

BIOLOGICAL NITROGEN FIXATION

Fixation of elemental nitrogen in the atmosphere by the micro organism through a reductive process into ammonia is called as BNF. A variety of prokaryotic organism have the ability to reduce the atmosphere N_2 BNF accounts for about 70% of the total N fixed in the biosphere. The ability to reduce atmosphere N is restricted only to bacteria, which are belonging to the diverse groups. The root nodule associations were the first to be recognized for their ability to fix atmosphere N_2 . Rhizobia are the first group of organism realized for its potential of nitrogen fixation.

Nitrogen fixing bacteria

Nitrogen fixing bacteria are classified according to their mode of fixation.

1. Free living N fixers – capable of fixing mol. N_2 to cellular N independently of other living organism.
2. Associative N fixers
3. Endophytic N fixers
4. Symbiotic N fixers

Rhizobium is predominant symbiotic N_2 fixing bacterium. Boussingault showed that leguminous plant can fix atmosphere N_2 . Then Hellriegel and Wilfarth – proved that N_2 is fixed by certain bacteria living in root nodules of leguminous plants. Latter isolated in pure culture by Beijerinck. Winogradsky isolated *Clostridium pasteurianum*. Which is an anaerobic N_2 fixer. Beijerinck isolated *Azotobacter* as free living aerobic N_2 fixing organisms.

Cross inoculation groups of rhizobium (CIG)

It (CIG) refers the groups of leguminous plants that will develop effective nodules when inoculated with the rhizobia obtained from the nodules from any member of that legume group.

I. Rhizobium

1.	<i>Rhizobium leguminosarum</i>	CIG	Host it can nodulate
	<i>bv. viceae</i>	Pea	Peas, lenfils, vicia
	<i>bv. phaseoli</i>	Bean	Phaseolus spp
	<i>bv. trifoli</i>	Clover	Trifolium spp
2.	<i>R. meliloti</i>	Alfalfa	Alfalfa, clover, fenugreek
3.	<i>R. loti</i>	Lotus	Trifoli, lupine,
4.	<i>R. fredii</i>	Soybean	Soybean
5.	<i>R. spp</i>	Cowpea group	Vigna, Arachis, Cajanus, Dolichus, Sesbania, Acacia, Prosopis, green gram and blackgram
6.	<i>R. sp</i>	Chickpea group	Chickpea



II. Bradyrhizobium

- *B. japonicum* Soybean
- *B. spp* Cowpea group

III. Azorhizobium - Stem nodulating – one. Nodulates *Sesbania rostrata*.

IV. Photorhizobia - Nodulants *aeschynomene* sp.

V. Sinorhizobium - fast growing soybean nodulator

I. Biological nitrogen fixation

Free living nitrogen fixers

- *Azotobacter* - Aerobic
- *Beijerinckia*
- *Clostridium* – Anaerobic
- *Cyanobacteria* (Blue green algae) etc.,

II. Associative symbiotic nitrogen fixer

Azospirillum

Herbaspirillum

III. Endophytic nitrogen fixer

Gluconacetobacter diazotrophicus

IV. Symbiotic nitrogen fixers

- *Rhizobium* (*Rhizobium* – legume association)
- *Bradyrhizobium* (*Bradyrhizobium* – soybean association)
- *Azorhizobium* (*Azorhizobium*- *Sesbania rostrata* association)
- *Anabaena azollae* (*Azolla* – *Anabaena* association)
- *Frankia* (*Frankia* – Casuarina association)

Species of *Azospirillum*

- *lipoferum*
- *brasiliense*
- *amazonense*
- *halopareferans*
- *irkense*

- *A.largomobilis*

Species of Azotobacter

- *chroococcum*
- *vinelandii*
- *beijerinckii*
- *paspali*
- *agilis*
- *insignis*
- *macrocytogens*

Important genera of blue green algae

Anabaena, Nostoc, Cylandrospermum, Rivularia, Oscillatoria, Plectonema, Aphanothece, Lyngbya, Scytonema, Calothrix etc.,

Species of Azolla

- *pinnata*
- *filiculoides*
- *microphylla*
- *caroliniana*
- *mexicana*
- *nilotica*

Nitrogen fixation

Process of N₂ fixation

The process of N₂ fixation is mediated by the enzyme, called nitrogenase (which mediates the reduction of N₂ to ammonia) first, this enzyme was extracted from the anaerobic di nitrogen fixer *Clostridium pasteurianum*. Latter, this has been isolated from most other N₂ fixing bacteria.

The mechanism of N₂ fixation appears to be quite similar in most N₂ fixing prokaryotes. The enzyme has been fairly well characterized and the enzymes from these different systems share common properties allowing a unified single description of nitrogenase.

Nitrogenase

Nitrogenase is a functional enzyme which reduces N_2 to ammonia and depends on energy source from ATP. The nitrogenase has two components one containing Mo-Fe, designated as Mo – Fe protein and the other Fe protein . Two components are necessary for the nitrogenase activity.

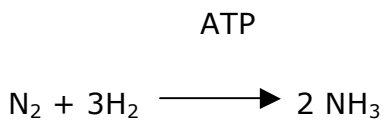
Mo-Fe protein

Consists of 4 subunits and having the molecular weight of 22,000 or 270,000 daltons and it is the big component.

Fe-protein

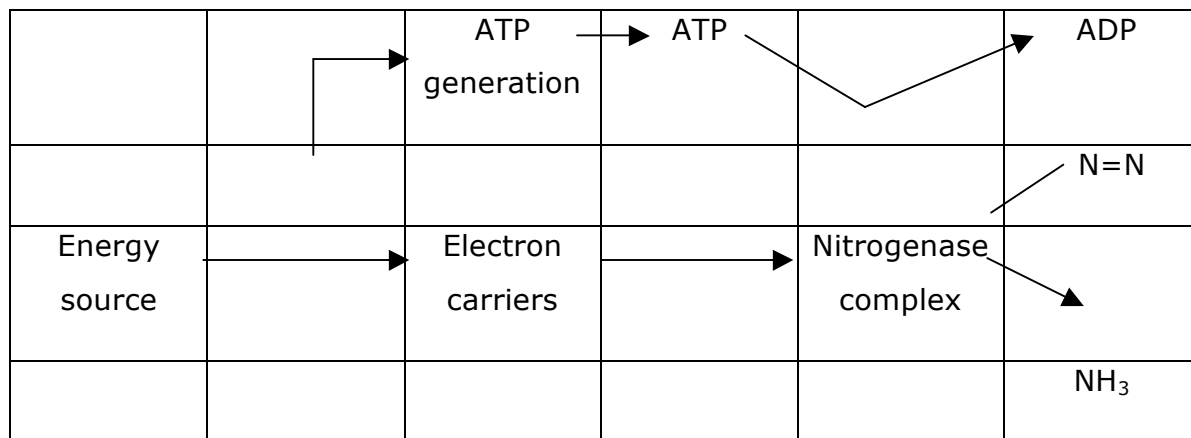
Smaller component, contains 2 subunits, molecular weight 60,000 daltons.

Ammonia is the end product of N_2 fixation. The overall reaction is as follows.



General pathway of N_2 fixation

This process requires a source of ATP and reductants, which are provided by photosynthesis. 16 molecules of ATP are required to fix a molecule of N_2 .

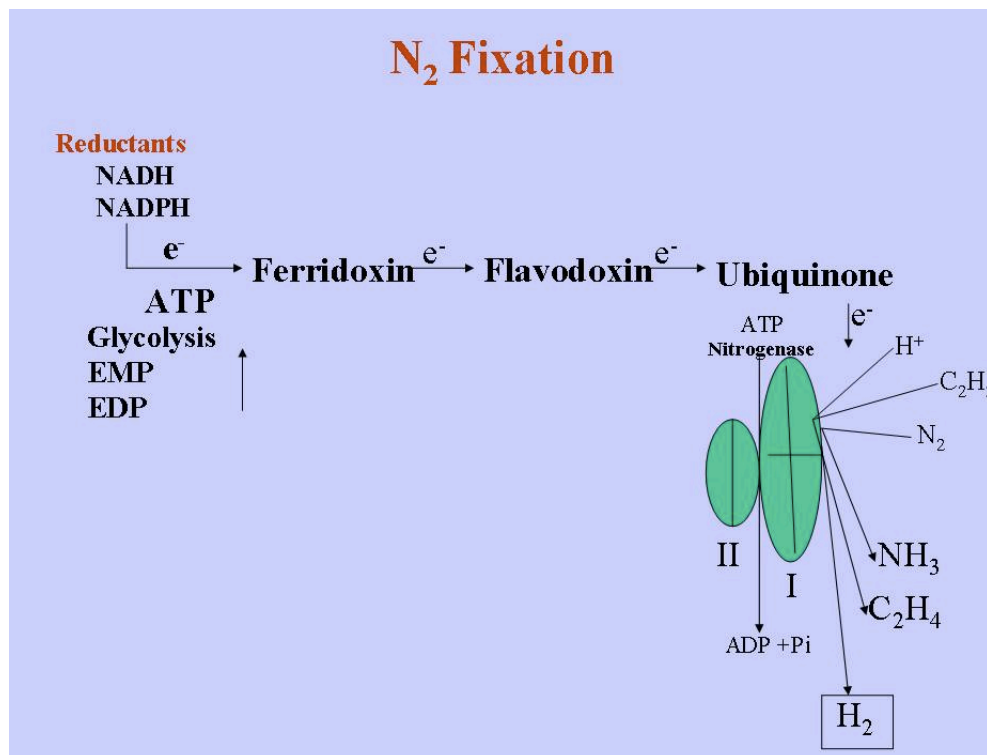


Nitrogenase can also reduce $C_2H_2 \rightarrow C_2H_4$

Hydrolysis of ATP into ADP with electron transfer from a reduced electron donor (Ferridoxin, Flavodoxin) is coupled to reduce N_2 to $2NH_3$. The ammonia is the first stable product of fixation and it is assimilated by GS-GOGAT pathway.

NADH									
NADPH	e^-		e^-		e^-		e^-		
	→	Ferridoxin	→	Flarodoxin	→	Ubiquinone			
APP									
EMP		e^- Carrier proteins						NII	NI
EDP									
Glycolysis									

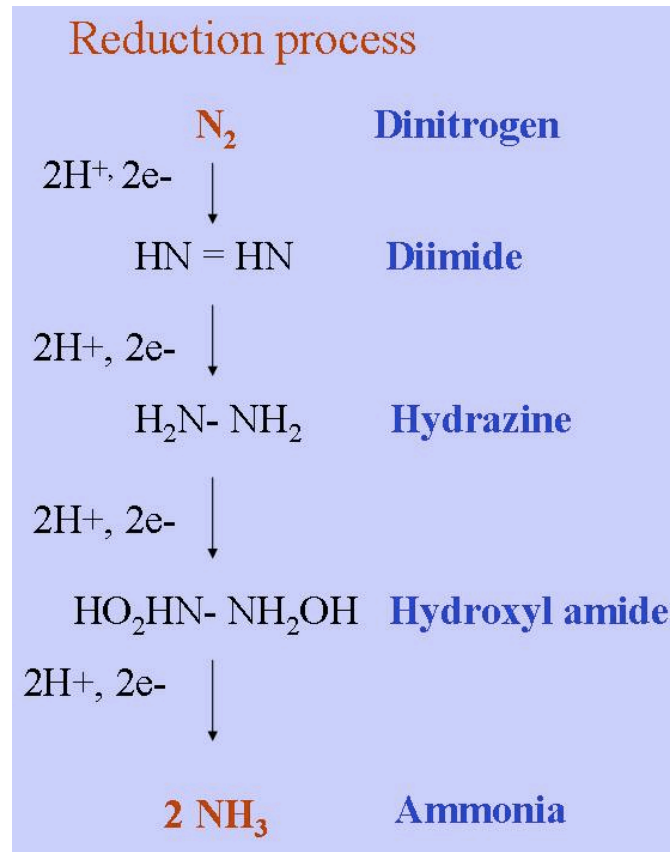
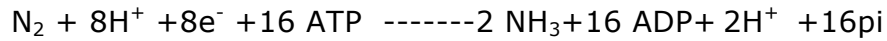
Nitrogenase is O_2 labile various protection mechanism are operating in different N_2 fixing systems.



Mechanism

Reduction takes place on the surface of the enzyme

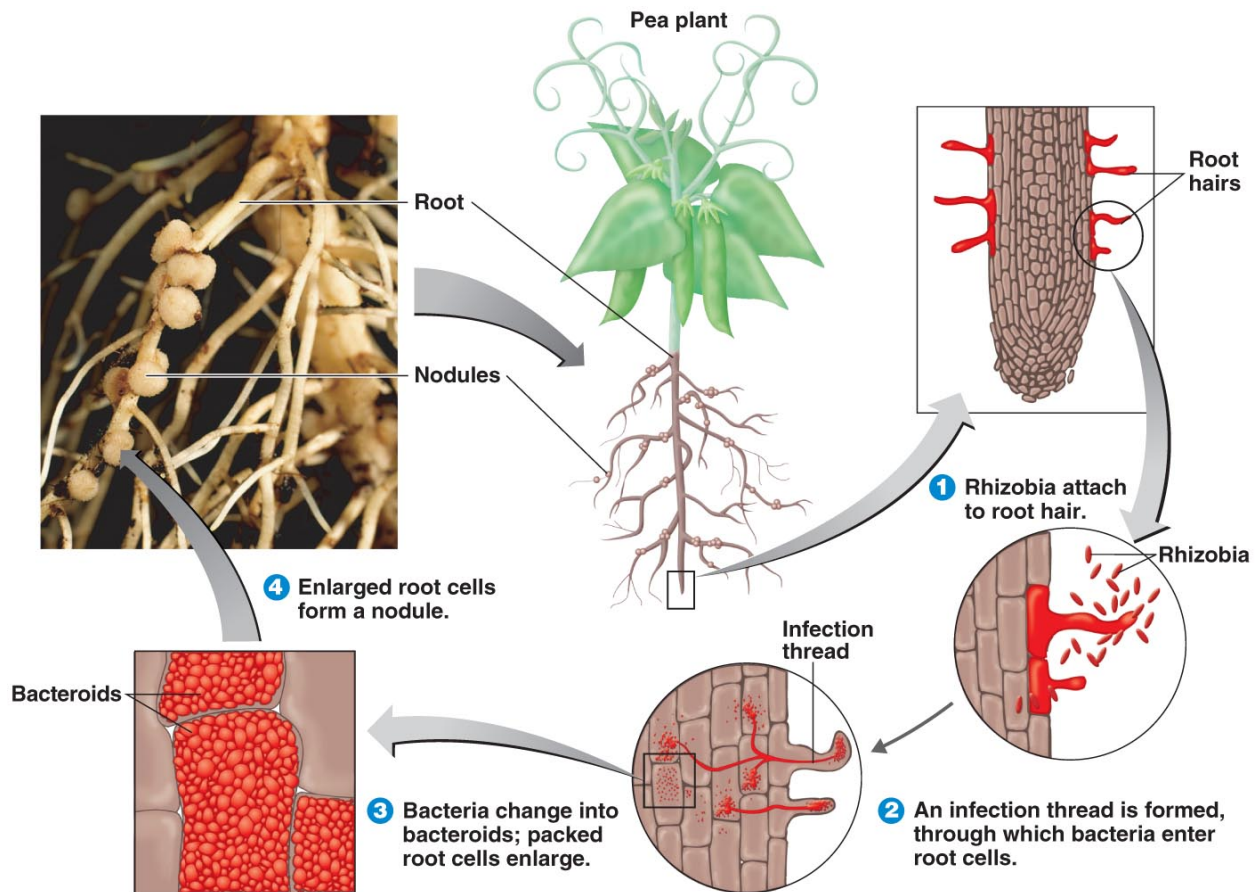
- Six electrons are required to reduce one mole of N to two moles of ammonia.



It is postulated that, atoms of N_2 are separated through charge in the valency of metal ion (mo) bound to the enzyme involved in reduction of N_2 . For every electron transfer, 4 ATP mole are required.

Hydrogenase -Uptake hydrogenase (HUP^+) converts the released hydrogen during N_2 fixation, and cycled back the Hydrogen for energy generation. by this they contribute 9-10 %ATP requirement for N_2 fixation process.

Formation of a Root Nodule



Factors affecting N_2 fixation

1. Presence of nitrate or ammonium : More N_2 , No, N_2 fixation
2. Presence of certain inorganic substances
Ca, Co, Mo – influence N_2 fixation along with P
3. Availability of energy source – addn. of C source increase N_2 fixation
4. pH : Neutral – favours *Azotobacter* – Acidic- *Beijerinckia*
5. Soil moisture : Adequate is good for fixation
6. Temperature: Mesophilic – 30°C .

The energy requirement for BNF is very high and it is a major factor determines the amount of N_2 fixed. In, *Azotobacter* the rate depends on amount of available carbon. In symbiotic N_2 fixers since photosynthesis is the ultimate source

of energy the rate of N_2 fixation is influenced by the factors that effect photosynthesis and rate of translocating photosynthates to the N_2 fixing system.

Nitrogenase protection mechanisms

1. Leghaemoglobin scavenges O_2 to protect nitrogenase in legume rhizobium symbiosis
2. Confirmatory protection in *Azotobacter* as well as the higher respiratory rate.
3. Thick walls of Heterocyst protect O_2 in BGA, since Nitrogenase are present in the heterocyst.
4. Microaerophilic nature in *Azospirillum*

Losses of N by non biological ways

Leaching

20 to 50% of fertilizer N. The most striking loss of N in rice soils where more than half of the fertilizer N applied get lost through leaching.

Volatilization

Another factor is the volatilization of ammonia in soil 5-20%.

Fixation of ammonium in soils is the minor contributory factor to overall loss of N_2 available for plant growth.

Such losses of N by physical causes and by nitrification and denitrification process can be controlled by the application of certain chemicals. Some chemicals have been designed to control the rate of release of nutrient from nitrogenous fertilizers, while others retard nitrification in soil by controlling the activity of nitrifying bacteria.

a. Controlled release fertilizers

Urea from	}	Fertilizers, sparingly soluble in water can regulate the release of N from fertilizers
isobutyldene diurea		
Crotonilidene diurea		

S coated urea

b. Nitrification inhibitors

These are substituted with pyridines, pyrimidines, anilines and isothiocyanates,

Examples

1. 2 chloro 6 (tricholormethyl) – pyridine – (N serve)

2. 2 amino 4 chloro 6 methyl pyridine –(AM.)

N serve inhibits the growth of *Nitrosomonas europea* and *N. agilis*.

The seeds of neem contain lipid associates act as nitrification inhibitors and there by increases the efficiency of urea fertilizers.

Ammonia assimilation

N_2 fixation results in NH_4 formation which reacts with organic acids and form amino acids which is mediated by ammonia assimilating enzyme.

GS – Glutamine synthetase

GOGAT – Glutamate synthase

GDH – Glutamate dehydrogenase

Genetics

Nif genes are responsible for N_2 fixation.

Nif genes are 22, which are located in 7 or 8 clusters.

GENETICS OF NODULATION AND N FIXATION

Root nodule bacteria and symbiosis with legumes

One of the most interesting and important plant bacterial interactions is that between leguminous plants and certain gram negative nitrogen fixing bacteria. *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Azorhizobium* are gram negative motile rods. Infection of the roots of a leguminous plant with the appropriate species of one of these genera leads to the formation of root nodules that are able to convert gaseous nitrogen to combined nitrogen, a process called nitrogen fixation. Nitrogen fixation by legume Rhizobium, symbiosis is of considerable agricultural importance, as it leads to very significant increases in combined nitrogen in the soil. Because nitrogen deficiencies often occur in unfertilized bare soil, modulated legumes are at a selective advantage under such conditions and can grow well in areas where other plants cannot.

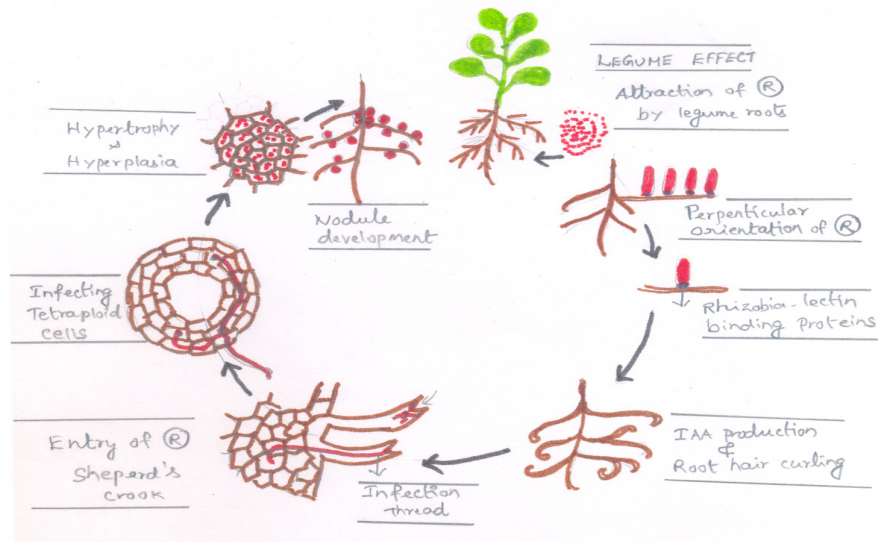
- CIG refers, the groups of leguminous plants that will develop effective nodules when inoculated with the rhizobia obtained from the nodules from any member of that legume group.

Stages in root nodule formation

The stages in the infection and development of root nodules are not fairly well understood. They include

1. **Recognition** of the correct parameter on the part of both plant and bacterium and **attachment** of the bacterium to the root hairs.
2. **Excretion** of nod factors by the bacterium.
3. **Invasion** of the root hairs by the bacterial formation of an infection thread.
4. Travel to main root via the infection thread.
5. Formation of deformed bacterial cells, **bacteroids**, within the plant cells and development of the nitrogen fixing state.
6. Continued plant and bacterial division and formation of the mature root nodule.

NODULATION IN LEGUMES



Nodulation events

- Normal root hair
- Exudation of organic substances
- Accumulation of rhizobia in the rhizosphere
- Orientation and binding of rhizobia
- IAA production
- Root hair curling and deformation
- Formation of infection thread by rhizobia
- Formation of sheperd's crook cells and entry of infection thread
- Thread containing bacteroids extending into root hair cells
- Entry of infection thread into cortex and branching
- Nodule development

Attachment and infection

The roots of leguminous plants secrete a variety of organic compounds that stimulate the growth of a rhizosphere micro flora. This stimulation is not restricted to the rhizobia but occurs with a variety of rhizosphere bacteria. If there are rhizobia in the soil, they grow in the rhizosphere and build up to high population

densities. Attachment of bacterium to plant in the legume Rhizobium symbiosis is the first step in the formation of nodules. A specific adhesion protein called **rhicadhesin** is present on the surface of all species of *Rhizobium* and *Bradyrhizobium*. Rhicadhesin is a calcium-binding protein and may function by binding calcium complexes on the root hair surface. Other substances, such as carbohydrate-containing protein called **lectins**, also play in plant bacterium attachment.

Initial penetration of Rhizobium cells into the root hair is via the root hair tip. following binding, the root hair curls as a result of the action of substances excreted by the bacterium called nod factor and the bacteria enter the root hair and induce formation by the plant of a cellulosic tube, called infection thread, which spreads down the root hair. Root cells adjacent to the root hairs subsequently become infected by rhizobia and nod factors stimulate plant cell division, eventually leading to formation of the nodule.

Bacterioids

Bacterioids are specifically referred to a swollen deformed *Rhizobium* cell found in the root nodule, capable of nitrogen fixation

The Rhizobium bacteria multiply rapidly within the plant cells and are transformed into swollen, misshapen and branched forms called bacterioids. When the plant dies, the nodules can be deteriorated, releasing bacteria into the soil. The bacterioid forms are incapable of division, but there are always a small number of dormant rod shaped cells present in the nodule. These now proliferate; using some of the products of the deteriorating nodule as nutrients, and the bacteria can initiate the infection in other roots or maintain a free living existence in the soil.

Lectins

Plant proteins which specifically bind to carbohydrate receptors (polysaccharides) in the rhizobial cell

Genetics of nodule formation

Genes directing specific steps in nodulation of a legume by a strain of *Rhizobium* are called nod genes. Many nod genes from different *Rhizobium* species are highly conserved and are borne on large plasmids called ***sym plasmids***. In addition to nod genes which direct specific nodulation events, sym plasmids contain specificity gene, which restrict a strain *Rhizobium* to a particular host plant. Indeed cross inoculation group specificity can be transferred across species of rhizobia by simply transferring the respective sym plasmid.

In the sym plasmid of *Rhizobium leguminosarum* bio var *viciae*, *nod* genes are located between two clusters of genes for nitrogen fixation the *nif* genes. Ten *nod* genes have been identified in this species. The *nod* ABC genes are involved in the production of oligosaccharides called *nod factors*, which induce root hair curling and trigger plant cell division, eventually leading to formation of the nodule. In *Rhizobium leguminosarum* bio var *viciae*, the gene *nodD* encodes a regulatory protein; this controls transcription of other *nod* genes.

Nod D genes

- Genes directing specific steps in nodulation of a legume by a strain of rhizobium are called Nod genes
- Nod genes are born on large plasmids ,called sym plasmids
- Nod genes are located between two clusters of genes for N₂ fixation called Nif genes
- Nod gene consists of 8 genes
- nod A,B,C,D,E,F,L,M
- nod D controls the function of all nod genes

Nif Genes

- Genes responsible for N fixation are called Nif genes
- 22 genes are involved, arranged in 7 /8 clusters
- Nif Q,B,A,L,F,M,Z,W,V,S,U,X,N,E,Y,T,K,D,H,J

- KDH – control Nitrogenase enzyme complex

Factors affecting nodulation

- Temperature and light
- Combined Nitrogen
- Hydrogen iron concentration
- Mineral nutrition-Co,Mo,P,Ca
- Genetic factors
- Ecological factors
- Salinity and alkalinity