Current Developments in Biological Nitrogen Fixation

Edited by
N.S. SUBBA RAO

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EDWARD ARNOLD

Foreword

From time immemorial legumes have been of prime importance in Indian agriculture and together with millets and rice (associated with nitrogen-fixing blue-green algae), they form the staple of primary production. With this background of history and continuing recognition of the value of legumes, a book from New Delhi on nitrogen fixation is specially welcome.

The editor is exceptionally well qualified to organise and assemble the material of this volume having worked throughout his career on many aspects of nitrogen fixation in nodulated legumes and by free-living microorganisms. He has also been personally involved in a number of projects for legume improvement, notably the 'All India Coordinated Pulse Improvement Programme', the 'International Biological Programme on Nitrogen Fixation' and the 'Coordinated Project on Biological Nitrogen Fixation' of the Indian Council of Agricultural Research.

Over the last two decades there has been a remarkable resurgence of interest and research in biological nitrogen fixation caused by mounting worldwide concern over the depletion of energy resources. This was sharply accentuated by the 1973 and subsequent oil price rises and the consequent several-fold increases in the cost of nitrogen fertilizer, which has had a particularly serious impact upon the agriculture of developing countries. The research on nitrogen fixation stimulated by the energy crisis has led to a better assessment of its potential, and an appreciation of the need to promote its efficient use in agriculture by all possible means: by extending the areas of leguminous and non-leguminous nitrogen-fixing plants especially those with special relationships with free-living nitrogen-fixing microorganisms. Equally important is the need to increase the amounts of nitrogen fixed in these crops by the application of this newly gained knowledge as well as by ensuring their optimal nutrition and pest and disease control by better agronomy.

Agricultural statistics show that most crop legumes and forage plants fall far short of their potential yields and that in general those of the tropics fix appreciably less nitrogen than those of temperate agriculture. This disparity may reflect the larger environmental stresses experienced in the tropics, especially respecting temperature and water relation. It may also be partly a consequence of problems caused by symbiotic specificity. Because many tropical legumes belong to the 'cowpea miscellany', they are readily cross-infected and the consequent symbioses are not always the most effec-

tive. The widespread occurrence of naturally existing strains of *Rhizobium* of less than elite status present considerable problems in their replacement with better ones by inoculation.

Recent research has brought us to the verge of complete understanding of some of the most intractable problems of symbiosis and nitrogen fixation: the mechanism and biochemistry of the infection processes, the nature of specificity, the economy of nitrogen transportation and use within the plant and the interrelation of bacterial and host physiology and its genetic control. Some of these advances have come from the use of new techniques, notably the acetylene reduction assay for nitrogenase, the use of isotopes and the relatively new and powerful tools of microbial genetics. Some also have come from originality in outlook and interpretation.

New ideas and methodologies will doubtless promote new advances, but if the past is any guide, progress is most likely to be fostered by the multidisciplinary approach either by research teams or by individuals expert in more than one field.

This volume gives an up-to-date assessment of the present knowledge in some of these aspects of special interest, viz. the genetics, ecology, physiology and metabolism of certain nitrogen fixing systems; necessarily a single volume can cover only a part of so large a subject. As appropriate to a work of this scope the authors are cosmopolitan and do not restrict their interest to the tropical scene. Nevertheless students, research workers and agriculturalists attracted by the challenging problems of biological nitrogen fixation will find this volume valuable and stimulating.

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Preface

This volume on 'Current Developments in Biological Nitrogen Fixation' is an extension of the objectives with which the earlier volume on 'Recent Advances in Biological Nitrogen Fixation' was brought out a few years ago. The objective was to focus attention on the many research and developmental aspects of the unique process of microbiologically mediated reduction of dinitrogen of the atmosphere into ammoniacal form of nitrogen. In recent years, while the developing countries of the world are eagerly attempting to harness the practical aspects of the biological nitrogen-fixing processes on the farm front by field oriented technologies as a specific measure to relieve the stress on chemical fertilizers, the affluent nations are equally engaged in understanding the genetic control and the mechanism of fixation in biological nitrogen-fixing systems in general so as to pave the way for a new bio-technology to render the crop plants self-sufficient with regard to their nitrogen nutrition. As a result of these increased research activities, it was felt desirable and appropriate to take stock of the current situation again by summarizing the present state of art on some aspects of biological nitrogen fixation in the form of a book.

The nitrogen nutrition of nodulated legumes is dependent upon the effective rhizobial symbiosis with the appropriate host plant. In rain-fed tropical soils where most of the grain legumes are nodulated by *Rhizobium* sp. (cowpea miscellany), the competition with native ineffective rhizobia together with the stress factors operating on both the symbionts by way of high soil temperature and drought often tend to limit the formation of effective root nodules. These aspects of rhizobial survival in tropical soils form the basis of the first chapter written by Eaglesham and Ayanaba. A related subject is the interaction of nitrogen-fixing microorganisms in soil with other soil microorganisms. The associative and antagonistic factors operating in *Rhizobium* survival and performance in soil is governed by the activity of other microorganisms which perform other functions such as the solubilization of bound phosphates, provision of auxiliary hormones and growth factors or secretion of antibacterial substances. Among the beneficial associates, the vesicular-arbuscular mycorrhizal (VAM) fungi appear to be significant. The non-symbiotic nitrogen-fixing species, *Azotobacter chroococcum* is known to secrete an antifungal antibiotic substance active against plant pathogens. Many viral infections of leguminous plants diminish the benefits of root nodulation. Therefore, an inte-

grated view of these interactions is taken by Subba Rao who has written the second chapter on microbial interactions with reference to biological nitrogen fixation.

The meeting point between a nitrogen-fixing microorganism and the host plant benefiting from root associations is the root surface. There are biochemical events which are very significant in the pre- as well as post-penetration stages of interactions between roots of plants and nitrogen-fixing microorganisms. One of the widely discussed factors operating in the pre-penetration stages is the specific protein called lectin which binds the surface of two symbionts of legume-*Rhizobium* symbiosis and determines the attachment of nitrogen-fixing bacteria to the root surface. Recently, however, an intense controversy has emerged on the involvement of lectins in legume-*Rhizobium* infection, a process which still remains highly enigmatic. Nevertheless Dazzo and his associate, Truchet who uphold the lectin mediated recognition phenomenon explain the recent developments on this aspect in the third chapter.

Nitrogen-fixing nodules on stems of plants would indeed be a convenient way to increase the surface area for plant-*Rhizobium* interaction and stem nodulation has indeed been recorded in the genera *Aeschynomene* and *Sesbania*. The fourth chapter on stem nodules by Subba Rao and Yatazawa highlights the current status of work on this aspect and points out the importance of extending research in this area.

Ironically, nitrogen fixation is an anaerobic process even in highly aerobic systems such as *Azotobacter chroococcum*. While this is acknowledged universally, precise explanations for oxygen control mechanisms have not come forth in any of the free-living systems. Needless to say, an understanding of the oxygen control mechanisms is a necessary prelude to achieving the transplantation of nitrogen-fixing bacteria into highly aerobic plant protoplasts. A review of the current knowledge on oxygen control mechanisms has been given by Shaw of New Zealand in the fifth chapter.

Genes responsible for nodulation and nitrogen fixation situated in mega-plasmids have been demonstrated in *Rhizobium meliloti*. Being extra-chromosomal in occurrence, plasmids offer tremendous potentiality as vehicles for *nif* gene transfer to higher plants. Although there is yet a long way to go in this exercise, Kondorosi and his colleagues explain in the sixth chapter how plasmids work in the expression of symbiosis in legumes.

Apart from cultivated legumes, forest trees such as alder (*Alnus* spp.) and *Casuarina* spp. benefit from nitrogen-fixing symbiosis through an actino-mycetous microsymbiont designated as *Frankia*. The microsymbiont has been isolated successfully in *Alnus* and *Comptonia* and many of the physiological aspects of symbiosis have now been well understood. The seventh chapter by Wheeler summarizes the recent advances in the knowledge on *Frankia* symbiosis.

Lichens are composite structures consisting of blue-green algae and fungal symbionts, the nitrogen-fixing blue-green alga helping the fungal partner in nitrogen nutrition while the fungal partner provides protection for the survival of the alga even under desiccated condition. Lichens have been recognized as primary colonizers of rocks and exhibit an extreme capacity to survive under environmental hazards. They also serve as an excellent example of symbiosis. The intricate aspects of nitrogen fixation in lichens have been covered by Millbank in the eighth chapter.

In Brazil, sugar cane is grown on virgin soil requiring very little mineral nitrogen fertilizer. Biological nitrogen fixation has been demonstrated by finite methods in this crop and several non-symbiotic nitrogen-fixing bacteria have been implicated in this process. The role of bacteria in nitrogen fixation in sugar cane has been dealt with by Ruschel and Vose in the ninth chapter.

Wetland rice fields provide an ideal microhabitat for photosynthetic as well as heterotrophic nitrogen-fixing microorganisms which live and fix nitrogen under submerged conditions. The International Rice Research Institute, Manila, Philippines has carried out intensive work on this system and Watanabe and Roger provide an integrated information on this subject in the tenth chapter.

Graminaceous plants have nitrogen-fixing bacteria in their root system among which *Azospirillum* has been highlighted in recent years. The role of *Azospirillum* and other diazotrophic nitrogen-fixing bacteria in the nitrogen nutrition of grasses such as sorghum and millets has been substantiated from more than one laboratory in recent years. Many of the recent developments in this exciting area have been elucidated by Boddey and Döbereiner in the eleventh chapter.

The genetics of *Azotobacter* and *Azospirillum* is receiving increasing attention in recent years, more particularly on plasmid controlled molecular biology. From the world famous Pasteur Institute, Claudine Elmerich explains the methodology and results connected with this area of research in the twelfth chapter.

Admittedly, it is difficult to bring home all the developments in biological nitrogen fixation in a single handy volume but nevertheless, the present exercise highlights potential areas where significant advances are being currently made. In this task, I owe a great deal to the various contributors for their prompt compliance with my request in providing the manuscript. I wish to express my indebtedness to Dr. O.P. Gautam, Director-General, Indian Council of Agricultural Research, Dr. N.S. Randhawa, DDG(SAE), and Dr. H.K. Jain, Director, Indian Agricultural Research Institute, New Delhi for their kind encouragement. Finally, I will be failing in my duty if I do not say a word of thanks to my wife Gowri Subba Rao and my daughters

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1. Tropical Stress Ecology of Rhizobia, Root Nodulation and Legume Fixation

A.R.J. Eaglesham and A. Ayanaba

INTRODUCTION

To adequately feed the world's increasing population it is essential that food production be increased in the Third World where it will be most needed. This aim is the mandate of the International Institutes funded by the Consultative Group on International Agricultural Research and the many national agricultural institutes throughout the tropical world. Moreover, an increasing number of scientists in the developed countries are becoming involved in solving problems which relate directly to tropical agriculture.

The potentially important role of legumes in maintaining soil fertility is well established. Grain legumes provide valuable nutritious seed and, when effectively nodulated, can yield in nitrogen-deficient soils where cereals and other non-leguminous crops would barely survive. It is no coincidence that legumes are a component of many of the traditional farming systems throughout the tropics. If food production is to be increased with more productive farming systems, the package of improvement practices is likely to include an increased input of biologically fixed nitrogen to complement the use of fertilizer nitrogen if it is available. It is important to bear in mind, however, that contrary to popular opinion, the growing of a legume crop does not necessarily result in a nitrogen gain for a farming system, except where an effectively nodulated forage legume is ploughed under as a green manure. If more nitrogen is removed in the harvested grain than was fixed in the nodules, a net depletion results, even if all vegetative residues are ploughed under. The greater the amount of soil nitrogen that is available to inhibit nodulation and fixation, and the higher the harvest index for nitrogen, the more is the likelihood that a nitrogen depletion would occur [1]. The correct grain legume should be selected for a specific use, be it high grain yield at the possible expense of

some soil nitrogen or less grain yield with the expectation of nitrogen accretion to the soil.

Those involved with rhizobia and legumes should not assume that "improving" nitrogen fixation is necessarily the main priority in increasing legume production [2]. With judicious use of fertilizers, water management, insect control, disease control, superior cultivars etc., the inputs of biologically-fixed nitrogen may increase concomitantly and spontaneously. On the other hand there is no room for complacency: the natural environment is constantly changing and field crops are subjected to stresses throughout the growth cycle. There exist too many gaps in the understanding, particularly in terms of the responses of biological nitrogen-fixing systems to these stresses. The objective of this review is to focus on the main environmental stresses commonplace in tropical agriculture and on how they may affect survival of rhizobia in soil, the root-nodulation process, and the functioning of the effectively nodulated legume.

For background reading and to gain access to related aspects of *Rhizobium* and legume research the reader is referred to the excellent reviews on *Rhizobium* ecology [3], nodule initiation and development [4, 5], functioning of legume nodules [6], environmental effects [7], legumes in acid soils [8, 9], legume nutrition [10–13], and tropical agricultural legumes [14].

ACIDITY

In wet equatorial zones rainfall exceeds evapotranspiration for much of the year and as a result soils become thoroughly leached of calcium and magnesium leaving them markedly acid. Acid soils are characterized by high concentrations of hydrogen ions and free aluminium, and low concentrations of calcium and available phosphate. Some acid soils contain manganese at phytotoxic levels and in some molybdenum is unavailable.

It appears that acidity is less of a constraint to cowpea rhizobial survival in soil than is desiccation or high temperature [15]. Thus, in an acid soil at Onne in Nigeria (pH 4.6, annual rainfall 2,500 mm) the cowpea rhizobial count was 4.3×10^4/g soil, whereas at Maradi in the sahel-savannah zone in Niger Republic (pH 6.1, annual rainfall 600 mm) the count was 4.9×10^2/g soil. Laboratory studies of the effects of acidity on rhizobia from soils such as these have been based on growth in synthetic media. However, because rhizobia vary in the ability to withstand conditions associated with acid soils, the acid tolerance of rhizobia cannot be predicted from the growth rate or acid production characteristics in liquid media at higher pH [16]. Moreover, because slow-growing rhizobia produce an alkaline reaction in most growth media [9] caution is needed when testing their ability to tolerate low pH-using conventional techniques. Growth media may be modified by changing the carbon source to arabinose from mannitol which is customarily recommended [17], so that pH is stabilized and remains at the initial low value throughout much of the growth cycle [18]. Alterna-

tively, studies may be made during the early phase of growth up to visible turbidity before significant pH changes occur [19]. Although slow-growing rhizobia are in general more tolerant of low pH than the fast-growers, strain to strain differences exist [20]. *Rhizobium meliloti* is particularly sensitive to acid conditions [21]. Some slow-growing rhizobia native to acid soils are acid-requiring and grow only at approximately pH 4.5 [18]. On the other hand in a survey of 65 strains of slow-growing rhizobia of mixed origin, in liquid media acidity (pH 4.5 or 4.8) prevented the growth of 29 per cent of the strains and slowed the growth of most of the rest. Low phosphate levels limited the growth of some strains but with less severity than did acid. Aluminium (50 μM) was the most severe stress factor, stopping growth of 40 per cent of the strains. Tolerance of acidity was not necessarily correlated with tolerance of aluminium, since aluminium increased the lag time or slowed the growth rate of almost all of the strains which were tolerant of low pH [22]. A complementary study showed that while high manganese (200 μM) and low calcium (50 μM) had adverse effects on slow-growing rhizobia which varied from strain to strain in severity, neither was as severe a stress as aluminium, and strains which were tolerant of aluminium were also tolerant of manganese and low calcium [23]. The adverse effects of acid and aluminium on rhizobial growth appear to be bacteriostatic rather than bacteriocidal [24]. Unfortunately because of the lack of information in these surveys of rhizobial responses to acid-associated stresses, no correlation can be drawn between patterns of resistance or susceptibility and the type of soil from which the rhizobia originated.

The ability to grow in liquid media which mimic acid soil conditions may indicate an ability to survive in such soils with the potential to later colonize the host rhizosphere. An agar plate method for the rapid screening of rhizobia for tolerance to acidity and aluminium has recently been developed and is amenable for screening large numbers of rhizobial isolates [25]. This rapid screening technique may be used to reduce numbers for subsequent more critical examination in liquid media. Rhizobia which had been identified as tolerant of acid (pH 4.5) and aluminium (50 μM) in liquid media nodulated better and were more effective on cowpeas in an acid (pH 4.6) cum high aluminium soil than strains which had been identified as sensitive [26]. However, the pre-screening of rhizobia in acid liquid media was less useful when mung bean was used as the host in a soil of pH 5.0. The main cause of symbiotic failure in mung bean was sparsity of nodules which occurred even with some of the strains which grew in acid-defined media containing aluminium. These strains nodulated cowpea reasonably well in the same soil adjusted to pH 4.6, indicating a greater acid sensitivity in the nodulation process of mung bean [16]. Clearly, when testing the nodulating ability of rhizobia in acid conditions, an acid tolerant host is required.

When growing on mineral nitrogen, most legume species are only slightly adversely affected by acidity down to pH 4.0. Indeed some species actually grow better at pH 4.0 than in less acidic conditions, e.g. *Stylosanthes humilis* [27]. Legumes dependent on the root nodule symbiosis for nitrogen showed a range of responses to low pH, but in general nodulation was reduced or eliminated at pH values below 5 [27, 28]. In a survey of the effects of liming on eight soils of pH 3.4-4.25 the critical pH for nodule initiation and development in soybean was in the range 4.5-4.8 [29]. The inhibition of nodulation appears to result from a combination of low calcium and low pH since it was alleviated by increasing either calcium or pH [9]. Nodule initiation was more restricted when pea plants were exposed to pH 4.5 at two or three days after inoculation than at one or four days [30]. The lesion in the infection process which is induced by calcium deficiency and acidity has not been identified [9]. The nitrogen fixing activity of nodules is also adversely affected by acidity in many species [27, 28].

The presence of available aluminium in acid soils inhibits nodulation directly [31] and indirectly by stunting root growth, and also tends to compound the effects of low levels of calcium by inhibiting its uptake [8]. The mean nodule number of twelve soybean cultivars was highly correlated with primary root calcium content and inversely correlated with level of available aluminium in the soil. Exposure of nodulated roots of *Phaseolus vulgaris* to aluminium, however, had no effect on nodule development or function [31]. The inhibitory effect of the aluminium-calcium interaction on soybean nodulation varied with soil type. In two soils of higher Ca : Al ratio, mean growth of thirteen soybean cultivars at pH 4.5, although reduced in comparison with plants at pH 6, was the same whether they were relying on mineral nitrogen or nodule-fixed nitrogen. With 2×10^6 rhizobia/seed as inoculum nodule number and weight were the same at pH 4.5 as at pH 6 [33]. These findings indicate that at least for soils of this type, improvement of aluminium tolerance is more likely to be achieved by manipulating the plant rather than the *Rhizobium*. However, taking pains to use rhizobia that are stress tolerant has proven to be a wise precaution with soybean. In the acid, high aluminium soil at Onne in Nigeria mentioned earlier, soybean supplied with 150 kg N/ha gave a 74 per cent increase in grain yield in response to a lime application of 1 t/ha. A prior screening of effectiveness of rhizobia in the same soil led to the identification of two superior strains of *R. japonicum*. Plants inoculated with either of these strains did not respond to liming and yielded 1.9-2.1 t/ha of seed, approximately 43 per cent higher than the N + lime treatment [34].

Phosphorus deficiency is common in the acid soils of the tropics and in clay soils of high iron and aluminium content, phosphorus may be strongly adsorbed making the use of fertilizers uneconomic [12]. In some soils liming alleviates phosphorus deficiency but in others it may exacer-

bate it [35]. Although species and cultivars differ in their nutritional needs, legumes have a relatively high phosphorus requirement for optimum growth [36]. Some require significantly more phosphate to reach optimum yields when relying on symbiotically fixed nitrogen in comparison to when supplied with fertilizer nitrogen [37].

Little work has been done on root nodulation at low phosphorus levels. Indications are that phosphorus deficiency limits nodulation indirectly by limiting legume growth rather than the infection process *per se* [8, 38], although there is evidence to imply that some rhizobia are more able to nodulate at lower phosphate levels than others [11]. Nodule development requires adequate phosphorus [39, 40] and nodules accumulate a higher phosphorus content than roots [41]. A number of experiments in which sterile soils were inoculated with vesicular-arbuscular mycorrhizal fungi have established their important role in the phosphorus nutrition of plants, particularly in phosphorus-deficient soils [41, 42–46]. In comparison to the gramineae the legume root system is typically restricted in its geometry, making it particularly dependent on mycorrhizal infection [11]. For example, with a phosphate-deficient Brazilian soil (sterilized, pH 5.3) applications of rock phosphate or mycorrhiza increased the vegetative yield of *Stylosanthes guyanensis* by factors of 2.2 and 8 respectively and increased whole-plant per cent phosphorus by factors of 1.6 and 3 respectively; increased nodulation and nitrogen fixation were concomitant with improved phosphorus nutrition [41]. Very little mycorrhizal work has been done in very acid soils and although their efficiency is known to be influenced by pH [47] at least some mycorrhizas do function below pH 5. Inoculation of a sterile soil (pH 4.5) with mycorrhiza increased the growth of *Pueraria phaseoloides* by 12-fold. Addition of rock phosphate further improved yield by only 10–25 per cent, showing that without amendments phosphate was unavailable rather than grossly deficient [46].

The scant evidence available indicates that high manganese levels are unlikely to inhibit the growth or survival of rhizobia in acid soils [9]. Manganese toxicity mainly affects legume growth *per se* rather than nodulation in particular [8, 9, 48], and tolerance of manganese varies considerably between and within legume species [9]. However, variations among strains of *R. phaseoli* were found in their ability to nodulate and fix nitrogen in conditions where manganese was marginally phytotoxic [49]. The adverse effects of manganese were alleviated by liming [49].

Legume species also vary in the capacity to tolerate molybdenum deficiency in acid soils. In a Brazilian acid soil *Stylosanthes* grew well without molybdenum fertilization, whereas siratro and *Centrosema* responded positively to molybdenum fertilization. In the same soil six cultivars of *Phaseolus vulgaris* responded to molybdenum fertilization only in conjunction with liming to a pH greater than 5.4. Further liming in excess of pH 5.8 caused a sufficient desorption of molybdenum in the soil that a response to