TEN DECADES OF RESEARCH ON BIOLOGICAL NITROGEN FIXATION

N. S. SUBBA RAO
Senior Scientist, INSA, University of Agricultural Sciences, Bangalore 560 065, India.

ABSTRACT

Biological nitrogen fixation (BNF) was first discovered in legume-*Rhizobium* symbiosis one hundred years ago and subsequently in heterotrophic and photosynthetic nitrogen fixing bacteria. The discovery of BNF in cell-free extracts of *Clostridium pasteurianum* initiated indepth studies on the nitrogenase complex. Similarly, discoveries of the acetylene reduction technique as a facile method to assess the extent of nitrogenase activity and the accurate method to quantify the amount of N_2 fixation by ^{15}N enrichment procedure have helped to enlarge the scope of investigations in BNF. The genetic determinants of N_2 fixation (*nif* genes) in bacteria have been understood as a cluster of 17–19 genes in *Klebsiella pneumoniae* and by homology experiments, the *nif* genes of other N_2 fixing micro-organisms are also being unravelled. The importance of plasmids controlling nodulation and N_2 fixation in rhizobia has been emphasized and strategies to clone *nif* genes into higher plants envisaged. Our knowledge of *Frankia*, nodulating several non-leguminous plants increased with the isolation and characterization of the nitrogen fixing actinomycete in the root nodules of *Comptonia perigrina*. Nodules on stems of *Aeschynomene* and *Sesbania rostrata* have been highlighted to give a new dimension to the old practice of green manuring in rice cultivation. The practice of legume inoculation with rhizobia and the application of blue green algae and *Azolla* in rice cultivation as biofertilizers have been found useful. A new biofertilizer containing *Azospirillum* for millet crops has been tested and recommended. Of late, dual inoculation effects of rhizobia and VAM fungi (both in symbiosis with legumes) to augment nitrogen as well as phosphorus nutrition of pulse crops are being field tested.

INTRODUCTION

The elemental nitrogen of the atmosphere is reduced to ammonia by the nitrogenase enzyme complex in several bacteria, some free-living and others occurring in symbiotic associations with plants. The discovery of nitrogen fixation by nodule bacteria was made a century ago and it is worthwhile to look back and take stock of the significant achievements in research in BNF processes because BNF is currently relevant in the context of our increasing reliance on renewable sources of energy for part of the inputs on the farm rather than continuing to depend totally on fossil fuels to produce nitrogenous fertilizers.

THE DISCOVERY OF LEGUME FIXATION

It was September 1886 and the venue was Berlin where at the 58th Conference of German scientists, Hellriegel[1] read a paper entitled what is the source of Plant's nitrogen? Two years later Hellriegel and Wilfarth[2] published detailed account of carefully conducted pot experiments on peas and clovers to show that nodules on roots of these legumes fixed N_2 from the atmosphere. In 1888, a Dutch scientist Beijerinck[3,4] isolated the causative bacterium in legume root nodules and named it *Bacillus radicicola* (presently known under the generic name *Rhizobium*). Prior to these two milestones in our knowledge, remarkable work on the differential abilities of legumes and cereals to accumulate plant nutrients, more particularly nitrogen was done by Boussingault[5] in France and Lawes and Gilbert[6] in England despite the fact that they had overlooked the role of root nodules in nitrogen fixation and hence not really understood the reasons for the vexed question of how legumes alone could accumulate more of nitrogen than cereals. Very soon confirmations of these findings came from all over Europe and North America followed by the descriptions of nodule morphology and the formation of bacterial infection thread (which was earlier confused with fungal hyphae) by Marshall–Ward[7] and physiology including application to agriculture by Nobbe and Hiltner[8].

FREE-LIVING HETEROTROPHIC NITROGEN FIXERS

The discovery of symbiotic N_2 fixation was soon followed by the isolation and identification of several free-living N_2 fixers. The first was the anaerobic free-living *Clostridium pasteurianum* isolated by the Russian microbiologist Winogradsky[9]. The ubiquitous aerobic *Azotobacter chroococcum* was isolated by Beijerinck[10]. Starkey and De[11] isolated *A. indicum* from paddy fields of India which was later relegated to a new genus *Beijerinckia* by Derx[12]. De *et al*[13] isolated a new bacterium from West Bengal soils which was named as *Derxia*.

Lohnis and Pillai[14,15] detected N_2 fixation in *Klebsiella pneumoniae* and Becking[16] from Netherlands isolated the nitrogen fixing members of microaerophilic *Spirillum* which was later highlighted by Bulow and Dobereiner[17] from Brazil as *Azospirillum* living in associative symbiosis with graminaceous plants. It was again Dobereiner[18] who described the specialized association of *Azotobacter paspali* with the roots of a grass *Paspalum*.

FREE-LIVING PHOTOSYNTHETIC NITROGEN FIXERS

De[19] from West Bengal pointed out for the first time that cyanobacteria (blue-green algae) play a role in the BNF processes in low-land rice soils which was confirmed and documented in the UK[20], Japan[21] and India[22].

That anaerobic photosynthetic purple sulphur bacteria fix nitrogen was established in *chromatium*[23,24]. Likewise, the ability of *Rhodopseudomonas* and *Rhodospirillum*, the anaerobic non-sulphur photoheterotrophs to fix N_2 was also simultaneously established[25]. Among the anaerobic photosynthetic green sulphur bacteria, *Rhodospirillum rubrum* has been studied extensively after the initial publication of Gest and Kamen[26] on the ability of the species to fix N_2.

There were many claims to point out that several micro-organisms fix N_2. It was however discovered that some claims could not be considered unequivocal because ^{15}N enrichment tests proved to be negative in many instances.

ENTRY OF RHIZOBIUM INTO PLANTS — AN ENIGMA

It is clear that rhizobia either enter root hairs or directly penetrate the epidermal cells depending on the plant species but the enigmatic question is how they[27] enter? No enzymatic actions on the root surface have been clearly linked with infection except that a lectin mediated recognition due to host-bacterium interaction has been proposed[28,29]. Trifolin, a lectin from root hairs of clovers has been shown to bind to specific polysaccharide receptors on *Rhizobium trifolii* cell surface, thereby functioning as a 'cell recognition molecule'[30]. This host-bacterial specificity is plasmid encoded[31].

THE NITROGENASE ENZYME COMPLEX

Unlike the energy intensive Haber Bosch's ammonia process (requiring 500°C temperature and pressures over 350 atm), nitrogenase reduces dinitrogen to ammonia at ambient pressure and normal temperature. This unique reaction can be carried out in the absence of bacterial cells as has been shown for the first time by Carnahan *et al*[32] in cell-free extracts of *Clostridium pasteurianum*. Since that time, the universality of nitrogenase in all the N_2 fixing systems has been shown by purifications and reconstitution studies of the enzyme from more than 15 systems ranging from free-living to symbiotic ones. The observation that C_2H_2 is an inhibitor of N_2 fixation and is reduced to C_2H_4, by Schollhorn and Burris[33] and Dilworth[34] provided a facile tool for assaying the extent of N_2 fixation. This acetylene reduction method helped greatly to quicken the pace of research on nitrogenase because the method is less expensive and far more sensitive than the ^{15}N enrichment method[35]. The interchangeability of the smaller low molecular weight Fe protein fraction I from one N_2 fixing micro-organism with the larger high molecular weight Mo-Fe protein of another N_2 fixing micro-organism is possible without reduction in activity provided anaerobic conditions are maintained (See Burris *et al*[36] and Burgess[37] for details). To reduce one mole of N_2 to NH_3, the requirement of energy varies from 5 to 29 ATP, depending on the bacterial strain and conditions; electrons flow from ferredoxin (Shethna *et al*[38]) to the Fe protein which when complexed with ATP serves to reduce the Mo-Fe protein (and release inorganic phosphate) which eventually passes electrons to reducible substrates N_2 and $6H^+$ to form $2NH_3$ and $3H_2$. The discovery that free-living rhizobia (*R. japonicum*, *Rhizobium* sp. of the cowpea group) exhibit nitrogenase activity under defined conditions[39-43] has shown that nodule bacteria possess the complete genome for N_2 fixation.

The majority of the root nodulated legumes lose 30–50% of their nitrogenase electron flux as H_2 (Schubert and Evans[44] and Evans *et al*[45]), a finding which is an outcome of the initial observation of Dixon[46] who noted that nodules formed by *R. leguminosarum* had a hydrogenase system. Those nodules formed by uptake hydrogenase possessing rhizobia do not lose H_2 because of an active recycling of electrons lost by H_2 production back into the $N_2 \rightarrow NH_3$ reaction. This finding that rhizobial strains are either Hup^+ or Hup , depending respectively on their ability to lose H_2 or not, can be potentially exploited to augment legume yields for which field experimental evidence is yet equivocal probably due to competition from indigenous native soil rhizobia[45].

ASSIMILATION OF FIXED N_2

Glutamate and glutamine have been found to contain the highest amino acid label from either $^{15}N_2$ or $^{15}NH_3$ assimilation[46]. When NH_4 level is high glutamate dehydrogenase (GDH) is active and when the level of NH_4 is low both glutamine synthetase (GS) and glutamine-amide-2 oxoglutarate amino transferase or glutamate synthase (GOGAT) become operative in the process of assimilation of ammonia. This was shown by Nagatani *et al*[47] in *Klebsiella*, and Kondorosi *et al*[48] in *R. meliloti*. Subsequently many other findings have confirmed the operation of these two routes[49].

GENETICS

By following classical approaches of mutations, deletion mapping, cloning vectors and transposons, it is now known that seven distinct operons together known as *nif* totalling to at least 17–19 genes on the chromosome of *Klebsiella pneumoniae* exist which control the BNF process[50]. With this knowledge and by DNA homology experiments, various aspects of genetic control of nitrogen fixation have been deciphered in other N_2 fixing micro-organisms. In cyanobacteria, *nif* genes are also located on the chromosome but not clustered as in *K. pneumoniae*[51,52] whereas in *Rhizobium*[50] and in *Frankia*[53] (nodulating roots of non-leguminous plants) *nif* genes are borne on plasmids. Johnston *et al*[54] provided the first convincing evidence for the location of genes for nodulation (*nod* genes) in a self-transmissible plasmid pRL1J1 in a *R. leguminosarum* field isolate. Mutation in these genes results in non-nodulating (nod^-) phenotype. There are 'common *nod* genes' required for the nodulation of all legumes and *hsn* genes which appear to be required for recognition of a specific leguminous species. Similarly nitrogen fixation (*fix*) genes have been identified on the chromosome as well as on plasmids of *Rhizobium* which can be transferred to other *Rhizobium* species and *Agrobacterium tumefaciens*[55] or *Escherichia coli*[56]. Brewin *et al*[57] transferred the determinants for nodulation ability (nod^+), nitrogen fixation (fix^+) and hydrogen uptake (hup^+) from pRL6J1, a plasmid that was not self-transmissible on to a replicon that was transmissible at high frequency and carried a selectable drug marker. While plasmids control *nod* and *nif* functions in the fast growing rhizobia (*R. meliloti*, *R. leguminosarum*, *R. trifolii*), the slow growing ones (*R. japonicum*) have them either on their chromosome or on plasmid which needs further study[58,59].

By homology studies with 17 genes of *K. pneumoniae*, 10 *nif* genes have been identified in a chromosome cluster of *Azotobacter chroococcum* and *A. vinelandii*[60]. Hybridization between *K. pneumoniae* *nif* probes covering the entire cluster and total DNA of several *Azospirillum* strains revealed homology with *nif* HDK and *nif* A, which code for an activator of *nif* transcription[61]. All *Azospirillum* strains examined so far contain at least one plasmid even though no phenotypic property was demonstrated as plasmid-borne[62].

In filamentous cyanobacteria, ammonia induces only vegetative cell formation whereas on nitrogen-free media some of the vegetative cells transform into heterocysts, the seats of N_2 fixation due to induction of nitrogenase. The genes coding this enzyme (*nif* H, D and K) have been identified in *Anabaena* 7120 as one contiguous unit in an operon by hybridization and heterologous probes obtained from *K. pneumoniae*[63].

One of the fast growing fields of research is the genetics and molecular biology of N_2 fixing micro-organisms and we have no doubt come a long way in our knowledge on this subject since the time of Balassa[64] who first described transformation mechanism in *Rhizobium*.

NODULINS

Nodulins are nodule-specific proteins which are controlled by plant genes. Several nodulins have been characterized from soybean, pea and alfalfa. They determine the structure, maintenance and overall metabolism of the root nodule. According to

Verma and Nadler[65], nodulins could be sub-divided into proteins common to all legume nodules (common or C-nodulins) and species-specific or S-nodulins. Leghaemoglobin, a pink pigment in root nodules first recognized by Kubo[66] and which regulates O_2 supply to bacterioids (the seats of nitrogen fixation) is a C-nodulin. On the other hand, some enzymes for species-specific carbon-nitrogen metabolism[67] in the nodule which control the translocation of amides (glutamine, asparagine) as in pea and lupin or ureide[68] as in mungbean, cowpea and soybean can be classified as S-nodulins.

N_2 FIXING PLANTS?

If the *nif* gene cluster which controls the ability of N_2 fixing micro-organisms to reduce N_2 to NH_3 can be cloned into higher plants, the use of fertilizer nitrogen to crop plants may become superfluous. At the present time, far from being even remotely considered as successful in rendering plants self-sufficient with regard to its nitrogen needs, one can only mention the strategies envisaged in the transfer of *nif* DNA to higer plant cells. These strategies may involve the chloroplast DNA, the *Agrobacterium* Ti plasmid and the yeast cell which is the simplest of all eukaryotes[69].

The chloroplast DNA which is 80–100 md[70] in size has no doubt large ATP power necessary for N_2 fixation but is highly oxygenic, not so conducive to achieve anaerobiosis so essential for N_2 fixation. The oxygenation factor can, however, be overcome by controlling RuBP carboxylase activity or by the creation of mutant chloroplasts lacking in oxygenation which can function for N_2 fixation and the normal chloroplasts doing the function of photosynthesis.

A large indigenous plasmid called T_i plasmid required for the initiation of tumours has been identified in *Agrobacterium tumefaciens*, the incitant of 'Crown gall' in dicotyledonous plants[71,72]. When a bacterial transposon is introduced into the T_i plasmid, the entire transposon is also transferred to the plant and is stably inherited as part of the genome of the crown gall tumour[73] and hence once the crown gall is initiated by the tumour inducing DNA (T-DNA), galling can proceed without the need for fresh infection by the pathogen. The next step is to construct a T_i plasmid mutant devoid of all oncogenic functions of T-DNA which can still be efficiently transferred and integrated into the plant genome. Indeed, such a cointegrate plasmid pGV3850 useful in transformation of plant cells has been constructed[74–77] which needs further refinement so as to be a useful vector (in size) for the introduction of foreign DNA such as *nif* DNA into plant cells. However, the T_i plasmid strategy can only be adopted to dicots only because *A. tumefaciens* is not known to infect monocotyledonous plants (maize, rice, wheat, rye, barley, sugarcane, etc.) which is indeed a limitation for adoption of the strategy to food crops.

Yeast (*Saccharomyces cerevisiae*) is the simplest of eukaryotes whose genome is well understood and which can readily be transformed by plasmids[78]. Further, yeasts can be grown anaerobically which is advantageous to protect the O_2 sensitive nitrogenase from being inactivated. So far, *nif* genes from *K. pneumoniae* have been successfully cloned into yeast cells but the expression of N_2 fixation is however lacking[79].

FRANKIA

Janse[80] reported the occurrence of nodules on roots of *Casuarina* which was followed by reports from Miehe[81] and Narasimhan[82] from India. The first demonstration of the ability of extracts of *Casuarina* nodules in reproducing nodulation by inoculation experiments was done by Aldrich-Blake[83] in Dehra Dun, India. Other reports followed soon on *Casuarina* as well as other non-leguminous plants[84,85] capable of forming root nodules. Currently, Becking[86] describes 173 species of dicotyledonous plants from diverse families of the genera *Casuarina*, *Myrica*, *Comptonia*, *Alnus*, *Elaeagnus*, *Hippophae*, *Shepherdia*, *Ceanothus*, *Discaria*, *Colletia*, *Trevoa*, *Coriaria*, *Rubus*, *Dryas*, *Purshia*, *Cerocarpus* and *Datisca* which are known to be nodulated. The early confusion about the claims of isolation and nomenclature of the microsymbiont in non-leguminous plants was settled with the isolation of pure cultures of an actinomycete *Frankia*[87] from nodules of *Comptonia perigrina* by Callaham *et al*[88] in 1978 by enzymatic digestion of nodular tissue.

PARASPONIA

The only singular instance of nodulation of roots of non-leguminous plants by *Rhizobium* has been reported[89] in the family Ulmaceae from *Parasponia rugosa*, *P. parviflora* and *P. andersonii*, which were described earlier[89] under the genus *Trema*.

LEAF NODULES

The genera, *Ardisia* of Myrsinaceae and *Pavetta* and *Psychotria* of Rubiaceae are known to possess nodules on leaves. From time to time, several bacteria have been isolated from these nodules and described differently as *Streptomyces*, *Bacillus*, *Enterobacter*, etc even though nodules do not fix N_2 as tested by ^{15}N enrichment procedures or by acetylene reduction tests. On the other hand, cytokinin-like substances have been speculated to be responsible for the observed beneficial influence of leaf nodules on plants[90].

STEM NODULES

There are two genera of legumes which are known to bear typical nodules on stem due to rhizobial infection. They are *Aeschynomene* and *Sesbania*[91]. Jaensch[92] in 1884 observed stem nodules on *A. elaphroxylon* for the first time but subsequently such observations have been made in several countries in other species. Arora[93] from India made the first detailed structural analysis of these nodules. One of the significant observation in recent years has been the report of stem nodulation in *Sesbania rostrata*[94], which has great potentialities as green manure in lowland rice. What is more interesting is the possibility of inducing similar stem nodulation in pulse crops by genetic manipulation[91].

SEED AND SOIL INOCULANTS

Nobbe and Hiltner[8] in 1896 may be acredited as the first investigators to prepare an inoculant from pure cultures of rhizobia for different leguminous crops under the patented name 'nitragin'. The use of phosphates with rhizobia was first suggested by Thornton and Gangulee[95] in 1926. The classification of rhizobia based on the cross inoculation groups by Fred *et al*[96] in 1932 was infact the basis by which inoculants are even now prepared all over the world. Of the multitude of inoculants that are being produced for legumes, some of the standard ones come from USA[97] and Australia[98], and they set the quality control standards which have been adopted to the Indian situation by the Indian Standard Institution. In the USSR[99], *Azotobacter chroococcum* inoculant under the name 'Azotobakterin' was marketed in the early part of this century. *Azotobacter* inoculants are being used in India for cotton, maize and vegetable crops[100]. The use of *Azospirillum* inoculant for millets like bajra and ragi for saving fertilizer nitrogen input up to 20% level has been demonstrated and advocated under Indian conditions[100,101], which has also been substantiated in Israel[102]. Soil based blue green algal and *Azolla* inoculants have proved successful in India, China and Vietnam for rice cultivation and can save chemical inputs up to 30–40 kg N/ha[103–105]. Obligate endosymbiotic fungi known as vesicular-arbuscular mycorrhizal (VAM) fungi are known to inhibit the roots of legumes as well as non-legumes and help primarily in the phosphorus nutrition of plants[106]. One of the recent developments has been the added beneficial effect of dual inoculation of *Rhizobium* as well as VAM fungi despite the limitation that VAM fungi are not amenable to culturing on artificial media and one has to carry out experiments with soil and root based inoculum[107]. However, clearcut field results to merit large scale application of VAM inoculum to crops are yet to emerge.

One of the primary requirements for success in the use of any of these inoculants is the ability of inoculated N_2 fixing bacteria to survive in soil by withstanding competition from indigenous strains already present in soil and the adverse conditions of the soil. Nevertheless, given the ideal conditions, it can be safely inferred that these artificially applied inoculants containing live N_2 fixing bacteria (biofertilizers) can serve to replace fertilizer nitrogen up to 30–40 kg N/ha but the non-availability of these products at the time of sowing and the lack of zeal to polularise these inexpensive inputs in agriculture have limited their extensive use on the farm.

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ANNOUNCEMENTS

FLOOD PROBLEMS OF NORTH BIHAR

A two-day seminar on the Flood Problems of North Bihar has been sponsored by the L. N. Mithila University, Darbhanga and will be held in the University Department of Physics on the 6th and 7th of August 1988. Experts in the field of meteorology, oceanography, hydrography, water resources management, flood control, geography, forest denudation in the himalayan belt, and voluntary bodies associated with protection and preservation of healthy environment in the country and persons interested in this topic are invited to participate and exchange views on this vital problem which recurs almost every year in the monsoon season and takes heavy toll of plant, animal and human lives in the vastly populated North-eastern India.

Interested persons may contact Prof. Dhirendra Kumar Jha, Head of the University Department of Physics, L. N. Mithila University, Darbhanga 846 004 latest by 20th July 1988.

ILZIC SILVER JUBILEE CONFERENCE

Indian Lead Zinc Information Centre (ILZIC) — the Indian branch offices of the Lead and Zinc Development Associations of London — has completed (in 1987) 25 years of service to the lead, zinc and cadmium using industries in India and adjoining countries.

In commemoration of the Silver Jubilee of ILZIC, an International Conference with the theme "Lead, Zinc and Cadmium into the 90's" is being organised at the Taj Palace Hotel, Sardar Patel Marg, New Delhi 110 021 during 1–3 November 1988.

Comprehensive presentations on various aspects of the three metals by reputed experts both from India and overseas will be presented and discussed during the three-day deliberations. For more details please contact: The Secretariat, Silver Jubilee Conference, Indian Lead Zinc Information Centre, No. 7, Shopping Centre, Block B-6, Safdarjung Enclave, New Delhi 110 029.