** The process begins with the release of flavonoids by pea plant roots into the soil. These flavonoids attract *Rhizobium* bacteria to the root system.
- **Bacterial Attachment and Infection:** Upon encountering the flavonoids, *Rhizobium* bacteria produce Nod factors, which initiate the infection process. The bacteria enter the root hairs through infection threads, which are specialized structures formed by the invagination of the root hair cell membrane.
- **Nodule Initiation:** Once inside the root cortex, the bacteria are released from the infection threads. This triggers the formation of a nodule through the division and differentiation of plant cells in the cortical region of the root.
- **Development of Symbiotic Relationship:** Within the nodule, the bacteria transform into bacteroids, which are capable of fixing atmospheric nitrogen.

1.6 PRACTICAL EXERCISE-1

1.6.1 Exercise-1: To study and understand the root system of leguminous plants, focusing on the formation and function of root nodules involved in nitrogen fixation.

1.6.2 Materials Required

Leguminous plants (e.g., **Kidney Bean, Pea, Soybean, Alfalfa, Clover);** Garden soil or, spade, secateurs; Watering can; Magnifying glass or hand lens; Dissecting microscope; Sterile gloves; Labels and markers; Notebook and pen; Camera for documentation; Pen; Some papers, Fork, Ruler; Some plastic sheets etc.

1.6.3 Procedure

1. Fill the pots or containers with garden soil for the sowing of leguminous such as **Kidney Bean, Pea, Soybean, Clover, Alfalfa etc**.
2. Label each pot with the plant species and the date of planting.
3. Check the proper growth of the plant for several weeks until they develop a mature root system with visible nodules. Watering and care for the plants as needed during this perio

4. **Preparing for root examination:** After the plants are fully grown, carefully take plants out of its pot so as not to damage the roots.
- Use water to carefully remove contaminants from the roots.
- Take care not to separate any root nodules. To avoid infection, put on sterile gloves.

5. **Examining the root system:** Examine the overall structure of the root system. Note the primary root, lateral roots, and any fine root hairs.

- Search for root nodules, which are usually spherical, tiny, and can be pink, white, or reddish-brown in colour.

6. **Observation of root nodules**

- Use a magnifying glass or hand lens to examine the nodules closely. Note their size, shape, and distribution on the root system.
- If available, use a dissecting microscope for a more detailed examination of the nodules' surface and internal structure.
- Cut and open a nodule with a sterile blade to observe the internal color and texture, indicating the presence of nitrogen-fixing bacteria.
- If the infecting strain of *Rhizobium* is able to fix nitrogen in the given species of legume, the inside of the nodule wall will be pink in colour. The pink colour indicates the presence of plant protein necessary to fix nitrogen.

- If the bacteria cannot fix nitrogen, the inside will be green.

7. Record your observations in your notebook, noting the number, size, and condition of the nodules.

8. Take photographs of the root system and nodules for documentation and comparison. Also draw diagrams of the root system and nodules, labeling all significant parts.

1.6.4 Results

- Compare the root systems of different leguminous plants.
- Discuss the role of the nodules in nitrogen fixation and how they benefit the plant.
- Analyze any differences in nodule formation and distribution among the species studied.

1.6.5 Conclusion

Summarize your findings on the root system and nodule formation in leguminous plants. Reflect on the importance of leguminous plants in agriculture, particularly their role in enhancing soil fertility through nitrogen fixation.

1.7 SUMMARY

Studying the root systems of leguminous plants are complex and multifunctional, playing a vital role in plant health, soil fertility, and ecological balance. Understanding these characteristics enables better management of leguminous crops in agricultural systems, enhancing productivity and sustainability. Thus, the study of characteristics of legumes root system through field observations, laboratory experiments, molecular techniques, and advanced imaging technologies, researchers and farmers can optimize the benefits of legumes in various environments.

1.8 SELF ASSESSMENT QUESTIONS

1.8.1 Multiple Choice Questions

1. What is the primary function of the taproot in leguminous plants?

   a) Seed dispersal
   b) Photosynthesis
   c) Nitrogen fixation
   d) Uptake of water and nutrients from deep soil layers

2. Which of the following is a characteristic feature of leguminous root systems?

   a) Aerial roots
   b) Fibrous roots
   c) Root nodules
   d) Stilt roots

3. What symbiotic relationship is crucial for nitrogen fixation in leguminous plants?

   a) Algae

   b) Nematodes

   c) Mycorrhizal fungi

   d) Rhizobia bacteria

4. Which type of root is primarily responsible for the absorption of water and nutrients in leguminous plants**?**

   a) Aerial roots

   b) Adventitious roots

   c) Lateral roots

   d) Taproot

5. Root nodules in legumes are primarily associated with which process?

a) Photosynthesis

b) Nitrogen fixation

c) Water absorption

d) Carbon sequestration

6. What is the primary advantage of using leguminous plants in crop rotation systems?

a) Increased pest resistance

b) Enhanced soil nitrogen levels

c) Reduced water requirements

d) Faster plant growth

7. Which of the following is NOT a method used to study leguminous root systems?

a) Computational modeling

b) Field observations

c) Grafting techniques

d) Laboratory experiments

8. What is the main function of root hairs in leguminous plants?

a) Facilitate gas exchange

b) Store nutrients

c) Support the plant structurally

d) Increase surface area for water and nutrient absorption

1.8.2 Fill in the Blanks

1) …….. is the microsymbiont , which nodulates and infects legume roots.

2) If the infecting strain of *Rhizobium* is able to fix nitrogen in the given species of legume, the inside of the nodule wall will be …….

3) The pink colour indicates the presence of plant protein necessary to fix……...

4) If the bacteria cannot fix nitrogen, the inside will be …….. in colour.

5) *Rhizobium* bacteria convert atmospheric N_2 into NH_4 through the enzyme ……

Answer key

1.8.1: *1-(d); 2-(c); 3-(d); 4-(c); 5-(b); 6-(b); 7-(c); 8-(d)*

1.8.2**:** 1) *Rhizobium*; 2) pink in colour; 3) nitrogen; 4) green; 5) nitrogenase

1.9 REFERENCES

Esau, K. (1977). *Anatomy of Seed Plants*. Wiley. A comprehensive text on plant anatomy that includes detailed sections on root structures.

Sprent, J. I. (2009). Legume Nodulation: A Global Perspective. Wiley-Blackwell.

Fageria, N. K., Baligar, V. C., & Jones, C. A. (1997). Growth and Mineral Nutrition of Field Crops. CRC Press.

Graham, P. H., & Vance, C. P. (2003). "Legumes: Importance and constraints to greater use." Plant Physiology, 131(3), 872-877.

Smith, D. L., & Hume, D. J. (1987). "Comparison of three soybean (*Glycine max* (L.) Merr.) genotypes from the F4 generation in root system characteristics and growth and yield responses to drought stress." Plant and Soil, 98, 123-132.

1.10 SUGGESTED READINGS

https://gml.noaa.gov/education/lesson_plans/Nitrogen%20Fixation%20in%20Root%20Nodules.pdf.

Pommeresche & Hansen (2017): Examining root nodule activity on legumes. FertilCrop Technical Note. Download at www.fertilcrop.net.

Jones, A. J. (2015). *The Role of Leguminous Root Systems in Soil Fertility*. Ph.D. dissertation, University of California, Davis.

1.11 TERMINAL QUESTIONS

1.11.1 Short Answer Type Questions

1. How do root nodules contribute to the nitrogen content in the soil?
2. What is the symbiotic relationship between leguminous plants and *Rhizobium* bacteria?
3. How can the study of leguminous plant root systems benefit agricultural practices?
4. What factors might affect the formation and effectiveness of root nodules in leguminous plants?

1.11.2 Long Answer Type Questions

1. Examine the structure, function, and symbiotic processes of the root system of kidney beans.
2. What differences did you observe in the root systems of different leguminous plants?

UNIT-02 ISOLATION OF *RHIZOBIUM* FROM ROOT NODULES OF LEGUMES

Contents:

2.1 OBJECTIVES

After reading this unit you will be able to:

- Know about the procedure of isolation of *Rhizobium* from legumes root.
- Easily isolate *Rhizobium* from the roots of legumes.

2.2 INTRODUCTION

Rhizobium is a genus of soil bacteria that fix nitrogen. Certain *Rhizobium* species associate with the roots of legumes and *Parasponia* to fix nitrogen in an endosymbiotic manner. Symbiotic relationship between *Rhizobium* and legume plants are the most important biological supplier of fixed nitrogen in soil based ecosystem.

Rhizobium is the microsymbiont, which nodulates and infects legume roots. There are currently six genera in the family Rhizobiaceae like *Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium,* and *Bradyrhizobium*, which are generally referred to as Rhizobia (Vance, 1998). Rhizobia are aerobic, gram negative, non-sporulating rod shaped bacteria that live in symbiotic partnerships with legumes, forming nodules on their roots or stems and fixing atmospheric nitrogen (Quatrini et al. 2002). They are motile nature and they can travel through the water films around the soil particles in moist soil (Hamdi, 1971).The different plant species have different nodule morphologies and sizes. On clover, the nodules are rounded or oval in form and quite small, whereas the nodules on cowpea, common beans, and soybeans are comparatively big, spherical, and tightly attached to the root. The nodules on vetch, lucerne and peas are typically longer and resemble fingers.

Symbiotic nitrogen fixation is dependent upon the infection of the host root by the suitable microbial symbiont and the subsequent development of the necessary enzymes. Plant cells within root nodules are colonised by bacteria, where they convert atmospheric nitrogen into ammonia using the enzyme nitrogenase and then provide organic nitrogenous compounds like Glutamine or Ureides to the plant. Organic molecules produced by photosynthesis are subsequently supplied to the bacterium by the plant.

Rhizobium can be isolated from the root nodules after cleaning and surface sterilizing the nodules. The nodules can be crushed in small quantity of sterile water and the nodules contents streaked on the plate containing Congo red yeast extract mannitol agar (CRYMA). Dye Congo red in medium are used to distinguish rhizobia from other contaminating bacteria.

2.3 PROCEDURE OF ISOLATION OF RHIZOBIUM

The isolation of *Rhizobium* from root nodules of leguminous plants, involves a series of steps to obtain pure cultures of these nitrogen-fixing bacteria. The isolation of *Rhizobium* from root

nodules of leguminous plants, involves a series of steps to obtain pure cultures of these nitrogen-fixing bacteria. Here we will study about different steps of the isolation of *Rhizobium* from the fresh nodules of Legumes. Here we will study about different steps of the isolation of *Rhizobium* from the fresh nodules of Legumes (Fig 2.1).

2.3.1 Materials required

- Well developed legume nodule
- Mercuric chloride (0.1%) and Alcohol (Ethnol 70% or 90%)
- Forceps, Glass rod , Petri dishes and sterile water blanks
- Inoculation needle
- Congo red stock solution, yeast extract mannitol agar medium
- Yeast Extract Mannitol Agar (YEMA) (Vincent, 1970) composition contains:-

Mannitol	:	10 g/l
K2HP04	:	0.5 g/l
MgS04 .7H20	:	0.2 g/l
NaCl	:	0.1 g/l
Yeast Extract	:	0.5 g/l
Agar	:	15 g/l
Distilled Water	:	1000 ml
PH		7±0.1

They were autoclaved at 121^0C for 15 minutes.

2.3.2 Collection and preservation of root nodules

i) Equipment for collection and preservation of root nodules in field trips

- **Sampling vials with desiccated silica gels:** During the field trips, the collected root nodules must be prevented from decomposing and prevented from invasion by soil microorganisms which inhibit further isolation procedures. Therefore, the root nodules are freshly collected and preserved in a vial with desiccated silica gels. If the gel colour turns pink, a blue desiccant must be used in its place as soon as possible.
- It is very difficult to collect root nodules from wild legumes, because soil growing the wild legumes is very hard. Therefore, the sturdily built shovel is essential for digging up legume roots.
- Field note book and pencil, Detailed road maps, , Compass and GPS, Camera, Knife, Forceps, Small medium and large plastic Ziplocs bags for soil, root samples and herbarium specimen collection , Paper towels, Permanent marker.

ii) Points to consider before to sampling

- **Site identification:** Use a GPS device to record altitude, latitude and longitude and other related parameters. You should noted nation, region, landmark, distance, and orientation if at all possible.
- **Host plants identification:** It is strongly advised that you go with a taxonomist who specialises in bean plants. To determine the genus, species, and cultivar of the legume plant, gather a sample for your herbarium specimen. Capture images of the legume-producing plants.
- **Soil identification:** Collect soils in small plastic ziplocs bags and examine soil analysis

iii) Collection and preservation of root nodules

- Dig up entire plants to get root nodules.
- Carefully remove soil around root nodules.
- Exposed root nodules can be collected with forceps.
- One unit of collected material is represented by all the nodules from a single host plant, which is kept in the same vial. Root nodules from different plants of the same species should not be combined because they may represent different soil environments even if only several metres apart.
- The vials containing dried root nodules are kept in a refrigerator at 4oC until isolations of *Rhizobium*.

2.3.3 Procedure

- Fresh roots of legume crops collected from field are washed with tap water to remove soil and organic particles.
- The roots with the associated nodules are cut 2-3 mm on either side of the nodules, which are kept in place using forceps.
- Whole and, undamaged nodules are immersed for 10 second in ethanol (95%) or isopropanol (to release the tissue's surface tension and remove air bubbles); transferred to a 2.5-3.0% (v/v) solution of sodium hypochlorite or chlorox (commercial bleach) 1:1 (v/v) and soaked for 4-5 min.
- The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse the sterilizing chemicals (Lupwayi and Haque, 1994). Forceps may be sterilized quickly by dipping in alcohol and flaming. Sterile glass or plastic Petri dishes may be used as containers for the alcohol, sodium hypochlorite, and water. Otherwise, nodules may be placed into a 125 ml conical flask.
- Mercuric chloride solution (0.1% weight/volume) or solution of hydrogen peroxide (3% W/V) can be used for the sterilization of nodule.
- Followed by nodules were transferred into sterile Petri-dishes and crushed with alcohol flamed sterile glass rod in a drop of normal saline solution (0.85% NaCl) inside a laminar air flow hood (Somasegaran and Hoben, 1994).

- Then after 0.1ml (loopful) of the suspensions were streaked on plate containing Yeast Extract Mannitol Agar (YEMA) and incubated at 28 ± 2^0C from 3-5 days.

2.3.4 Observation

- Describe the observations during the incubation period. Record colony characteristics such as appearance, color, shape, size, and texture.
- Appearance of circular, raised and white translucent colonies indicates *Rhizobium*.
- Red colored, small colonies are *Agrobacterium.*

2.3.5 Purification and preservation

- After 3-5 days of growth, single dome-shaped colonies were chosen with sterile inoculating loop and streaked on sterile YEMA plates and incubated at 28 ± 20C. After a thorough examination of the purity and homogeneity of the colony types by repeated re-streaking, one well-isolated colony was selected, moved on a YEMA slant containing 0.3% (W/V) CaCO3, and incubated at 28±2^0C. When sufficient growth was observed, the culture was transferred to be preserved at 4^0C for future use (Vincent, 1970). Then, in accordance with Jordan (1984), the isolated native strains were categorised based on morphological, biochemical and physiological traits.

2.3.6 Result: You can give an in-depth account of the isolation's outcomes on the basis of colony morphology, Gram-staining, biochemical test and microscopic tests.

a. Colony Morphology: The appearance of *Rhizobium* colonies on the selective medium can be described as follows:

- Colony color (typically white, creamy, or translucent)
- Shape (circular, irregular)
- Surface texture (smooth, rough)
- Margin (entire, undulate)

b. Gram Staining: Specify that *Rhizobium* is Gram-negative according to the results of the Gram staining procedure.

c. Biochemical Tests: Summarise the results if any biochemical tests (catalase, oxidase, nitrate reduction, etc.) were carried out.

- Catalase Test: Positive (bubbling observed)
- Oxidase Test: Positive (color change to dark purple)
- Nitrate Reduction: Positive (red color after adding reagents)

d. Microscopic Examination: Discuss the results of microscopic examination, describing the shape and arrangement of the bacteria (e.g., rod-shaped, pleomorphic).

2.3.7 Precautions

1. Media should be prepared carefully.
2. Experiment should be carried in sterile conditions.

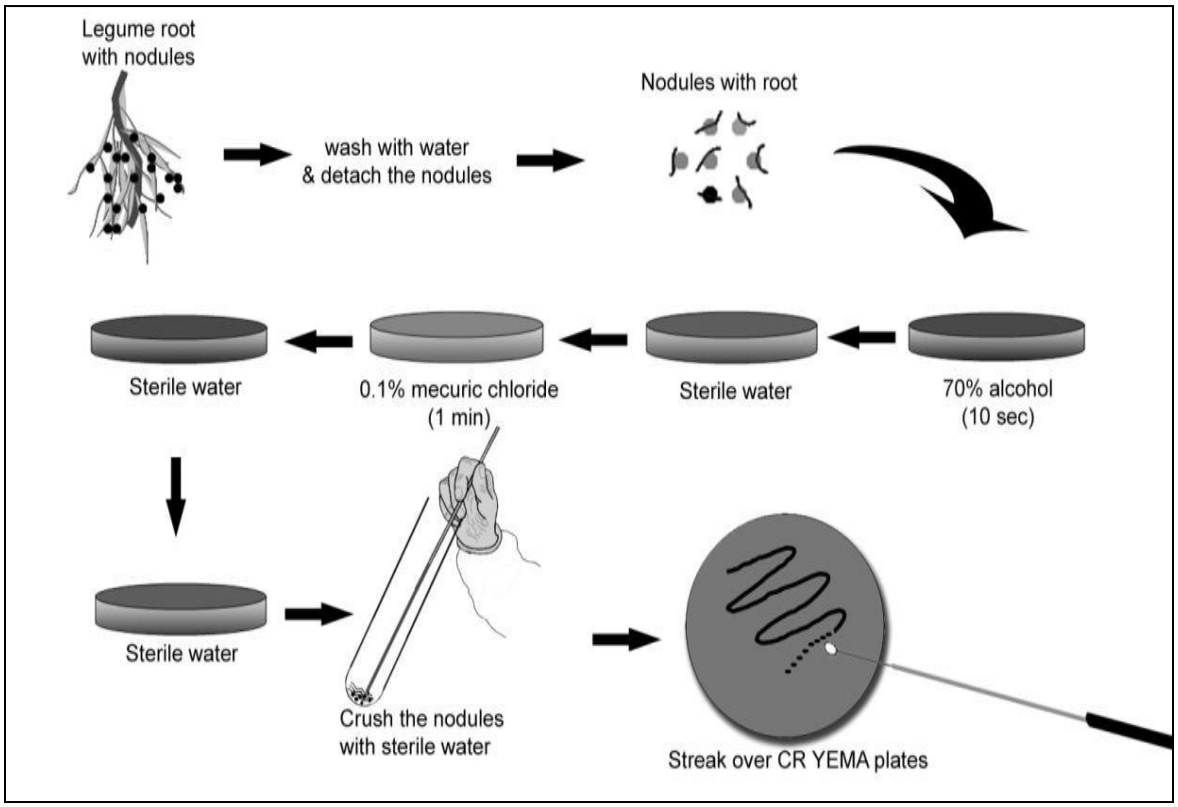


Fig 2.1: Steps of isolation of *Rhizobium* from fresh root nodule of legume (Source: Karthikeyan and Sivasakthivelan, Practical manual cum record CAG – AGM)

2.3.8 Discussion: You will write the discussion in your own words. As an example, here, given an explanation of the discussion:

Colonies of *Rhizobium* with distinctive morphological and biochemical characteristics were successfully obtained by the isolation process. The colonies were Gram-negative rods, catalase and oxidase positive, confirming their identity as *Rhizobium*. The experiment highlighted the importance of proper sterilization and selective media in isolating *Rhizobium*.

2.3.9 Conclusion

The experiment successfully isolated *Rhizobium* from root nodules. The isolated bacteria exhibited typical characteristics of *Rhizobium*, contributing to our understanding of its role in nitrogen fixation.

2.4 SUMMARY

To isolate *Rhizobium* from legume root nodules, first collect healthy nodules from leguminous plants and thoroughly wash them to remove soil. Whole and, undamaged nodules are immersed for 10 second in ethanol (95%) or isopropanol (to release the tissue's surface tension and remove air bubbles); transferred to a 2.5-3.0% (v/v) solution of sodium hypochlorite or chlorox (commercial bleach) 1:1 (v/v) and soaked for 4-5 min. Crush the sterilized nodules in a sterile mortar, and transfer the extract onto Yeast Extract Mannitol Agar (YEMA) plates containing Congo Red using a sterile inoculating loop. Incubate the plates at 28°C for 5-7 days. Observe for the growth of characteristic Rhizobium colonies (white, creamy, smooth, entire margins) and confirm their identity through Gram staining (Gram-negative), biochemical tests (catalase and oxidase positive, nitrate reduction positive), and microscopic examination (rod-shaped bacteria).

2.5 SELF ASSESSMENT QUESTIONS

2.5.1 Multiple Choice Questions

1. What is the primary purpose of isolating *Rhizobium* from legume root nodules?
    a) To study its pathogenic properties
    b) To examine its role in nitrogen fixation
    c) To use it as a food preservative
    d) To investigate its antibiotic resistance
2. In the process of surface sterilising root nodules, which sterilising agent is applied first?
    a) Ethanol
    b) Distilled water
    c) Hydrogen peroxide
    d) Sodium hypochlorite
3. What is the incubation temperature for YEMA plates inoculated with *Rhizobium* extract?
    a) 25°C
    b) 28°C
    c) 30°C
    d) 37°C
4. What color do *Rhizobium* colonies typically exhibit on YEMA plates?
    a) Red
    b) Pink
    c) Yellow
    d) White to creamy
5. Which staining technique is used to confirm *Rhizobium* as Gram-negative bacteria?
    a) Capsule staining
    b) Acid-fast staining

c) Gram staining
d) Endospore staining

6. Which of the following is a characteristic feature of *Rhizobium* colonies on YEMA plates?
   a) Irregular shape
   b) Spiky margins
   c) Rough texture
   d) Smooth texture with entire margins
7. What type of media is YEMA considered to be for the isolation of *Rhizobium*?
   a) Differential media
   b) Enriched media
   c) Minimal media
   d) Selective media
8. What is the role of Congo Red in the YEMA plates used for *Rhizobium* isolation?
   a) To act as a nutrient source
   b) To differentiate *Rhizobium* from other bacteria
   c) To provide a color indicator for pH changes
   d) To inhibit the growth of non-target bacteria

2.5.2 Fill in the Blanks

1) *Rhizobium* is the …….which nodulates and infects legume roots.

2) CRYMA stands for…….

3) Mercuric chloride solution (0.1% weight/volume) can be used for the ……

4) Appearance of circular, raised and white translucent colonies indicates……..

5) Plant cells within root nodules are colonised by bacteria, where they convert atmospheric nitrogen into ammonia using the enzyme …….

Answer keys:

2.5.1: 1-(b); 2-(a); 3-(b); 4-(d); 5-(c); 6-(d); 7-(d); 8-(b)
2.5.2: 1) microsymbiont; 2) Congo red yeast extract mannitol agar; 3) sterilization of nodule; 4) *Rhizobium*; 5) nitrogenise

2.6 REFERENCES

- Hamdi, Y. A. (1971). Soil-water tension and the movement of rhizobia. Soil Biol. Biochem. 3, 121–126. 10.1016/0038-0717(71)90004-6.
- Lupwayi, N. and Haque, I. (1994). Legume-Rhizobium Technology Manual: Environmental sciences division, international livestock center for Africa, Addis Ababa, Ethiopia. pp.1-93.

- Quatrini, P., Scaglion, G., Cardinale, M., Caradonna, F. and Puglia, A.M. (2002). *Bradyrhizobium* sp. nodulatig the mediterranean shrub, Spanis broom (*Spartium junceum* L.). J .Appl.Microbiol.92:13-21.
- Solomon Legesse and Fassil Assefa (2014). Symbiotic and phenotypic characteristics of rhizobia nodulating faba bean (vicia faba) from tahtay koraro, northwestern zone of Tigray Regional State, Ethiopia. IJTEEE: 2(11), 15-23.
- Vance, C.P. (1998). Legume Symbiotic nitrogen fixation: Agronomical aspects. Kluver Academic Publishers, Dorderecht, 509- 530.
- Vincent, J.M. (1970). A Manual for the Practical Study of Root Nodule Bacteria. Blackwell, Oxford and Edinburgh, pp.164.

2.7 SUGGESTED READINGS

- Karthikeyan, B. and Sivasakthivelan, p. Practical manual cum record CAG – AGM 227 Biofertilizer Technology. Department of Agricultural Microbiology Faculty of Agriculture, Annamalai University.
- Yadav, A. N. (2021). Production Technology for Bioagents and Biofertilizers: A Laboratory Manual. DKSGACA Manual No. 07/2021. Eternal University, Baru Sahib, Sirmour-173101, Himachal Pradesh, India

2.8 TERMINAL QUESTIONS

2.8.1 Short Answer Type Questions

1- What are the main steps involved in the isolation of *Rhizobium* from legume root nodules?

2- Why is surface sterilization of root nodules necessary in the isolation process?

3- Describe the appearance of *Rhizobium* colonies on YEMA plates.

4- What is the significance of using Congo Red in YEMA plates?

2.8.2 Long Answer Type Questions

1- Explain the procedure of isolating *Rhizobium* from legume root nodules, highlighting the importance of each step.

2- Discuss the importance of *Rhizobium* in agriculture and the benefits of isolating and studying these bacteria.

UNIT-3 COLLECTION OF CYANOBACTERIA AND *AZOLLA* FROM RICE FIELDS

Contents:

3.1 OBJECTIVES

After reading this unit learners will be able:

- To understand the isolation and collection of BGA from rice fields,
- To understand the isolation and collection of *Azolla* from rice fields.

3.2 INTRODUCTION

Algae are prevalent in environments with sufficient light and moisture content. While many algae species are present on earth, only a few types of blue green algae (BGA) have the ability to fix atmospheric nitrogen, which greatly increases soil fertility. Algae that fix nitrogen and release vitamin B12, auxins, and ascorbic acid, which may aid in plant growth, are classified as cyanobacteria and are commonly known as blue green algae. The various groups of cyanobacteria are filamentous, non heterocystous *(Oscillatoria , Spirulina),* filamentous, heterocystous (*Anabaena*, *Nostoc*), unicellular (*Microcystis, Microcoleus)*

On the other hand, *Azolla* is a free-floating water fern that attaches itself to the nitrogen-fixing blue green algae *Anabaena azollae* in order to fix atmospheric nitrogen. *Azolla* is known to contribute 40–60 kg N ha-1 per rice crop when used as a biofertilizer for wetland rice. *Azolla* has a large amount of agronomic potential, especially for rice crops, and it is frequently utilised as a biofertilizer to raise rice production. In the Philippines, Thailand, Sri Lanka, and India, the use of *Azolla* as a dual crop with wetland rice is becoming more and more significant.

The fast decomposition of *Azolla* in the soil and the rice's effective uptake of its nitrogen make it a valuable biofertilizer for rice crops. When applied to tropical rice soils, *Azolla* quickly mineralizes and releases nitrogen into the rice crop quite rapidly. *Azolla microphylla, Azolla filiculoides, Azolla pinnata, Azolla caroliniana, Azolla nilotica, Azolla rubra* and *Azolla mexicana* are the most common species.

3.3 ISOLATION AND COLLECTION OF BGA

3.3.1 By employing various medium and the serial dilution approach, BGA can be extracted from soil.

- We take 10 g of rice field soil and keep it in 100 ml sterile water and mit it thoroughly.
- Then 10 ml of the soil mixture should be transferred aseptically to another flask containing 90 ml sterile water to make 10^{-2} dilution.
- We prepare BG 11 medium and pour into a series of petridishes.
- With the help of micropipette we pipette out 1 ml of soil suspension from 10^{-2} dilution over the BG 11 medium plates and spread it

- The plates should be kept in light chamber or partial sunlight and will be incubated for 3-4 weeks.
- We will observe the blue green colour growth on the agar surface of the petriplates.

Chemical composition of BG – 11 medium

Compound	Amount (gms/Litre)
Sodium nitrate (Na NO_3)	1.5
Dipotassium hydrogen phosphate(K_2HPO_4)	0.04
Magnesium sulphate ($MgSO_4.7H_2O$)	0.075
Calcium chloride dehydrate ($CaCl_2.2H_2O$)	0.036
Sodium carbonate ($NaCO_3$)	0.020
Disodium magnesium EDTA.Na_2.2 H_2O	0.001
Ferric ammonium citrate	0.012
Citric acid	0.005
Trace metal solution	1 ml/l
Final pH after sterilization (at 25°C)	07.1

3.3.2 BGA can also be isolated from soil using liquid medium

- For isolation in liquid medium we prepare Chu –10 liquid medium in 100 ml quantities
- We add 1 g sieved soil to each flask containing Chu-10 liquid medium, mix it well
- We incubate it in light or in partial sunlight
- After 3-4 weeks after incubation the algal growth can be seen as floating biomass on the surface.

3.3.3 Multiplication of BGA under field condition

Materials required

- Rice field
- Super phosphate
- Carbofuran
- Composite BGA starter culture

Procedure

- We select an area of 40 m^2 (20m x 2m) near a water source which is directly exposed to sunlight.
- We will make a bund all around the plot to a height of 15 cm and give it a coating with mud to prevent loss of water due to percolation.
- The plot is well prepared and leveled uniformly and water is allowed to a depth of 5-7.5 cm and left to settle for 12 hrs.

- We will apply 2 kg of super phosphate and 200 g lime to each plot uniformly over the area.
- The soil based composite starter culture of BGA containing 8-10 species @ 5 Kg / plot is powdered well and broadcasted.
- 200 g Carbofuran is also applied to control soil insects occurring in BGA.
- We will maintain the water level to a height of 5 cm.
- After 15 days of inoculation, the plots are allowed to dry up in the sun and the algal flakes are collected and stored.

Observations

- The floating blue green algal mass will be observed.
- We will determine the mass of algal flakes in each harvest (the plot yields 30 to 40 kg of dry algal flakes in each harvest).

3.4 COLLECTION AND MASS MULTIPLICATION OF AZOLLA

3.4.1 Mass multiplication of *Azolla* under field conditions

For large-scale *Azolla* multiplication in the field, an easy *Azolla* nursery technique has been developed so that farmers may easily implement it on farming.

Materials required

- An area of 40 square meter
- Cattle dung
- Super phosphate
- Furadan
- Fresh *Azolla* inoculum (can be collected from rice fields)

Procedure

- Choose a wetland area and level it evenly and completely.
- Mark the area of 20 x 2m plot by providing suitable bunds and irrigation channels.
- Keep the water level at or above 10 cm.
- Sprinkle 10 kg of cattle dung in a field after mixing it with 20 litres of water.
- Use 100 g of superphosphate as the baseline dosage.
- Inoculate each pot with 8 kg of fresh *Azolla* biomass.
- Apply 100 g of superphosphate as a top dressing fertiliser on the fourth and eighth day following the *Azolla* inoculation.
- On the seventh day following the *Azolla* inoculation, apply 100 g/plot of carbofuran (furadan) granules.

- During the two or three weeks of growth, keep the water level at a height of 10 cm.
- Harvest the *Azolla* for a maximum of 50 kg in 10 to 15 days, at a pace of 8 to 10 kg per plot per day.

Observation

You will observe a green leafy mass on the top of water that is *Azolla*.

Fig. 3.1: Azolla in paddy fields

3.5 SUMMARY

It is indisputable that cyanobacteria can be used in agriculture to increase productivity because of their important nitrogen fixation characteristic. The application of dried cyanobacteria as a soil inoculant to promote fertility has been the subject of numerous research; the impact of cyanobacteria addition on rice yield was initially investigated in Japan during the 1950s. Nowadays, the process of inoculating soil with a particular combination of cyanobacterial species is referred to as "algalization" and research on the subject is being conducted in all of the major rice-producing nations.

The N requirement of rice crops is well known. To overcome acute N deficiency in rice soils, this element is usually supplied to the rice crop as the commercially available fertilizer urea. But unfortunately a substantial amount of the urea-N is lost through different mechanisms causing environmental pollution problems. Cyanobacteria offer an economically attractive and ecologically sound alternative to chemical fertilizers for realizing the ultimate goal of increased productivity, especially in rice cultivation. In a wetland rice ecosystem, nitrogen fixation by free living cyanobacteria also significantly supplements soil nitrogen.

It is well-known that rice crops require nitrogen. The commercial fertiliser urea is typically used to provide the element of nitrogen (N) to rice crops in order to address severe deficiencies in rice soils. Unfortunately, urea-N is lost in significant amounts through a variety of methods that lead to pollution issues in the environment. In order to achieve the ultimate goal of enhanced

production, particularly in rice farming, cyanobacteria present an economically and environmentally safe alternative to chemical fertilisers. Nitrogen fixation by free-living cyanobacteria also considerably increases soil nitrogen in a wetland rice habitat.

For thousands of years, the Far East has cultivated *Azolla* in large quantities in rice paddies as a way to increase rice production by over 50%. This is due to *Azolla*'s symbiont, anabaena, having the ability to accumulate atmospheric nitrogen. For the rapidly growing rice plants, less than 5 percent of the nitrogen stored by *Azolla* is readily available. Until the *Azolla* plant dies, the remaining 95% is contained in its biomass. The organic nitrogen in the plant is quickly metabolised and released as ammonia during its decomposition. This ammonia can then be used as a biofertilizer by the developing rice plants.

3.6 GLOSSARY

- ***Azolla:*** A small fern with a triangular stem measuring up to 2.5 centimeters in length that floats on the water
- **Biofertilizer:** Biofertilizers are substance that contains microbes, which helps in promoting the growth of plants and trees by increasing the supply of essential nutrients to the plants.
- Blue Green Algae (BGA): A heterogeneous group of prokaryotic photosynthetic nitrogen fixing organisms which contain chlorophyll 'a'. They are obligate phototrophs and store cyanophycean starch.
- **Carbofuran:** It is a pesticide that is widely used to control insects and nematodes on a variety of agricultural crops
- **Cyanobacteria**: also called Cyanobacteriota or Cyanophyta, are a phylum of autotrophic gram-negative bacteria that can obtain biological energy via oxygenic photosynthesis.
- **Fertilizer:** A fertilizer or fertiliser is any material of natural or synthetic origin that is applied to soil or to plant tissues to supply plant nutrients.
- **Inoculants:** beneficiary microorganisms applied to either the soil or the plant in order to improve productivity and crop health

3.7 SELF ASSESSMENT QUESTIONS

3.7.1 Multiple choice questions

1. Which of the following nitrogen fixers is found in rice fields associated with *Azolla*?

(a) *Tolypothrix* (b) *Frankia*

(c) *Anabaena* (d) *Spirulina*

2. Which of the following is a pair of biofertilizers?

(a) *Salmonella* and *E.coli* (b) *Rhizobium* and grasses

(c) *Nostoc* and legume (d) *Azolla* and BGA

3. Which of the following fern is a biofertilizer?

(a) *Salvinia* (b) *Azolla*

(c) *Pteridium* (d) *Marsilea*

4. A biofertilizer involving a pteridophyte host is

(a) *Azotobacter* (b) *Clostridium*

(c) *Anabaena* (d) *Rhizobium*

3.7.2 True and false

1. A*zolla* contains nitrogen fixing cyanobacteria in its leaf cavities.

2. The water fern *azolla* harbouring *Anabaena* is an important green manure for rice cultivaton.

3. Cyanobacteria are autotrophic prokaryotes which are used as biofertilizers in paddy fields.

4. Azolla is used as biofertilizer because it has association with mycorrhizae

Answer Key:

3.7.1 1. b; 2. d; 3. b; 4.c.

3.7.2 1. True; 2. True; 3. True; 4. False.

3.8 REFERENCES AND SUGGESTED READINGS

- https://www.researchgate.net/figure/Composition-of-BG-11-medium_tbl1_308692839
- https://the*Azolla*foundation.org/*Azolla*s-uses/in-rice-production/
- https://www.shivajicollege.ac.in/sPanel/uploads/econtent/3c122bcaae2bd0d108786a988bcd075e.pdf
- https://www.researchgate.net/publication/326369217_STUDY_OF_CYANOBACTERIA_AS_BIOFERTILIZER_FROM_THE_RICE_FIELD

3.9 TERMINAL QUESTIONS

1. Describe the collection and isolation of blue green algae from soil.
2. Describe the procedure of multiplication of BGA under field conditions.
3. Discuss the procedure of mass multiplication of *Azolla* under field conditions.

UNIT-04 STUDY THE MORPHOLOGY OF *AZOLLA*

Contents:

4.1 OBJECTIVES

After reading this unit learners will be able to:

- know the morphological characteristics of *Azolla*
- Identification of *Azolla*

4.2 INTRODUCTION

Azolla is a small, free-floating freshwater fern found in tropical and subtropical regions of the world (Mishra and Dash, 2014). The word *Azolla* is derived from Greek word azo (to dry) and allyo (to kill) meaning that plant dies when it dries (Svenson, 1944) and established by Lamarck in 1783. Total seven species of *Azolla* was reported i.e., *A. caroliniana*, *A. cristata*, *A. filiculoides*, *A. imbricata*, *A. mexicana*, *A. microphylla* and *A. pinnata* (Hills and Gopal, 1967; Konar and Kapoor, 1972).

The ability of Azolla-Anabaena system to fix atmospheric nitrogen at faster rates makes it an outstanding agronomic choice for the cultivation of rice under tropical conditions (Yadav et al., 2014). Therefore, *Azolla* considered as an excellent biofertilizer and green manure having global distribution. It has the property to multiply faster at very high rates and covers the surface of water bodies; thus, it forms a thick mat and helps in reducing the volatility of ammonia in the fields. The fern appears as a green floating mat over water, which becomes reddish due to excess anthocyanin pigmentation. The blue-green algae, cyanobacteria (*Anabaena azollae*), present as a symbiont with this fern in the lower cavities actually fixes atmospheric nitrogen. The detail morphology and morphology characteristics are describe in this chapter in detail.

4.3 MORPHOLOGY OF AZOLLA

The genus *Azolla* is small, floating fern, forming a dense covering on the surface of permanent ponds. Mature plants of *Azolla* impart characteristic reddish colour to the pond. Three species from the genus *Azolla* are found in India in which *A. imbricata* is restricted to the Eastern Himalayas, while *A. pinnata* and *A. filiculoides* are found throughout the country.

Sporophyte: *Azolla* plant, often called mosquito fern, duckweed fern, fairy moss, water fern, consist of a main rhizome rarely exceeding 3-4 cm in length. The plant is a sporophyte. It grows freely in ponds and ditches. The sporophyte can be divided into stem, leaves and roots despite its minuscule size.

- **Stem:** It is modified stem known as rhizome. The rhizome is thin and delicate and floats horizontally on the water surface. It is pinnately branched, the branches are extra-axillary.

The dense, overlapping leaves hide the whole rhizome. From this emerge at regular interval secondary rhizomes having the same general characteristics as the principal rhizome and being able in turn to produce rhizome of third order which subsequently produce the fourth. 'T" growth pattern gives the plant a triangular or circular shape depending on the species.

- **Leaves:** The leaves are minute (0.5 mm or less long) cover entire rhizome and its branches. These are alternate and are arranged in two overlapping rows. Each leaf is divided into two lobes of approximately equal size. The upper or aerial lobe is thick and green. It is somewhat obliquely placed and only one of the edges touches the water. The thin lower or submerged lobe is nearly colourless. The lower lobe is thin and colourless, and is completely submerged in water. The absorption of water is believed to take place through this lobe. The dorsal lobe encloses large mugilage filled cavities. Inhabiting these mucilage cavities is found a Cyanophycean alga-Anabaena azollae. According to Oes (1913), the relationship between alga and *Azolla* is symbiotic. While the alga provides nitrogen to the plant the latter gives it shelter. The same species of Anabaena occurs in Azolla all over the world. The rhizome on its lower surface produces simple roots either singly or in clusters. These roots help in stabilising the plants in water.

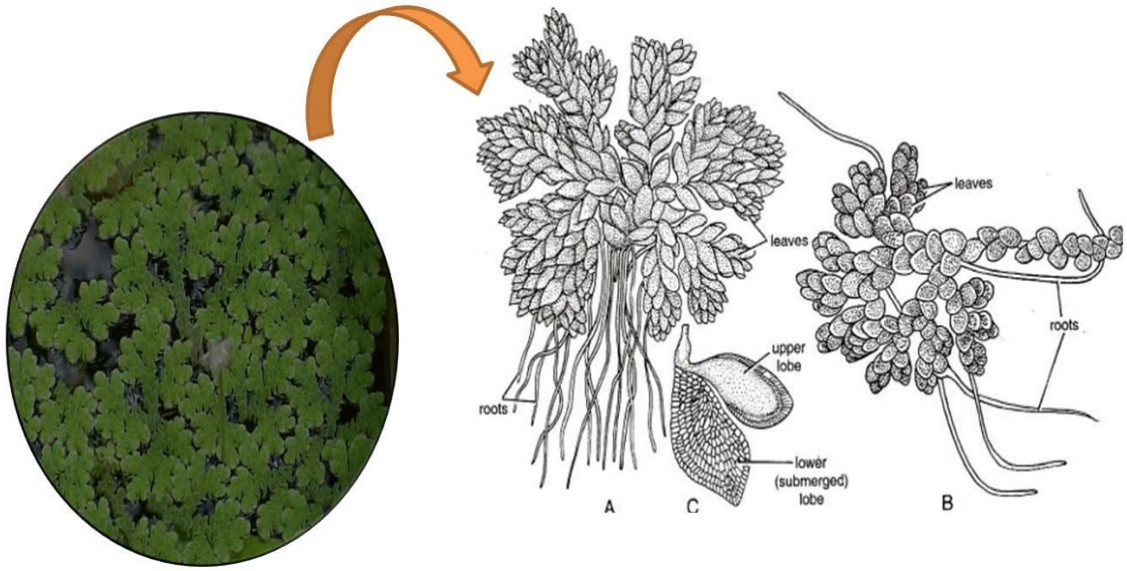


Fig. 4.1: Azolla: External morphology;
(A). *Azolla microphylla*, (B). *Azolla filiculoides*, (C). Leaf

- **Roots:** The roots are usually close to the point of branching and are pendent in water. Roots are unbranched, adventitious are formed on the lower part of the rhizome and are remain submerged in water. Roots have fine lateral rootlets giving a feathery appearance in the water.

4.4 INDENTIFICATION OF AZOLLA

Division: Pteridophyta

1. True roots generally present (except in Psilopsida),
2. True vascular strand present.

Sub-division: Pteropsida

1. Vascular cylinder siphonostelic, with leaf gaps.
2. Plants macrophyllous, leaves compound, with rachis.
3. Leaves bear sporangia in sori.
4. Gametophytes small, green and free living.

Class: Leptosporangiatae

1. Sporangium with a jacket layer one cell in thickness.
2. Definite number of spores.

Order: Salviniales

1. Sporocarp is a single sorus enclosing either megasporangia or microsporangia.
2. Sporocarp walls formed by the indusia.

Family: Salviniaceae (Single family)

Genus: *Azolla*

1. Presence of endophytic blue green algae *Anabaena* in the leaf
2. Each leaf divided into two lobes
3. Megasporocarp with only one megasporangium.

4.5 SUMMARY

This unit described the morphology, or external characteristics, of the genus Azolla. It is a small, free-floating freshwater fern found in tropical and subtropical regions of the world. Total seven species of *Azolla* was reported in which *A. imbricata* is restricted to the Eastern Himalayas, while *A. pinnata* and *A. filiculoides* are found throughout the India. Azolla is use as the biofertilizers due to the ability of Azolla-Anabaena system to fix atmospheric nitrogen at faster rates makes it an outstanding agronomic choice for the cultivation of rice under tropical conditions.

The plant body is sporophyte and can be divided into stem, leaves and roots. The stem is modified stem and known as rhizome. The rhizome is thin and delicate and floats horizontally on

the water surface. The leaves are minute (0.5 mm or less long) cover entire rhizome and its branches. These are alternate and are arranged in two overlapping rows. Each leaf is divided into two lobes of approximately equal size. The roots are unbranched, adventitious are formed on the lower part of the rhizome and are remain submerged in water. Roots have fine lateral rootlets giving a feathery appearance in the water.

4.6 GLOSSARY

- **Adventitious roots:** Plant roots that form from any nonroot tissue and are produced both during normal development
- ***Azolla:*** A small fern with a triangular stem measuring up to 2.5 centimeters in length that floats on the water
- **Biofertilizer:** It is a natural fertilizer that helps to provide all the nutrients required by the plants and to increase the quality of the soil with a natural microorganism environment.
- **Morphology:** a particular form, shape, or structure
- **Rhizome:** It is a modified subterranean plant stem that sends out roots and shoots from its nodes.
- **Sporophyte:** It is a diploid multicellular spore producing phase in the life cycle of plant body which exhibits alternation of generation

4.7 SELF ASSESSMENT QUESTIONS

4.7.1 Multiple choice questions

1. Azolla is a
   a. Pteridophyte b. Bryophyte
   c. Algae d. Fungi

2. Azolla used as
   a. Nitrogenous biofertilizer b. Phosphorus solubilizing biofertilizer
   c. None of the above d. All of the above

3. Plant body of Azolla is
   a. Gametophyte b. Sporophyte
   c. Unicellular sd. Microscopic

4.7.2 True and false

1- *Azolla* serves as main decomposer of organic matter in the soil.
2- The leaves of Azolla show the symbiosis with green algae.

3- The stem of Azolla is known as rhizoid.

4.7.3 Fill in the blanks

1. Azolla belongs to division _____
2. The species of *Azolla* named _____ is restricted to the Eastern Himalayas.
3. Azolla is commonly (in English) known as _____

Answer keys:

4.7.1: 1.a; 2.b; 3.b

4.7.2: 1. False; 2. True; 3. False

4.7.3: 1. Pteridophyta; 2. *A. imbricate*; 3. mosquito fern/duckweed fern/fairy moss/water fern

4.8 REFERENCES AND SUGGESTED READINGS

- Hills, L. V. and Gopal, B. 1967. *Azolla primaeta* and its phylogenetic significance. *Canadian Journal of Chemistry*, 45: 1179-1191.
- Konar, R. N. and Kapoor, R. K. 1972. Anatomical studies on *Azolla pinnata*. *Phytomorphology*, 22: 211-223.
- Mishra, P. and Dash, D. 2014. Rejuvenation of Biofertilizer for Sustainable Agriculture and Economic Development. *The Journal of Sustainable Development*, 11 (1): 41-61.
- Svenson, H. K. 1944. The new world species of *Azolla*. *American Journal of Ferns*, 61: 1-13.
- Yadav, R. K., Abraham,G., Singh, Y. V. and Singh,P.K. 2014. Advancements in the Utilization of *Azolla-Anabaena* System in Relation to Sustainable Agricultural Practices. *Proceedings of the Indian National Science Academy*, 2: 301-316.
- *https://www.florafondness.com/azolla-pteridophyta/#EXTERNAL_MORPHOLOGY_OF_THE_SPOROPHYTE_OF_AZOLLA*

4.9 TERMINAL QUESTIONS

1- Describe the habitat of *Azolla*.

2- Discuss the morphological features of *Azolla* in detail.

3- How will you identify whether the given plant material is Azolla? Write briefly its identical features.

UNIT-5 DEMONSTRATION OF BIOFERTILIZER PREPARATION

Contents

5.1-OBJECTIVES

After reading this unit student will be able to:

- know about the preparation of biofertilizers,
- learn about mass production of bacterial biofertilizers.

5.2-INTRODUCTION

The term "biofertilizer" most often refers to the use of soil microorganisms to enhance availability and uptake of mineral nutrients for plant. In simpler form, "Biofertilizer" is defined as a compound that, when applied to seed, plant surfaces, or soil, contains living microorganisms that colonise the rhizosphere or the interior of the plant and enhance growth by increasing the supply or availability of primary nutrients to the target crop.

Biofertilizers are the products of the fermentation process constituting effective living soil microorganisms. They enhance plant growth and productivity by providing easily utilisable nutrients.

The relationship between a number of microorganisms and crop plants is being used to produce biofertilizers. Specific strains of beneficial soil microorganisms cultured in a lab and packed in an appropriate carrier to improve the availability or uptake of nutrients by plants.

5.3-DEMONSTARTION OF BIOFERTILIZER PREPARATION

5.3.1 Mass Production of bacterial biofertilizers

Biofertilizers are carrier based preparations containing effective strain of nitrogen fixing or phosphate solubilizing microorganisms. Usually, carrier-based inoculants are used in the formulation of biofertilizers. The organic carrier materials are more effective in the preparation of bacterial inoculants. More bacterial cells are present in the solid inoculants, which also help the cells survive for longer periods of time.

The mass production of carrier based bacterial biofertilizers includes three stages.

1. Culturing of Microorganisms

2. Processing of Carrier material

3. Mixing the carrier and the broth culture and packing

1. Culturing of Microorganisms

Culturing is the process of multiplying microorganisms in the laboratory. Growth media, which can be solid or liquid, provides a surface on which microorganisms can grow.

Although many bacteria can be used beneficially as a biofertilizer the process of mass production is standardized for *Rhizobium*, *Azospirillum*, *Azotobacter* and *phosphobacteria*. The media used for mass culturing are as follows:

(a) *Rhizobium*: Yeast extracts mannitol broth

Growth on Congo red yeast extract mannitol agar medium

Components	Amounts (g l^{-1})
Mannitol	10.0 g
K2 HPO4	0.5 g
Mg So4 7H2 O	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Agar	20.0 g
Distilled water	1000.0 ml

10 ml of Congo red stock solution (dissolve 250 mg of Congo red in 100ml water) to 1 liter after adjusting the PH to 6.8 and before adding agar.

On this medium Rhizobium form white, translucent, glistening, elevated and comparatively small colonies. In addition, Rhizobium colonies do not absorb the colour of congo red dye added in the medium. Those colonies which readily absorb the congo red stain are not rhizobia but probably Agrobacterium, a soil bacterium closely related to *Rhizobium*.

(b) *Azospirillum*: Dobereiner's malic acid broth with NH4Cl (1g per liter)

- **Composition of the N-free semisolid malic acid medium**

Components	Amounts (g l^{-1})
Malic acid	5.0g
Potassium hydroxide	4.0g
Dipotassium hydrogen orthophosphate	0.5g
Magnesium sulphate	0.2g
Sodium chloride	0.1g
Calcium chloride	0.2g
Fe-EDTA (1.64% w/v aqueous)	4.0 ml
Trace element solution	2.0 ml
BTB (0.5% alcoholic solution)	2.0 ml
Agar	1.75 g
Distilled water	1000 ml
pH	6.8
Trace element solution	
Sodium molybdate	200 mg
Manganous sulphate	235 mg
Boric acid	280 mg
Copper sulphate	8 mg
Zinc sulphate	24 mg
Distilled water	200 ml

c) Waksman medium No.77 (N-free Mannitol Agar Medium for *Azotobacter*)

Components	Amounts (g l^{-1})
Mannitol	10.0 g
Ca CO_3	5.0 g
K2HPO4	0.5 g
Mg SO4.7H2O	0.2 g
NaCl	0.2 g
Ferric chloride	Trace
MnSO4.4H2O	Trace
N-free washed Agar	15.0 g
pH	7.0
Distilled Water	1000 ml

(d) Phosphobacteria: Pikovskaya's Broth

Components	Amounts (g l-1)
Glucose	10.0 g
Ca3(PO4)2	5.0 g
(NH4)2SO4	0.5 g
KCl	0.2 g
MgSO4. 7H2O	0.1 g
MnSO4	Trace
FeSO4	Trace
Yeast Extract	0.5 g
Distilled Water	1000 ml

Prepare suitable broth in 50 ml flasks and inoculate the mother culture into the flasks. Grow the culture under shaking conditions at 30 ± 2^0C until maximum cell population of 1010 to 1011 cfu/ml is reached. Under ideal conditions this population level could be attained within 4 to 5 days for *Rhizobium*, 5 to 7 days for *Azospirillum*, 2 to 3 days for Phosphobacteria, and 6-7 days for *Azotobacter*. The culture obtained in the flask is called **starter culture**. For large scale production of inoculant, inoculum from starter culture is transferred to large flasks/seed tank fermentor and grown until required level of cell count is obtained.

Inoculum preparation

Inoculum preparation includes obtaining organisms in an ideal state for inoculation into cell culture, tissue culture, media, and fermentors.

Procedure for Inoculum preparation as follows:

1) Prepare the suitable media for particular to the bacterial inoculant in 250 ml, 500 ml, 3 litre and 5 litre conical flasks and sterilize them.
2) Under aseptic condition the media in 250 ml flask is inoculated with effective bacterial strain.

3) Put the flask under room temperature in rotary shaker (200 rpm) for 5 to 7 days.
4) Observe the flask for growth of the culture and measure the population, which serves as the starter culture.
5) Using the starter culture (at log phase) inoculate the larger flasks (500 ml, 3 litre and 5 litre) with the media, after achieving growth in each flask.
6) The above media is prepared in large quantities in fermentor, properly sterilised, cooled and then stored it ready.
7) The media in the fermentor is inoculated with the log phase culture grown in 5 litre flask.
8) Generally 1 -2 % inoculum is enough however inoculation is done up to 5% depending on the growth of the culture in the larger flasks.
9) The cells are grown in fermentor by supplying aeration (passing sterile air through compressor and sterilizing agents like glass wool, cotton wool, acid etc.) and given regular stirring.
10) During the growth period the broth is examined for the population of inoculated organism and any contamination.
11) After incubation period the cells are harvested with the population load of 109 cells ml-1.
12) There should not be any fungal or any other bacterial contamination at 10-6 dilution level.
13) It is not recommended to store the broth after fermentation for more than 24 hours. Even at $4^{o}C$, the number of viable cells starts to decrease.

2. Processing of carrier material: The use of ideal carrier material is essential in the production of good quality biofertilizer. Peat soil, lignite, charcoal, vermiculite, press mud, farmyard manure and soil mixture can be used as carrier materials. The neutralized peat soil/lignite are found to be better carrier materials for biofertilizer production.

Characteristics of an Ideal carrier

1) Cheaper in cost
2) Should be available locally.
3) High organic matter content
4) No harmful chemicals
5) A water-holding capacity of higher than 50%
6) Easy to process, fragile and vulnerability.

Preparation of carrier material

1) Grind the carrier material (lignite or peat) to a fine powder so as to pass through 212 micron IS sieve.
2) Neutralize the pH of the carrier material with the help of calcium carbonate (1:10 ratio) , since the peat soil / lignite are acidic in nature (pH of 4 - 5)
3) Sterilize the neutralized carrier material in an autoclave to eliminate the contaminants. The sun drying method and gamma irradiation are used for large-scale production.

3. Mixing the carrier and the broth culture and packing: The inoculant packets are made by mixing the broth culture (broth culture is any kind of liquid used to grow bacteria) obtained from the fermentor with sterile carrier material as explained below:

Preparation of Inoculants packet

1) Spread the neutralized, sterilized carrier material in a clean, dry, and sterile metallic or plastic tray.
2) The bacterial culture transfer from the fermentor is added to the sterilized carrier and thoroughly mixed by hand (by wearing sterile gloves) or by mechanical mixer. The culture suspension should be added to a level of 40 – 50% water holding capacity depending upon the population.
3) Seal the inoculant packet of 200 g in polythene bags with an electric sealer and allow to cure for 2-3 days at room temperature (curing can be done by spreading the inoculant on a clean floor/polythene sheet/ by keeping in open shallow tubs/ trays with polythene covering for 2 - 3 days at room temperature before packaging).

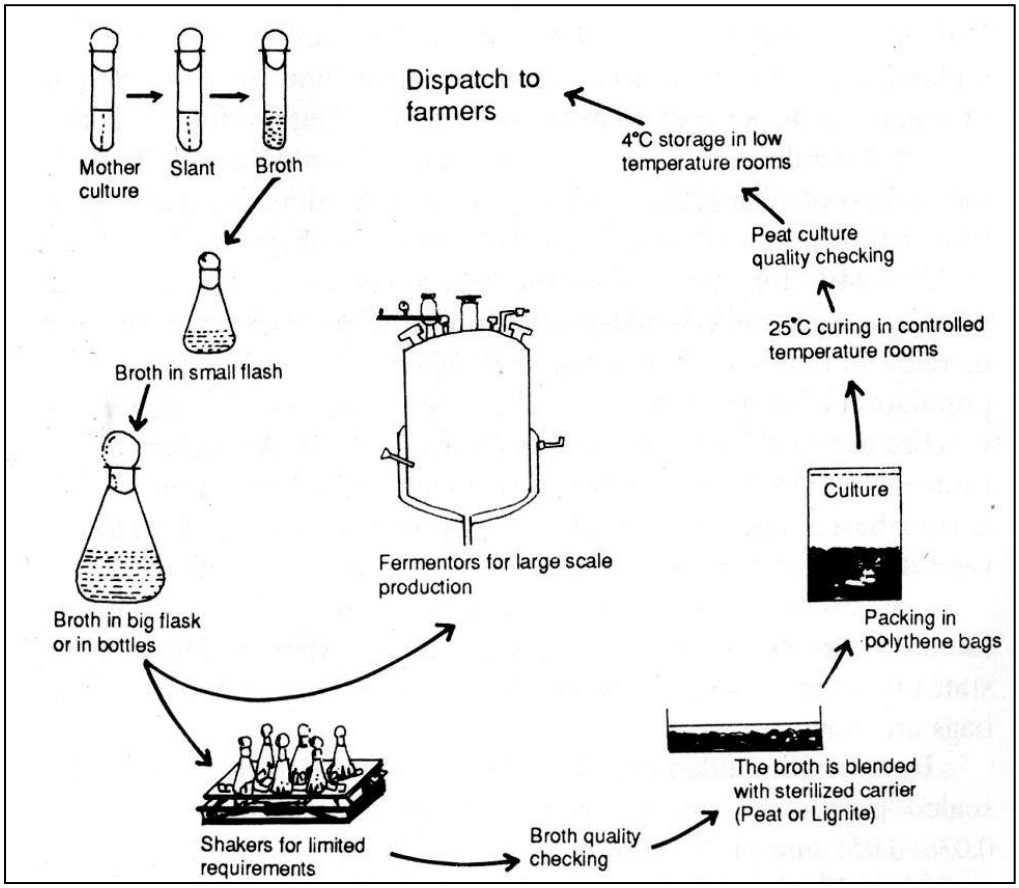


Fig. 5.1 Schematic representation of mass production of bacterial biofertilizers

Specification of the polythene bags

1. The polythene bags must be of low density grade.
2. The bag thickness should be between 50-75 microns.
3. Each packet should be marked with the manufacturer's name, product name, strain number, the crop to which recommended, method of inoculation, date of manufacture, batch number, date of expiry, price, full address of the manufacturer and storage instructions etc.

Storage of biofertilizer packet

1. The packet should be kept in a cool place away from the direct sunlight or heat.
2. The packets can be stored at room temperature or in cold storage in plastic crates or polythene / gunny bags in batches.

5.4- SUMMARY

"Biofertilizer" is defined as a compound that, when applied to seed, plant surfaces, or soil, contains living microorganisms that colonise the rhizosphere or the interior of the plant and enhance growth by increasing the supply or availability of primary nutrients to the target crop. The mass production of carrier based bacterial biofertilizers includes three stages-Culturing of microorganisms, Processing of carrier material, mixing the carrier and the broth culture and packing.
Culturing is the process of multiplying microorganisms in the laboratory. Many bacteria, including Rhizobium, Azospirillum, Azotobacter, and Phosphobacteria, can be utilised as biofertilizers in the mass-production process. The use of a suitable carrier material is of great importance in the production of high-quality biofertilizer. Peat soil, lignite, charcoal, vermiculite, press mud, farmyard manure, and soil mixture can all be utilised as carrier materials. The inoculant packets are prepared by combining broth culture collected from the fermentor with sterile carrier material.

5.5 GLOSSARY

Mother culture: A pure growth of any organism on a small scale is called as a mother culture.

Inoculum: The pathogen that lands on or is otherwise come in contact with the plant is called the inoculums.

Broth: *a liquid medium containing types of nutrients which is used to grow cultures of* bacteria and other microorganisms.

Fermenter: A fermenter is an enclosed and sterilized vessel or an apparatus that maintains ideal conditions for the growth of microorganisms such as bacteria and fungi and used for the

fermentation of microorganisms to be produced in huge quantities for commercial use. After the completion of the fermentation process the product is collected.

pH meter: An instrument for measuring pH of the solution using a 0-14 scale in which seven denotes neutral points, less than seven is for acidity (excess of H‘ over OH-) and more than seven is for alkality (excess of OH- over H‘) useful in adjusting the pH of the growth medium.

Autoclave: It is an apparatus that uses air-free saturated steam under pressure to sterilise materials at temperatures higher than 1000 degrees Celsius. The autoclave's internal temperature will rise to 121°C if the steam pressure is increased to 15 psi. This is enough to destroy all vegetative cells. The autoclave is often used to sterilise all growth medium.

Laminar air flow chamber: A uniform flow of filtered air is produced by a laminar air flow chamber. This regular flow of air will prevent settling of particles in the work area. Air borne contamination is avoided in this chamber. Culture transfers and inoculation can be done here.

Rotary shaker: It is used with a variable speed control to shake culture flasks in a circular motion. Shaking provides aeration for growth of cultures. Shakers with a capacity of 20–50 flasks are usually used. If it's a double-decker shaker, its capacity might be increased.

Slants: Solid medium made of agar together with different nutrients and indicators called Slants.

5.6- REFERENCES AND SUGGESTED READINGS

- https://biologyreader.com/biofertilizer-production.html
- https://annamalaiuniversity.ac.in/studport/download/agri/agmic/resources
- https://www.fnca.mext.go.jp/bf/bfm/pdf/Biofertilizer_Manual.pdf
- A handbook of organic farming by Arun.K.Sharma published by Agrobios (India), Jodhpur.
- A text book of Soil Science by R.K. Mehra published by Indian Council of Agricultural Research, Pusa, New Delhi.
- Handbook of Composite Organic Farming by Himadri Panda and Dharamvir Hota, published by Gene-Tech Books, New Delhi.

5.7-TERMINAL QUESTIONS

1-What is biofertilizers?
2- Discuss the process of culturing of microorganism.
3. Describe the process of biofertilizer preparation.
4. What are the Characteristics of an Ideal Carrier?
5- Draw a labeled diagram of representation of mass production of bacterial biofertilizers.

UNIT-6 STUDY OF CELL STRUCTURE OF CYANOBACTERIA

Contents

6.1 OBJECTIVES

After reading this unit learners will be able:

- To understand the cell structure of cyanobacteria
- To know the general characters of *Anabaena*

6.2 INTRODUCTION

Owing to a particular colouring in their cells, the members of the class cyanophyceae or myxophyceae are also referred to as cyanobacteria or blue green algae. There are over 2,000 species in this class, organised into 150 genera. Most of the species are found in freshwater environments, although some are also semi-terrestrial and marine. Example: *Anabaena, Scytonema, Spirulina.*

6.3 STRUCTURAL COMPONENTS OF CYANOBACTERIA

The components of a typical blue-green algal cell are as follows:

1. The outer covering layer of cells.
2. Cytoplasm.
3. Nuclear substance.

The outer covering of cells includes:

(a) Mucilaginous sheath
(b) Cell wall
(c) Innermost plasma membrane.

(a) Mucilaginous sheath

The outermost layer that covers the cell wall is called the mucilaginous sheath. The mucilaginous layer may be clearly evident in some cases and form a mucilaginous sheath, but it may also be less noticeable in other cases. It protects the cell from harmful environmental factors.

(b) Cell wall

The cell wall is located directly beneath the mucilaginous layer. The structure of the cell wall is relatively complex, as demonstrated by electron microscopy. The inner layer of the two or three-layered cell wall occurs between the plasma membrane and the outer wall layer. Both polysaccharides and mucopeptides constitute the cell wall.

(c) Plasma membrane:

The plasma membrane is a lipoprotein-based, selectively permeable biological membrane that surrounds the cytoplasm.

2. Cytoplasm

The groundplasm, which is visible beneath the plasma membrane, is made up of variously structured and functional components. Lamellae containing pigments are found on the cytoplasmic periphery (Fig. 6.1). It is evident from fine structure studies that the pigmented lamellae are not arranged in plastids. Plasma membrane is the source of lamellae, or membranes.

Chlorophylls, carotenes, xanthophylls, c-phycoerythrin, and c-phycocyanin are among the pigments found in lamellae; the latter two are primarily present in blue-green algae. Apart from lamellae, the cytoplasm can also contain a number of membrane-bound vesicles, which are occasionally arranged in layers. Furthermore, ribosomes can be dispersed throughout the groundplasm.

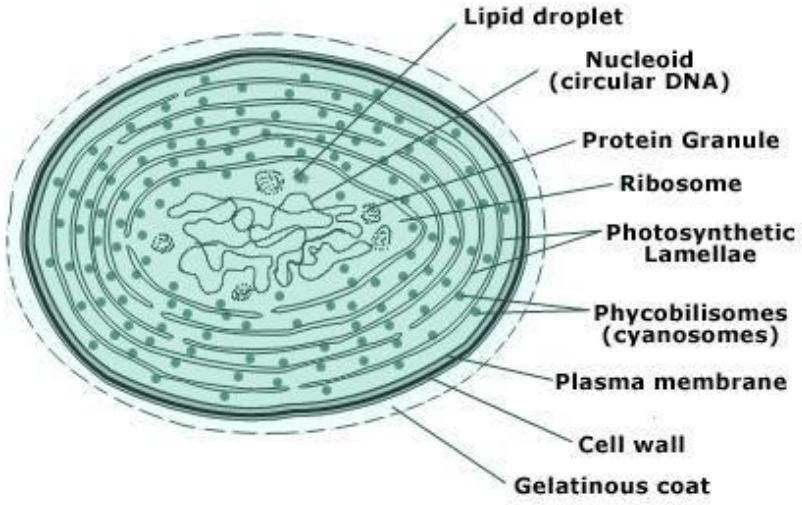


Fig. 6.1: Cyanobacteria cell
(Source: https://id.pinterest.com/pin/35-ga-cyanobacteriacell-structure)

3. Nuclear Material

The nucleoplasm, also known as the DNA-containing area, is located in the centre of the cell and has a fibrillar shape. There is no nuclear border and no nucleolus in the nucleoplasm, which is feulgen-positive but not arranged into an electron micrograph of the cell. No spindle apparatus is involved in the division of the nucleoplasmic material that is distributed throughout the cell during division.

6.4 ANABAENA: CLASSIFICATION AND GENERAL CHARACTERS

6.4.1 *Anabaena*

Classification:

Class – Cyanophyceae

Order – Nostocales

Family – Nostocaceae

Genus – *Anabaena* (Gr: anabaino, I will go up)

There are about 25 species of *Anabaena* known to exist in India. *Anabaena plantorica, Aphanizomenoides, A. spiroides, A. sphaerica, A. gelatinicola, A. iyengarii* are some of the important species. These are free-floating, freshwater forms that are frequently seen in pools, ponds, rice fields, and other damp areas. Certain species exhibit symbiotic relationships with coralloid roots found in *Cycas* (*Azolla cycadacearum*), *Azolla* (*Anabaena azollae*) leaves, and *Anthoceros* thallus. It forms water blooms as well.

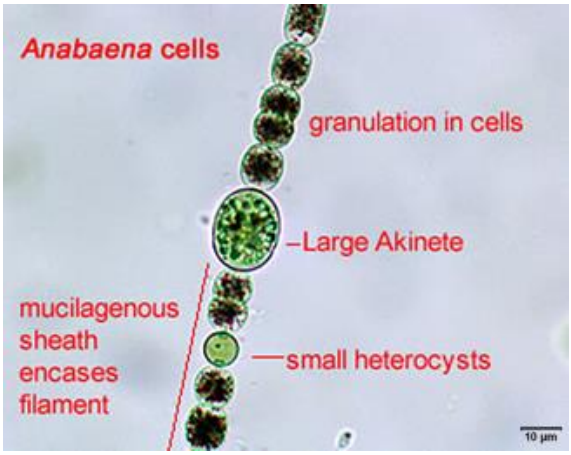


Fig. 6.2: *Anabaena sp.* (Source: https://cfb.unh.edu/CyanoKey/Anabaena.html)

General characters

1) The thallus is unbranched, filamentous (usually straight but can be circinate or irregular), and it grows singly inside a hyaline, watery gelatinous sheath but it is typically not enclosed in a mucilaginous sheath.
2) The filament is made up of a series of sub-cylindrical, barrel-shaped, or spherical cells.
3) The trichome contains a number of terminal and intercalary heterocysts. Heterocysts resemble vegetative cells in form. Whereas intercalary heterocysts have two polar nodules, terminal heterocysts only have one.

4) A particular kind of cells known as akinetes, which have thick walls and greater sizes, can be found either on one side of the heterocyst, on both sides, or outside of it.
5) Every vegetative cell has three layers, representing the normal cyanophycean cell structure: an outside mucilage layer, a middle pectic layer, and an inner thin cellular layer.
6) The components of protoplasm include pigment-containing peripheral chromoplasm, colourless material similar to the centre nucleus called centroplasm or chromatin granules, and gas vacuoles.
7) Heterocysts, acinates, and horngones all reproduce asexually rather than sexually.
8) Heterocysts and akinetes are involved in nitrogen fixing.

6.5 SUMMARY

Prokaryotic oxygenic phototrophs known as cyanobacteria are pigmented with phycobilins, a blue pigment that is produced during photosynthetic reactions, and chlorophyll, a green pigment. Being prokaryotic indicates that they lack a membrane-bound nucleus, mitochondria, or other membrane-bound organelle, unlike true algae. An organism that synthesises organic compounds for food using solar energy is called a phototroph. Being one of the biggest and most significant groups of bacteria on the planet, they hold the distinction of being the oldest fossils ever discovered, dating back more than 3.5 billion years. These are the only living things that can both fix nitrogen and carry out oxygenic photosynthesis. Cyanobacteria are thought to be responsible for a large number of Proterozoic oil deposits.

They are also significant suppliers of nitrogen fertiliser for the growing of beans and rice. Throughout the history of the world, the cyanobacteria have also had a significant impact on how evolution and ecological change have progressed. In the course of the Archaean and Proterozoic Eras, a variety of cyanobacteria produced the oxygen atmosphere on which we live. Prior to that time, the atmosphere's chemistry was drastically different and unsuited for modern life. The cyanobacteria are also largely responsible for the origin of plants. In reality, the chloroplast that plants use to produce their own food is a cyanobacterium that resides inside their cells. In certain eukaryotic cells, cyanobacteria started to live at some point during the late Proterozoic or early Cambrian, providing nourishment for their hosts in exchange for a place to live. The eukaryotic mitochondrion came from this process, which is called endosymbiosis. Cyanobacteria are commonly referred to as "blue-green algae" due to their aquatic and photosynthetic nature.

6.6 GLOSSARY

Azolla: A small fern with a triangular stem measuring up to 2.5 centimeters in length that floats on the water.

Blue Green Algae (BGA): A heterogeneous group of prokaryotic photosynthetic nitrogen fixing organisms which contain chlorophyll 'a'. They are obligate phototrophs and store cyanophycean starch.

Cyanobacteria: also called Cyanobacteriota or Cyanophyta, are a phylum of autotrophic gram-negative bacteria that can obtain biological energy via oxygenic photosynthesis.

DNA: Deoxyribonucleic acid (DNA) is the molecule that carries genetic information for the development and functioning of an organism.

Feulgen stain: It is a staining technique discovered by Robert *Feulgen* and used in histology to identify chromosomal material or DNA in cell specimens.

Phototrophs: An organism that uses energy from sunlight to synthesize organic compounds for nutrition.

6.7 SELF ASSESSMENT QUESTIONS

6.7.1 Multiple choice questions

1. Cyanobacteria is a
   (a) Category of bacteria (b) New name of myxophyceae or blue green algae
   (c) New name of all bacteria (d) None of the above

2. Prokaryotes are identified by
   (a) Absence of chloroplast (b) Absence of mitochondria
   (c) Absence of nuclear membrane (d) Absence of cell membrane

3. Blue algae belongs to
   (a) Myxomycetes (b) Eukaryota
   (c) Prokaryota (d) Neither eukaryote nor prokaryota

4. Atmospheric nitrogen is fixed by
   (a) Funaria (b) *Anabaena*
   (c) Chlamydomonas (d) Fern gametophyte

6.7.2 True and false

1. Cyanobacteria are algae and their cells have nuclei.
2. Cyanobacteria perform oxygenic photosynthesis.
3. Anabaena belongs to kingdom Monera.
4. Anabaena is found as endosymbiont in the thallus of Anthoceros.

Answer keys:

6.7.1 1.b; 2.c; 3.c; 4. b

6.7.2 1. False; 2.True; 3. False; 4. True;

6.8 REFERENCES AND SUGGESTED READINGS

- http://bgsscienceacademy.ac.in/EducationalNotes/StudyMaterial/BOTANY/1st%20Sem%20Botany%20Notes%20BGSSARC.pdf
- http://bgsscienceacademy.ac.in/EducationalNotes/LABORATORY%20MANUALS/BOTANY/1st%20B.Sc.%201st%20Semester%20BOTANY%20LABORATORY%20MANUAL.pdf
- https://en.wikipedia.org/wiki/Cyanobacteria
- https://en.wikipedia.org/wiki/Anabaena

6.9 TERMINAL QUESTIONS

1. Write a note on structural components of cyanobacteria.
2. Write about the general characters of Anabaena.
3. Draw a well labeled diagram of a cyanobacterial cell.

UTTARAKHAND OPEN UNIVERSITY

Teenpani Bypass Road, Behind Transport Nagar,
Haldwani- 263139, Nainital (Uttarakhand)
Phone: 05946-261122, 261123; Fax No. 05946-264232
Website: www.uou.ac.in; e-mail: info@uou.ac.in
Toll Free No.: 1800 180 4025