NITROGEN FIXATION

For centuries together it was a well-known fact that by growing leguminous crops the reduced fertility of land can be increased again. The reason for this increase in fertility was not known for long. Later on, it was found that the fertility of the soil increases due to some bacteria which are found in root nodules of leguminous crops and soil and these bacteria are capable of fixing atmospheric nitrogen. Nitrogen fixation can be defined as the *phenomenon of conversion of free nitrogen into nitrogenous salts to make it available for absorption by plants.*

The fixation of nitrogen can be either a physical fixation or a biological nitrogen fixation. Biological nitrogen fixation is more common in nature.

About 200 million metric tonnes of nitrogen is fixed annually by natural processes. Only 5 percent of this natural fixation occurs by physical process (physical nitrogen fixation) and about 95 per cent occurs through certain micro-organisms (biological nitrogen fixation).

(*I*) Physical Nitrogen Fixation

It occurs in several steps and starts with combination of atmospheric nitrogen with oxygen under the influence of electric discharge and thunder to produce nitric oxide:

$$\text{Nitrogen } (N_2) + \text{Oxygen } (O_2) \xrightarrow[\text{and thunder}]{\text{Electric discharge}} \text{Nitric oxide } (2NO)$$

The nitric oxide is then oxidised to nitrogen peroxide in the presence of oxygen

$$\text{Nitrogen oxide } (2NO) + \text{Oxygen } (O_2) \xrightarrow{\text{Oxidation}} \text{Nitrogen peroxide } (2NO_2)$$

During rains, the nitrogen peroxide combines with rain water to form nitrous acid and nitric acid which come to ground along with rains.

$$\text{Nitrogen peroxide } (2NO_2) + \text{Rain water} \rightarrow \text{Nitrous acid } (HNO_2) + \text{Nitric acid } (HNO_3)$$

On ground the alkali radicals of the soil react with nitric acid to produce nitrites and nitrates which are soluble in water and which can be absorbed by the plants through roots:

$$\text{Ca or K salts} + HNO_3 \xrightarrow{\text{Soil}} \text{Ca or K-nitrates}$$

(*ii*) Biological Nitrogen Fixation

The biological nitrogen fixation may be categorised into following two types:

(*a*) Symbiotic nitrogen fixation

(*b*) Asymbiotic nitrogen fixation

This classification is maintained to refer to two different types of organisms involved in N_2 fixation.

SYMBIOTIC NITROGEN FIXATION

This kind of nitrogen fixation is carried out by bacteria frequently found in the root nodules of leguminous plants. The most common bacterium is *Rhizobium*. It has two types of strains, (a) fast growers that include *R. leguminosarum* or *Bacillus radicicola, R. faciolii, R. trifolii, R. mollilotii* and (b) slow growers that include *R. japonicum* and *R. lupinii*. Rhizobia are commonly found in the soil of crop fields where bacteria and plant both specifically affect each other's growth.

Nodule formation. The development of nodule is rather a complicated process. In the initial stage, host cells have to recognise the correct strain of symbiotic bacteria. A critical specificity is involved in the association of various species of root nodule bacteria and leguminous plants. This step is controlled by specific proteins found in a host cell called lectin. Bauer (1981) proposed that these lectins interact selectively with microbial cell carbohydrates (or glycoproteins) found in the capsule of bacteria and serve as determinants of recognition or host specificity. This interaction induces some responses in host plants and may result in exclusion or inclusion of bacteria in the host cell. Thus, lectins are the key proteins involved in the Rhizobium-legume symbiotic association and in the process calcium plays a critical role in modifying the ability of root to absorb rhizobia.

Nodule formation is initiated by the infection of root hair by free living rhizobia. Since these bacteria cannot digest cellulose it enters the root hair from the tip region where cellulose is nearly absent. The process of nodule formation is very peculiar. Leguminous plants release tryptophan in the soil which is absorbed by *Rhizobium* and is metabolised to produce IAA (indole acetic acid). The Rhizobia produce another characteristic substance called *root hair curling factor* that causes deformation and twisting of root hairs. The root hair gets penetrated by a large number of rhizobia which collectively convert into an infection thread lined by cellulose which is provided by the host cell. The infection thread intrudes, settles and liberates its contents

in a cortical cell which is usually a polypoid (tetraploid). The rhizobia are liberated either individually or in small groups enclosed by a membrane. The cells of bacteria enlarge, assume pleomorphic shape and are called *bacteroids.* These bacteroids stimulate polyploid cell to undergo repeated divisions. These also induce the surrounding cells (diploid cells) to divide which ultimately form the cortex of a nodule. The chemical nature of inducer has been found to be free and active IAA or a substance secreted by rhizobia (Truchet *et al.* 1980).

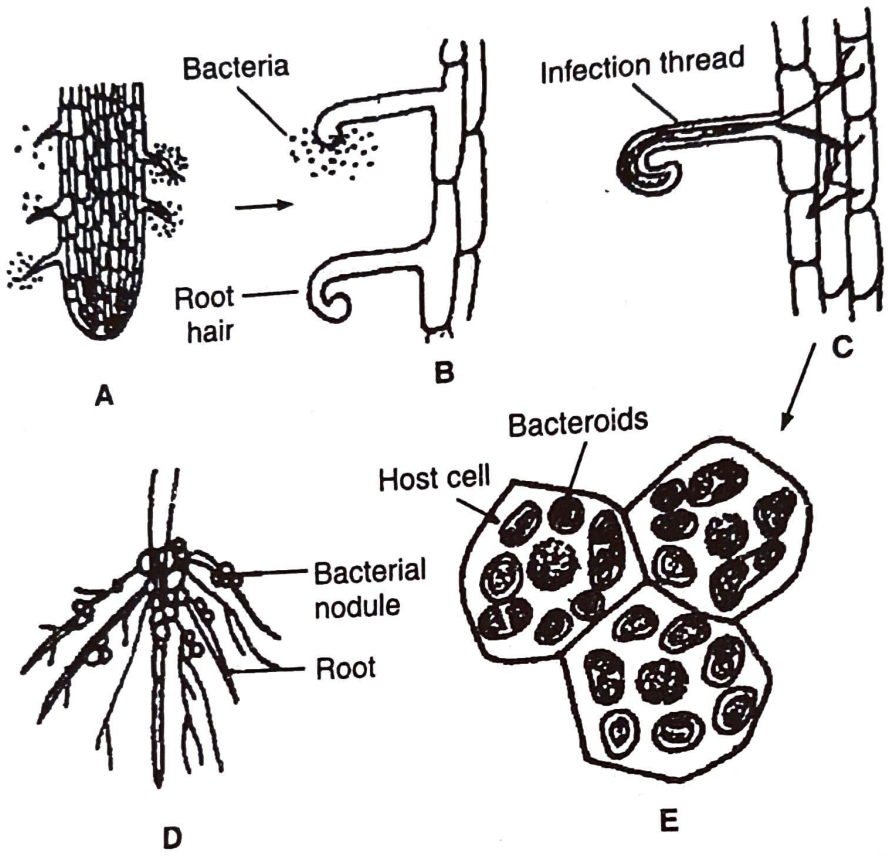

Fig. 17.2. Stages in the formation of root nodule. A—Rhizobia bacteria growing in the soil near the root, B—entrance of bacteria through sub-apical region of root hair, C-infection thread formed by bacteria, D-roots of a plant containing several nodules and E—bacteroids in membrane bounded sacs in the host cells.

The nodule contains a pink coloured *leghaemoglobin* pigment which like true haemoglobin combines with oxygen and CO_2 and gets readily oxidised into brown form with a trivalent iron. The development of leghaemoglobin and nitrogen fixing capacity are outcome of symbiotic interaction, since individually neither leguminous plants produce leghaemoglobin nor bacteria carry out nitrogen fixation. The symbiotic relationship is so essential that in the absence of *Rhizobium* and combined nitrogen in soil leguminous plants do not grow. There are two views about the location of leghaemoglobin that it is either located in the space between bacteroids and the membrane enclosing them or is present outside the membrane and in the host cytoplasm.

Nitrogenase. It has now been fully accepted that the process of nitrogen fixation, whether in symbiotic or asymbiotic forms, involves reduction of atmospheric nitrogen to ammonia (NH_3) by the enzyme *nitrogenase.*

Nitrogenase is made up two protein components, one containing iron and molybdenum, known as Mo-Fe protein or molybdo-ferredoxin (component I) and

the other containing only iron called Fe-protein or azoferredoxin (component II). Mo-Fe protein contains 1 to 30 iron atoms, a similar amount of sulphur and one or two atoms of molybdenum for every 2,00,000 to 3,00,000 units of molecular weight and is made up of four sub-units grouped in two types and contains two iron-containing electron carriers. The mol. wt. of Fe-protein ranges from 55,000 to 65,000 and has about four iron and four sulphur atoms per 60,000 mol. wt. In addition to nitrogen, *nitrogenase* can reduce a variety of multiple bonded substrates like N_2O and those with general formulae RCN, RNC, RCCH as well as acetylene. The acetylene reduction is the quick procedure to determine *nitrogenase* activity. *Nitrogenase* is extremely sensitive to oxygen. It is for this reason that N_2-fixing organisms have evolved variety of ways for protecting *nitrogenase* from O_2 induced inactivation. The development of leghaemoglobin pigment in nodule cells and heterocysts in blue green algae are few examples. In general, the protection mechanism has been categorised into two types, (a) *conformation protection* (by change in the shape of enzyme in the presence of O_2 to become insensitive to it) and (b) *respiratory protection* (by utilizing excess of oxygen in oxidising various substrates). However, a considerable amount of work has yet to be done to precisely understand the exact nature, composition and function of *nitrogenase*.

The enzyme remains active under anaerobic conditions. The leghaemoglobin regulates the course of O_2 and provides suitable atmosphere to the enzyme. The pigment leghaemoglobin combines rapidly with O_2 to avoid any inhibitory effect of it on enzyme. At the same time it is able to make O_2 available to bacteroids for ATP production, required for nitrogen fixation.

Mechanism of nitrogen fixation. It is believed that during the process of nitrogen fixation, the free, atmospheric nitrogen first bound to the enzyme surface is not released until it is completely reduced to ammonia. Nitrogen bound to the enzyme surface is reduced in stepwise reactions before N-N bond is ruptured. The total reduction of nitrogen occurs on an enzyme complex without release of intermediates less reduced than ammonia.

The pathway of electron transport in nitrogen fixation in nodule bacteroids may be summarised as follows:

The fixation of atmospheric free nitrogen is carried out by enzyme *nitrogenase*. The reduction of nitrogen into ammonia by *nitrogenase* in bacteroids depend upon the availability of ATP and reduced substrate capable of donating hydrogen atoms to nitrogen. ATP is generated in bacteroid respiratory chain system and reduced substrate is obtained from host cells. Glucose-6-phosphate is considered to be the reduced substrate for the process and reduced NADP together with ferredoxin function as electron carriers.

The ATP interacts with non-heme iron (NHI) protein component of *nitrogenase* and brings about conformational change to convert it to a powerful reductant. This powerful reductant becomes capable of transferring electrons to reduce N_2 into NH_3. The summary of the process is given in Fig. 17.3.

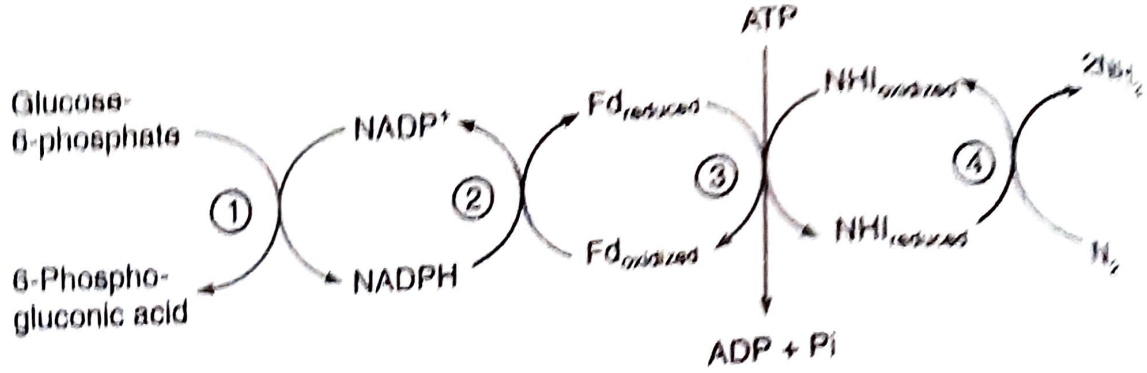

Fig. 17.3. The proposed pathway of N_2 fixation. (Fd = ferredoxin, NHI = non-heme iron, 1 = enzyme *glucose-6-phosphate dehydrogenase*, 2 = enzyme *NADPH-ferredoxin oxido-reductase*, 3 = enzyme iron containing protein component of *nitrogenase* and 4 = enzyme iron-molybdenum protein component of *nitrogenase*)

The *in vitro* studies on the process revealed that at least four molecules of ATP are hydrolysed for each pair of electrons transferred to nitrogen. Thus, the reduction of one molecule of nitrogen into two molecules of ammonia requires twelve molecules of ATP because six electrons are required per molecule of nitrogen reduced.

$$N_2 + 6H^+ + 6e^- + 12ATP \longrightarrow 2NH_3 + 12ADP + 12Pi$$

Several other substrates in addition to nitrogen can be reduced by *nitrogenase* complex, *e.g.* proton is also a substrate for it.

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

Due to this nature of *nitrogenase*, there may be evolution of additional H_2 by bacteroids during nitrogen fixation. Such hydrogen evolution causes a waste of ATP and reducing power. However, some strains of *Rhizobium* do not evolve hydrogen and are thus more efficient in nitrogen fixation. Scientists are effortful to develop such strains.

Nitrogen fixing symbionts with non-leguminous plants. Nodule formation is not only found in leguminous plants but also in quite a few other plants. Symbiotic bacteria have been found to occur in the root nodules of *Casuarina, Ceanothus, Cycas, Podocarpus, Dioscoria, Coriaria, Camptonia, Alnus, Myria, Hippophae* etc. and in leaf nodules of different species of *Pavetta, Psychotria, Dioscorea* etc. Some members of Rubiaceae also develop root nodules, the bacterium being *Mycobacterium rubiacearum.* Species of *Klebsiella* and *Enterobacter* have been detected in the leaves of *Psychotria* and white fir. Similarly, blue green algae, *Anabaena azollae* form symbiotic association with a water fern (*Azolla*), *A. cycadae* with *Cycas* (gymnosperm) and *Nostoc* with *Gunnera macrophylla* (angiosperm).

Bacteria *Azotobacter paspali* form colonies below mucilaginous root sheath of *Paspalum notatum. Spirillum notatum* is found associated with *Digitaria, Zea mays, Sorghum etc.*

ASYMBIOTIC NITROGEN FIXATION

Many free living bacteria and blue green algae are capable of fixing atmospheric nitrogen.

Bacteria (*i*) Anaerobic forms, *e.g. Bacillus polymyxa, of Clostridium pasteurianum, Azospirillum brassilense, Klebsiella pneumoniae;*

(*ii*) Aerobic forms, *e.g. Azotobacter chroococcum, A. gilis, A. agile, Azomonas, Beijerinckia;*

(*iii*) Photosynthetic forms, *e.g. Rhodospirillum, Chromatium, Chlorobium, Rhodopseudomonas, Rhodomicrobium Chloropseudomonas;*

(*iv*) Chemosynthetic forms *e.g. Thiobacillus, Desulphovibrio;*

Fungi–*Pullularia* and yeasts.

Blue green algae (*i*) Unicellular forms, *e.g. Gloeothece, Synechococcus;*

(*ii*) Filamentous non-heterocystous forms, *e.g. Oscillatoria erythraea* (syn. *Trichodesmium erythraeum*), *Lyngbya-Phormidium-Plectonema* group;

(*iii*) Filamentous heterocystous forms *e.g. Nostoc, Anabaena, Cylindrospermum, Calothrix, Tolypothrix, Aulosira, Scytonema, Gloeotrichia etc.*

Process of Nitrogen Fixation (Studies on Cell Free Nitrogen Fixation)

The process remains basically the same in non-symbiotic nitrogen fixation, which has been supported by studies on cell free extracts of free living nitrogen fixing bacteria. Some points are considered here.

Studies made on cell-free preparations to understand biological nitrogen fixation have opened a new aspect on the subject. McNary and Burris (1962) extended the first experimental evidence that ATP was involved in nitrogen fixation and Mortenson (1964) while studying cell-free extracts of *Clostridium pasteurianum* showed clearly the necessity of ATP in the process. Similarly, Dilworth (1965) found that the addition of 5 mM ATP to an extract of *C. pasteurianum* supported nitrogen fixation for 5 minutes at a rate of 3.8 mμ molecule N_2/mg protein/minute.

Hardy and his coworkers (1965) explained the sequence of nitrogen fixation as follows:

In this scheme ferredoxin plays an important role which is helpful in reducing X compound. This X-reduced compound utilising energy from ATP forms ammonia in presence of the enzyme *nitrogenase.*

Burris (1966) proposed working hypothesis for nitrogen fixation suggesting the function of ATP and ferredoxin at each step in the reduction of nitrogen. The pretty function of ATP donor is furnished by pyruvate which also acts as an electron donor for N_2 reduction.

Pyruvate on one hand acts as an ATP donor while on the other hand it supplies hydrogen ions and electrons for nitrogen reduction via $NADH_2$ and ferredoxin. The enzyme requires 15 or even more ATP molecules, 6 hydrogen ions and 6 electrons to reduce one molecule of nitrogen to two NH_3 molecules. The reduction takes place step by step as shown in Fig. 17.4.

Explaining the mechanism of *nitrogenase* activity, it is now believed that electrons are transferred from the reducing agents (Ferredoxin, Flavoprotein or Dithionite) to a complex of Mg-ATP and Fe-protein (component II). From here electrons flow to Mo-Fe protein (component I) and then to substrate (nitrogen) which is finally reduced (to NH_3).

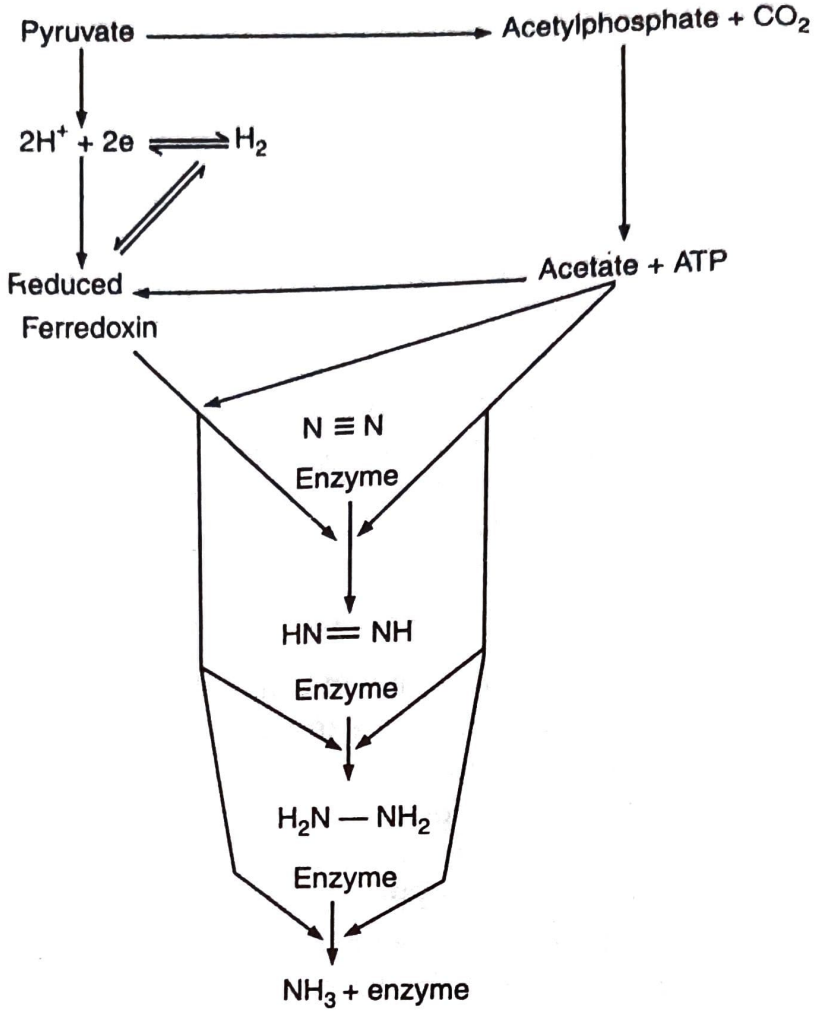

Fig. 17.4. Scheme suggesting the role of ATP and ferredoxin at each step in the reduction of nitrogen. Enzyme is *Nitrogenase* (Burris, 1966).

In most diazotrophs (N_2-fixing organisms), ferredoxin and flavodoxin are probably the natural electron carriers for the reduction of Fe-protein. The reduced Fe-protein binds to Mg-ATP ($Mg^{2+}ATP$), creating a complex with Mo-Fe protein. Dissociation of two proteins occur between electron transfer events. The oxidised Fe-protein dissociates and becomes reduced again which recombines randomly with another *nitrogenase* until all electrons needed for the reduction of substrate (*e.g.* 6 for N_2) are accumulated.

Genetical studies. The genetical approach to study the mechanism of *nitrogenase* activities in recent and successful studies have been made on *Klebsiella pneumoniae*. The gene responsible for the synthesis of *nitrogenase* is called *nif* gene. Streicher and his coworkers (1972) successfully mapped *nif* regions in this bacteria. The genetic map of *nif* regions indicated closeness of four *nif* genes to *his* D (the region of DNA). Merrick (1979) has been successful in identifying 14 *nif* genes in *K. pneumoniae* which were organised into eight transcriptional units. Recent studies indicated that *nif* region has been found flanked by two sets of inverted repeat units which suggested the recent origin of *nif* genes during the course of evolution in the organisms (diazotrophs). Besides, it is further supported by the fact that *nif* DNAs of widely separated diazotophs have a close relationship. A segment of *nif* operon of *K. pneumoniae* has been found to hybridise with DNA from eight

different N_2^- fixing species. Bacteriophages have been successfully used in studying similarities of *nif* genes and in their transferring (transferring of *nif* genes) to other bacteria. N_2 fixing genes of *K. pneumoniae* have been successfully transferred to *Escherichia coli* (a non-nitrogen fixing bacterium) through transduction using Phage 424, and this hybrid produced *nitrogenase* similar to that of *K. pneumoniae.* This achievement is now considered as a great success of genetic engineers (biotechnologists) that offers prospects for a bright future. If somehow *nif* genes can be manipulated and successfully introduced in crop plants, *e.g.* paddy plants, the latter would develop an enormous capacity for high yield without any external supply of nitrogen fertilisers. In this way, biotechnologists would offer double benefits to the future world, *i.e.* high yield of rice and economy of nitrogen fertilisers.