CHAPTER 2

Aspects of Biological Nitrogen Fixation

1. Introduction

The earth's population is expected to reach 8.3 billion by 2025, and to maintain the current level nutritional intake, crop production will have to increase dramatically. Seen against the backdrop of deteriorating environmental conditions, which is in part due to the injudicious use of fertiliser, this will be an almost impossible task.

The effective management of nitrogen (N_2) in the environment is an essential element of sustainable agriculture. It usually involves some use of biologically fixed nitrogen, which is less susceptible to volatilisation, denitrification and leaching (Graham & Vance, 2000b). It is estimated that approximately 80% of biologically fixed nitrogen involves the symbiosis between rhizobia and leguminous plants (Vance, 1998). Worldwide, legumes are grown on approximately 250 Mha and fix close to 90 Tg of N_2 per year (Fink *et al.,* 1999). Other organisms are also increasingly recognised as major contributors to overall nitrogen fixation. These include actinorhizal symbiosis and associative relationships, including sugarcane and *Acetobacter.* However, most of the investigations of such N_2-fixing organisms reflect a molecular rather than a field orientation (Graham & Vance, 2000b).

Scientific discoveries over the last three decades have led to a better understanding of the processes involved in nodulation and nitrogen fixation. Despite these developments, little impact has been made on the field level. This is clearly illustrated by the total absence or lack of good quality inoculants in many parts of the world (Graham & Vance, 2000a).

The capacity of soil to supply nitrogen declines rapidly as soon as agricultural activities starts. This represents a major problem to farmers since nitrogen should then be supplemented from other sources. Biological nitrogen fixation presents an environmentally sound alternative if farmers are going to meet the increased demand of food production.

Despite the obvious advantages of biological nitrogen fixation, a number of factors influences agricultural dependence. These include the variation in farmers' acceptance of inoculation technologies, the availability of cheap nitrogen fertiliser and the availability of land for lower-yielding crops, etc. Where there is wider acceptance for this approach, factors impacting on the symbiosis will need to be examined. These include breeding and selection

programmes aimed at alleviating nutrient deficiencies, pH and drought tolerances, etc. (Graham & Vance, 2000b).

2 Nitrogenase and Biological Nitrogen Fixation.

Elemental nitrogen is abundant in the earth's atmosphere and is essentially inert at room temperatures in the absence of a suitable catalyst. The reduction of N_2 to form ammonia requires a large amount of activation energy, as has been indicated in the industrial fixation of nitrogen to ammonia by the Haber-Bosch process. The abundance of N_2, contrasted with the difficulty of chemically utilising this source, creates a paradox, which nature has ingeniously solved through the process of biological nitrogen fixation. A relatively small number of microorganisms, termed diazotrophs, are capable of carrying out this process, contributing annually approximately 60% of the earth's fixed nitrogen (Kim & Rees, 1994).

Biological nitrogen fixation is catalysed by the nitrogenase enzyme system which consists of two metalloproteins: the iron (Fe-) protein and the molybdenum-iron (MoFe-) protein. These enzymes mediate an ATP dependent reduction of dinitrogen to ammonia. Under optimal conditions the overall stoichiometry of dinitrogen reduction has been established as (Kim & Rees, 1994):

$$N_2 + 8\,H^+ + 8e^- + 16\,MgATP \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16\,P_i$$

3 Phylogenetic perspectives on nodulation

Nitrogen-fixing symbiosis is best known from its associations between leguminous plants and a diverse group of gram-negative soil bacteria, collectively called rhizobia. However, this nitrogen-fixing symbiosis is also known in several other plant groups. *Parasponia* (Ulmaceae family) is also nodulated by rhizobia, while cyanobacteria such as *Anabaena* and *Nostoc*, form symbiosis with plants such as the water fern *Azolla*, cycads and flowering plant *Gunnera* (Gunneraceae). The Gram-positive actinomycete *Frankia* nodulates nearly 200 species belonging to eight different families and it is considered that actinorhizal symbiosis fixes as much nitrogen as the legume-rhizobia symbiosis (Doyle, 1998).

Not all genera of legumes are nodulated. Most members of the subfamily Papilionoideae (including the typical beans) and Mimosoideae (acacias and mimosas) appear to be capable

of nodulation. By contrast, only few members of the third subfamily, Caesalpinioideae are known to nodulate (de Faria *et al.*, 1989). The observation that rhizobia can infect the roots of non-nodulated legumes and that such plants show nitrogenase activity, led Bryan *et al.* (1996) to speculate that all legumes are capable of rhizobial symbiosis though not all are able to form nodules. This further compounds the issue of the origin of nodulation. This phenomenon of nodulation could have arisen once or have had multiple independent origins. The multiple origin theory of nodulation is supported by the following:

1. Based on chloroplast gene phylogeny, the subfamily Caesalpinioideae is composed of diverse elements including earliest diverging lineages of the Leguminosae (Doyle *et al.*, 1997), it is therefore suggested that nodulation occurred once in each of these subfamilies.
2. The actinorhizal nodules from different families are structurally diverse as would be expected in the case of multiple origins of nodulation (Doyle, 1998)

On the other hand, an assessment of the major nodule types in the Leguminosae revealed that the unbranched indeterminate "caesalpinioid" nodule type is scattered throughout the family (Doyle *et al.*, 1997). This is consistent with a single origin of nodulation within the family, with the caesalpinioid nodule type being the common ancestor.

It is clear that many arguments can be raised on whether nodulation arose once or more times. The other related key issue is: how did they evolve? To examine this issue, Gualtiery & Bisseling (2000) investigated the nodule-specific proteins, called nodulins. These proteins were expressed in other parts of the plant, suggesting that these may have been recruited from other developmental processes. Determining the ancestral expression patterns of these nodulins could perhaps give better insight into the evolution of nodulation. The hypothesis of an independent origin of nodulation would therefore mean an independent recruitment of nodulin genes. Different legumes would therefore have different copies of the nodulin genes. In contrast, the common ancestor species possessing homologous nodules would have recruited the same type of nodulin genes. These nodulin genes, as well as transcription factors, remain to be investigated in greater detail and could so provide insight into the origin of nodulation.

4 The *Rhizobium* legume symbiosis

Most higher plants have the ability to form arbuscular endomycorrhiza (AM), a symbiotic association between the plant root and fungi of the order *Glomales*. These fungi grow toward the inner cortical cells of the root, where they differentiate into the highly branched structures, the arbuscules. Additionally, the fungus also forms hyphae outside the plant, which facilitate the uptake of nutrients. The *Rhizobium*-plant symbiosis is more complex and more specific, interacting symbiotically with only a few plant species. This interaction results in the formation of a new organ, the root nodule in which the bacteria are hosted in an ideal environment where they can reduce atmospheric nitrogen (Albrecht *et* al., 1999).

The process of rhizobium-legume infection consists of successive, discrete recognition events involving interactive, complementary plant and bacterial functions. Rhizobia in the rhizosphere are chemoattracted to the plant roots by compound such as amino acids, sugars organic acids and plant secondary metabolites. This is followed by bacterial attachment to the root hairs, which is considered a complex process involving multiple mechanisms which include plant lectins and bacterial fimbriae (Roth & Stacey, 1991). A study by van Workum *et al.* (1998) showed that exopolysaccharides of rhizobium accelerates root hair curling and infection to such an extent that rhizobial root penetration precedes a plant defense response. Root nodule formation involves the redirection of the development of fully differentiated plant cells and the responsible bacterial signals are the so-called nodulation (Nod) factors. The common basic Nod-factor structure (Fig. 2.1) of different rhizobia is: β-1,4-linked *N*-acyl-D-glucosamine backbone of almost four or five units, containing a fatty acid at the non-reducing terminal sugar (Carlson *et al.*, 1994).

The Nod-factor secreting rhizobia induce morphological changes of the root hairs, commonly referred to as the "shepherd's crook'-like curling of the root hairs. Although a curled root hair is not essential for infection , it is thought to facilitate infection. The microenvironment within such curls are used by the rhizobia to establish an infection site, where they locally degrade the plant cell wall and enter the root hair via invagination of the plasma membrane (Albrecht *et al.*, 1999). New cell wall material is deposited to form the infection thread. The infection thread continues to grow beyond the root hair cell and penetrates the cortex of the root (Dixon & Wheeler, 1986).

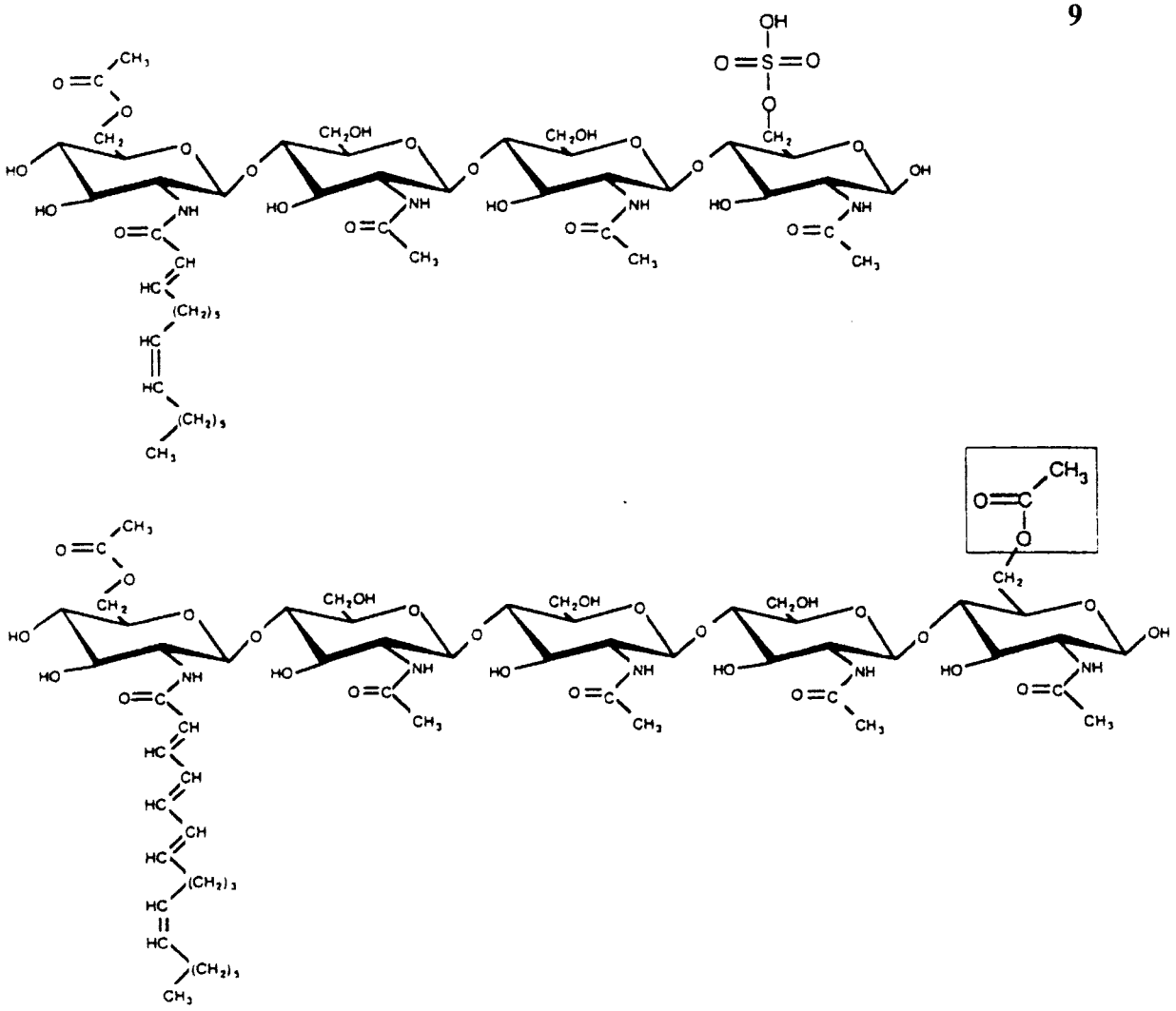

Figure 2.1. The major Nod factors produced by *Sinorhizobium meliloti* (top) and *Rhizobium leguminosarum* bv. *vicae* (bottom). The major differences concern the specific decoration at the reducing terminal sugar unit and the structure of the acyl chain. The *S. meliloti* Nod factor contains four glucosamine units, an acyl chain of 16C-atoms, an acetyl group at the non-reducing end and a sulphate group at the reducing terminal sugar residue. *R. leguminsarum* : the glucosamine backbone is four or five units carrying an acyl chain of 18 C-atoms (Carlson *et al.*, 1994)

Clusters of cortical cells, as determined by the host species, are mitotically activated by Nod-factors to form a nodule primordium. The infection thread grows, reaches the primordium and the bacteria are released. The bacteria enter the cytoplasm via an invagination of the host-cell membrane, forming a so-called symbiosome. Within the host cytoplasm the bacteria remain surrounded by a host membrane (also termed peribacteriod membrane) and is never in direct contact with the host cytoplasm. Upon infection, the nodule primordia form a meristem as well as different tissues that form a nodule. Bacteria within the symbiosome are referred to as bacteroids due to the series of biochemical and cellular changes they have undergone (Albrecht *et al.*, 1999; Roth & Stacey, 1991).

A differentiation is made between effective and ineffective nodules. In the former nitrogen is fixed effectively to the benefit of the host plant, while in the latter no nitrogen is fixed or the level of fixation is so low that it is of no benefit to the plant. This ineffectivity might be ascribed to the inability of the bacteria to emerge from the infection thread or the lack of production of nodule tissue. Senescence of nodules is also a well-known occurrence, typified by a total disintegration of nodules and is essentially controlled by the plant (Roth &Stacey, 1991).

5 Molecular basis for nodulation

In rhizobia the nodulation genes are organised in several operons, located either on the chromosome or on a large symbiotic (Sym) plasmid. The nodulation genes may be classified into three groups: the regulatory *nodD* gene, the so-called "common" *nod*-genes and host specific nodulation genes (Albrecht *et al.*, 1999).

These *nod* operons are preceded by a conserved *cis*-regulatory element, the *nod* box. The regulatory NodD–protein, induced by plant flavonoids, binds to the *nod* box and activates the transcription of the *nod* genes. The interaction of NodD-protein with specific plant flavonoids of the host plant represents the first level host-specific interaction (Schultze & Kondorosi, 1998). Some rhizobial species appear to have more than one *nodD* gene e.g. *Bradyrhizobium* species, *Sinorhizobium meliloti* and *Sinorhizobium fredii*. In contrast, *R. leguminosarum* bv. *vicae* contains only a single *nodD*-gene. These copies are generally highly homologous producing NodD proteins capable of interacting with a range of different inducer molecules (Roth & Stacey, 1991).

In response to the release of the appropriate inducers by the host plant, the rhizobia synthesise and secrete a family of Nod-factors. The synthesis of the basic Nod-factor structure is catalysed by the bacterial NodA-, NodB- and NodC-proteins. NodC (*N* –acetyl glucosaminyl-transferase) catalyses the synthesis of the chitin oligomer and controls the length of the backbone, NodB deacetylates the terminal non-reducing glucosamine unit of the oligomer, which is substituted by an acyl chain by NodA (Albrecht *et al.*, 1999; Perret *et al.*, 2000). According to Carlson *et al.* (1994), several other species-specific nod-proteins modify the terminal sugar residue or determine the nature of the acyl chain. Such modifications determine biological activity and host specificity of these Nod factors. The products of the

other *nod* genes play subtle roles in nodulation, perhaps by permitting interaction with certain plants or by protecting the Nod factors from degradation (Perret *et al.*, 2000). An outline of the signal exchange in the *Rhizobium*-plant symbiosis is given in Fig. 2.2.

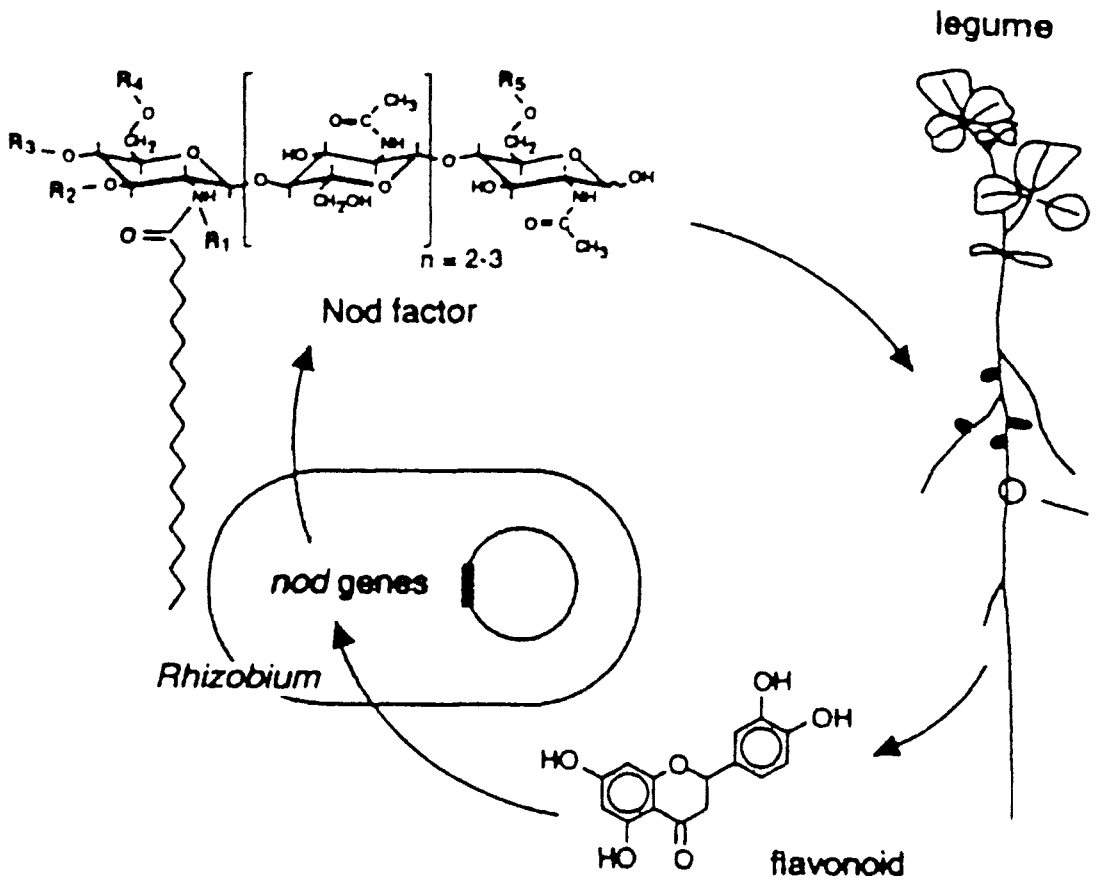

Figure 2.2. Signal exchange in the *Rhizobium*-legume symbiosis. Flavonoids induce the rhizobial *nod* genes. This leads to the production of nodule-inducing (Nod) factors, lipochitooligosaccharides (LCO), which are differently modified depending on the *Rhizobium* species (Schultze & Kondorosi, 1998).

6 Conclusion

Developing countries are faced with the dilemma of increasing food production due to escalating population numbers. However, the problem is compounded by the low nutrient status of agricultural soils. The demand for chemical nitrogen fertiliser is therefore expected to increase with a consequent increased negative effect on the environment. The challenge is therefore to find an alternative to chemical nitrogen fertiliser. Biological nitrogen fixation is an approach to meet this goal. It is therefore important to have an understanding microbes involved in biological nitrogen fixation and the signal exchange between the symbiotic partners. In this way symbiotic interactions of agricultural significance may possibly be identified.