3-5 BIOLOGICAL NITROGEN FIXATION

The conversion of molecular nitrogen to ammonium is a reduction process and is carried out by certain microorganisms like bacteria and cyanobacteria. This type of nitrogen fixation is known as *biological nitrogen fixation.* It occurs in fresh as well as marine waters, on dunes, salt marshes, estuarine muds, Arctic and Antarctic regions, high alpine regions besides agricultural and non agricultural lands. *Trichodesmium*, a cyano bacterium occurs in the Pacific ocean. It is an efficient nitrogen fixer in the marine environment. Biological nitrogen fixation accounts for large quantity of nitrogen supply to the soil. While biological nitrogen fixation adds about 200 million metric tonnes to the earth's surface every year, man produces about 28 million metric tons in the form of synthetic fertilizers.

Main Points of Biological Nitrogen Fixation :

The conversion of molecular nitrogen into ammonia by microorganisms is called ***biological nitrogen fixation.***

(a) The organisms fixing nitrogen are called ***nitrogen fixers.***

(b) The nitrogen fixers include ***blue green algae, bacteria*** and a ***fern.***

Eg :

Nostoc, *Anabaena*, *Oscillatoria*	Blue green algae
Rhizobium, *Azotobacter*, *Azospirillum*	Bacteria
Azolla	— Fern

(c) The nitrogen fixers may be ***autortophs*** or ***heterotrophs.***

(d) They may be ***free living*** or ***symbiotic.***

(e) The free living nitrogen fixers are ***non-symbiotic.*** They live in the soil.

Biological nitrogen fixation is of two types : Asymbiotic and symbiotic.

1. Asymbiotic or non symbiotic nitrogen fixation : Nitrogen fixation is carried out by free living soil micro organisms. These include a number of aerobic, anaerobic, photosynthetic and cyano bacteria.

(i) Aerobic bacteria : eg. *Azotobacter*

(ii) Anaerobic bacteria : eg. *Clostridium, Klebsiella*

(iii) Photosynthetic bacteria : eg. *Rhodospirillum*

(iv) Cyano bacteria or blue green algae :

(a) *Forms with heterocyst :* eg. *Anabaena, Nostoc, Scytonema*

(b) *Forms without heterocyst :* eg. *Oscillatoria, Lyngbya, Gleotrichia*

Free living organisms of the above mentioned groups are capable of reducing nitrogen to amino group. *Nitrogenase* is the key enzyme in nitrogen fixation.

Azotobacter grows in soils with pH 6·0. Brown (1974) reported that inoculation of soil with Azotobacter is effective in increasing the yield of crops in well manured soil having high organic matter. Experiments conducted in temperate regions showed that nitrogen fixation in *Azotobacter* inoculated soils is about 15 Kg of Nitrogen/ hectare/ year. *Azotobacterin* is the trade name of the bacterial inoculant used in East European countries and Russia for increasing the yield in crops like wheat, barley, maize, sugar beet, carrot, cabbage and potato. *Clostridium* is the dominant anaerobic nitrogen fixer. Blue green algae (Cyanobacteria) play important role in agriculture. Most of the nitrogen fixing blue green algae belong to genera *Anabaena, Aulosira, Cylindrospermum, Nostoc, Calothrix, Scytonema* and *Tolypothrix*. The amount of nitrogen fixed by blue green algae may be 35 to 195 Kg/ hectare per season. Blue green algae also add organic matter to the soil. Further they synthesize and secrete several vitamins and growth substances which improve plant growth.

Azospirllum has gained importance because of its association with rhizosphere of grasses and monocotyledons. The amount of nitrogen fixed is estimated about 42 to 80 kg/ hectare per crop season. It has been observed recently *Azospirllum* exhibits associative nitrogen fixation in cortical cells of roots of Maize. Other nitrogen fixing bacteria that are gaining importance are *Bejerenckia, Pseudomonas* and *Flavobacterium.*

The nif genes are the controlling agents of nitrogen fixation. Much attention is directed towards understanding of organisation of nif genes in free living microbes. Certain mutants of *Klebsiella* and *Azotobacter* are not capable of nitrogen fixation. Especially *Klebsiella pneumoniae* can grow under aerobic as well as anaerobic conditions. However nitrogen fixation occurs only under anaerobic conditions. It has been suggested that in *Klebsiella* 17 genes concerned with nitrogen fixation are clustered and linked to the operator end of histidine (his) operon. Currently plasmids are used in genetic engineering of nitrogen fixation. Cannon and coworkers from England constructed an amplifiable plasmid that carries 14 nif genes of *Klebsiella pneumoniae* that code also for Mo and Fe proteins of nitrogenase. Transfer of nif genes could be effected from nitrogen fixing genera like *Klebsiella pneumoniae* to *Escherishia coli.* Although transfer of nif genes has been successful, replication and nitrogen fixation are not taking place in higher plants.

Main Points of Non-symbiotic Nitrogen fixation :

The conversion of nitrogen into ammonia by free living soil microbesis called non-symbiotic nitrogen fixation.

Eg : *Oscillatoria ; Anabaena ; Nostoc ; Clostridium ; Azotobacter.*

(1) They contain ***nitrogenase*** enzyme is their cytoplasm.

(2) They have the ability to fix atmospheric nitrogen. Hence these microbes are called ***nitrogen fixers.***

(3) They may be ***anaerobes*** or ***aerobes.***

(4) They may be ***autotrophs*** or ***heterotrophs.***

(5) The nirogenase enzyme is present in the cytosol.

(6) It is transcribed by the *Nif gene.*

(7) The enzyme becomes active in the absence of oxygen. Oxygen inactivates this enzyme.

(8) The enzyme catalyzes the reacton of ***nitrogen reduction.***

(9) Nitrogenase is a ***protein*** enzyme.

(10) It consists of 4 ***polypeptide chains, molybdenum, iron*** and ***sulphur.***

(11) The enzyme receives electrons from ***ferredoxin*** or *$NADPH_2$*.

(12) *ATP* supplies the energy.

(13) The enzyme binds to ***nitrogen.***

(14) The activated nitrogenase enzyme supplies hydrogen to nitrogen and the nitrogen is reduced to ammonia.

(15) The ammonia is released into the ***cytoplasm.***

(16) Ammonia is converted into ***ammonium*** ions (NH_4).

(17) Ammonium ions are used for the biosynthesis of ***amino acids.***

Symbiotic Nitrogen Fixation : The most important method of nitrogen fixation is carried out by symbiotic nitrogen fixation. Symbiosis refers to a partnership of two organisms in which each partner is benefitted by the association. The symbiotic association occurs in a wide range of plant groups such as Lichens, Bryophytes, Pteridophytes, Gymnosperms and Angiosperms. The nitrogen fixer in the symbiotic association may be *Rhizobium* or *actinomycetes* or *blue green algae.* The legumes are the main group of plants which contain the bacterium namely *Rhizobium* in root nodules. Root nodules are formed due to infection by the soil bacterium *Rhizobium.* Interestingly neither the host nor the bacterium is capable of fixing up molecular nitrogen. Moreover the symbiotic relationship between the legumes and *Rhizobium* seems to be species specific. In the legume - rhizobium association, the *Rhizobium* supplies the host plant the fixed nitrogen (reduced nitrogen) while the host plant provides the Rhizobium with soluble carbohydrates. Legumes fix about 100 - 200 kg of nitrogen per hectare per year. Certain species of *Rhizobium* form nodules on stems of *Sesbania rostrata* and *Aeschynomene indica.* eg. *Azorhizobium caulinodans.* The nitrogen fixing capacity of stem nodules of *S. rostrata* is very high i.e., about 150 kg of nitrogen fixed per hectare in 52 days. It can be used as a very good green manure for rice crops. In rare instance Rhizobium is associated with a non legume plant, *Parasponia.*

Certain non legume plants also bear nodules on their roots. These nodules contain actinomycetes which fix nitrogen eg. Alder (*Alnus* sp), *Myrica, Purshia, Casuarina. Casuarina equesetifolia* fixes 50 to 200 kg / hectare / year. In non leguminous trees the nodulating organism is mostly *Frankia*, a genus belonging to actinomycetes.

Symbiotic nitrogen fixation occurs in corolloid roots of *Cycas* and in leaf cavities of Azolla, a water fern. The nitrogen fixers in these systems are *Anabaena* and *Nostoc* which belongs to Cyanophyceae. In Lichens, the algal partner is usually a blue green alga. In some tropical plants like *Psychotria* nodules are present on leaves and bacteria (*Klebsiella*) inhabit the nodules.

Physiology of nodule formation :

Rhizobium is an aerobic bacillus type of bacterium occurring in the soil. It remains saprophytic in the soil until it infects a root hair or damaged epidermal cell. The soil bacteria accumulate in the vicinity of plant roots due to secretion of growth factors from the root.

The bacteria invade the root hair and move into the cortex as a thread like structure known as infection thread till they reach the endodermis. The infection thread is made up of mucilage which contains large number of bacteria. The infection thread stimulates cortical cells to divide and these divisions result in some tetraploid cells. The nodule structure takes shape due to repeated divisions of these tetraploid cells. So a mature nodule is made up of largely tetraploid cells containing bacteria and some diploid cells without bacteria.

The nodule establishes contact with the root by means of vascular supply. Bacteria inside the infected host cells multiply rapidly and are transformed into enlarged non motile bacteria called *bacteriods*. The bacteriods carry out nitrogen fixation as they contain necessary enzymes.

Nodules of leguminous plants contain a pink pigment called *leghemoglobin*. It is essential for nitrogen fixation. It is synthesised in the host cell and localized within the envelope that surrounds the bacteroids. Neither legume nor the bacterium Rhizobium can synthesize independently leghemoglobin. Hence it is considered as a product of symbiotic interactions at the genetic level.

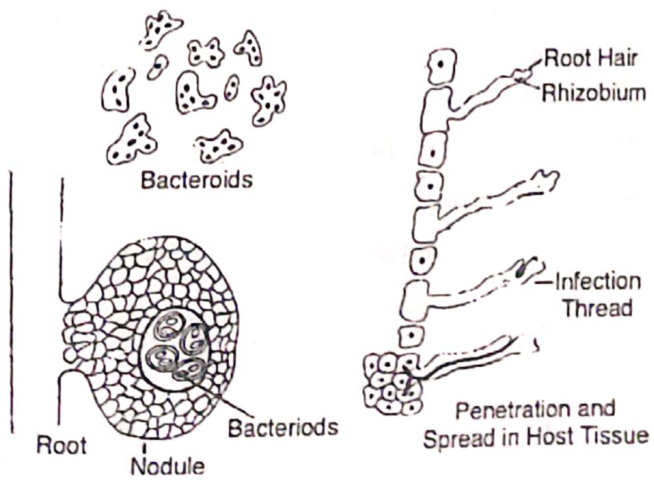


Fig. 3·1 : Nodule formation in Rhizobium- legume symbiosis

Leghemoglobin is similar to mammalian hemoglobin. This molecule is red because of hemegroup attached as a prosthetic group to the globin protein. There is a correlation between leghemoglobin and symbiotic nitrogen fixation. The pigment appears to be involved as an oxygen carrier in the process of nitrogen fixation in the nodules. It transports oxygen to the *Rhizobium bacteroids* at carefully controlled rates, as too much oxygen inactivates the enzyme nitrogenase and at the same time oxygen is essential for bacteroid respiration.

Besides being an oxygen carrier, leghemoglobin protects the nitrogenase from the effects of high concentration of atmospheric oxygen. However leghemoglobin has not been recorded in other symbiotic associations. So it is now cosidered to be helpful but not a necessary requirement.

Nitrogen fixation in root nodules takes place within the bacteroids. The formation of leghemoglobin and *nodulin* (nodule inducing proten) is coded by plant genes. But with regard *nif* genes (nitrogen fixing genes) which code for nitrogenase, it is not clear whether the plant or the bacteroid is responsible for organizing the *nif* genes.

In the legume rhizobium symbiotic association, the host plant provides the bacterium with carbohydrates. The bacterium oxidizes the sugars and obtains energy. The ATP and electrons produced during this oxidation in the bacteroids are utilized to reduce nitrogen to ammonium. The essential micronutrients required in nitrogen fixation are iron, molybdenum and cobalt. Iron is an essential constituent of leghemoglobin. Cobalt is an essential part of vitamin B_{12} which is required in the formation of leghemoglobin. Molybdenum is an electron acceptor or donor in the reduction of nitrogen to ammonia.

Main Points of Symbiotic Nitrogen Fixation

The conversion of nitrogen into ammonia by symbiotic organisms is called symbiotic nitrogen fixation.

Eg: *Rhizobium* ; *Frankia*

(1) They contain *nitrogenase* enzyme.

(2) They have the ability to fix atmospheric nitrogen in the soil. Hence these microbes are called *nitrogen fixers*.

(3) They are *soil bacteria*.

(4) Rhizobium leads a symbiotic life with the roots of *leguminous* plants.

(5) The microbes absorb *nutrients* from the plants.

(6) The leguminous plants are benefited by getting *fixed nitrogen* from the bacteria.

(7) The bacterium enters the root cells and produces *root nodules*.

(8) The bacteria *multiply* in the *root nodules*.

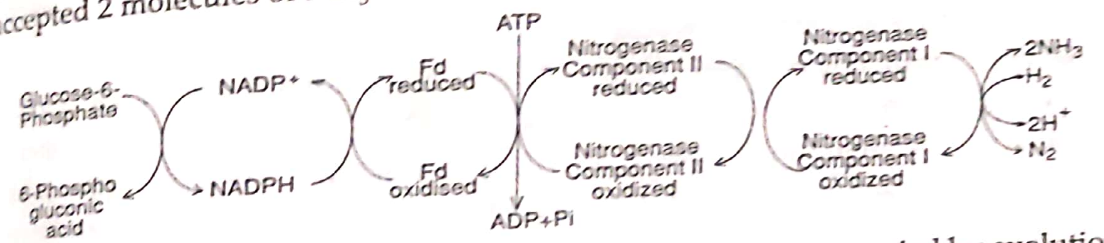
(9) The bacteria use the root nodules as the *nitrogen fixing factory*.

3-6 MECHANISM OF NITROGEN FIXATION

Rhizobium contains the enzyme *nitrogenase*. This enzyme catalyses the reduction of nitrogen to ammonia. Though ammonia occupies a key position in the pathway of nitrogen fixation, the biochemical changes from molecular nitrogen to ammonia are not exactly known. By using ^{15}N in nitrogen fixing cell preparations from bacteroids, it is confirmed that the nitrogen is converted to ammonia. But the intermediate products of this reaction are not detected. The enzyme nitrogenase consists of two protein components : Component I contains both iron and molydbenum. component II is a non heme iron protein. The overall reaction of reduction of nitrogen to ammonia can be represented as :

$$N_2 + 6H^+ + 6e^- \longrightarrow 2\,NH_3$$

As noted in the equation, the reduction of nitrogen to ammonia requires 6 protons and 6 electrons. Besides it requires two ATP for each of six electrons are involved. The source for these electrons and protons is the carbohydrate (Sucrose) translocated from the leaves. Glucose 6 phosphate is the substrate and oxidation of glucose 6 phosphate generates NADPH or NADH. NADH or NADPH reduces ferredoxin. Both molydenum and iron become reduced and then oxidized as nitrogenase accepts electrons from ferredoxin. Ultimately electrons are transferred to nitrogen which is reduced to ammonia Nitrogenase accepts electrons from reduced ferredoxin. component II of ATP interacts (binds) with non heme component of Nitrogenase(component II) so that component II acts as strong reducing agent. Component II transfers electrons to component I. Component I transfers electrons to N_2 . ATP becomes ADP. When six electrons and $8H^+$ and are accepted 2 molecules of NH_3 are released.



In certain species of Rhizobium, nitrogen fixation is accompanied by evolution of H_2 . This evolution of hydrogen is considered as waste of energy.

$$N_2 + 8H^+ + 8e^- \longrightarrow 2NH_3 + H_2$$

Some species of Rhizobium contain enzyme hydrogenase which oxidizes H_2 to H_2O before it escapes. These strains are more efficient in N_2 fixation as they contain a mechanism (hydrogenase) whereby H_2 evolved by nitrogenase is recycled.

$$H_2 \xrightarrow{\text{hydrogenase}} 2H^+ + 2e^-$$

Protons and electrons are reutilized by the electron carriers.

3-7 OVERALL VIEW OF MECHANISM OF NITROGEN FIXATION

Nitrogen fixation is the conversion of nitrogen into ammonia in the cells by nitrogenase enzyme.

1. Nitrogen fixation occurs in some ***bacteria, blue green algae*** and a ***fern.***
2. These organisms contains ***nitrogenase*** enzyme.
3. The nitrogenase enzyme provides the ability to fix nitrogen.
4. The organisms are called ***nitrogen fixers.***

   Eg : *Oscillatoria ; Anabaena ; Nostoc ; Rhizobium ; Azospirillum ; Azotobacter ; Azolla, etc.*

5. The nitrogen fixers may be ***free living (non-symbiotic)*** or ***symbiotic.***
6. The non-symbiotic nitrogen fixers live in the ***soil.***
7. The symbiotic nitrogen fixers lead a symbiotic life with the root system of ***leguminous plants.*** Eg. Rhizobium.
8. *Rhizobium* enters the root through root hairs and produces swellings on ***root nodules.***
9. Symbiotic nitrogen fixers fix ***nitrogen*** in the root nodules.
10. The nitrogenase is a ***protein*** enzyme.
11. It consists of 4 ***polypeptide chains, molybdenum, iron*** and ***sulphur.***
12. It is made up of two subunits namely a large ***molybdenum-ferrous - protein*** subunit and a small ***ferrous-protein*** subunit.
13. In nitrogen fixation, nitrogenase enzyme reduces nitrogen into ammonia.
14. It is a ***redox*** reaction.
15. The mechanism of nitrogen fixation is the same in symbiotic and non-symbiotic nitrogen fixers.

3-8 NITROGEN C...