DINITROGEN FIXATION-WITH PARTICULAR REFERENCE TO NON-LEGUMINOUS FLOWERING PLANTS-A REVIEW

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CERTIFICATE

This dissertation entitled "Dinitrogen Fixation-with Particular Reference to Non-Leguminous Flowering Plants-A Review" embodies the work carried out at the School of Environmental Sciences, Jawaharlal Nehru University, New Delhi. This work has not been submitted in part or in full for any degree or diploma of any university.

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Chapter 1

Introduction

Nitrogen forms the basic constituent of proteins, enzymes, nucleic acids, vitamins and hormones, all of which are essential for living organisms. Unlike other essential elements, no shortage of nitrogen should ever occur as the atmosphere contains an inexhaustible amount of dinitrogen, i.e. 78 volume per cent. In spite of the wide occurrence of dinitrogen in the atmosphere, it is usually the most critical limiting factor in the productivity of ecosystems.

Early in the nineteenth century, the superiority of legumes over cereals was a perplexed question. The idea of biological dinitrogen fixation was conceived by Sir Humphery Davis in 1813, who advanced the idea that legumes might be able to utilize the atmospheric source of dinitrogen. Jean Baptiste Boussingault, a French Chemist, in 1838 was the first to make an enduring commitment to this problem. From chemical analysis he found that nitrogen content in legumes was higher as compared to cereals. On the experience of crop rotation and experimental studies with clover and wheat, Boussingault proclaimed that atmospheric air is the source of extra nitrogen found in clover. Later, he also showed that legumes enriched the soil with fixed nitrogen. He, however, could not ascertain the nature of nitrogen whether the atmosphere contained ammonia or gaseous nitrogen.

In 1840, Liebig hypothesized that ammonia is the most active atmospheric species of available nitrogen. This

suggestion was accepted by all those who believed in the theory of mineral nutrition. In 1850, Georges Ville thwarted the ammonia hypothesis by presenting experimental evidence of dinitrogen fixation by potted plants maintained in ammonia free atmosphere.

Lachmann in 1858 found 'vibro-like' bodies in the legume root nodules which were described by Woronin in 1862 as 'bacterial-like'. In 1862, Jodin observed nitrogen enrichment by certain free living microorganisms in the nitrogen free medium. Two famous German experimentalists namely, H. Hellriegel and H. Wilfarth in 1888 did the pioneering work on biological dinitrogen fixation. They found that legumes differ from other plants in three respects namely, 1. they are capable of utilizing atmospheric dinitrogen, 2. the dinitrogen fixation occurs as long as the symbionts are in association with legumes roots and, 3. the legumes possess root nodules which are the sites of dinitrogen fixation.

A great Dutch microbiologist, M.W. Beijerinck in 1888 successfully isolated Rhizobium radicicola from the roots of leguminous plants which was subsequently renamed as Rhizobium leguminosarum on account of its obligatory symbiotic nature with the roots of legumes plants. Winogradsky in 1893 successfully isolated a free living anaerobic dinitrogen fixing bacterium, Clostridium pasteurianum from soil. The

Table 1. A broad classification of dinitrogen fixing organisms.

Biological N_2-fixing system	N_2-fixing organism	Associated plant	Habitat
1	2	3	4
Bacteria-Freeliving Non-photosynthetic			
Anaerobic	Clostridium		Live in soil; some can live in more acidic conditions than Azotobacter
Aerobic	Azotobacter		Found in soil; pH optimum around 7.0
Facultative anaerobic	Klebsiella, Enterobacter, Bacillus		
Photosynthetic	Rhodospirillum Chromatium		
Bacteria-Associative symbiotic			
Leguminous association	Rhizobium sp.	Legumes	Nodules form on roots
	R. Melilotii	Alfalfa	
	R. trifolii	Clover	
	R. lupini	Lupines	
	R. Japonicum	Soybeans	

Contd.../-

ability of blue-green algae to fix atmospheric dinitrogen was demonstrated by Frank in 1889. Beijerinck in 1901, reported dinitrogen fixation in an aerobic free living soil bacterium *Azotobacter chroococcum*. Later, in 1923 he isolated another facultative anaerobic dinitrogen fixing bacterium namely, *Spirillum lipoferum* from tropical and subtropical soils.

The capability of utilizing atmospheric dinitrogen is confined only to a limited number of organisms. So far, only the prokaryotes some bacteria and blue-green algae, either in free living or in symbiosis with higher plants have been shown to possess this unique property of dinitrogen fixation. Earlier dinitrogen fixation was thought to be the monopoly of symbiotic *Rhizobium*-legumes, a few free living bacteria and blue-green algae. However, it has been now shown that a wide range of ecologically and physiologically different groups of microorganisms can fix dinitrogen. Recently, Postgate (1971) has suggested a specific term 'diazotrophs' for organisms capable of fixing atmospheric dinitrogen. A broad classification of the dinitrogen fixing organisms is given in Table 1.

Biological dinitrogen fixation is one of the most fascinating ecosystem processes involving the reduction of dinitrogen to ammonia by dinitrogen fixing microorganisms. The fixation of dinitrogen is a reductive process catalyzed by the enzyme nitrogenase and is accompanied by the utilization of energy obtained from ATP generated by the oxidation

1	2	3	4
Non-leguminous association	Believed to be members of the genus Actino-mycete	Alnus (alder tree) Ceanothus Myrica Casuarina	Nodules form on roots
	Rhizobium sp.	Trema aspe^ra	Nodules form on roots
	Azotobacter paspali	Paspalum notatum (Brazilian tropi-cal grass)	Colonies form below mucilagenous sheath on root
	Spirillum lipoferum	Digitaria (C_4-grass) Maize Sorghum Pearl millet	Location undefined, could live either around or in root
Blue-green algae			
Free living	Anabaena sp. Nostoc sp.		Alga found in streams, ponds, lakes
Associative symbiotic	Anabaena azolle Nostoc punctifo-rme	Azolla (waterfern) Gunnera macrophylla (tropical herb)	Alga lives in pockets of leaves Stem nodules form at the basis of leaves

From Skinner (1976).

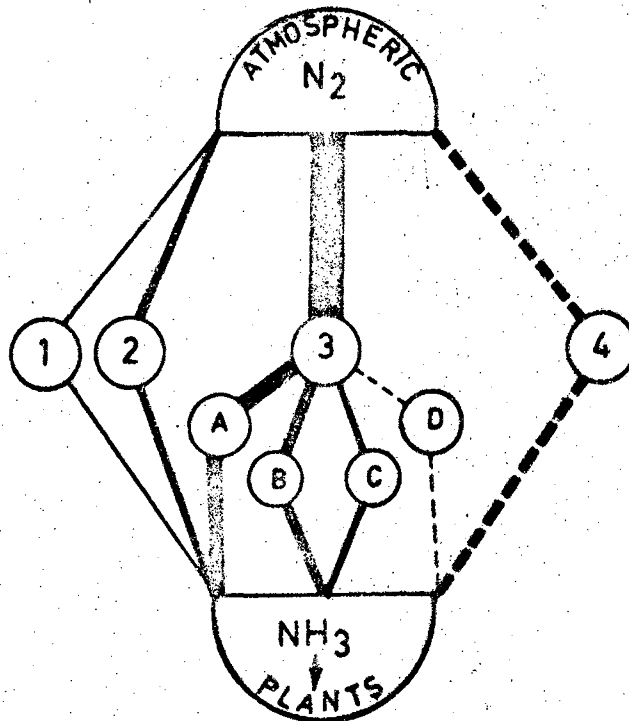
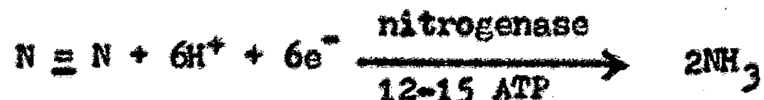


Fig. 1: Various Pathways of Atm N_2 Fixation

1. Lightning Fixation (4 %)
2. Combustion (7 %)
3. Biological Fixation (69 %)
   A. Free Living Microorganisms (48 %)
   B. Non-Legume associations (32 %)
   C. Legume Symbioses (20 %)
   D. Nif^+ Gene Transfer (?)
4. Industrial Fixation (20 %)

of carbohydrates. A generalized equation for biological dinitrogen fixation can be represented as follows:



$$N \equiv N + 6H^{+} + 6e^{-} \xrightarrow[12\text{-}15\ ATP]{nitrogenase} 2NH_3$$

Biological dinitrogen fixation amounts to about 69 per cent (i.e., 175 x 10^6 metric tons yr^{-1}) of the total nitrogen fixed in the biosphere which is approximately 255 x 10^6 metric tons yr^{-1}(Skinner, 1976). In addition to biological dinitrogen fixation, there are two more ways of fixing atmospheric dinitrogen in the nature namely, 1. industrial fixation, and 2. lightning discharge as shown in Fig. 1.

Apart from biological dinitrogen fixation, industrial fixation by the Haber-Bosch process developed in 1914 is equally important. A mixture of dinitrogen and hydrogen in the ratio 1 : 3 is passed under 500 C and 200 atm over finely divided iron as a catalyst and molybdenum as a promotor. The industrial fixation of dinitrogen amounts to about 20 per cent (i.e., 50 x 10^6 metric tons yr^{-1}) of the total dinitrogen fixed in the nature. The manufacture of nitrogen fertilizers by industrial fixation is a high energy consuming process since the conversion of one mole of dinitrogen to ammonia requires 147 kcal of energy. It is difficult to be dependent on industrial fixation solely due to the soaring cost of petroleum and other energy resources. This has given a new impetus to exploit the

Table 2. Estimates of dinitrogen fixed in the nature through various processes.

Type of fixation	Dinitrogen fixed (metric tons x 10^6 yr^{-1})
Biological fixation	
Agricultural	35
Legumes	35
Non-legumes	
Rice	4
Other	5
Permanent meadows	45
Forest and woodland	40
Unused land	10
Total land	139
Sea	36
Total biological	175
Non-biological fixation	
Lightning	10
Combustion	20
Industrial	
Fertilizer	40
Industrial uses	10
Total non-biological	80
Total dinitrogen fixed	255

From Skinner (1976).

natural mechanism of biological dinitrogen fixation to meet the requirement of fixed nitrogen for agricultural production.

Another means of dinitrogen fixation in the nature is through the lightning discharge. The oxides of nitrogen formed in the atmosphere are washed down with rain water in the form of dilute nitrous and nitric acids. The gain of nitrogen through this process amounts to about 4 per cent (i.e., 10×10^6 metric tons yr^{-1}) of the total dinitrogen fixed in the nature. The amount of dinitrogen fixation through lightning discharge cannot be augmented, as a suitable technology for this purpose is yet to be developed. The latest estimates of the dinitrogen fixed in nature through various processes are given in Table 2.

By the end of twentieth century, the world's population seems to increase from 4 billion to 7 billion thus the demand for food will be approximately double. Therefore, it naturally requires heavy inputs of nitrogen fertilizers whose consumption has increased eight-times, i.e., from 5.2 million tons to 40 million tons between 1954 to 1974.

In India, the production of nitrogen fertilizers has increased eight-times, i.e., from 1.5×10^4 metric tons to 12.5×10^4 metric tons between 1961 to 1977. The increasing demand for nitrogen fertilizers and the impending energy crisis has led to a steep rise in the prices of nitrogen fertilizers throughout the world and has created a serious

difficulty in increasing agricultural production. Increasing use of nitrogen fertilizers not only adds to economic costs but also causes serious problems of water pollution. According to Child (1976) only 50 per cent of the nitrogen fertilizers applied in the field is utilized by the crops, while the remainder is lost either with the run off waters or percolates down into the soil causing pollution problems of subsoil water sources. The growing awareness of environmental quality and scarcity of non-renewable resources may aggravate the problem of production of nitrogen fertilizers. Thus, the increasing attention is being paid to the process of biological dinitrogen fixation to meet the growing demand of fixed nitrogen.

Till recently, the interest in biological dinitrogen fixation was largely confined to leguminous plants because of their obvious agronomic importance. The study of non-leguminous flowering plants from the view point of dinitrogen fixation remained neglected for a prolonged period on account of many reasons. One of the reasons has been the general lack of interest and knowledge of microbiologists and biochemists about the non-legume flowering plants. Another important factor has been the absence of a suitable and sensitive technique for evaluating dinitrogen fixation. The newly developed ^{15}N-technique (Burris & Wilson, 1957) and acetylene reduction assay (Dilworth, 1966) have greatly facilitated the studies

of dinitrogen fixation in non-legume flowering plants.

The first report on a non-legume dinitrogen fixing flowering plant, Elaeagnus angustifolia was made by Nobbe in 1892. Before IBP only 13 root nodulated non-legume flowering plants were known to fix dinitrogen. The IBP under its sub-section 'Production Processes of nitrogen' (PP-N) has provided a major stimulus to the study of biological dinitrogen fixation in non-legume flowering plants. Systematic surveys under the IBP were organized with the following three objectives : 1. to secure full information on the regularity of nodulation in the field of species already known to bear nodules, 2. to investigate all the species of those genera known to bear nodules and 3. to encourage a search for the presence of nodules in genera not previously known to be included in the list of nodule bearing genera, particularly of Rhamnaceae, a family of about 60 genera of which two namely, Ceanothus and Discaria are known to bear nodules and the family Rosaceae comprising of 100 genera of which only three, Cercocarpus, Dryas and Purshia have been known to develop nodules.

One of the recent developments in the field of biological dinitrogen fixation, is the discovery of an interesting and simple symbiotic system of tropical C_4-grasses in loose association with soil bacteria which have been shown to fix atmospheric dinitrogen (Döbereiner et al.,

1972). The dinitrogen fixing bacteria develop a loose association with the roots of C_4-grasses described as 'associative symbiosis'.

Döbereiner _et al._ (1972) were the first to demonstrate the dinitrogen fixation by _Paspalum notatum_ in loose association with the _Azotobacter paspali_ bacteria. The bacteria have been found to form a membrane-like structure around the roots. However, An symbiosis between _Digitaria decumbens_ and _Spirillum lipoferum_ Beijerinck the bacteria have been found in the intercellular spaces of the inner cells of root cortex (Dart & Day, 1975). It has been reported that the nitrogenase activity in _Digitaria-Spirillum_ and corn-_Spirillum_ associations is as high as 1 kg N ha^{-1} day^{-1} and 2 kg N ha^{-1} day^{-1} respectively (Döbereiner _at al._, 1975). The dinitrogen fixation has also been shown in rice in association with _Beijerinckia_ and some aquatic plants and weeds roots associated with unidentified dinitrogen fixing microorganisms (Postgate, 1974).

A new potential area for increasing biological dinitrogen fixation has been thrown open by the discovery of dinitrogen fixation in tropical C_4-grasses (Döbereiner _at al._, 1972), and some aquatic macrophytes (Patriquin & Knowles, 1972; Bristow, 1974). However, these grasses-bacterial systems have been reported as efficient in dinitrogen fixation as legumes but the reports are available only from a few tropical regions of the world, i.e., Brazil, Nigeria and Ivory Coast.

These findings of associative symbiotic dinitrogen fixing activity in grasses-bacterial systems have not received much support from other parts of the world. Investigations on dinitrogen fixation in grasses and their importance to the nitrogen economy of tropical and subtropical regions of Asia require a careful study in view of their likely potential of increasing biological productivity.

The Rhizobium-nonlegume, Trema aspera (Trinick, 1973) symbiosis has paved the way for the possibilities of extending the symbiosis between Rhizobium and non-legume agronomically important crops. The fixation of dinitrogen has been shown by cowpea Rhizobia strains with the cell culture of non-legumes namely, wheat, rape grass, brome grass and tobacco (Child, 1975; Schoeraft & Gibson, 1975). The success has been achieved even in persuading different Rhizobial strains of cowpea, soybean and pea to fix dinitrogen in the pure culture media in complete absence of plant cells (Kurz & LaRue, 1975; McComb et al., 1975; Tjepkema & Evans, 1975). However, these achievements have not been extended well and supported much so their practical implications in the near future seem to be uncertain.

Another most interesting and sophisticated means of biological dinitrogen fixation seems to be through the transfer of nif^{+} genes. The structural and regulatory genes determining the synthesis of enzyme nitrogenase

catalysing the fixation of dinitrogen are referred to as *nif*$^{+}$ genes. However, the transfer of *nif*$^{+}$ genes has been successfully carried out in lower organisms, i.e.,from a dinitrogen fixing bacterium, *Klebsiella pneumoniae* to *Escherichia coli* (Dixon & Postgate, 1972) but no such transfer of *nif*$^{+}$ genes to higher plant has been possible so far. Dunican and Tierney (1974) have obtained functional *nif*$^{+}$ genes from *Rhizobium trifolii* to *Klebsiella aerogenes*. The possibilities of successfully intergeneric transfer of *nif*$^{+}$genes from dinitrogen fixing prokaryotes to eukaryotes may provoke the hope for introducing dinitrogen fixing activity in cereal crops, which are heavily reliant upon nitrogen fertilizers. However, the implication regarding *nif*$^{+}$genes transfer to eukaryotes is still unclear and does not seem certain that this would be feasible in the near future.

In this review an attempt has been made to present an uptodate information on the biology and ecology of dinitrogen fixation with particular reference to non-leguminous flowering plants.

Table 3. Methods of measuring biological dinitrogen fixation and their underlying strategies.

Sr. No.	Method	Strategy
1.	Kjeldahl method	Estimation of total nitrogen
2.	^{15}N-technique	Estimation of ^{15}N-enrichment
3.	Acetylene reduction assay	Reduction of alternative substrate, i.e., C_2H_2 by the enzyme nitrogenase

Chapter 2

Methods of Measuring Dinitrogen Fixation

The study of biological dinitrogen fixation has been greatly influenced by the improvements in the methodology of biological dinitrogen fixation. A review of the historical developments amply demonstrates the importance of improving the techniques of quantitative estimation of biological dinitrogen fixation. Early in 1883, only the Kjeldahl method was known to estimate nitrogen enrichment. Since then a number of important developments have taken place in the methodology of estimating biological dinitrogen fixation. Three methods are commonly used for measuring dinitrogen fixation based on different strategies as given in Table 3.

Kjeldahl method

This method was introduced by J. Kjeldahl in 1883 for estimating total nitrogen in the organic materials. The plant material is digested with concentrated sulphuric acid for reducing combined nitrogen to ammonia which is subsequently fixed as ammonium sulphate. Later, ammonia is evolved by distilling with concentrated solution of sodium hydroxide and finally absorbed into a known volume of standard hydrochloric acid. The unused acid is estimated titrimetrically using standard sodium hydroxide solution.

Winkler in 1913, modified the Kjeldahl method by replacing standard hydrochloric acid with a standard solution of boric acid to absorb ammonia liberated from the distillation of

digested material. The ammonia may then be titrated directly with standard hydrochloric acid because boric acid is too weak to affect the pH to an appreciable extent during the titration. The advantages of this modification are that it needs only one standard solution namely, hydrochloric acid, it saves time, and the boric acid needs to be measured approximately. However, care must be taken that the receiver of the distillate be kept cool during the distillation, for ammonium borate is somewhat volatile.

The Kjeldahl method in spite of being the oldest is still widely used for estimating total nitrogen in organic materials, due to its simplicity and cheapness. It, however, suffers from a number of drawbacks. For example, it is not possible to distinguish between the absorption of combined nitrogen from the substratum and the dinitrogen fixed by the plant. A relatively large amount of material is required and it usually takes long time to accumulate. Nitrogen enrichment below one per cent cannot be satisfactorily detected by this method. The sulphuric acid digestion does not reduce all combined nitrogen into ammonia and thus a small but varying amount of nitrogen goes unestimated.

^{15}N-technique

The isotope technology applied to the studies of biological dinitrogen fixation in 1957 has greatly improved the method of estimating biological dinitrogen fixation. The

^{15}N-technique as it is now popularly known was developed by Burris and Wilson in 1957. It is the most reliable and sensitive technique available for estimating biological dinitrogen fixation. The use of $^{15}N_2$ for studying animal metabolism by Urey et al. in 1930 indicated the possibility extending the use of $^{15}N_2$ for studying biological dinitrogen fixation. Burris and Miller (1941) found that the enzyme nitrogenase cannot distinguish between the $^{15}N_2$ and $^{14}N_2$ isotopes and nor it can catalyze mutual exchange between the two isotopes. Burris and Wilson (1957) took advantage of this discovery and developed the ^{15}N-technique for determining biological dinitrogen fixation which basically involves the following seven steps:

1. Preparation of $^{15}N_2$ gas.
2. Incubation of the plant material in $^{15}N_2$ enriched in N_2.
3. Conversion of fixed nitrogen into ammonia using Kjeldahl digestion method.
4. Collection of ammonia by distilling the digested mixture.
5. Oxidation of ammonia to dinitrogen.
6. Mass spectrometric analysis of $^{15}N : ^{14}N$ isotope ratio, and
7. Calculation of excess as atom per cent ^{15}N.

The ^{15}N-incorporated by a dinitrogen fixer is detected by mass spectrometer as an increase in the abundance of masses

28 and 30 above the background value of 0.37 atom per cent and the results are expressed as excess ^{15}N atom per cent. Burris and Wilson (1957) established that a sample giving an atom per cent ^{15}N excess by 0.015 could be assessed positively the evidence for biological dinitrogen fixation. $^{15}N_2$ can be prepared in the laboratory by reacting $^{15}NH_4NO_3$ with sodium hydroxide solution (Burris & Wilson, 1957) or can be purchased commercially. The $^{15}NH_3$ evolved is passed over the hot copper oxide in a gas-tight system which oxidizes $^{15}NH_3$ to $^{15}N_2$. To obtain pure gas it is necessary to pass the gas through alkaline potassium permangnate solution. It is advisable to pass the $^{15}N_2$ through hydrochloric acid to remove any remaining traces of ammonia.

The plant material is incubated in a gas-tight chamber under $p^{15}N_2$ of 0.1-0.3 atm enriched in N_2 which is normally sufficient to saturate the enzyme nitrogenase. A sample of the gas mixture is withdrawn from the incubation chamber with a gas-tight hypodermic syringe and injected into a mass spectrometer to determine the per cent of $^{15}N_2$ in the gas mixture. After incubation for a desirable period of time the system is inactivated by injecting about 0.5 ml of 5 N-sulphuric acid. The plant material is digested following Kjeldahl method and ammonia is liberated by adding sodium hydroxide solution. An aliquot of the liberated ammonia is analyzed by Chaykins method (1969) to determine the total nitrogen in the sample. The colour response is linear from

0.02 to 1.40 μ moles of ammonia. The remainder of ammonia is concentrated and oxidized to dinitrogen with alkaline hypobromite solution (Burris & Wilson, 1957) which is then transferred into the mass spectrometer for the determination of $^{14}N/^{15}N$ ratios.

By determining the total nitrogen and ^{15}N in the sample the amount of ^{15}N-fixed can be calculated and expressed as atom per cent ^{15}N excess, using the following relation:

$$\text{Atom per cent } ^{15}N \text{ excess} = \frac{100}{2R + 1}$$

$$R = {}^{14}N^{14}N / {}^{14}N^{15}N$$

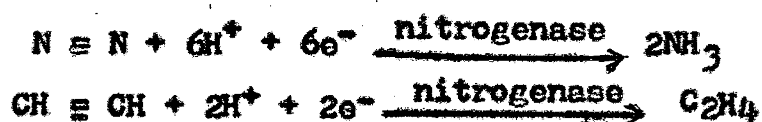
where R is the ratio of intensities of the ionic currents corresponding to ^{14}N and ^{15}N atoms. Mass spectrometer can assess the positive evidence for biological dinitrogen by measuring as low as 0.003 atom per cent ^{15}N excess. The ^{15}N-technique is 10^3-times more sensitive than the Kjeldahl method (Burris & Wilson, 1957). This technique has made a great stride in the field of biological dinitrogen fixation by discovering a large number of non-leguminous flowering plants as efficient dinitrogen fixers which could have never been detected with the Kjeldahl method. However, the application of the ^{15}N-technique is limited as it requires extensive chemical manipulations, highly sophisticated mass spectrometer, and expensive $^{15}N_2$ gas.

Recently, the ^{15}N-technique has been improved by utilizing the optical emission spectroscopy in place of mass

spectrometer for analysing $^{14}N/^{15}N$ ratios by using relatively much $^{15}N_2$ during the plant exposure to N_2 gas. It is more sensitive over the mass spectrometer and can measure smaller samples upto 1 μg of ^{15}N-enrichment (Porksch, 1972).

Acetylene reduction assay

In 1966, an important discovery was made that the enzyme nitrogenase could also reduce substances other than dinitrogen. Earlier in 1965, R.W.F. Hardy had discussed with H.J. Evans and W. Silver, the possibilities of using cyanide (-CN) reduction as a quantitative measure for estimating the nitrogenase activity. Dilworth (1966) for the first time detected ethylene as the reduction product of acetylene and later Schöllhorn and Burris (1967) established the acetylene inhibition of dinitrogen fixation in cell free extracts of *Clostridium pasteurianum*. This unique characteristic of the enzyme nitrogenase has been the basis of developing the acetylene reduction assay which is now widely employed for estimating biological dinitrogen fixation. The qualitative and quantitative estimation of ethylene has been greatly facilitated through the use of a gas chromatograph fitted with a hydrogen flame ionization detector (FID). Rigorous studies made by Dilworth (1966) has shown that the reduction of acetylene to ethylene is analogous to the reduction of dinitrogen to ammonia, as



$$N \equiv N + 6H^+ + 6e^- \xrightarrow{\text{nitrogenase}} 2NH_3$$

$$CH \equiv CH + 2H^+ + 2e^- \xrightarrow{\text{nitrogenase}} C_2H_4$$

Stoichiometry of this reaction clearly shows a quantitative relationship between acetylene reduction and dinitrogen fixation. These studies have shown that acetylene reduction assay could be successfully used for estimating biological dinitrogen fixation (Hardy et al., 1968). Upto 1 μμ moles of C_2H_4 can be successfully detected with the help of a gas chromatograph. All types of dinitrogen fixing systems can be evaluated for their dinitrogen fixing activity without any major difficulty.

The following characteristics of the acetylene reduction assay qualify it for quantitative determination of nitrogenase activity:

1. Ethylene has been identified as the only product of nitrogenase catalyzed acetylene reduction by using mass spectrometer and gas chromatograph.
2. Small concentrations of ethylene do not inhibit the nitrogenase activity and also it does not undergo any further reduction (Dilworth, 1966).
3. Acetylene-ethylene reduction by nitrogenase is same both *in vitro* and *in vivo*.
4. All nitrogenase preparations from different dinitrogen fixers reduce acetylene to ethylene (Bergersen, 1970).
5. Nitrogenase requires a strong reductant, and ATP as energy source.
6. N_2/C_2H_4 conversion factor -- a theoretical conversion factor of three is used as two electrons are required

for acetylene-ethylene reduction whereas six electrons are required for the reduction of dinitrogen to ammonia.

7. Michaelis-Menten constant (Km) - the Km of acetylene for nitrogenase varies from 0.01 to 0.04 atm C_2H_2 in comparison with Km of N_2 varying from 0.1 to 0.2 atm N_2.

8. The nitrogenase activity is limited by reductant and ATP hydrolysis coupled with e^{-*} (activated electron) transfer.

9. The electron transfer from nitrogenase is not influenced by the type of reducible substrate.

10. The assay measures the nitrogenase activity but not the concentration of nitrogenase.

Hardy et al. (1968) have outlined the following steps to carry out the acetylene reduction assay as shown in Fig. 2.

1. Sample preparation : The soil samples containing roots or dinitrogen fixing plants are taken with a great care to avoid distrubances which may not influence the dinitrogen fixing activity of the system (Hardy et al., 1968; Paul et al., 1971). The roots are sampled following excavasion as nodulated roots or excised nodules (Schwinghammer et al., 1970; Sprent, 1969, 1971). The nodulated roots are generally more active than the excised nodules. The nodulated roots or root soil cores are immediately kept in sealed glass bottles used as assay chambers.

In case of C_4-grasses roots are assayed for their nitrogenase activity by preincubating overnight at a low

pO_2 of 0.02 atm in a sealed assay chamber. Most commonly, disposable hypodermic plastic syringes of 50 to 100 ml capacity have been used for assaying root soil cores and nodulated roots (Hardy _et al._, 1968; van Straten & Schmidt, 1975). Several other assay chambers have also been used such as serum bottles or vials (Minchin & Pate, 1975), plastic bags (Burris, 1974) and Mason jars (Hardy _et al._, 1973; Streeter, 1974).

2. Gas exchange phase : The assay chamber is evacuated with a vacuum pump and filled with argon at 0.65 atm, C_2H_2 0.15 atm, O_2 0.20 atm and CO_2 0.04 atm through serum stopper with a gas-tight syringe. However, in recent studies the gas exchange phase has been replaced by direct injecting C_2H_2 at 0.15 atm equal to the volume of air drawn from the assay chamber, because of the non-competitive inhibition of dinitrogen fixation and much lower Km of C_2H_2 for nitrogenase than that of dinitrogen (Akkermans, 1971; Sprent, 1971; Hwang _et al._, 1973).

3. Incubation : The root nodules or root soil cores are incubated in a gas-tight assay chamber for a period of time depending upon the dinitrogen fixing activity of the sample. The time of incubation should not be prolonged because high levels of ethylene may inhibit the reduction of dinitrogen.

4. Termination of assay : The assay is terminated by adding trichloroacetic acid (Stewart, 1970) or 0.5 ml of 5N-sulphuric acid into the assay chamber. Without inactivating

the plant material, the direct gas samples can also be drawn from the assay chamber and can be stored in evacuated bottles.

5. Analysis of C_2H_4 : A gas chromatograph fitted with a column of Porapak-T is used for the separation and quantitative measurement of acetylene and ethylene gases using hydrogen-flame ionization detector. Nitrogen is commonly used as a carrier gas approximately at a constant flow rate of 40 ml min^{-1}. Gas chromatograph is standardrized with ethylene and a standard curve is plotted taking peak heights vs concentration of the ethylene. However, acetylene can also be used as an internal standard for the indirect measurement of ethylene by determining the quantity of acetylene left in the assay chamber (Hardy et al., 1968). The Porapak-T column gives maximum reproducibility over 60-90 C. The amount of ethylene is represented as m moles of C_2H_4 gm^{-1} dry weight min^{-1}.

The acetylene reduction assay is 10^3-times more sensitive over the ^{15}N-technique, because of C_2H_2 reduction to C_2H_4 is accompanied by a single electron pair transfer whereas $^{15}N_2$ reduction to ammonia is three electron pairs transfer. Due to low Km of C_2H_2 (0.01 to 0.04 atm C_2H_2) for nitrogenase than that of N_2 (0.10 to 0.20 atm N_2) 100 per cent of the electrons are transferred from nitrogenase to acetylene but in the case of dinitrogen reduction only 75 per cent are effective. The measurement of ethylene by

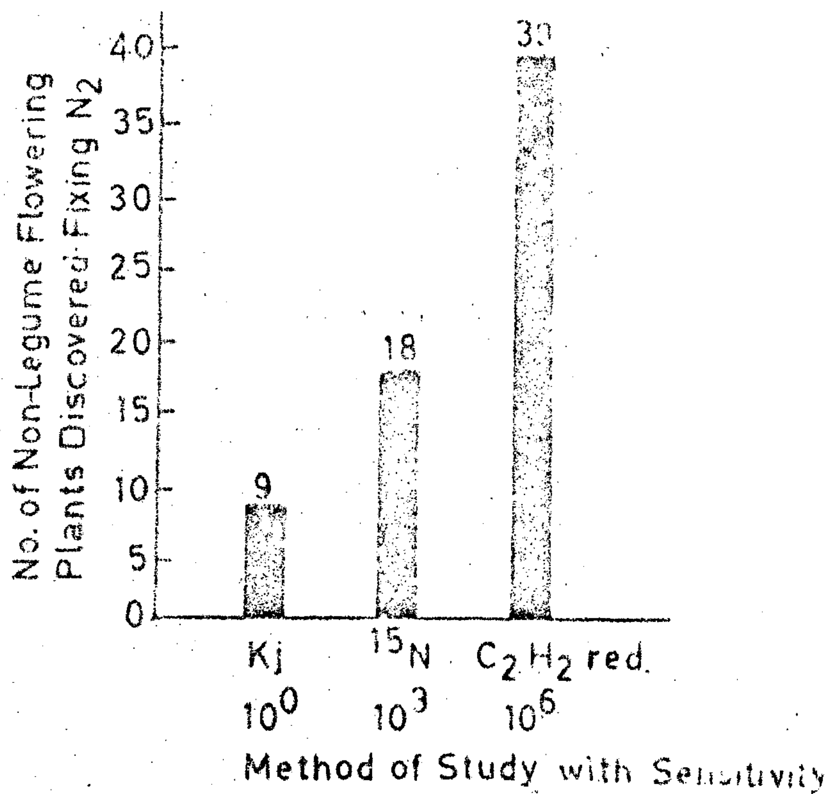


Fig. 3: A relationship between the sensitivity of three methods and the number of non-leguminous flowering plants discovered fixing dinitrogen.

gas chromatograph using the hydrogen flame ionization detector makes it more sensitive which can detect upto 1 $\mu\mu$ moles of ethylene. Furthermore, the solubility of acetylene in water is 80-times more than that of dinitrogen.

The following precautions should be given due consideration when employing acetylene reduction assay for measuring dinitrogen fixing activity:

1. Acetylene is explosive and highly inflammable in nature.
2. Acetylene-ethylene reduction assay is an indirect way of measuring biological dinitrogen fixation.
3. Ammonia produced requires certain keto-acids for immediate metabolization into amino acids and amides whereas ethylene does not undergo any metabolism.
4. Presence of ammonia in low concentration represses the nitrogenase activity, but that of ethylene has no effect.
5. Ethylene is a well known plant hormone.
6. Soils rich in organic content may produce ethylene naturally which may introduce an error upto 5 kg N ha^{-1} yr^{-1}.

During the past decade the application of acetylene-ethylene reduction assay has made a great stride to the studies of biological dinitrogen fixation and a large number of non-leguminous flowering plants have been discovered as efficient dinitrogen fixers. A relationship between the sensitivity of three methods and the number of non-leguminous flowering plants discovered fixing dinitrogen is shown in Fig. 3.

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Table 4. An evaluation of the three methods used for the measurement of biological dinitrogen fixation.

Sr. No.	Characteristic	Kjeldahl method	^{15}N-technique	Acetylene reduction assay
1.	Discoverer	J. Kjeldahl (1883)	R.H. Burris & P.W. Wilson (1957)	M.J. Dilworth (1966). R. Schöllhorn & R.H. Burris (1967)
2.	Underlying strategy	Reduction of the total combined nitrogen into NH_3 by digesting the plant material with concentrated sulphuric acid and estimating the NH_3 volumetrically	The enzyme nitrogenase neither differentiates nor catalyzes an exchange between the $^{14}N_2$ and $^{15}N_2$ isotopes of nitrogen.	The enzyme nitrogenase has the capability of reducing surrogate substrates, e.g., C_2H_2
3.	Nature of reaction	Direct, $N_2 \rightarrow 2NH_3$	Direct, $^{15}N_2 \rightarrow {}^{15}NH_3$	Indirect, $C_2H_2 \rightarrow C_2H_4$
4.	Experimental factor	No	0.37 ^{15}N atom per cent blackground value	A conversion factor of three, i.e., $\sum_{0}^{t} \frac{\frac{n \text{ moles of } C_2H_2 \rightarrow C_2H_4}{3}}{\text{mg N g}^{-1} \text{ dry weight}} \times 2$[illegible]

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A summary of the three different methods with their advantages and disadvantages used for the measurement of biological dinitrogen fixation is given in Table 4.

Sr. No.	Characteristic	Kjeldahl method	^{15}N-technique	Acetylene reduction assay
5.	Efficiency	Inefficient, 5-8 samples day^{-1}	Inefficient, 3-5 samples day^{-1}	Highly efficient, about 80 incubations and 160 gas chromatograms day^{-1}
6.	Sensitivity	Insensitive, incapable of measuring increase in nitrogen less than one per cent	10^3-times more sensitive than Kjeldahl method, 0.015 ^{15}N atom per cent excess can be measured successfully	10^3-times more sensitive than ^{15}N-technique and 10^6 times than Kjeldahl method ≥ 1 μμ moles of C_2H_4 can b detected
7.	Stability of product	NH_3 slowly undergoes changes in storage	$^{15}NH_3$ slowly metabolises in storage	C_2H_4 is quite stable and does not undergo any chang in storage
8.	Ease of operation	Simple and easy to handle	Sophisticated and tedious handling	Simple and easy to handle
9.	Application to *in vitro* systems	No	Applicable	Highly applicable
10.	Application in field	No	No	Highly applicable
11.	Economy	Cheap	Very expensive	Inexpensive

Chapter 3

The Nitrogenase

The enzyme nitrogenase catalyzes the reduction of dinitrogen to ammonia (Burris, 1942) through a series of complex reactions. Attempts to extract enzyme nitrogenase were started in 1930 but prior to 1960 all nitrogenase preparations gave poor activity and the reproducibility of the results was unsatisfactory. The main difficulty in obtaining active nitrogenase preparations was due to the lack of knowledge about its: 1. oxygen sensitivity, 2. cold-lability, and 3. high energy requirement in the form of ATP for reducing dinitrogen. After thirty years of continuous efforts Carnahan et al., (1960) were successful in obtaining cell free extract of nitrogenase from Clostridium pasteurianum using vacuum drying process. Since then, nitrogenase has been extracted from about sixteen different dinitrogen fixing organisms. Nevertheless, its precise composition, properties, and mechanism of action are still obscure.

Methods of extraction

1. Vacuum drying process : Carnahan et al. (1960) used the vacuum drying process and successfully obtain the cell free nitrogenase preparations from Clostridium pasteurianum. The cells were vacuum dried at relatively high temperature about 35 C and the dried cells were autolyzed by 0.1 M tris buffer at approximately pH 7.5 which allows nitrogenase to pass out of the cell into the medium because of the changes

in the permeability of cell membrane. This eliminates the need for breaking the cell wall. Usually nitrogenase is soluble and remains in the supernatant. The crude nitrogenase preparations are extremely sensitive to oxygen inactivation. This process could not succeed with other dinitrogen fixers such as *Azotobacter*, *Rhizobium* and *Anabaena* because of the denaturation of their nitrogenases.

2. French Press method: The French Press method was developed by Bulen *et al.* (1965) to obtain active nitrogenase preparations from *Azotobacter vinelandii*. The active nitrogenase preparation was obtained by passing the frozen cells through a French Press. A thick slurry of the cells is passed through a narrow orifice at 10,000 to 20,000 pounds per square inch. A sudden decrease in pressure together with the shearing force of passing through the orific disrupt cell wall releasing the enzyme nitrogenase. The crude nitrogenase preparation obtained by this method is of particulate nature and is also relatively stable towards oxygen inactivation in comparison with that of *Clostridium pasteurianum* obtained by vacuum drying process. The French Press method requires a large amount of material which makes it unsuitable for wide application to various other organisms.

3. Osmotic shock method: Oppenheim *et al.* (1970) employed the property of osmotic pressure to device a new method called "Osmotic shock" which is very convenient

and applicable to a wide range of dinitrogen fixing organisms. They applied this method to *Azotobacter vinelandii* for obtaining crude nitrogenase extract which was found to be similar in properties to that of *Clostridium pasteurianum* obtained by vacuum drying process. Therefore, from the foregoing methods of extraction it has been concluded that crude nitrogenase preparations of *Azotobacter vinelandii* differ in their particulate size and oxygen sensitivity obtained by French Press and Osmotic shock methods. It is, therefore, highly desirable to examine whether the variations observed in the properties of nitrogenase are due to the methodology of extraction or due to the differences in the subcellular organization of nitrogenase obtained from different source organisms.

The active nitrogenase preparation from the actinomycete-like endophyte of non-legume flowering plants such as *Alnus*, *Casuarina*, *Ceanothus* and *Myrica* has not been obtained as yet by any of the available methods. A suitable method is needed to be developed to obtain active nitrogenase preparations from these microorganisms so as to ascertain similarities and differences in the properties of nitrogenase obtained from other microorganisms. Most probably due to the variation in molecular weight, atomic composition and oxygen sensitivity of nitrogenase so far any of the methods of extraction has not been applicable to all the dinitrogen fixing organisms

Table 5. A comparative account of the activity of nitrogenases obtained from various dinitrogen fixers using different methods of extraction.

Sr.No.	Organism	VD	FP	OS	Reference
1.	*Anabaena cylindrica*	-	+	±	Cox *et al.*, 1964
2.	*Azotobacter chroococcum*	-	+	-	Kelly, 1968
3.	*Azotobacter vinelandii*	-	+	+	Bulen *et al.*, 1965
4.	*Chromatium* sp.	±	+	-	Oppenheim *et al.*, 1970
5.	*Clostridium pasteurianum*	+	-	±	Winter & Arnon, 1970 Carnahan *et al.*, 1960
6.	*Klebsiella pneumoniae*	+	-	±	Mahl & Wilson, 1968
7.	*Rhizobium japonicum*	-	+	+	Evans *et al.*, 1970
8.	*Rhizobium rubrum*	-	+	+	Davis & Brill, 1972

VD = Vacuum drying process; FP = French Press method; OS = Osmotic shock method; + = active nitrogenase; ± = less active nitrogenase; - = inactive nitrogenase.

for extracting nitrogenases as shown in Table 5.

Purification of nitrogenase:

Most of the experiments can be performed with the crude extracts of nitrogenase. However, for studying composition, properties and mechanism of action it is necessary to obtain a pure sample of nitrogenase. The procedure of purification of the crude extracts is almost the same irrespective of the source organisms.

All steps in purification, without any exception, are performed under strictly anaerobic conditions. *Azotobacter* nitrogenase can be sedimented by differential centrifugation method because of its particulate nature. Partially purified nitrogenase preparations are usually contaminated with nucleic acids and other inactive proteins, which are treated with protamine sulphate to reduce the contamination. Excessive addition of protamine sulphate should be avoided, but otherwise, it may precipitate nitrogenase as well. After centrifugation the supernatant can be treated with RNAase and DNAase to hydrolyse any remaining nucleic acids. However, the removal of about 50 per cent of the inactive proteins can be achieved by heating the preparations at about 50 to 60 C for 5 to 10 minutes.

The application of polyethylene glycol (PEG) provides another good method for purifying nitrogenase preparations.

Different concentrations of PEG 6000 have been applied to crude nitrogenase preparations of _Clostridium pasteurianum_. The initial treatment with 10 per cent PEG precipitates all the inactive proteins alongwith nucleic acids. Higher PEG concentration of about 30 per cent precipitates active nitrogenase enzyme (Tso _et al._, 1972).

More commonly, the purification of nitrogenase is obtained by liquid chromatography using DEAE-cellulose (diethyl aminoethyl-cellulose) column (Zumft & Mortenson, 1973). After treating the crude extract with 5 per cent protamine sulphate the supernatant is run through a DEAE-cellulose column by differential concentrations of sodium chloride solution. The crude nitrogenase enzyme extract separates into three brown bands on the DEAE-cellulose column when eluted with 0.25 M sodium chloride solution (Mortenson _et al._, 1967).

The components of each band were subjected to atomic absorption spectrophotometer for analysing their metallic constituents. The first band was found to contain Mo and Fe atoms, the second band contained Fe atoms and the third band was also found to contain Fe atoms. Individual components lack the catalytic property, however, it could be restored by combining the components of first and second bands. This shows that the constituents of the third band are not an essential component of nitrogenase. Further

colorimetic analysis revealed it to be ferredoxin on the basis of its characteristic peak absorption spectrum.

In order to achieve a very high purity of nitrogenase Sephadex G200 and G100 columns are employed for individual nitrogenase components. Sephadex G200 is used for MoFe-protein and Sephadex G100 is used for Fe-protein (Mortenson et al., 1967).

Terminology

Investigations following the extraction and purification of nitrogenase soon revealed that it is composed of two protein components named after their metallic constituents and reduction property as "molybdoferredoxin" and 'azoferredoxin' (Mortenson et al., 1967). This terminology has not been accepted widely due to the lack of evidence whether molybdoferredoxin alone could act as a binding site for the substrate reduction. Mortenson et al. (1967) assigned these terms on the basis of their metallic constituents as MoFe-protein and Fe-protein respectively. Hardy et al. (1971) named the MoFe-protein as 'azofermo' and Fe-protein as 'azofer'.

On the basis of priority of elution of the protein components from a DEAE-cellulose column, the MoFe and Fe-proteins have been designated as 'Fraction I' or 'Component I' Fraction 2' or 'Component 2' respectively (Walker & Mortenson, 1973). Sussex group has used abbreviations to convey the information about the protein components and their source organisms. For example, MoFe-protein and Fe-protein components

of nitrogenase obtained from Klebsiella pneumoniae are designated as Kp1 and Kp2 respectively. This terminology is not in common use because the MoFe-protein is incapable of reducing dinitrogen by itself and, therefore, the terminology MoFe-protein and Fe-protein is relatively preferred. Recently in 1976, the enzyme Commission of the International Union of Biochemistry has assigned a systematic name to nitrogenase (nitrogen :acceptor) oxidoreduction, (EC 1.7. 99.2) (Davis & Orme-Johnson, 1976).

Properties of nitrogenase

Enzyme nitrogenase irrespective of its source organism has the same physical and chemical properties, except slight variations. Neither of the two protein components is functional by itself and the properties attributed to the nitrogenase are the collective properties of the two protein components. Important physical and chemical properties of the nitrogenase are described in the following paragraphs.

A. Physical properties:

1. Composition : Enzyme nitrogenase consists of two protein components namely, MoFe-protein and Fe-protein in the ratio of 1 : 1 per nitrogenase molecule. On the basis of differential rate of flow of the two protein components through a DEAE-cellulose column chromatograph, the average molecular weight of the MoFe-protein has been found to vary between 220,000 to 270,000 and that of the Fe-protein from

55000 to 65000, obtained from various source organisms (Mortenson *et al.*, 1967). The MoFe-protein has been reported a tetramer composed of four identical subunits each with a molecular weight ranging from 50000 to 60000. The Fe-protein also has been found to be a dimer which consists of two identical subunits each with a molecular weight varying from 27500 to 32500 (Skinner, 1976).

2. Oxygen sensitivity : Nitrogenase enzyme is very sensitive to O_2-inactivation, especially, the Fe-protein is much more sensitive than the MoFe-protein (Kelly *et al.*, 1967). Most probably nitrogenase protein fractions undergo irreversible oxidation in presence of molecular oxygen (Bulen *et al.*, 1965). The nitrogenase obtained from aerobic dinitrogen fixers is generally less sensitive to O_2-inactivation. The oxygen sensitivity of crude preparations of nitrogenase obtained from different bacteria can be arranged in the following sequence of decreasing order *Clostridium* < *Bacillus* < *Klebsiella* < *Azotobacter*. The activity varies from no loss with *Azotobacter* to 75 per cent loss with *Clostridium* even by exposing the crude nitrogenase extracts to air for about 10 minutes at 20 C (Kelly, 1969). Nitrogenase activity in *Alnus glutinosa* is inhibited within 10 to 15 minutes when plants are exposed to an atmosphere containing 90 per cent oxygen (Skeffington & Stewart, 1976).

3. Cold-lability : The loss of catalytic activity around 0 C appears to be a general property of nitrogenase.

Only the Fe-protein component of nitrogenase is inactivated at low temperatures (Moustafa & Mortenson, 1969). The Fe-protein component of _Klebsiella_ nitrogenase is an exception which is not inactivated even in the pure state (Eady _et al._, 1972). The degree of cold-lability varies with the source organism, method of extraction, and degree of purity. Crude nitrogenase preparations of _Azotobacter_ are cold-stable but purification enhances cold-lability (Bulen _et al._, 1965). An addition of 5 mM magnesium chloride has been reported to protect the purified preparations of _Azotobacter_ at 0 C for a period of one month (Silverstein & Bulen, 1970). _Clostridium pasteurianum_ nitrogenase is completely inactivated within 10 minutes at 0 C (Dua & Burris, 1965). So far, no mechanism has been worked out to explain the cold-lability of nitrogenase.

4. Crystallization : Enzyme nitrogenase has not been crystallized so far from any of the dinitrogen fixers. However, the MoFe-protein component has been successfully crystallised from the bacterium, _Azotobacter vinelandii_ (Burns _et al._, 1970). Although the Fe-protein component has been highly purified from _Azotobacter_ and _Clostridium_ (Moustafa, 1970) but crystallization has not been possible so far due to its excessive cold-lability and O_2-inactivation (Kelly _et al._, 1967).

B. Chemical properties:

1. Acidic nature : Enzyme nitrogenase as well both the protein components are acidic in nature. This acidic property

Table 6. Some properties of protein components of nitrogenase.

	Cp1	Kp1	Av1	Rj1	Cv1	Cp2	Kp2
Mol wt	220000	218000	270000	200000	-	55000	66700
Sub-units	50700	51300	54000		(2 types)		
	59500	59600	60000	50000		27500	34000
Mo/mol	2	2	2	1.3	1.3	1.3	0
Fe/mol	22-24	30-36	32-36	29	17	4	4
S^{2-}/mole	22-24		28	26	14	4	3.8
O_2-sensitivity ($t_{\frac{1}{2}}$ in min)	+	10	+	4.5	+	++	0.75

Cp = *Clostridium pasteurianum*; Kp = *Klebsiella pneumoniae*;
Av = *Azotobacter vinelandii*; Rj = *Rhizobium japonicum*;
Cv = *Chromatium vinosum*; 1 = MoFe-protein; 2 = Fe-protein;

From Postgate (1976).

has been utilized in their purification from crude extracts (Stewart, 1975). The reggents basic in nature such as protamine sulphate and polyethylene glycol are used to precipitate the nitrogenase components from the crude extracts. The acidic nature of both MoFe-protein and Fe-protein also enables their separation on DEAE-cellulose column (Mortenson et al., 1967).

2. Atomic composition : There is a great degree of variation in the atomic composition of nitrogenase from organism to organism. No two identical nitrogenases have been obtained from different source organisms with respect to their Mo atoms, Fe atoms and acid-labile sulphide groups associated with it. On the average MoFe-protein consists of one or two molybdenum atoms, 15-30 iron atoms and 15-30 acid-labile sulphide groups per 200,000 to 300,000 molecular weight (Dalton & Mortenson, 1972). The Fe-protein has also been found to contain about four iron atoms, four acid-labile sulphide groups and no molybdenum atom per 60,000 molecular weight. A brief account of some of the properties of protein components of nitrogenase obtained from different bacteria is given in Table 6.

3. Catalytic activity : The enzyme nitrogenase loses its catalytic activity on fractionation into its protein components. It can, however, be restored on recombination of the two protein components but with a slight variation

Table 7. Cross-reactions of nitrogenase components from bacteria.

	Av1	Ac1	Rj1	Kp1	Rr1	Mf1	Bp1	Cp1	Dd1	Reference
Av2	+		+	+			-	-		Murphy & Koch, 1971
Ac2		+		+	±	±	+			Kelley, 1969
Rj2	+		+							Murphy & Koch, 1971
Kp2	+	+		+	+	+	+	-	+	Kelly, 1969
Rr2					+					Biggins *et al.*, 1971
Mf2		+		+	+	+	+			Biggins *et al.*, 1971
Bp2	+	tr	+	+	+	+	+	±	+	Murphy & Koch, 1971
Cp2	-		-	-	-	-	+	+		Murphy & Koch, 1971

Ac = *Azotobacter chroococcum*
Av = *Azotobacter vinelandii*
Bp = *Bacillus polymyxa*
Cp = *Clostridium pasteurianum*
Dd = *Desulfovibrio desulfuricans*
Kp = *Klebsiella pneumoniae*
Mf = *Mycobacterium flavum*
Rj = *Rhizobium japonicum*
Rr = *Rhodospirillum rubrum*

+ = activity from about 50% to 100%
± = activity over 20% but less than about 50%
tr = trace
- = no complementation

From Dilworth (1974).

from the activity before fractionation (Detroy _et al._, 1968). The catalytic activity of nitrogenase can be restored by combining the two protein fractions irrespective of the source organisms but not to the extent as observed in the original combination of the two protein components in a given dinitrogen fixer (Murphy & Koch, 1971). The incidence of catalytic activity of nitrogenase protein components obtained from different organisms is given as under in Table 7.

4. Alternative substrate : The enzyme nitrogenase has an unusual versatility with respect to its ability to reduce a wide variety of substrates (Dilworth, 1966; Hardy & Knight, 1967). It catalyzes the reduction of N ≡ N, a triple bonded dinitrogen molecule. As early as in 1965, Hardy _et al._ have pointed out the possibility of reduction of a triple bonded compound other than dinitrogen. For the first time they observed the reduction of cyanide (-C ≡ N) to methane and ammonia. Later in 1966, Dilworth observed the reduction of acetylene to ethylene. Schöllhorn and Burris (1967) established the acetylene inhibition of dinitrogen fixation in cell free nitrogenase extracts obtained from _Clostridium pasteurianum._ In addition to cyanide and acetylene, a wide range of alternative substrates has been reported namely, isocyanides, nitriles, isonitriles, azide, nitrogen oxide and hydrogen ions (Hardy _et al._, 1971)

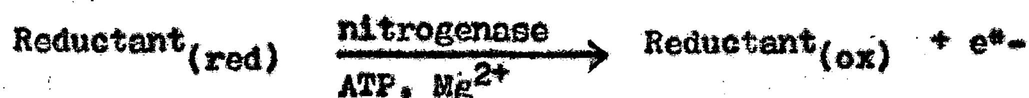
Table 8. A list of alternative substrates reduced by the enzyme nitrogenase.

Sr. No.	Substrate	Chemical formula	Product	Km (mM)	Reference
1.	Acetylene	C_2H_2	C_2H_4	0.1-0.3	Dilworth, 1966
2.	Azide	N_3^-	NH_3+N_2	0.2-1.3	Hardy & Knight, 1967
3.	Cyanide	CN^-	CH_4+NH_3	0.4-4.0	Hardy & Knight, 1967
4.	Hydrogen ion	H^+	H_2	0.04-0.4	Koch *et al.*, 1967
5.	Methyl isocyanide	CH_3NC	CH_3NH_2+ CH_4	0.2-1.0	Hardy & Knight, 1967
6.	Nitrogen	N_2	$2NH_3$	0.03-0.1	Koch *et al.*, 1967
7.	Nitrogen oxide	N_2O	N_2+H_2O	1.2	Hardy & Knight, 1967

A list of alternative substrates for nitrogenase alongwith their Km values and end products is given in Table 8.

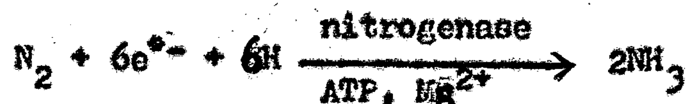
Mechanism of action

Since 1960, when the crude nitrogenase preparation was obtained from Clostridium pasteurianum (Carnahan et al., 1960), a substantial advancement has been made in understanding the mechanism of action of nitrogenase. A critical examination of nitrogenase catalyzed reaction shows that it performs two distinct functions. The first involves the transfer of electrons from a strong reductant to nitrogenase, as



$$\text{Reductant}_{(red)} \xrightarrow[\text{ATP, } Mg^{2+}]{\text{nitrogenase}} \text{Reductant}_{(ox)} + e^{-}$$

and the second step provides the active site for the reduction of substrate, i.e., N_2 or C_2H_2 and transfer of electrons to it for its reduction, as



$$N_2 + 6e^{-} + 6H \xrightarrow[\text{ATP, } Mg^{2+}]{\text{nitrogenase}} 2NH_3$$

It is not clear whether the substrate reduction is accomplished by a complex of two proteins or the MoFe-protein once reduced is capable of affecting the substrate reduction. It has been shown that nitrogenase requires a strong reductant, i.e., ferredoxin or flavodoxin which could be replaced in vitro by a non-physiological reductant, sodium dithionite ($Na_2S_2O_4$). Progress in understanding the mechanism of action of nitrogenase has been made by applying Mössbauer spectroscopy and EPR spectroscopy for studying the protein components

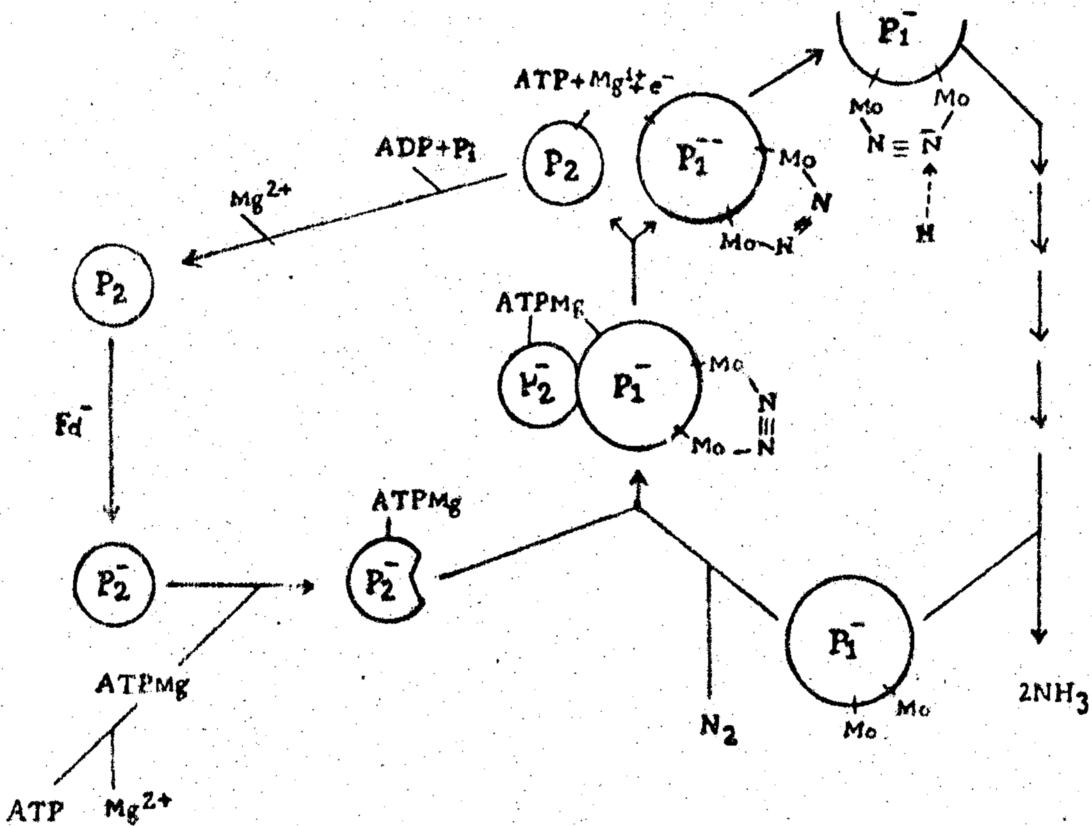


Fig. 3. A MECHANISM FOR NITROGENASE FUNCTION From Postgate (1976).

of nitrogenase (Orme-Johnson et al., 1972; Zumft & Mortenson, 1975). The addition of Mg-ATP to the Fe-protein, confers a conformational change in the Fe-protein since the EPR spectrum changes from rhombic to axial symmetry. This furnishes an evidence for specific binding of Mg-ATP to the Fe-protein, as no comparable shift in the EPR spectrum occurs when Mg-ATP is added to the MoFe-protein (Winter & Burris, 1976). This specific binding of Mg-ATP results in a substantial decrease in redox-potential of Fe-protein (Zumft et al., 1974). The high negative potential or low redox-potential confers on the Fe-protein a unique characteristic of reducing the MoFe-protein. The MoFe-protein generally remains in a partially reduced state as supported by the spectra of partially reduced and completely reduced states at the time of electron transfer (Winter & Burris, 1976). A most probable mechanism of nitrogenase action is given in Fig. 3.

Initially the oxidized dimer Fe-protein denoted as P_2 is reduced to $P_2^{=}$ by a strong electron donor, sodium dithionite or ferredoxin (Mortenson, 1964) which later reacts with Mg-ATP to form a $P_2^{=}$-Mg-ATP complex of low redox-potential accomplished by a conformational change (Zumft & Mortenson, 1973). This $P_2^{=}$-Mg-ATP combines with partially reduced tetramer MoFe-protein denoted as $P_1^{=}$. The binding of dinitrogen takes place at the transition Mo atoms of $P_1^{=}$.

There have been many suggestions indicating that electrons are transferred from Mo of the MoFe-protein to the various reducible substrates. However, experimental evidence has shown that it is much more likely that electron transfer takes place between Fe atom of the MoFe-protein and the substrate to be reduced. The binding of CO takes place especially with the MoFe-protein and blocks the electron transfer and this binding of CO is with Fe atom of the MoFe-protein rather than Mo atom (Winter & Burris, 1976). In the complex Mg-ATP-P_2^- -P_1^- $(Mo)_2N_2$ the electron transfer occurs from P_2^- to P_1^- giving fully reduced P_1^{--} species. The oxidized P_2 releases ADP + Mg^{2+} and again reduced by ferredoxin and reacted by Mg-ATP to form P_2^--Mg-ATP complex (Thorneley, 1975). The electron transfer continues repeatedly consuming two ATP per electron transfer until the stoichiometry of the end product formation is completed, i.e., 6 electrons for N_2, and only after that the end product dissociates from P_1 leaving the partially reduced P_1^- . Thus the substrate remains bound to a single molecule of P_1^- until the end product is formed and, therefore, no free intermediate dinitrogen complex is detected (Postgate, 1976).

Chapter 4

Biochemistry of Dinitrogen Fixation

The biochemistry of dinitrogen fixation is one of the most important areas of investigation. It involves a myriad of biophysicochemical reactions catalyzed by the enzyme nitrogenase. Our knowledge of the biochemistry of dinitrogen fixation is mainly based on the studies of a limited number of dinitrogen fixers namely, *Clostridium*, *Azotobacter*, *Rhizobium*, *Klebsiella*, *Rhodospirillum*, *Nostoc* and *Anabaena*. Burris (1942) was the first to make a concerted effort by employing a heavy isotope of nitrogen, $^{15}N_2$, for studying the biochemistry of dinitrogen fixation. However, a substantial advancement has been made in elucidating this process, but the understanding is still far from complete.

The biological dinitrogen fixation is a reductive process requiring two different energy sources namely, 1. a strong reductant as electron donor, and 2. ATP molecules. In addition, it also involves electron carriers, some intermediate dinitrogen complexes, and evolution of molecular hydrogen as a side reaction to the process of biological dinitrogen fixation. Each of these aspects is discussed below:

Electron donors

The fixation of dinitrogen requires a continuous supply of electrons obtained through the breakdown of strong reducing substrates. The origin and nature of electron donors vary

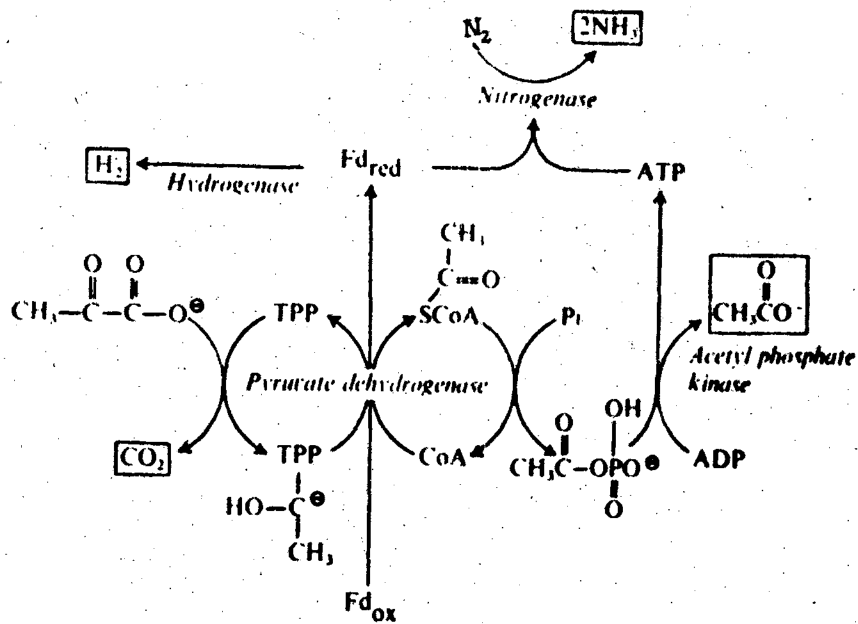


Fig. 4: The phosphoroclastic reaction in nitrogen fixation in Clostridia. Fd indicates Ferredoxin. From Benemann & Valentine (1972).

among different physiological and ecological groups of dinitrogen fixers (Carnahan *et al.*, 1960; Benemann & Valentine, 1972). *In vivo*, studies have shown that different groups of dinitrogen fixing organisms utilize different types of electron donors (Streicher & Valentine, 1973). However, *in vitro* studies have shown that sodium dithionite ($Na_2S_2O_4$) a non-physiological inorganic compound can act as a strong electron donor in cell free extracts of different dinitrogen fixing organisms (Bulen *et al.*, 1965). In spite of their diverse mode of synthesis through different metabolic pathways in different groups of microbes, all electron donors are potentially strong reducing agents which are able to reduce the low redox-potential electron carriers namely, ferredoxins and flavodoxins.

The anaerobic dinitrogen fixing *Clostridia*, most commonly utilize pyruvate as an electron donor generated from the fermentation of sugars (Carnahan *et al.*, 1960). The phosphoroclastic (substrate-level phosphorylation) breakdown of pyruvate reduces low redox-potential ferredoxin (Mortenson *et al.*, 1963) which in turn, reduces the enzyme nitrogenase. The reduced nitrogenase finally reduces dinitrogen to ammonia accompanied by the hydrolysis of ATP generated by the dephosphorylation of acetyl-phosphate according to the reaction scheme given in Fig. 4. In addition to pyruvate, formate and molecular hydrogen have also been reported as electron donors in *Clostridium pasteurianum*

(Mortenson, 1966), Klebsiella pneumoniae (Benemann & Valentine, 1972) and Bacillus polymyxa (Fisher & Wilson, 1970).

In Azotobacter vinelandii, and symbiotic Rhizobium-legume the electron donor is $NADPH_2$ generated during the Kreb cycle (Streicher & Valentine, 1976). The utilization of $NADHH_2$ as a potential electron donor has also been reported in the cell free extracts of Clostridia (D'Eustachio & Hardy, 1964) and Anabaena cylindrica (Smith et al., 1971). The discovery of $NADPH_2$ driven dinitrogen fixation in extracts of various physiologically different groups of dinitrogen fixers has led to the conclusion that $NADPH_2$ might be among the most important electron donors linking cellular reducing power to nitrogenase (Streicher & Valentine, 1973).

In photosynthetic bacteria the nature of electron donors supporting the fixation of dinitrogen is still unknown. However, Yoch and Arnon (1970) illuminated the chromatophore fragments of Chromatium in the presence of ferredoxin which provided a powerful reductant. Later, Evans and Smith (1971) supported them by using another photosynthetic bacterium, Chloropseudomonas ethylicum.

The actinomycete-like endophyte in non-legume woody plants is believed to utilize $NADPH_2$ produced by metabolizing isocitrate, glucose-6-phosphate and malate (Winter & Burris,

Table 9. A list of electron donors utilized by different groups of dinitrogen fixing organisms.

Sr. No.	N_2-fixing organism	Habit	Physiology	Electron donor	Reference
1.	Actinomycete	Symbiotic	Aerobe and non-photosynthetic	$NADPH_2$	Winter & Burris, 1976
2.	Anabaena cylindrica	Free living	Photosynthetic	$Na_2S_2O_4$, pyruvate and $NADPH_2$	Bulen et al., 1965 Smith et al., 1971
3.	Azotobacter vinelandii	Free living and associative symbiotic	Aerobe and saprophyte	$NADPH_2$	Benemann et al., 1971
4.	Bacillus polymyxa	Free living	Facultative anaerobe and saprophyte	Formate, H_2 and pyruvate	Fisher & Wilson, 1970
5.	Clostridium pasteurianum	Free living	Anaerobe and saprophyte	Pyruvate, formate, H_2 and $NADPH_2$	Carnahan et al., 1960 Mortenson, 1966
6.	Chromatium	Free living	Photosynthetic	Pyruvate and chromatophore?	Bennett et al., 1964 Yoch & Arnon, 1970
7.	Klebsiella pneumoniae	Free living and associative symbiotic	Facultative anaerobe and saprophyte	Pyruvate formate and H_2	Fisher & Wilson, 1970 Benemann & Valentine, 1970
8.	Chloropseudomonas ethylicum	Free living	Photosynthetic	Chromatophre?	Evans & Smith, 1971.
9.	Rizobium sp.	Symbiotic	Aerobe and non-photosynthetic	$NADPH_2$	Benemann et al., 1971
10.	Spirillum lipoferum	Associative symbiotic	Facultative anaerobe and non-photosynthetic	Malate and aspartate	Dart & Day, 1975

1976), however, it requires further investigations. The facultative anaerobic dinitrogen fixing bacterium, *Spirillum lipoferum* in loose association with roots of C_4-grasses has been shown specifically to utilize malate and aspartate as electron donors which can be easily met through the C_4 pathway of photosynthesis (Dart & Day, 1975). A list of electron donors utilized by different groups of dinitrogen fixing organisms is given in Table 9.

Electron carriers

The transfer of electrons from electron donors to dinitrogen is mediated through low redox-potential reductants called electron carriers which are generally of the ferredoxin and flavodoxin type (Streicher & Valentine, 1973). These electron carriers have been purified from most of the dinitrogen fixers on DEAE-cellulose column as coloured acidic proteins (Yoch *et al.*, 1970; Yoch & Valentine, 1972). Ferredoxins are iron-sulphide containing proteins which help in the transfer of electrons from one enzyme to another. Unlike other iron-sulphide containing enzymes, ferredoxins have no enzymatic function of their own (Streicher & Valentine, 1973).

Mortenson *et al.* (1962) were the first to discover bacterial ferredoxin from *Clostridium pasteurianum* and established a link between pyruvate oxidation and nitrogenase. *Azotobacter* ferredoxin has been purified and crystallized

as a dark acidic protein and with the help of X-ray differaction studies it has been shown that it contains eight atoms of iron and eight sulphide groups (Yoch & Arnon, 1972) The reduced *Azotobacter* ferredoxin has been found to be reluctant to oxidation when exposed to air. This has been suggested as an evolutionary adaptation which enables the bacterium to retain the reducing power needed for dinitrogen fixation under aerobic environment (Yoch *et al.*, 1969).

Rhizobium ferredoxin has also been isolated from the bacteroids of soybean root nodules but has been found relatively difficult to purify because of its high O_2-sensitivity which could be protected to some extent by adding sodium sulphite and ferrous ammonium sulphate (Koch *et al.*, 1970). Ferredoxin isolated from *Anabaena cylindrica* differs from that of *Azotobacter* ferredoxin in composition and consists of two atoms of iron and two sulphide groups with a molecular weight of about 10,000 (Yamanaka *et al.*, 1969). Shanmugam *et al.* (1972) have extracted two types of ferredoxins from a photosynthetic bacterium, *Rhodospirillum rubrum* and named them as Type I and Type 2 respectively. Type I possesses six atoms of iron and six sulphide groups while Type 2 has two iron atoms and two sulphide groups. The ferredoxins also differ in amino acid composition and molecular weight of 8700 and 7500 respectively (Streicher & Valentine, 1973).

A new class of electron carriers called flavodoxins, has been isolated and crystallized from different groups of dinitrogen fixing bacteria (Yoch & Valentine, 1972). Initially, flavodoxin was isolated as a flavoprotein capable of replacing ferredoxin in pyruvate oxidation in the cell free extracts of *Clostridium pasteurianum*. Flavodoxins have FMN as a redox group, which are generally yellow in their oxidized state having an absorption spectra similar to that of flavoproteins (Knight & Hardy, 1966). These neither contain Fe atoms nor labile sulphide groups. The only property they share with ferredoxins is their acidic nature and the ability to transfer electrons even at a low redox-potential than ferredoxins. Flavodoxins have low redox-potential, i.e., -373 mV in comparison with that of ferredoxins, -420 mV. Flavodoxins are the smallest known proteins among the flavoproteins.

Recently, two more electron carriers have been discovered which are supposed to mediate transfer of electrons from photosystem I of spinach chloroplast to the enzyme nitrogenase in the cell free extracts of *Azotobacter vinelandii* (Benemann *et al.*, 1971). One of these is a flavoprotein referred to as 'azotoflavin' while the other appears to be a non-heme Fe(III)-protein possessing typical properties of a ferredoxin (Yoch *et al.*, 1970; Shethna, 1970).

The transfer of electrons from $NADPH_2$ to nitrogenase in *Azotobacter* has been reported to be dependent on four

Table 10. Properties of ferredoxins and flavodoxins linking nitrogenase in different ecological classes of microorganisms.

	Ferredoxins				Flavodoxins		
	Molecular weight	Redox. potential (mV)	Iron-sulfide (atom/molecule)	Reference	Molecular weight	Redox potential	Reference
Fermentative							
Clostridium pastenrianum	6,000	-390	8	Yoch & Valentine, 1972	14,600	-132, -419	Mayhew, 1971
Bacillus polymyxa	9,000	-390	4	Shethna et al., 1971			
Aerobic							
Azotobacter vinelandii FdI	14,600	-420	8	Yoch & Arnon, 1972	23,000	-270, -460	
Azotobacter venelandii FdII	-	-460	8	Yoch & Arnon, 1972			
Rhizobium japonicum	9,400	-	-	Koch et al., 1970			
Photosynthetic							
Chromatium	10,000	-490	[illegible]	Buchanan & Arnon, 1970			

Contd/-

factors namely, *Azotobacter* ferredoxin, azotoflavin, a component replaceable by ferredoxin-NADP reductase of spinach chloroplast, and a soluble, heat-labile component which is yet to be chemically characterized (Benemann *et al.*, 1971). These four components probably constitute an electron transport chain between $NADPH_2$ and nitrogenase in *Azotobacter vinelandii* (Yoch & Valentine, 1972). The ferredoxins and flavodoxins isolated from different ecological groups of dinitrogen fixing organisms differ in many respects particularly, molecular weight, iron-sulphide contents, and redox-potentials as shown in Table 10.

Production and use of ATP

McNary and Burris (1962) were the first to show the utilization of ATP in dinitrogen fixation. Subsequent work has ravelled that ATP required for dinitrogen fixation may be produced through different metabolic pathways. For example, in *Clostridia*, ATP is generated by phosphoroclastic (substrate-level phosphorylation) breakdown of pyruvate. Pyruvate is oxidized and forms acetyl-phosphate through a series of reactions, which on dephosphorylation produces ATP molecules as shown in Fig. 4, (Streicher & Valentine, 1973).

Photosynthetic and non-photosynthetic dinitrogen fixers namely, *Rhodospirillum*, *Nostoc*, *Azotobacter* and *Rhizobium*

1	2	3	4	5	6	7	8
Rhodospirillum rubrum FdI	8,700	-	6	Shanmugam *et al.* 1972	23,000	-	
Rhodospirillum rubrum FdII	7,500	-	2	Shanmugam *et al.*, 1972			
Anabaena cylindrica	10,000	-	2	Yamanaka *et al.*, 1969			

From Streicher & Valentine (1973).

obtain their ATP requirements, for dinitrogen fixation, from oxidative phosphorylation in Kreb cycle. However, the photosynthetic dinitrogen fixers obtain at least a part of their ATP requirement through photophosphorylation (Yoch & Arnon, 1970). The implication of ATP in dinitrogen fixation was visualized from the studies of EPR spectrum and Mössbauer spectroscopy (Orme-Johnson et al., 1972; Zumft & Mortenson, 1975). These studies have shown that ATP molecules combine with Mg^{2+} ions and form Mg-ATP complexes. The Mg-ATP reacts specifically with Fe-protein component of nitrogenase and forms Mg-ATP-Fe-protein complex (Tso & Burris, 1973). The binding of Mg-ATP on Fe-protein lowers its redox-potential and confers upon it a unique ability to reduce the MoFe-protein component of nitrogenase (Walker & Mortenson, 1973; Thorneley, 1975).

Studies in vitro have indicated that during the transfer of every two electrons at least four or more ATP molecules are hydrolyzed and a total of about 12-15 molecules of ATP are utilized for reducing each molecule of dinitrogen (Dixon, 1975). It is not yet precisely known whether the ATP hydrolysis occurs at the site of electron transfer between the Fe-protein and MoFe-protein or between the MoFe-protein and dinitrogen (Winter & Burris, 1976).

Possible dinitrogen intermediates

The reduction of N_2 to NH_3 is accomplished by a gain of six electrons and six protons from a strong reductant. In biochemical reactions, conventionally, only two electrons and two protons are transferred at a time during the reduction of a substrate. Thus by demonstrating NH_3 as the end product of biological dinitrogen fixation in cultures of *Clostridium* and *Azotobacter* it is likely that reduction of N_2 to NH_3 might involve a series of intermediate dinitrogen complexes.

Hypothetical schemes have been proposed giving the sequence of reduction through various intermediate complexes. But none of the proposed schemes has been experimentally validiated. Chatt and Richards (1971) hypothesized two schemes explaining the possible sequence for the reduction of dinitrogen mediated through, 1. nitride formation, and 2. diimine and hydrazine formation. According to the first scheme, the reduction of dinitrogen occurs through nitride formation with metal atoms, Mo or Fe of the enzyme nitrogenase. The second scheme is mediated through diimine and hydrazine formation before ammonia is formed.

The first step in the reduction process seems to be the formation of dinitrogen complex with either of the two atoms as $M - N \equiv N$ (M = Mo or Fe). Molybdenum atom consists of redox-potential just appropriate for two electron transfer in biological systems and it can confer an acidic property

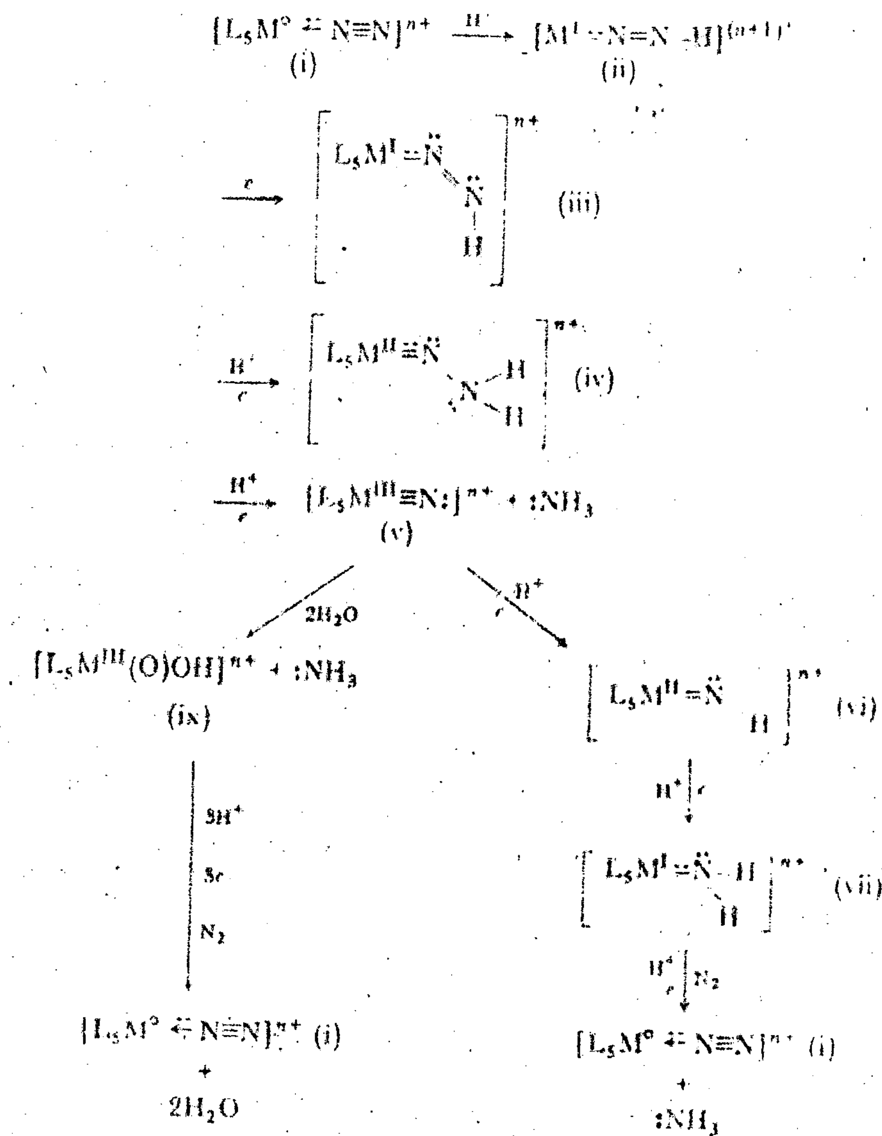


Fig. 5: Mode of reduction via a nitride complex. From Chatt & Richards, (1971).

to the neighbouring ligands that may enable it to control proton as well electron transfer. It is well known that the energy of dinitrogen molecule in the complex ($M - N \equiv N$) is lowered and the terminal nitrogen atom is charged negatively so high that if it is attacked by protons that it may result in the formation of a diimide complex with the stoichiometry of atoms, $M = N = NH$. Thus the reduction of dinitrogen could proceed by feeding electrons through metal atoms and protons from the aqueous medium, according to the reaction schemes proposed by Chatt and Richards (1971) as shown in Fig. 5 and 6.

Ligands surrounding the metal atom are denoted by L_5 and the metal atom is given oxidation state zero in the initial complexes (i). Dotted lines represent the partial bonds. The sequence of reactions involves strong multiple bonding between the intermediates and metal atom at all the stages from initial dinitrogen complex till the final hydrolysis to release ammonia free into the medium.

The reaction sequence via nitride formation provides the preferential reduction of terminal nitrogen atom. This sequence of dinitrogen reduction via nitride formation suffers from a serious drawback that so far no terminal nitrogen atom in a dinitrogen complex has been reduced by any of the reducing agents. The reduction sequence via diimine and hydrazine formation involves a rapid degradation of imido complex through a diimide complex (v) and hydrazido

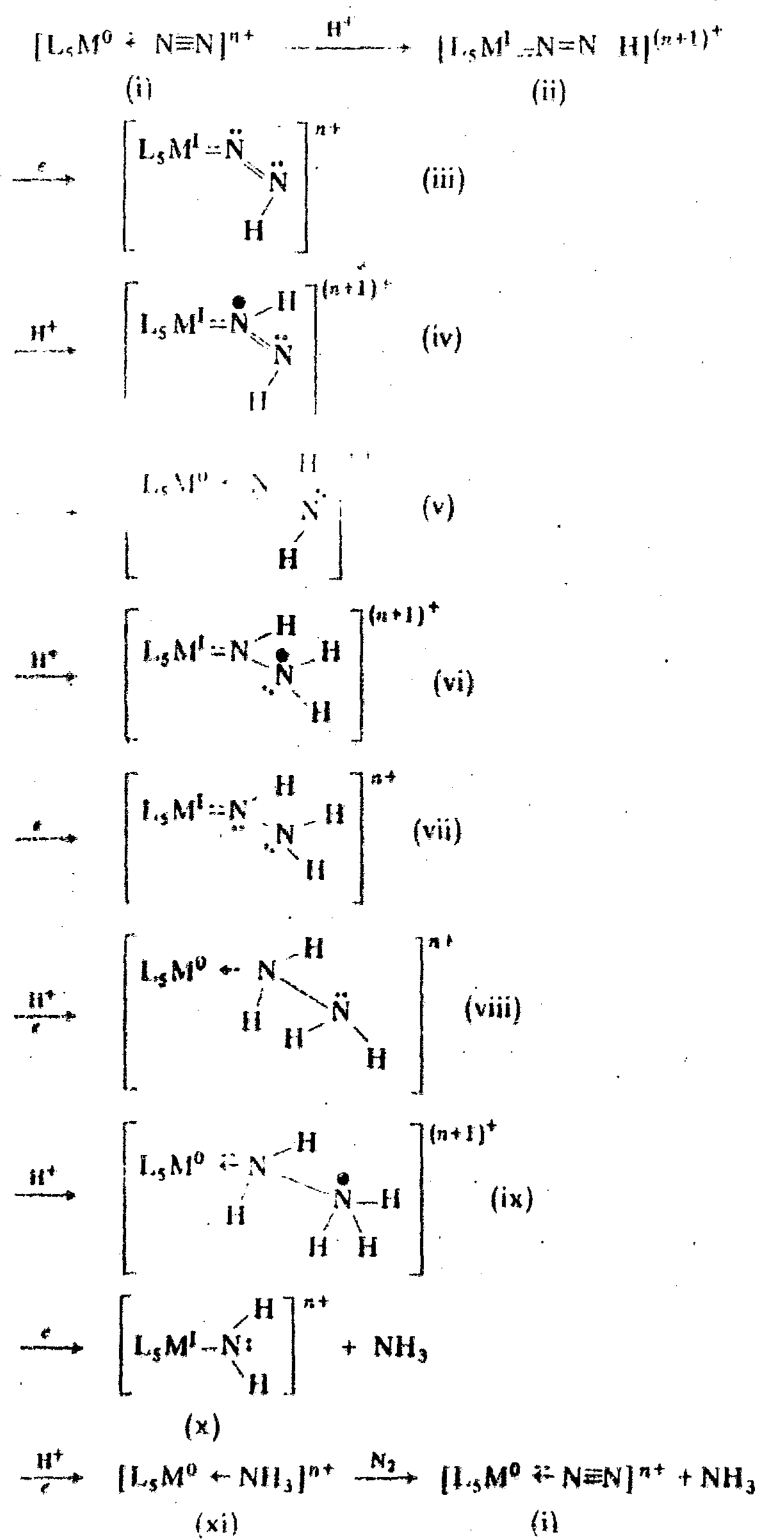


Fig. 6 : Mode of reduction via diimine and hydrazine. Chatt & Richards (1971).

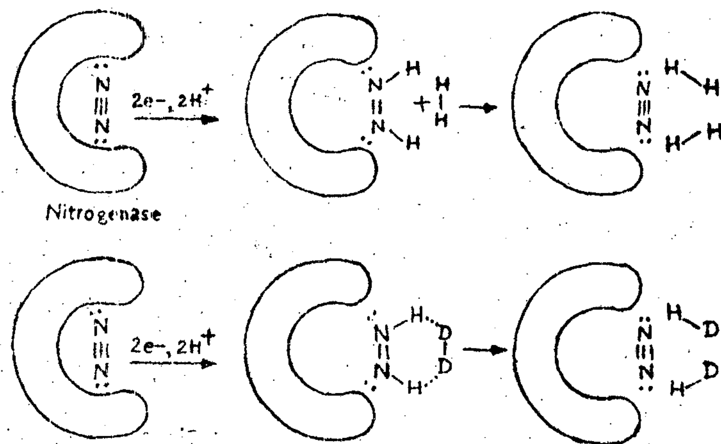


Fig. 7: **Diimide species implicated in nitrogenase reaction**
From Skinner (1976)

complex (vii) to give a hydrazine complex (viii). Since the reduction sequence involves the replacement of ammonia by dinitrogen from (xi) and that of hydrazine from (viii). It should be equally feasible, if hydrazine was added to the system it should have displaced dinitrogen from (i) to form directly hydrazine complex (viii) which then could have been reduced to ammonia.

Bulen (1976) has accumulated evidence in support of bound diimine and bound hydrazine as intermediates in the reduction of dinitrogen to ammonia. On the basis of *in vitro* studies they have indicated that deuterium gas inhibits dinitrogen fixation but results in the formation of HD. Such a characteristic may be explained by the reaction of deuterium with a diimine intermediate complex as diagrammatically shown in Fig. 7. The inhibition of dinitrogen fixation and HD formation in the presence of deuterium explain the inhibitory effect of molecular hydrogen and also identifies diimine as an intermediate complex during ~~during~~ the reduction of dinitrogen. Bulen and co-workers *in vitro* studies have also shown that nitrogenase can reduce a very low concentration of hydrazine (the reduced product of diimine) to ammonia.

Hardy *et al.* (1971) have proposed a model for nitrogenase possessing an active site for binding dinitrogen. The reduction sequence of dinitrogen to ammonia proceeds

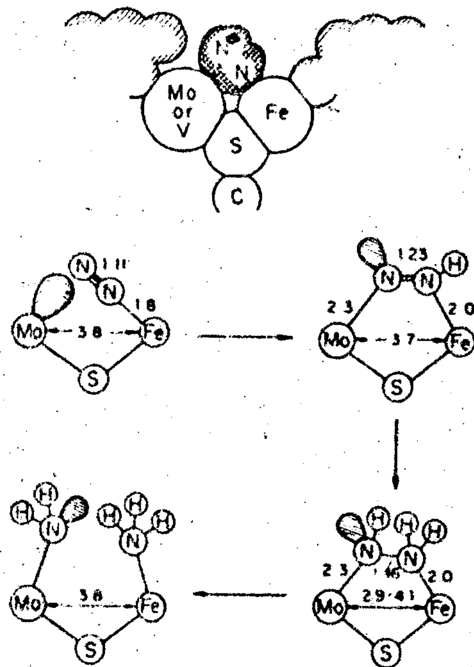


Fig. 8 : Proposed site of reducible substrate complexation and reduction. From Burns & Hardy (1975).

through bound diimine and hydrazine as intermediate complexes accompanied by two electron transfer per step as shown in Fig. 8.

It has been generally believed that the transfer of six electrons in the course of dinitrogen reduction may be carried with such a speed as to preclude the separation of intermediate dinitrogen complexes from the strongly bonded metal atom of the enzyme nitrogenase (Winter & Burris, 1976).

Production and utilization of ammonia

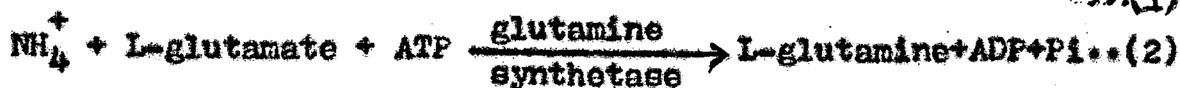
Burris (1942) applied $^{15}N_2$ isotope tracer technology to *Azotobacter vinelandii* in a closed system to identify the product of dinitrogen fixation. The cells were exposed to an atmosphere of 60 per cent $^{15}N_2$ and incubated for a period of 90 min before they were hydrolyzed with sulphuric acid. The hydrolysate was analyzed for identifying ^{15}N-labeled organic compounds. Glutamate formed most abundant followed by glutamine and asparagine. Burris on the basis of higher accumulation of ^{15}N in glutamate concluded that ammonia might be the end product of dinitrogen fixation which could be immediately and directly incorporated into α-ketoglutarate to give highest concentration of ^{15}N-glutamate catalyzed by glutamate dehydrogenase. The additional supporting evidence for ammonia to be the end product of dinitrogen fixation was obtained from the studies with the culture of *Azotobacter vinelandii* supplied with ^{15}N-labeled ammonia. The distribution of ^{15}N in the amino

acids was almost the same in the bacteria incubated with $^{15}N_2$ (Burris and Wilson, 1945). Similarly, it was also observed that with $^{15}N_2$ incubated <u>Clostridium pasteurianum</u> if the supply of dicarboxylic acid, oxaloacetic acid was low, the bacteria excreted recently fixed ^{15}N-compounds. Ammonia was the richest in ^{15}N content followed by glutamine and asparagine (Wilson and Burris, 1953).

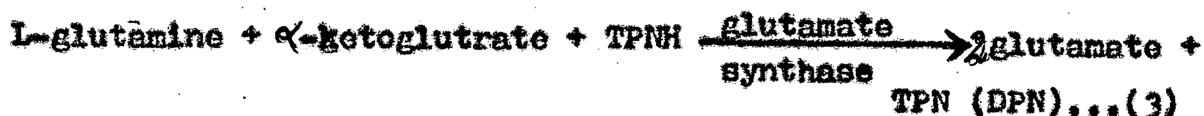
Studies with ^{13}N radioisotope for a short period of time and rapid isolation of the early ^{13}N-labeled metabolites have shown that all dinitrogen fixing microorganisms possess an additional pathway of ammonia assimilation (Nagatin <u>at al.</u>, 1971). The presence of this new pathway came to light when high levels of ^{13}N-labeled glutamine in comparison to glutamate were obsered. Thomas <u>at al.</u> (1975) have applied ^{13}N to <u>Anabaena cylindrica</u> for 2 min to 15 sec. The analysis of ^{13}N-labeled compounds showed that the ratio of glutamate to glutamine decreases from 1.64 to 0.19 as the time of exposure decreased from 2 min to 15 sec.

The central enzyme of this new pathway is glutamate synthase (L-glutamate : NAD(P) oxidoreducatase (deaminating, glutamate forming). The sequence of reactions is as follows:

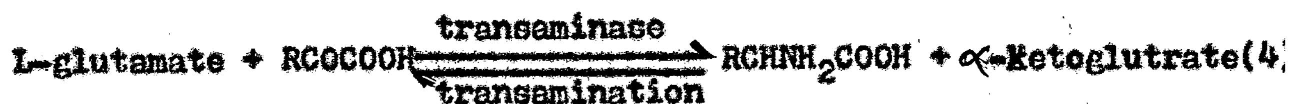
$$N_2 + Fd_{(red)} + 6\ ATP\ (?) \xrightarrow{\text{nitrogenase}} 2NH_4^+ + 6\ ADP + 6\ Pi + Fd_{(ox)} \quad ...(1)$$



$$NH_4^+ + \text{L-glutamate} + ATP \xrightarrow[\text{synthetase}]{\text{glutamine}} \text{L-glutamine} + ADP + Pi \quad ..(2)$$



$$\text{L-glutamine} + \alpha\text{-ketoglutrate} + TPNH \xrightarrow[\text{synthase}]{\text{glutamate}} 2\ \text{glutamate} + TPN\ (DPN) \quad ...(3)$$



$$\text{L-glutamate} + RCOCOOH \underset{\text{transamination}}{\overset{\text{transaminase}}{\rightleftharpoons}} RCHNH_2COOH + \alpha\text{-Ketoglutrate} \quad (4)$$

The above reactions indicate that nitrogenase catalyzed NH_4^+ produced (1) is initially assimilated in L-glutamate to from L-glutamine, irreversibly and ATP dependent catalyzed by glutamine synthetase (2). The L-glutamine undergoes deamination with α- ketoglutarate to form two moles of L-glutamate catalyzed by glutamate synthase (3). One of the two moles of L-glutamate is reutilized in reaction (2) for the incorporation of another NH_4^+ ion (Streicher & Valentine, 1973).

These observations find support from the fact that concentration of NH_4^+ ions in the medium increases by adding methionine sulphoximine which inhibits glutamine synthetase (GS, EC 6.3.1.2) which has a high affinity for ammonium as substrate (Km for NH_4^+ $<$0.2 mM). This pathway of ammonia assimilation is much more efficient than the pathway of ammonia assimilation catalyzed by glutamate dehydrogenase in non-dinitrogen fixers (Km for NH_4^+, 1.5-3 mM; Miller & Stadtman, 1972). This new pathway of ammonia assimilation seems to be an adaption to dinitrogen fixing organisms for the immediate assimilation of ammonia (Streicher & Valentine, 1973). It is well known that ammonium and nitrate ions inhibit the biological dinitrogen fixation. However, the exact mechanism of inhibition in still obscure. The available evidence indicates that enzyme glutamine synthetase is implicated as promotor for the biosynthesis of nitrogenase. The increased concentration of

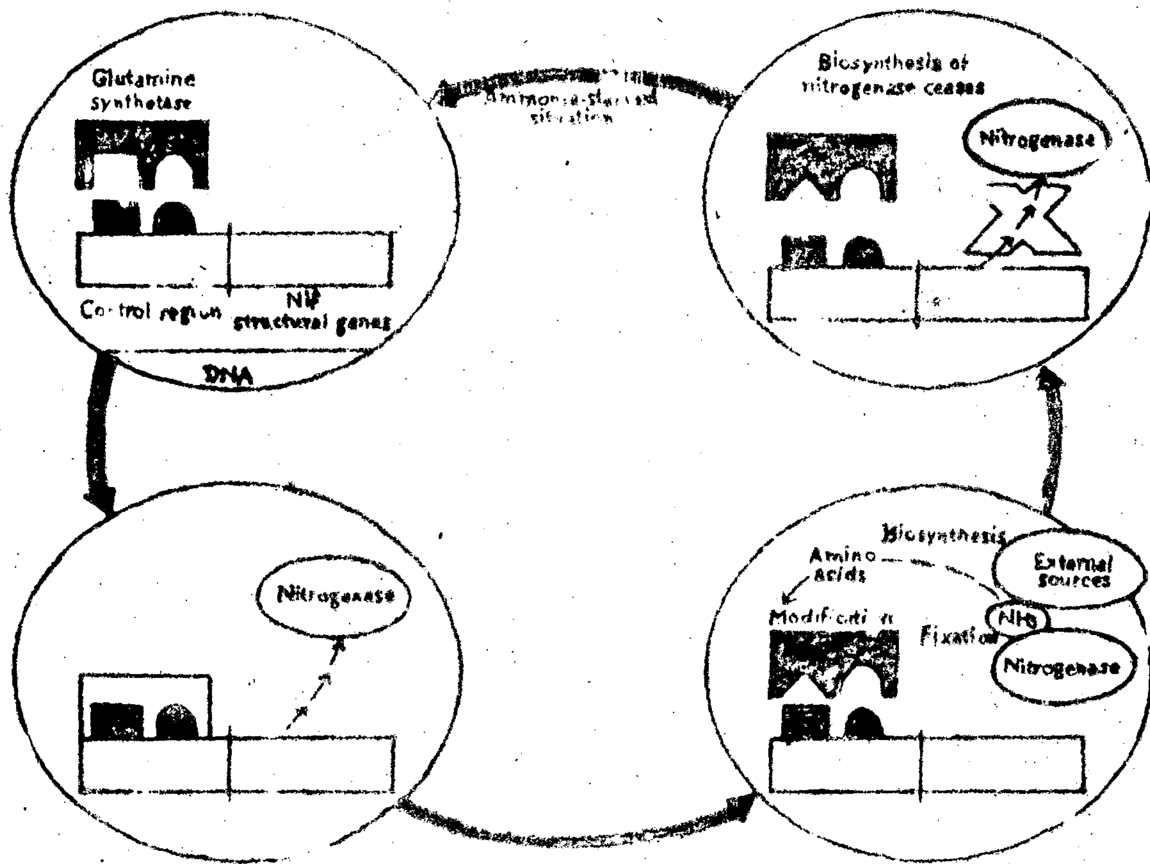


Fig. 9: **Glutamine synthetase is implicated in control process**

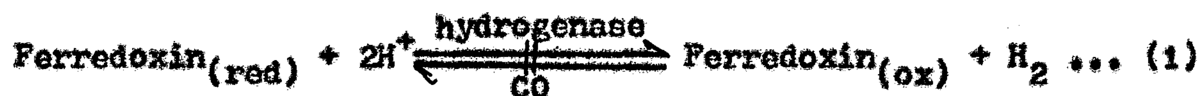
From Skinner (1976).

ammonium ions bring about conformational changes in glutamine synthetase and thus fails to act as promotor for the biosynthesis of nitrogenase. On depletion of NH_4^+ ions glutamine synthetase regains its original shape and starts acting as a promotor. The model given below explains the implication of glutamine synthetase in controlling the biosynthesis of nitrogenase as shown in Fig. 9.

Hydrogen metabolism.

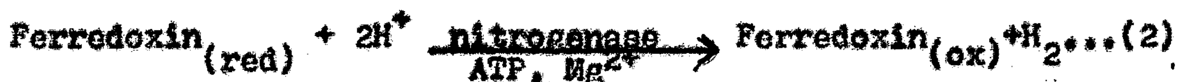
Hydrogen evolution has been shown to be a general phenomenon associated with most of the dinitorgen fixers. Hydrogen production for the first time was observed in the root nodules of soybean and the hydrogen formed in this process competitively inhibits dinitrogen fixation (Lockshin & Burris, 1965).

Bulen *et al.* (1965) on the basis of their studies on *Azotobacter vinelandii* using sodium dithionite as electron donor concluded that nitrogenase-dependent hydrogen evolution is accomplished by utilizing ATP as energy source. Hydrogen evolution in dinitrogen fixers can follow two independent pathways. The first pathway is hydrogenase catalyzed, reversible, ATP independent and CO inhibited (Kleiner & Burris, 1970; Nakos & Mortenson, 1971) as,



$$\text{Ferredoxin}_{(red)} + 2H^+ \underset{CO}{\overset{\text{hydrogenase}}{\rightleftharpoons}} \text{Ferredoxin}_{(ox)} + H_2 \quad \ldots (1)$$

The second pathway is nitrogenase-dependent, irreversible, ATP-dependent and CO uninhibited (Winter & Burris, 1968) as,



$$Ferredoxin_{(red)} + 2H^{+} \xrightarrow[ATP,\ Mg^{2+}]{nitrogenase} Ferredoxin_{(ox)} + H_2 \ldots (2)$$

Experiments with both cell free nitrogenase preparations and intact cells of *Clostridia* and nitrogenase preparations of *Azotobacter* have shown that hydrogen is evolved both through hydrogenase catalyzed and nitrogenase-dependent (Lockshin & Burris, 1965).

Postgate (1971) found no hydrogen evolution by *Azotobacter* in which hydrogenase catalyzed was inhibited with CO. It was thought that enzyme nitrogenase within the cell did not produce molecular hydrogen. These observa-tions have not been accepted unequivocally and subsequently shown to be erroneous by Smith *et al.* (1976), who showed that the apparent lack of nitrogenase-dependent hydrogen evolution from *Azotobacter*, *in vivo*, is due to the utilization of hydrogen gas by a specialized system of hydrogenases which is yet to be chemically characterized. It appears that system of hydrogenases becomes non-functional while making cell free preparations of nitrogenase. The lack of hydroger evolution, *in vivo*, has also been observed from *Vigna sinesis* and *Alnus rubra* while the hydrogen evolution has been observed in cell free nitrogenase preparations (Schubert and Evans, 1976). In these symbiotic systems the utilization of hydrogen as electron donor for consequent synthesis of ATP by specialized hydrogenases which enable them to recoup with some of the energy expended in wasteful nitrogenase-dependent production of hydrogen gas (Dixon, 1975). On account of utilization of nitrogenase-

dependent hydrogen evolved, it has been suggested that *Azotobacter*, *Vigna sinensis*, and *Alnus rubra* have their high efficiency of dinitrogen fixation (Hill *et al.*, 1972; Dixon, 1976; Schubert & Evans, 1976).

Chapter 5

Non-leguminous Dinitrogen Fixing Flowering Plants

Until the end of nineteenth century, only the legumes were known to possess the ability to fix atmospheric dinitrogen. In 1892, F. Nobbe for the first time reported dinitrogen fixation in *Elaeagnus angustifolia* a non-legume flowering plants. However, the first scientific report was made by R. Dinger, who in 1895 showed the importance of another non-legume, *Alnus glutinosa* in enriching soil nitrogen. He showed a positive correlation between the number of root nodules, the nitrogen content in the leaves and growth of the plant. The study of non-leguminous flowering plants from the view point of their dinitrogen fixation remained neglected till recently because of the following reasons:

1. a general lack of interest of microbiologists and biochemists,
2. the plants are of little direct agronomic importance,
3. occur mainly in non-agricultural lands and confined to forests,
4. the unavailability of sensitive and suitable technique for field study.

Later, from 1895 to 1957, using Kjeldahl method seven more non-legumes namely, *Ceanothus*, *Coriaria*, *Dryas*, *Casuarina*, *Hippophae*, *Myrica* and *Cercocarpus* were shown to fix dinitrogen. The availability of sensitive analytical ^{15}N-technique (Burris & Wilson, 1957), and acetylene

reduction assay (Dilworth, 1966) provide a stimulus to the study of dinitrogen fixation in non-leguminous flowering plants.

Before 1967, only thirteen non-legume genera belonging to eight families of angiosperms namely, Betulaceae, Casuarinacead, Coriariaceae, Elaeagnaceae, Ericaceae, Myricaceae were known to fix dinitrogen (Bond, 1967). A great impetus to the study of biological dinitrogen fixation in non-leguminous flowering plants was provided by the interest in dinitrogen fixation under the section of Production Processes (PP) of the International Biological Programme (IBP). The IBP surveys have revealed 36 new species, belonging to eight genera of non-legume flowering plants possessing root nodules (Bond, 1976). Recently, Döbereiner et al. (1972) have extended the range of non-legume dinitrogen fixers significantly by showing a high nitrogenase activity in the root-bacterial associations of a number of tropical C_4-grasses. Since 1967, a wide range of non-legume flowering plants about 50 in number has been discovered, to fix atmospheric dinitrogen belonging to nine familes namely, Cyperaceae, Dioscoreaceae, Gramineae, Haloragidaceae, Hydrocharitaceae, Myrsinaceae, Rubiaceae, Typhaceae and Ulmaceae. At present in all 650 species belonging to 61 genera of non-legumes spreading over 17 familes of angiosperms are know to fix dinitrogen in association with a wide range of prokaryotic microsymbionts.

Microorganisms involved

The dinitrogen fixing activity is mostly confined to the

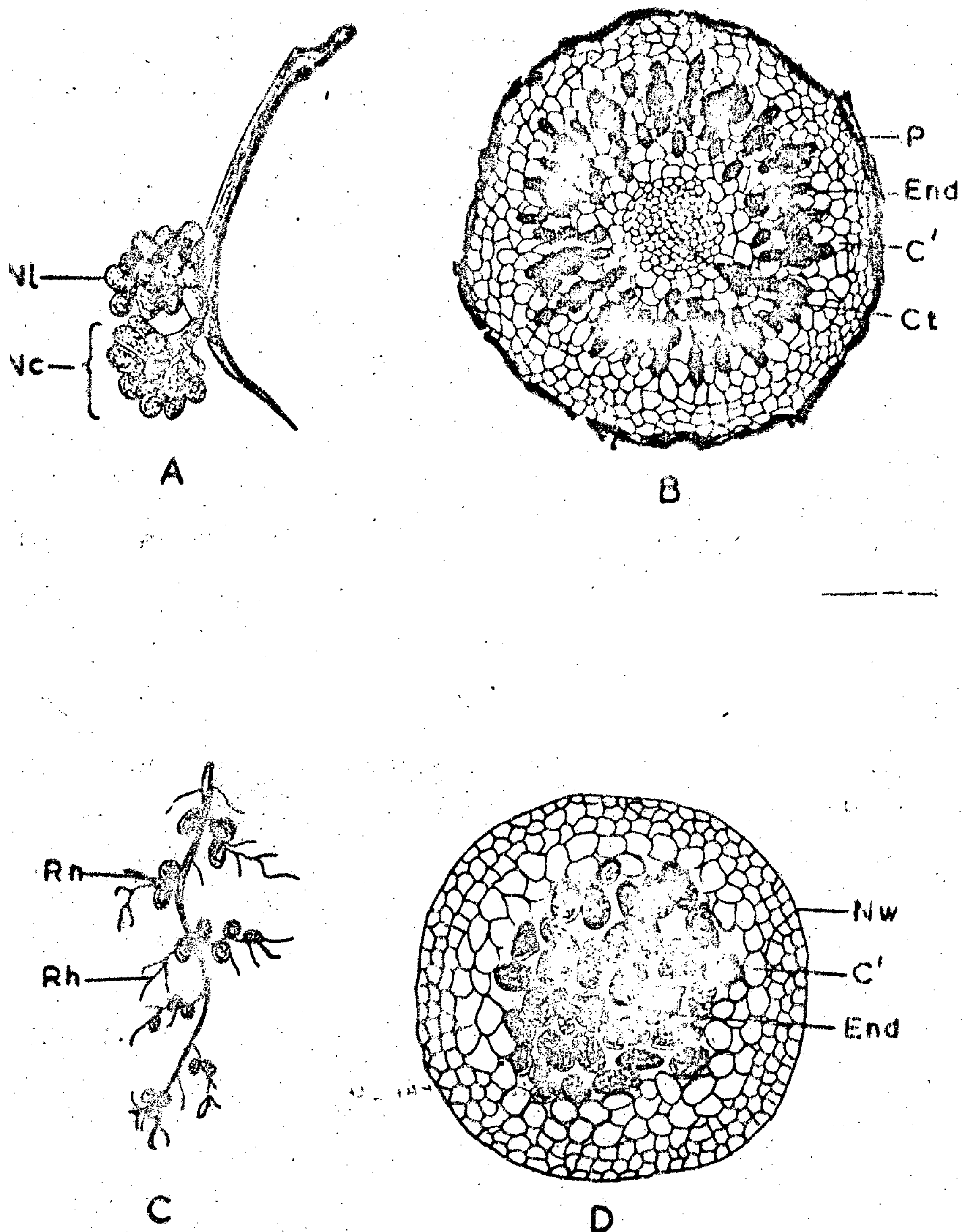


Fig. 10 : Differences between the *Alnus*-type and legume-type root nodules. A = *Alnus*-type root nodule, C = legume-type root nodule, C' = Cortex, Ct = conducting tissue, End = Endophyte, Nc = Nodule cluster, Nt,= nodule, Nw = nodule wall, Rn = Root nodule; Rh,=Root hair.

prokaryotic microorganisms. However, symbiotic systems involving higher plants and prokaryotic microorganisms are able to fix dinitrogen efficiently. The symbiotic associations between these two types of organisms result in the formation of a specialized structure on roots, stems and leaves are called nodules. The microsymbionts associated with non-legume flowering plants are actinomycete-like endophyte, bacteria and blue-green algae.

The actinomycete-like symbiont probably belongs to genus *Frankia* and has been shown to form symbiotic association with sixteen different non-legume flowering plants. The structure and function of the non-legume root nodules are similar to those found on *Alnus* roots which are one of the most extensively studied symbiotic systems. For the sake of convenience these are called *Alnus* - type root nodules (Bond, 1967). The another type of root nodules known to fix dinitrogen are of the legume-type formed in association with the *Rhizobium*. The *Alnus*-type and legume-type of root nodules differ from each other in morphology, anatomy and physiology as shown in Fig. 10 and Table 11.

Actinomycete-like endophyte has been reported to form root nodules in many non-legumes, generally but in the case of *Dioscorea*, it forms leaf nodules (Schaede, 1962). The *Rhizobium*, which is a common symbiont in the legume roots has been recently reported to form nodules in a non-legume namely, *Trema cannabina* (Trinick, 1973). The *Trema* nodules resemble with the

Table 11. A comparison of *Alnus*-type and legume-type root nodules.

Alnus-type	Legume-type
1. Branched nodules in coralloid type clusters	Unbranched and rounded nodule
2. A nodule cluster has central conducting tissue, periderm with enlarged cells of the cortex filled with a dense growth of actinomycete-like endophyte	Central conducting tissue and periderm are absent, only outer nodular wall and central enlarged cortical cells filled with extensive growth of *Rhizobium* bacteroids
3. Cell divisions are initially restricted to the infected cells and a few neighbouring cells resulting in the forming of a primary nodule which after two or three weeks starts developing into a coralloid type of nodule cluster	After infection the cells divisons immediately spread over a large number of neighbouring cells and form developed root nodule
4. The actinomycete-like endophyte passes through three different stages of life cycle namely, 1. hyphal 2. vesicular, 3. endophyte bacteroid	*Rhizobium* consists of single stage in its life cycle called Rhizobium bacteroids
5. Vesicles are the site of dinitrogen fixation	Bacteroids are the site of dinitrogen fixation

root nodules of Vigna sinensis. However, this species of Rhizobium remains to be identified. An endophyte possessing septate hyphae has been recently reported to form extensive Alnus-type root nodules in two non-legumes namely, Colletia and Rubus belonging to families Rhamnaceae and Rosaceae respectively (Bond, 1976). The microsymbiont responsible for the formation of root nodules is yet to be identified. The hyphae of actinomycete-like endophyte are septate, ranging from 0.5 - 1.0 µm and exhibit true branching. They are surrounded by an outer cell wall and inner plasma membrane containing granular cytoplasm with typical bacterial nucleoids. The function of hyphae is considered to infect the host plant and subsequently on repeated branching result in the formation of primary nodule (Becking, 1970). After two or three weeks the hyphae are transformed into spherical vesicles of about 2 µm in diameter and divided by a septum into two distinct subunits each with its own plasma membrane and nucleoid region (Gardner, 1976).

Clusters of vesicles in Alnus-type nodules have been shown to be the site of dinitrogen fixation ^{15}N-technique (Akkermans, 1971). However it is interesting to note that there is a considerable similarity between the formation of vesicles in non-legume root nodules and bacteroid formation in the nodules of legumes roots. During nodule senescence the vesicles are transformed into small granular structures called endophyte bacteroids. These endophyte bacteroids are surrounded by a

thin plasma membrane enclosing a dense cytoplasm where location of nucleoid region is difficult. The endophyte bacteroids resemble the cysts of Azotobacter and are believed to help in the dispersal of the endophyte (Gardner, 1976).

The bacterium, Klebsiella rubiacearum has been found to form leaf nodules in eight genera of the family Rubiaceae such as Psychotria, Pavetta, Gardenia etc.(Grobbelaar et al., 1971). The claim of leaf nodules ~~dinitrogen~~ fixing dinitrogen remains to be conclusively settled. The significance of the leaf nodules formation is probably related to the production of growth hormones, kinetin by the Klebsiella rubiacearum (Becking, 1971). The Klebsiella, however, the species has not been identified also forms leaf nodules on Ardisia belonging to the family Myrsinaceae. A blue-green alga, namely, Nostoc punctiforme has been reported to form symbiosis with Gunnera dentata belonging to the family Haloragidaceae (Silvester & Smith, 1969). The alga inhibits the intercellular spaces in stem nodules formed at the bases of leaves of Gunnera dentata.

In contrast to Rhizobium and actinomycete-like endophyte which form different kinds of root nodules, there are some other microorganisms such as Azotobacter, Bacillus, Beijerinckia, Spirillum etc., which develop a loose associations with the root surface of some tropical C_4-grasses and aquatic macrophytes (Döbereiner & Day, 1975; Patriquin & Knowles, 1972; Bristow, 1974; Silver[illegible] & Jump, 1975). These

bacteria living in loose association of about 30 tropical C_4-grasses such as *Andropogon*, *Cynodon*, *Paspalum*, *Pennisetum*, *Zea*, etc. and three aquatic plants namely, *Eichhornia*, *Hydrilla*, and *Thalassia* (Neyra & Döbereiner, 1977) have been shown to possess high nitrogenase activity. However, it has been reported that *Spirillum lipoferum* forms a semi-symbiotic association with the roots of *Digitaria decumbens* cv. *transvaala*, because of root cells fixing dinitrogen actively have been shown to in pure cultures (Marx, 1974; Döbereiner & Day, 1976).

Nitrogen economy and ecological importance

A significant proportion of the dinitrogen fixed from the atmosphere is contributed by the nodulated legumes but non-legume dinitrogen fixing angiosperms also appear to be quite significant. The distribution of non-legume root nodulated plants differs with that of legumes in tropical and temperate regions. In countries such as Scandinavia, Canada and New Zealand particularly at high altitudes, where legumes are either absent or insignificant in the native vegetation, the non-legume root nodulated plants are of great ecological significants. Most of the non-legume flowering plants so far known to fix dinitrogen dominate the non-cultivated land in tropical and temperate regions. Majority of the root nodulated genera are confined to the forest land in temperate regions from 1200 - 3500 m. *Dryas* and *Arctostaphylos* are being restricted to the arctic-alpine areas and *Casuarina* grows throughout the tropical and western Pacific regions with

C. equisetifolia alongwith the coastal area of the Indian Ocean.

Studies of Bond (1951) have largely initiated the interest in the non-legume diazotrophs namely, *Alnus*, *Dryas*, *Myrica*, *Coriaria* and *Casuarina*. He has demonstrated the importance of these plants in soil development and primary and secondary plant succession. It is interesting to know whether all the first non-legume angiospermic plants colonizing during the primary and secondary succession possess the capability of fix dinitrogen or it is limited to a small number of plants only.

As the time passes newer non-legume dinitrogen fixers are being discovered. A careful evaluation of the relative ecological importance of legumes and non-legumes in the nitrogen economy of the nature is very desirable. However, it is quite difficult to assess the contribution of non-legumes to nitrogen economy of the nature because of their diverse habitats and random distribution.

Rhamnaceae

Three genera out of 58 belonging to this family namely, *Ceanothus*, *Discaria* and *Colletia* are known to derive their nitrogen requirements through symbiotic dinitrogen fixation. *Ceanothus* consists of 55 species, all endemic to N. America out of which 31 have been reported to be nodulated. Delwiche *et al.* (1965) have reported that *C. azureus* under field conditions is fixes 60 kg N $ha^{-1}yr^{-1}$. Nodulated *C. integerrimus*

grew nine-times larger than those without nodules in deficient soils. *Discaria* another genus of the family disjunctly spread through south temperate areas such as New Zealand and *D. toumatou* of the only species known to be nodulated. However, the genus is important is plant succession and no quantitative assessment has been made for the nitrogen economy of the nature. Bond (1976) under IBP survey has reported the formation of well developed root nodules on an unidentified species of *Discaria*, and subsequently supported by Curtis at Royal Botanic Garden, Edinburg. Recently, *Colletia* native to S. America in its three species namely, *C. cruciata*, *C. paradoxa*, and *C. armata* have also been shown to develop root nodules (Bond, 1976). The plants generally grow well in nitrogen deficient soils.

Coriariaceae

The monogenric family consists of 15 species of *Coriaria* out of which 12 have been shown to bear root nodules. It is widely distributed over Mediterranean to Japan, New Zealand and Mexico to Chile. *Coriaria* grows vigorously on nitrogen poor sandy lowland and subalpine habitats. Dinitrogen fixation by *C. myrtifolia* in the field has been estimated upto 192 kg N ha^{-1} yr^{-1} (Silvester, 1974). *Coriaria* plays an important role in plant succession of forest in New Zealand and Japan. *Coriaria* was the first plant found to colonize Mount Tarawera, New Zealand after a volcanic eruption in 1886. The first woody

species to reinvade was *C. arborea* which also stimulated the growth of native species but at the same time it behaves as a weed in pasture lands where it is considered as a hazard to cattle due to its poisonous nature. The two species, *Coriaria nepalensis* and *C. terminalis* found in India (Missouri and Simla) have not been investigated and need examination for their capability to fix dinitrogen.

Rosaceae : Before IBP survey, the *Cercocarpus*, *Dryas* and *Purshia* restricted to Oregon to Mexico were known to bear root nodules. Recently under this programme, *Rubus elliptica* a native to Indonesia has also been reported to possess root nodules in association with an unidentified septate hyphal microorganism (Bond, 1976).

Dryas has a circum-polar distribution and it is only in Alaska and Canada that plants are known to bear root nodules. *Dryas drummondii* has a great ecological importance during secondary succession where it is the first vascular plant to colonize the recently deglaciated areas in Alaska and Canada and fixes 61.5 kg N ha^{-1} yr^{-1} under field conditions (Bond, 1971). *Purshia* consists of two species namely, *P. glandulosa* and *P. tridentata* which are restricted to the western states of N. America, have been reported to bear nodules, however, quantitative data on dinitrogen fixation are lacking.

Haloragidaceae : *Gunnera* is widely distributed through southern hemisphere consisting of 50 species out of which

40 have been reported to develop stem nodules at the bases of leaves in association with a blue-green alga, Nostoc punctiforme (Silvester & Smith, 1969). The symbiotic system is of a considerable significance as N. punctiforme being capable of supplying all nitrogen requirements of the plant which has been estimated to be 72 kg N ha^{-1} yr^{-1} in the field (Silvester & Smith, 1969). The alga inhabits the intercellular spaces in the stem and possesses eight-times more heterocysts which bring about 10-times increase in the dinitrogen fixation. In symbiotic conditions it seems that all cells of the laga behave like heterocysts and entirely depends on the host for carbohydrate supply (Silvester, 1976).

Rubiaceae : The importance of dinitrogen fixation in the leaves of some genera of Rubiaceae was highlighted IBP during the IBP surveys. Well developed leaf nodules containing the bacterium, Klebsiella rubiacearum (Silver & Centifanto, 1963; Bond, 1967; Mishustin & Shil'nikova, 1971). Silver and Centifanto (1963) have reported that nodulated Psychotria grows well on nitrogen free media, but dinitrogen fixation has not been confirmed for P. bacteriophylla when the detached nodulated leaves were exposed to $^{15}N_2$ and acetylene gas in a closed chamber (Backing, 1971). The analytical tests using ^{15}N-technique have given positive results for dinitrogen fixation in the leaf nodules of Coprosma (Silvester & Astridge, 1971) and Pavetta assimilis

(Grobbelaar et al., 1971).

The other genera of the family forming leaf nodules are Neorosea, Heterophyllaea, Lecanosperma (van Hove, 1972), Hodgkinsonia and Gardenia (Stevenson, 1957). The recent evidence indicates that leaf nodules are incapable of dinitrogen fixation. The significance of leaf nodules is probably related to the production of growth hormone kinetin by the bacterium (Silver & Astridge, 1971; Becking, 1971).

Ericaceae : This family has 50 genera of which only Arctostaphylos restricted to arctic-alpine regions has been reported to fix dinitrogen Arctostaphylos uva-ursi is the only out of 70 species in this genus known to bear root nodules which has been shown to fix dinitrogen using ^{15}N-technique (Bond, 1967). An assessment of the contribution of this species to the nitrogen economy of the region has not been made so far.

Myrsinaceae : Ardisia of the family Myrsinaceae develops marginal leaf nodules. Bacillus foliicola is the associated microsymbiont in the leaf nodule and has also been shown to fix dinitrogen in pure culture (Gordon, 1963). Three species of Ardisia namely, A. crenata, A. crispa and A. hortorum have been shown to fix dinitrogen but at a slow rate (Becking; unpublished data). In India, 10 species of Ardisia are found and they need to be examined for their potential dinitrogen fixation.

Ulmaceae : *Trema cannabis*, a member of Ulmaceae, forms root nodules similar to the legume root nodules in association with an unidentified species of *Rhizobium*(Trinick, 1973). The nodule extract examined under phase contrast microscope contained rods-like structures resembling the bacteroids of *Rhizobium* obtained from *Vigna sinensis*. *Trema* also formed extensive nodulation on inoculation with *Rhizobium* strain NGR 231 isolated from *Vigna sinensis*. The plants fix dinitrogen efficiently as indicated by vigorous growth in nitrogen deficient soils, and contributes a substantial amount of fixed dinitrogen estimated to be 86.5 kg N ha^{-1} yr^{-1} in the field (Trinick, 1973). The effective root nodule formation by *Trema cannabis* is of considerable interest due to its association with *Rhizobium* which generally forms symbiotic association with legumencus.

Elaeagnaceae : The three genera namely, *Elaeagnus*, *Hippophae* and *Shepherdia* have been shown to bear root nodules fixing dinitrogen. Before IBP survey, 10 species of *Elaeagnus* were known to form root nodules but six more species bearing extensive root nodules have been discovered by the IBP surveys (Bond, 1976). *Elaeagnus* was the first non-legume root nodulated plant to be studied by F. Nobbe in 1892; *Elaeagnus commulata* and *E. angustifolia* are weeds in N. America but also stimulate the growth of associated herbaceous species. In Japan, *E. umbellata* and in Europe *E. angustifolia* are used for the stabilization of sand dunes. In India seven species

of *Elaeagnus* are found but have not been studied dinitrogen fixation view point. *Hippophae rhamnoides* is the only nodulated species occurring in eastern Europe and Asia on coastal sand dunes. Nitrogen accumulation by *Hippophae* has been made U.K. has been estimated to be 179 kg N ha^{-1} yr^{-1} in 13 years old plants containing 642 kg nodule dry wt. ha^{-1} (Stewart & Pearson, 1967). *Hippophae* colonizes bare sandy soils where temperature may be upto 40 C. *Hippophae salicifolia* is found in India (Lauhal) needs examination for ascertaining its dinitrogen fixing potential. The three species of *Shepherdia* which are confined to N. America, possess root nodules (Bond, 1957). Nothing is known about the contribution of this plant to the nitrogen economy of nature, although, it grows vigorously in nitrogen poor soils of *Alaska Shepherdia canadensis* is a poineer species in secondary succession on recently deglaciated areas at Glacier Bay, Canada.

Casuarinaceae : *Casurina* consists of 45 species out of which 17 have been known to bear dinitrogen fixing root nodules. It is distributed widely throughout tropical western Pacific coast line of Australia, Malaysia and *C. equisetifolia* on the border of the Indian Oceans. In the course of IBP surveys seven new species of *Casuarina* have been found to form root nodules extensively. *Casuarina* has been designated as one of the most drought-resistant species suitable for desert plantation. It is a pioneer colonizer in the secondary

succession. Dommergues (1963) has made a quantitative estimate of the dinitrogen fixed is sandy soils of Cape Verde Islands of Africa, dominated with C. equisetifolia. He found that the bare soils contained 80 kg N ha^{-1} and after 13 years of plantation the soil nitrogen increased to 309 kg N ha^{-1}. In addition, the standing crop of Casuarina was estimated to contain 531 kg N ha^{-1} giving an average rate of dinitrogen fixation 58.5 kg N ha^{-1} yr^{-1}.

Betulaceae : Alnus is one of the most extensively studied dinitrogen fixing non-legume woody plant. About 33 species of Alnus out of 35 are known to possess root nodules, fixing dinitrogen actively. Crocker and Dickson (1957) studied adequately the implication of alders in plant succession and soil formation in deglaciated soils of Alaska. Alnus crispa is one of the first woody plants to appear in cleared areas after mossess and herbs have colonized. The litter fall is reported to contribute about 62 kg N ha^{-1} yr^{-1}. During the course of plant succession a thick growth of Alnus occurs in 25-40 years but it forms a transient community which is soon overtopped by spruce (Picea) plants. Mishustin and Shil'nikowa (1971) have reported that A. glutinosa can fix upto 100 kg N $ha^{-1}yr^{-1}$ in the field under favourable conditions. In wet sandy soils of the Netherlands, A. glutinosa develops root nodules biomass weighing upto 44 kg ha^{-1} can fix 56-130 kg N ha^{-1} yr^{-1} (Akkermans, 1971). A great significance of Alnus has been recognized that it stimulated the growth

of *Populus trichocarpa* by 22-times at Glacier Bay, Alaska (Lawrence, 1958) and estimated that litter fall of *A crispa* may account for 157 kg N ha^{-1} yr^{-1} under 5 years old plants. Nitrogen content of *Alnus* is much higher than the most other non-legume flowering plants and the rate of litter fall is extremely high. In India, two species of *Alnus* namely, *A. nepalensis* and *A. dioeca* are found in (Kumaon, Himalaya and Khasi Hills) and their potential for dinitrogen fixation needs to be evaluated.

Myricaceae : Three genera namely *Comptonia*, *Gale* and *Myrica* have been reported to bear root nodules. *Comptonia peregrina* form nodules in association with actinomycete-like endophyte is restricted to Canada. It occurs in sandy and peaty soils and significantly contributes to nitrogen to the substratum (Silver and Hague, 1970). Another genus, *Gale palustris* which also dominates the sandy soil in temperate N. America, N.W. Europe and N.E. Siberia has been shown to fix dinitrogen under field conditions at a rate of 9 kg N ha^{-1} yr^{-1} (Bond, 1971). *Myrica* the most extensively studied genus of the family is distributed through Asia, Europe, Africa and N. America. Fourteen new species of *Myrica* have been discovered during the IBP surveys and thus 26 species out of 35 are now known to develop root nodules (Bond, 1976). *Myrica gale* occurs around many British lakes and contributes substantially to the nitrogen economy of fresh water. *Myrica cordifolia* is a dominant species in sand dunes in S. Africa (Grobbelaar *et al.*, 1971). In Florida, *M. cerifera* acts as a sere stage to *Pinus*

forest and in Indonesia, *M. javanica* is an active colonizer of of soils exposed to fire or volcanic activity (Becking, 1970). Bond (1951) has estimated that *M. gale* may contribute 9 kg N ha^{-1} yr^{-1}. In India, *Myrica integrifolia* occurs in (Silhet and Khasi Hills) merits attention for evaluating its dinitrogen fixing potentiality.

Dioscoreaceae : *Dioscorea macroura* has also been reported with little evidences fixing atmospheric dinitrogen in its foliar environment. The microsymbiont is thought to be an actinomycete-like endophyte embedded in nodules found on the upper leaf surface. Schaeda (1962) claims that *Dioscorea*, like other leaf nodulated species fixes atmospheric dinitrogen but it has not been conclusively proved. In India (Simla) four species of *Dioscorea* are found from 1200 to 3000 m altitude need to be carefully studied for dinitrogen fixing potentiality.

Hydrocharitaceae : Acetylene reduction assay (Hardy *et al.* 1973) has revealed that dinitrogen fixation occurs in the rhizosphere of a number of marine, *Thalassia*, (Patriquin & Knowles, 1972), and freshwater plants particularly *Hydrilla* and *Eichhornia* (Bristow, 1974). These plants are believed to have loose assocations with unidentified epiphytic bacteria responsible for their high nitrogenase activities. *Thalassia testudinum*, a tropical marine angiosperm, abundantly found in Indian Pacific Oceans has the dinitrogen fixing capacity varying from 100-500 kg N $ha^{-1}yr^{-1}$ (Patriquin & Knowles, 1972).

Hydrilla verticillata and water hyacinth (Eichhornia carssipes)have anaerobic environment in their growing root zones favouring dinitrogen.fixation. However, it has not been shown that as to how the nitrogen requirement of these plants can be obtained from dinitrogen fixed in the rhizosphere (Silver & Jump, 1975). The importance of dinitrogen fixation in aquatic environment, where nitrogen is considered a productivity limiting factor the importance of dinitrogen fixation cannot be under estimated.

Typhaceae : Typha an emergent freshwater plant, forms a loose root association with an unidentified dinitrogen fixing bacteria. The report shows that the dinitrogen fixed in the rhizosphere in Typha sp. may fulfil 10-20 per cent of the nitrogen requirement of the plant (Bristow, 1974).

Cyperaceae : Four genera namely, Bulbostylis, Cyperus, Fimbristylis and Juncus are known to fix dinitrogen Azoto-batter and Beijernckia isolated from the roots of these plants are believed to be responsible for the high nitrogenase activity. Cyperus tetragonus, an aquatic plant has been shown to fix dinitrogen in the rhizosphere (Silver & Jump, 1975) and Balandreau et al. (1973) have estimated that C. obtusiflorus growing in tropical warm regions of Brazil and Nigeria can fix as much as 253 mg N ha^{-1} yr^{-1}. The other two species, Bulbostylis aphyllanthoides and Fimbristylis sp. (Day & Dart, unpub.) have also been shown to fix

dinitrogen approximately 30 and 77 kg N ha^{-1} yr^{-1} respectively.

Gramineae : Recent studies in Brazil, Nigeria and Ivory Coast have discovered a loose symbiotic association between the root of tropical C_4-grasses and dinitrogen fixing bacteria (Döbereiner *et al.*, 1972; Bülow & Döbereiner, 1975). Under favourable conditions these grass-bacterial associations may contribute substantially to the nitrogen economy of many forage and grain crops. De-Polli *et al.* (1977) have demonstrated that the $^{15}N_2$ isotope has been incorporated by the two C_4-grasses namely, *Digitaria decumbens* and *Paspalum notatum* and enrichments of 0.15 and 0.563 ^{15}N atom per cent excess were obtained in roots of *D. decumbens* cv. *transvaala* and *P. notatum* systems in three days. The tropical grasses which are able to support significantly dinitrogen fixation have been found to possess efficient C_4 pathway of photosynthesis (Day *et al.*, 1975). The root exudate of these plants in the rhizosphere plays an important role in the establishment and maintenance of the populations of the dinitrogen fixing bacteria (Neyar & Döbereiner, 1977).

In most of the dinitrogen fixing systems the availability of photosynthate is considered to be a major limiting factor to dinitrogen fixation. However, due to the efficient C_4 pathway of photosynthesis the C_4-grasses are considered to be in a better conditions with regard to the photosynthate supply for dinitrogen fixation (Neyra & Döbereiner, 1977). However, most of the studies of dinitrogen fixing grass-bacterial asso-

ciated systems have been conducted under laboratory conditions. Barber et al., (1976) have shown that there is a 30-fold increase in the number of dinitrogen fixing bacteria onto the roots of excised maize plants, when samples are preincubated overnight at low pO_2. Therefore, their results show that dinitrogen fixing activity measured in the laboratory are about 100-times more than the rates measured under field conditions. Thus, the grass root-bacterial loose associations need a critical evaluation of their dinitrogen fixing activity under laboratory and field conditions.

The first report of C_4-grass-bacterial association relates to Paspalum notatum (Döbereiner, 1966; Döbereiner & Campelo, 1971). Azotobacter paspali has been reported as the microorganism localized in the mucilagenous sheath around the root (Döbereiner et al., 1972). Estimate of dinitrogen fixation in intact root soil cores of Paspalum notatum has been measured to be 124 kg N ha^{-1} yr^{-1} (Neyra & Döbereiner, 1977). Digitaria decumbens-Spirillum lipoferum possess high nitrogenase activity and the bacteria have been located in the intercellular spaces of the inner cortex of the root (Döbereiner & Day, 1976). The nitrogenase activity of Digitaria decumbens root soil cores measured by acetylene reduction assay indicate a gain 548 kg N ha^{-1} yr^{-1} (Neyra & Döbereiner, 1977).

Seedlings of sugarcane exposed to $^{15}N_2$ have been shown to fix dinitrogen actively (Döbereiner, 1961). The acetylene reduction assay has shown that only a part of the dinitrogen

fixed occurs on the root surface of the plant, otherwise, mostly it occurs in the rhizosphere. Dinitrogen fixed by sugarcane has been reported to be 8 kg N $ha^{-1} yr^{-1}$ (Döbereiner, 1961). Maize, a major grain crop of the tropical world has been reported to have the highest nitrogenase activity reaching tupto 9000 n moles C_2H_4 g^{-1} root hr^{-1} (Bülow & Döbereiner, 1975). However, the results have been obtained under laboratory conditions after pre-incubating the roots at low pO_2, 0.025 to 0.05 atm. Döbereiner et al. (1975) have shown that nitrogenase activity both in *Pennisetum purpureum* and maize is as high as 365 and 730 kg N ha^{-1} yr^{-1} respectively. A detailed study to ascertain the localization of *Spirillum lipoferum* in the roots of these plants is yet to be made. A number of tropical C_4 forage grasses namely, *Andropogon*, *Brachiaria*, *Cenchrus*, *Cynodon*, *Hyparrhenia*, *Melinis* and *Sorghum*, have also been shown to possess high nitrogenase activities in their rhizosphere (Neyra & Döbereiner, 1977).

The C_3-grasses have been also shown to make some contribution to the nitrogen economy of nature. Rice and wheat are the two important grain crops which are able to support symbiotic dinitrogen fixation. Blue-green algae such as *Nostoc*, *Anabaena* and *Aleusira* and the bacteria *Beijerinckia* sp and *Enterobacter cloacae* are mainly responsible for a large part of dinitrogen fixed in the paddy fields (Balandreau, 1975; Neyra & Döbereiner, 1977). Acetylene reduction assays with excised roots of field grown rice have

Table 12. A list of non-leguminous dinitrogen fixing flowering plants. (Plants are arranged according to Benthem & Hooker (1862-1883) system of classification).

Sr. No.	Family/ genus	Geographical distribution	No. of sp. in genus**	No. of sp. fix. N_2	Method of study	kg N ha^{-1} yr^{-1}	Micro-symbiont	Micro-symbiont habitat	Reference
1	2	3	4	5	6	7	8	9	10
	Rhamnaceae								
1.	Ceanothus azureus	Canada, India (Dehru Dun)	55	31	KM	60.0	AM	RN	Delwiche *et al.*, 1965
2.	*Colletia paradoxa	Subtrop. and temp. S.America	17	3	ARA	+	USHM	RN	Bond, 1976
3.	*Discaria toumatou	Australia; New Zealand; S. Andes; Brazil	10	2	^{15}N	+	AM	RN	Morrison, 1961
	Coriariaceae								
4.	Coriaria myrtifolia	Medit. to Japan; New Zealand; Mexico to Chile; India (Missouri, Simla)	15	13	KM	192.0	AM	RN	Silvester, 1974

*= Genus not found in India; **= According to Willis (1973); + = fixation of dinitrogen confirmed; ± = fixation is doubtful; AM = Actinomycete-like endophyte; Ap = *Azotobacter paspali*; AZ = *Azotobacter* sp.; ARA = Acetylene reduction assay; Bj = *Beijerinckia* sp.; Bf = *Bacillus folliicola*; Bp = *Bacillus polymyxa*; Ent = *Enterobacter* sp.; GNB = Gram negative bacteria; KM = Kjeldahl method; Kr = *Klebsiella rubiacearum*; LN = Leaf nodule; Np = *Nostoc punctiforme*; Nt = *Nostoc* sp.; Rh = *Rhizobium* sp.; RN = Root nodule; RS = Rhizosphere; Sl = *Spirillum lipoferum*; SN = Stem nodule; StN = Stipular nodular; UB = Unidentified bacteria.

confirmed the fixation of dinitrogen in the rhizosphere and at the flowering stage it has been estimated that 50-60 kg N $ha^{-1}yr^{-1}$ is fixed by the association bacteria (Balandreau, 1975).

The nitrogenase activity of root soil cores containing wheat is significantly higher in comparison with the bare soils (Day et al., 1975). In Broadbalk field wheat experiment at Rothamsted, U.K., from 1843 to 1967, it has been shown that on an average there was a gain of 34 kg N ha^{-1} yr^{-1} (Jenkinson, 1973). However, the root soil cores of wheat tested with acetylene reduction method gave a value of 2-3 kg N ha^{-1} yr^{-1} (Day et al., 1975). In Broadbalk field wheat experiment a major part of dinitrogen fixed was contributed by the blue-green algae. *Enterobacter cloacae* and *Bacillus polymyxa* have been reported to be the most active dinitrogen fixing bacteria on the roots of wheat (Neyra & Döbereiner, 1977). The *Bacillus polymyxa* has been isolated from the wheat root surface as well as from the intercellular spaces of the cortex (Larson & Neal, 1976). The follwoing Table 12 provided an uptodate information on the non-leguminous dinitrogen fixing flowering plants in a concise manner.

	2	3	4	5	6	7	8	9	10
	Rosaceae								
5.	*Cercocarpus betuloides	S.W. USA; Oregon to Mexico	20	4	ARA	+	AM	RN	Vlamis et al., 1964
6.	*Dryas drummondii	Arctic-alpine U.S.A.; N. Temp. zone of Europe	2	2	KM	61.5	AM	RN	Bond, 1971
7.	*Purshia tridentata	Pacific U.S.A. Cosmpp; especially N. temp. regions	2	2	^{15}N	+	AM	RN	Wagle & Vlamis, 1961
8.	Rubus ellipticus	Cosmop; especially N. temp. regions	250	1	ARA	+	USHM	RN	Bond, 1976
	Haloragidaceae								
9.	*Gunnera dentata	Malaysia; Tasmania	50	40	MM	72.0	Np	SN	Silvester & Smith, 1969
	Rubiaceae								
10.	Psychotria mucronata	Trop. S. Africa; Malagassy; Zaire; Sweden; Ivory Coast; India (Sikkim, Assam, Nicobars)	700	410	^{15}N	±	Kr	LN	Becking, 1971
11.	Pavetta assimilis	N. Australia; B Burma; Malayan Peninsula to China; India (Sikkim, Andamans)	427	339	ARA	+	Kr	LN	Grobbelaar et al., 1971

Contd../-

1	2	3	4	5	6	7	8	9	10
12.	*Neorosea andogensis	Trop. W. Africa; Columbia	12	1	^{15}N	±	Kr	LN	van Hove, 1972
13.	*Coprosma robusta	Australia; New Zealand; Malaysia; Chile; Polynes	90	27	^{15}N	+	Kr	StN	Silvester & Astridge, 1971
14.	*Hodgkinsonia ovatifolia	E. Australia	2	1	^{15}N	±	Kr	LN	Stevenson, 1957
15.	Gardenia thumbergia	Trop. Asia; India (Bundelkhund, Sikkim, Dehra Dun)	250	15	^{15}N	±	Kr	LN	Stevenson, 1957
16.	*Heterophyllaea sp.	Argentina; Bolivia	4	1	^{15}N	±	Kr	LN	van Hove, 1972
17.	*Lecanosperma sp.	Bolivia	1	1	^{15}N	±	Kr	LN	van Hove, 1972
	Ericaceae								
18.	*Arctostaphylos uva-ursi	N.W. & C. America; N. temp. and arctic regions	71	1	^{15}N	+	AM	RN	Bond, 1967
	Myrsinaceae								
19.	Ardisia crispa	U.S.A.; Trop. Asia; Japan; India (Assam, Sikkim, Orissa, Manipur) Chile; Europe	400	13	^{15}N	±	Br	LN	Becking (unpub.)

Contd.../-

1	2	3	4	5	6	7	8	9	10
	Elaeagnaceae								
20.	Elaeagnus angustifolia	Asia; India (Simla; W. Bengal) Canada; Europe	45	16	KM	+	AM	RN	Gardner, 1958
21.	Hippophaé rhamnoides	Temp. Eurasia; India (Kumaon, Lahaul)	3	1	KM	179.0	AM	RN	Stewart & Pearson, 1967
22.	*Shepherdia canadensis	Alaska to N. Mexico; U.K.; Colombia	3	2	^{15}N	+	AM	RN	Bond, 1957
	Ulmaceae								
23.	Trema cannabis	Trop. and Subtrop. Australia; New Guinea; Malaya; Asia; Africa; N. America	30	1	ARA	86.5	Rh	RN	Trimick, 1973
	Casuarinaceae								
24.	Casuarina equisetifolia	Trop. Australia; Malaysia; Polynes; Africa; Asia; India (Andamans)	45	24	KM	58.5	AM	RN	Becking, 1970
	Betulaceae								
25.	Alnus glutinosa	Alaska; Indonesia; Japan; India (Srinagar; Kumaon; Khasihills)	35	33	KM	100.0	AM	RN	Mishustin & Shilnikova, 1971

contd.../-

1	2	3	4	5	6	7	8	9	10
	Myricaceae								
26.	*Comptonia peregrina	N.E. America (Canada)	1	1	^{15}N	+	AM	RN	Silver & Mague, 1970
27.	*Gale palustris	Temp. N. America; N.W. Europe; N.E. Siberia	2	1	ARA	9.0	AM	RN	Bond, 1971
28.	Myrica cordifolia	Trop. and subtrop. U.S.A.; N.E. Asia; Malaya; China; Japan; India (Simla)	35	26	KM	9.0	AM	RN	Bond, 1971
	Dioscoreaceae								
29.	Dioscorea macroura	Trop. and subtrop. S.W. Africa; India (Simla)	600	10	^{15}N	±	AM	LN	Schaede, 1962
	Hydrocharitaceae								
30.	Eichhornia crassipes	S.E.U.S.A. to Argentina	7	1	ARA	+	Bj	RS	Silver & Jump, 1976
31.	Hydrilla verticillata	Eurasia; Africa to Australia	1	1	ARA	+	Az Bj	RS	Silver & Jump, 1976
32.	Thalassia testudinum	Indian Pacific Oceans; Atlantic Oceans	2	1	ARA	500.0	UB	RS	Patriquin & Knowles, 1972

Contd.../-

1	2	3	4	5	6	7	8	9	10
Typhaceae									
33.	Typha sp. -C_3	Trop. and temp. regions	20	2	ARA	+	Az Bj	RS	Bristow, 1974
Cyperaceae									
34.	*Bulbostylis aphyllanthoides	Trop. countries; S. America; S. Africa	100	11	ARA	30.0	Az	RS	Day & Dart, unpub.
35.	Cyperus obtusiflorus	Trop. and warm temp. regions; Brazil; Nigeria; India; Sri Lanka	550	13	ARA	253	Bj	RS	Balandreau et al. 1973
36.	Fimbristylis sp.	Trop. and subtrop. regions of Indomalaya to Australia	300	4	ARA	77.0	Bj	RS	Balandreau et al. 1973
37.	Juncus balticus	Cosmop. mainly in cold and wet places	300	12	ARA	+	Sl	RS	Barber et al., 1976
Gramineae									
38.	Andropogon gayanus - C_4	Trop. and subtrop.	113	10	ARA	110.0	Sl	RS	Döbereiner et al. 1975
39.	Alopecurus pratensis Len	Temp. Eurasia; S. America; U.K.	50	4	ARA	+	GNB	RS	Krasil'nikova-Krainava, 1962.

Contd.../-

1	2	3	4	5	6	7	8	9	10
40.	*Ammophila arenaria	Atlantic N. America; N. Africa, Europe; U.K.	2	1	ARA	+	Az	RS	Hassouna & Wareing, 1964
41.	Brachiaria regulosa-C_4	Trop. regions	50	5	ARA	59.0	Sl	RS	Döbereiner & Day, 1973
42.	Cenchrus regulosa - C_4	Trop. and warm temp. regions	25	3	ARA	6.5	Az	RS	Döbereiner & Day, 1973
43.	Cymbopogon giganteus-C_4	Trop. and subtrop. Savannas of Africa; Asia	60	3	ARA	34.7	Az	RS	Döbereiner & Day, 1973
44.	Cynodon dactylon-C_4	Trop. and warm temp. regions	10	2	ARA	110.0	Bj	RS	Döbereiner & Day, 1973
45.	Digitaria decumbens-C_4	Trop. and subtrop. Savannas of Africa; Asia	380	6	ARA	500.0	Sl	RS	Neyra & Döbereiner, 1977.
46.	Eleusine caracana-C_4	Trop. and subtrop. Savannas of Africa; Asia	9	2	ARA	31.0	Az	RS	Döbereiner, et al., 1975
47.	Glyceria borealis	Cosmop. especially in N. America	40	2	ARA	60.0	UB	RS	Bristow, 1974

Contd.../-

1	2	3	4	5	6	7	8	9	10
48.	*Hyparrhenia rufa-C_4	Medit.; Africa; Trop. and subtrop. regions.	75	5	ARA	12.0	Az Sl Bj	RS	Döbereiner & Day, 1973
49.	Loudetia simplex	Trop. S. Africa; S. America; Malagassay	41	1	ARA	22.0	UB	RS	Day *et al.*, 1975
50.	*Melinis mitif- [nu] lora - C_4	Trop. S. America; W. Indies; Trop.S. Africa; Malagassy	18	2	ARA	16.5	Az Sl Bj	RS	Döbereiner & Day, 1973
51.	Oryza sativa-C_3	S.E. Asia; Burma; Japan; China; U.S.A; U.S.S.R., Phillipines	25	1	ARA	120.0	Nt Bj Ent	RS	Watanabe & Kukki-Lee, 1975
52.	Pānicum maximum - C_4	Trop. and subtrop. regions	500	4	ARA	122.0	Sl	RS	Döbereiner & Day, 1975
53.	Paspalum notatum C_4	Trop. and subtrop. regions	250	5	ARA	329.0	Az Sl	RS	Neyra & Döbereiner, 1977
54.	Pennisetum perpureum-C_4	Trop. countries; extensively cultivated in India	130	3	ARA	365.0	Az Sl	RS	Döbereiner, *et al.*, 1975

Contd.../-

1	2	3	4	5	6	7	8	9	10
55.	Saccharum officinale-C_4	Trop. E. Asia; Cuba; Java; Hawaii; Brazil	5	1	ARA	8.0	Dj	RS	Döbereiner, 1961
56.	Setaria anceps-C_4	Trop. and subtrop. S. Europe to Japan Australia	140	4	ARA	49.0	Az Sl	RS	Tjepkema& Burris, 1976
57.	Spartina alterniflora-C_4	Temp. America; Europe; Africa	16	1	ARA	+	Sl	RS	Patriquin, 1976
58.	Sporobolus heterolepis-C_4	Trop. and warm temp. regions	150	3	ARA	1.0	Az	RS	Tjepkema & Burris, 1976
59.	Sorghum bicolor-C_4	Trop. and subtrop. regions	60	2	ARA	63.0	Sl	RS	Döbereiner et al., 1975
60.	Triticum vulgare-C_3	W. Asia; W. Australia; Medit. region; U.S.A.; U.S.S.R.; Canada; India.	20	1	ARA	3.0	Ent BB	RS	Neyra & Döbereiner, 1977
61.	Zea mays-C_4	N.E. Asia; N. America; Argentina; Brazil, Mexico; India.	1	1	ARA	730.0	Sl	RS	Döbereiner et al., 1975

Chapter 6

Factors Affecting Dinitrogen Fixation

Allarge part of nitrogen requirement in agricultural production is supplied by dinitrogen fixing symbiotic systems namely, legumes and non-legumeinous flowering plants which utilize photosynthetically stored solar energy for reducing atmospheric dinitrogen to ammonia. The fixation of dinitrogen is not dependent nonrenewable resources, therefore, its use in agricultrual production should be maximized. The process is greatly influenced by many environmental factors, thus, the complete understanding of the factors limiting biological dinitrogen fixation deserve serious attention. Environmental stresses at the early stages of symbiosis formation either have adverse effects or stop dinitrogen fixation completely. A critical evaluation and a proper understanding of the environmental factors influencing the process will greatly help in developing suitable strategy for optimizing biological dinitrogen fixation. The factors influencing the process of biological dinitrogen fixation can be broadly classified into the following four categories:

1. Atmospheric factors
    - i. Light
    - ii. Carbon dioxide
    - iii. Oxygen, and
    - iv. Temperature

2. Edaphic factors

i. Inorganic combined nitrogen

ii. Mineral nutrition

iii. Soil pH and calcium

iv. Water stress, and

v. Ethylene

3. Biological factors

i. Host-microsymbiont specificity

ii. Injury

4. Physiological factors

i. Hydrogen evolution

1. Atmospheric factors

A number of atmospheric factors namely, light, carbon dioxide, oxygen, and temperature affect the process of biological dinitrogen fixation profoundly. The various effects of these factors are described in the following paragraphs.

i. Light : Light influences the process of biological dinitrogen fixation both directly and indirectly.. The direct effects of light are due to the intensity, its duration and quality of the incident light. Indirectly, the light affects dinitrogen fixation by influencing the process of photosynthesis through the carbohydrates supply to the microsymbiont which is described under the effect of carbon dioxide.

There is a direct relationship between light intensity and dinitrogen fixing activity of a plant. The increasing

Table 13. Daily fluctuations in acetylene reduction by the nodules of first-year and second-year alder plants.

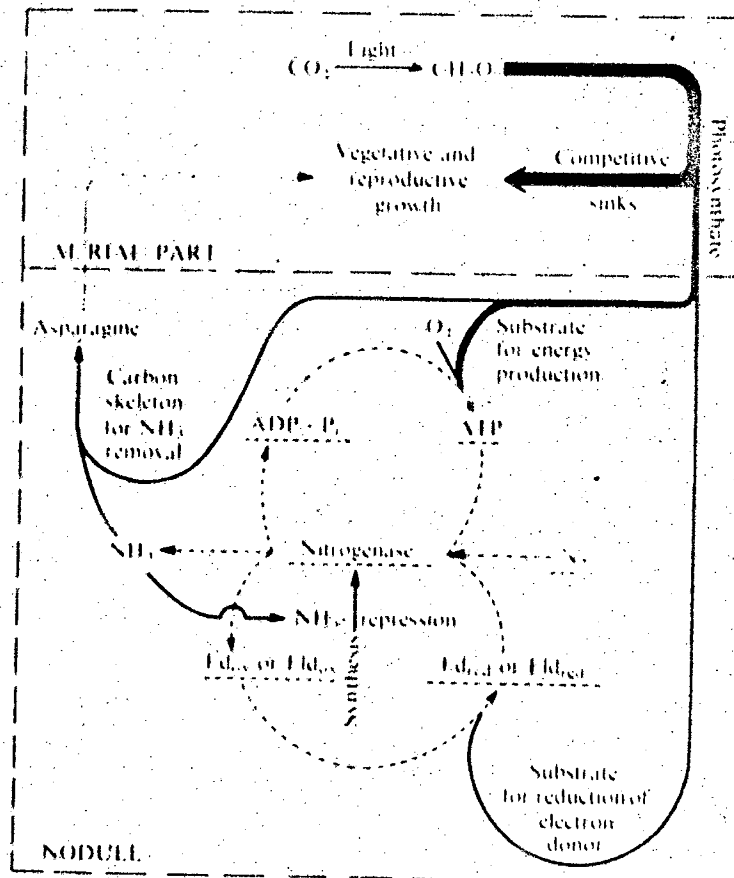
Date	Time of day (h)	C_2H_4 (μ mole/g fresh weight nodules/h)	Least significant different between means. P = 0.05	Temperature variation in glasshouse during experiment (C)	Total sunshine (h)
First-year alders					
21 Aug. 1969*	12.00	6.5	0.60	19-23	4.6
	20.15	3.1			
1 Sept.1969**	12.00	8.8	1.88	20-3	8.2
	24.00	6.2			
12 July 1973	13.00	3.41	1.82	19-21	Nil
	24.00	4.33			
Second-year alders					
29 Aug. 1969	13.00	2.22	0.41	20-4	10.1
	24.00	1.92			
12 July 1973	13.00	1.92	0.25	19-21	Nil
	24.00	1.74			

*From Wheeler (1969) and **Wheeler (1971).

effects on specific dinitrogen fixing activity of soybean and *Alnus* plants have been observed with the exposure of high light intensity and longer photoperiod. It has been shown that self-shading and mutual-shading depress the dinitrogen fixing activity considerably in the older plants. Fifty per cent shading imposed at the end of flowering stage in soybeans decreased the fixation from 125 to 91 kg N ha^{-1} $season^{-1}$ while supplemental light increased the fixation to 165 kg N ha^{-1} $season^{-1}$ (Sloger et al., 1975). Wheeler (1971) has observed a drastic curtailment in the rate of acetylene reduction by the young *Alnus* plants in complete darkness within 24 hr. The diurnal effect on the acetylene reduction due to variations in light intensity by the nodules of one and two years old *Alnus* plants is given in Table 13. Bond (1971) studied the diurnal fluctuations in dinitrogen fixing activity of glasshouse grown *Casuarina* plant and found that fixation was double at midday as compared with rates in the early mornings and late evenings. A large part of the diurnal variations is thought to be attributed to the differences in temperature. In contrast, Wheeler (1969) observed that the variations in dinitrogen fixing activity of glasshouse grown *Alnus glutinosa* and *Myrica gale*, is due to change in the light intensity since the temperature was kept constant. Silvester (1976) has shown that excised stem nodules of *Gunnera denatata* inhabited by a blue-green alga, *Nostoc punctiforme*

readily responded to light intensity by ten-fold increase in the nitrogenase activity with nodules kept at 40 Wm^{-2} over those kept in dark. The light response was found to be almost linear over the range 0-40 Wm^{-2}. The different wavelengths of light affect the dinitrogen fixing activity of plants considerably (Lie, 1971). The dinitrogen fixing activity of soybeans increased two the three-fold when they were transferred from blue to red light. The initiation of nodule formation and nodule development is completely inhibited if the roots were exposed to direct sunlight even for a brief period of 5-10 min (Lie, 1971). However, the mechanism has not been elucidated so far.

ii. Carbon dioxide: The partial pressure of atmospheric carbon dioxide has a strong influence upon nodule formation and dinitrogen fixing activity of the plants. It has been shown that Glycine max var. Kent in an atmosphere enriched with CO_2 ranging from 800-1200 ppm (approx. 0.5 kg CO_2 hr^{-1}) has increased the dinitrogen fixing activity by more than three times over the control. Soybean plants exposed to an atmosphere with three-times the normal concentration of CO_2 increased the dinitrogen fixing capacity from 75 kg N ha^{-1} to 425 kg N ha^{-1} (Hardy & Havelka, 1974). The availability of carbohydrates to Rhizobium in legumes has been regarded as an important factor limiting dinitrogen fixation. The



ig. 11 : Schematic diagram of symbiotic nitrogen fixation system to show possible limiting factors. From Hardy & Havelka (1976).

relative supply of carbohydrates to dinitrogen fixing nodules and other physiological processes in the plant is shown diagrammatically in Fig.11. Soybeans grown in relatively nitrogen rich soils derive only 25 per cent of their nitrogen requirement by fixing the atmospheric dinitrogen. The acetylene reduction assay has revealed that the plants obtained about 80 per cent of its nitrogen requirement by fixing atmospheric dinitrogen (Hardy & Havelka, 1975). It would be interesting to examine the effect of carbon dioxide enrichment on non-legume flowering plants.

The tropical C_4-grasses possessing root-bacterial loose associations such as corn-*Spirillum*, *Paspalum*-*Azotobacter* and *Digitaria*-*Spirillum* have been found to be efficient in dinitrogen fixation. The carbohydrate supply in case of these C_4-grasses does not seem to be a limiting factor as already they possess an efficient C_4-pathway of photosynthesis (Day & Dart, 1976). However, detailed studies have not been made to show whether carbohydrate supply is a limiting factor or not in case of grass-bacterial dinitrogen fixing systems.

iii. Oxygen : The partial pressure of oxygen affects biological dinitrogen fixation severely as it is a reductive process requiring relatively low oxygen tension. It affects dinitrogen fixation in two ways, 1. by irreversible inactivation of enzyme nitrogenase presumably due to oxidation and, 2. by inhibiting the reduction of dinitrogen (Burns & Hardy, 1975). Both protein components of the enzyme nitrogenase are sensitive

to oxygen inactivation but the Fe-protein is relatively more sensitive (Kelly _et al._, 1967). Different mechanisms have been evolved by the dinitrogen fixers to protect their enzymes from oxygen inactivation.

The _Clostridia_ have overcome the problem of oxygen sensitivity by adopting anaerobic mode of respiration. The aerobic bacteria, _Azotobacter_, _Beijerinckia_ and _Derxia_ maintain their dinitrogen fixing activity through respiratory protection, i.e., reducing the ambient pO_2 by maintaining exceedingly high rates of respiration (Postgate, 1974).

The dinitrogen fixing blue-green algae possess specialized cells called heterocysts which are responsible for dinitrogen fixation. It has been shown that these cells possess photosystem I, but lack photosystem II responsible for oxygen evolution (Postgate, 1974). There are, however, certain non-heterocystous blue-green algae, e.g., _Microcystis_, _Gloeocapsa_ and _Anacystis_ which also fix dinitrogen efficiently. These are believed to fix dinitrogen only under low oxygen tension provided by low illumination or by secreting large amounts of slime around their cells which dissolve excess oxygen (Postgate, 1974).

In root nodulated plants the nodule wall and the bacteroid membrane are believed to reduce the oxygen tension (Bergersen, 1971). Leghaemoglobin, a non-haeme iron-protein, is a prominent feature of nodulated legumes which has been shown to possess high affinity for oxygen and serves as an oxygen carrier at required pO_2 to the bacteriods (Wittenberg _et al._, 1974).

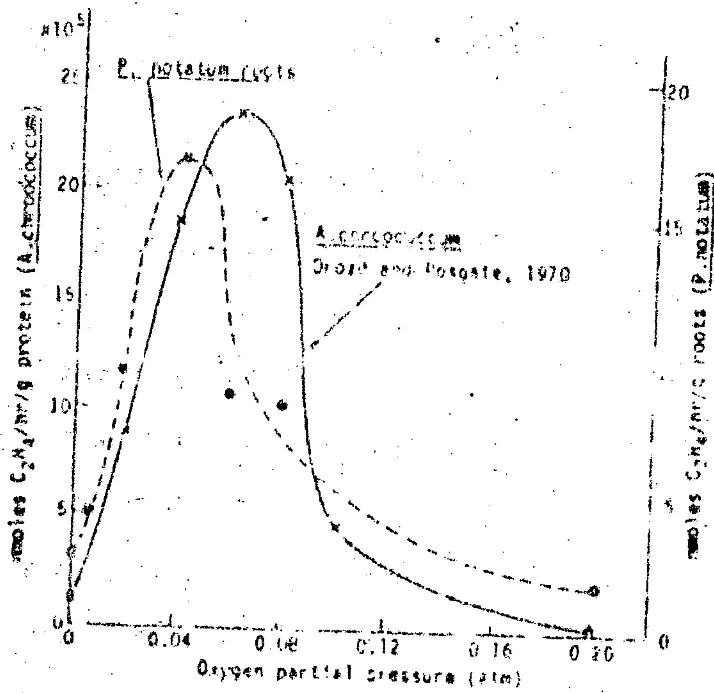


Fig. 12: Effect of oxygen tension on the nitrogenase activity of field-grown *Paspalum notatum* roots extracted from the soil (Döbereiner *et al.*, 1973) and on nitrogenase activity of continuous cultures of *Azotobacter chroococcum* at pO_2 0.09 atm (Drozd & Postgate, 1970).

However, no such oxygen carrier has been reported from the actinomycete-like endophyte of the non-legume flowering plants. Almost nothing is known of the mechanism that how these microorganisms protect their enzyme nitrogenase from the oxygen inactivation.

The tropical dinitrogen fixing grass root-bacterial associations such as *Paspalum - Azotobacter* and *Digitaria-Spirillum* require a low pO_2 ranging from 0.015 to 0.04 atm for optimum dinitrogen fixing activity as shown in Fig. 12 (Döbereiner *et al.*, 1973; Döbereiner, 1975; Abrantes, 1975). The mechanism involved in maintaining such a low pO_2 at the site of dinitrogen fixation has not been studied so far. However, it is likely that respiratory protection, excessive slime production resulting in the formation of a capsule around the bacteria may provide the necessary protection against oxygen damage (Abrantes, 1975). In case of low availability of carbohydrates for respiratory protection the protein components of bacterial nitrogenase appear to undergo some conformational change in which they are passively protected from oxygen damage. Conformational protection probably implies some temporary changes in the hydrogen bonding and subcellular compartmentation in which membranes are involved (Postgate, 1974).

iv. Temperature : Soil temperature influences biological dinitrogen fixation by affecting the metabolic activities of the microorganisms involved. The optimum dinitrogen fixation by legume-*Rhizobium* symbiosis occurs at 25 C.

Different stages of legume root nodules formation take place at different temperatures thus effective infection and nodule formation take place at relatively low range of temperature varying from 20 to 22 C (Dart et al., 1976). Breeding experiments with soybean have shown that nodule formation is temperature dependent and is determined by one major gene present in the host plant (Lie, 1971). The root nodulated non-legume flowering plants have the range of temperature for optimal dinitrogen fixation from 19 to 21 C. *Alnus glutinosa*, shows its maximum dinitrogen fixing activity at 21 ± 1 C (Akkermans & VanDijk, 1976).

The ra-nge of temperature for optimum dinitrogen fixation is 31 C to 40 C in C_4-grass bacterial systems in comparison with legumes having the upper limit of 27 C (Dart et al., 1976). Neves et al. (1975) have shown that the optimum nitrogenase activity of pure culture of *Spirillum lipoferum* isolated from corn roots takes place at 31 C. The *Spirillum* strains isolated from corn roots and *Digitaria* roots do not show much appreciable differences in their nitrogenase activities at 31 C but their behaviour is totally different at low temperature. *Spirillum* strain isolated from corn roots showed five-times more nitrogenase activity at 22 C than that of isolated from *Digitaria* roots at the same temperature (Döbereiner et al., 1975). The cause of variation in the nitrogenase activity of *Spirillum* strains at low temperatures has not been explained as yet and deserves careful study.

2. Edaphic factors

Soil factors have the most profound influence on the process of biological dinitrogen fixation. Availability of inorganic combined nitrogen, soil nutrient status, soil pH and calcium, water stress, and ethylene production are important parameters affecting the fixation of dinitrogen.

I. Inorganic combined nitrogen : Availability of inorganic combined nitrogen, most commonly ammonium and nitrate ions have an inhibitory effect on biological dinitrogen fixation. The degree of inhibition depends on the concentration of inorganic nitrogen compounds, time of their application and the types of host-symbiont strains. However, it is well known that ammonia is the end product of biological dinitrogen fixation, but it never accumulate in high concentrations as it is immediately incorporated into amino acids and amides. It has been shown that the transfer of pea plants grown in a medium free from inorganic combined nitrogen to a culture solution containing 315 ppm nitrate ions has resulted in complete cessation of dinitrogen fixation within 48 hours (Oghoghorie, 1971). In *Alnus* and *Hippophaë* it has been shown that the application of small quantities of inorganic nitrogen between 10 to 15 kg N ha^{-1} reduces the fixation of dinitrogen to one fifth of the values without applying the inorganic combined nitrogen (Akkermans, 1971).

However, the behaviour of tropical C_4-grass root-bacterial associations is quite different from that of root

nodulated legumes and non-legume flowering plants with regard to the supply of inorganic nitrogen. Döbereiner *et al.* (1975) have shown that the tropical C_4-forage grasses namely, *Digitaria* and *Pennisetum* have no effect on their dinitrogen fixing activity when 20 kg N ha^{-1} was added to the soil. This observation in these C_4-grasses is of fundamental importance because of the possibility of taking simultaneous advantage of both fertilizer nitrogen added and biological dinitrogen fixation.

Generally, it is believed that the inhibition of biological dinitrogen fixation is caused due to the repression of biosynthesis of nitrogenase by ammonium ions. However, recently it has been shown that the enzyme glutamine synthetase is involved as promotor in the biosynthesis of nitrogenase (Skinner, 1976). High concentration of ammonium ions induces conformational changes in glutamine synthetase which fails to act as a promotor for the synthesis of nitrogenase. In the absence of ammonium ions glutamine synthetase restores to its original structure and starts acting as a promotor for the biosynthesis of nitrogenase as shown in Fig. 9 (Skinner, 1976).

II. Mineral nutrition : Biological dinitrogen fixation requires a set of mineral nutrients.

Following are the important nutrient elements which are involved in biological dinitrogen fixation:

a. Molybdenum

b. Iron

c. Cobalt, and

d. Phosphorus

a. Molybdenum : The involvement of molybdenum as a micronutrient in biological dinitrogen fixation was known long before the enzyme nitrogenase was isolated. It forms an essential constituent of the MoFe-protein component of nitrogenase. Jensen and Spencer (1947) have shown that vanadium may partly substitute for molybdenum, but vanadium-nitrogenase is relatively less active in comparison with molybdenum-nitrogenase. Mo atom probably provides an active site for the binding of dinitrogen and by undergoing repeated oxidation-reduction mediates the transfer of electrons from various strong reductants to dinitrogen and reduces it to ammonia (Streicher & Valentine, 1973). In molybdenum deficient soils dinitrogen fixation ceases completely and plants can survive only when supplied with molybdenum in the form of molybdate ions or with fertilizer nitrogen (Postgate, 1974).

b. Iron : Iron constitutes an essential part of both the MoFe-protein and Fe-protein components of nitrogenase. The severe deficiency of Fe ions affect the biosynthesis of nitrogenase significantly. Fe atom also forms an essential constituent of the ferredoxin which acts as an electron carrier for transferring electrons from the electron donors of low redox-potential to enzyme nitrogenase (Quispel, 1974). Repeated oxidation-reduction of Fe atoms of ferredoxin and nitrogenase plays the vital role in transferring electrons to dinitrogen (Streicher & Valentine, 1973).

c. Cobalt : Cobalt is known to facilitate the process of infection and nodule formation both in legume and non-legume flowering plants. The importance of cobalt in low concentrations, 10-15 ppm was realized by observing promoted nodule development and dinitrogen fixation in the potted plants of _Glycine max_, _Alnus oregana_ and _Ceanothus velutinus_ (Kliewer & Evans, 1963). Information is lacking on the mechanism of the involvement of cobalt in enhancing the process of infection, and healthy growth of root nodules.

d. Phosphorus : Phosphorus plays an important role, as ATP which supply the energy for the reduction of dinitrogen. The free living bacteria of the family Azotobacteriaceae namely, _Azotobacter chroococcum_, _Derxia_ and _Klebsiella_ require large amounts of phosphate ions on account of their high respiratory activities to reduce ambient pO_2 and also to provide necessary ATP molecules for dinitrogen fixation. Becking (1962) has reported that _Azotobacter chroococcum_ requires upto 4.5 mg P g^{-1} of mannitol as a metabolite for respiration.

Since the respiration is regulated by the ADP : ATP ratios, therefore, the phosphorus deficient soils are generally found to be devoid of dinitrogen fixing microorganisms (Yates, 1970), and such soils seriously suffer from dinitrogen fixed biologically.

III. Soil pH and calcium : Soil pH is one of the important parameters influencing biological dinitrogen fixation.

Low pH inhibits the fixation of dinitrogen both in asymbiotic and symbiotic dinitrogen fixing systems. In acidic soils the availability of manganese and aluminium ions increases which are very toxic to plants (Döbereiner, 1966). Liming helps in increasing soil pH which reduces the solubility of both Mn^{2+} and Al^{3+} ions and at the same time it increases the solubility of molybdenum and iron ions which are essential for the biosynthesis of nitrogenase (Bould & Hewitt, 1963). The optimum pH varies from 6.5 yo 7.5 for optimum dinitrogen fixation both in legumes (Lowther & Loneragan, 1968) and grasses-bacterial symbiotic systems (Döbereiner et al., 1975). The role of soil pH on dinitrogen fixing activity of root nodulated non-legumes seems to be insignificant. As a survey of 650 plants of *Alnus glutinosa* at 244 sites in northwest France has reported that effective nodulation has been found in soils of pH 4.0 to 8.0. At most sites nodulation was reported as good, as nodule clusters ranging upto 7 cm in diameter even at pH 4.0 but at fifteen sites nodulation was found to be poor and at five sites no nodulation could be found (Bond, 1976).

Acidic soils are generally found to be devoid of micro-organisms especially, *Rhizobium meliloti*. Lowther and Longragen (1968) have reported that root nodule formation was inhibited in soybean plants in a culture medium ~~with~~ at pH 5.2 and low calcium ions concentration of 0.5 mM. At the same

Table 14. Effect of water stress on nodule number, size and acetylene reducing activity of *Phaseolus vulgaris* (44 days old).

	Rhizobium strain 3601		*Rhizobium* strain 3605	
	Control	Stressed	Control	Stressed
p moles C_2H_4 mg^{-1} min^{-1}	16.45	1.75	37.45	3.15
Nodule number	28.4	8.3	18.5	4.5
Average nodule wt (mg)	1.44	0.95	1.72	1.16
Water content of sand (%DW)	6.54	0.71	6.34	0.81

From Sprent (1976).

pH they also showed that effective nodule formation took place it increased the concentration of calcium ions from 0.5 to 0.8 mM. It has been suggested that higher amount of calcium is needed in the soils of low pH for the effective nodulation (Munns, 1970). However, the mechanism to explain the involvement of calcium ions in root nodule formation is not known so far.

IV. Water stress: The water stress influences the biological dinitrogen fixation by depressing the activity of developed root nodules and by inhibiting the nodule formation. The severe paucity of water may lead to stop dinitrogen fixation completely (Sprent, 1976). However, the adverse effects of water stress on root nodulated plants have been reported to be reversible provided that water losses from the root nodules do not exceed more than 20 per cent of their maximum fresh weight (Engin & Sprent, 1973). The legumes have been found fixing dinitrogen optimally at field capacity. In maize, it has been shown that at the wilting point of the dinitrogen fixing activity ceases completely (Vlassak *et al.*, 1973; Day *et al.*, 1975). It has been shown that during the dry days of March-April when two plots containing wild species of *Vicia*, *Melilotus* and *Medicago* mixed with tropical C_4-grasses were watered, the rate of acetylene reduction in these plots increased more than ten-fold over the control ones (Sprent, 1976). Some effects of water stress on *Phaseolus vulgaris* have been shown in Table 14.

Table 13. Effect of waterlogging on nodule number, size, water content and acetylene reducing activity of *Phaseolus vulgaris* cv. *Glamis*.

Treatment	Age (days)	Nodule no. $plant^{-1}$	Average nodule size(mg)	Water content (fresh: dry wt)	p mole: C_2H_4 mg^{-1} fresh wt min^{-1}
Waterlogged	29	15.1	3.63	2.72	4.10
Waterlogged	37	44.2	3.65	7.46	6.09
Waterlogged	43	94.5	4.49	9.67	6.30
Control	29	53.5	3.76	2.54	11.73
Control	37	58.4	5.36	5.42	15.47
Control	43	77.5	6.38	6.74	15.44

From Sprent (1976).

Waterlogging affects biological dinitrogen fixation adversely by reducing the nodule formation and their dinitrogen fixing activity. Generally, the adverse effects ofw waterlogging have been found to be reversible on the restoration of normal conditions. Waterlogging depresses the process of infection, nodule formation and their number to a great extent in legumes as well non-legume flowering plants. It has not been yet critically assessed whether the lowering of nodule formation is due to the unavilability of microsymbionts or due to its inhibition of the process of infection or both. However, Mague and Burris (1972) have reported that waterlogging depresses nodule formation and dinitrogen fixation largely due to the oxygen depletion required in respiration by both the plant roots and the microsymbionts. Nodule number, size, water content and acetylene reducing activity of *Phaseolus vulgaris* are greatly affected by waterlogging as shown in Table 13.

v. Ethylene : Ethylene at very low partial pressure has no inhibitory effect on dinitrogen fixation. However, at high concentrations of about 4 ppm it has been shown to inhibit nodule formation and dinitrogen fixation in *Phaseolus vulgaris* (Grobbelaar *et al.*, 1971).

Ethylene has been found to be the most active gaseous emanations from peat soils and waterlogged habitats which are comparatively rich in organic matter. If the ethylene produced in the soil was removed continuously, an appreciable increase in dinitrogen fixing activity may take place. In a long-term

experiment, Grobbelaar *et al.* (1971) have demonstrated that a continuous removal of ethylene gas from excised nodulated roots of *Phaseolus vulgaris* resulted in a more than ten-times increase in dinitrogen fixing activity.

3. Biological factors

In case of symbiotic dinitrogen fixing systems there exists a great degree of specificity between the host and its microsymbiont. The host-microsymbiont specificity is the result of mutual fulfilment of the specific requirements of both the partners. Injury is another important biological factor which influences the process of biological dinitrogen fixation considerably. The host-microsymbiont specificity and the effect of injury on dinitrogen fixation are discussed in the following paragraphs.

I. Host-microsymbiont specificity : The degree of specificity between the host and the microsymbiont is very high in root nodulated plants. The investigations made so far indicate that *Rhizobium* symbiosis is highly specific. However, there is an exception to this in which an unidentified species of *Rhizobium* forms foot nodules on a non-legume flowering plant namely, *Trema cannabis* that fixes dinitrogen efficiently (Trinick, 1973). *Rhizobia* are classified on the basis of their restricted invasiveness and the strains are named on the basis of their selectivity towards different species and strains of legumes plants (Child, 1976). For example, *Rhizobium leguminosarum* forms root nodules only

with pea plants and not with soybean and clover plants. *Rhizobium* sp. are further classified into strains on the basis of some characteristic of host or some other special feature of the life cycle of *Rhizobium* sp., e.g., *Rhizobium meliloti* strain U_{45} forms different colony with *Melilotus*.

Previously, it was considered that specificity of legume-*Rhizobium* symbiosis is due to the presence of leghaemoglobin which is formed by the combination of leghaeme and globin components contributed by the bacterium and the host plant respectively (Postgate, 1971). Recently, the work of Hambin and Kent (1973) have suggested that lectins which are chemically phytochemeglutins produced by the legume roots are responsible for the specificity of legume-*Rhizobium* symbiosis. They hypothesized that lectins are involved in binding *Rhizobia* to legume roots. Bohlool and Schmidt (1974) tested this hypothesis by labelling soybean lectins with fluorescein isothiocyanate to make the protein fluorescent. They found that the lectin bound to 23 out of 25 strains of *Rhizobium japonicum*. They have suggested that an interaction between legume lectins and *Rhizobia* is responsible for the specificity of symbiosis.

The non-legumes such as *Alnus*, *Casuarina*, *Ceanothus*, and *Hippophaë* have been reported to fix dinitrogen in specific association with actinomycete-like endophyte most probably the genus *Frankia*. The specificity is so high that neither the microsymbiont has been possible to cultivate *in vitro* possessing

consistent dinitrogen fixing activity nor it forms symbiosis with plants other than the specific host plant. Advancement in the elucidation of the symbiotic interactions and understanding of the factors responsible for dinitrogen fixation in this type of symbiosis can only be possible after axenic cultues of the endophyte become available. The problems involved in obtaining such cultures are still to be solved. The lichen, genus Peltigera aphthosa contains Nostoc species, a green alga Coccomyar sp., and a fungus (an ascomycete) as symbiotic partners. Nostoc contains 20 per cent heterocysts in symbiotic conditions while the free living Nostoc contains only 4-5 per cent. This tendency to form more heterocysts by Nostoc in lichen appears to be developed because of the availability of enough ready made food provided by the green alga (Stewart & Rowell, 1977).

The bacteria, Spirillum lipoferum and Azotobacter paspali form associative symbioses with roots of tropical C_4-grasses such as corn, Pennisetum, Digitaria and Paspalum. However, these grass root-bacterial symbioses are not so specific as root nodulation in legumes and non-legume plants. The factor responsible for these grass root-bacterial associations appears to be the specific requirement of bacteria for malate and aspartate as food supply which could be easily met through the C_4-photosynthetic pathway (Dart & Day, 1975). This may be one of the factors but other physiological factors also seem

to be involved in establishing these symbiotic associations.

ii. Injury : An injury caused to a dinitrogen fixing plant or a system lowers the dinitrogen fixing activity. Intact root nodules fix dinitrogen more efficiently in comparison with detached nodules and, furthermore, the intact plant is more efficient as compared to the detached nodulated root (Pate, 1971).

In tropical C_4-grasses a lag phase of 8-18 hr is introduced before they resume their dinitrogen fixing activity in the assay chamber (Harris & Dart, 1973). It is believed that disturbance of relatively anaerobic conditions attained by the microorganisms in association with grass roots is responsible for the lag period.

4. Physiological factors

There are several physiological factors which directly or indirectly influence the process of biological dinitrogen fixation. In addition, hydrogen evolution a side reaction to dinitrogen fixation is an important physiological factor which substantially affects dinitrogen fixing activity of the plants.

I. Hydrogen evolution : Dinitrogen fixers have been shown to evolve hydrogen gas as a byproduct in the process of biological dinitrogen fixation (Burns & Hardy, 1975). Nitrogenase-dependent hydrogen evolution has been studied both _in vitro_ and _in vivo_, but the overall effect of hydrogen evolution has not been yet critically assessed. During the electron transfer to dinitrogen by the enzyme nitrogenase some of the electrons

are trapped by protons present in the medium resulting in the evolution of hydrogen gas (Schubert & Evans, 1976). In most of the symbiotic systems only 40-60 per cent of the electrons are transferred from nitrogenase to dinitrogen while the remainder are lost through hydrogen evolution.

Hydrogen evolution may severely reduce the dinitrogen fixing efficiency of many agronomically important plants especially where carbohydrate supply is considered a limiting factor to biological dinitrogen fixation (Hardy & Havelka, 1973). Jones and Burris (1972) have shown that *in vivo* about four ATP molecules are hydrolyzed per hydrogen molecule is produced. In contrast, Burns and Hardy (1975) have suggested that no net ATP- dependent hydrogen gas is produced via nitrogenase. Thus, the question has not been resolved adequately and requires further evaluation. *Azotobacter* and symbiotic *Vigna sinensis* *Vicia sativa* and *Alnus rubra*, *Elaeagnu angustifolia* and *Ceanothus california* have developed a mechanism for utilizing the hydrogen to recoup a part of their energy wasted in its production, by a specialized system of hydrogenases (Dixon, 1972). It is now well recognized that hydrogen evolution accompanied by dinitrogen fixation is a major factor to affect the process of dinitrogen fixation and thus ultimately the productivity of many agronomically important crops (Schubert & Evans, 1976).

Chapter 7

Future Aspects

The biological dinitrogen fixation is driven by the solar energy through the supply of carbohydrates. Adequate supplies of fixed nitrogen are essential for increasing the food production required by the growing population. Scarcity and the price rise of raw materials needed for the manufacture of nitrogen fertilizers and the problem of environmental degradation resulting from the manufacture and use of nitrogen fertilizers have greatly promoted interest in the biological dinitrogen fixation.

The International Biological Programme which lasted from 1964 to 1974 and the use of new technical developments in the methodology of dinitrogen fixation have provided a great stimulus to the study of dinitrogen fixation in non-leguminous flowering plants. The non-legume flowering plants fixing dinitrogen are of two types. Type I are woody shrubs with the microsymbionts in specialized structures called nodules and Type II are recently discovered non-nodulated tropical C_4-grasses which develop a loose association with dinitrogen fixing bacteria. Alnus is the most extensively studied among the non-legume flowering plants.

Through the work of Bond (1967) the root nodulated non-legumes have received substantial attention. Some of the root nodulated plants namely, Alnus, Casuarina, Ceanothus, Coriaria, Elaeagnus, Hippohhaë, and Myrica play an important role in soil

development and plant succession by colonizing nitrogen deficient habitats such as bare slopes and sand dunes. It would be interesting to examine non-legume vascular plants inhibiting the nitrogen deficient soils for their ability to fix dinitrogen. In India, there are found fifty species belonging to twelve different root nodulated genera of non-legumes which are still to be investigated for their capability to fix dinitrogen. There is little information on the amount of dinitrogen fixed by leaf nodulated plants of the families Rubiaceae, Dioscoriaceae, Myrsinaceae and by the dinitrogen fixing bacteria that occur in other plant structures such as *Coprosma* stipular glands.

Döbereiner *et al.* (1975) have reported high rates of dinitrogen fixation in some forage grasses and maize upto 1 kg N ha^{-1} day^{-1}, and 2 kg N ha^{-1} day^{-1} respectively. The most active dinitrogen fixing bacteria forming loose associations with roots of maize, *Sorghum* and some other C_4-grasses are *Spirillum lipoferum*. However, the grasses-bacterial loose associations have not been supported and gained much importance from different parts of the world. Only a detailed study with regard to microorganisms associated and *in situ* measurement of dinitrogen fixing potential may reveal the true importance of the C_4-grasses-bacterial loose associations. The factors involved in C_4-grasses root-bacterial loose associative systems have not been known so far.

Host-microsymbiont specificity determines the possibility of dinitrogen fixation in higher plants. The actinomycete-like endophyte of root nodulated non-legumes is still to be cultivated in axenic cultures fixing dinitrogen. The true nature of the endophyte has not been identified so far which probably seems to be the genus, *Frankia*.

Except, *Trema aspera* which forms extensive root nodules (Trinick, 1973), *Rhizobium* has been known to form root nodules with legumes. However, recently the cowpea *Rhizobium* strain has been shown to fix dinitrogen in association with cell cultures of non-legumes namely, wheat, rape grass, brome grass and tobacco (Child, 1975). Different *Rhizobial* strains of soybean and cowpea have also been shown to fix dinitrogen in pure cultures (Kurz & LaRue, 1975; McComb et al., 1975). Why *Rhizobium* does not form symbiotic associations with cereals and other non-legumes is a challanging question.

Rapid progress has been made in understanding of composition, properties and catalytic activity of nitrogenase. Some sixteen different dinitrogen fixers have yielded their nitrogenases. The understanding of these aspects with regard to the nitrogenase of the microorganisms which form symbiotic associations with non-leguminous flowering plants is lacking. Characterization of nitrogenase from the actinomycete-like endophyte of the root nodulated non-legumes, and *Spirillum lipoferum* which forms loose association with the roots of C_4-

grasses has not been done. A comparative study of nitrogenase obtained from different diazotraphs is important to ascertain the similarities and differences with regard to composition, oxygen sensitivity, cold-lability, and catalytic activity. Study

The biochemistry of dinitrogen fixation has been confined to a limited number of microorganisms, e.g., *Azotobacter*, *Clostridium*, *Rhizobium*, *Klebsiella*, *Anabaena*, and *Nostoc*. However, the biochemistry of dinitrogen fixation in actinomycete-like endophyte and *Spirillum lipoferum* which are the important microsymbiots of non-legume flowering plants n especially with regard to ATP as energy source, electron donor and electron carrier have not been made successfully so far. It has been known that ammonium ions inhibit the biosynthesis of nitrogenase. However, the exact mechanism is not known. A peculiar phenomenon associated with dinitrogen fixers is the nitrogenase-dependent hydrogen evolution which greatly affects the efficiency of dinitrogen fixation. At the same time, Schubert and Evans (1976) have observed that some legumes, i.e. *Vigna sinensis* and *Vicia sativa* and non-legumes, e.g., *Alnus rubra*, *Ceanothus velutinus*, *Elaeagnus angustifolia* and *Myrica californica* do not evolve hydrogen. The mechanism of lack of hydrogen evolution and its quantitative effect on the efficiency of dinitrogen fixation need to be critically assessed.

The important environmental factors which greatly influence biological dinitrogen fixation are light, carbon

dioxide, temperature, water stress, and inorganic combined nitrogen. Increased light intensity and carbon dioxide concentration result in the increased dinitrogen fixation through the supply of photosynthate to the associated symbiont. Supply of photosynthate to *Rhizobium* in the nodules of legume roots is frequently a limiting factor for dinitrogen fixation. The study of the effect of increased carbon dioxide concentration and photosynthate supply to microorganisms associated with non-legumes is yet to be made.

The dinitrogen fixation is a reductive process and is sensitive to molecular oxygen. The physiologically and ecologically different groups of dinitorgen fixers have developed suitable mechanisms to protect their nitrogenases from O_2-inactivation. The protection of nitrogenase from oxygen in the case of root nodulated non-legumes has not been studied. However, nodular wall and certain membranes are thought of providing necessary protection. Free living bacteria, e.g., *Azotobacter*, *Beijerinckia*, *Derxia*, *Klebsiella*, and *Spirillum* in loose associations with the roots of tropical C_4-grasses are believed to protect their nitrogenase from O_2-inactivation by respiratory protection, excessive slime production, and conformational protection.

Effect of temperature on dinitrogen fixation requires a careful study. The nitrogenase activity of *Spirillum* strains isolated from the root surfaces of corn and *Digitaria* is same at 31 C but at 22 C the corn strain exhibits five-times more

activity in comparison with *Digitaria* strain (Döbereiner *et al.*, 1975).

The inorganic combined nitrogen namely, ammonium and nitrate ions inhibit the fixation of dinitrogen in both root nodulated legumes and non-legumes. However, in contrast, it has been shown that fertilization of grasses, *Digitaria* and *Pennisetum* with 20 kg N ha^{-1} had no effect on their dinitrogen fixing activity (Döbereiner *et al.*, 1975). Absence of inhibitory effect by fertilizer nitrogen in C_4-grasses-bacterial associations is of practical importance for taking the advantage of both fertilizer nitrogen and biological dinitrogen fixation.

The discovery of non-legume diazotrophs has provided a new hope of proving the fixed nitrogen required for increasing di crop fertility. A proper understanding of symbionts associated with non-legumes and the factors responsible for symbiosis, in the enzyme nitrogenase, the various factors affecting dinitrogen fixation, and which influence their contribution to the nitrogen economy of the nature is an important area of investigation. In view of the above the non-legume diazotrophs deserve intensive study on priority basis.

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